Inhibition of c-kit in Late Cirrhosis may Restore TGF-β Inhibitory Effect on Somatic Liver Stem Cells and Prevent Development of Hepatocellular Carcinoma.

Tamer A. Al-Shafie[1, 2*], Alaa Ismail[3], Abeer M. Ashmawy[1], Abeer M. Elsayed[4], Motawa E. El-Houseini[1]

[1]Department of Cancer Biology, National Cancer Institute, Cairo University, Cairo, Egypt
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy and Drug Manufacturing, Pharos University, Alexandria, Egypt
[3]Department of Surgery, Faculty of Medicine, Ain Shams University, Egypt
[4]Department of Pathology, National Cancer Institute, Cairo University, Cairo, Egypt
tamershafie@yahoo.com

Abstract: Background: Hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer worldwide and the second leading cause of cancer-related death in the world**.** It occurs within an established background of chronic liver disease and cirrhosis. Liver fibrosis is a wound healing-like event stimulated by TGF-β following repeated and/or chronic insult and can grow into cirrhosis. Proliferation of somatic liver stem cells (SLSCs) and increased TGF-β production during chronic liver disease has been linked to the risk for HCC development. The cross-talk between TGF-βreceptor (serine/threonine) and tyrosine kinase receptors drew attentions to a probable relationship between TGF-β and c-kit gene expressed by SLSCs which might explore the biology and etiology of HCC if it is uncovered. **Aim**: SLSCs were isolated from tumor and non-tumor (cirrhotic) liver tissue to study the effect of TGF-β on tumor- and non-tumor-derived SLSCs. **Material and Methods:** Tumor- and non-tumor SLSCs were isolated from liver tissues excised from patients with HCC. Such cells were cultured and characterized using different stem cell markers then treated with TGF-β. Cell morphology, CD90 and CD117 expression were detected before and after treatment.C-kit gene expression was performed using PCR. **Results:** C-kit gene expression was positive in tumor liver tissue, while it was not expressed in non-tumor liver tissue. After TGF-β treatment a significant decrease in both CD90 and CD117 expression in non-tumor-derived stem liver cells, whereas non-significant change was detected in the expression of both markers in tumor-derived liver stem cells. **Conclusion:** Loss of TGF-β inhibitory effect on SLSCs enhances the conversion of SLSCs into tumor stem cells and up-regulation of c-kit gene. HCC can be prevented in the setting of chronic liver injury by suppressing the formation of tumor liver stem through restoring TGF-β inhibitory effect and/or inactivating c-kit gene expression in liver stem cells at this stage.

[Tamer A. Al-Shafie, Alaa Ismail, Abeer M. Ashmawy, Abeer M. Elsayed and Motawa E. El-Houseini. **Inhibition of c-kit in Late Cirrhosis may Restore TGF-β Inhibitory Effect on Somatic Liver Stem Cells and Prevent Development of Hepatocellular Carcinoma.** *Life Sci J* 2014;11(11):1080-1089]. (ISSN:1097-8135). http://www.lifesciencesite.com. 185

Key words: *Somatic liver stem cells (SLSCs), Isolation, Culture, Characterization, TGF-β, c-kit (CD117)*
Abbreviations: SLSCs; somatic liver stem cells, NPC; non-parenchymal cell, HCC; hepatocellular carcinoma, *TGF-β; transforming growth factor-beta, c-kit (CD117); stem cell factor receptor*.

1. Introduction:

Ranking the fifth most frequently diagnosed cancer worldwide and the second leading cause of cancer-related death in the world, hepatocellular carcinoma (HCC) has been a grievous scourge of humanity for a long time and can be considered as a major threat to human being [1]. HCC is unique in that it largely occurs within an established background of viral or non-viral-linked chronic liver disease and cirrhosis. However, none of present therapeutics has shown tangible disease regression [2]. Conventional therapies might not be sufficient to eradicate total tumor because they target mainly the differentiated cells. Until now, transplantation of liver or its parts remains the prevailing method for treating chronic liver diseases. Liver stem cells-targeted therapy should be considered to increase the efficiency and safety of the treatment [3]. Bone marrow; the rich source of stem cells, is a possible sources of multipotent stem cells for liver repopulation. Unfortunately, bone marrow-derived stem cells have the potential to differentiate into endothelial cells and fibroblasts within the liver, and, as such, they might exert a profibrogenic effect [4].Transforming growth factor-β (TGF-β) is a potent growth inhibitory and pro-fibrotic cytokine. It exerts its biological effects via Smad and non-Smad pathways. In Smad pathway, TGF-β binds to receptor serine/threonine kinases, while in non-Smad pathways it utilizes other intracellular signaling pathways [5]. It plays a pivotal role in the physiological process of wound healing and the pathogenesis of organ fibrosis [6]. Liver fibrosis is a wound healing-like event which

occurs following repeated or chronic insult and can develop into cirrhosis. Kupffer cells-derived TGF-β stimulates the hepatic stellate cells to secrete extracellular matrix proteins to replace damaged liver cells [7]. Proliferation of liver progenitor cells (LPCs) parallel with increased TGF-β production during chronic liver disease has been linked to the risk for development of HCC [8]. Recently, rat-derived liver progenitors were impaired and converted into tumor-initiating cells after long exposure to TGF-β while in the absence of TGF-βno tumor-initiating cells were formed; hence TGF-β can be hypothesized to serve as an important link between liver progenitor cells and chronic injury, cirrhosis, and HCC. However, mechanism by which such impairment had occurred is not known [9].

In addition, proteomics-based studies discovered a novel paradigm in the crosstalk between serine/threonine and tyrosine kinase receptors in which tyrosine kinase receptors was detected in a complex with serine/threonine kinase BMPR-II receptor [10]. Human c-Kit (stem cell factor receptor/CD117) is type-III trans-membrane tyrosine kinase receptor that is expressed by somatic liver stem cells. Over-expression and/or constitutive expression of c-kit alone or concurrently with SCF leading to autocrine stimulation of several solid tumors [11].

Aim of the Work

Because we believe that better understanding of the molecular events regard to etiology that lead to oncogenesis of HCC can be the clue to the discovery of novel therapeutic agents, this study was designed to isolate SLSCs from tumor and non-tumor (cirrhotic) liver tissue to elucidate the effect of TGF-β on tumor- and non-tumor-derived somatic liver stem cells.

