UNIVERSITÁ DEGLI STUDI DI TRIESTE

Scuola di Dottorato in Biomedicina Molecolare

Ciclo XXIII

THE CANCER STEM CELLS AND HUMAN LIVER CANCER: identifications, expressions, and drug resistance

Dottoranda:

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Settore Scientifico - Disciplinare: BIO/11

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Anno Accademico 2009/2010

UNIVERSITÀ DEGLI STUDI DI TRIESTE

Sede Amministrativa del Dottorato di Ricerca

UNIVERSITÀ DEGLI STUDI DI Trieste ISTITUTO/CENTRO RICERCHE ETC Centro Studi Fegato

Sedi Convenzionate (1)

Posto di dottorato attivato grazie al contributo del (2) Ministero degli Affari Esteri

XXIII (3) CICLO DEL

DOTTORATO DI RICERCA IN (4)

BIOMEDICINA MOLECOLARE

The cancer stem cells and human liver cancer: identifications, expressions, and drug resistance

Settore scientifico-disciplinare BIO/11 (5)

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I would like to thank the Ministero Affari Esteri and the Istituto Italiano di Cultura Jakarta for the PhD fellowship 2008 – 2011, in particular for the support of Prof. Ostelio Remi, Dott.ssa Giovana Jatropelli and Dott.ssa Livia Raponi.

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THE CANCER STEM CELLS AND HUMAN LIVER CANCER: identifications, expressions, and drug resistance

Caecilia H.C. Sukowati

Summary

The advance information on molecular biology and medicine had suggested a new theory of the initiation of the cancer. Cancer is composed in a hierarchy of many types of cells with various degree of differentiation in which there is only a small proportion of cells can initiate and sustain the tumor growth. These cells are known as the cancer stem cells (CSCs). The CSCs still possess the whole capacity as the normal stem cells to self-renew, to differentiate into multiple types of cells and to proliferate extensively. In the liver cancer, the characteristics of the hepatic CSCs remain unclear. They might be resident hepatic stem cells or derived/migrated stem cells from bone marrow. Several molecular markers such as CD90, CD44, CD133, EpCAM, and so-called side populations with ABCG2 expression had been proposed. The ABCG2 expression is closely related with drug resistance in cancers.

The general objective of this study is to better understand the heterogeneity of cancer stem cells in hepatic system. This general objective was specified into three interrelated projects: 1) to isolate and characterize the stem cells from different tissues parts of several human liver malignancies, mainly HCC and CC; 2) to assess the expression of several stem cells markers in clinical samples tissues; 3) to study the expression of a drug transporter ABCG2 in relation with cells differentiation and drug resistance.

We had isolated cells populations from distant pair of neoplastic and distal tissues of HCC and CC patients. These isolated cells co-expressed CD90 and CD44 but not hematopoietic stem cells markers CD34. Further analysis showed that they express mesenchymal surface markers and also pluripotency factors. This population had cells with cytokeratin expression and low albumin expression. When they were plated in low density, they cloned and formed fibroblastic-like colonies. These data suggested that these isolated cells populations contained mesencyhmal stem cells. Interestingly, when they were induced into insulin producing cells, they showed up-regulations of markers of pancreatic cells, indicating their potential to transdifferentiate to endodermal lineage. One line had capacity to differentiate into adipogenic lineage, demonstrated by the fat droplets accumulation in the cytoplasm and high expression of peroxisome proliferator-activated receptor gamma (PPARG). These results had provided the evidences that stem cells from cancerous tissues had capacity to trans-differentiate into insulin producing cells and adipocytes. As expected, both stem cells from neoplastic and distal tissues both shared similar phenotypic markers. We assumed that the differences between them might be on different mechanisms of molecular pathways of those cells.

From our study in clinical tissues samples, we found that the distribution of stem cells markers genes from liver malignancies tissues were highly variable. Interestingly, it showed that there was a tendency that the expressions of these genes were higher in the diseased tissues than in normal tissues and in the lesion than distal tissues. These results may imply that there was active proliferation of the stem cells in the cancer and may represent the tumorigenesis process from normal to disease tissues.

Concerning the drug resistance issue, we found that the expression of ABCG2 in clinical samples was higher in neoplastic tissues compared to distal and young normal tissues. To support data *in vivo*, we employed several hepatic cell lines for data *in vitro*. We observed that ABCG2 was significantly highest in the most undifferentiated cell lines JHH-6. The Hoechst 33342 efflux assay demonstrated that this line also had the highest capacity to pump out the substrate from the cells. After exposure to doxorubicin, a common anti-neoplastic agent, all hepatic cell lines showed up-regulations of ABCG2, the highest up-regulations were detected in cell lines which had low basal ABCG2 expression.

All the data shown in this study has supported the presence of stem cells in liver cancer. This summary might be only small contributions in the knowledge of hepatocarcinogenesis and cancerogenesis in general. However, more data in the characterizations of both normal SCs and CSCs are important for better understanding their mechanisms in cancer initiation, maintenance, and treatment. This should be achieved by several steps. First, it is important to know how the CSCs initiate the cancer. Second, the identification of specific markers for these cells for a specific targeting of CSCs while sparing normal SCs, might be some structural protein markers or specifically different mechanisms in cell pathways. Third, it is important to understand the better handling of drug resistance problem in cancer.

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CELLULE STAMINALI TUMORALI E TUMORE EPATICO PRIMITIVO UMANO: identificazione, espressione e chemioresistenza

Caecilia H.C. Sukowati

Riassunto

Le scoperte più recenti in campo biomedico hanno proposto una nuova teoria sull"inizio del cancro. Secondo queste ipotesi il tumore è composto da una popolazione eterogenea di cellule a diverso grado di differenziamento in cui solo una piccola percentuale è in grado di avviare e sostenere la crescita del tumore. Queste cellule sono conosciute con il nome di cellule staminali tumorali. Queste cellule, come le cellule staminale normali, hanno la capacità, di auto-rinnovarsi, di differenziare in diversi tipi di cellule e di proliferare estensivamente. Nel tumore del fegato, le caratteristiche delle CSC rimangono poco chiare. Queste cellule potrebbero derivare da cellule staminali epatiche residenti o da cellule staminali del midollo osseo. Per la loro identificazione sono stati proposti diversi marcatori molecolari tra i quali CD90, CD44, CD133, EpCAM, e il metodo della 'side population' mediante l'espressione di ABCG2 . L'espressione di ABCG2 sembra anche essere correlata con la resistenza ai farmaci.

L'obiettivo generale di questo studio è di meglio capire l'eterogeneità delle cellule tumorali nel sistema epatico. A tale scopo sono stati sviluppati tre progetti interconnessi: 1) isolamento e caratterizzazione delle cellule staminale in diverse parti di tessuti epatici neoplastici umani, in particolare HCC e CC, 2) valutazione dell'espressione di marcatori di

cellule staminali in diversi campioni clinici, 3) analisi dell'espressione del trasportatore ABCG2 in relazione alle cellule staminali e alla resistenza ai farmaci.

Abbiamo isolato popolazioni di cellule sia dai tessuti neoplastici che distali dei pazienti con HCC e CC. Queste cellule esprimono i marcatori di superficie CD90 e CD44 ma non il marcatore per le cellule staminali emopoietiche CD34. Ulteriori analisi hanno dimostrato che queste cellule esprimono marcatori di superficie mesenchimali e anche fattori per la pluripotenza. Alcune sottopopolazioni esprimono anche le citocheratine e a basso livello l'albumina. Quando vengono coltivate a bassa densità, queste cellule formano colonie simili a quelle formate dai fibroblasti suggerendo che queste popolazioni contengono anche cellule mesenchimali. È interessante notare che, quando sono state indotte a trans-differenziare in cellule che producono insulina, si è osservata una up-regolazione dei marcatori delle cellule pancreatiche. Inoltre una linea ha dimostrato la capacità di differenziare in adipociti, in quanto, se opportunamente indotta, è in grado di accumulare lipidi nel citoplasma oltre a mostrare un aumento del gene PPARG. Questi risultati hanno fornito indicazioni del fatto che le cellule staminali da tessuti cancerosi poterebbero mantenere la capacità di trans-differenziare in diverse linee cellulari. In particolare in cellule pancreatiche e adipociti. Come atteso, le cellule staminali dai tessuti neoplastici e distali, condividono simili marcatori fenotipici. Abbiamo ipotizzato che le differenze tra di esse possano esse a livello intracellulare nelle vie e nei meccanismi molecolari di tali cellule.

Dal nostro studio sui campioni di tessuto, emerge che la distribuzione dei marcatori delle cellule staminali nei tessuti tumorali è molto variabile. Ciò nonostante si è osservata una tendenza: l'espressione di questi geni è più elevati nei tessuti malati rispetto ai tessuti normali e nelle lesioni rispetto ai tessuti distali. Questi risultati potrebbero indicare l'esistenza di cellule staminali in attiva proliferazione nel tumore e questo processo potrebbe rappresentare il processo di tumorigenesi che sottende alla trasformazione neoplastica.

Per quanto riguarda la resistenza ai farmaci, abbiamo osservato che l'espressione di ABCG2 in campioni clinici è maggiore nei tessuti neoplastici rispetto ai tessuti distale e ai campioni normali derivanti da pazienti giovani. Per supportare i dati *in vivo*, abbiamo impiegato diverse linee cellulari epatiche *in vitro*. Abbiamo osservato che l'espressione di ABCG2 è significativamente più alta nella linea cellulare JHH-6: una linea cellulare epatica

derivante da un tumore poco differenziato . Il test dell' efflusso di substrato Hoechst 33342 ha inoltre dimostrato che questa linea cellulare ha anche la maggior capacità di eliminare il substrato. In seguito al trattamento con doxorubicina, un comune agente anti-neoplastico, è stato osservato un aumento di ABCG2 in tutte le linee cellulare epatiche, sopratutto nelle linee cellulari con l'espressione basale più bassa.

Tutti i dati riportati in questo studio hanno rinforzato le prove a favore del'ipotesi della presenza di cellule staminali nel tumore al fegato. Questo lavoro potrebbe rappresentare solo un piccolo contributo nella conoscenza dell'epatocarcinogenesi e cancerogenesi in generale. Tuttavia, una maggior caratterizzazione delle cellule staminali normali e tumorali sarebbe molto importante per una miglior comprensione dei meccanismi di trasformazione e crescita neoplastica. A tale scopo andrebbero chiariti principalmente alcuni aspetti ancora poco chiari: per prima cosa sarebbe importante capire come le cellule staminali tumorali sono coinvolte nella trasformazione maligna. In secondo luogo sarebbe importante identificare marcatori specifici per distinguere le cellule staminali tumorali dalle cellule staminali normali, questi marctatori potrebbero essere proteine strutturali o proteine di vie metaboliche differenzialmente espresse. Un terzo aspetto molto importante è poi la comprensione dei meccanismi che sottendono la chemoresistenza al fine di sviluppare trattamenti più efficaci.

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Publications during PhD program 2008 – 2010

Full articles

1. Sukowati CHC, Rosso N, Crocè LS, Tiribelli C. Hepatic cancer stem cells and drug resistance: relevance in targeted therapies of hepatocellular carcinoma. W. J. Hepatol. 2010 (3): 114-126
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4. Sukowati CHC, Rosso N, Pascut D, Anfuso B, Torre G, Francalanci P, Crocè LS, Tiribelli C. The expression of the breast cancer resistance protein BCRP/ABCG2 transporter in human liver and cells models of hepatocellular carcinoma. *Submitted*

Conference poster and oral presentations

1. Sukowati CHC, Anfuso B, Crocé L, Tiribelli C. The identification of cancer stem cells from human primary liver cancer. Keystone Symposia: Cancer Stem Cells and Metastasis. Colorado, USA. 2011
2. Sukowati CHC, Anfuso B, Crocé L, Tiribelli C. The cancer stem cells from primary liver cancer: identification and expression. Eijkman-Nehcri Joint Symposium. Jakarta, Indonesia. 2010
3. Sukowati CHC, Anfuso B, Tiribelli B. Cancer stem cells and drug resistance: The BCRP expression in human hepatocellular carcinoma. The ESTOOLS International Scientific Symposium: Stem Cells in Biology and Disease. Lisbon, Portugal. 2010
4. Pascut D, Rosso N, Sukowati CHC, Tiribelli C. Modulation of ABC transporters expression and multidrug resistance in HCC. The 3rd Conference of International Liver Congress Association. Milan, Italy. 2009
5. Sukowati CHC, Rosso N, Pascut D, Tiribelli C. The BCRP expression and drug resistance in hepatocellular carcinoma. The 3rd Conference of International Liver Congress Association. Milan, Italy. 2009
6. Sukowati CHC and Tiribelli C. The future direction for the stem cells therapy in liver cancer. Liver International Congress. Cairo, Egypt. 2008

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ABBREVIATIONS

ABC transporters	ATP binding cassette transporters
ABCG2	ABC binding cassette transporter, subfamily G (alias: BCRP)
AFP	Alpha fetoprotein
au	Arbitrary unit
BA	Biliary atresia
BCRP	Breast cancer resistance protein
CAFs	Cancer associated fibroblasts
CC	Cholangiocarcinoma
CD	Cluster of differentiation
CFU-F	Colony forming unit - fibroblast
CK	Cytokeratin
CoH	Canal of Hering
CSCs	Cancer stem cells
EMT	Epithelial to mesenchymal transition
FACS	Fluorescence activated cells sorting
hASCs	Human adult stem cells
HB	Hepatoblastoma
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
hESCs	Human embryonic stem cells
HSCs	Hematopoietic stem cells
iPS	Induced-pluripotent stem cells
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
N	Normal tissues
O	Other liver malignancies
OV-6	O volvulus 6

PLC	Primary liver cancer
qRT-PCR	Quantitative reverse transcription polymerase chain reactions
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reactions
SCs	Stem cells
SP	Side populations
α-SMA	Alpha smooth muscle actin

Chapter I

General Introduction

1.1. Primary Liver Cancer

1.1.1. Epidemiology and risk factors

Primary liver cancer (PLC) is the fifth most common neoplasms in the world and the third most common cause of cancer-related death. PLC accounted for around 1% of all death worldwide (Parkin *et al.* 2001). Approximately more than 500,000 new cases are diagnosed per year, with an age-adjusted worldwide incidence of 5.5–14.9 per 100,000 populations (Llovet, Burroughs, and Bruix 2003).

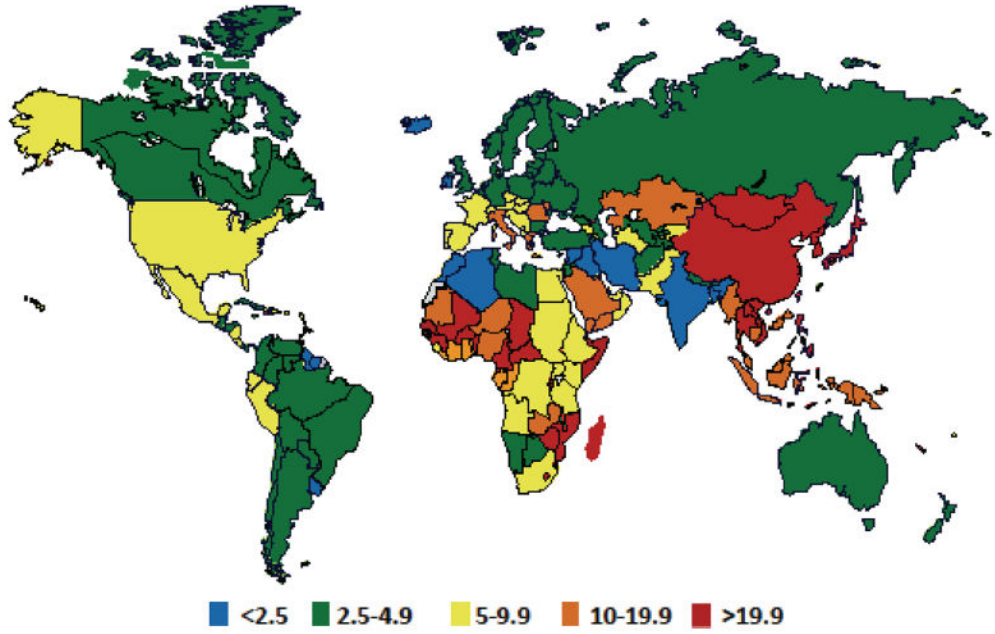

Figure 1.1. Age-standardized incidence rates of the primary liver cancer worldwide. Source: GLOBOCAN 2002, picture is taken from (Nordenstedt, White, and El-Serag 2010).

PLC prevalence is variable distributed, found to be high in Asia and low to high in Europe and low to moderate in America continents. Figure 1.1 shows the global distribution of PLC with age standardized rate (ASRs). Highest ARS is found in China (ARS > 20.0), Southeast

Asia which is endemic to Hepatitis B Virus (HBV) and Japan with Hepatitis C Virus (HCV), and sub-Saharan African countries such as Cameroon and Mozambique. Italy has the highest incidence in Southern Europe with ASR of 15.9 in men and 5.1 in women (Nordenstedt *et al.* 2010). The PLC could be classified into several types based on the primary site of the cancer as described in Table 1.1.

Table 1.1. Types of human primary liver cancer

PLC types	Primary site	Frequency
Hepatocellular Carcinoma	Liver parenchyma	85 -90% from all PLC cases
Cholangiocarcinoma	Bile duct	3 -20% from all PLC cases
Hepatic angiosarcoma or hemangiosarcoma	Blood vessel	2% from all PLC cases
Hepatoblastoma	Liver parenchyma in infant and neonatal	1-4% from all solid childhood tumors

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) accounts for 85% to 90% of PLC cases (El Serag and Rudolph 2007). However as shown in Fig.1.1, HCC is not distributed evenly in the world, in some area in Asia, HCC is the most common cause of cancer-related mortality (Llovet *et al.* 2003).

Several main risk factors for HCC are widely known. About 80% of all detected cases HCC develops in chronic liver disease and cirrhosis is the strongest factor (El Serag and Rudolph 2007;Llovet *et al.* 2003) mainly correspond to viral infections Hepatitis B virus (HBV) and Hepatitis C virus (HCV), also chemical exposure like excess alcohol consumption, aflatoxin B and vinyl-chloride monomer (Mastrangelo *et al.* 2004), obesity-related disease, and familial-related disorder such as primary haemochromatosis (Niederau *et al.* 1985). In almost all populations, males have higher cancer rates than females, with male:female ratios usually

averaging between 2:1 and 4:1. In HCC relation with ages, female rates peak in the age group 5 years older than the peak age group for males (El Serag and Rudolph 2007). Until now, the relation between smoking tobacco and HCC is not clearly established.

In Asia and Africa, HBV infection is the highest risk factor of HCC. The annual mortality from hepatitis B infection and its relation is 1-2 million people worldwide (Zuckerman and Zuckerman 2000). Until now, over two billions people worldwide have been infected, of whom over 350 million are chronic carriers. Some 25% of carriers develop progressive liver disease. Chronic HBV carriers have a 100-fold relative risk of developing HCC compared with non-carriers, which decreases if infection is acquired in adulthood (Beasley *et al.* 1981). The HBV infections can be prevented by vaccination. The universal Taiwanese HBV vaccine program launched in 1984 had significantly decreased the HCC incidence 20 years after vaccination (Chang *et al.* 2009).

In Europe and North America 50-70% of HCC cased is caused by HCV infection (Llovet *et al.* 2003;Bosch, Ribes, and Borras 1999). About 150 million people (3% of the world's population) harbor long-term (chronic) infections with the HCV and about 3–4 million people become infected with this virus every year (Magiorkinis *et al.* 2009). HCV increases HCC risk by promoting fibrosis and eventually cirrhosis. The estimation of HCC development from HCV infections is from 1% to 3% after 30 years. If cirrhosis is established, HCC develops at an annual rate of 1% to 7% (El Serag and Rudolph 2007). Until now, potent HCV vaccine is not yet available and HCV infections can be treated with combination of drugs, but they are not real effective in all patients.

Some other established HCC risk factor is chemical toxins, such us heavy alcohol intake, aflatoxin, and vinyl chloride. Among alcohol drinkers in Italy, HCC risk increased in a linear fashion with daily intake more than 60 g. The effect of alcohol drinking was evident even in the absence of HBV or HCV infection (Donato *et al.* 2002). Aflatoxin B1 (AFB_1) is a mycotoxin produced by *Aspergillus* fungus. This toxin is mainly found in Asia and sub-Saharan Africa in which climatic factors and food storage techniques support the fungus to contaminate foods, such as grain, corn, peanuts and legumes (Gomaa *et al.* 2008). Once ingested, AFB_1 is metabolized to an active intermediate which can bind to DNA and cause

damage (Garner, Miller, and Miller 1972). In China, the urinary excretion of aflatoxin metabolites was associated with a 4-fold increase in HCC risk. However, in its interaction with HBV carriers, the presence of AFB_1 had a 60-fold increase risk in HCC (Qian *et al.* 1994). Even though vinyl chloride has been a risk factor, its association with development of HCC remains unclear.

Correlations between HCC and fatty infiltration had been also predicted, represented severe forms of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). Several reports had been published proposing the incidences of HCC without any identifications of viral infection or heavy alcohol intake. In some way, the development of cancer is more likely occur from disease associated with NASH such as diabetes and obesity, in which their presence with HCC risk is strongly associated (El-Serag, Tran, and Everhart 2004;Calle *et al.* 2003).

A familial genetic disorder primary haemochromatosis (iron overload), mainly caused by C282Y mutation of gene HFE, allows excess iron to be absorbed from the diet. The deposition of iron might cause liver fibrosis leading to cirrhosis; however its progression to HCC remains unclear. A study from East Anglia showed that only a very small proportion of homozygotes for the C282Y mutation developed hepatocellular carcinoma. However, individuals with this genotype have a significantly increased risk of this rare disease relative to those who do not carry the mutations (Willis *et al.* 2005).

Cholangiocarcinoma

Cholangiocarcinoma (CC), a hepatic tumor originating from bile duct cells, is the second-most common PLC. It is estimated to be accounted as much as 15% and its incidences varies by region (3 – 20%) (Shin *et al.* 2010). Anatomically, CC can be classified as intrahepatic CC, hilar CC, and distal extrahepatic bile duct cancers. Most cases of CC occur sporadically and the exact etiology is still unclear. However, the development of CC might be strongly associated with chronic inflammation and biliary duct injury, primary sclerosing cholangitis or infestation with liver flukes (*Opistorchis viverrini, Clonorchis sinensis*) (Mosconi *et al.* 2009). In Thailand, CC is the most common PLC instead of HCC due to high exposure to liver

flukes. In recent years, viral infection might increase the risk of CC in relation with liver cirrhosis (Okuda, Nakanuma, and Miyazaki 2002). Surgery is the only potential curative treatment of CC.

Angiosarcoma

Angiosarcoma is a type of cancer that starts in the blood vessels of the liver, accounting 2% of all PLCs (Mani and Van Thiel 2001). This cancer is very aggressive and grows fast. Risk factor of angiosarcoma is usually unknown, but its development is associated with occupational exposure to carcinogens, hemochromatosis and von Recklinghausen disease (Bhati *et al.* 2008;Forbes *et al.* 1987). In factory workers, vinyl chloride exposure has been established with angiosarcoma of the liver, but not other histologics of the liver (El Serag and Rudolph 2007;Boffetta *et al.* 2003). Treatment may be used to slow the progression the disease but life expectancy and prognosis are usually low.

Hepatoblastoma

Hepatoblastoma (HB) is the most common liver cancer in children and infant, approximately more than 1% of all childhood malignancies. This tumour is rare and usually not detected on early stages until they formed a large abdominal mass and spread to other tissues (Finegold *et al.* 2008). Immunnohistologically, HB cells shows various epithelial or mesenchymal lineages of differentiation (Abenoza *et al.* 1987). The epithelial components of hepatoblastomas exhibit features of embryonal and fetal liver differentiation (Ishak and Glunz 1967). Like adult liver cancers, complete liver resection is necessity for the cure and the goal of therapy.

1.1.2. Treatments

The HCC staging system had been described according to Barcelona-Clinic-Liver-Cancer (BCLC) (Llovet, Bru, and Bruix 1999), Cancer of the liver Italian Program (CLIP) (1998), TNM (tumor, node, and metastasis) (Lei *et al.* 2006), Okuda (Okuda *et al.* 1985), and Japanese Integrated Staging (JIS) (Kudo, Chung, and Osaki 2003) score. Recently, the BCLC

system has became the basis for international guideline for HCC treatments (Llovet *et al.* 2003).

Until now, main curative treatments for the PLC are surgical procedures such as liver transplantation or partial liver resection, local ablation and trans-arterial therapies. However, these treatments are curative only for a small number of patients with early stages and monofocal diseases when patients have good life expectancy. Furthermore, potential candidate patient for surgical treatment must be carefully selected based on liver function.

Most of the patients with more advanced stages and severe cirrhosis could only receive systemic chemotherapies or supportive treatments. For HCC patients who cannot receive any surgical interventions, the survival has not significantly increased in the past 30 years (Blum 2005). Many chemotherapeutic agents have been tested but the response rate was still low, ranging between 10% and 15% (Abou-Alfa *et al.* 2008). Unfortunately, most PLC patients have poor survival prospect with median survival is estimated at less than 1 year. Low survival rate is associated with delayed observation that most of cases are detectable in late stages, aggressive disease and therapy failure.

One of the most studied chemotherapeutic agents for HCC treatment for more than 30 years is doxorubicin. A report from phase III trial in un-resectable HCC patients compared the administration of doxorubicin as single-agent therapy and combination regimen therapy PIAF (cisplatin/interferon/doxorubicin [Adriamycin]/5-fluoruacil [5-FU]). Although patients on PIAF showed a higher overall response rate than patients on doxorubicin alone, the difference was not significant (Yeo *et al.* 2005). The exact mechanism of action of doxorubicin is supposed to intercalate with cell's DNA and inhibit biosynthesis (Momparler *et al.* 1976). Doxorubicin restrains the progression of the enzyme topoisomerase II, which relaxes supercoils in DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication.

Many chemotherapy drugs as a single agent or combinations therapy have been introduced to treat HCC. Some of the most studied drugs are described in Table 1.2.

Table 1.2. Several current chemotherapy agents for HCC treatment

Agent	Mechanisms
Doxorubicin	Inhibits the progression of the enzyme topoisomerase II and discontinue replication of the cells
Etoposide	Inhibits the enzyme topoisomerase II
Cisplatin	Crosslinks DNA and interferes cell division
Gemtacitabine	Act as nucleoside analog and target the enzyme enzyme ribonucleotide reductase
Mitoxantrone	Inhibits the enzyme topoisomerase II
Paclitaxel	Hyper-stabilizes the microtubule and induces apoptosis by binding to protein Bcl-2
Irinotecan	Inhibits the enzyme topoisomerase I

1.2. Cancer Stem Cells Theory

1.2.1. Sources of stem cells

The stem cells are the unspecialized cells which give rise to multiple cell types in the body. The stem cells have specific properties of ability to self-renew through cell division for long period, capability to differentiate into multiple lineages, and potential to proliferate extensively. There are three main sources of stem cells that can be obtained from human organism: embryonic stem cells, adult stem cells, and induced-pluripotent stem cells as shown in Figure 1.2. These cells have their own characteristic and potentials.

The human embryonic stem cells (hESCs) are derived from the embryo, typically from inner cell mass in the blastocyst. These cells are considered to be most pluripotent and can become all cell types in the body. The isolation and cultivation of hESCs have opened the

prospect of cell and tissue engineering in human body, mainly in future treatment of untreatable diseases. Due to its pluripotential, the hESCs would be potent tools in regenerative medicine such as Parkinson's disease, spinal cord injury, myocardial infarction, and many more (Mountford 2008). During the embryonic development, pluripotent hESCs give rise to somatic stem cells that further differentiate into multipotent tissue-specific stem or progenitor cells.

The adult stem cells or somatic stem cells (hASCs) are small number of undifferentiated cells found in a specific area of tissue or organ. They are activated by disease or severe tissue injury and function to repair that specific tissue from severe damage. The multipotency of the hASCs is less than hESCs, mainly they can differentiate into cells of their host tissues. For example, haematopietic stem cells give rise to all blood cells; bone marrow stromal cells (mesenchymal cells) to osteocytes, chondrocytes, and adipocytes; neural stem cells to neurons, astrocytes, and oligodendrocytes. However, many kinds of stem cells from adult tissues seem to have the ability to differentiate into different cells. Reported human multipotent adult stem cells (hMASCs) from liver, heart, and bone marrow had shown to exhibit a wide range of differentiation potential, both in morphological and functional level (Beltrami *et al.* 2007).

The most recent induced pluripotent stem cells (iPS) from adult cell were first reported by Yamanaka & Takahashi in 2006. By using four defined transcription factors Oct3/4, Sox2, c-Myc, and Klf4, mouse fibroblasts were reprogrammed into embryonic stage. These iPS cells exhibited ES morphology and growth properties and ES cell marker genes. Furthermore subcutaneous transplantation of iPS cells into nude mice resulted in tumors containing a variety of tissues from all three germ layers (Takahashi and Yamanaka 2006). Consequently, many studies have been carried out based on this technique of reprogramming human cells from fetal, neonatal and adult human primary cells, including dermal fibroblasts, to pluripotency states (Park *et al.* 2008). Because of its embryonic stem cells-like pluripotency, the iPS might be valuable tools for the research of the mechanisms of tissue formation, cells therapy, and patient-specific cells development in the futures.

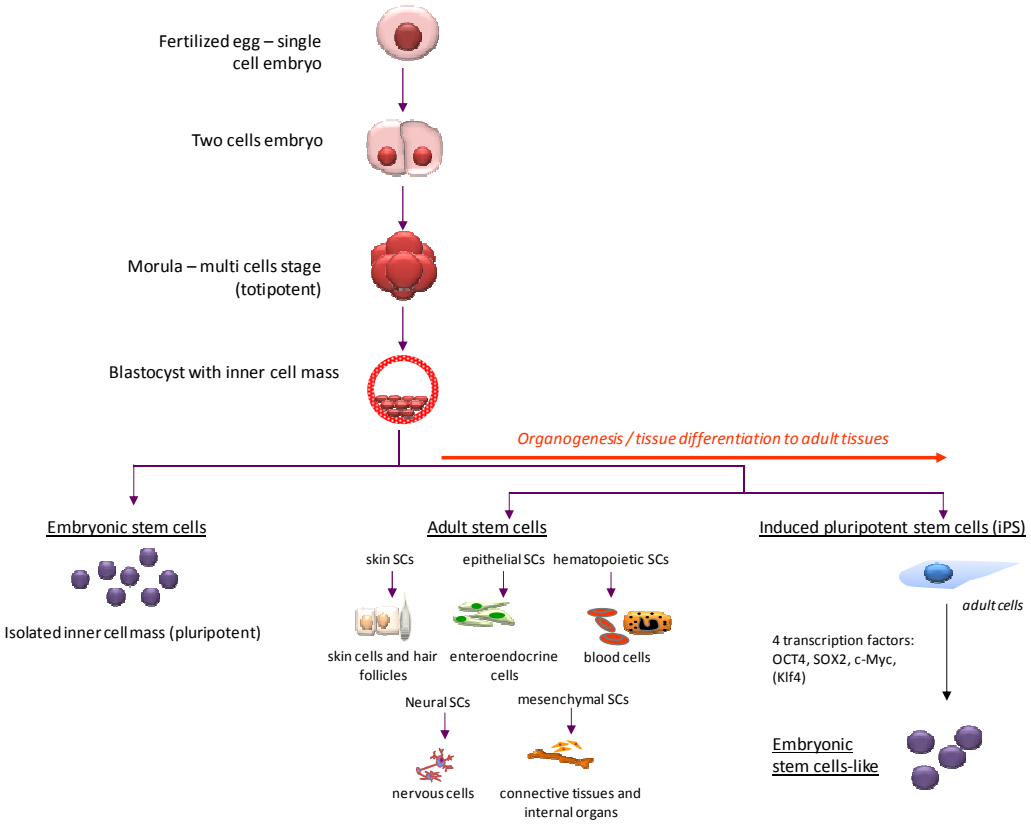

Figure 1.2. Sources of embryonic, adult stem cells, and induced pluripotent stem cells. Human stem cells can be classified based on their sources as hESCs from the embryo, hASCs from adult tissues, and iPS from re-programmed mature cells to embryonic-like cells.

