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Isolation and Characterization of Quiescent Human Hepatic Stellate Cells For Physiological Modeling of Liver Metabolism and Function

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ABSTRACT

Human hepatic stellate cells (HSC) are liver non-parenchymal cells (NPC's) that are present in the peri-sinusoidal tissue space of Disse. In normal liver, stellate cells store vitamin A in the form of retinol droplets and are deemed quiescent (q-HSC). Following hepatic injury, HSCs become activated, proliferate and produce extracellular matrix (ECM) which accumulates in chronic condition leading to liver fibrosis. In addition, liver cell-cell interactions, including ones between hepatocytes and stellate cells, have important functions in stabilization of hepatic function. This can be recreated in vitro where the co-culturing of primary hepatocytes with HSC maintains and stabilizes the differentiated phenotype of hepatocytes such as albumin secretion and cytochrome P450 expression. To facilitate these studies we have made attempts to isolate, purify and characterize quiescent hepatic stellate cells from human liver tissue. Activated HSCs were also generated from q-HSC and benchmarked against activated HSC cell line LX2. Cells were characterized for presence of retinol droplets by phase and fluorescent microscopy. Further characterization was carried out by ICC, flow-cytometry and microscopic analysis. Gene expression was characterized by q-RT PCR for various stellate and mesenchymal cell markers including collagen and vimentin. Activated HSC were co-cultured with primary human hepatocytes to study their effect on hepatic function and metabolism. Isolated cells were positive for GFAP and negative for α-SMA staining. Flow cytometry analysis indicated enrichment of approximately 85% or more cells positive for GFAP. As expected, culturing these cells for more than ten days activated them to myofibroblasts (A-HSC) indicated by positive staining for α-smooth muscle actin. Activated HSCs (A-HSC) were also positive for Vimentin and CD271 indicating their mesenchymal origin. Expression of TGFβ and PDGFrβ was used to determine their functionality. Comparative analysis with LX2 indicated that our activated stellate cells were found to express all stellate cell markers at significantly higher levels. When combined with hepatocytes in co-culture HSCs were able to stabilize the culture of primary human hepatocytes. In this study, we have generated primary quiescent and activated hepatic stellate cells from human liver tissue with high purity and function. Our data indicates that mono- and co-culture of hepatic stellate cells with hepatocytes will mimic physiologically relevant models of stellate cell activation and their effect on hepatic function.

INTRODUCTION

Hepatic stellate cells (HSCs) are liver non-parenchymal cells located in the space of Disse between the sinusoidal endothelial cells and hepatocytes, and account for 5%–8% of the cells in the liver. In normal uninjured liver they are site for vitamin A storage however, when activated by chronic liver injury these cells acquire a myofibroblastic state secreting ECM matrix, leading to scarring of the tissue and liver fibrosis.

Hepatic stellate cells being a major non-parenchymal cell (NPC) of the liver are the essential part of organotypic liver disease models aimed towards recapitulating the intricate autocrine and paracrine signaling that occurs between liver cell types.

MATERIALS AND METHODS

Cell Isolation: Hepatic stellate cells were isolated from resected or whole liver human tissue. Briefly, tissue were digested with a two step Pronase and Collagenase/Protease solution followed by purification of non-parenchymal liver cell using a density gradient.

Immunostaining: Isolated cells were characterized by intracellular staining for GFAP and α-SMA. In brief, cells were fixed, permeabilized, and following blocking with 10% goat serum incubated overnight in primary antibodies. Following three washes, bound primary antibody were detected with alexa fluor labeled secondary antibody. Cells were characterized either by microscopy or by flow cytometry.

Gene Expression analysis: Cells were lysed using Trizol® and RNA was purified using the Purelink® RNA Mini kit (Life Technologies, 12183018A). Five hundred nanograms of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies, 4368813). Gene expression was assessed using a custom designed TaqMan® Array card containing either 48 hepatocyte-specific genes of interest to ADME/Tox studies or 28 genes to study hepatic stellate cell specific effects.