2. Material and Methods:

Human liver tissue specimen collection

In this study somatic liver stem cells were separated from liver tissues obtained from patients with HCC (no. = 24) who were candidates for partial therapeutic hepatectomies at Ain Shams University Hospital (Table 1). Safety margins (non-tumor part) were used as controls.Liver tissue specimens were received in the operation room, immersed in sterile 50 ml-falcon tubes containing 20-25 ml tissue culture medium and brought within 2 hours to the tissue culture unit at National Cancer Institute. All liver resected tissues contained tumor part and non-tumor part. Low quality and small-sized tissues were avoided because the techniques used for human liver progenitor cell isolation as well as cellular outcome are strongly influenced by the quality and size of tissue [12& 13].

Histopathology

Histopathological examinations were carried out to confirm the source of SLSCs either from tumor liver tissue (tumor-derived) or cirrhotic liver tissue (non-tumor-derived) before starting tissue processing and stem cell disaggregation. Portions of collected liver tissue were fixed in 10% phosphate-buffered neutral formalin, routinely processed and stained with Hematoxylin and Eosin (H&E) for histolopathological examination.

C-kit gene expression in non-tumor and tumor liver tissues

RNA extraction from non-tumor and tumor liver tissue was performed using Invisorb® RNA kit-I Invitek GmbH, Germany.**Reverse transcription** of extracted RNA was carried out using RevertAid^{TM} Premium First Strand cDNA Synthesis Kit obtained from Fermentas Life Sciences, USA. **PCR primers** for c-kit gene were obtained from Sigma Scientific Services Co., USA with the following sequence:

***Forward**: 3' CAG-GCA-ACG-TTG-ACT-ATC-AGT 5'

***Reverse**: 5' ATT-CTC-AGA-CTT-GGG-ATA-ATC 3'

PCR products were detected at 288 bp.**MasterMix** Dream Taq Green PCR Master Mix was obtained from (ThermoScientific, USA). **DNA ladder 100bp** obtained from Sigma Chemical Co., USA was used as a molecular weight marker along with PCR products.

Somatic liver stem cell isolation, Culture and Characterization

Whole Liver cell isolation

As shown in (Fig. 1) tumor and non-tumor portion of liver tissue specimens were processed separately to obtain tumor and non-tumor liver cell suspension [14]. Liver tissues were washed with hypotonic saline and equal volume of Dulbecco's Modified Eagle Medium 16 (DMEM; CellGro, Mediatech Inc, Manassas, VA) comprised of 10% fetal bovine serum (FBS; Thermo Scientific Hyclone, Logan, UT) and 5% antibioticantimyocotic solution (CellGro, MediatechInc, Manassas, VA). Each specimen was then minced to obtain very small pieces (1x1 mm) then was digested by type-I collagenase solution (300U/ml, Sigma Aldrich) for 30 min at 37^{o}C in a shaking incubator followed by incubation at 4^{o}C. Supernatants containing the released cells were passed through 350 um followed by 100 um metal meshes and centrifuged at 1,000xg for 8 min.

Each of the two pellets were washed twice with PBS then divided into two parts; the first part was cultured as whole liver cell culture and the second part was undergone cell fractionation to isolate SLSCs [17].

Liver Cell Fractionation to obtain NPC fraction

Fractionation technique depends on two bases; first, variation in size and density between mature liver cells (hepatocytes) and liver stem cells (Fig. 2 B-D). Second, the exclusion of liver immune cells through changing culture media as such cells are non-adherent

cells leaving pure adherent somatic liver stem cells. Non-parenchymal liver cells were separated from both tumor- and non-tumor liver tissues using repeated differential centrifugation to remove the majority of mature hepatocytes, followed by density gradient separation by layering the supernatant on density gradient medium (ficoll-hypaque) to purify NPC fraction from contaminating debris and dead cells (Fig. 2 A).

Whole liver cells and NPCs culture

Cell cultures were carried out in DMEM medium with glutamine and non-essential amino acids, supplemented with 20 ng/ ml basic fibroblast growth factor (b-FGF), epidermal growth factor (EGF) obtained from Biowest Inc.-France. Other additives included FCS (10%), antibiotics (2%) and (all from Sigma Aldrich, St Louis, MO, USA). T-25 tissue culture flasks were used for liver cell culture and six-well plates were used for non-parenchymal cells culture. The cultures were maintained at 37˚C in a humidified incubator in a mixture of 95% air and 5% CO_2.

Morphologic characterization of cultured liver cells

Morphologic evaluation of cultured cells was performed regularly during the entire experiment to monitor stem cell differentiation and proliferation by phase contrast light microscopy. The cells were characterized on the basis of cell shape, size, nucleus to cytoplasm ratio and number. The pictures were acquired, and processed using the digital imaging software. Cultured cells were harvested after 1-2 min treatment with 0.25% of trypsin-EDTA solution for the following steps.

Flow cytometric characterization of SLSCs

Flow cytometric characterization of liver stem/progenitor cells was performed using FITC- and PE-conjugated CD49f, CD90, CD117 and CD133 monoclonal antibodies all were obtained from (MiltenyiBiotec Inc., Germany). It has been reported that isolated liver NPCs express liver stem cell markers including CD90, CD49f and CD133 [15-17]. Human liver stem cells were cultured in DMEM/10% FCS for 2 days followed by flow cytometric analysis. Cells were washed in 2% inactivate FCS/0.05% sodium Azide/PBS and were re-suspended in 100 ml of the same buffer and incubated at 4^{o}C for 1 hour in thepresence or absence of primary unconjugated rat anti-human SSEA3 (EMD Millipore; Billerica, Massachusetts). Cells were then washed twice with the same buffer and incubated with the corresponding secondary FITC-conjugated anti-rat IgM (BD Biosciences; San Diego, CA) for 45 minutes at 4^{o}C. After two consecutive washes, cells were incubated with PE-mouse anti-human CD133 (BD Biosciences, San Diego, CA) at 4^{o}C for 1 hour. Cells were then washed and resuspended in 200 ml of the same buffer. Analysis of count and cell type was performed using flowcytometric analysis. Flowcytometer used was Calibur flow cytometer with cEllQuest-Pro

Transforming Growth Factor-β Treatment

Recombinant protein of human TGF-β (25 KD) obtained from Koma Biotech Inc., Korea was added to cultured liver cells in concentration (5ng/ml) at day 12 for 24 h to study its effect on somatic liver stem cells.

Flow cytometric determination of CD90 and CD117 expression

Flow cytometricdetermination of CD90 and CD117 was performed before and after TGF-β treatment using human CD90 and CD117 monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC) obtained from (MiltenyiBiotec Inc., Germany).