1.2.2. Hepatic stem cells

Liver is a largest internal organ in human body which has high regenerative potential. In minor damage, hepatocytes can divide and repair the damage. In adult liver, mature hepatocytes seldom proliferate and have a life span of over a year. However, after partial hepatoctomy, proliferation of quiescent hepatocytes and cholangiocytes, followed by proliferation of hepatic stellate cells and endothelial cells, quickly restores the liver into its original mass (Mishra *et al.* 2009). In severe major damage, hepatic stem cells will be activated to replenish the injury in the liver. Moreover, these cells rarely acquire age-related genetic defects associated with cancer induction that may imply their protective mechanism against genetic damage (Mishra *et al.* 2009). The origin of stem cells in the liver has been a subject of discussion on whether they are real resident hepatic stem cells or derived from bone marrow stem cells migrated to the liver.

The common normal hepatic stem/progenitor cells have been proposed to be localized at the junction of the bile ducts and hepatic cords, known as canal of Hering (CoH) (Theise *et al.* 1999;Sell 1993;Sell and Leffert 2008). The Canal of Hering (CoH) represents anatomic and physiological link between intralobular canalicular system of hepatocytes and the biliary tree (Figure 1.3). The reside cells in CoH showed shared morphology and immunophenotye between hepatocytes and cholangiocytes. They are positive for cytokeratin CK7 and CK19, oval cell markers OV6 and OV1, neuroendocrine markers chromogranin-A, neural cell adhesion molecule, and many others.

The hepatic/progenitor stem cells give rise to hepatoblast, dominant cell type in fetal and neonatal liver, and along organ maturation will differentiate into hepatocytes and cholangiocytes (Schmelzer, Wauthier, and Reid 2006). When mature hepatocytes or cholangiocytes are damaged or inhibited in their replication, this reserve compartment of hepatic stem/progenitor cells is activated, referred as 'ductular reaction' in human and 'oval cell' reaction in rodents. Alpha fetoprotein (AFP) is one of the earliest markers detected in the liver bud developed from ventral foregut. Recent studies also showed that EpCAM, an epithelial adhesion molecule, is a biomarker for hepatic stem cells and hepatoblasts.

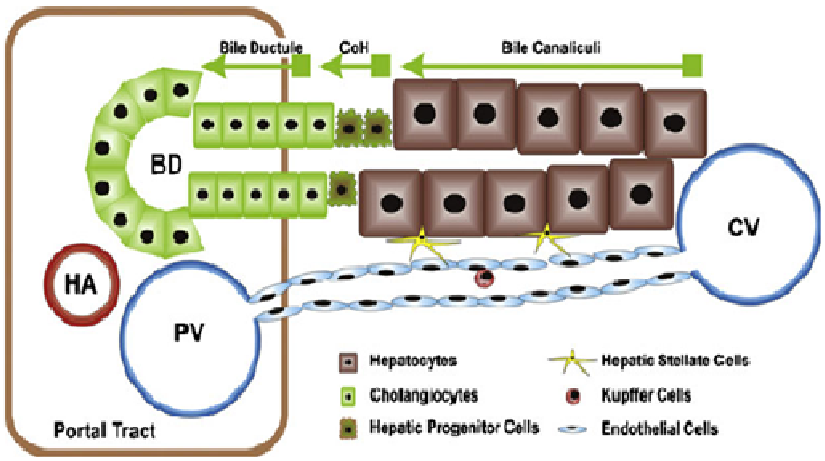

Figure 1.3. The Canal of Hering as resident hepatic stem cells niche in human liver. The Canal of Hering (CoH) which represents connection between bile canaculi and bile duct is the location and niche for stem/progenitor cells. Scheme of CoH is taken from Peng *et al.* Stem Cell (2010).

However, a subpopulation of ductular cells also expresses markers of haematopoietic cells such as CD34 and CD90 which might be directly originated from bone marrow (Roskams 2006). Bone marrow-derived cells could be source for oval cells (Petersen *et al.* 1999) and may contribute in liver regeneration through positive selective pressure through trans-differentiation capacity or fusion from bone-marrow derived cells with hepatocytes (Lee *et al.* 2009).

Furthermore, the identification and distribution of MSCs in many adult human tissues been demonstrated. Interestingly, these MSCs reside in a diverse host of tissues and posses the ability to generate tissue-specific cell types. The characteristic of MSCs, self-renewal, differentiation capacity and cell niche is reviewed in (Kolf, Cho, and Tuan 2007). In human adult liver, isolated MSCs have capacity to differentiate into mature hepatocytes, even to osteogenic and endothelial lineages. Furthermore they contributed to the regeneration of liver parenchyma in immunodeficient mice (Herrera *et al.* 2006).

1.2.3. CSCs theory in liver cancer

Carcinogenesis (development and progression of cancer) is consisted of many steps and long term courses from normal to malignant tissues. There are two main theories of initiation of cancer. The stochastic model proposes that cancer occurs due to a random process and all the cells in the body of tumor have capacity to initiate cancer.

A more recent theory proposed that cancer is composed in a hierarchy and only a small population of the cells in the cancer has capacity to initiate and maintain tumor growth. Just as normal stem cells in normal tissue, CSCs perform as stem cells in cancerous tissue. These cells act as the main players in the highest level of the cancer hierarchy and may still have stem cells properties such as self-renewal and ability to multiple cell types. According to this CSCs theory, cancer mass is assembled in heterogeneous populations of cells: malignant CSCs as central populations with the capacity to divide and differentiate and partial or full differentiated cancer cells derived from CSCs that comprise the majority of cancer mass. This hierarchy model proposes that only CSCs population is gifted with special and unique protective mechanisms to be responsible for the maintenance and propagation of the tumor (Ma, Chan, and Guan 2008).

Many cancers are found to be very heterogeneous and contain many cell types. The first conclusive evidence of cancer stem cells was demonstrated by the group of John Dick in mid 1990's in acute myeloid leukemia (AML) cells in which a $CD34^{+}/CD138^{-}$ subpopulation is capable in initiating tumors in NOD/SCID mice (Lapidot *et al.* 1994;Bonnet and Dick 1997). After this breakthrough, many reports had demonstrated the proofs of tumor initiating cells both in hematopoietic cancer and solid tumors. In solid tumors, the cancer stem cells have been found in breast tumor, brain tumor, colon cancer, pancreatic tumor, ovarian cancer, melanoma, lung cancer and many others. The CSCs had been identified in almost all human cancers.

In liver cancer, CSCs with various markers had been purified from established cancer cell lines and primary tumor. However until now, there is no definite agreement of specific CSCs markers for liver cancer due to wide risk factors, cancer types, and prognostic groups. Several studies had reported the association of these cells with clonal activity, aggressive growth,

tumor induction capacity and chemoresistance. However, one of the important stem cells characteristics, the multilineages differentiation potential, is still unclear.

The first evidence of CSCs existence in liver cancer was reported in 2005 by Haraguchi and colleagues when they isolated side population (SP) cells of HCC cell lines Huh7 and Hep3B. Huh7 SP cells were found to be more resistant to anticancer drugs such as doxorubicin, 5-flouracil, and gemcitabine, compared to non-SP cells (Haraguchi *et al.* 2006b;Haraguchi *et al.* 2006a). Another evidence of SP in HCC was also provided by Chiba *et al.* in which transplantation of as few as 1000 cells successfully induced tumor in NOD/SCID mouse (Chiba *et al.* 2006).

The search of CSCs in liver had progressed and developed. In late 2006, CD133 was proposed to be a CSCs surface marker to isolate CSCs population from HCC cell line. Further evidences of CD133 as hepatic CSCs marker had been also exhibited in several reports (Ma *et al.* 2007;Ma *et al.* 2008a;Ma *et al.* 2008b;Yin *et al.* 2007;Zhu *et al.* 2010). In 2008, Yang *et al.* give a wider outlook and exhibit that CD90 cells isolated from hepatic cell line, primary cancer, and peripheral blood have also distinct characteristic as CSCs (Yang *et al.* 2008b;Yang *et al.* 2008a).

Because CSCs are important in the initiation and maintenance of the cancer, their resistance to anticancer drugs is an obstacle for the total eradication of cancer. Conventional chemotherapies may recognize and kill most of bulk (differentiated) tumor cells but spare the CSCs. Therefore to achieve a complete response in liver cancer therapy it is crucial to target the CSCs first to eradicate the source of the cancer, and then the more differentiated tumor cells (Figure 1.4). Total eradication of the tumor will not only reduced the differentiated tumor size, but more importantly to prevent the reoccurrence of cancer (Dean, Fojo, and Bates 2005).

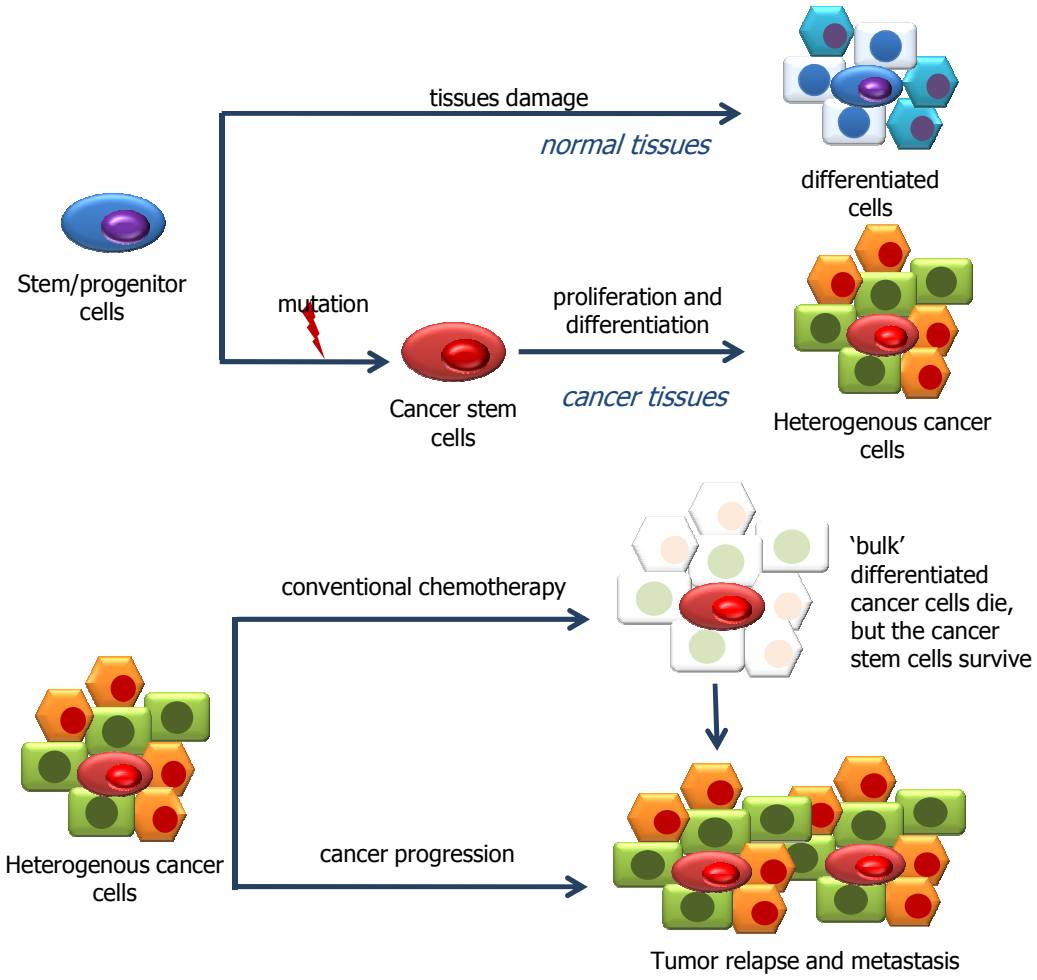

Figure 1. 4. Initiation and progression of the CSCs in cancer. The CSCs might be derived from mutation in normal SCs developing cancerous SCs with SCs potentials. Consequently, CSCs induce tumors, maintain it, and create metastasis. The CSCs were also thought to be resistant to conventional chemotherapies.

1.2.4. Hepatic CSCs marker

Stem cells marker is a specialized signaling molecule or protein receptor that mainly coating the surface of cell which allow the identification and differentiation one cell to other cells. As had mentioned, the definite characteristic of the CSCs in liver cancer is still debatable and unclear. Because of the wide variability of HCC risk factors and long term development, the characteristic of HCC between individuals are sometimes unique and variable. Besides that, the heterogeneity is not only between HCCs but also within given tumor, in which the more-differentiated cells usually occur via the emergence of less-differentiated clonal areas within the tumor (Saeki *et al.* 2000).

Table 1.3. Several markers for hepatic CSCs identification

Models	Markers	Function	Frequency	Origin
Cell lines	$CD90^+$	Cell-cell interaction, adhesion	0.1 – 1.9 %	MSC/HSC/HPSC
	$CD133^+$	unknown	0.1 – 90.0 %	Immature HSC
	$CD133^+$ $ALDH^+$	ALDH is responsible for oxidation of intracellular aldehydes	0.9 – 55.7 %	HSC
	$EpCAM^+$	Adhesion molecule	58.1 – 99.2%	Epithelial/early progenitor
	$OV6^+$ antibody	Recognize cytokeratin	0.2 – 3.0 %	Biliary cells/ SEC
	Side population	Cells defense mechanism against chemotherapies	0.3 – 0.8%	Diverse
Primary cancer	$CD90^+$ $CD45^-$	Cell-cell interaction, adhesion	0.7 – 4.2%	MSC
	$CD90^+$ $CD133^+$	Cell-cell interaction, adhesion	1 sample	MSC/HSC

Several studies had proposed different markers to identify CSCs populations such as surface markers CD90 (Thy-1), CD133 (Prom-1) and EpCAM, oval cells antibody OV-6, and side populations (SP) phenotype. The list of hepatic CSCs is summarized in Table 1.3.

CD90 (THY-1)

CD90 is a 25-37 kDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein expressed in many cell types such as T cells, thymocytes, neurons, endothelial cells, and fibroblast. It has function as an important regulator of cell-cell and cell-matrix interaction, apoptosis, adhesion, migration, cancer, and fibrosis (Rege and Hagood 2006). CD90 is also expressed in bone-marrow derived stem cells (Dennis *et al.* 2007), hepatic stem/progenitor cells both in adult or fetal livers, but not in adult hepatocytes (Herrera *et al.* 2006;Dan *et al.* 2006;Lazaro *et al.* 2003). From fetal liver, $CD90^{+}CD34^{+}CD117^{+}$ cells can be differentiated into adult liver cells (hepatocytes and cholangiocytes) and mesenchymal lineage adipogenic and osteogenic, and endothelial differentiation (Dan *et al.* 2006). From normal adult liver, cells with phenotype $CD90^{+}CD44^{+}CD29^{+}CD73^{+}$ had also osteogenic and endothelial potential differentiation, and even could be induced into pancreatic islet-like structures (Herrera *et al.* 2006).

In systemic cancer T-acute lymphoblastic leukemia (T-ALL), a small subpopulation of CD90+/CD110+ which expressed in haematopoietic stem cells were shown to correlate with stem cell properties both *in vitro* and in transplantation experiments. These markers are useful positive-selection markers for the isolation of CSCs in some cases of T-ALL (Yamazaki *et al.* 2009).

In solid tumor like HCC, cells expressed CD90+ had been purified from hepatic cancerous cell lines and primary cancers. The CD90+ cells but not CD90- cells from HCC cell lines HepG2, Hep3B, PLC, HuH-7, MHCC97L, and MHCC97H displayed tumorigenic capacity when they were injected into immunodeficient mice (Yang *et al.* 2008a). From primary tumors, CD90+CD45- cells from the tumor tissues and blood samples of liver cancer patients also have capacity to generate tumor nodules in mice. Furthermore, CD90+CD45- cells were detectable in 90% of blood samples from cancer patients but none in normal

subjects or patients with cirrhosis indicates circulating CSCs in human liver cancer (Yang *et al.* 2008b).

CD133 (Prominin-1)

CD133 (prominin-1) is a member of pentaspan transmembrane glycoprotein family. Human surface antigen AC133, a homologue for mouse prominin-1, was discovered by generating a monoclonal antibody to CD34+ hematopoietic stem cells isolated from fetal liver, bone marrow, and cord blood (Yin *et al.* 1997). Human prominin-1 consisted of 865 amino acids (aa) with a total molecule weight of 120 kDa (115 kDA in mouse). Prominin-1 has a unique structure composed of an N-terminal extracellular domain, five transmembrane domains with two large extracellular loops, and a 59 aa cytoplasmic tail. Until now, the main function of prominin-1 remains unclear (Shmelkov *et al.* 2005).

CD133 is one of the most common CSCs markers in solid tumors. It is often expressed in adult stem cells and has function in suppressing cells differentiation. The evidence of CD133 as CSCs marker had been demonstrated in solid tumor such as brain cancer (Singh *et al.* 2004;Singh *et al.* 2003), prostate cancer (Miki *et al.* 2007), colon cancer (O'Brien *et al.* 2007), and pancreatic cancer (Hermann *et al.* 2007).

In liver cancer, CD133 is proposed to be a marker mainly in hepatic cell lines. Cells CD133+ purified from cell lines SMMC-7721, Huh-7 and PLC8024 were able to induce tumors in xenograft models in contrast with their CD133- counterpart (Suetsugu *et al.* 2006;Ma *et al.* 2007;Yin *et al.* 2007). Following cells-directed differentiation, CD133+ cells showed a dramatic increase of angiomyogenic markers, suggesting potential to skeletal and cardiac features differentiation (Ma *et al.* 2007). In combination with aldehyde-dehydrogenase (ALDH), CD133+ ALDH+ cells were significantly more tumorigenic than their CD133+ALDH- or CD133-ALDH- (Ma *et al.* 2008a). In connection with chemoresistance, CD133+ cells activate the preferential Akt/PKB and Bcl-2 as survival response (Ma *et al.* 2008b). A recent report exhibited that combination CD133+CD44+ cells have extensive proliferation, self-renewal, and differentiation into the bulk of cancer cells. *In vivo* xenograft experiments

revealed that the highly tumorigenic capacity was primarily attributed to CD133+CD44+ cells instead of their CD133+CD44- (Zhu *et al.* 2010).

EpCAM

The name EpCAM derives from the original functional description as a glycoprotein epithelial cell adhesion molecule. Structurally, EpCAM is closely related to one transmembrane glycoprotein GA733-1, both are novel proteins in structure and likely function (Litvinov *et al.* 1994). The gene EpCAM contains nine exons with extracellular, transmembrane, and cytoplasmic components (Linnenbach *et al.* 1993). EpCAM has been shown to be expressed on the basolateral cell surface of selected normal epithelia and many carcinomas (Armstrong and Eck 2003).

EpCAM is a recent marker used to identify the CSCs in liver cancer. EpCAM gene encodes a carcinoma-associated antigen and is expressed on most normal epithelial cells and gastrointestinal carcinomas and functions as a homotypic calcium-independent cell adhesion molecule. The antigen is being used as a target for immunotherapy treatment of human carcinomas.

Yamashita *et al.* reported that EpCAM expression in HCC displayed a distinct molecular feature with features of stem markers, whereas HCC without EpCAM expression displayed genes with features of mature hepatocytes (Yamashita *et al.* 2008). The isolation of $EpCAM^+$ cells had also been performed from both HCC cell line and primary cancers. These isolated cells had displayed hepatic CSCs-like capacity including self-renewal capacity, differentiation, and tumor induction in NOD/SCID mice (Yamashita *et al.* 2009;Kimura *et al.* 2010). Furthermore, EpCAM has been shown to be a direct transcriptional target in the Wnt/β-catenin pathway that has been suggested to have an important in the self-renewal of cancer cells (Yamashita *et al.*2007).

OV-6 antibody

The OV-6 monoclonal antibody had been developed after treatment with hepatotoxins or hepatocarcinogens in rat and is a useful marker to oval cells and hepatoma cells (Dunsford

and Sell 1989;Dunsford *et al.* 1989). The positivity of OV-6 had been observed in fetal liver and adult biliary disease, adult liver disease, and pediatric liver disease. The antigen target of OV-6, the oval cells, shared epitope with CK14 and CK19 (Libbrecht *et al.* 2001). Several studies have shown a progenitor cell with OV-6 reactivity in a substantial number of HCCs. These cells are thought to be derived from hepatic stem cells in the intraportal area and they have ability to differentiate into hepatocytes and biliary cells.

ABCG2

Simultaneously with the progression of molecular techniques, the ABCG2 expression had been closely related with cells differentiation and stem/cancer stem cells. This marker is related with a distinct phenotype of stem cells, commonly known as side population, associated with its function as protective protein against chemotherapies. More detail of ABCG2 as a marker of CSCs in hepatic system is further described in chapter 1.4.

1.2.5. Plasticity

Until now, many reports demonstrated capacity of SCs from both fetal and adult tissues to differentiate into other cells, even other lineages. In the other hand, trans-differentiation data of CSCs or SCs from cancerous tissues into other lineages are still limited. This restriction capacity of the CSCs might be caused by changes in cell 'stemness' pathway which constrain cells plasticity. However, it is important to study the possible trans-differentiation potency of the CSCs (Table 1.3). This capacity is associated with function of these cells in the cancerous tissues and also might be related with their metastatis capacity.

As reported, cells with phenotype CD90+, CD44+, CD29+, CD73+ from normal adult liver tissues can be differentiated into mesenchymal lineage osteogenic cells, even to endodermal cells such as pancreatic islet. From fetal liver, cells CD90+, CD34+, CD117+ can be induced into adult liver cells, mesenchymal lineages, and endothelial cells.

From HCC cell line, CD133 cells also have potency to up-regulate several marker genes of angiomyogenic cells. A recent report showed that CD133+ cells purified from rat hepatic stellate cells could trans-differentiated into several other cell types (Kordes *et al.* 2007).

Hepatic stellate cells possess signaling pathways important for maintenance of stemness and cell differentiation, such as hedgehog, beta-catenin-dependent Wnt, and Notch signaling, and are resistant to CD95-mediated apoptosis. Moreover, stellate cells display a differentiation potential as investigated *in vitro* and *in vivo*. Collectively all these properties are congruently found in stem/progenitor cells and support the concept that stellate cells are undifferentiated cells, which might play an important role in liver regeneration (Kordes, Sawitza, and Haussinger 2009). Stellate cells are activated and responsible to develop liver fibrosis and progress to cirrhosis. These data indicate that in humans, liver cirrhosis leads to recruitment of various populations of hematopoietic progenitor cells that display markers of intrahepatic progenitor cells (Gehling *et al.* 2010).

Table 1.4. Current data of trans-differentiation study of SCs from fetal and adult liver

	Sources	Phenotype	Differentiation	Reference
Normal SCs	Adult human liver	$CD90^+$, $CD44^+$, $CD29^+$, $CD73^+$	Endothelial, osteogenic, and islet-like structures	(Herrera *et al.* 2006)
	Fetal human liver	$CD90^+$, $CD34^+$, $CD117^+$	Liver cells; adipogenic, osteogenic, endothelial	(Dan *et al.* 2006)
	Fetal human liver	$CD34^+$, $CD117^+$	Mature liver cells	(Nowak *et al.* 2005)
	Adult rat liver	$CD90^+$	Pancreatic cells	(Yang *et al.* 2002)
Cancer SCs	Cell lines and primary cancers	$CD90^+$	no data	(Yang *et al.* 2008b)

Cell lines	$CD133^+$	Angiomyogenic cells	(Ma *et al.* 2007)
HepG2 cell line (+ plasmid)	no data	Pancreatic cells	(Li *et al.* 2005)

1.3. Tumor Microenvironment: CAFs

Stroma is interposed between cancerous cells and normal tissues. Stroma is induced as a result of tumor-host interaction or cross-talk and it is essential to support tumor growth by providing access to nutrients or disposal. The components of the stroma are vital for cancer initiation, growth and progression. Furthermore they might be sources of information for prognostic or targeting response-predictive (Pietras and Ostman 2010). This information leads to a further details of cancer biology that cancer cells itself might be not enough to invade its surrounding and carcinogenesis needs environment support.

Recent studies have demonstrated the importance of cross-talk between cancer cells and its surrounding stromal microenvironment in liver cancer. Tumor environment mainly consists of cellular components such as fibroblasts, stellate cells, immune cells, and endothelial cells. These cells provide non-cellular components: growth factors including transforming growth factor β1 (TGF- β1) and platelet derived growth factor (PDGF), proteolytic enzymes such as matrix metalloproteinases (MMPs) and tissue inhibitor metalloproteinases (TIMPs), extracellular matrix (ECM) proteins and inflammatory cytokines. The non-cellular components modulate signalling pathways, tumor invasion, and metastasis (Yang, Nakamura, and Roberts 2010).

Hepatocarciogenesis involves alteration of cell signalling molecules and might be associated with epithelial to mesenchymal transition (EMT). EMT is a process when epithelial cells changed their characteristics to be closer to mesenchymal cells. EMT is more well-known in embryonic development, but currently is also considered to play an important role in

cancer, specifically in invasion and metastasis. Studies of EMT are mainly performed in breast cancer.

Hepatic stellate cells are major cell type for collagen synthesis in the liver. These cells will be activated in response to damage in the liver and trans-differentiated into myofibroblast-like cells resulting to liver fibrosis and extensive accumulation of ECM. Interestingly, hepatic stellate cells also promote proliferation HCC cells. Conditioned medium collected from stellate cells significantly induced HCC proliferation and migration in vitro and promoted tumor growth in vivo (Amann *et al.* 2009).

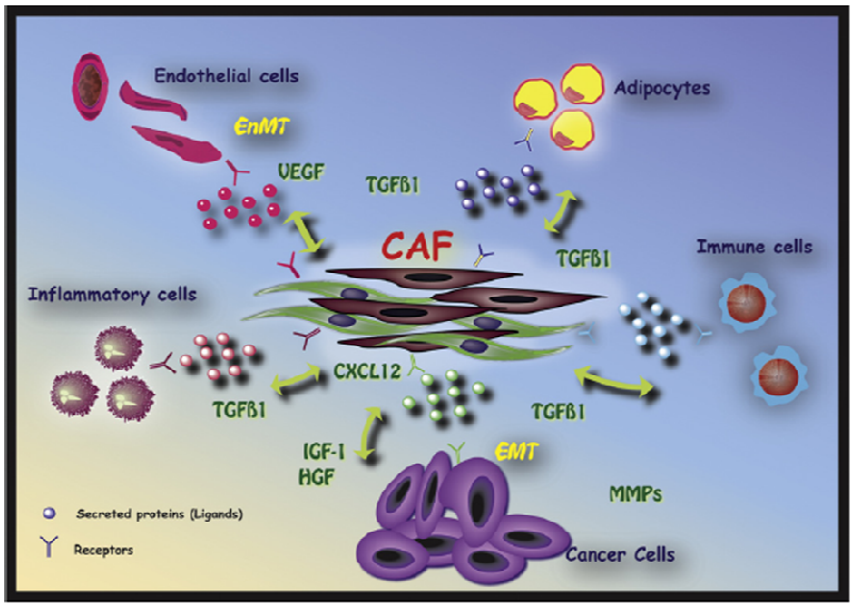

Figure 1.5. The role of cancer-associated fibroblasts in cross-talk interaction between cancer cells and tumor microenvironment. Scheme is taken from (Franco *et al.* 2010)

Cancer-associated fibroblasts (CAFs) or sometimes acknowledged as cancer stromal cells are the most important cell type in the stroma. They are activated by TGF-β and are responsible for the synthesis, deposition, and modelling of excessive ECM (Yang *et al.* 2010). Furthermore, they secrete soluble factors that influence all cells in the stroma including

angiogenesis enhancement and tumor immunity (Rasanen and Vaheri 2010). However, CAFs characteristic remains poor defined even though usually it is defined by the expression of vimentin and α-smooth muscle actin (α-SMA). Moreover, the origin of these cells is still unclear and might be derived from several sources.

First evidence of tumor-stimulating property of the CAFs was demonstrated from human prostate cancer by group of Tlsty and Cunha in 1999. The mixture of fibroblasts from cancer and initiated, nontumorigenic prostate epithelial cells dramatically stimulated growth and altered histology in immunodeficient mice, while this result was not detected of mixture with normal fibroblasts. However, CAFs did not affect growth of normal epithelial cells (Olumi *et al.* 1999). Further study in breast cancer showed similar properties in inducing tumor growth significantly by secreting stromal cell-derived factor 1. Moreover, cultured CAFs expressed traits of activated fibroblasts (myofibroblasts) with increased of α-SMA (Orimo *et al.* 2005).

However, information of tumor-stromal interaction in HCC is still limited. A recent study demonstrated a cross-talk between malignant hepatocytes and myofibroblasts in co-transplantation *in vivo*. They demonstrated that tumor cells invasion diminished after TGF-β and PDGF interference, proposing a very important function of these two factors in tumor growth and EMT (van *et al.* 2009). Hepatic myofibroblasts are proposed to be originated through activation of hepatic stellate cells, portal fibroblasts and bone marrow-derived MSCs during liver injury and liver cancers. However, the nomenclature of myofibroblast is complex. Several reports use the term myofibroblast for both myofibroblast-like and myofibroblast and this may cause misunderstanding in tracing the origin of these cells. CAFs express α-SMA which is a characteristic of hepatic myofibroblasts in chronic liver injury. It is reported in rat liver fibrosis that CD90 is expressed in myofibroblasts or activated hepatic stellate cells during cells proliferation (Dezso *et al.* 2007).

The sources of hepatic myofibroblasts are thought to be originated from activated hepatic stellate cells, portal fibroblasts, or bone marrow-derived stem cells during liver injury. A recent paper using mouse model demonstrated cross-talk between malignant hepatocytes and myofibroblasts in co-transplantation *in vivo*. Furthermore, they showed that tumor cells

invasion diminished after TGF-β and PDGF interference, proposing a very important function of these two factors in tumor growth EMT (van *et al.* 2009).

Adult MSCs are a potential candidate cell type for the study of regenerative medicine. The existence of MSCs as bone-marrow derived cells in normal adult liver and maybe in cancerous liver may provide new understanding in the maintenance and homeostatis of the tumor. Phenotipically and functionally, they fit with requirements of supporting cells CAFs (Franco *et al.* 2010). For example, in pancreatic cancer, MSCs can contribute to one forth of total myofibroblast of total stroma (Ishii *et al.* 2003).

From clinical point of view, interaction between tumor and stroma has been interesting target for HCC treatment. The basis of this system is to attack property of tumor microenvironment that acts as supplier of the tumor needs. Targeting this specific molecule is conducted simultaneously with conventional chemotheraphy that focus directly tumor cells or CSCs.

Several drugs inhibit angiogenesis and disrupt tumor-stromal interaction is one of the promising therapeutic agents for HCC. Sorafenib, a multi-tyrosine kinase inhibitor including VEGFR-2, VEGFR-3 targeting, as well raf kinase, is currently standard of care for patients with advanced stages. It was demonstrated prolong median survival and time to progression by nearly 3 months in patients with advanced HCC in a large phase III trial (Llovet *et al.* 2008). Another inhibitor on phase II clinical trial sunitinib had demonstrated tolerability and efficacy in patients with advanced HCC (Faivre *et al.* 2007). Bevacizumab, a recombinant monoclonal antibody against VEGF is also used as single or combination therapy agent (Siegel *et al.* 2008). Combination of bevacizumab and erlotinib in advanced HCC patients showed significant anti-tumor activity (Thomas *et al.* 2009). Still, further evaluation is needed to avoid the negative side effects of agents. More recently, LY2109761 was shown to inhibit tumor specific neoangiogenesis and furthermore, to interrupt the cross-talk between HCC cells and CAFs, thus inhibiting tumor progression (Mazzocca *et al.* 2010).