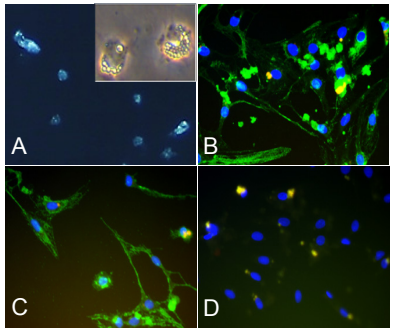
Co-culture of Hepatic stellate cells with human primary hepatocytes: Human primary hepatocytes (Life Technologies, HMCPIS) were co-cultured along with activated hepatic stellate cells at 1:5 ratio of stellate cells/hepatocytes in 6 well plates. Cells were co-cultured either in trans-well or in direct cell to cell contact.

CYP3A4 Enzymatic Activity: On day 4, cultures were washed with fresh William's E Maintenance Media and incubated for 15 minutes with testosterone for CYP3A4 activity. After incubation, supernatants were collected and frozen until analysis. Metabolites were detected using LC/MS/MS or HPLC. Results are normalized to cell numbers and incubation time.

ELISA for human Albumin and TGF-β: Expression of human albumin and TGF-β was measured using commercially available kits from Abcam (Cambridge, MA) and Life Technologies (Cat# KAC1688) respectively. The Abcam kit specifically measures human albumin.

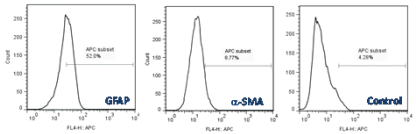
RESULTS

Figure 1. Morphological and Immuno-characterization of quiescent human hepatic stellate Cells



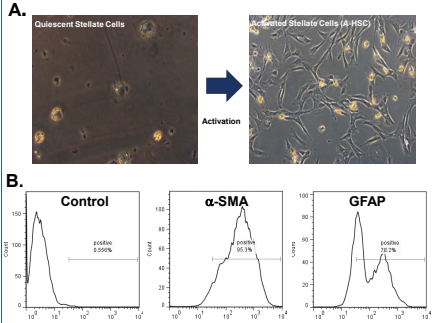
Immuno-characterization of human hepatic stellate cells: Cells were isolated from human liver tissue and Immunostained for stellate cell markers. (A) Vitamin A autofluorescence. Inset figure shows magnified view of stellate cells in phase contrast. (B) GFAP (C) Desmin (D) α-SMA.

Figure 2. Flow cytometry analysis of quiescent stellate cells



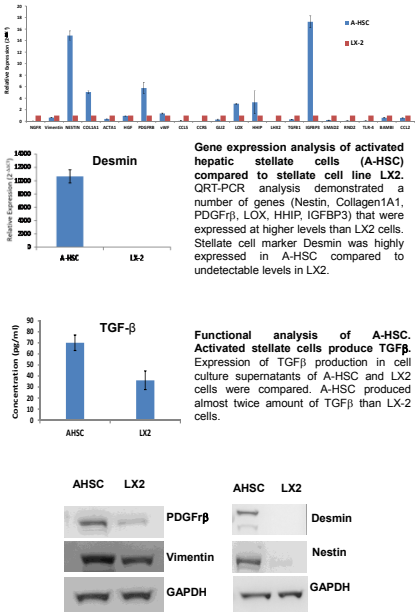
Representative flow cytometry analysis of isolated human quiescent stellate cells: Cells were isolated and labeled with anti-GFAP antibody (stellate cell marker) and α-SMA (activated stellate cell marker) followed by detection with alexa fluor 633 labeled secondary antibody.

Figure 3. Activation of quiescent hepatic stellate cells to A-HSC *in vitro*



Generation of activated HSCs from quiescent cells. (A). Quiescent hepatic stellate cells were grown in uncoated tissue culture plates. After 7-10 days in culture cells turned myofibroblastic and started proliferating. **(B).** Immunostaining followed by flow-cytometry analysis demonstrated that more than 90% of cells were positive of α-SMA. Cells were also positive for Vimentin and CD271.