3. Results:

Somatic liver stem cell culture and characterization

Morphologic characterization of cultured liver cells

Cultured liver cells isolated from tumor and non-tumor liver tissues included different types and morphology. Hepatocytes were shown to be large polygonal cells and represented the majority (~75-80%) of total liver cells. Non-parenchymal cells (NPCs) were small rounded cells and constituted ~ 20-25% of total cultured liver cells (Fig. 2).

Proliferation and differentiation of cultured SLSCs

In this work, differentiation of tumor and non-tumor-derived SLSCs occurs in the presence of EGF and b-FGF, while in the absence of such growth factors, both tumor and non-tumor-derived SLSCs undergo proliferation. In accordance with Laurson *et al.* [18] and Kon *et al.* [19] who stated that EGF, b-FGF, or HGF can trigger the hepatocytic differentiation of SLSCs, our SLSCs cultured in a medium supplemented with EGF and b-FGF showed a change in morphology after 7-10 days represented by an increase in cytoplasm size relative to the nucleus and spindle-shape cells. On day 14-20 cells started to differentiate into epithelial-like morphology. Such morphological changes can be interpreted as a sign of beginning of differentiation of SLSCs (Fig. 3A-C). SLSCs showed *in vitro* proliferation during time course of culture free of EGF and b-FGF indicated by increased cell number between day 0 and day 12 by about 5 times in non-tumor stem cells and about 18 in tumor stem cells (Fig. 3 D-E, Suppl. data S1).On the other hand, proliferation of tumor- and non-tumor-derived SLSCs cultured in a of EGF and b-FGF-free medium was recognized to increase in number of cultured detected by phase contrast microscopic images on day 12 in comparison with day 0.

Flow cytometric characterization of SLSCs

SLSCs proliferation was also recognized by flow cytometric determination of liver stem cell markers expression in tumor- and non-tumor-derived liver stem cells on day 0 and day12. CD90, CD133 and CD49f

stem cell surface markers were selected to characterize liver stem cells. The choice of such markers was based on Weiss *et al.* [20], Clayton and Forbes [21], Tanaka *et al.* [22] and others, who reported that flow cytometric analysis of isolated liver NPCs revealed the expression of liver stem cell markers including CD90, CD49f and CD133. Flowcytomeric analysis showed significant increase in liver stem cell markers expression on day 12 in comparison with day 0 ($P < 0.013$). The expression of CD49f was significantly higher in tumor stem cells than in non-tumor stem cells in both day 0 and day 12 and (Fig. 4 A-B, Fig. 5). Flowcytometric analyses data of other liver stem cell markers expression (CD90 and CD133) are shown in (Suppl. data S2-S3).

C-kit gene expression in non-tumor and tumor liver tissues

The expression of c-kit (stem cell factor receptor) gene revealed high expression in liver tissue of tumor origin, while expression was absent in non-tumor liver tissue (Fig. 6)

Expression of c-kit protein (CD117) before treatment with TGF-β

The determination of CD117 expression in non-tumor- and tumor-derived liver stem cells revealed a significant increase in CD117 expression in non-tumor-derived liver cells at day 12 compared to day 0 ($p = 0.04$). Also, the expression of CD117 in tumor-derived liver cells increased significantly at day 12 compared to day 0 ($p = 0.016$) (Fig. 7). Flow cytometric analyses are shown in (Fig.10A).

Effect of TGF-β on tumor- and non-tumor-derived somatic liver stem cells

Liver cell morphology

In non-tumor-derived cells, a large number of cytoplasmic vacuoles appeared in hepatocytes and NPCs after treatment with TGF-β which is considered to be a sign of celldegeneration and confirmed the apoptotic effect of TGF-β on non-tumor liver cells. However, in tumor-derived cells only mature cells (hepatocytes) were affected by TGF-β and developed cytoplasmic vacuoles, while NPCs were not affected and did not develop any cytoplasmic vacuoles (Fig. 8 A-B).

Expression of CD90

CD90 expression was (4.64±0.55) before treatment and (2.74±0.75) after treatment in non-tumor-derived liver cells which showed a significant decrease (*p=0.004*). However, in tumor-derived liver cells CD90 expression was (5.77±0.65) before treatment and (5.98±0.74) after treatment showing non-significant change (*p=0.36*) (Fig. 9A). Flow cytometric analyses are shown in (Fig. 10B)

Expression of c-kit protein (CD117)

CD117 expression was (18.23%±0.71) before treatment and (7.35%±0.60) after treatment in non-tumor-derived liver cells which showed a significant decrease ($p = 0.018$). On the other hand, in tumor-derived liver cells CD117 expression was (2.42%±0.61) before treatment and (3.80%±0.25) after treatment showing non-significant change ($p=0.11$) (Fig. 9B). Flow cytometric analyses are shown in (Fig. 10C).

Table (1) Main demographic and clinical investigations of patients have undergone therapeutic hepatectomy.

No of Cases	24 Case
Age	48-65±2.5 y
Sex	
Male	16/24
Female	8/24
Lab Investigations	
ALT (U/L)	55.0 +/- 7.0
AST (U/L)	104.0 +/- 10.0
γ-GT (U/L)	102.0 +/- 14.0
Albumin (g/l)	3.0 +/- 0.3
αFP (ng/ml)	410±90
HCV	8/24
HBV	6/24
Non-viral	10/24
Radiological Investigations	
Hepatomegaly	4/24
Splenomegaly	2/241
Cirrhosis	9/24
Neoplastic focal lesions	20/24
Epigastric mass	7/24

Schematic presentation for isolation and cultivation of stem cells from tumor and non-tumor liver tissue. Liver stem cells were obtained after 16 hours of tissue collection, then incubation with collagenase in DMEM medium without FCS at 4°C. Hepatocytes were separated from non-parenchymal cell (NPC) preparations by repeated short time (1 min) and low speed (50 xg) centrifugation rounds. These centrifugation steps are followed by density gradient separation using density gradient medium (e.g. Ficoll) which also has been used for cell fractionation and isolation of a distinct cell fraction from a mixture of cells including hematopoietic cells. To avoid cellular stress that affects the cell viability, collagenase was added just for 15 min, RBCs were removed by adding water for injection for only 15 seconds then immediately tonicity was restored by adding HBSS solution (2X), finally centrifugation steps were adjusted at low speed and short time (See Methods and Discussion).