1.4. CSCs Markers and Clinical Significances in Liver Disease

Beside the use of stem cells markers as described above to identify and isolate population of CSCs in hepatic cell lines and primary cancers, these markers might have also clinical significances in liver diseases, correlating with bad or good prognosis in patients. However, due to the PLCs wide risk factors and individual uniqueness, they may consist of many distinct molecular characteristics. As predicted, the expressions of CSCs in human liver diseases were found to be highly variable. But collectively, their expressions whether weak- or strong-correlated with liver cancer compared to normal liver may support the CSCs hypothesis.

An elegant study by Lee *et al.* demonstrated new prognostic types of HCC. Using a global integrated gene expression data, individuals with HCC who shared a gene expression pattern with fetal hepatoblast (HB subtype, cluster A) had poor prognosis compared to individuals with hepatocyte pattern (HC subtype, cluster B). The expression of expression of well-known markers of hepatic oval cells, the early progenitors of adult liver stem cells, is found in the HB subtype of HCC. HCC with fetal hepatoblast subtype may arise from hepatic progenitor cells (Lee et al. 2006).

EpCAM had been reported to be expressed mostly in CC and less in HCC (de Boer *et al.* 1999). A high throughput analysis identified that based on this marker, HCC can be divided into EpCAM+ subtype with molecular signature of hepatic progenitor cells (Hepatic Stem-like HCC; HpSC-HCC) and EpCAM- subtype with features of mature hepatocytes (Mature Hepatocyte-like HCC; MH-HC). In addition, by determining the level of AFP, HCC could be sub-classified into four groups with prognostic implications. EpCAM+ AFP+ HCC has poor prognosis compared to EpCAM−AFP− HCC (Yamashita *et al.* 2008).

Nonetheless, even the use of single CSCs marker such as CD90, CD133, and EpCAM had also been attempted in its correlation with clinical prognostic significance. Based on literature

reviews in current biomedical database, the association between CSCs marker and their clinical significance is summarized in Table 1.5.

Table 1.5. The CSCs marker in human liver cancer tissues and their clinical significances.

Markers	Expression in liver cancer tissues	Clinical significance	References
CD90	(+) majority in poor-differentiated HCC	Poor prognosis	(Yu *et al.* 2011;Lingala *et al.* 2010; Lu *et al.* 2011)
CD133	(+/-) found frequently	↓ disease survival	(Yu *et al.* 2011;Song *et al.* 2008)
EpCAM	(-) majority HCC ; (+) CC	↑ poor prognosis	(Yamashita *et al.* 2008;de Boer *et al.* 1999;Kim *et al.* 2004)
ABCG2	(+); ↑ after treatment and tumoral		(Zen *et al.* 2007;Vander *et al.* 2008;Vander *et al.* 2006;Sun *et al.* 2010)
OCT4	↑ tumoral tissues	↓ disease survival	(Yuan *et al.* 2010;Huang *et al.* 2010)
CK7	protein (+/-)		(Durnez *et al.* 2006)
CK19	protein (+/-)	worse prognosis, recurrence	(Durnez *et al.* 2006;Uenishi *et al.* 2003)

In neuroblastoma CD90- patients have a significantly impaired overall survival compared to CD90+ patients (Fiegel *et al.* 2008). In contrast, $CD90^{+}$ expression in acute myeloid leukemia (AML) is related with unfavorable clinical and biological features, indicating the use

of CD90 as an additional marker of AML prognostic value (Buccisano *et al.* 2004). In liver disease, information of CD90 with its clinical significance is not available, however CD90 expression was found preferably in poor differentiated HCC (Yu *et al.* 2011). A recent report from immunohistochemical data showed that CD90 protein was increased in 73% of HCC samples. CD90 expression was not influenced by chronic alcohol exposure or cirrhosis. Over-expression in CD90 was correlated with age, HBV infection, and histological grade but not with alcohol or cirrhosis. Patients with highest level of CD90 expression showed the poorest prognosis (Lu *et al.* 2011).

In glioma patients, multivariate analysis showed that proportion of CD133+ cells were significant to adverse progression-free survival and overall survival. Moreover it was an independent risk factor for tumor regrowth and time to malignant progression (Zeppernick *et al.* 2008). In HCC, immunohistochemical analysis also showed that CD133 cells were frequently present. Increased CD133 levels were correlated with increased tumour grade, advanced diseases stage, and elevated serum alpha-fetoprotein (AFP). Further analysis also showed that patients with high CD133 expression was correlated with shorter overall survival and higher recurrence compared to patients with low CD133 expression (Song *et al.* 2008).

As CD90, in HCC case with and without bile duct tumor thrombi (BDTT), CD133 expression was closely associated with tumor differentiation. In HCC with BDTT, most of these cells were poorly differentiated. This result may indicate that these cells had the characteristics of presumed liver cancer stem cells and support the CSCs hypothesis (Yu *et al.* 2011). In contrast, Lingala *et al.* reported that the expression of individual or a combination of CSCs markers in HCC is not unique and these markers were also expressed in inflamed or nearly normal liver tissues. However, many HCC were positive for ALDH and CD133-positivity was identified mostly in ADLH+ cells and in nearly two third of specimens. The positive rate of CD133 in HCC was similar to viral hepatitis specimens and not all adjacent to HCC tissues were positive for CD133, CD90, CD44, and ALDH (Lingala *et al.* 2010).

OCT4, an embryonic pluripotency transcription factor, is closely-associated with embryonic stage and pre-fetal. It has been found to be expressed in HCC cell lines and HCC specimens (Yuan *et al.* 2010). Furthermore, OCT4 can mediate chemoresistane through a

potential OCT4/AKT-ABCG2 pathway and has clinical significance regarding to expression patterns in HCC tumors (Wang *et al.* 2010). In other cancers, OCT4 over-expression was found in gastric cancer tissues compared to non cancerous tissues, athropic gastritism and gastric ulcer tissues. OCT4 expression was correlated with differentiation status and was proposed as one of markers for human gastric cancer (Chen *et al.* 2009). In rectal cancer, together with CD133 and SOX2, OCT4 may be used to predict recurrence and poor prognosis after preoperative chemoradioresistance (Saigusa *et al.* 2009).

Stem cells identified by immunohistochemical markers using OV-6 and RNA-FISH markers using several antibodies including CK19 were found scattered in the liver parenchyma of cirrhotic livers and within hepatocellular carcinomas (HCCs). Pre-cirrhotic alcoholic or non alcoholic steatohepatitis all stained negative for these stem cells (Oliva *et al.* 2010).

Durnez *et al.* reported that around 30% of HCC expressed CK7 and/or CK19. These cells were potentially derived from malignant degeneration of hepatic progenitor cells. The expression of CK7 was significantly associated with hyperbilirubinemia. HCC expressing CK19 had a higher incidence of AFP expression, elvated serum AFP, and less advanced liver fibrosis. Higher recurrence rate of CK19+ in HCC after transplantation suggested a worse prognosis compared to CK19- and might be used as prognostic marker (Durnez *et al.* 2006).

1.5. ABCG2 Expression: Drug Resistance and CSCs

1.5.1. ABCG2 and drug resistance

The ATP Binding Cassette (ABC) transporter is one of the largest families of membrane transport proteins. These proteins utilize a pair ATP (Adenosine-5'-triphosphate) molecule to export specific compounds or to flip them from inner to outer leafs of the membranes (Higgins 1992). In humans, there are 49 members of ABC transporters gene which are classified into seven subfamilies based on the sequence homology and ATP-binding proteins (Dean, Hamon, and Chimini 2001).

The Breast Cancer Resistance Protein (ABCG2/BCRP/ABCP/MXR) is one member of the ATP-Binding Cassette (ABC) transporters superfamily proteins (Dean *et al.* 2001). ABCG2 protein is composed of 665 aa resulting a 72 kDa protein. It has an N-terminal ATP-binding domain (NBF) and a C-terminal transmembrane domain (TMD), a structure half the size and in reverse configuration to most other ABC proteins comprising two NBFs and two TMDs as shown in Figure 1.6 (Doyle & Ross 2003;Robey *et al.* 2009;Bailey-Dell *et al.* 2001). The ABCG2 gene is highly conserved and has been found in all sequenced vertebrates to date, including birds, reptiles, and fish. In most species there is a single gene present (Annilo *et al.* 2006). The exceptions to this are the rodents which contain one or more copies of a closely related gene, Abcg3; and fish which have 3 or more ABCG2 genes (Mickley *et al.* 2001).

ABCG2 protein is widely expressed in tissues, mainly in placenta, epithelium of small intestine and colon, and breast. Furthermore it is also expressed in the epithelium of the prostate and bladder, endocervical cells of uterus, kidney tubules and others tissues. In liver, ABCG2 is expressed in liver canalicular membrane (Maliepaard *et al.* 2001) and hepatocytes (Fetsch *et al.* 2006). In the same way, ABCG2 is also found in bile ducts, reactive bile ductules, and blood vessel endothelium of human liver (Vander *et al.* 2006).

One of the main functions of ABCG2 is related with the cell resistance to exposure of external compounds, exporting the drug out of the cells thus maintaining the intracellular drug compound below toxic level. Regarding this underlying principle, extensive studies have

been carried out to find out the relationship of ABCG2 with drug resistance, especially in chemotherapy-treated cancers (Figure 1.6).

The spectrum of anticancer drugs transported by ABCG2 includes mitoxantrone, camptothecin-derived and indolocarbazole topoisomerase I inhibitors, methotrexate and flavopiridol (Doyle and Ross 2003). ABCG2 is one of the chemosensitivity determinants of irinotecan hydrochloride (CPT-11), an effective anticancer drug (Takahata *et al.* 2008).

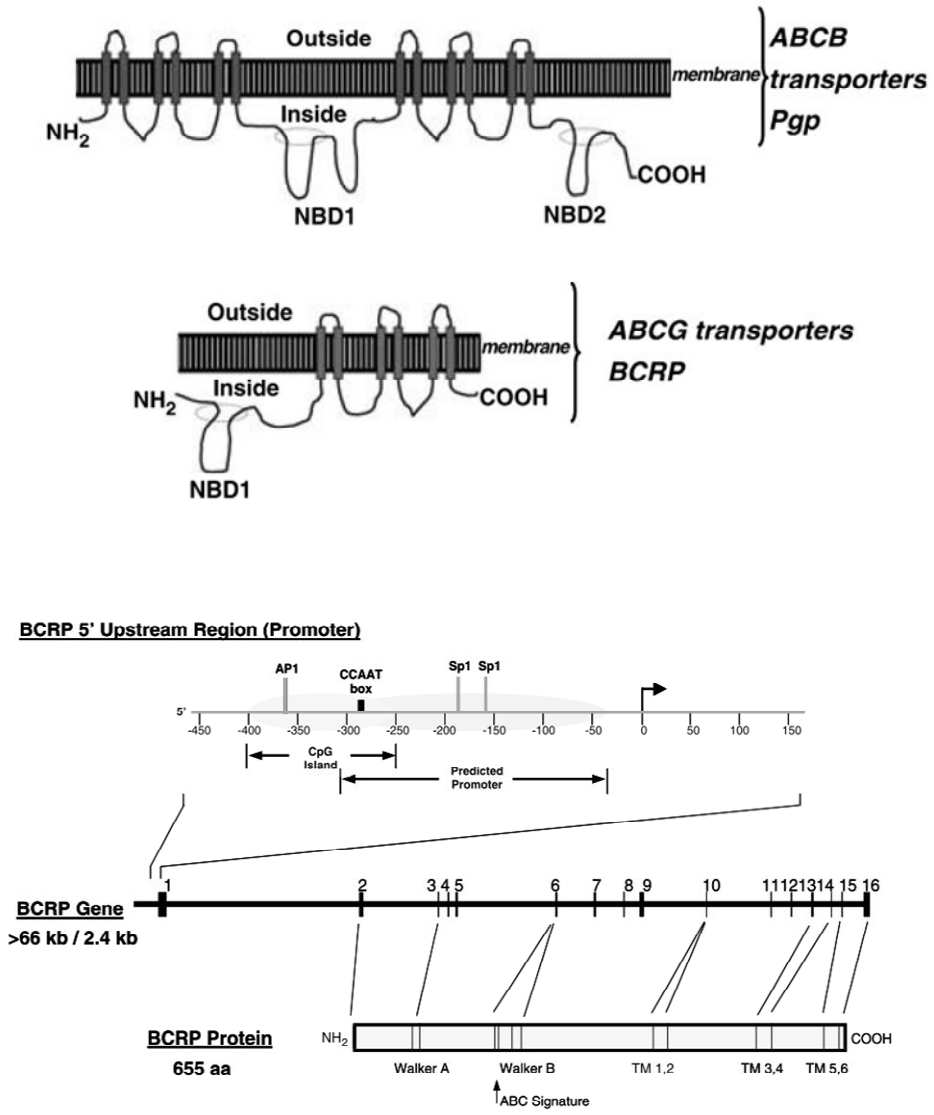

Figure 1.6. Physical structure of the ABCG2 gene and promoter. Picture was taken from Doyle & Ross 2003 (adapted from Bailey-Dell *et al.* 2001).

In leukemia, ABCG2 may associate with drug resistance and survival (Ross *et al.* 2000). In solid cancers, ABCG2 expression is extensively studied in breast cancer as its first case of finding. Its expression is found to be common in digestive system, lung, and melanoma (Diestra *et al.* 2002). However, whether its expression increase or decrease in cancers is still debatable due to many different reports.

In resistant cancer cells, ABCG2 expression is associated to many chemotherapy agents. In liver cancer cells, treatment of mitoxantrone, doxorubicin, epirubicin, and gefitinib resulted in induction of ABCG2 and low sensitivity to the drugs (Cusatis *et al.* 2006;Kamiyama *et al.* 2006;Li *et al.* 2007). The ABCG2 expression was found to be high in hepatocellular carcinoma samples(Sun *et al.* 2010) and was reported to increase following chemotherapy in hepatoblastoma patients (Vander *et al.* 2008).

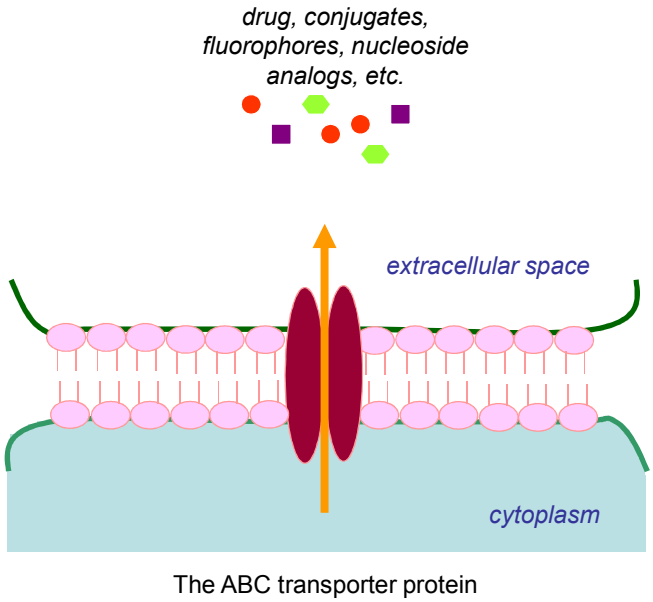

Figure 1.7. Main function of ABC transporter protein in cell defense mechanism. One of the main roles of ABC transporters including the ABCG2 protein is to export chemotherapies drugs from intra- to extracellular compartment and to maintain drug concentration under cytotoxicity level.

1.4.2. ABCG2 and side population phenotype

In 1997, Goodell and colleagues pioneered a technique to purify a small population of cells which is rich with stem cells. These cells, more known as side population (SP) phenotype, had capacity to export the Hoechst 33342 dye out of the cells and recognized as Hoechst$^{null/low}$ in FACS instrument as shown in Figure 1.7. Hoechst 33342 is a fluorescent dye agent binds to double stranded DNA and has been reported to be a substrate of ABCG2. The purified SP cells had been obtained from many solid tumors, including isolation of stem/progenitor cells from cancer originating from prostate, pancreas, stomach and liver (Chiba *et al.* 2006).

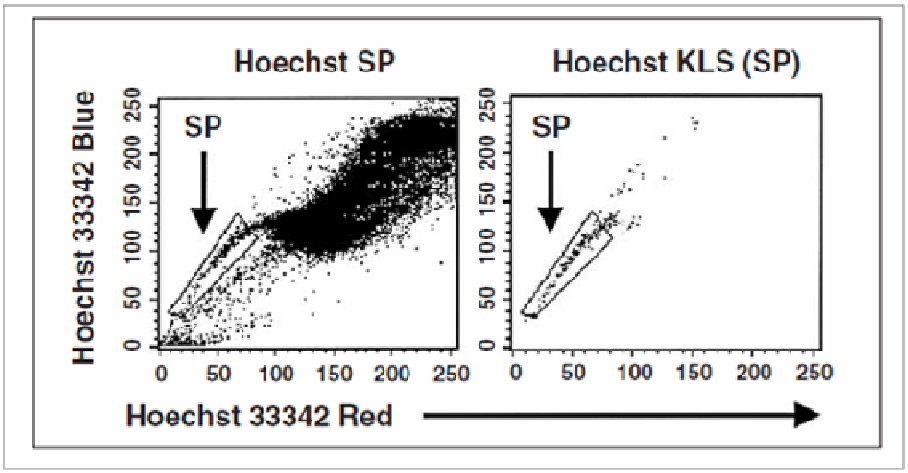

Figure 1.8. Side population phenotype of stem cells with Hoechst 33342$^{null/low}$ identified by FACS instrument. The SP cells population from hematopoietic stem cells has capacity to pump out Hoechst dye out of the cells. This population is rich with stem cells. Sample picture is taken from (Bunting 2002).

An elegant study from Zhou *et al.* demonstrated that bone marrow cells from Mdr1a/1b$^{-/-}$ mice contained a normal number of SP cells, indicating that Mdr1a/1b is not required for SP phenotype. By contrast, a significant reduction of SP cells in bone marrow and skeletal muscle was observed in Bcrp1$^{-/-}$ mice, suggesting Bcrp1 as molecular phenotype of SP (Zhou *et al.* 2001;Zhou *et al.* 2002).

In HCC, the SP population had also been reported. As mentioned previously, the first evidence of CSCs existence was found in isolated SP cells of HCC cell lines. Huh-7 SP cells were found to be more resistant to anticancer drugs such as doxorubicin, 5-flouracil, and gemcitabine, compared to non-SP cells (Haraguchi *et al.* 2006b;Haraguchi *et al.* 2006a). The SP from cell lines PLC/PRF/5 (0.80%) and HuH7 (0.25%) showed high proliferations, anti-apoptotic properties and capabilities to initiate tumor formation in non-obese diabetes/severe combined immunodeficiency (NOD/SCID) mice (Chiba *et al.* 2006). Moreover, SP cells sorted from HCC cell lines HCCLM3, MHCC97-H, MHCC97-L and Hep3B harboured CSCs-like might be related to the metastasic potentials and therapeutic-resistance (Shi *et al.* 2008). However, this tumorigenesis capacity of ABCG2+ cells is in contrary with a report demonstrated that ABCG2^{+} had similar tumorigenicity to ABCG2^{-} cells in glioma, breast, and prostate cancer (Patrawala *et al.* 2005).

Further studies on ABCG2 expression in these cell lines showed that the sorted ABCG2^{+} cells generated both ABCG2^{+} and ABCG2^{-} cells while ABCG2^{-} cells only gave ABCG2^{-} cells, indicating higher hierarchy of ABCG2^{+} compared to ABCG2^{-} cells. Additionally, GATA6, an essential factor of earliest phase of hepatic development was intensely expressed in ABCG2^{+} cells and C/EBP$_{\beta}$, a factor for late phase of liver development, was more expressed in ABCG2^{-} cells (Zen *et al.* 2007). A study using drug treatment showed that ABCG2 expression significantly influenced the levels of drug efflux. The SP cells were importantly involved in the drug efflux-related chemotherapy resistance and the SP analysis was found to be an efficient method to evaluate the functional activity of ABCG2 (Hu *et al.* 2008).

The relationship between cells differentiation and ABCG2 expression has been also reported in previous studies performed in other cells. High level of functional ABCG2 was detected in undifferentiated human embryonic cells and decreased during cellular

differentiation (Apati *et al.* 2008). In hematopoietic system, the ABCG2 expression was restricted to the most immature progenitor cells and down-regulated at the committed progenitor level (Scharenberg, Harkey, and Torok-Storb 2002). These studies have indicated the significance of ABCG2 in human malignancies and its association with drug resistance and cells differentiation.

1.4.3. ABCG2 inhibitors

As mentioned previously, one of the most important appearances of the CSCs is they may be resistant to many standard chemotherapies. Tumor relapse often occur and it may spread and cause metastasis. The combination of chemotherapy drugs and specific inhibitors targeting ABC transporters, including ABCG2, could be a potential strategy to eliminate both tumor cells and the CSCs (Dean *et al.* 2005).

Bench and clinical studies focus on increasing the sensitivity of cancer cells to anticancer drugs is ongoing. Some strategies include the specific drug delivery system, transporters inhibitors and targeted antibodies. The use liposome-encapsulated adriamycin through hepatic arterial administration improve the anticancer efficacies in hepatoma compared to adriamycin solution alone (Sun *et al.* 2006). Recent studies proposed also the use of nucleic acid constructs on ABC transporters gene to be efficient and safe system. Adenoviral delivery of the ABCC2 antisense sequence reduced the inhibitory concentration (IC50) for doxorubicin, vincristine, cisplatin and etoposide in HepG2 cell line. A significant tumor regression was also observed in nude mice after vincristine treatment (Folmer *et al.* 2007). Combination between modulators and antibody against transporters protein has been performed as another approach (Goda *et al.* 2007).

Many inhibitors have been attempted in clinical studies against ABCB1 including competitive inhibitors verapamil and cyclosporine A (the first and second generation), and blocker GF120918 (third generation). ABCB1 modulators cyclosporine analog PSC 833, GG918 and verapamil was reported improve hepatoblastoma response to doxorubicin *in vitro*

(Warmann *et al.* 2002). A clinical trial of biricodar (VX-710), a ABCB1 and ABCC1 inhibitor, is reported sensitize antracycline-resistant sarcoma to doxorubicin (Bramwell *et al.* 2002).

A high throughput cell-based screen for ABCG2 has been established and is being used to identify new inhibitors (Henrich *et al.* 2006). Fumitremorgin C (FTC) and Kol43 are potent inhibitors of ABCG2. FTC at 5 μM concentration almost completely reversed resistance mediated by ABCG2 to mitoxantrone, doxorubicin and topotecan (Doyle and Ross 2003). Kol43, the tetracyclic analog of FTC, is a specific ABCG2 inhibitor without the neurotoxicity that is a principal side effect of FTC (Allen *et al.* 2002). Novobiocin, a coumermycin antibiotic, is reported to effectively overcome ABCG2-mediated drug resistance (Yoshikawa *et al.* 2004). GF120918, a ABCB1 inhibitor, is reported to be less specific than FTC but without neurotoxicity impact, increases drug accumulation and cytotoxicity in cells expressing ABCG2 (de *et al.* 1999). A natural substrate naringenin, a flavonoid in grapefruit juice, showed also an anti-ABCG2 effect. The addition of naringenin increased the chemosensitivity and intracellular accumulation of drugs CPT-11 and SN-38 in HCC cells expressing abundant ABCG2 (Takahata *et al.* 2008).

Nevertheless, serious toxicity of those inhibitors to the cells needed a careful inspection. Some considerations should also be taken on drug sensitization, biochemical characterization, and transport studies (Henrich *et al.* 2006). Combination regiments therapy containing low concentration of anticancer drugs and inhibitors with a better targeting system will be useful for the successful of the therapy. A report of combined application of UIC2 monoclonal antibody and certain ABCB1 modulators including cyclosporine resulted in a completely restored steady-state accumulation of various Pgp substrates and inhibited the pump activity almost completely. The low concentration of modulators to approximately 20 times significantly decreased cytotoxicity (Goda *et al.* 2007).

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Chapter II

Objectives of the Study

The cancer stem cells (CSCs) theory as cancer initiation models had been proposed by many studies. Evidences of tumor initiating cells had been demonstrated both in circulating and solid cancers. These cells have the specific capacity just as normal stem cells to proliferate and differentiate into other cells, but more importantly, a unique ability to promote and invade tumor in animal model.

Primary liver cancer, one of the most fatal cancers in the world, is caused by various known risk factors. Besides extrinsic factors, intrinsic individual variations may present wide disease characteristic, subtypes, and prognostic significances. Morphologically, liver is consisted of many types of cells and during its development, liver organogenesis takes place by joint coordination between endoderm and mesoderm layers. Furthermore, hepatocarcinogenesis is also a process of interaction between cancer cells and cancer microenvironment, a state that provides supportive niche for cancer growth and invasion.

Regarding treatment strategy of liver cancer, low response of cancer cells to the conventional treatment has been an important issue in cancer biology and biomedicine. Whereas surgical treatment is considered as the best choice for patients with early disease, chemotherapy is given to eliminate cancer cells and avoid metastasis in unresectable patients with higher stages of disease. However, survival of this group has not significantly increased in several decades. Chemoresistance plays role to therapy failure and relates with the presence of CSCs.

The main objective of this thesis is study the cells heterogeneity in hepatic system to have a comprehensive outlook of hepatic CSCs theory. It is pathologically and clinically important to gain more information in the heterogeneity of liver cancer cells, the distribution of stem cells in the liver, and whether there is an association between stem cells or CSCs and drug resistance in liver cancer.

Main objective is specified into three inter-related projects with several experimental models, both *in vivo* and *in vitro*. The first task is to isolate and characterize a population with stem cells characteristic from tumoral and distal parts of human primary liver cancers hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). Second task is to assess the expression of several stem cells markers in transcriptional level in clinical samples tissues. And the third task is to study the expression of a drug transporter ABCG2 in relation with cells differentiation and drug resistance. Further explanation of these tasks are described in the chapter Results and General Discussion.

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Chapter III

Materials and Methods

3.1. Materials

3.1.1. In vitro models: hepatic cell lines

Human liver cell lines IHH, HepG2, HuH-7, and JHH6 were used as *in vitro* models. The immortalized non tumoral liver cells IHH was a kindly provided from Dr. Didier Trono (Mai *et al.* 2005). Human HCC cell lines HuH-7 (JCRB0403) and JHH-6 (JCRB1030) were obtained from the Japan Health Science Research Resources Bank (HSRRB, Tokyo, Japan). The HepG2 cell line was obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER, Brescia, Italy). The morphology of the cell lines used in this study was shown in Figure 3.1.

The IHH cells were grown in DMEM-F12 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% antibiotics, 1% L-glutamine, 1 uM dexamethasone and 5 ug/mL insulin. The HepG2 and HuH-7 cells were grown in DMEM medium (high glucose) supplemented with 10% (v/v) heat-inactivated FBS, 1% L-glutamine and 1% antibiotics. The JHH6 cells were grown in Williams' E medium supplemented with 10% (v/v) heat-inactivated FBS, 1% L-glutamine and 1% antibiotics. The cultures were maintained at 37°C in a humidified 5% CO_2 incubator and were routinely passaged with 0.05% Trypsin in PBS when they reached 85% - 95% confluence.

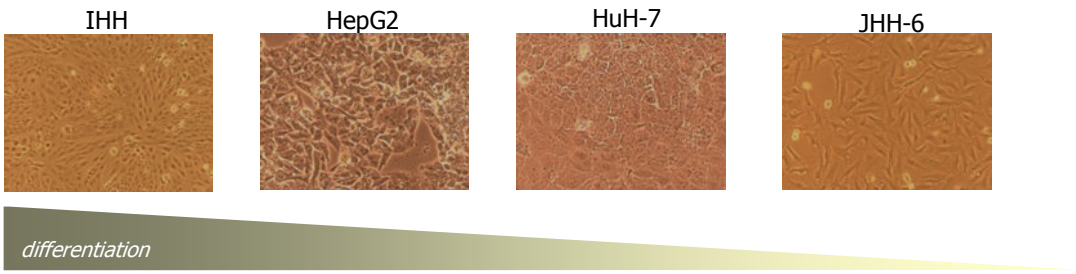

Figure 3.1. Morphology of hepatic cell lines for study *in vitro*. Cell lines IHH, HepG2, HuH-7, and JHH-6 with different degree of morphologic differentiations were used as models. Magnification 100x.

3.1.2. Human primary liver cells

Fresh liver tissues as the sources of primary cells cultures from 11 patients undergoing liver surgery were obtained from Surgical Department, Hospital of Cattinara, Trieste in period June 2008 – September 2010. Informed consent was obtained from each patient before surgery. Six patients were diagnosed with HCC, three with CC, one with cystic hepatic, and one with steatosis and not confirmed as HCC. Female and male ratio is 6:5 with age mean 70 ± 9 years old (range 54 – 81 years old) and three samples were HCV positive. From those tissues, three parts were selected: the neoplastic (tumoral), peripheral (peritumoral), and distal ('normal') region.

3.1.3. Clinical samples: human liver tissues

A total of 59 human liver tissues were obtained from patients undergoing liver resections or liver transplantations. Several liver pathologies included hepatocellular carcinoma (HCC), cholangiocarcinoma (CC), biliary atresia (BA), hepatoblastoma (HB) and normal donor tissues as control. Most of those included the tumoral, peripheral, and non-tumoral regions. All tissues were snap-frozen in liquid nitrogen and stored in -80°C before processing.

3.1.4. Xenotransplantation animals

Athymic nude Foxn1(nu/nu) homozygotes mice for *in vivo* xenotransplantation studies were bought from Harland Laboratories, SRL (Udine, Italy). The mice were 7 weeks age, male and maintained in animal house facility University of Trieste. Xenotransplantation study was approved by Ministero della Salute, Direzione Generale della Sanità Animale e del Farmaco Veterinario, no. 107/2010 – B (07 June 2010).