Figure 4. Comparison of activated hepatic stellate cells (A-HSC) to hepatic stellate cell line LX2

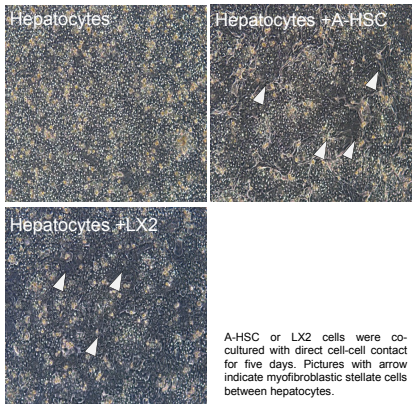


Gene expression analysis of activated hepatic stellate cells (A-HSC) compared to stellate cell line LX2. QRT-PCR analysis demonstrated a number of genes (Nestin, Collagen1A1, PDGFrβ, LOX, HHIP, IGFBP3) that were expressed at higher levels than LX2 cells. Stellate cell marker Desmin was highly expressed in A-HSC compared to undetectable levels in LX2.

Functional analysis of A-HSC. Activated stellate cells produce TGFβ. Expression of TGFβ production in cell culture supernatants of A-HSC and LX2 cells were compared. A-HSC produced almost twice amount of TGFβ than LX-2 cells.

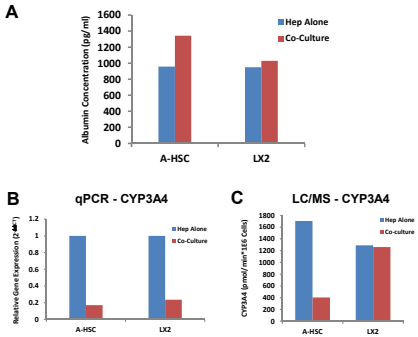
Western blot analysis of activated hepatic stellate cells (A-HSC) compared to stellate cell line LX2. Western blot analysis of A-HSC and LX2 cells demonstrated that majority of the activation markers e.g. Nestin, PDGFrβ and mesenchymal cell marker Vimentin were expressed at significantly higher levels than LX2 cells. Levels of expression of quiescent marker Desmin was unusually high in AHSC supporting q-PCR data.

Figure 5. Co-culture of A-HSC and LX2 with primary human hepatocytes .



A-HSC or LX2 cells were co-cultured with direct cell-cell contact for five days. Pictures with arrow indicate myofibroblastic stellate cells between hepatocytes.

Figure 6. Co-culture of activated stellate cells (A-HSC or LX2) stabilizes albumin production while lowering CYP3A4 activity



Co-culture of activated stellate cells (A-HSC or LX2) along with human primary hepatocytes (A). Albumin expression measured by ELISA at 24 hours of co-culture demonstrate increased albumin production by co-cultures compared to hepatocyte cultures alone. **(B &C)**. CYP3A4 activity measured at day 4 by qRT-PCR gene expression analysis (B) and by LC/MS (C) . The data indicates that co-culture of hepatocytes along with activated stellate cells lowered CYP3A4 activity

CONCLUSIONS

- Our data indicates that we were able to isolate quiescent hepatic stellate cells from human liver tissue as indicated by positive staining for GFAP and Desmin and negative staining for α-SMA .
- Quiescent hepatic stellate cells trans-differentiated to myofibroblast like cells that expressed α-SMA. These cells produced TGF-β and had up-regulated expression of PDGFrβ indicating their activated state. Comparison of primary activated hepatic stellate cell to stellate cell line LX2 indicated enhanced expression of some of the activation marker in A-HSCs.
- Co-culture of activated primary stellate cells or LX2 cells with hepatocytes stabilized hepatic function as evident by increased albumin synthesis. However, a decreased expression of CYP3A4 activity was observed by both qRT-PCR and LC/MS analysis. Similar decrease in CYP3A4 activity has been previously shown in NAFLD induced liver fibrosis (Woolsey SJ *et al*).

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