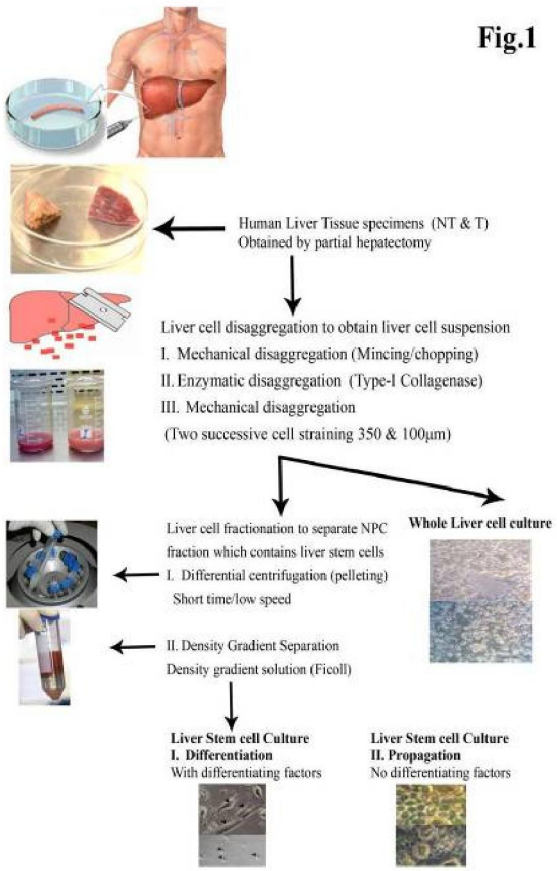


Figure 1. Isolation and morphologic characterization of Human Liver Stem Cells.

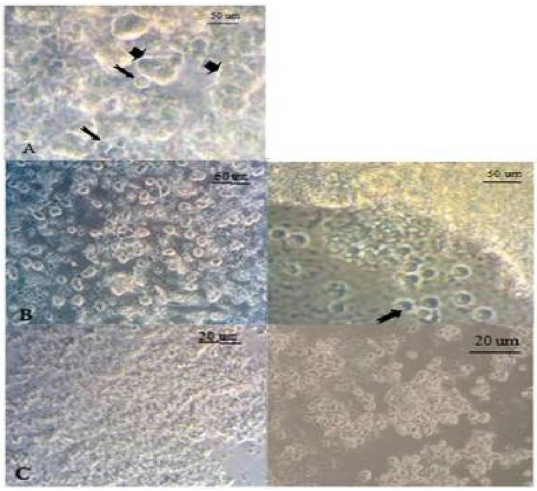


Figure 2. Fractionation of liver cells isolated from tumor and non-tumor tissues

(A) Non-parenchymal liver cells were separated from both tumor- and non-tumor liver tissues using repeated differential centrifugation followed by density gradient separation to purify NPC fraction from contaminating debris and dead cells. Thin arrows indicate representatives of non-parenchymal cells. Thick arrows indicate representatives of parenchymal cells. (B) Liver cell morphology: large polygonal hepatocytes and small rounded NPCs. (arrow indicates NPCs). (C) Non-tumor-derived liver cells did not develop a full sheet of confluent monolayer at day 5 (right figures), tumor-derived liver cells (left figures) developed a full sheet of confluent monolayer at day 5 due to higher rate of proliferation.

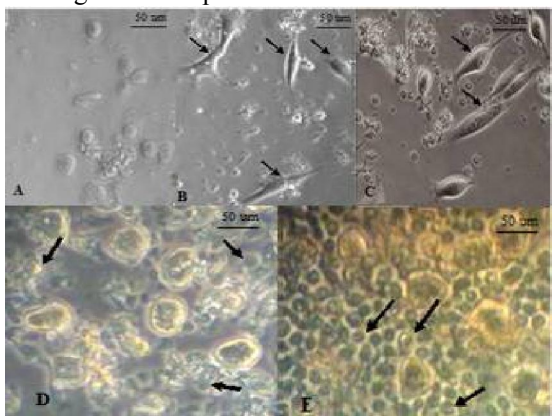


Figure 3. Differentiation and proliferation of cultured liver stem/progenitor cells

Morphological variations of isolated cultured liver stem cells indicated differentiation of liver stem cells. (A) Liver stem cells showed small (about 10 um) spherical to oval shaped cells in day 0. (B) Liver stem cells showed relatively larger (about 30 um) spindle shaped cells in day 7-10. (C) Liver stem cells becomes large (up to 50 um) epithelial-like shape resembling mature liver cells in day 14-20. (D) Non-tumor-derived Liver stem cells in day 12. (E) Tumor-derived liver stem cells in day 12.

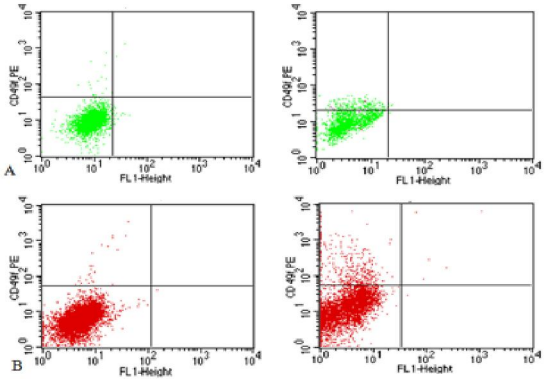


Figure 4. Flow cytometric characterization of liver stem/progenitor cells

Flow cytometric analysis showed significantly higher expression of CD49f marker in both non-tumor- and tumor-derived liver stem cells (21 fold increases). (A) Left panel showed 1.5 fold increases in day 0 between tumor- (upper panel) and non-tumor-derived liver stem cells (lower left panel). (B) Right panel

showed CD49f marker expression in day 12 in both tumor- (upper panel) and non-tumor-derived (lower panel) liver stem cells.

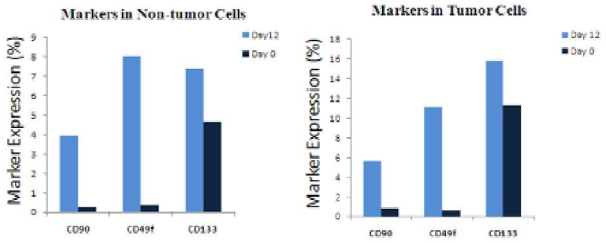


Figure 5. Comparison between liver stem cell markers expression of tumor and non-tumor cells at Day 0 and Day 12

CD49f expression showed 1.5 fold increase between tumor and non-tumor cells at day 0 or day 12. Moreover, CD49f expression showed 21 fold increase between tumor or non-tumor cells at day 0 or day 12 ($P < 0.013$). CD90 and CD133 markers expression confirmed the higher proliferation rate of tumor-derived liver stem cells than non-tumor-derived liver stem cells in both day 0 and day 12.