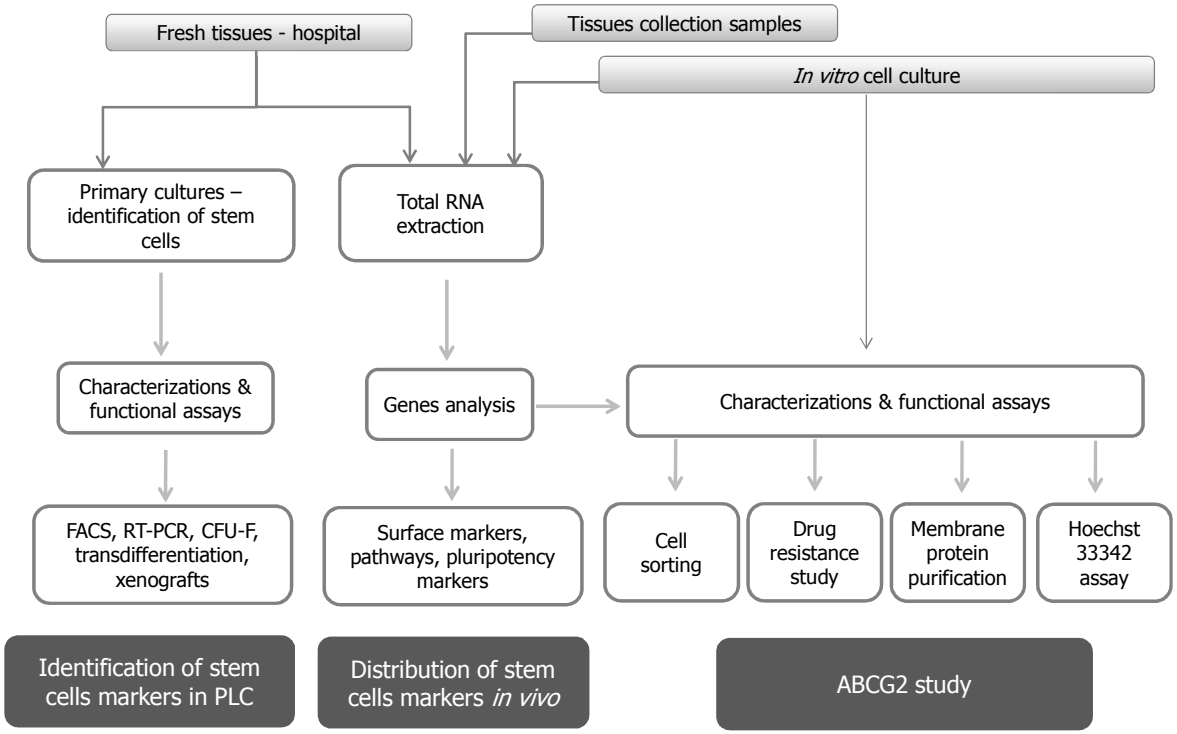

Figure 3.2. Work flow of general methods. Collaborative approaches using both *in vivo* and *in vitro* sample models and cellular and molecular biology techniques were performed for three objectives of the study: identification of stem cells in the PLCs, expression of stem cells markers in tissues samples, and ABCG2 study.

3.2. Methods

3.2.1. General techniques

Fluorescence activated cells sorting (FACS)

The presence of surface marker antigens in the cell lines and primary cultures were detected by FACS. The cells were detached from the wells by a short treatment with trypsin–EDTA and placed in centrifuge tubes. After centrifugation at 1000 rpm for 5 minute at room temperature, the supernatant was discarded and the pellet was washed two times with sterile phosphate buffer saline (PBS) with 0.5% bovine serum albumin (BSA) (w/v) and filtered with 40 μM cell strainer (BD BioScience). At least 2 x 10^6 cells/mL cells were incubated with specific first antibodies for 30-60 minutes on ice in the dark. After two PBS-0.5% BSA washes, if necessary, the cells were incubated with secondary antibody for 30-60 minutes on ice in the dark. Flow cytometric analysis was performed immediately on a FACSCalibur flow cytometer (Becton Dickinson, NJ, USA). A total of 10,000 events were analyzed per sample. Nonspecific staining for secondary antibody was assessed.

Cells separation of JHH-6 by magnetic sorting

CD90+ cells positive selection of HCC cell line JHH6 was performed by indirect method using magnetic cell sorting (Miltenyi Biotec GmbH, Germany). A total of 20 millions cells were detached from cell culture flasks by treatment with trypsin–EDTA and placed in centrifuge tubes. After filtration with 40 μm cell strainer, the cells were suspended in 200 μL of prepared buffer (PBS pH 7.2, 0.5% BSA, 2 mM EDTA) with 20 μL CD90-FITC (10 μL first antibody in 10 millions of cells) and were incubated for 10 minutes on ice in the dark. After two washes 1000 rpm, 10 minutes, 4°C, the cells were re-suspended in 180 μL of buffer and 20 μL anti-FITC microbeads (Cat.no. 130-048-701, Miltenyi Biotec GmbH, Germany) and incubated for 15 minutes on ice. After washing, the cells were re-suspended in 500 μL buffer and proceed to magnetic separation.

The cells were passed in MS column of a magnetic field separator with three washings, the labeled cells remained in the column and unlabeled cells passed through in collection tube. Immediately, positive fraction were removed from the magnetic field and collected in separate tube. This procedure might be conducted for three times until separation purity was reached. After sorting, the cells were washed and plated back in the presence of antibiotics. The success of cells separation was directly checked by FACS.

Protein extraction

For total protein extraction, cells were dissolved in cell lysis buffer (PBS containing 1% v/v of a protease inhibitor cocktail [Sigma, P8340] and 2 mM phenyl-methylsulfonylfluoride). Cells were then placed on ice for 10 minutes and disrupted by scrapping and pipetting. Protein lysate were obtained by centrifugation at 14,000 rpm for 10 minutes in 4°C.

The protocol for membrane protein purification was based on Paulusma method (Paulusma *et al.* 1999). Cells were lysed and scraped in ice-cold 2 mM EDTA and 200 μM PMSF in PBS and then centrifugated for 5 minutes at 1000 g in 4°C. Cells pellet was dissolved in 1 mM bicarbonate and kept in ice for 30 minutes and then subsequently homogenized in a glass potter (tight pestle, 50 times) in ice and centrifugated for 8 minutes at 1000 g in 4°C. The supernatant was centrifugated for 1 hour at 100,000 g in 4°C and the resulted pellet was dissolved in 250 mM sucrose/10 mM Hepes/Tris pH 7.4 and re-homogenized using glass potter. Total protein extract was stored in -80°C and membrane protein in liquid nitrogen. Protein concentration was determined by copper (II) sulphate solution (Sigma-Aldrich, C2284) and bicinchonic acid ((Sigma, B9643) protein assay following the manufacturer's instructions.

Western Blot

Proteins of desired quantity (in μg) were size-separated, together with molecular weight standards (Fermentas, SM1811), by (SDS–PAGE) on 10% polyacrylamide gel, using a Mini Protein III Cell (Bio-Rad, Hercules, CA, USA). After SDS–PAGE, proteins were electro-transferred with a semi-dry blotting system at 100V for 90 min onto immune-blot PVDF

membranes (Bio-Rad) using a Mini Trans-Blot Cell (Bio-Rad). Membrane was incubated overnight at 4°C with first antibody at dilution 1:50 to 1:1000 in 4% skim milk or BSA in T-TBS buffer (Tris 20 mM, Tween 20, 0.2%, NaCl 500 nM, pH 7.5). After three washes with T-TBS the membranes were incubated with secondary antibodies with peroxidase conjugate at dilution 1:1000 to 1:4000 in 4% skim milk or BSA in T-TBS for 1 hour in room temperature. The peroxidase reaction was obtained by exposure of membrane in the ECL-Plus Western blot detection system solutions (ECL Plus Western blotting Detection Reagents, GE-Healthcare Bio-Sciences, Italia).

Total RNA isolation

Total RNA from the cell lines and tissues was extracted using the TriReagent solution according to the manufacture's protocol (T9424, Sigma–Aldrich, Milan, Italy). The cells on 25 cm^2 culture flask were grown until they reached ~85% confluence. The cells were washed two times with PBS and lysed with 1 mL of TriReagent. After scrapping and pipetting, the lysate was removed to a microtube and homogenized by vortexing. After addition of 200 µL chloroform per 1 mL TriReagent, the tube was centrifuged at 12,000 rpm for 15 minutes at 4°C and the upper layer containing RNA was removed to a new microtube. For RNA precipitation, 500 µL isopropanol per 1 mL TriReagent was added. The tube was centrifuged at 12,000 rpm for 10 minutes at 4°C and the supernatant was discarded. The RNA pellet was washed with 1 mL of cold ethanol 75% and centrifuged at 8,000 rpm for 5 minutes at 4°C. After air-drying for 15 minutes, the pellet was diluted in 10 – 50 µL of sterile water.

Total RNA samples were quantified in a spectrophotometer at 260 nm. Absorbance ratio at 260/280 nm and 260/230 nm was used to asses the purity of the samples. Agarose gel electrophoresis and staining with ethidium bromide, indicated that the RNA preparations were of high integrity.

Reverse transcription (RT) and real time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The RT using 1 ug of total RNA was performed with an iScript cDNA synthesis Kit (170–8891, Bio-Rad) according to the manufacture's suggestions. A total of 20 uL volume reaction was conducted in a thermocycler (Gene Amp PCR System 2400, Perkin-Elmer, Boston, MA, USA) at 25 °C for 5 min, 42 °C for 45 min, 85 °C for 5 min. The final cDNA was conserved at -20 °C until used.

The qRT-PCR was performed according to the iQ SYBR Green Supermix (Bio-Rad) protocol. PCR amplification was carried out in 15 uL reaction volume containing 25 ng of cDNA, 1x iQ SYBR Green Supermix containing 100 mM KCL; 40 mM Tris–HCl; pH: 8.4; 0.4 mM each dNTP; 50 U/mL iTaq DNA polymerase; 6 mM MgCl2; SYBR Green I; 20 mM fluorescein; and stabilizers (Bio-Rad Laboratories) and 250 nM gene specific sense and anti-sense primers. Reactions were run and analyzed on a Bio-Rad iCycler iQ real-time PCR detection system (iCycler IQ5 software, version 3.1; Bio-Rad) together with reference genes. Cycling parameters were determined and analyzed using the Pfaffl modification of the ΔΔCt equation with taking accounts to the efficiency of the reaction (Pfaffl 2001).

Primer design

The primers for qRT-PCR were designed using software Beacon Designer Version 7.9 (Premier Biosoft International, Palo Alto, CA, USA). Primer sets were built across two exons to avoid contamination of genomic DNA. Nucleotide BLAST was performed to check the specificity of the sequences. Melting curve analysis and agarose gel electrophoresis were carried out to asses templates products. The list of the primers description and sequences were shown in Table 3.2 and Table 3.3.

Statistical analysis

Box plot graphics and statistical analysis were constructed using software SigmaPlot Version 11.0 (Systat Software, Inc., Chicago, USA). The student's *t*-test was performed for statistical comparison between groups. Value of $p<0.05$ was regarded as statistically significant.

3.2.2. Identification of stem cells from human liver tissues

Primary cell cultures

The primary cell cultures of human liver were obtained from patients undergoing partial hepatic resection. Directly from the hospital, the tissues were kept in a falcon tube without any buffers and keep it in ice for maximum 3 hours before processing.

Under the sterile hood, the tissue (about 1 cm^3 in size) was washed twice with pre-warmed PBS. After the tissue was cut into small pieces using blade and scalpel and dissociated using collagenase type 4 (C5138, Sigma) for 5-15 minutes in a CO_2 incubator 37°C, 95% humidity, 5% CO_2. The enzyme activity was blocked with a minimum an equal volume of PBS + 10% FBS. After centrifugation (1,800 rpm, RT, 5 minutes) they were washed twice with pre-warmed PBS and filtered through a 40 μm cell strainer gradually several times. The flow through and the remain pellets on the filter were again washed and plated on 100 mm dishes in MyeloCult® medium (StemCell Tech., Vancouver, BC, Canada) in the presence of 10^{-6} M hydrocortisone sodium succinate and 1% of antibiotics. The cells were grown in a CO_2 incubator: 37°C, 95% humidity, 5% CO_2 with media changes every 3-4 days.

Characterization of stem cells

The surface markers of isolated cancer stem cells were checked by FACS using antibodies CD90, CD133, CD45, and CD44 (Table 3.1). Wider analysis to characterize the primary cells was done by real time RT-PCR. The detail of procedure and methods for FACS and RT-PCR were explained in previous subchapters. The expression of genes studied including: Cluster of Differentiation (CD) CD90, CD133, CD117 (c-kit), CD34, CD45, CD29, CD31, CD105, and CD166; marker for hepatocytes and epithelial biliary albumin, cytokeratin 7 (CK7) and cytokeratin 19 (CK19); pluripotency markers OCT4 and SOX2; ABC transporter ABCG2/BCRP. The description of genes identification is listed in Table 3.2 and Table 3.3.

The Colony Forming Unit – Fibroblast (CFU-F) assay

The isolated cells from passage °1 – passage °6 were plated in low density on 25 cm^2 flask for 4 weeks. After two washing with PBS, the cells were fixed by cold methanol for 10 minutes. The staining was done with Wright-Giemsa staining solution (WG16, Sigma) for 10 minutes. The CFU-F colonies were identified macroscopically and microscopically under light microscope.

Differentiation to adipocytes

The primary cultures were plated on 35 mm tissue culture dishes with 1.5 mL of AdipoDiff medium (130-091-677, Miltenyi Biotec). The medium was changed each 4 days and the cells were maintained for 2 – 3 weeks in culture. The identification of adipocytes was performed by qRT-PCR on gene PPARG (peroxisome proliferator-activated receptor gamma), master regulation of adipocytes differentiation (Table 3.3. and Table 3.4). Fat deposits in the cytoplasm were stained using Nile Red, a particular intercellular lipid staining. Cells on coverslip were washed and fixed with methanol for 15 minutes. After two times washing, cell nucleus was stained with Hoechst 33328 for 10 minutes at room temperature. Nile Red (1:50 in acetone) was added and the plate was gently shaken on a plate shaker for 20 minutes. Immediately after washing with de-ionized water, cells were examined under fluorescence microscope.

Differentiation to pancreatic cells

The protocol for pancreatic cells differentiation was performed according previous reports (Yang *et al.* 2002;Herrera *et al.* 2006). Briefly, primary cultures were plated on 35 mm tissue culture dishes with 3 mL DMEM-HG medium supplemented with 10% FBS, 1% antibiotics and 1% L-glutamine. After 7 days of plating, 10 mM of nicotinamide (N0636, Sigma) was added and medium was routinely changed for 2-3 days and cells were maintained until 3 weeks. The identification of cells was performed by qRT-PCR on genes GIP and somatostatin (Table 3.3 and Table 3.4).

Xenograft assay: pilot study

The capacity of isolated stem cells from human liver tissues to induce tumor in model *in vivo* in male nude mice Foxn1(nu/nu) age 7 – 10 weeks old was performed and currently is under evaluation. Briefly, single cells suspension was prepared using trypsin-EDTA detachment from several 75 cm^2 cell culture flasks. After washing, the cells were counted using a counting chamber to reach a desired concentration. After centrifugation 1000 rpm, 4°C for 5 minutes, cells pellet was re-suspended in 400 µL cold PBS and placed in ice. The injection was injected subcutaneously into left and right abdomen of the mouse. Duplicates were performed in the same mouse. Viability of the cells was checked by tryphan blue staining dye. The xenotransplantated mice together with control were observed for four months after injection. Mouse body weight was measured every week.

3.2.3. Expression of stem cells markers in human liver tissues

The gene expression study of several markers in human liver tissues was performed using qRT-PCR. The following genes were studied: stem cells surface markers CD90, CD133 and EpCAM, pluripotency markers OCT4, and cytokeratin 7 and cytokeratin 19. The target genes mRNA expressions were normalized to reference genes 18S-rRNA, β-actin, and GAPDH. The description of primers is listed in Table 3.2. Box plot graphics and statistical analysis were constructed using software SigmaPlot Version 11.0 (Systat Software, Inc., Chicago, USA).

3.2.4. ABCG2 expression: drug resistance and CSCs

In vitro drug cytotoxicity test

The cytotoxic effects of doxorubicin hydrochloride (D1515, Sigma-Aldrich), verapamil hydrochloride (V4629, Sigma-Aldrich) and Hoechst 33342 (B2261, Sigma-Aldrich) were assessed by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; M2128, Sigma-Aldrich) dye reduction test. The cells were seeded in concentration 20,000 cells/cm² in 24-well plates for corresponding time. The dose ranges of doxorubicin, verapamil and Hoechst used in this study were 0.0 – 10.0 μM, 0.0 – 20 μM and 0.0 – 50 μg/mL, respectively. For doxorubicin the exposure time is 24 hours whereas for verapamil and Hoechst, exposure time of the compounds was tested on 30, 90 and 270 minutes as required time for Hoechst exclusion assay.

Briefly, when MTT was taken up by living cells, it was converted from a yellow to a water insoluble blue-colored precipitate by cellular dehydrogenases (Mosmann 1983). After treatment, the medium was removed and the treatment was followed by addition of 0.5 mg/mL of MTT and incubation for 1 h at 37°C. The cells were then lysed and the resulting blue formazan crystals were solved in DMSO. The absorbance of each well was read on a microplate reader (Beckman Coulter LD 400C Luminescence detector) at 562 nm. The absorbance of the untreated controls was taken as 100% survival. The data represented the mean ± SD of three independent experiments.

ABCG2 mRNA expression

The ABCG2 mRNA expression was studied both in clinical samples and cell lines in the basal condition and after exposure to 5 μM doxorubicin for 24 hours. The ABCG2 mRNA expression was normalized to reference genes 18S-rRNA, β-actin, and GAPDH. The details of total RNA extraction and real time RT-PCR had described in previous subchapters.

Protein expression

For ABCG2 protein expression, membrane protein fractions were purified from basal condition and after exposure to 5 μM doxorubicin on hepatic cell lines IHH, HuH-7, and JHH-6. A total of 30 μg membrane protein was subjected to Western Blot. ABCG2 antibodies used in this experiment is anti-BCRP BXP-53 (Ab24115, abcam), dilution 1:200, with second antibody anti-rat IgG peroxidase (A5795, Sigma) dilution 1:1000, resulted protein band with molecular weight 72 kDa. As control, housekeeping protein actin was also checked with antibodies anti-actin (A2066, Sigma), dilution 1:1000 with second antibody anti-rabbit IgG peroxidase (P0448, Dak0) dilution 1:2000, resulted protein band 42 kDa. Details of Western Blot procedure was described in previous subchapter.

Hoechst 33342 efflux assay

The activity of ABCG2 in cell lines HepG2, HuH-7 and JHH-6 was assessed using Hoechst 33342 efflux assay with modification from previous report (Plumb, Milroy, and Kaye 1990). Single cells suspension was filtered with 40 μm cells strainer (BD Falcon). Sterile 1 μM or 20 μM final concentration of verapamil hydrochloride (V4629, Sigma-Aldrich) were added and the cells were incubated for 30 minutes in 37°C. After incubation, 5 μg/mL Hoechst 33342 (B2261, Sigma-Aldrich) was added and the cells were incubated for 90 minutes in 37°C. Subsequently, the reaction was stopped by incubating the cells on ice for 5 minutes. The Hoechst 33342 efflux was measured by spectrofluorometer (Jasco FP-770, Maryland, USA) on 355 nm excitation and 460 nm emission wavelength.

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Table 3.1. List of antibodies for protein identification

Markers	Clone	Cat. No.	Company	Applications
First antibody				
CD90-FITC	5E10	#10427	StemCell Tech.	FACS, cell sorting, Immunofluorescence
CD44	F10-44-2	Ab6124	Abcam	FACS, Immunofluorescence
CD133	AC133	130-090-422	Miltenyi Biotec	FACS
CD117	AC126	130-091-735	BD Biosciences	FACS
CD34	My10	348050	BD Biosciences	FACS
CD45-PE	5B1	130-080-202	Miltenyi Biotec	FACS
CD90	5E10	14-0909	eBioScience	Western Blot
ABCG2/BCRP	BXP53	Ab24115	Abcam	Western Blot
Actin		A2066	Sigma	Western Blot
Secondary antibody				
Anti-mouse-FITC		715-096-150	Jackson ImmunoResearch	FACS, IF
Anti-mouse IgG		P0260	Dako	Western Blot

Anti-rat IgG HRP	A5795	Sigma	Western Blot
Anti-rabbit IgG HRP	P0448	Dako	Western Blot

Table 3.2. List of the genes for the identification of isolated cells populations

Gene	Official name	Alias	Function
<u>Reference genes</u>			
18S	18S ribosomal RNA	RN18S1	rDNA repeating units
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	G3PD; GAPD; MGC88685	catalyzed an important energy-yielding step in carbohydrate metabolism
ACTB	actin, beta	PS1TP5BP1	involved in cell motility, structure, and integrity
<u>Cell surface markers</u>			
CD90	Thy-1 cell surface antigen	FLJ33325; THY1	adhesion molecules', found in stem cells
CD133	prominin 1	RP41; AC133; MCDR2; STGD4; CORD12; PROML1; MSTP061; PROM1	often expressed on adult stem cells, where it is thought to function in maintaining stem cell properties by suppressing differentiation
CD117	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	PBT; SCFR; C-Kit; KIT	a type 3 transmembrane receptor for MGF (mast cell growth factor, also known as stem cell factor)

CD29	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	FNRB; MDF2; VLAB; GPIIA; MSK12; VLA-BETA; ITGB1	involved in cell adhesion and recognition in a variety of processes
CD31	platelet/endothelial cell adhesion molecule	PECAM-1; FLJ58394; PECAM1	platelet/endothelial cell adhesion molecule
CD34	CD34 molecule		selectively expressed on human hematopoietic progenitor cells
CD44	CD44 molecule (Indian blood group)	IN; LHR; MC56; MDU2; MDU3; MIC4; Pgp1; CDW44; CSPG8; HCELL; MUTCH-I; ECMR-III; MGC10468	cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration
CD45	protein tyrosine phosphatase, receptor type, C	LCA; LY5; B220; T200; CD45R; GP180; PTPRC	regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. This gene is specifically expressed in hematopoietic cells.

CD105	Endoglin	END; ORW; HHT1; ORW1; FLJ41744; ENG	a major glycoprotein of the vascular endothelium. a component of the transforming growth factor beta receptor complex and it binds TGFB1 and TGFB3 with high affinity.
CD166	activated leukocyte cell adhesion molecule	MEMD; FLJ38514; MGC71733; ALCAM	activated leukocyte cell adhesion molecule
EpCAM	epithelial cell adhesion molecule	EGP; ESA; KSA; M4S1; MK-1; EGP-2; EGP34; EGP40; KS1/4; MIC18; TROP1; CO-17A; Ep-CAM; hEGP-2; CO17-1A; GA733-2; TACST-1; TACSTD1;	is expressed on most normal epithelial cells and gastrointestinal carcinomas and functions as a homotypic calcium-independent cell adhesion molecule.
<u>Hepatic and biliary markers</u>			
Albumin	Albumin	PRO0883; PRO0903; PRO1341; DKFZp779N1935	a soluble, monomeric protein synthesized in the liver which comprises about one-half of the blood serum protein.

AFP	alpha-fetoprotein	FETA; HPAFP	a major plasma protein produced by the yolk sac and the liver during fetal life. Alpha-fetoprotein expression in adults is often associated with hepatoma or teratoma
CK19	keratin 19	K19; K1CS; MGC15366; KRT19	intermediate filament proteins responsible for the structural integrity of epithelial cells
CK7	keratin 7	K7; SCL; K2C7; MGC3625; MGC129731; KRT7	is specifically expressed in the simple epithelia lining the cavities of the internal organs and in the gland ducts and blood vessels.
<u>ABC transporter gene</u>			
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	MRX; MXR; ABCP; BMDP; MXR1; ABC15; BCRP1; CD338; CDw338; EST157481; MGC102821; BCRP	function as a xenobiotic transporter which may play a major role in multi-drug resistance.
<u>Pluripotency markers</u>			
OCT4	POU class 5 homeobox 1	OCT3; OTF3; OTF4; MGC22487; POU5F1	is associated with self-renewal in embryonic stem cells
SOX2	SRY (sex determining region Y)-box 2	ANOP3; MCOPS3; MGC2413	a transcription factor involved in the regulation of embryonic development

Table 3.3. List of primer sequences for the identification of isolated cells

Gene	Acc. No.	Length	Sequence F	Sequence R	exons	Size (bp)	position	Reference
18S	NR_003286.2	1869	TAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	1	150	1578 - 1727	(Schmittgen and Zakrajsek 2000)
GAPDH	NM_002046.3	1310	CCCATGTTCGTCATGGGTGT	TGGTCATGAGTCCTTCCACGATA	4	145	460 - 604	(Yoshida *et al.* 2001)
ACTB	NM_001101.3	1852	CGCCGCCAGCTCACCATG	CACGATGGAGGGGAAGACGG	1 - 2	120	70 - 189	CSF
CD90	NM_006288.2	1791	AGAGACTTGGATGAGGAG	CTGAGAATGCTGGAGATG	4	178	1338 - 1515	CSF
CD133	NM_006017	3794	CATCTGCTCTCTGCTGAC	AACTTAATCCAACTCCAACC	13 – 15	152	1532 - 1683	CSF
CD117	NM_000222	5190	AGGCTCTTCTCAACCATCTG	ATTCATTCTGCTTATTCTCATTCG	1 – 2	179	153 - 331	CSF
CD29	NM_002211.3	3879	GCCTTGGTGTCTGTGCTGAG	AGTCGTCAACATCCTTCTCCTTAC	14 - 15	189	2128 - 2316	CSF

CD31	NM_000442	3754	GAGTCCAGCCGCATATCC	TGACACAATCGTATCTTCCTTC	6	183	1132 - 1314	CSF
CD34	NM_001025109	2621	CTGATACCGAATTGTGACTC	TTGGGCGTAAGAGATGTC	9	120	1400 - 1519	CSF
CD44	NM_000610	5748	CTCATACCAGCCATCCAATG	GAGTCCATATCCATCCTTCTTC	10 – 11	133	1726 - 1858	CSF
CD45	NM_002838.3	5330	CGGCTGACTTCCAGATATGAC	GCAGTGGTGTGAGTAGGTAAG	2 - 5	183	159 - 341	CSF
CD105	NM_001114753.1	3072	CTTCCTCCTCCACTTCTAC	GGACTTCCTGGTCTTGAG	13 - 14	101	2006-2106	CSF
CD166	NM_001627.2	4760	GAAGGAGGAGGAATATGGAATC	GTCAAGTCGGCAAGGTATG	1 - 2	152	527 - 678	CSF
EPCAM	NM_002354.1	1731	GAATAATAATCGTCAATGCCAGTG	CGCTCTCATCGCAGTCAG	2 - 3	188	478 - 665	CSF
Albumin	NM_000477.3	2215	GGCATCCTGATTACTCTGTCG	AATTCTGAGGCTCTTCCACAAG	9 - 10	150	1121 - 1270	CSF
AFP	NM_001134	2032	GCGGCTGACATTATTA	TTGGCACAGATCCTTATG	11 - 12	192	1473 -	CSF

			TCG	G			1664	
CK19	NM_002276.4	1490	TGAGTGACATGCGAA GCCAATAT	GCGACCTCCCGGTTCAAT	4 - 5	103	888 - 990	(Dimmler *et al.* 2001)
CK7	NM_005556.3	1753	TGAATGATGAGATCA ACTTCCTCAG	TGTCGGAGATCTGGGACT GC	4-5	76	750 - 855	(Dimmler *et al.* 2001)
ABCG2	NM_004827	4445	TATAGCTCAGATCATT GTCACAGTC	GTTGGTCGTCAGGAAGA AGAG	9 - 10	124	1389-1512	CSF
OCT4	NM_002701	1411	AGCGAACCAGTATCG AGAAC	TTACAGAACCACACTCGG AC	3 - 4	142	752 - 893	(Park *et al.* 2008)
SOX2	NM_003106.2	2518	AGCTACAGCATGATG CAGGA	GGTCATGGAGTTGTACTG CA	1	126	935 – 1060	(Park *et al.* 2008)
PPARG	NM_138712.3	1892	ACGAAGACATTCCATT CACAAG	TCTCCACAGACACGACAT TC	7 - 8	199	396 - 594	CSF
GIP	NM_004123.2	711	CCCTCAACCTCGAGGC CCCA	CCCGAGCCTCCCTCTGGG TG	3 - 4	161	230-390	CSF
SST	NM_001048.3	665	CCCAGACTCCGTCAGT TTCT	AGCCTGGGACAGATCTTC AG	1 - 2	144	207 - 350	CSF

Table 3.4. List of the genes for the identification of cells-directed differentiation to adipocytes and pancreatic cells

Markers	Gene	Official name	Alias	Function
Adipocytes differentiation	PPARG	peroxisome proliferator-activated receptor gamma	GLM1; CIMT1; NR1C3; PPARG1; PPARG2; PPARgamma	The protein encoded by this gene is PPAR-gamma and is a regulator of adipocyte differentiation.
Pancreatic differentiation	GIP	gastric inhibitory polypeptide	-	It encodes an incretin hormone and belongs to the glucagon superfamily. It maintains glucose homeostasis as it is a potent stimulator of insulin secretion from pancreatic beta-cells following food ingestion and nutrient absorption.
	SST	somatostatin	SMST	This hormone is an important regulator of the endocrine system through its interactions with pituitary growth hormone, thyroid stimulating hormone, and most hormones of the gastrointestinal tract.

Chapter IV – Result 1

Identifications of Stem Cells from Human Primary Liver Cancers

4.1. Primary Cells

From June 2008 – September 2010, a total 11 liver cancer patients was obtained from the Department of Surgery, Hospital Cattinara Trieste. The patients underwent surgical procedure after indication by hepatologist. Age of the patients ranged from 54 – 81 years old (70 ± 9 y) with female vs male ratio 6:5. Three patients were HCV positive.

Three parts of the liver were taken: neoplastic (tumoral), peripheral (between tumoral and 'normal') and distal ('normal') as visualized in Fig 4.1. However, distal tissues were also cirrhotic.

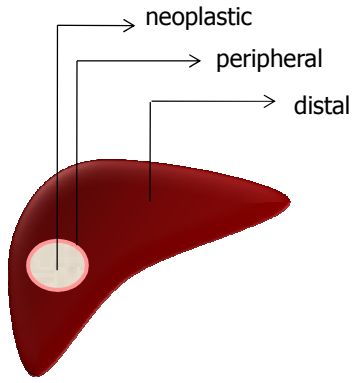


Fig. 4.1. Sources of tissues for the isolation of stem cells. The cells were obtained from three parts of liver: neoplastic, peripheral, and distal tissues.

4.2. Morphology of the Cells

Cells population lines were obtained by culturing single cells suspension after enzymatic treatment. Hepatocytes usually died 1 to 2 weeks after plating. Surviving cells attach to tissue-culture plates were routinely expanded by trypsin detachment and characterized by FACS and RT-PCR under sixth passage.

All the isolated cells from HCC and CC have fibroblastic-like morphology and attach strongly to the plastic tissue culture flasks. Generally, all cells have the capacity to fast proliferate and make colonies when they were plated in low density, indicating clonal potency of these cells (Fig. 4.2.A). To confirm a clonogenic anchorage-independent of cancer cells in 3 dimensional matrixes, we diluted cells in thick gel of matrigel (BD Bioscience). As shown in Figure 4.3, both for dilution 1:30 and 1:60 (usually the cells were expanded in dilution 1:3), single clone is observed to be highly clonogenic and colonies with minimum 4 cells were identified.

Growth curve of the cells were studied by plating 10,000 cells/mL in a 24-wells cell culture plate. Cells count was directly performed using a counting chamber under microscope. Growth curve of cells populations are variable among cancerous populations (data not shown). However, when we compared the growth of cells originated from cancers with one line from non-tumoral liver, the growth are significantly faster.

The colony forming unit – fibroblast (CFU-F) is a heterogeneous cell populations derived from bone marrow which showed nonhematopoietic differentiation potential. These cells are also named as mesenchymal stem cells (MSCs). multipotent stromal cells, mesodermal stem cells or marrow stromal cells (all abbreviated as MSCs), mesenchymal progenitor cells (MPCs), multipotent adult progenitor cells (MAPCs), stromal precursors, bone marrow stromal stem cells (BMSSCs) or bone marrow stromal cells (BMSCs).These cells have characteristic to form fibroblastic colonies in small density plating. After Giemsa staining, these colonies could be observed macroscopically and identified as CFU-F colonies.

The CFU-F assay was performed 3-4 weeks after plating. Macroscopically, they can be visualized and observed directly by the presence of purple clusters/colonies with diameter more than 1 mm. Microscopically, they are stained purple both in nucleus (dark purple) and cytoplasm (slightly light purple), and consisted at least 8 cells as shown in Fig. 4.2.B.

In total, 20 isolated cells were obtained consist of 7 neoplastic, 4 peripheral, and 7 distal regions. Only one cell populations (CSF08N) showed morphology of hepatocyte and further analysis was performed to identify this cells population in following results.

Table 4.1. Isolated cells populations from human liver tissues

Parameter	Description	n
Period	June 2008 – September 2010	
Disease	Hepatocellular carcinoma	6 *
	Cholangiocarcinoma	3
	Not cancer	2 *
Sex (F : M)	6 : 5	
Age	69.8 ± 8.9 y.o (54 – 81 y.o)	
Serology	Negative	8
	HCV (+)	3
Cells populations obtained	Neoplastic (N)	7
	Peripheral (P)	4
	Distal (D)	7

* 1 line could not be maintained after 1st passage

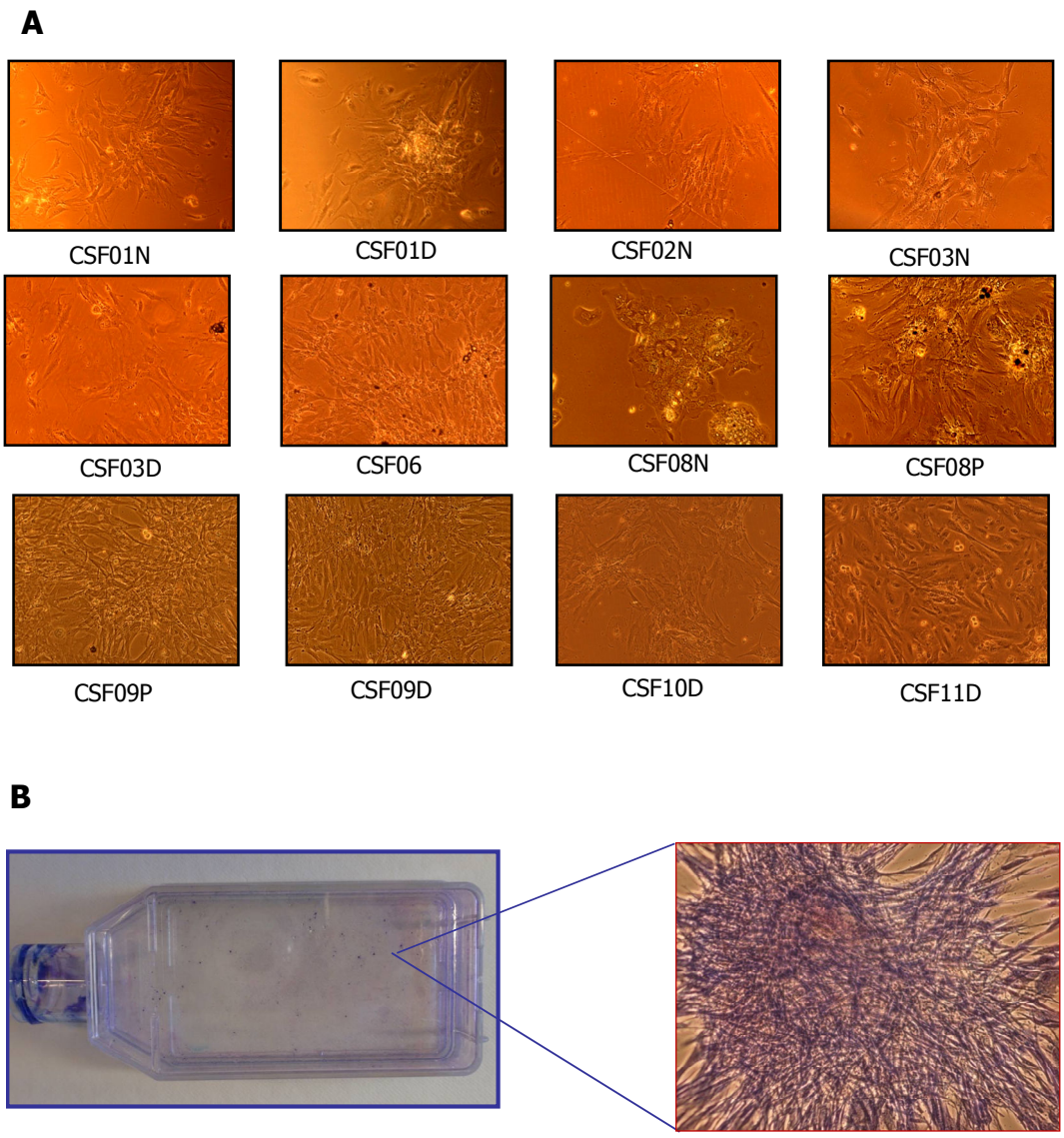


Figure 4.2. Morphology of isolated cells. A. The isolated cells from HCC and CC have fibroblastic-like morphology and capacity to attach strongly to cell culture flask. Furthermore, they are able to clone when plated in small density. **B.** The Giemsa staining showed CFU-F colonies both macroscopically and microscopically. Magnification 100x.

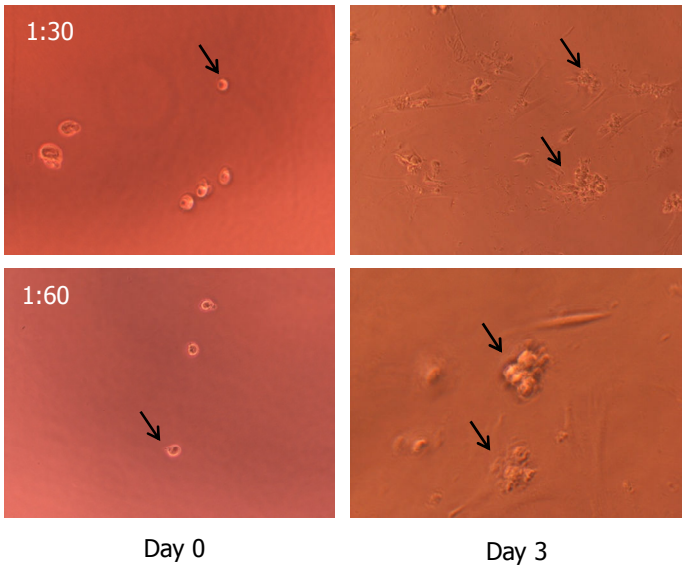


Figure 4.3. Clonogenic capacity in 3 dimensional matrix of matrigel. Cells from cancerous tissues have a clonal capacity in anchorage-independent assay in low density concentration with dilution 1:30 and 1:60. Arrows indicated cell and cell colonies. Magnification 100x.

4.3. Phenotype of the Cells

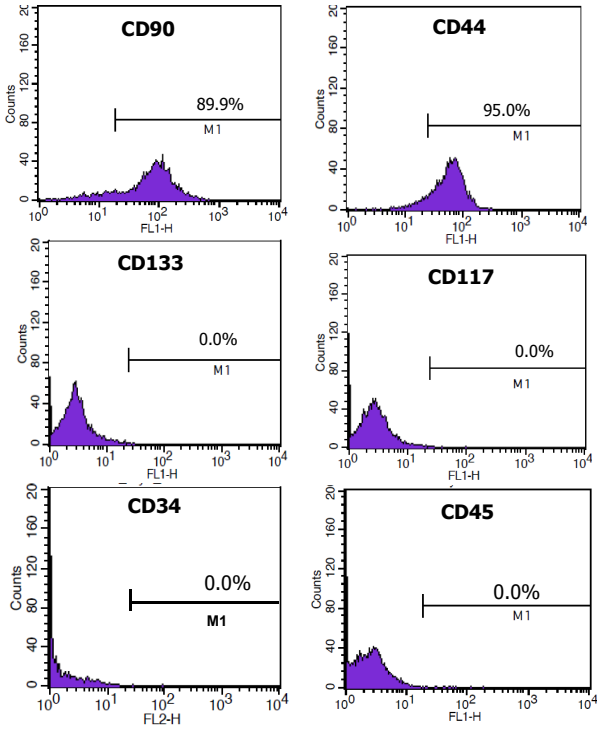
The surface markers protein of primary cells were examined by flow cytometer with direct or indirect staining as described in chapter Materials and Methods. All the cells in first passages showed the expression of proteins CD90 and CD44 with different percentage (0.4% to 89.9% and 3.8% to 94.8% for CD90 and CD44, respectively) but all were negative for hematopoietic markers CD34 and CD45. The majority of cells lacked CD133 and CD117, suggesting that they are not circulating hematopoietic cells. The CD45, a marker for

lymphocytes, was checked to avoid the presence of lymphocyte with CD90 expression with because the cells were obtained from primary tissues. The localization of surface proteins CD90 and CD44 were also checked by fluorescence microscope (Figure. 4.5).

To have a wider analysis of these primary cells, further characterizations of cell surface markers were carried out using real time RT-PCR on CD90 (Thy-1), CD133 (Prominin-1), CD45, CD44, CD34, CD29, CD31, CD105, CD117 (c-kit), and CD166 (ALCAM) as previously reported (Herrera *et al.* 2006). As control for the specificities of primer sets we used total RNA extract from IHH cells for CD90, Huh-7 cells for CD133 (Ma *et al.* 2007;Suetsugu *et al.* 2006), Jurkat cells for CD34, and human liver cancer tissues and blood samples for CD34, CD44, CD45, CD29, CD31, CD105, and CD166. The description and function of these CDs are reported in Table 4.2 while the primer sequences are listed in Table 4.3.

The qualitative PCR showed that all the cells are positive for mRNA CD90, CD44, CD29, CD105 and CD166, and negative for hematopoietic cell markers CD34, and CD45. They are also lack the expression of CD31, an endothelial molecule. An interesting observation indicated that high expression of CD133 and OCT4 mRNA were observed in cells with HCV infection compared to hepatitis-free liver cancer.

For CSF08N cells that are morphologically almost identical to hepatocytes, RT-PCR results demonstrated that these cells produced high albumin and lower CD90 expression compared to other cells. Furthermore, they have no ability to form CFU-F colonies.



Percentage of stem cells in isolated cells populations

Surface markers	Frequency
CD90 (Thy-1)	0.4% - 89.9%
CD44	3.8% - 94.8%
CD133 (Prom-1)	0.0% - 1.0%
CD117 (c-kit)	0.0% - 0.5%
CD45	0.0%
CD34	0.0%

Figure 4.4. Frequency of cell surface marker proteins using FACS. The isolated cells were examined for antigens CD90, CD44, CD133, CD117, CD34, and CD45 to see the proportion of stem cells in the PLC. The percentage of stem cells positivity is listed in the table.

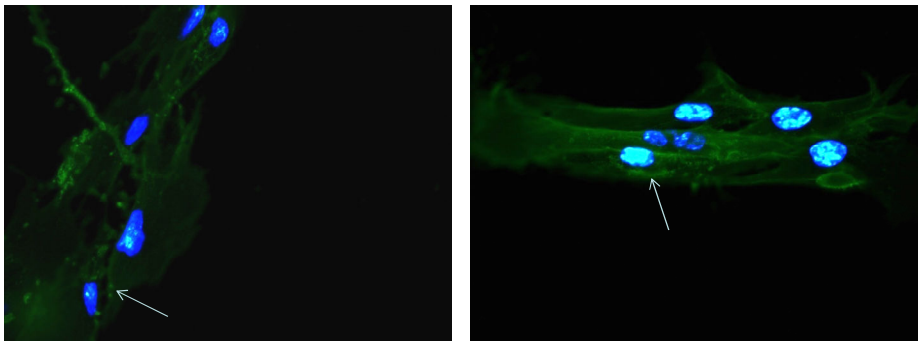


Figure 4.5. Localization of CD90 and CD44 surface protein in primary cells. The isolated cells were immunostained with **A)** CD90-FITC and **B)** CD44 and antimouse-FITC. The surface marker proteins are localized in surface of the fibroblastic-like cells. Arrow indicates the proteins.

4.4. Expression of Albumin and AFP Genes

Albumin and alpha-fetoprotein (AFP) are two plasma proteins synthesized by the liver and the yolk sac. Production of albumin and alpha-fetoprotein (AFP), marker of adult hepatocytes and immature liver cells, respectively, were examined by RT-PCR. For the specificities of both primer sets we used total RNA extract from HepG2 as control positive. Albumin is not/very weak expressed in majority of isolated cells, except in CSF08N and CSF10D (both are HCV+). The expression of AFP in isolated cells is found to be weak and more variable than albumin. Previously, Yamashita *et al* reported that AFP– cell lines had a subpopulation of CD90+, but AFP+ cell line did not (Yamashita *et al*. 2009).

4.5. Expression of Pluripotency Markers Genes

Expression of OCT4 and SOX2, embryonic pluripotency transcription factors, were also examined by RT-PCR. For the specificities of both primer sets we used total RNA extract from immortalized IHH and HCC cell line HepG2 as controls positive. It had been reported that OCT4 is expressed in cancer and SV40 immortalized cell line (Tai *et al.* 2005). All isolated cells showed positivity of both markers, indicating a possible embryonic-like potency of these cells.

Table 4.2. Characteristic of isolated cells from PLCs using RT-PCR analysis

Samples		**01**	**02**	**03**		**06**	**08**			**09**		**10**		**11**
Tissues portions		**N**	**N**	**N**	**D**	**D**	**N**	**P**	**D**	**P**	**D**	**P**	**D**	**D**
Control	B-actin	+	+	+	+	+	+	+	+	+	+	+	+	+
	18S-RNA	+	+	+	+	+	+	+	+	+	+	+	+	+
Surface markers	CD90	+	+	+	+	+	+	+	+	+	+	+	+	+
	CD44	+	+	+	+	+	+	+	+	+	+	+	+	+
	CD29	+	+	+	+	+	+	+	+	+	+	+	+	+
	CD105	+	+	+	+	+	+	+	+	+	+	+	+	+
	CD166	+	+	+	+	+	+	+	+	+	+	+	+	+
	CD133	-	-	-	-	-	+	-	+	-	-	+	+	+
	CD117	-	-	-	-	+	-	+	+	-	-	+	+	-
	CD34	-	-	-	-	-	-	-	-	-	-	-	-	-
	CD45	-	-	-	-	-	-	-	-	-	-	-	-	-
	CD31	-	-	-	-	-	-	-	-	-	+	-	+	
Puripotency marker	OCT4	+	-	+	+	+	+	+	+	+	+	+	+	+
	SOX2	+	+	+	+	+	+	+	+	+	+	+	+	+
Hepatic lineage	Albumin	-	-	-	-	-	+	-	+	-	-	-	+	-
	CK19	+	+	-	+	+				+	+	+		
	AFP	-	-	-	+	-					+	+	-	

4.6. Plasticity

4.6.1. Differentiation to adipocytes

Seven primary cells (CSF01N, 02N, 03N, 03D, 08P, 08D, 09P) were subjected to differentiation to adipogenic lineage with specific medium. After 4 weeks, induction of gene PPARG, a master regulator for adipocytes differentiation, was examined using qRT-PCR and fat droplet in the cells cytoplasm was stained using Nile Red staining. In lipid vacuole staining, Nile Red dye was used instead of standard Oil Red. Both dyes are used to stain intracellular lipid, and Nile Red could be easily observed using both red and green fluorescence light under microscope.

From all seven primary cells, only one cell CSF03D showed a clear differentiation potential to adipocytes as demonstrated with drastic up-regulation of PPARG gene and deposition of lipid vacuoles in the cytoplasm (Figure 4.6). CSF03D cell is originally obtained from distal part or a CC patient with a histological moderate degree of cancer differentiation. We assume that this cell might be most closely related to 'normal' compared to the others which allows differentiation. Even though adipogenic-differentiation capacity is observed in only one cell population, this data demonstrated the capacity of the SCs or CSCs obtained from liver cancer and cirrhotic. This data is concordant with previous one that SCs with CD90+ phenotype from fetal liver have capacity to transform into adipocytes (Dan *et al.* 2006). Furthermore, this data gives a wider comprehension from previous reports of normal hLSCs and CSCs (Herrera *et al.* 2006;Yang *et al.* 2008) that $CD90^{+}$ cells from adult diseased tissue may still have capacity for trans-differentiation.

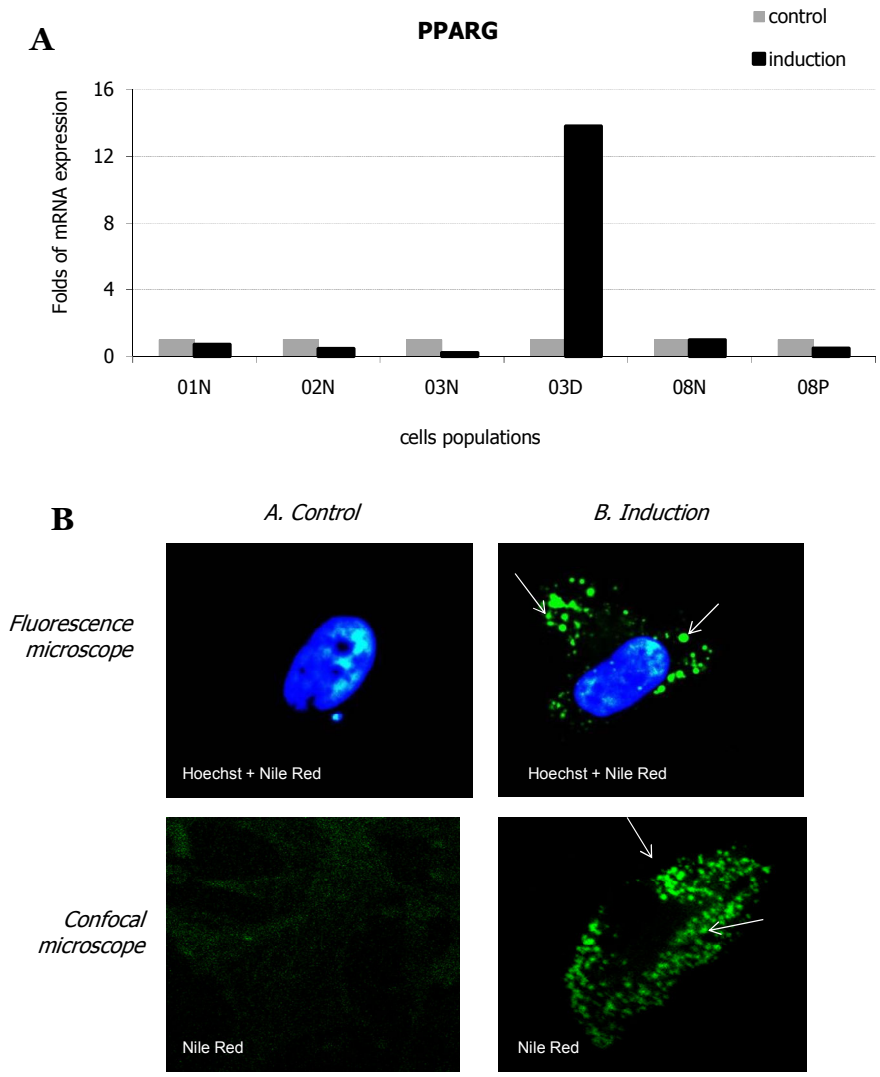


Figure 4.6. Cells-directed differentiation to adipogenic lineage. A. Quantitative RT-PCR result of gene PPARG showed an up-regulation of PPARG in one cells population 03D. The expression of basal 03D as control= 1.00. The target mRNA expression was normalized to reference gene β-actin. **B.** Nile Red staining of lipid vacuoles in the cytoplasm under fluorescence microscope of cells CSF03D. Arrows indicates lipid vacuoles.

4.6.2. Differentiation to pancreatic cells

To check whether the isolated cells have capacity to differentiate into endodermal lineage, we induced the cells towards pancreatic cells. Data of pancreatic differentiation presented in this report is an ongoing study and a further examination is still under analysis.

Seven primary cells (CSF01N, 02N, 03N, 03D, 08P, 08D, 09P) were subjected to differentiation into pancreatic cells by exposure to 10 mM nicotinamide (Herrera *et al.* 2006;Yang *et al.* 2002). The sorted JHH-6 with similar phenotype JHH6 CD90+CD44+ and its counterpart JHH6 CD90-CD44+ were used as phenotype control. Further description of these sorted cells may be found in subchapter 4.9. After 12-18 days, qRT-PCR on genes related with endocrine differentiation somatostatin (SST) and gastric inhibitory poplypeptide (GIP) were examined. A control universal mRNA from pancreatic tissues was used as SST primer control (data not shown).

As shown in Figure 4.7, five from seven cells population showed expression and up-regulation of SST and GIP with variable folds of up-regulation. In CC samples, the SST gene is expressed even in basal condition.

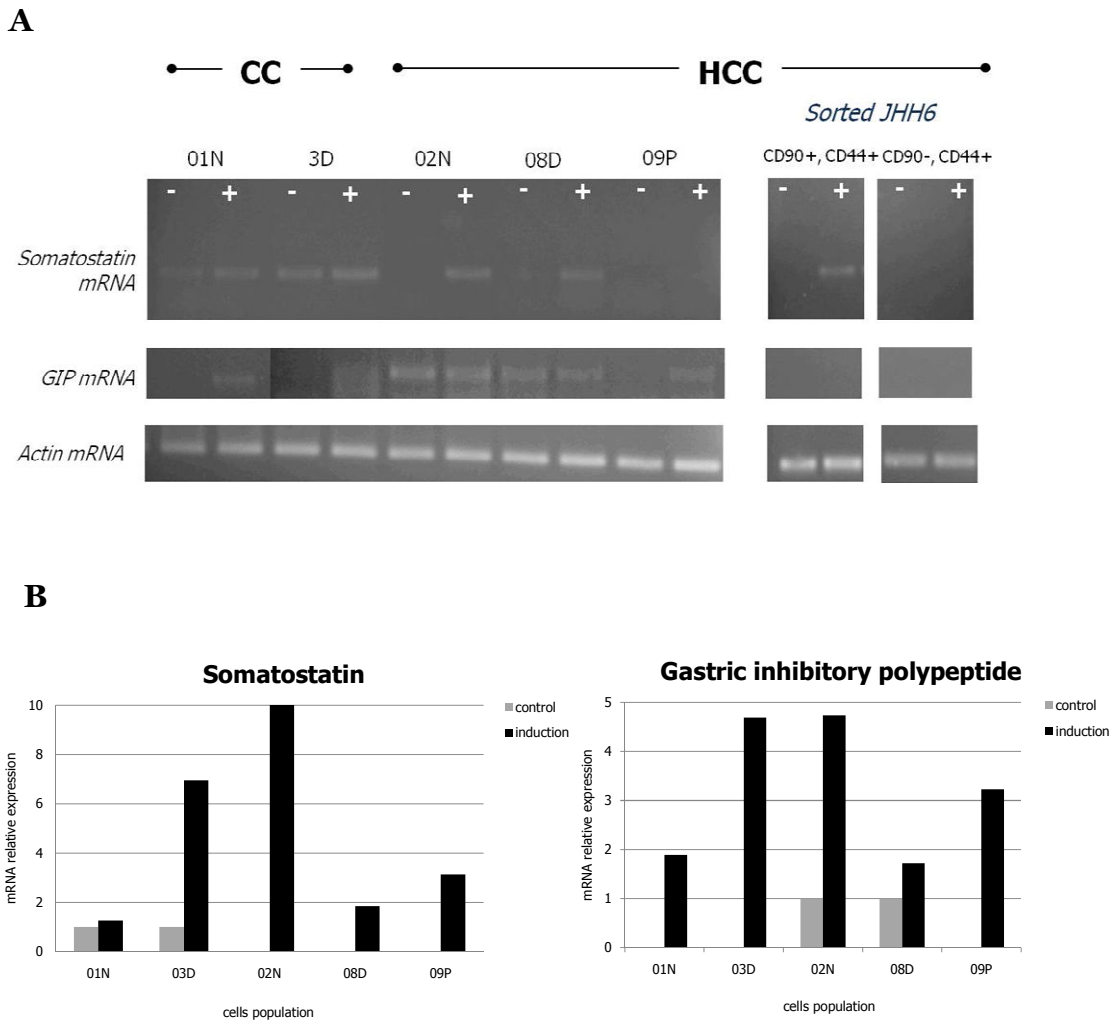


Figure 4.7. Cells-directed differentiation to pancreatic cells. After nicotinamide induction, 5 primary cells showed induction and up-regulation of SST and/or GIP mRNA. The sorted JHH6 CD90+ and CD90- cells were used as control. **A**. The qualitative result based on gel electrophoresis, actin mRNA was employed as internal reaction control. **B.** The quantitative result based on qRT-PCR, expression of basal 01N and 02N were control = 1.00 on gene SST and GIP, respectively. The target mRNA expression was normalized to reference gene β-actin.

4.7. Xenograft Assay: Pilot Study

To check whether primary cells with CD90+CD44+ phenotypes have the capacity to act as real CSCs in liver cancer we injected subcutaneously these cells with a minimal 80% positivity into athymic male nude FOXN1(nu/nu) homozygotes mice. Cells CSF01N (poorly differentiated CC) and CSF09P (HCC with cirrhosis) were selected for this pilot study for their fast growth capacity as compared to the other cells. One million of sorted cells from established cell line JHH6 CD90+ and JHH6 CD90- were used as positive and negative control. The viability of cells was checked using tryphan blue exclusion assay after injection and showed more than 95% viability. The xenograft assay is described in Table 4.3.

Four months after injection, no sign of tumor nodules were observed in all injected mice. Positive control mouse (1 million cells CD90+) died 10 days after injection, but negative control mouse (1 million CD90-) survived. Those mice were injected with same origin cells JHH-6 but with different CD9 phenotype. All mice did not show any particular behavior. Interestingly, body weights of all mice injected with CD90+CD44+ were 10% higher than mouse injected with CD90- cells and control mice without treatment (data not shown).

Table 4.3. Pilot study of xenograft assay: preliminary result

Control	Cells ID	Sources	Serology	Concentration	Tumor after 4 mos
Control mouse	-	-	-	-	0/2
Control (+)	JHH6 CD90+	Cell line HCC	HCV(-) HBV(-)	1,000,000	† day 10
Control (-)	JHH6 CD90-	Cell line HCC	HCV(-) HBV(-)	1,000,000	0/1
Primary cancers	CSF01N	CC	HCV(-) HBV(-)	10,000	0/1
				50,000	0/1
				500,000	0/1
	CSF09N/P	HCC	HCV(-) HBV(-)	10,000	0/1
				50,000	0/1
				500,000	0/1

4.8. Expression of ABCG2 Gene

To explore the expression of drug-resistant-protein ABCG2 in isolated cells populations, qRT-PCR was performed. As predicted, ABCG2 expression is found to be variable, just as the result of tissues samples (please refer to Chapter VI). As shown in Figure 4.8, the distribution of ABCG2 may be higher in peripheral or distal tissues. However, variations of ABCG2 expression may vary among individuals due to different tissues types and intrinsic factors, such as age and type of HCC treatment exposure.

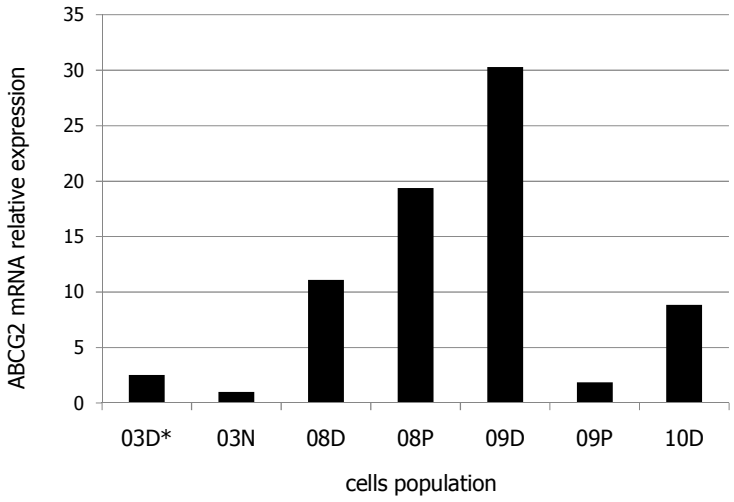


Figure 4.8. ABCG2 mRNA expression in isolated cells populations. The ABCG2 expression is found to high variable among samples. ABCG2 expression was normalized to reference gene β-actin and expressed as au compared to sample 03D.

The result of ABCG2 mRNA expression in isolated each cells populations is separated from result from whole tissues samples because ABCG2 is also expressed in mature cells such as hepatocytes, cholangiocytes, and liver blood vessel endothelium (Maliepaard *et al.* 2001) (Fetsch *et al.* 2006;Komuta *et al.* 2008). Regarding this reason, a direct comparison between isolated cells which are 'more' homogenous with liver tissues which are more 'heterogeneous' may give a bias result. Result of ABCG2 mRNA expression in tissues samples is reported in Chapter VI.

4.9. Progression to Cancer Heterogeneity: JHH-6 Model

JHH-6 (JCRB1030) is a hepatoma cell line originated from a female Japanese HCC patient with hepatitis B surface antigen serogenative HBs-Ag(-), HBs-Ag(-). These cells have undifferentiated morphology. Albumin, AFP and CEA were not observed in the supernatant of the cell culture but ferritin was found to be secreted (6.7 ng/ml).

In basal level, JHH-6 cells express CD90 and CD44 mRNA. When they were checked by FACS, they have a frequency of 0.1% CD90 and almost 100% of CD44 (data not shown). To see the difference between CD90+ and CD90- cells, they were sorted based on its CD90 phenotypes using a MACS magnetic sorter with 99% purity as mentioned in Materials and Methods section. The sorted cells has phenotypes JHH-6 $CD90^{+}CD44^{+}$ and its counterpart $CD90^{-}CD44^{+}$. By RT-PCR, both cells express CD29, CD105, and CD166, negative for CD34 and CD45, and weak expressed CD31. This phenotype is similar with the isolated cells in primary culture.

The purity of the CD90 cells at $P^{o}1$ after sorting was confirmed by RT-PCR of total RNA, immunofluorescence of fixed cells, and Western blot of membrane fraction protein. As shown in Figure 4.9, sorted cells showed distinct characteristics. RT-PCR analysis demonstrated that positive fraction cells expressed around 45-folds more CD90 mRNA, while negative cells have similar expression of control. Immunofluorescence data showed that only positive fraction had a clear CD90 protein in cells membrane. Similar result was also obtained by Western blot where a band of CD90 25-35 kDa is observed in membrane extract of positive but not negative fraction. Immortalized cell line IHH is used as control.