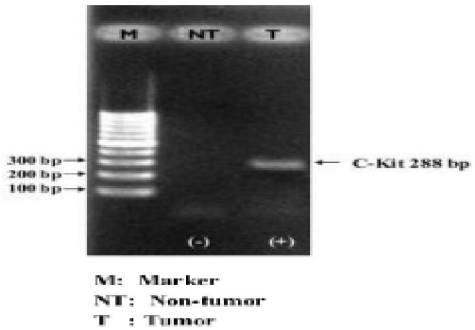


Figure 6. Expression of c-kit gene in non-tumor and tumor human liver tissue detected by Reverse Transcription-PCR.

The expression of c-kit (stem cell factor receptor) gene did show positive expression in liver tissue of tumor origin, while expression was absent in non-tumor liver tissue.

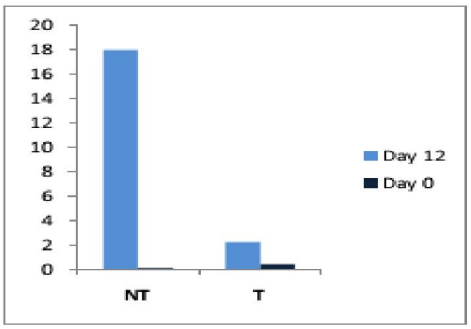


Figure 7 Comparison between the expression of CD117 at day 0 and day 12 in tumor- and non-tumor-derived liver cells.

CD117 expression showed a significant increase in both tumor-and non-tumor-derived liver cells at day 12 compared to day 0.

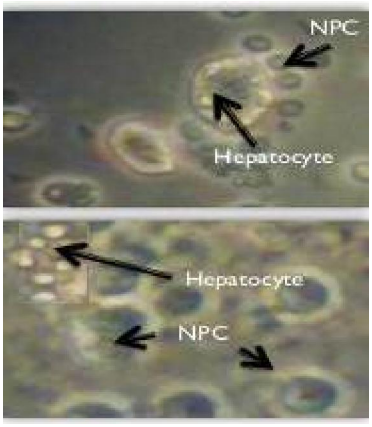
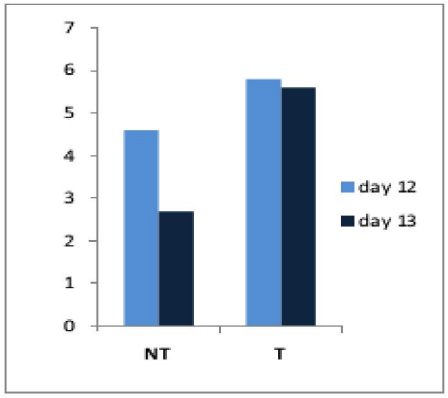
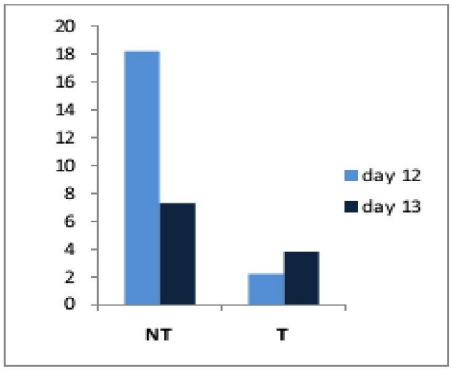


Figure 8 The impact of TGF-β on liver cell morphology

Phase contrast microscopic images show the development of cytoplasmic vacuoles (A) in hepatocytes and NPCs of non-tumor tissue after 24 h of treatment with TGF-β (5 ng/ml) and is considered as a sign of cell death whereas, NPCs of tumor tissues did not affected by such treatment (B).



A



B

Figure 9. The effect of TGF-β on CD90 and CD117expression

(A) The expression of CD90 in liver stem cells before and after 24h of TGF-β (5 ng/ml) treatment in tumor- and non-tumor-derived liver stem cells. After TGF-β treatment, CD90 expression significantly decreased in non-tumor cells, whereas non-significant change was detected in tumor cells. (B) The expression of CD117 in liver stem cells before and after treatment with TGF-β (5 ng/ml) for 24h in tumor- and non-tumor-derived liver stem cells. After treatment TGF-β, CD117 expression significantly decreased in non-tumor liver stem cells whereas, non-significant change in CD117 expression was detected in tumor cells.

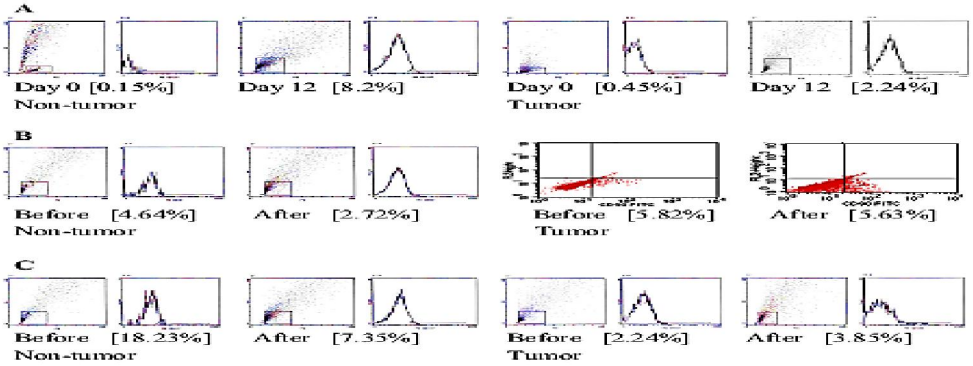


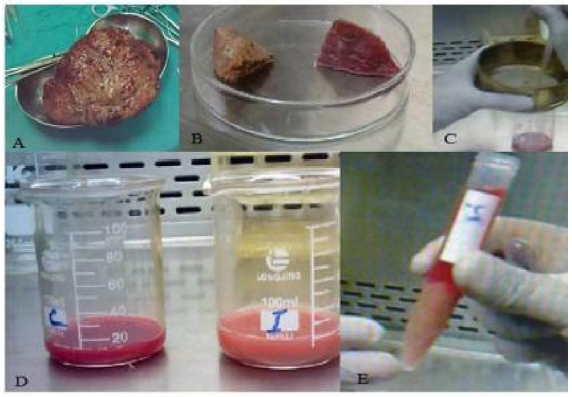
Figure 10 Flow cytometricanalysisof CD117 and of CD90

Flow cytometric analysis patterns of CD117 and CD 90. (A) Flow cytometric analysis of CD117 at day 0 and day 12 in both tumor- and non-tumor-derived liver cells. (B) Flow cytometric analysis of CD90 before and after treatment with TGF-β in both tumor- and non-tumor-derived liver cells. (C) Flow cytometric analysis of CD117 before and after treatment with TGF-β in both tumor-and non-tumor-derived liver cells.