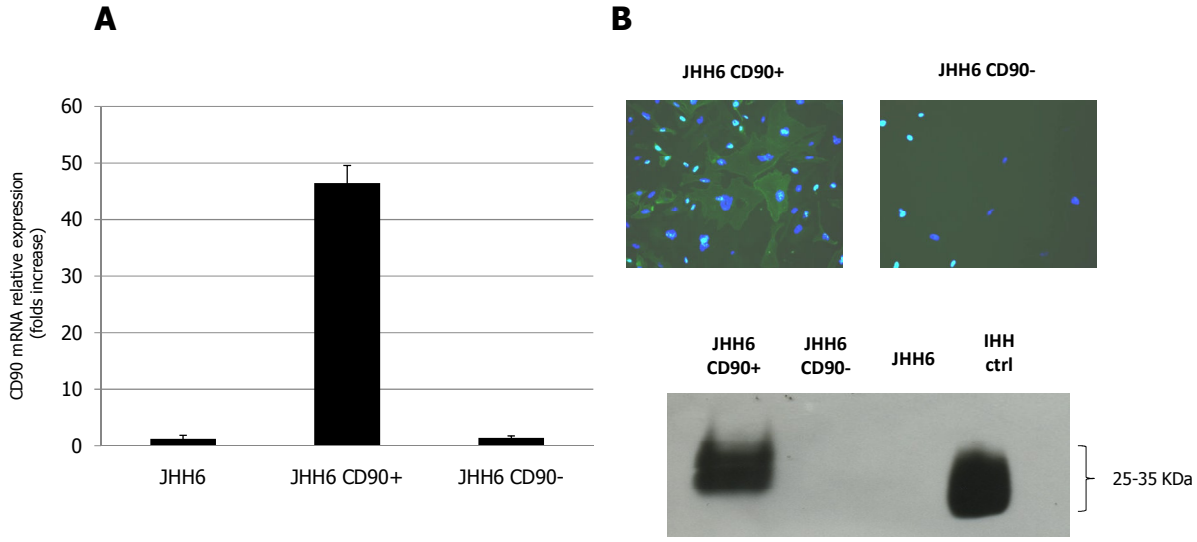


Figure 4.9. Purity of sorted JHH-6 CD90+ and CD90-. A. Quantitative RT-PCR result. The expression of unsorted JHH-6 as control = 1.00. The mRNA expression was normalized to reference gene 18S-rRNA and β-actin. **B.** Immunofluorescence andWestern blot of CD90 protein from membrane extract fraction resulting a 25 – 35 kDa protein band [*Experiments were performed together with B. Anfuso].*

After sorting and confirmation of the purity, the cells phenotype from both positive and negative fractions was followed-up until the 15th passage. An interesting change is observed as CD90^{+} cells could differentiate into CD90^{+} and CD90^{-}. In contrast, the CD90^{-} cells can harbour only CD90^{-} cells, as shown in Figure 4.10. Until the 21st passage after sorting, CD90+ cells reached a composition of positive and negative fraction of about 20% and 80%, respectively. Until passage 21st, CD90- cells can be only 100% CD90- cells. There are no morphological differences in our JHH6 CD90^{+} and CD90^{-} cells, checked by FACS and microscopy.

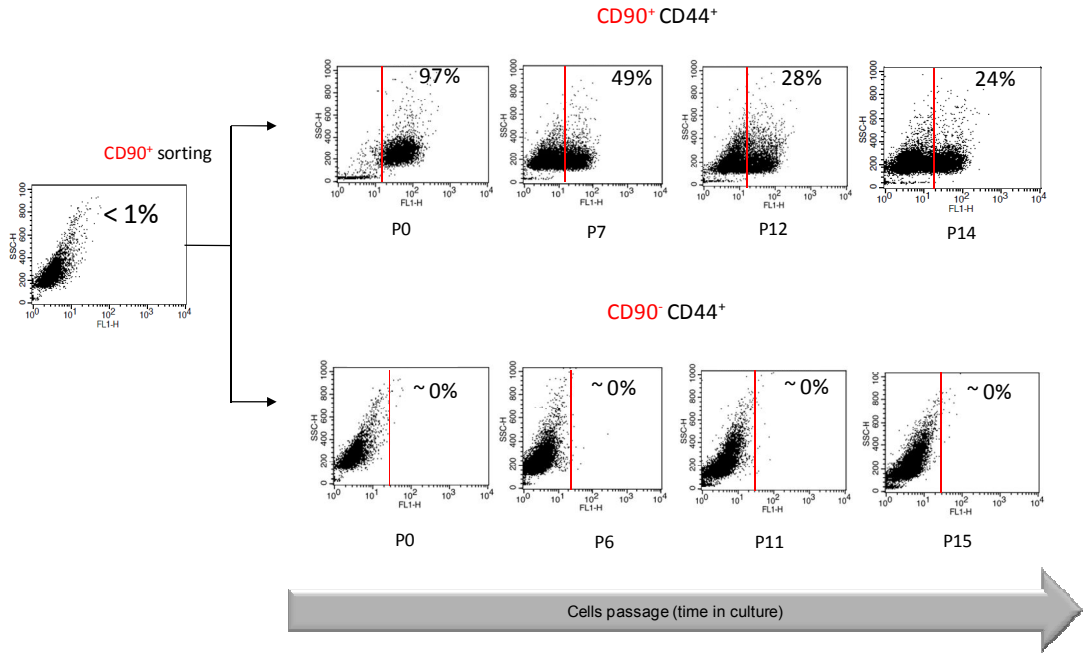


Figure 4.10. Progression of JHH-6 CD90+ cells to comprise cancer heterogeneity. Cells JHH-6 CD90+ could differentiate into both phenotypes CD90+ and CD90-, its positivity diminished along cells passages; in contrast CD90- could harbor only CD90- *[Experiments were performed together with B. Anfuso].*

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Chapter V – Result 2

Distributions of Stem Cells mRNA Expressions in Human Liver Tissues

5.1. Human Liver Tissues

As mentioned in the General Introduction, beside the use of the stem cells markers in the isolation of putative CSCs from human disease, they might have clinical significances in human disease. From this viewpoint, the expression of stem cells markers might be also useful in the clinical settings. To search a possible relationship between these markers with the disease progression in human liver diseases, a gene expression study on several most common hepatic stem cells markers was performed. The genes examined consist of CD90, EpCAM, CD133, OCT4, CK19, and CK7.

We examined several liver malignancies: HCC (n=28), CC (n=3), pediatric hepatoblastoma HB (n=7), other benign tumors O (n=5), biliary atresia BA (n=5), and young and adult normal tissues N (n=12). Other tumors included were hamartoma and pseudotumor. The number of patients and total tissues size is described in Table 5.1. Biliary atresia (BA) is a non neoplastic neonatal liver disease in which the bile ducts is absent. As the biliary tract is disrupted, the liver cannot deliver bile into the intestine and its accumulation resulted in liver fibrosis and eventually liver cirrhosis. Due to small number of CC samples, they are not included in the distribution graphs and used as positive control for expression of EpCAM, CK19 and CK7.

Table 5.1. Description of tissues types for SCs markers mRNA distribution analysis

Disease groups	Patients no.	Samples no.	Tissues types
HCC	12	28	Neoplastic, peripheral, and distal tissues
CC	2	3	Neoplastic and distal tissues
HB	4	7	Neoplastic and distal tissues
O	4	5	Diseased and 'normal' tissues
BA	5	5	All cirrhotic tissue
N	12	12	Normal tissues

5.2. Distribution of CD90 (Thy-1)

The CD90 mRNA levels from the 52 tissues samples analyzed, estimated by qRT-PCR, are expressed as arbitrary units (au), compared to a normal sample (1.00 au). The CD90 gene is expressed in all tissues examined. In normal donor livers, CD90 mRNA expression is more limited and homogenous, ranges from 0.05 to 1.00 au and in BA from 0.24 to 1.91 au. In both HB and HCC, CD90 mRNA expression is found to be more variable than in normal tissues. The lowest is identified in the distal tissues of both tumors with an expression value of 0.29 and 0.11 au, respectively, and to be highest in neoplastic tissues of 6.05 and 5.99 au, respectively. As shown in the Figure 5.1, the overall distribution of CD90 mRNA in hepatic malignancies has a clear distinguished pattern. The CD90 expression in cirrhotic and liver cancers is significantly difference with normal liver tissues ($p<0.05$ for HB and BA, $p<0.01$ for HCC).

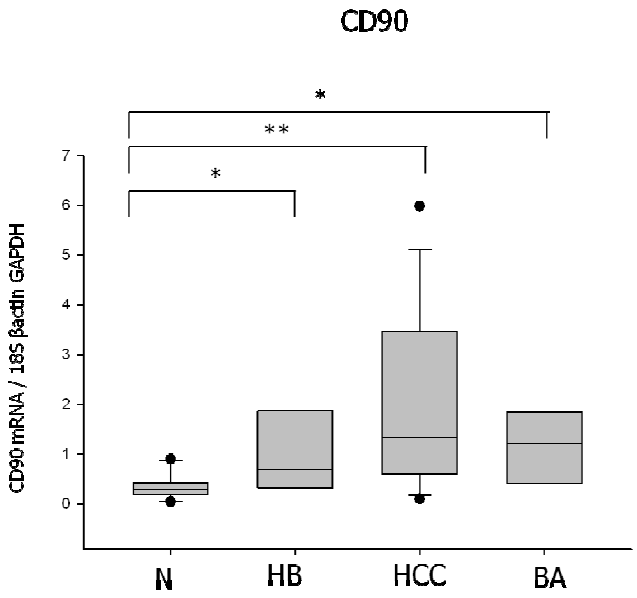

Figure 5.1. Distribution of CD90 mRNA in human liver. CD90 expression is normalized to three reference genes 18S-RNA, β-actin, and GAPDH, and is expressed as au compared to one normal tissue. Samples size: Normal N = 11; Hepatoblastoma HB = 7; Hepatocellular Carcinoma HCC = 17; Biliary atresia BA= 5. Student's *t*- test * $p<0.05$, ** $p<0.01$.

5.3. Distribution of EpCAM

The EpCAM mRNA levels in the of 43 tissues samples analyzed and estimated by qRT-PCR, are expressed as au compared to a normal sample (1.00 au). The EpCAM gene is expressed in all tissues but one HCC neoplastic sample in which EpCAM expression was very low. As a positive control, EpCAM expression in CC tissues was more than 1000 folds compared to control was used. In normal donor livers, its expression ranges from 0.11 to

10.37 au and in BA 3.55 to 18.94 au In liver cancers, it is found to be very high variable, ranging from 0.85 to 201 au and 0.093 to 557 au in HB and HCC, respectively. This variability had been also reported in HCC where protein expression was assessed by IHC (Yamashita *et al.* 2009). As shown in Figure 5.2, the distribution of EpCAm mRNA in hepatic malignancies has a distinguished pattern similar to the CD90 distribution. EpCAM expression in normal tissues is more homogenous than liver diseases and EpCAM expression in cirrhotic and liver cancers BA, HB, and HCC are significantly difference with normal liver tissues ($p<0.05$).

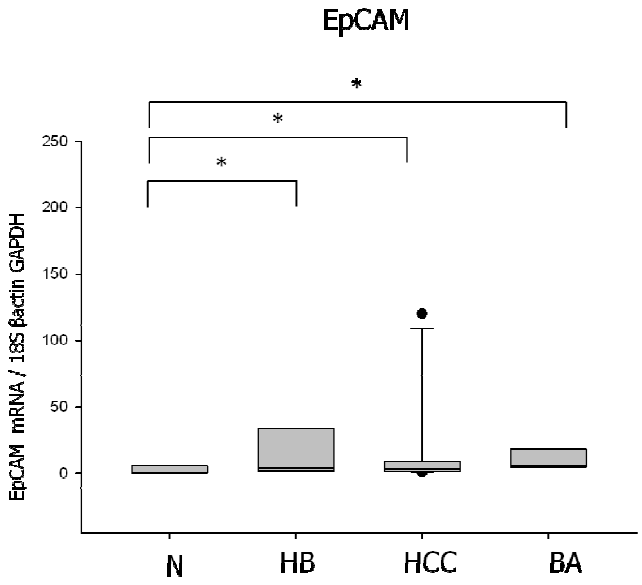

Figure 5.2. Distribution of EpCAM mRNA in human liver. EpCAM expression is normalized to three reference genes 18S-RNA, β-actin, and GAPDH, and is expressed as au compared to one normal tissue. Samples size: Normal N = 6; Hepatoblastoma HB = 7; Hepatocellular Carcinoma HCC = 18; Biliary atresia BA= 5. Student's *t*- test * $p<0.05$.

5.4. Distribution of CD133 (Prominin-1)

The CD133 mRNA levels of a total of 59 tissues samples were analyzed, by qRT-PCR. Interestingly, not all the samples express CD133 mRNA, even in neoplastic tissues. This result is concordant with previous report that showed CD133 positivity only in about 70% of HCC tissues (Lingala *et al.* 2010). Figure 5.3 shows the distributions of the CD133 in positive 41 samples. In normal donor livers, CD133 expression ranges from 0.07 to 1.00 au and in BA ranges from 0.40 to 2.60 au In liver cancers, its expression was highly variable ranging from 0.03 to 2.93 and 0.05 to 1.21 au in HB and HCC, respectively.

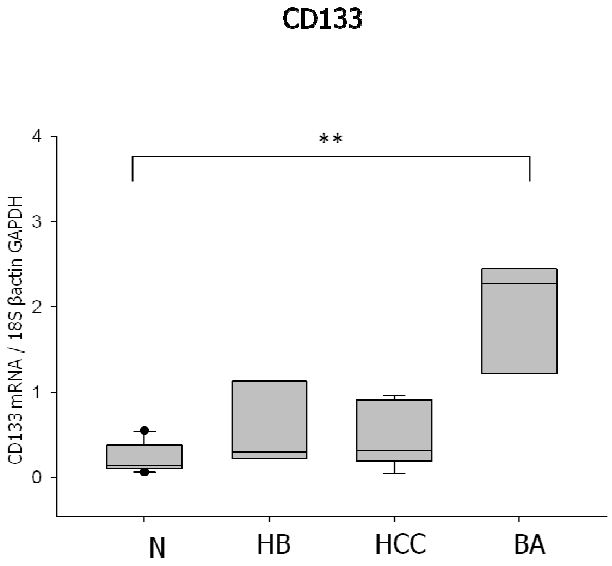

Figure 5.3. Distribution of CD133 mRNA in human liver. CD133 expression is normalized to three reference genes 18S-RNA, β-actin, and GAPDH, and is expressed as au compared to one normal tissue. Samples size: Normal N = 12; Hepatoblastoma HB = 7; Hepatocellular Carcinoma HCC = 9; Biliary atresia BA= 5. Student's *t*- test ** $p<0.01$.

As shown in Figure 5.3, the distribution of CD133 mRNA in hepatic malignancies is found to be more unevenly distributed as compared to CD90 and EpCAM. There is no significant difference between CD133 expression between normal tissue and liver cancers. However, CD133 mRNA is preferably expresses in cirrhotic liver BA ($p<0.05$ to N).

5.5. Distribution of OCT4

OCT4, an embryonic pluripotency transcription factor, is closely-associated with embryonic stage and pre-fetal. The OCT4 mRNA levels from a total of 45 tissues samples analyzed in this study, estimated by qRT-PCR, are expressed as au, compared to a normal sample as 1.00 au In normal donor livers, its expression ranges variably from 0.01 to 1.00 au and in BA from 0.53 to 2.60 au In liver cancers, its expression is found also to be scattered and ranges from 0.26 to 3.49 and 0.28 to 1.31 au in HB and HCC, respectively. There is no significant difference between OCT4 expression between normal and liver cancers (Figure 5.4).

However, it is really important to pay intention that the identification of OCT4 mRNA is debatable, especially in adult tissues. Even though this data may represent distribution of OCT4 in transcription level, further analysis in protein level such as immunostaining must be conducted.

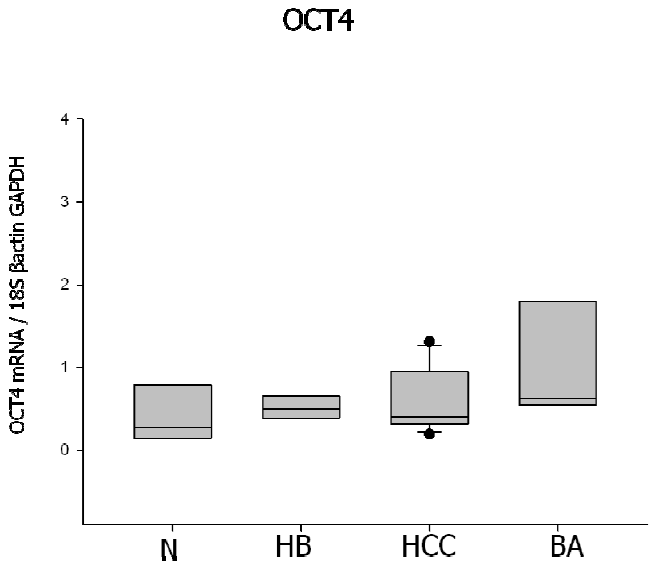

Figure 5.4. Distribution of OCT4 mRNA in human liver. OCT4 expression is normalized to three reference genes 18S-RNA, β-actin, and GAPDH, and is expressed as au compared to one normal tissue. Samples size: Normal N = 9; Hepatoblastoma HB = 7; Hepatocellular Carcinoma HCC = 13; Biliary atresia BA= 5.

5.6. Distribution of Cytokeratin 19 and Cytokeratin 7

Beside studies of surface markers expression, we decided also to examine cytokeratins (CK) CK19 and CK7 mRNA distribution in liver. CK19, and perhaps CK7, are epitopes recognized by OV6 antibody together with CK14. Expression of CK19 and CK7 are found to be high in bile duct cancer as a result of high proliferation of cholangiocytes . Based on this knowledge, we used three tissues of CC as positive controls of the reaction. The CK19 and CK7 mRNA levels were assessed in in 48 tissues samples by qRT-PCR, expressed as au compared

to a normal sample with value 1.00 au For CC samples, the mRNA levels of CK19 and CK7 were 118.97 and 10.47 au, respectively.

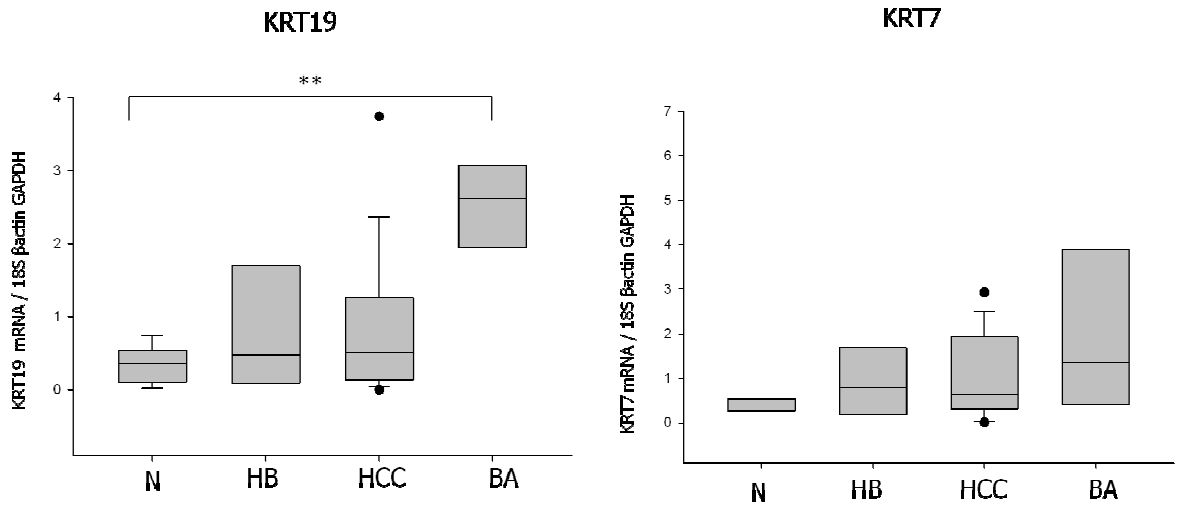

Figure 5.5. Distribution of cytokeratin 19 and cytokeratin 7 mRNA in human liver. mRNA expressions are normalized to three reference genes 18S-RNA, β-actin, and GAPDH, and are expressed as au compared to one normal tissue. Samples size: Normal N = 10; Hepatoblastoma HB = 7; Hepatocellular Carcinoma HCC = 17; Biliary atresia BA= 5. Student's *t*- test ** $p<0.01$.

As shown in Figure 5.5, in normal livers CK19 expression ranges from 0.02 to 1.00 au and from 1.77 to 3.39 au in BA. In HCC, it ranges from 0.10 in distal and 1.94 in neoplastic tissue and from 0.03 to 2.52 au in HB. There is a significant difference of CK19 expression between BA and normal tissues ($p<0.01$). For CK7, its expression ranges in normal liver from 0.03 to 1.36 au and in 0.81 to 4.12 au BA. In HB and HCC, it ranges from 0.05 to 5.85 and 0.06 to 2.93, respectively. There is no significant difference among all groups.

To summarize, the list of values of gene expression result of stem cells markers examined in hepatic malignancies is described in Table 5.2, as compared to a normal liver sample, In the case of HB and HCC, data report both neoplastic tissues and cirrhotic tissues.

Table 5.2. Gene expression data expressed as arbitrary unit in several hepatic malignancies. Data report the median value and the interval for each category

	mRNA expression values in arbitrary unit (median [min-max])			
	Normal	BA	HB	HCC
CD90	0.31 (0.05-1.00)	1.22 (0.24-1.91)	0.71 (0.29-6.05)	1.34 (0.11-13.33)
EpCAM	0.73 (0.11-1.00)	6.20 (3.55-18.94)	3.82 (0.85-201.40)	2.92 (0.41-557.57)
CD133	0.18 (0.07–1.00)	2.27 (0.40–2.60)	0.30 (0.03-2.93)	0.32 (0.05-1.21)
OCT4	0.33 (0.01 – 1.00)	0.62 (0.53–2.60)	0.49 (0.26-3.49)	0.50 (0.25-1.31)
CK19	0.37 (0.02-1.00)	2.63 (1.77-3.39)	0.48 (0.03-2.52)	0.63(0.10-1.94)
CK7	0.53 (0.03-1.36)	2.50 (0.81-4.12)	0.80 (0.05 – 5.85)	1.22 (0.06-2.93)

5.7. Differential Expressions between Neoplastic and Distal Tissues

To observe the differential expression due to the genes up-regulations in the tumoral compared to non tumoral tissue (distal) of liver cancers, we studied the mRNA expressions of several SCs markers described above in paired samples of the same patient. As we assumed that the basal expression of these genes varies in every patient, the comparison in each individual will give a more meaningful and relevant information on the possible regulation. In total, we checked 8 paired tissues from cancer patients, consisting in 3 HB (HB-1, HB-2, and HB-3), 4 HCC (HCC-1, HCC-2, HCC-3, and HCC4), and 1 CC (CC-1). The differential expressions between neoplastic to distal were calculated using the ratio: neoplastic mRNA / distal mRNA. Ratio more than 1.00 indicated folds of higher expression in neoplastic tissues in individual sample.

As shown in Figure 5.6, the most interesting pattern is observed for CD90 and EpCAM. Almost all samples showed drastic up-regulations in neoplastic compared to distal tissues. Expressed as median (min – max), folds of up-regulations are 9.27 (0.56 – 18.24) and 41.51 (0.04 – 235.83) for CD90 and EpCAM, respectively. The data of CD90 mRNA was also verified by protein expression by Western Blot (Figure 5.7). For OCT4, all samples showed a homogenous though modest up-regulation with a median value 1.13 (0.19 – 3.66). For CK19, the difference is more variable with values ranging from 0.07 – 27.56 (median 1.82). Interestingly, in HCC samples, differential expressions of CK19 and CK7 seem to be complementary: high up-regulation of CK19 is accompanied with lower up-regulation of CK7, and vice versa.

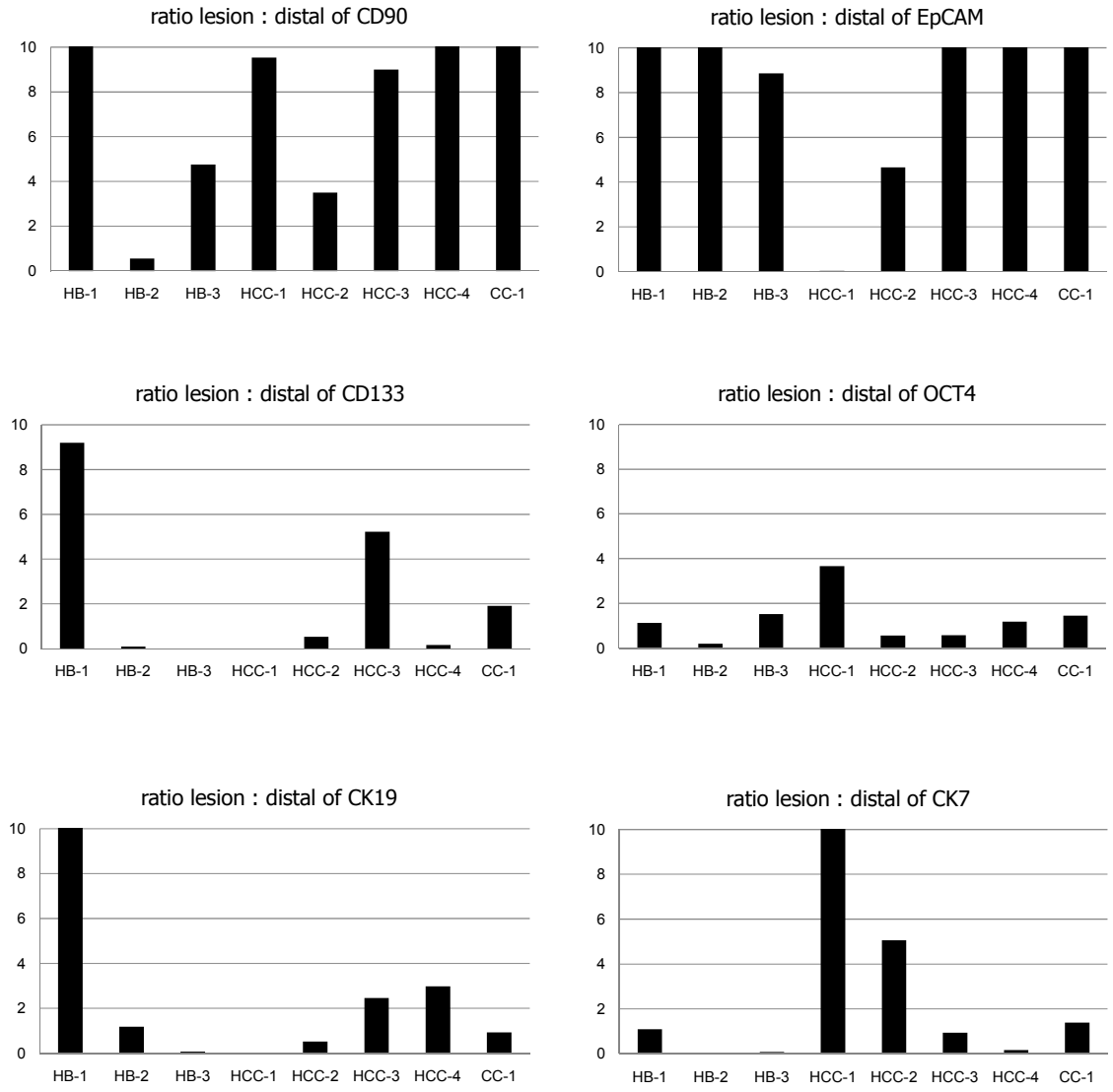

Figure 5.6. The mRNA differential expression between tumoral and distal tissues in human liver cancer. Stem markers genes analyzed CD90, EpCAM, CD133, OCT4, CK19 and CK7. Value of ratio > 1.00 indicated higher expression in tumoral (neoplastic) than distal. HB = hepatoblastoma, HCC = hepatocellular carcinoma.

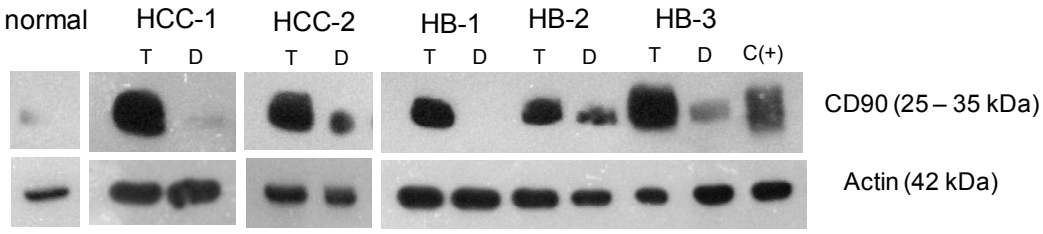

Figure 5.7. The CD90 protein differential expression between tumoral and distal tissues in human liver cancer. Normal = normal liver, HCC = hepatocellular carc inoma, HB = hepatoblastoma.

5.8. Distribution of Ct values

Another helpful way to check the variability of target genes expression in the sample is by checking the distribution of cycle threshold (Ct) values in the PCR reaction. Even though gene expression analysis must be calculated with normalization to reference genes and the efficiency of the primers, graphic of Ct would give a direct comparison between two samples groups within same gene. For qRT-PCR we used similar quantity 25 ng of cDNA for all genes.

We compared the ct values of all target genes CD90, EpCAM, CD133, OCT4, CK19, and CK7 in groups of normal liver and neoplastic nodules. Neoplastic nodules examined in this study included HCC, CC, and HB. The scheme in Figure 5.8 shows the minimum and maximum Ct of the samples:

i. The Ct values of three housekeeping genes used are relative stabile in normal and diseased tissues.

ii. The distribution of Ct values of normal tissues is more limited than neoplastic nodules for all genes, particularly for the CD90 gene.

iii. Lower Ct values in neoplastic nodules means higher template in the samples, i.e. higher expression of the target gene.

The mean values of Ct of these groups are described in Table 5.3, (mean ± SD). In concordance with data of gene expression of CD90 and EpCAM, the difference of the Ct between normal liver and neoplastic nodules is clear for both genes. It is important to put attention that the PCR condition, experimental set up, and efficiency of primer sets of different target genes are variable, and the direct comparison of Ct distribution is only valid for sample in the same genes and not among different genes.

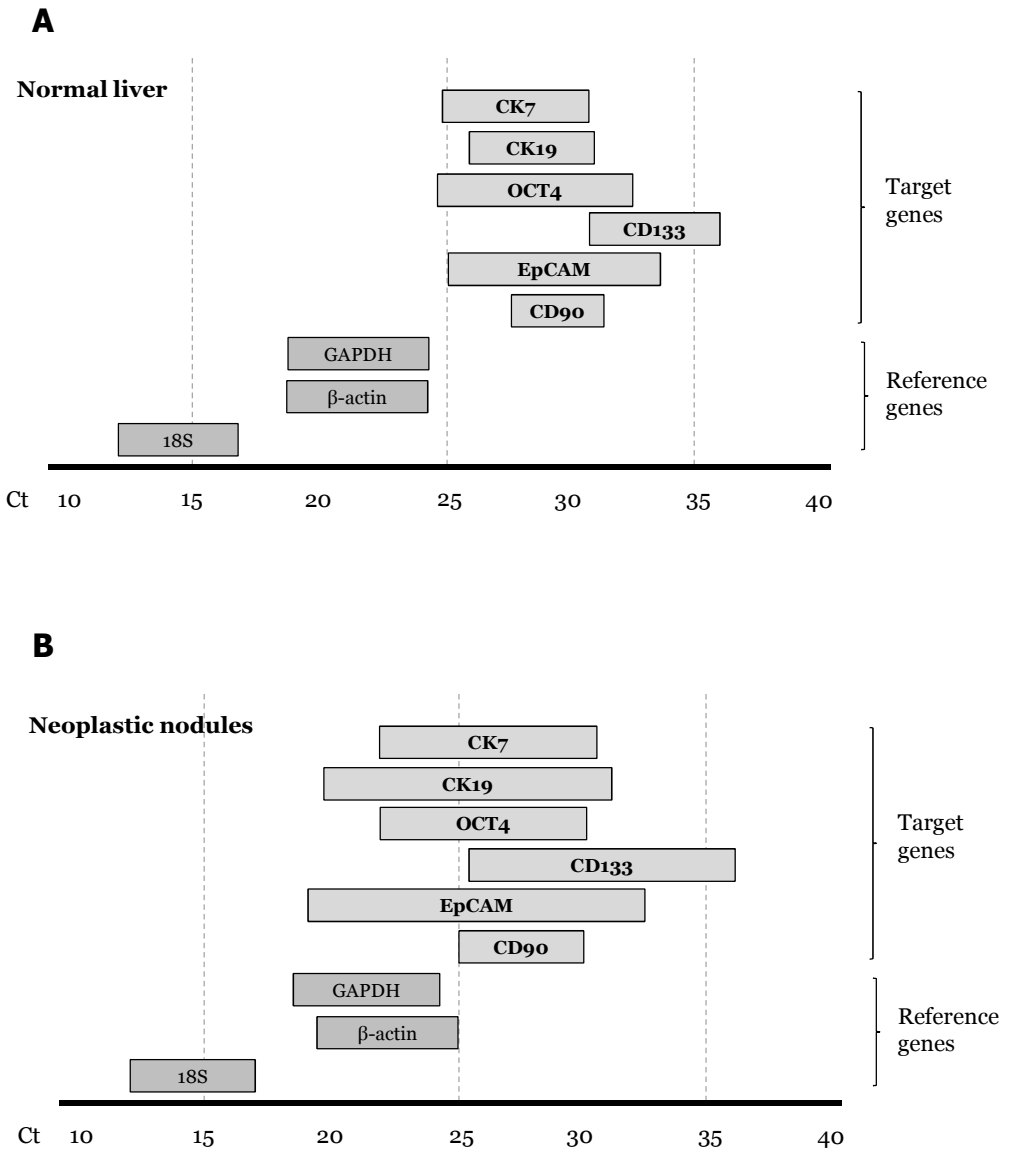

Figure 5.8. The distribution of cycle threshold (Ct) values of qRT-PCR in liver cancers. A scheme of distribution of Ct values of reference genes and target genes of **A.** normal liver donor, and **B.** neoplastic nodules of PLCs HB, HCC, and CC. Bar graphic represents minimum and maximum Ct.

Table 5.3. Cycle threshold values of qRT-PCR of normal tissues and neoplastic nodules of human PLCs. Values are expressed as mean ± SD

	Ct values (mean ± STD)	
Target genes	Normal tissues	Neoplastic nodules
CD90	30.54 ± 0.94	27.48 ± 1.65
EpCAM	30.31 ± 3.37	27.00 ± 4.56
CD133	33.51 ± 1.90	32.22 ± 2.79
OCT4	27.34 ± 2.37	26.62 ± 1.55
CK19	28.75 ± 1.46	27.72 ± 2.87
CK7	27.16 ± 1.88	26.40 ± 2.37

Chapter VI – Result 3

ABCG2 Expression: Drug Resistance and CSCs

6.1. Distribution of ABCG2 mRNA in Human Liver Tissues

Even though ABCG2 is associated with CSCs marker, the ABCG2 study was separated from previous analysis because the use of ABCG2 is more based on its functional activity. ABCG2 is also expressed in mature cells. In this chapter, we report the study on the expression of ABCG2 both *in vivo* in clinical tissues samples and *in vitro* in hepatic cell line models.

The ABCG2 mRNA levels from 59 tissues samples was analyzed and estimated by quantitative real time PCR. Values are expressed in arbitrary units (au), compared to a normal sample to which a value of 1.00 au is given.

As shown in Figure 6.1, the ABCG2 mRNA expression in normal liver is related with age. ABCG2 expression in children (age 3 to 14 y) is lower compared to adults (age 25 to 68 y) with mean value of 2.84 ± 2.38 and 13.77 ± 8.95, respectively, with expressions ranging from 0.92 – 7.26 in children and 4.08 – 23.07 in adults ($p<0.05$). This consequence is likely due to the function of ABCG2 itself to protect the cells from extra compounds and person with older age are tend to receive more exposure to drugs and other compounds than children.

The ABCG2 mRNA expression is observed to be highly variable (au. ranged from 0.41 in a BA patient to 56.63 in a HCC). The ABCG2 expression in BA is the most homogenous compared to other groups. Low levels in ABCG2 expression are found mainly distributed in BA patients and young normal livers, whereas the highest ones are observed in tissues originating from HCC, HB, and other malignancies (Figure 6.2). The expression of group HCC to group normal liver is significantly different ($p<0.05$).

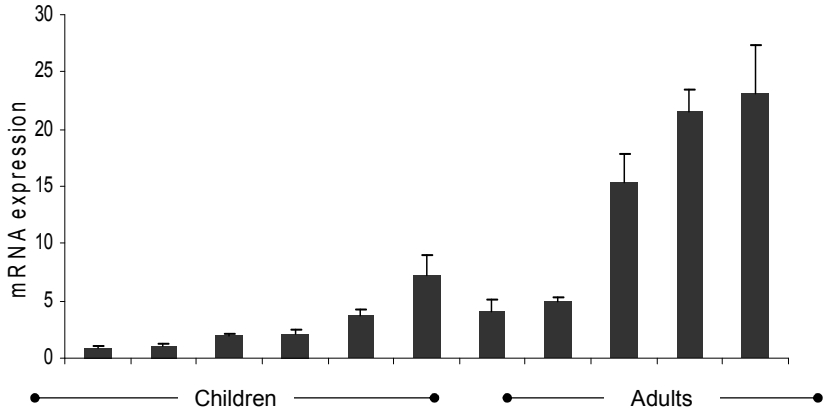

Figure 6.1. ABCG2 mRNA expression in normal liver tissues. The ABCG2 expression is concordant with age (n = 11, children: 3 – 14 y, adult: 25 – 68 y). ABCG2 expression is normalized to three reference genes 18S-RNA, β-actin, and GAPDH, and expressed as au compared to one child tissue = 1.00 au.

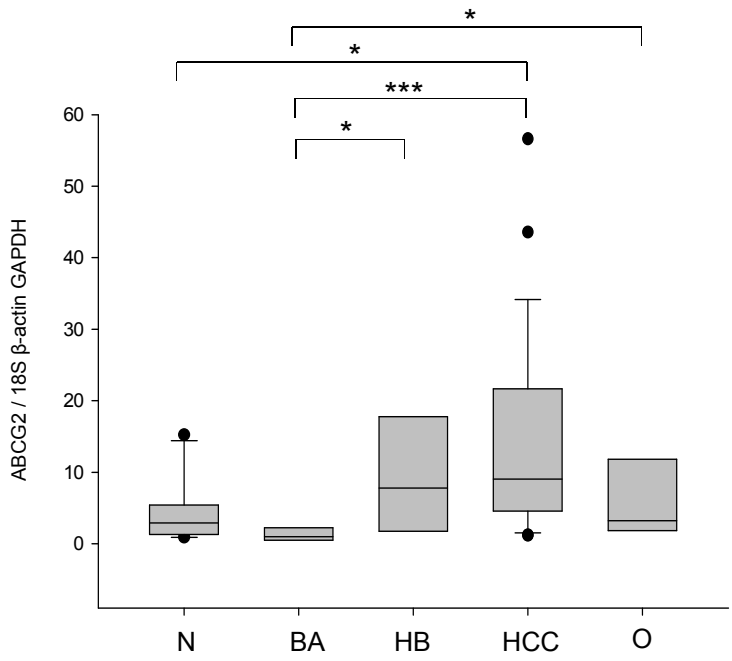

Figure 6.2. ABCG2 mRNA expressions in several hepatic malignancies. ABCG2 expression was normalized to three reference genes 18S-RNA, β-actin, and GAPDH, and expressed as au compared to one normal tissue. Sample size: Normal N = 10, Biliary atresia BA = 5, Hepatoblastoma HB = 7, Hepatocelular carcinoma HCC = 28 and Other tumors O = 5. Student's *t*- test * $p<0.05$, *** $p<0.001$

6.2. Expression of ABCG2 against drug therapy: models in vitro

Previous data demonstrated the expression of ABCG2 in human tissues. However, the limitation of the result is that as treatments may influence the expression of the transporter and samples without this intrinsic and unavoidable bias are not available, the real "normal" value cannot be assessed. To overcome this issue, several hepatic cell lines were used to see the effect of the treatment. For this reason the use of cell lines derived from human liver cancer may help in unravel this limitation.

Four human hepatic cell lines with different stage of differentiations were analyzed (Figure 3.1). We used immortalized hepatocytes IHH, well-differentiated HCC HepG2 and HuH-7, and poor-differentiated HCC JHH-6. We checked albumin transcription markers for hepatic markers on these cell lines. Albumin mRNA was detected in HepG2 and HuH-7, but not in JHH-6 cells. We assume that differentiated cancer cells HepG2 and HuH-7 may still function to produce albumin, but not undifferentiated cells JHH-6 (data not shown).

As shown in Figure 6.3, a close relation is observed between cells differentiation and ABCG2 expression. With the expression in IHH cells defined as 1.00 au, the highest level of ABCG2 mRNA expression was observed in the most undifferentiated cells JHH-6 (76.27 ± 6.00), followed by the more differentiated cells HepG2 (53.52 ± 19.06) and HuH-7 (35.07 ± 10.96). In line with the mRNA results, the ABCG2 protein (72 kDa) is detected in membrane fraction of tumoral cell lines HuH-7, HepG2, and JHH-6, but not in IHH (Figure 6.4).

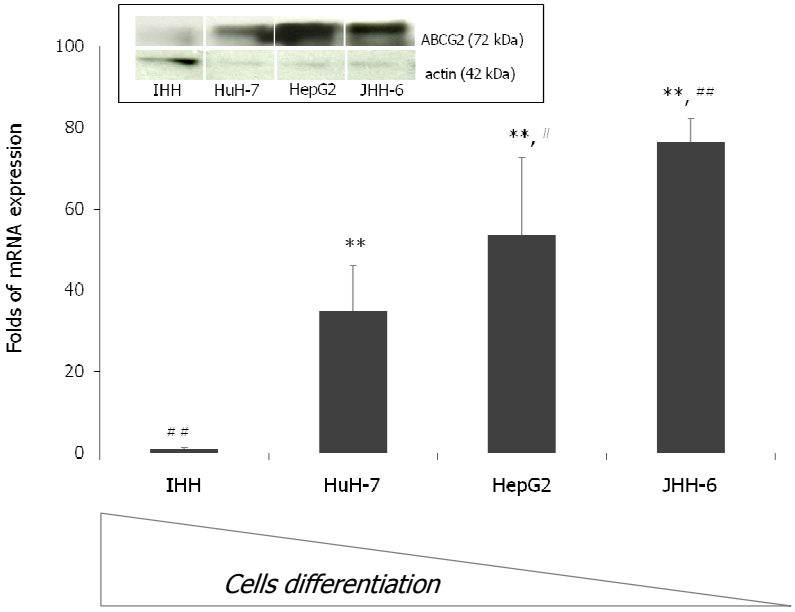

Figure 6.3. Basal ABCG2 mRNA and protein expression in hepatic cell lines. The basal ABCG2 mRNA was found to be highest in less differentiated cells JHH-6 than differentiated cells HuH-7 and JHH-6 and immortalized hepatocytes IHH. IHH expression was used as control (1.00 au). Student's *t*- test: ** $p<0.01$ to cells IHH; # $p<0.05$ and ## $p<0.01$ to cells HuH-7.

To functionally asses the ABCG2 activity in response to drug exposure, we assessed the cytotoxicity effect to these cells by a 24 hour exposure to 5 µM doxorubicin, a drug used in the treatment of liver cancer. As shown in Figure 6.4, after 24 hours of incubation, this dose is cytotoxic to about 50% cells populations as assessed by MTT assay. To explore the effect of similar dose and similar treatment time of doxorubicin to different cell lines, we decided to expose these cell lines to mentioned dose and duration.

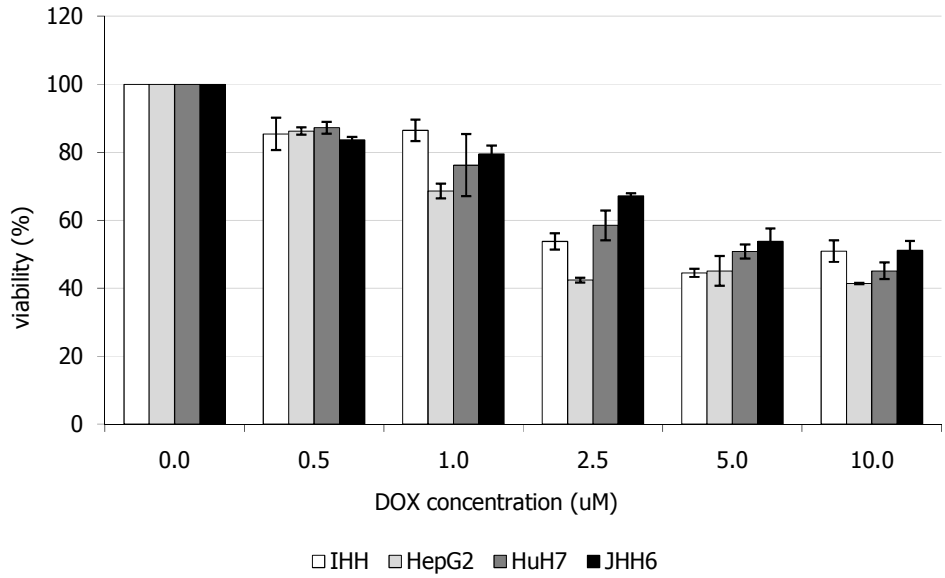

Figure 6.4. Cells viability after dose-dependent DOX treatment for 24 hours. The cells were treated with 0.0 – 10.0 μM of doxorubicin for 24 hours. All cells showed more less 50% viable in 5 μM dose. Viability of cells were assayed using MTT dye reduction test with un-treated cells used as control.

As shown in Figure 6.5, the exposure to doxorubicin induces an up-regulation of ABCG2 mRNA in all the cell lines tested. Interestingly, the extent of the up-regulation is inverse to the basal level of expression as IHH and HuH-7 show a significant increment of gene expression (15.25 ± 4.45 and 9.36 ± 0.48 folds, respectively) compared to HepG2 and JHH-6 (1.14 ± 0.68 and 1.38 ± 1.58 folds, respectively). As in the human tissues, qualitative protein blots from total membrane fraction followed the level of the gene expression. In basal condition of IHH cells, the ABCG2 protein band (72 kDa) is not detected, but it is noticeable after exposure to doxorubicin. The same occurred in HuH-7 while no difference is observed in

and HepG2 and JHH-6 cells where the treatment does not significantly induce the over-expression of the transporter (Figure 6.5).

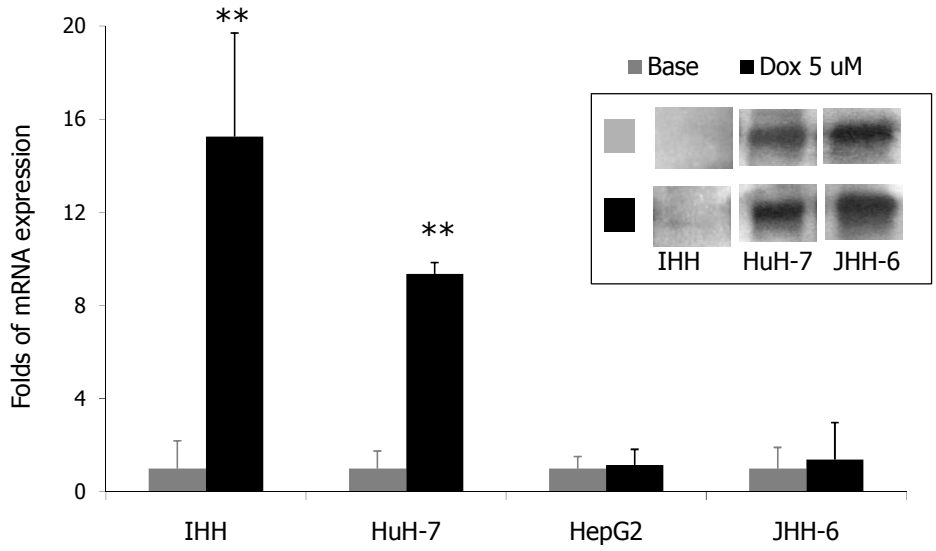

Figure 6.5. ABCG2 mRNA and protein expression after exposure to 5 µM doxorubicin for 24 hours. mRNA up-regulations of ABCG2 were observed in all cell lines with different folds. Student's *t*- test ** $p<0.01$ compared to basal expression (inset: protein blots).

6.3. Activity of ABCG2

To assess the activity of the ABCG2 protein, we used the Hoechst 33342 efflux functional assay where lower intracellular fluorescence indicates smaller dye content due to a higher efflux from the cell. To establish the potential role of ABCG2 in this system, the assay was performed in the absence and presence of verapamil, a potent ABCB1 inhibitor but a weak ABCG2 inhibitor (Zhang *et al.* 2005).

Preliminary experiments demonstrated no effect on cell viability (MTT test) by either Hoechst 33342 or verapamil up to at concentration of 20 μM and 10 μg/mL, respectively. Moreover, 3 hours time frame also does not affect cells viability (data not shown). The intake fluorescence dye value is defined as au with normalization with the amount of total protein using a complex Copper-BCA assay. The IHH cell line is excluded in functional test because of a very low ABCG2 basal expression.

The efflux potential is concordant with the increase concentration of Hoechst (2.5 – 10.0 μg/mL) in all three cell lines tested. Considering the intracellular dye intake of 2.5 μg/mL Hoechst as 100% (1.00 au), the dye content decreases for as much as 47%, 49%, and 46% in HuH-7, HepG2, and JHH-6 cell lines (Table 6.1).

In the presence of 1 μM verapamil, the difference of the intracellular dye intake with and without verapamil indicates the ABCG2 efflux activity. Higher amount intracellular intake fluorescence indicates lower efflux capacity. Among all three cell lines, the smallest difference with and without verapamil is observed in JHH-6 indicating that ABCG2 efflux capacity was dominant in JHH-6 compared to HepG2 and HuH-7 (Figure 6.6).

Table 6.1. The Hoechst efflux capacity of the HCC cell lines

Hepatoma cell lines	The intracellular dye intake of Hoechst 33342 (% au)		
	2.5 µg/mL	5.0 µg/mL	10.0 µg/mL
HuH-7	100.0 ± 6.4	88.4 ± 5.5	53.0 ± 2.8
HepG2	100.0 ± 6.2	74.7 ± 3.1	51.2 ± 2.8
JHH-6	100.0 ± 5.8	80.1 ± 2.1	53.7 ± 4.0

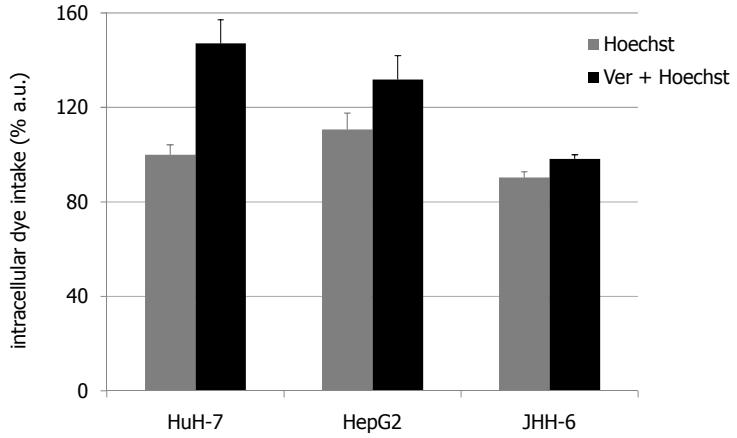

Figure 6.6. Functional capacity of ABCG2 to export the Hoechst 33342. Difference in intracellular content with and without the ABCB1 inhibitor verapamil was observed to be small in JHH-6 followed by HepG2 and HuH-7 indicating major functional activity of ABCG2 in JHH-6. The intracellular intake of HuH-7 with Hoechst 33342 was considered as 100 % au. All the results were normalized to total protein quantification.

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Chapter VII

General Discussion

7.1. The stem cells population in primary liver cancers

The evidences of CSCs hierarchy theory had been demonstrated by several studies, both in circulating or solid tumors. A small population of cells of the cancer with distinct phenotype has the ability to induce tumor in xenograft model and might have potency to differentiate into multiple lineages. Until now, evidences of the identifications in liver cancer are still very limited.

In this thesis, in period 2008 – 2010, we isolated a population of cells from 3 parts of liver cancer HCC and CC: neoplastic nodules, peripheral tissues, and distal tissues. As shown previously in Chapter 4, the cells have phenotypes $CD90^{+}$ and $CD44^{+}$, but however, they are also positive for MSCs markers mRNA CD90, CD44, CD105, CD166, and CD29, and negative for endothelial marker CD31, and hematopoietic cells CD34 and CD45. These phenotypes were found in all cells populations from all 3 parts liver cancers. Several populations positive for AFP and albumin indicate a partial commitment to hepatic lineage. In addition, samples with HCV positive seem also to express CD133 and higher OCT4.

As shown in Figure 4.2 and 4.3, these cells have spindle-shape like fibroblasts morphology and they have capacity to form clonal colonies, both on plastic surface and three dimensional matrix indicating anchorage-independent and clonogenic capacity. This phenotype had been reported as indicative of human liver stem cells (HLSCs) isolated from adult normal liver. The HLSCs were able to differentiate in mature hepatocytes, even osteogenic and endothelial cells. Furthermore, they contributed to regeneration of liver parenchyma in severe-combined immunodeficient mice. However, the HLSCs could not be differentiated into adipogenic lineage (Herrera *et al.* 2006).

MSCs or commonly known also as stromal stem cells are non-hematopoietic precursor cells, mainly found in bone marrow, which contribute to the maintenance and regeneration of wide varieties of mesoderm lineage such as bone, cartilage, muscle, and adipose tissues (Stagg 2008). Moreover, cells co-purified with MSCs and named as multipotent adult progenitor

cells (MAPCs) differentiated at single cell level not only into mesenchymal cells but also in cells with visceral mesoderm, neuroectoderm, and endoderm *in vitro* (Jiang *et al.* 2002). The MSCs are capable to adher to plastic cell culture flask and are identified as on specific surface markers that distinguish these cells from others.. The MSCs in human and mouse must positive for CD105 (endoglin), CD73 (ecto-5'-nucleotidase) and CD44 (hyaluronate receptor) and for negative for CD31 (endothelial cell marker) and CD45 (hematopoietic cell marker).

The MSCs are found not only in bone marrow, but also in many adult tissues. Because of their differentiation capacity, the MSCs become good tools in regenerative medicine. The MSCs isolated from bone marrow and cord-blood can be differentiated into hepatocytes-like *in vitro*, including production of albumin, glycogen storage, urea secretion, uptake of low density lipoprotein, and phenobarbital-induced cytochrome P450 activity (Lee *et al.* 2004). Furthermore, hepatocytes derived from adipose-MSCs could expand and integrate into liver parenchyma in mice model (Banas *et al.* 2007). Beside MSCs in normal tissues, the MSCs in human solid cancers have also been reported in various types of cancers such as breast cancer, glioma, and many others (Karnoub *et al.* 2007;Nakamura *et al.* 2004).

Based on the mRNA data, our cell populations are fit with suggested MSCs population identification from the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (Dominici *et al.* 2006; Horwitz *et al.* 2005). The committee proposed three minimal criteria to name cells as MSCs: 1) plastic adherent behavior in standard culture condition; 2) expression of CD105 and CD90, but not CD45, CD34; 3) possible potency to differentiate into mesenchymal lineage *in vitro*.

We performed also trans-differentiation experiments. As reported, cells with this phenotype obtained from normal liver could be differentiated not only into mesodermal lineages, but also into endodermal lineage (Herrera *et al.* 2006). However, because our cells populations were obtained from cancerous tissues, their multipotent capacity might be more restricted compared cells from bone marrow and normal tissues. Furthermore, these cells have limited growth when they were plated in specific medium for MSCs, indicating different behavior of these cells compared to normal MSCs obtained from normal bone marrow or tissues.

We tried to induce the cells into mesodermal lineage adipocytes and endodermal lineage pancreatic cells. Our result might indicate a possible role of these cells in the expansion of cancer and aggressive spread, and even metastasis, with regards to their potency. As shown in Figure 4.6, from seven cells populations of neoplastic and distal tissues, one cell population showed a clear differentiation into adipocytes. The cells showed drastic up-regulation of PPARG mRNA, a master regulator of adipocytes differentiation, and accumulation of lipid vacuoles in cells cytoplasm. This finding could be an indication that stem cells from cirrhotic distal tissue of liver cancer tissue might still be multipotent. This sample is obtained from a liver cancer with a moderate level of differentiation. We assume that this tissue is most 'normal' compared to the others tissues which allow cells-directed differentiation.

As far as the pancreatic differentiation is involved, we observed an induction of somatostatin and/or gastric inhibitory protein (GIP) a member of glucagon, marker for δ cell and α cell, respectively (Figure 4.7). The islets of Langerhans are small organs located in the pancreas that are crucial for glucose homeostasis. Islets typically consist of four types of secretory endocrine cells, namely, the insulin-containing β cells, the glucagon-containing α cells, the somatostatin-containing δ cells, and the pancreatic polypeptide producing (PP) cells (Cabrera *et al.* 2006).

In CC samples, the SST gene is expressed even in basal condition. A previous report showed that biliary epithelium is similar to the gastric or intestinal epithelia and many biliary tract carcinomas showed neuroendocrine differentiation (Marzioni, Fava, and Benedetti 2006). Interestingly, sample which can be differentiated into adipocytes also have higher up-regulations of SST and GIP after pancreatic induction. However, the expression of insulin, marker for β cells of pancreatic islets, is either absent or very low detected, at least at the mRNA level.

However, the only evidence that proves a cell populations as a CSCs population is by *in vivo* model. Our pilot *in vivo* study in nude mice showed that CD90 from cells with concentration up to 500,000 with at least 75% positivity and 95% viability were not able to induce tumor nodule in nude mice 4 months after injection (Table 4.3). Cells CSF01N and CSF09P were selected for this pilot study for their faster growth capacity compared the other

cells. These samples are originated from poor differentiated CC and HCC with cirrhosis. Previously it has reported that CD90 cells from primary tumors could promote tumor growth when they were injected orthotophically into the liver of the SCID/Beige mice (Yang *et al.* 2008a). In our model, we use nude mice FOXN1 (nu/nu) which is less immunodepressant than SCID/beige mice. Nude mouse with spontaneous or targeted deletion in the FOXN1 gene cannot generate mature T lymphocytes. In addition to lack of T lymphocytes, SCID/beige mouse cannot produce B lymphocytes and natural killer cells. Another additional point to be considered is that we injected the cells subcutaneously instead of ortothopically in the liver to check whether these cells are able to induce tumor in non-hepatic environment related with their metastasis potential.

Yang *et al.* reported that CD90 cells from PLC and MHCC97L cell lines (moderate to high metastatic potential) were successfully promoted tumor in nude mice when they were injected subcutaneously. However, in our JHH-6 cell line which is very aggressive and undifferentiated, a mouse injected with 1 million JHH-6 CD90+ cells died 10 days after injection, whether mouse with JHH-6 CD90- survived. However, in our pilot study we used only one mouse for positive and negative control, and the death of positive mouse might be or might be not related to injected cells. As mentioned previously both JHH-6 CD90+ and CD90- are also positive for mRNA markers CD29, CD105, and CD166, just as in our primary cells culture. It seems that tumorigenic capacity of CD90 cells is really dependent to the aggresiveness of the cells, but further studies are clearly necessary.

In summary, the absence of tumor 4 months after initiation may be caused by several reasons:

1. The isolated cells obtained from those two samples had limited capacity to induce a tumorigenesis. Their capacity might be related with aggressiveness of cancer and degree of differentiation, but more importantly because of the nature of the cells itself.

2. Longer duration or higher cells concentration is needed to induce tumorigenesis.

3. More immunodepressant mice models are needed, for example: NOD/SCID mice or SCID/Beige mice.

4. Several populations with shared CD90+CD44+ phenotypes in liver cancer. These different cells populations may act differently as CSCs or SCs in cancers.

Interesting result obtained from cell line JHH-6 showed a particular behavior. JHH-6 is a very aggressive HCC cell line with undifferentiated morphology. In basal level, these cells express CD90 mRNA with quantity less than 1% checked by FACS. After magnetic cell sorting and validation of the cells purity, we followed up the changes in cells phenotype during subcultures. Both fractions concurrently expressed CD44 protein. Sorted cells showed that CD90+ cells can generate two clearly distinct populations of CD90+ and CD90- cells, but CD90- cells could harbor only CD90- (Figure 4.10). We continued to subculture the cells until passage 21st after sorting until the CD90+ cells reached composition of positive and negative fraction for about 20% and 80%, respectively. In the other hand, until passage 21st, CD90- cells can be only 100% CD90- cells. This capacity of cells differentiation was also had been reported in ABCG2+ cells of HCC cell lines Huh-7 and PLC (Zen *et al.* 2007) in which ABCG2+ fraction generate ABCG2+ and ABCG2-, and on the other hand ABCG2- could only generate ABCG2- cells. In contrast of CD133, sorted CD133+ and CD133- from Huh-7 and HuCCT1 cells similarly produced CD133+ and CD133- cells during subculture (Yoshikawa *et al.* 2009).

This data shows that CD90+ cells might be at a the higher hierarchy level than CD90- cells in liver cancer and might be one of responsible cell types in generating cancer heterogeneity. However, we should consider the regulation of CD90 expression. Marker Stro-1 had been reported to decrease its expression during cells expansion (Gronthos *et al.* 2003). Regarding cells morphology, it has been reported that in murine pulmonary, CD90- fibroblasts had a more polygonal morphology than the more spindle-shaped CD90+ (Phipps *et al.* 1989). Although these differences had been credited to cells signaling activity, we have not seen any morphological differences in our JHH6 CD90+ and CD90- cells, checked by FACS and microscopy.

7.2. Tumor initiating and/or supporting cells?

Recent studies and further information in cancer cell biology open a new discussion of these cells populations. As mentioned in General Introduction, interaction and cross talk between tumor cells and their microenvironment is crucial for cancer cells maintenance and expansion. Further assays will be needed to study these isolated cells populations, whether they are associated with the cancer associated fibroblasts (CAFs).

Cancer-associated fibroblasts (CAFs) or cancer stromal cells are the most important cell type in the stroma. As described in General Introduction, tumor stroma is essential to support the tumor and the components of the stroma are vital for cancer initiation, growth and progression. The CAFs cells, the most important cells in the stroma, are important in the modulation of biological activities of the cancer, immunity, and expansion.

The presence of the MSCs have been thought to meet the requirements of the CAFs. In addition to their similar spindle-shape morphology, MSCs and tissues fibroblasts are considered to be dynamic cells from at least the same lineage. They might also share the capacity of de- and re-differentiation that allow an overlapping biological impact (Lindner *et al.* 2010). In skin, the MSCs share common surface antigens and exhibit common functions with dermal fibroblasts (Haniffa *et al.* 2007). Because of the existence of MSCs in many types of normal and cancer tissues, trans-differentiation capacity of these cells is thought to be the n key characteristic to reside in the tissue host. The immunosuppresive properties of these cells are inferred to be a specialized function associated to their multipotency.

The migration of MSCs from bone marrow to tumor in experimental sets has been demonstrated in several cancers such as breast, ovary and colon cancers (Dwyer *et al.* 2007;Coffelt *et al.* 2009;Shinagawa *et al.* 2010). A review of breast cancer described that the MSCs from bone marrow or other tissues responds to numerous attractant signals including TGF-β, VEGF, and IL-6 from the tumor. Then in the tumor, MSCs joined the tumor stroma and produced bioactive molecules. The interaction of MSCs and tumor cells promoted tumor growth and metastasis (El-Haibi and Karnoub 2010). The mixture of human bone marrow-

derived MSCs and weakly metastatic breast cancer cells caused the cancer cells increase metastatic potency in xenograft models. The cancer cells stimulate chemokine CCL5 from the MSCs which facilitate metastasis spread by a mutual interaction between stromal cells and cancer cells (Karnoub *et al.* 2007). In addition, a MSCs-CSCs niche in breast cancer has been studied. Both CSCs and MSCs are organized in a cellular hierarchy in which ALDH expressing mesenchymal cells regulate CSCs through cytokine networks (Liu *et al.* 2011).

This new information opens further questions whether all cells isolated, or partially MSCs cells, are associated with CAFs in HCC. As described previously, the isolated cells population from the PLCs expressed several MSCs markers. They might be still multipotent to differentiate into other cell types, shown by expression of somatostatin after induction. Plasticity is one important requirement to act as the CAFs.