Supporting Information

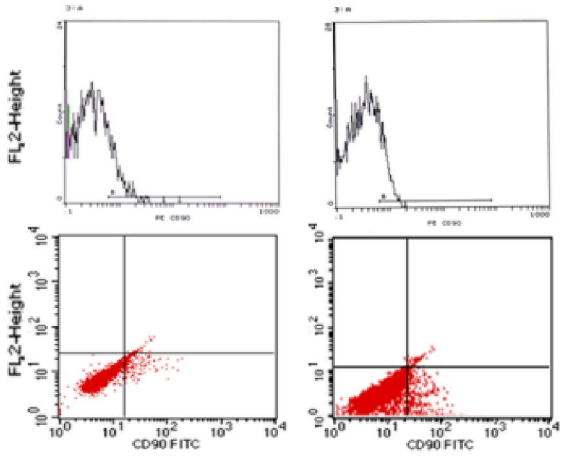
Steps of liver cell disaggregation. (A) Whole resected liver tissue obtained by partial therapeutic hepatectomy. (B) Right tissue is the malignant part and the left tissue is the non-malignant part. (C) Digestion of tumor and non-tumor liver tissue. (D) Right panel shows tumor and left panel shows non-tumor liver cell suspension. (E) Red blood cells-free liver cell suspension.

Supplemental Data S1



Supplemental Data S1

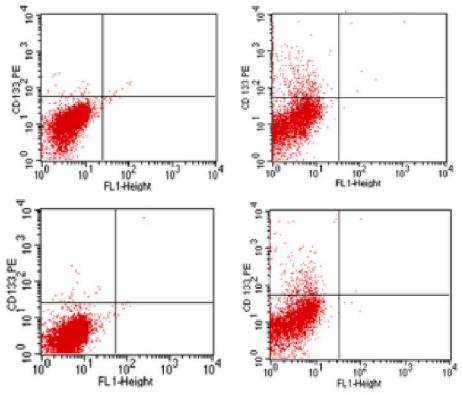
Supplemental Data S2



Supplemental Data S2

Flow cytometric analysis of CD90 marker expression in tumor- and non-tumor derived liver stem cells at day 0 and day 12. (A) Left panel showed 1.8 fold increases in day 0 between tumor- (upper panel) and non-tumor-derived liver stem cells (lower left panel). (B) Right panel showed CD90 marker expression in day 12 in both tumor- (upper panel) and non-tumor-derived (lower panel) liver stem cells.

Supplemental Data S3



Supplemental Data S3

Flow cytometric analysis of CD133 marker expression in tumor- and non-tumor derived liver stem cells at day 0 and day 12.(A) Left panel showed 1.27 fold increases in day 0 between tumor- (upper panel) and non-tumor-derived liver stem cells (lower left panel). (B) Right panel showed CD133 marker expression in day 12 in both tumor- (upper panel) and non-tumor-derived (lower panel) liver stem cells.

4. Discussion:

Treatment of liver diseases is still a significant problem of contemporary medicine. WHO Statistics revealed that more than 240 million people are diagnosed with various chronic and acute liver diseases and 600,000 people die annually. Conventional therapeutic approaches remain insufficient for treating liver pathologies. A large body of data accumulated over recent years attests to the fact that cell therapy can be considered as one of the priority areas in modern biomedicine. No risk of rejection, no need for sophisticated techniques of surgery, availability of long term storage and restoring the recipient own cell function are the major advantages of using somatic stem cells in liver diseases [23]. SLSCs (0.5–2.5% of the hepatic parenchyma cells) are already committed to hepatocytes capable of both *in vivo* differentiation into hepatocytes and population maintenance; this fact may prolong their therapeutic effect. They require no additional differentiation procedures. Specific attention is given today to the biology and isolation methods of SLSCs and searching for optimal cell populations possessing the highest regenerative potential [24].In this work, microscopic images of liver cell morphology are consistent with Wee [25] who reported that the liver parenchyma comprises a heterogeneous population of cells, namely, hepatocytes, bile duct and ductular epithelia; and non-parenchymal cells NPCs including Kupffer, endothelial, mesothelial and inflammatory cells.Also, Turner *et al.* [26] stated that hepatic progenitor cells are smaller in diameter in comparison with mature hepatocytes. In addition,results provided that SLSCs are activelyproliferatingcells induced in liver diseases including HCC. These data suggests the participation of tumor stem cells in hepatocarcinogenesis process. These results are in agreement with Clayton and Forbes [27] who reported that the intra-hepatic population of liver progenitor cells expands during liver injury when hepatocyte proliferation is inhibited. Also, these results agreed with Stachelscheid *et al.* [28], Oishi and Wang [29] and Mikhail and He [30] who stated that SLSCs compartment in several types of liver diseases are active and they proliferate as a regenerative response to tissue damage, while stem/progenitor cells in normal livers are quiescent.

The protooncogene c-kit thatencodes a receptor tyrosine kinase (stem cell factor receptor) has been demonstrated to be expressed in a wide variety of humanmalignancies, including gastrointestinal stromaltumors (GIST), colon carcinoma, salivary glandcarcinoma, testicular germ cell tumors and neuroblastoma [31]. In this study the analysis of c-kit gene expression in tumor and non-tumor liver tissue using RT-PCRrevealed that expression was absent in non-tumor liver tissue, while it was clearly expressed in tumor liver tissue. This isconsistent with previous studies reported that up-regulation of c-kit gene has been reported in a number of solid tumors [32-34] and that c-kit expression increases with severity of liver diseases particularly in carcinogenic conditions and it might play a certain role in HCC progression, and serves as a prognostic marker [33]. TGF-β signaling regulates diverse cellular processes. TGF-β pathways have been implicated in normal liver development as well as in cancer formation.The dysfunctionsof such pathway can result in various kinds of diseases, such as liverfibrosis and cancer [35 and 36]. The relationship between TGF-β and c-kit gene has been reported in some hematologic and solid tumors [32-34], but up to our knowledge, nothing has been studied about its effect in liver tumors. To examine such a relationship, TGF-β was added to cultured tumor- and non-tumor-derived liver cells and morphology as well as the expression of the stem cell marker;CD90 and CD117 (c-kit protein) were studied.In this work, TGF-β-treated liver cells developed a marked cell degeneration sign in the form of large number of cytoplasmic vacuoles in hepatocytes of both tumor and non-tumor tissue. This morphological observation was reported by Cheng *et al.* [37] who studied the effect of plasma of severe chronic hepatitis patients with high TGF-β levels on