In 2009, Mazzocca demonstrated a tumor-stromal interaction between CAFs and HCC cells. CAFs were essential for tumor growth and metastatis and mutually, tumor cells stimulated proliferation of the CAFs. HCC invasive cells produce high levels of connective TGF (CTGF) and generate tumors with high stromal component *in vivo*. The TGF-β inhibitor LY2109761 inhibited CTGF and consequently diminished tumor growth by inhibiting the proliferation of CAFs. In addition, by TGF-β1 stimulation, non-invasive HCC cells form tumor with high stromal content and CTGF expression, and again is inhibited with LY2109761 (Fransvea *et al.* 2009;Mazzocca *et al.* 2010).

Theoretically, cells with MSCs phenotypes may act as both tumor initiating and supporting cells in liver cancer. Their trans-differentiation and migration potency may allow them to be exist in the liver and replenish many type of cells. In regards to the CSCs theory, if mutations occur in this cell population, they could initiate and promote cancer and differentiate into cancer mass. On the other hand, by secreting soluble factors and creating supportive microenvironment on several difference stages, the MSCs may also responsible to maintain the expansion of the cancer.

However, the real question is how tumorigenic cancerous our isolated cells can be in the development of liver cancer. One limitation in this study that we did not perform a single

clone culture, therefore, the isolated cells conducted for plasticity and *in vivo* studies could be resulted from several different cells which could be derived from several cells populations which share similar phenotypes CD90 and CD44. Another hypothesis that could be taken is whether there is a small subpopulation of CSCs in the isolated cells or in MSCs population in the liver cancer.

Based on our preliminary data in nude mice after 4 months, we thought that the activity of these cells to initiate cancer and act as the real CSCs must be supported by a friendly environment that favors cancer development. As a preliminary data, when we checked for α-SMA mRNA, all cells isolated from PLCs showed higher expression of this gene compared to an established cell line with similar phenotype in standard culture medium. This observation may indicate an activation of fibroblasts with CAFs characteristics in primary tumor. Interestingly, even though variations of α-SMA were observed, in many samples, this gene is more expressed in cells populations isolated from PLCs than one population from non-tumoral tissues (data nor shown). The HLSCs from normal liver with similar phenotype with our cells population did not express α-SMA (Herrera *et al.* 2006). We assumed that there must be an activation of these cells in a supportive microenvironment of primary cancers. However, this argument must be proven with further studies.

In this study, we assumed that presence of both CSCs and MSCs in the liver cancers have several important concerns: 1) MSCs, CSCs, and activated-resident-fibroblasts in cancer share common markers and overlapping function; 2) the MSCs could be recruited and migrated from bone marrow or other tissues to give support for tumorigenesis; and 3) it is widely known that MSCs are also found in normal liver tissues. If mutations occurs and change the nature of the cells, they might promote tumor growth and at the same time alter their plasticity which may act as supportive components that favor microenvironment of the tumor. This last argument is a model of CSCs theory and thus will be correlated with small percentage of the CSCs in the whole liver cells population.

The evidence of mutated MSCs as CSCs was demonstrated in sarcoma, a non-epithelial non-hematopoietic cancer derived from embryonic mesoderm. MSCs were found to rapid proliferate and differentiate at locations where especially in children sarcomas are often

found. This capacity might increase the occurrence of transformations into CSCs (Mohseny and Hogendoorn 2011;Honoki 2010).

Taken this information all together, further studies, especially *in vivo* assay, to analyze our isolated cells population will be very important. The combination of the clonogenic MSCs from PLC and non-cancerous MSCs from normal tissues with differentiated HCC cells will give more comprehensive data of tumor-stromal interaction.

7.3. Stem cells marker gene in liver tissues

Even though global gene expression analysis is a potent tool to distinguish different subtypes and to predict prognostic value of HCC patients based on molecular markers, single CSCs marker had been attempted to study its association with clinical significance. Many reports also emphasize the importance of the expression of these markers with cells origin of the cancer and potential future therapy.

In the second task of my thesis we focus our attention to the study of several stem cells markers which are commonly identified in liver cancer. We decided to analyze surface markers CD90 and CD133, epithelial molecule EpCAM, embryonic pluripotency factor OCT4, and more-committed hepatic progenitor markers CK19 together with CK7. We used several groups of liver disease: HCC, HB, BA, and normal tissues. Several samples of CC were used as control for cytokeratins expression. In general, many studies reported their expression in protein level, but we perform the study from a different approach as the mRNA level of several hepatic malignancies simultaneously. This approach provided us information on: 1. the transcription stage of these genes and 2. direct comparison within groups and among groups because we used similar individual samples and RNA extract from each groups.

For quantitative real time PCR data, we used three reference genes 18s-rRNA, β-actin, and GAPDH to normalize the ABCG2 target gene expression. To recheck the result, the use of

recommended single reference gene 18S-rRNA separately for normalization gave similar trends in the result (Bustin *et al.* 2002). All the figures and data in this study represented normalization to three reference genes.

As shown in Figure 5.1 to Figure 5.5, we found that all the genes expressed variably in human liver tissues. However, generally, in normal liver tissues, their variations are more limited compared to diseased liver. This observation could be noticed based on mRNA relative expression compared to a normal control normal sample and also on the Ct distribution in the target gene (Table 5.2 and Table 5.3). Interestingly, the CD90 and EpCAM are expressed significantly higher in liver cancers than in normal tissue ($p<0.05$). Furthermore, these genes are expressed distinctly among malignant livers.

CD90 is a promiscuous molecule expressed in several cell types in the liver, including fibroblast, resident hepatic stem cells, and bone marrow derived cells. When gene study using total RNA extract of tissues specimens is performed, we should be aware of the origin of this expression. From our result, we observed that CD90 mRNA is expressed in all tissues samples although more homogenously in normal than that of diseased tissues. Its expression in HCC, HB, and BA are significantly higher than normal tissues ($p<0.05$ for HB and BA, $p<0.01$ for HCC). The overall distribution of CD90 mRNA in hepatic malignancies has a clear distinguished pattern (Figure 5.1). Based on this result, we assumed that the expression of CD90 in normal liver is relatively stable compared to diseased tissues, and in particularly to cancerous tissues.

The mRNA data from this study is concordant with a recent data demonstrating protein expression of CD90 in HCC with and without bile duct tumor thrombi (BDTT). CD90 protein was highly expressed in poorly differentiated tumors than in moderately or well differentiated tumors ($p<0.05$) (Yu *et al.* 2011). This data is also in agreement with a recent mRNA data that CD90 is more expressed in tumoral tissues than non-adjacent non-tumor tissues (Lu *et al.* 2011).

As mentioned previously, a higher expression of CD90 in tissues samples are also found in our isolated cells. In contrast, Linggala *et al.* reported that not all HCC tissues expressed

CD90 protein (Lingala *et al.* 2010). We assumed that due to variations in mRNA level, found to be high or low expressed, the protein expression might be not detected with low protein expression.

The general distribution of EpCAM mRNA expression in liver malignancies is found to be like the CD90. It is expressed in all tissues, even though its expressions are more variable. The EpCAM mRNA expression in CC is significantly higher than normal tissues, reached more than 1000 folds compared to a control (data not shown). This result is concordant with previous observation that EpCAM expression is found high in CC than in HCC (de Boer *et al.* 1999). The EpCAM expression in HCC, HB, and BA is significantly higher than normal tissues ($p<0.05$). Its expression in these 3 malignancies is also more variable than in normal liver (Figure 5.2).

Both EpCAM and CD90 are expressed in early hepatic stem cells, even though the origin of the hepatic stem cells itself is still unclear. Regarding this, we can assume that in liver cancer, there is a fast proliferation of different cells populations with CD90 and EpCAM phenotype, most probably activation of early progenitor cells. This over-expression might be due to so-called reaction of stem cells or fibroblast-like cells, migration of MSCs in response to hepatic injury, or circulating stem cells (both hematopoietic and mesencymal) in liver blood vessels. However, for CD90, it has been reported in rat liver fibrosis model using 2-acetylaminfluorene/partial hepatoctomy, CD90 is expressed not in oval cells during cells proliferation, but in myofibroblasts or activated hepatic stellate cells (Dezso *et al.* 2007).

Song and colleagues proposed a significance of CD133 expression to HCC prognostic (Song *et al.* 2008). In contrast, a recent article suggested that evaluation of CD133+ or CD133+/CD44+ cells in HCC and in colocteral carcinoma metastasis was not sufficient to serve as single prognostic parameter (Salnikov *et al.* 2009). We showed that in our samples not all of HCC samples expressed CD133 mRNA, and possibly also protein (Subchapter 5.4). This observation raises questions whether CD133 is a good marker for prediction of clinical significance. Our result is concordant with recent article from Lingala *et al.* that cancer tissues with CD133 expression was found in only two third of HCC specimens (Lingala *et al.* 2010).

An intriguing result of CD133 and OCT4 mRNA expression is found in cirrhotic sample of BA as shown in Figure 5.3 and Figure 5.4. Even though a linear relationship between expression of CD133 and OCT4 could not be demonstrated due to small sample size of BA, we notice that both of these markers were highly expressed in BA samples. Previous study in rectal cancer with chemoradiotherapy (CRT) showed there were significant linear correlations among CD133, OCT4, and SOX2 which may be associated with tumor relapse and metastatic growth after CRT (Saigusa *et al.* 2009).

Histologically, all BA tissues examined were cirrhotic. Based on the literature review, several recent articles reported a subpopulation in stellate cells expressed high CD133 and OCT4. As described in the General Introduction, subpopulation of CD133 cells was identified in hepatic stellate cells (Kordes *et al.* 2007). Moreover, stellate cells display a differentiation potential as investigated *in vitro* and *in vivo*. It indicates that stellate cells are undifferentiated cells, which might play an important role in liver regeneration (Kordes, Sawitza, and Haussinger 2009). Stellate cells are activated and responsible to develop liver fibrosis and progress to cirrhosis.

Pattern of CD133 and OCT4 in clinical tissues was simultaneously found in our isolated cells population from primary cancers (please refer to previous subchapter 7.1). Beside the presence of MSCs, we noticed that only in HCC with Hepatitis C Virus (HCV) cells, there are higher mRNA expressions of CD133 together with OCT4 compared to viral-free HCC. Moreover, HCC tissues with HCV infection also express high expression of CD133. It is widely known that HCV infection lead to liver cirrhosis. An interesting study reported the presence of CD133/Nanog-postive cells in liver tumors of alcohol fed in NS5 transgenic mouse. This synergism between HCV, alcohol, and stem cell marker Nanog is mediated by Toll-like receptor 4 (Machida *et al.* 2009). Further studies in the association between HCV, cirrhosis/HCC and hematopoietic cells interaction will be intriguing.

Tai *et al.* showed that using liver stem cells undergoing differentiation to mature hepatocytes, the expression of OCT4 diminished (Tai *et al.* 2005). In this study we still observe OCT4 mRNA in normal liver tissues because, however, normal tissues still contains normal stem/progenitor cells even in small number. However, unfortunately, the mRNA data

of OCT4 will be not sufficient. The observation of OCT4, especially in normal adult tissues, is controversial, therefore must be proven by at least protein assay. This study should be considered as a starting point in mRNA data. Further analysis on different genes exons and variants must be followed.

In conjunction with gene expression studies on proposed CSCs marker above, we decided also to check the expression of CK19 and CK7. We considered these genes even if CK19 and CK7 act as markers for fully mature cholangiocytes. CK19 together with CK14 antigen shares same epitope with OV-6 target antigen, an antibody to recognize hepatic progenitor cells (Bisgaard *et al.* 1993). For this reason, observation on the proliferation of more committed hepatic resident cells in tissues samples might be carried out.

As shown in Figure 5.5, we found that CK7 is distributed variably and there is no significant difference among tissues samples, at least in the mRNA level. The CK19 is also wide expressed, but is found to be higher in liver cancers and significantly highest in BA compared to normal tissues. Data in HCC is concordant with a recent report showing that CK19 antigen was found to be positive in HCC and cirrhotic tissues in which its expression in HCC to be higher. Moreover, this marker was also positive in non-tumor liver tissues (Oliva *et al.* 2010).

High expression of CK19 and CK7 in BA is thought to be an effect of bile duct cells proliferation. In BA samples, CK19 expression was found to be highest among other malignancies. This data is also in agreement with previous reported data that antibodies CK19 and OV-6 stained ductular proliferative cells extensively, and not in hepatocytes, in extra hepatic BA patients, although OV-6 staining was more selective in the number and type of cells that were immunopositive (Crosby *et al.* 1998). It showed higher proliferation of CK19 cells in the response of biliary disturbance compared with liver cancer. This proliferation might be explained by proliferation of bipotent hepatic progenitor cells and hepatoblasts, hepatic metaplasia, and abnormal proliferation of intrahepatic bile duct cells. Tan *et al.* reported that extra hepatic BA may be caused by a failure in the remodeling process in fetal stage development. Furthermore, proliferating ductules in this disease is similar with first trimester ductal plate-derived primitive bile ducts (Tan *et al.* 1994).

We thought that hepatoblastoma (HB) with the absence of essential other liver diseases, aging, viral infections, and abundance drugs exposure, might be the best candidate to study CSCs theory. In our HB samples, one HB neoplastic nodule showed highest expression of all CSCs marker CD133, CD90, EpCAM, CK7, and CK19. Cairo *et al.* divided molecular signature of HB into two groups: well-differentiated HB which is less aggressive with more CSCs markers (C1) and poor-differentiated HB which is more aggressive with fewer CSCs gene expression (C2). We predicted that this sample belongs to the C1 group (Cairo *et al.* 2008). Two HB neoplastic nodules showed a mutual high EpCAM and CK19 expression and considerably low β-catenin (CTNNB1) expression. Interestingly, we noticed that all highest expressions EpCAM and some of CD90 are found in HB patients and young HCC patients (data not shown).

As mentioned previously, the differential expression of the tumoral compared to non tumoral tissue (distal) of liver cancers might be a better approach to study the gene up-regulations related with progression of the diseases. We studied the mRNA expressions of several SCs markers described above in paired samples of the same patient. As we assumed that the basal expression of these genes varies in every patient, the comparison in each individual will give a more meaningful and relevant information on the possible regulation. As shown in Figure 5.6 and 5.7, in agreement with previous result, the most interesting pattern is observed for CD90 and EpCAM. Almost all samples showed drastic up-regulations in tumoral compared to distal tissues. In CC sample, tumoral samples showed differential expression >1.00 of all SCs markers gene under study.

Taken data all together from CD90, EpCAM, and CK19, markers for several early liver stem cells and progenitor cells, we assume that liver cancers might be associated with proliferation of hepatic stem cells. However, in regards to liver cancers, proliferation of more-primitive stem cells may be more than proliferation of more-committed progenitor cells.

Collectively, gene expression data of several stem cells markers in liver malignancies support the CSCs theory regardless the origin of the cells. It might be related also with poor prognostic of the patients. As mentioned before, beside various risk factors and individual intrinsic variations, proliferation of various cells might happen in the response of liver injury

or as the cause of the hepatocarcinogenesis itself. Despite of the ample promising data in this study for CSCs in liver cancer, the use of CSCs mRNA marker to screen tissues in liver diseases might still be limited by several factors:

1. The mRNA expressions of these markers were also identified in inflamed liver, normal liver, and nearly normal liver, even though more limited and homogenous. A recent article using immunohistochemistry reported that the expressions of individual or a combination of CSCs marker CD133, CD44, and ALDH in HCC was not a unique phenomenon. They were expressed randomly in HCC in spite of the presence of viral infection, advance fibrosis, and cirrhosis (Lingala *et al.* 2010).

2. The origin of the cells that are associated with mRNA expression should be identified. Hepatic stem cells, hepatic fibroblasts, myofibroblasts, and MSCs share a common marker CD90. Moreover, CD133 expression might be obtained from circulating hemotopoietic cells and proliferating subpopulation of hepatic stellate cells in liver injury.

3. Post-transcriptional and post-translational regulation and modification. In mouse cerebrum, CD90 mRNA expression was rapidly followed by protein expression, whereas a delay of several days occurred between CD90 mRNA and protein in mouse Purkinje cells (Xue and Morris 1992)

4. The possible influence of circulating cells in liver blood vessel, transit cells, and migrated cells. The presence of circulating CSCs CD90 in peripheral blood has been demonstrated (Yang *et al.* 2008b).

5. The expression patterns of various stem cell markers in tumor sites may be different among individuals, possibly also due to the heterogeneity of activated signaling pathways in normal stem/progenitor cells where the CSCs may originate. Therefore, it would be useful to comprehensively investigate the expression patterns of stem cell markers to characterize the population of CSC that may correlate with the activation of their distinct molecular pathways (Yamashita *et al.* 2008).

However, it is important to consider that both in both malignant and normal tissues, the existence of stem cells population shares similar phenotype markers. Hence, it is essential to study the disparity of these normal and tumorigenic stem cells.

7.4. ABCG2 as marker for hepatic CSCs

In this thesis, chapter of ABCG2 expression is separated from other CSCs markers. Even though ABCG2 has been associated with CSCs marker, we considered it is not the most desirable marker for CSCs by several reasons. First, its determination is based on the protein functional activity. Second, ABCG2 is expressed in wide variety of cells including in mature differentiated cells. And third, the method of isolation of side population (SP) cells by flow cytometry really depends on individual techniques, dyes, and experimental factors. We thought that expression of ABCG2 is not real CSCs *per se*, but more likely supportive dynamic for the cancer in response to drug therapy.

Based on our results, the ABCG2 mRNA expression in liver tissues is observed to be highly variable. The variations of ABCG2 intra- and inter-groups observed in this study had also been reported in acute leukemia (Ross *et al.* 2000), breast cancer (Burger *et al.* 2003) and lung cancer (Kawabata *et al.* 2003). We hypothesized that these variations were linked to individual variations such as age and severity of the disease, and drug therapy factors such as type of the drugs and duration of the treatments. Furthermore, regarding the main function of the liver, expression of ABCG2 is also related with individual lifestyle such as alcohol intake and chemical consumption. We noticed that ABCG2 expressions in normal liver tissues are related with age, to be low in children and high in adult (Figure 6.1). In liver cancers, ABCG2 mRNA expression is significantly higher than normal and BA livers (Figure 6.2). It is an interesting observation that normal liver, especially of children liver, and BA tissues are ones with minimal cytotoxic drugs exposure. In contrast, our cancer tissues were obtained from

liver transplantation and liver resection patients who received treatments, and consequently increase the defense cells reaction.

High variation in ABCG2 expressions demonstrated that its expression might be dependent on the tissues types, even in the same organ of an individual. Tumor proliferation and loss of cell differentiation may also induce ABCG2 up-regulations. Furthermore, a recent paper showed that knock-down of ABCG2 inhibited breast cancer and lung cancer cells proliferation, suggesting the role of ABCG2 in maintenance of the cancer cells (Chen *et al.* 2010). Our finding data is difference with one reported in colon and cervical cancer in which ABCG2 protein was observed to be down-regulated (Gupta *et al.* 2006). However our data was in good agreement with previous finding demonstrated that ABCG2 protein and mRNA was higher in HCC compared to control group (Sun *et al.* 2010), was up-regulated following chemotherapy in HB patients (Vander *et al.* 2008), and was also reported to be induced in human livers after acetaminophen overdose and primary biliary cirrhosis (Barnes *et al.* 2007).

The lack of naïve tissues before treatment in our tissues samples is an unavoidable limitation in this study. As mentioned before, basal ABCG2 expression in each individual may vary and we cannot compare the data before and after treatment. To support data *in vivo*, we used several hepatic cell lines with different degree of morphological differentiation (IHH, HuH-7, HepG2, and JHH-6) as shown in Figure 3.1. Highest basal ABCG2 expression was found in JHH-6, the poorest differentiated HCC cell line (Figure 6.3). Interestingly, when we exposed these cells with doxorubicin, highest up-regulation was noticed in cells with low basal ABCG2 expression, which are immortalized hepatocytes and differentiated HCC cells (Figure 6.5). We hypothesize that in these cells with low ABCG2 expression, in the presence of drugs, ABCG2 is highly induced to pump out the drugs and to overcome the toxicity of the compounds. The data in HepG2 cells supports a previous report from Li *et al.* in which ABCG2 mRNA was also up-regulated in doxorubicin-resistant HepG2 (Li *et al.* 2007). However in our data, the increase of ABCG2 in HepG2 is less (2-folds compared the reported 9-folds). It might be due to the different exposure time and dosing of doxorubicin on the cells.

As mentioned, the introduction of ABCG2 as CSCs is mainly due to its function in the cells protective mechanism. So we performed functional analysis of this transporter using Hoechst 33342 efflux assay in HCC cell lines. Immortalized hepatocyte IHH was excluded because of very low basal ABCG2 expression and no protein band was detected. We observed that the increase concentration of Hoechst 33342 raised cells efflux capacity in all cells, detected by spectro-fluorescence instrument (Table 6.1). Hoechst 33342 is a substrate for ABCB1/P-GP and ABCG2, thus, using this technique we could not distinguish which transporter has bigger role for Hoechst efflux.

To see only the ABCG2 activity, we subtracted the fluorescence of internal dye accumulations with and without verapamil. Verapamil is potent P-GP inhibitor but weak ABCG2 inhibitor. Among all three cell lines, the smallest difference is observed in JHH-6 indicating that ABCG2 efflux capacity was dominant in JHH-6 compared to HepG2 and HuH-7 (Figure 6.6). It shows that the high expression of ABCG2 transcription level also has an active functional role in the cellular response to the potentially toxic compounds.

Combining the data all together, we suppose that even though the ABCG2 is not an ideal marker for determining a CSCs population, there is an association of this transporter in the level of gene, protein, and functional activity with cells degree of differentiation. The relationship between cell differentiation and ABCG2 expression had been reported in studies performed in other cells. A high level of functional ABCG2 was detected in undifferentiated human embryonic cells and decreased during cellular differentiation (Apati *et al.* 2008). In hematopoietic system, the ABCG2 expression is restricted to the most immature progenitor cells and down-regulated at the committed progenitor level (Scharenberg, Harkey, and Torok-Storb 2002).

Nonetheless, drug resistance mechanism in cancer is a complex biological interaction that consisted of many transporters and activated signaling pathways. To fully understand this phenomenon, better information in the up-regulation of other transporters i.e. ABCB1, ABCC1, and ABCC3, as the most studied ABC transporters proteins in liver cancers, will be crucial.

More information of CSCs uniqueness and activation would be one of main keys in understanding initiation and development of cancer. Furthermore, to achieve a better strategy for a total elimination of HCC, several biological and clinical aspects should be considered for an effective CSCs-targeted therapy (Sukowati *et al.* 2010). The hepatic CSCs identifications and their functional significances, including multidrugs resistance behavior and aberrant signaling pathways should be considered. Together with CSCs markers, clinical aspects such as drug delivery system, single or combination therapy, drug dose and toxicity will support the potential of therapy.

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Chapter VIII

General Conclusion

8.1. Identification of stem cells population in PLCs

We had identified the presence of cells populations with $CD90^{+}CD44^{+}$ phenotypes in human primary liver cancers Hepatocellular Carcinoma and Cholangiocarcinoma and non tumoral liver. These cells also express mesenchymal stem cells characteristic, identified based on morphology, cell surface antigens mRNA, and functional capacity. These cells populations from cancerous tissues have characteristics: 1. clonogenic capacity in standard culture condition and three dimensional matrices; 2. effectiveness for cells expansion *in vitro*; 3. trans-differentiation potentials that may allow them to promote and to support cancer growth; and 4. a probable player in cancer heterogeneity.

Due to wide variations of liver cancers, the function of these cells populations may depend on many factors, such as severity of the disease, prognostic type, etiological factor, and exposure to the treatment. We thought that these cells may have capacity to play a role as tumor-initiating cells or tumor-promoting cells in liver cancer, however a more comprehensive xenograft *in vivo* assay will need to be further investigated.

8.2. The distribution of stem cells phenotypic markers in liver cancer tissues

Albeit an ideal global gene expression assay to study prognostic type of liver cancers, we presume that CSCs single marker might still be useful as support of the clinical significance of the cancers. From our data, we demonstrated that high expression of stem cells genes in disease tissues may suggest an active proliferation of CSCs in accordance with tumorigenesis, regardless the origin of CSCs. In more detail, our study showed that:

- In normal liver, the expressions of stem cells genes, especially CD90 and EpCAM, are more restricted than in diseased tissues.
- High variability of CD90 (Thy-1), EpCAM, and CD133 (Prom-1) expression in hepatic malignancies.
- The expression of CD90 and EpCAM is significantly higher in liver cancers than in normal liver. In paired tissues, differential expression between neoplastic nodules and distal tissues are also observed for these two genes. In addition, highest EpCAM expression is identified in HB and HCC samples from young patients with absence of viral infections.
- In liver cancers, proliferation of more-primitive stem cells is higher than proliferation of more-committed progenitor cells.
- High expressions of CD133 in biliary atresia and cirrhotic livers are observed compared to normal and liver cancer tissues.

8.3. ABCG2: drug resistance and CSCs

Regarding the expression of ABCG2 in regards to stem cells and side population study, we demonstrated that :

- High variability of ABCG2 mRNA in normal and diseased liver.
- An association of ABCG2 in the level of gene, protein, and functional activity with hepatic cells degree of differentiation, examined *in vitro* in hepatic cell line models.
- A close relation of ABCG2 expression with chemoresistance to doxorubicin.

All the data shown in this study has supported the presence of stem cells in liver cancer. This summary contributes in the knowledge of hepatocarcinogenesis and cancerogenesis in general. However, more data in the characterizations of both normal and cancerous stem cells together with their functions in liver cancer tissues are important for better understanding their mechanisms in cancer initiation, maintenance, and treatment.

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Supplement

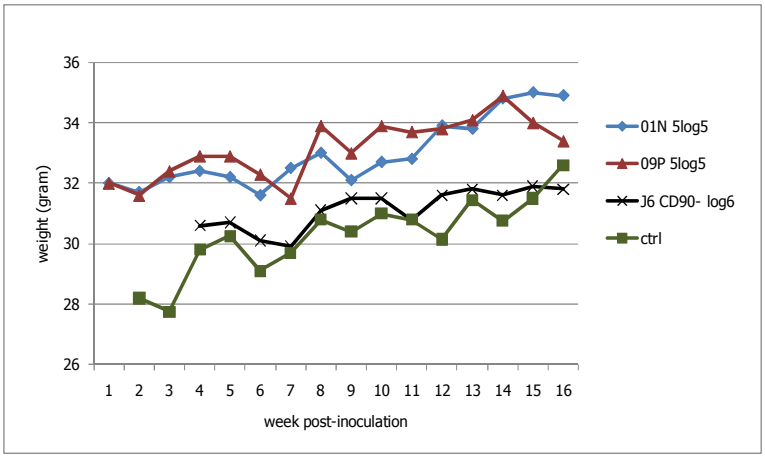
Supplemental data 1

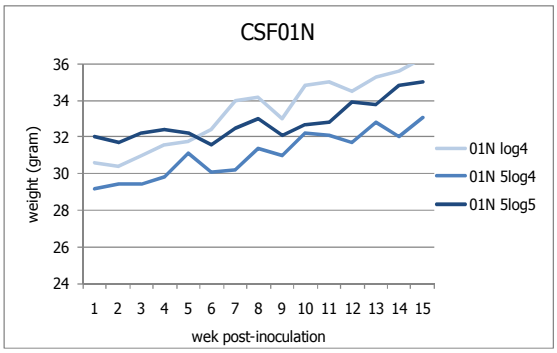
Characteristic of hepatic cell lines

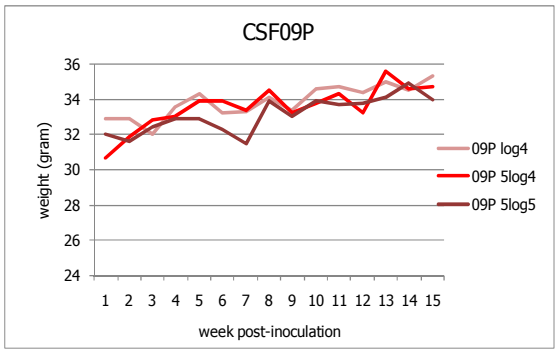
Cell Lines	Characteristics	Medium	Passaging
IHH	Hepatic non tumoral cells	DMEM/F12 1x medium with 15 mM Hepes buffer, L-glutamine, pyridoxine HCl; 1µM dexamethasone; 5 µg/mL insulin; 1% antibiotics; 10% FBS	0.05% trypsin-EDTA with centrifugation
HepG2	Hepatocellular carcinoma	DMEM-HG medium; 2 mM L-glutamine; 1% antibiotics; 10% FBS	0.05% trypsin-EDTA with centrifugation
HuH-7	Differentiated human hepatoma	DMEM-HG medium; 2 mM L-glutamine; 1% antibiotics; 10% FBS	0.05% trypsin-EDTA without centrifugation
JHH-6	Undifferentiated Hepatocellular carcinoma	Williams'E medium; 2 mM L-glutamine; 1% antibiotics; 10% FBS	0.05% trypsin-EDTA without centrifugation

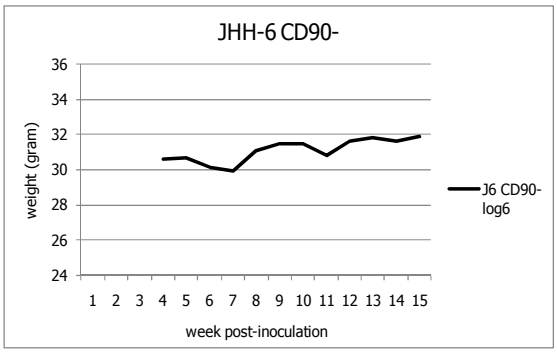
Supplemental data 2

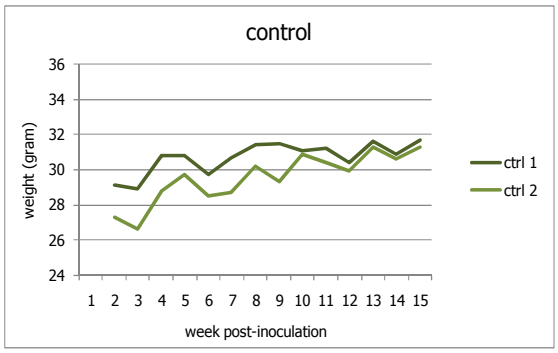
Body weight of xenograft mice post-inoculation











Acknowledgment

Fondazione Italiana Fegato

Prof. Claudio Tiribelli, Dr. Cristina Bellarosa, Sabrina Corsucci, Dr. Natalia Rosso, Beatrice Anfuso, Dr. Silvia Gazzin, Dr. Norberto Chavez-Tapia, Dr. Graciela Mazzone, Dr. Pablo Giraudi, Devis Pascut, Andrea Berengeno, Carlos Coda Zabetta, Celeste Robert, Elena Boscolo, Sandra Leal, Mohamed Qaisiya, Sabrina Gambaro

Dr. Lory Saveria Crocè, Dr. Flora Masutti, Dr. Elena Garlati-Costa

Department of Surgery, Ospedale Cattinara

Prof. Nicolò de Manzini, Dr. Adelino Adami, Dr. Michela Giuricin

Ospedale Pediatrico Bambino Gesù, Roma

Dr. Giuliano Torre, Dr. Paola Francalanci

Eijkman Institute for Molecular Biology, Jakarta

Dr. David Handojo Muljono

SDBM, Università di Trieste

Prof. Giannino Del Sal, Dr. Licio Collavin

Università di Udine

Dr. Antonio Paolo Beltrami, Prof. Carlo Alberto Beltrami

Istituto Italiano di Cultura, Jakarta

Prof. Ostelio Remi, Dr. Giovana Jatropelli, Dr. Livia Raponi