porcine hepatocytes morphology. Although, non-tumor-derived NPCs generated cytoplasmic vacuoles when treated with TGF-β, tumor-derived liver stem cells did not. This can be explained as resistance of tumor -derived liver stem cells to the inhibitory effect of TGF-β. Our resultswere also confirmed by the flow cytomertic analysis of liver stem cells marker; CD90. TGF-β-treated non-tumor-derived liver stem cells showed highly significant decrease in CD90 expression, whereas no considerable change in the expression of such marker in TGF-β-treated tumor-derived liver stem cells. The unchanged expression of CD90 in tumor-derived liver stem cells after TGF-β treatment might reflect an interrupted TGF-β signaling pathway in tumor liver stem cells which caused these cells to resist the inhibitory effect of TGF-β and escape from antiproliferative response enhancing migration and invasiveness. This suggestion is consistent with other studies [38 and 39] which observed a marked attenuation of TGF-β-mediated antiproliferative response in several human HCC cell lines and stated that tumor cells selectively lost their growth-inhibitory responsiveness to TGF-β may exhibit enhanced migration and invasion. Also, our hypothesis agreed with Tang *et al.* [40] who stated that loss of TGF-β responsiveness can switch TGF-β from tumor suppressor to pro-metastatic factor. Our flow cytometric analysis of CD117 of tumor- and non-tumor-derived TGF-β-treated liver stem cells showed a highly significant decrease in the percentage of CD117 expression in non-tumor-derived TGF-β-treated SLSCs. This result report that TGF-β inhibited non-tumor-derived SLSCswhich express no or very low CD117. On the other hand non-significant change in the percentage of CD117 expression was detected in tumor TGF-β-treated liver stem cellswhich suggested that TGF-β has lostits inhibitory effect on in tumor-derived SLSCswhich show highly increases c-kit expression.

4. Conclusion:

TGF-β loses its inhibitory effect on SLSCs due to an increase in c-kit expression. This suggests that up-regulation of c-kit; which is known to enhance carcinogenesis, in late chronic liver injury and cirrhosis might contribute to the resistance of SLSCs to TGF-β inhibitory effect leading to SLSCs survival and conversion into tumor stem cells. HCC can be prevented in the setting of late chronic liver injury by suppressing the formation of tumor liver stem cells through inactivating c-kit gene expression and restoring TGF-β inhibitory effect on SLSCs at this stage. However, more studies are needed to determine how these findings can be translated into a new therapeutic approach for HCC treatment.

Correspondence to:
Tamer A. Al-Shafie
Mailing Address: Pharmacology and Toxicology Department – 6th floor – Room (C616)
Faculty of Pharmacy and Drug Manufacturing – Pharos University in Alexandria (PUA).
Canal El-Mahmoudia Street, Green Plaza Compound, Smouha District- Alexandria.
E-Mail: tamershafie@yahoo.com

References:

1. Jemal A, Bray F, Center MM, *et al.* Global cancer statistics. CA Cancer J Clin 2011; 61:69.
2. Michael C Kew. Hepatocellular carcinoma: epidemiology and risk factors, Journal of Hepatocellular Carcinoma 2014:1 115–125.
3. Caecilia HC Sukowati, Natalia Rosso, Lory S Crocè, Claudio Tiribelli(2010): Hepatic cancer stem cells and drug resistance: Relevance in targeted therapies for hepatocellular carcinoma. World J Hepatol2010 March 27; 2(3): 114-126.
4. Piscaglia AC, Campanale M, Gasbarrini A, Gasbarrini G. Stem Cell-Based Therapies for Liver Diseases: State of the Art and New Perspectives. *Stem Cells Int.* 2010; 2010: 259461-259471.
5. Katsunori Yoshida, Miki Murata, Takashi Yamaguchi and Koichi Matsuza. TGF-β/Smad signaling during hepatic fibro-carcinogenesis (Review) International Journal of Oncology, 45: 1363-1371, 2014.
6. Novo, E. and Parola, M. (2008). Redox mechanisms in hepatic chronic wound healing and liver fibrogenesis. Fibrogenesis & Tissue Repair 1, 5.
7. Baranova A, La P, Birerdinc A and Younossi ZM. Non-Invasive markers for hepatic fibrosis. Gastroenterology 2011, 11:91.
8. Knight B, Tirnitz-Parker JE, Olynyk JK. C-kit inhibition by imatinibmesylate attenuates progenitor cell expansion and inhibits liver tumor formation in mice. Gastroenterology. 2008 Sep; 135(3):969-79, 979.e1.
9. Kun Wu, Jin Ding, Cheng Chen,Wen Sun, Bei-Fang Ning, Wen Wen, Lei Huang. Hepatic TGF-β Gives Rise to Tumor-Initiating Cells and Promotes Liver Cancer Development. Hepatology 2012 Dec; 56(6):2255-67.
10. Hassel S, Yakymovych M, Hellman U, Ronnstrand L, Knaus P,Souchelnytskyi S. Interaction and functional cooperation between the serine/threonine kinasebone morphogenetic protein type II receptor with the tyrosine kinase stem cell factor receptor. Cell Physiol 2006; 206:457-467.
11. Hassan HT. C-Kit expression in human normal and malignant stem cells prognostic and therapeutic implications. Leukemia Research 33 (2009) 5–10.
12. Wauthier E, Schmelzer E, Turner W, Zhang L, Cluyse EL, Ruiz J, Turner R, Furth ME, *et al.* (2008): Hepatic Stem Cells and Hepatoblasts: Identification, Isolation, and Ex Vivo Maintenance In: Mather JP Stem Cell Culture 1stedn. Vol 86 Ch. 8, p. 137.
13. Stachelscheid H, Urbaniak T, Ring A, Spengler B, *et al.* (2009): Isolation and Characterization of Adult

Human Liver Progenitors from Ischemic Liver Tissue Derived from Therapeutic Hepatectomies. Tissue Eng Part A. Jul; 15(7):1633-43.
14. Wauthier E, Schmelzer E, Turner W, Zhang L, LeCluyse E, Ruiz J, Turner R, Furth ME, Kubota H, Lozoya O, Barbier C, McClelland R, Yao HL, Moss N, Bruce A, Ludlow J, Reid LM. Hepatic stem cells and hepatoblasts: identification, isolation, and ex vivo maintenance. *Methods Cell Biol.* 2008; 86: 137-225.
15. Weiss TS, Lichtenauer M, Kirchner S, Stock P, Aurich H, Christ B, Brockhoff G, Kunz-Schughart LA, Jauch KW, Schlitt HJ, Thasler WE. Hepatic progenitor cells from adult human livers for cell transplantation. *Gut.* 2008; 57(8): 1129-1138.
16. Tanaka M, Itoh T, Tanimizu N, Miyajima A. Liver stem/progenitor cells: their characteristics and regulatory mechanisms. *J Biochem.* 2011; 149(3): 231-239.
17. Clayton E, Forbes SJ. The isolation and in vitro expansion of hepatic Sca-1 progenitor cells. Biochem *Biophys Res Commun.* 2009; 381(4): 549-553.
18. Laurson J, Selden C, Clements M, Mavri-Damelin D, Coward S, Lowdell M, Hodgson HJ. Putative human liver progenitor cells in explanted liver. *Cells Tissues Organs.* 2007; 186(3): 180-191.
19. Kon J, Ichinohe N, Ooe H, Chen Q, Sasaki K, Mitaka T. Thy1-positive cells have bipotential ability to differentiate into hepatocytes and biliary epithelial cells in galactosamine-induced rat liver regeneration. *Am J Pathol.* 2009; 175(6): 2362-2371.
20. Weiss TS, Lichtenauer M, Kirchner S, Stock P, Aurich H, Christ B, Brockhoff G, Kunz-Schughart LA, Jauch KW. Hepatic progenitor cells from adult human livers for cell transplantation. *Gut* 2008; 57: 1129–1138.
21. Clayton E, Forbes SJ. The isolation and in vitro expansion of hepatic Sca-1 progenitor cells. *Biochem.Biophys. Res. Commun.* 2009; 381(4): 549-553.
22. Tanaka M, Itoh T, Tanimizu N and Miyajima A. Liver stem/progenitor cells: their characteristics and regulatory mechanisms. *J. Biochem.* 2011; 149(3): 231–239.
23. Esrefoglu M. Role of stem cells in repair of liver injury: Experimental and clinical benefit of transferred stem cells on liver failure. *World J Gastroenterol.* 2013; 19(40): 6757-6773.
24. Schmelzer E, Zhang L, Bruce A, Wauthier E, Ludlow J, Yao HL, Moss N, Melhem A, McClelland R, Turner W, Kulik M, Sherwood S, Tallheden T, Cheng N, Furth ME, Reid LM. Human hepatic stem cells from fetal and postnatal donors. *J Exp Med.* 2007; 204(8): 1973-1987.
25. Wee A. Fine needle aspiration biopsy of the liver: Algorithmic approach and current issues in the diagnosis of hepatocellular carcinoma. *CytoJournal* 2005; 2:7-23.
26. Turner R, Lozoya O, Wang Y, Cardinale V, Gaudio E, Alpini G, Mendel G, Wauthier E, Barbier C, Alvaro D. Human hepatic stem cell and maturational liver lineage biology. *Hepatology*2011; 53(3): 1035-1045.
27. Clayton E, Forbes SJ. The isolation and in vitro expansion of hepatic Sca-1 progenitor cells. *Biochem. Biophys. Res. Commun.* 2009; 381(4): 549-553.
28. Lei-Bo Xu, Chao Liu. Role of liver stem cells in hepatocarcinogenesis *World J Stem Cells 2014 November 26; 6(5): 579-590.*
29. Oishi N, Wang XW. Novel therapeutic strategies for targeting liver cancer stem cells. *Int J Biol Sci.* 2011; 7(5): 517-535.
30. Mikhail S, He AR. Liver cancer stem cells. *Int J Hepatol.* 2011; 2011: 486954.
31. Chih-Yuan Chung, Kun-TuYeh, Nicholas C. Hsu, Julia Huei-Mei Chang,Jen-Tsun Lin, Huey-Ching Horng, Cheng-Shyong Chang. Expression of c-kit protooncogene in human hepatocellular carcinoma. Cancer Letters 217 (2005) 231–236.
32. Chen L, Shen R, Ye Y, Pu X-A, Liu X, *et al.* (2007). Precancerous Stem Cells Have the Potential for both Benign and Malignant Differentiation. PLoS ONE 2(3): e293.
33. Yan WW, Huang A, Li YG, Wang SS, Dai GH. (2011): Expressions of CD34 and CD117 in human hepatocellular carcinomas and the clinical significance. ZhonghuaGanZang Bing ZaZhi. Aug; 19(8):588-93.
34. Dubois CM, Ruscetti FW, Stankova J, Keller JR (1994): TGF-β Regulates c-kit Message Stability and Cell-Surface Protein Expression in Hematopoietic Progenitors. Blood, Vol 83, No 11 (June l), pp 3138-3145.
35. Huang and Chen.Regulation of TGF-β receptor activity Cell & Bioscience 2012, 2:9.
36. M, Carter KT, Baek JY, Koszarek A, Yeh MM, Knoblaugh SE, Grady WM.TGF-β signaling alters the pattern of liver tumorigenesis induced by Pten inactivation. Oncogene. 2014 Aug 18; 0.
37. Cheng YB, Wang YJ, Zhang SC, Liu J, Chen Z, Li JJ (2005): Response of porcine hepatocytes in primary culture to plasma from severe viral hepatitis patients. World J Gastroenterol; 11(48):7585-7590.
38. Kitisin K, Pishvaian MJ, Johnson LB, Mishra L (2007): Liver Stem Cells and Molecular Signaling Pathways in HCC. Gastrointest Cancer Res 1(suppl 2):S13–S21.
39. Amin R, Mishra L. (2008): Liver Stem Cells and TGF-β in Hepatic Carcinogenesis. Gastrointest Cancer Res 2(suppl 2): S27–S30.
40. Cindy Neuzillet, Annemilaï Tijeras-Raballand, Romain Cohen, JérômeCros, Sandrine Faivre, Eric Raymond and Armandde Gramont (2014): Targeting the TGF-β pathway for cancer therapy. Pharmacology & Therapeutics (2014).

11/22/2014