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CO-CULTURE OF PRIMARY HEPATIC STELLATE CELLS AND HEPATOCYTES IN MODELING OF LIVER FIBROSIS IN 2D AND 3D SPHEROIDS

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ABSTRACT

Human hepatic stellate cells (HSC) are the major contributor to collagen deposition following liver damage. During injury, hepatic stellate cells activate to a phenotype characterized by increased proliferation, motility, contractility, and synthesis of extracellular matrix components that results in progressive liver fibrosis. To understand the activation process of q-HSC and to facilitate cell-cell interactions and predict their effect on hepatocyte function, we have developed co-culture 2D and 3D spheroid systems to model the progression and reversion of liver fibrosis *in vitro*. Following isolation of q-HSC from human cadaveric tissue, their quiescence was indicated by the presence of vitamin A, the presence of GFAP and absence of a-SMA. As expected, after plating and culturing for 10 days on rigid plastic, q-HSC activated to the myofibroblastic (MF) phenotype, which is shown by increased a-SMA expression. Activated MF-HSCs were also positive for Vimentin and CD271 indicating their mesenchymal origin. In order to determine the role of various factors on the activation, q-HSC were maintained in culture until viable culture was establish after which they were cryopreserved at passage 5 and used for all subsequent experiments. Using 2D and 3D co-culture systems, TGF-b and *Methotrexate were used to induce MF-HSC to produce ECM and* to stimulate cytokine, chemokine, and growth factor production as observed in the physiological *in-vivo* setting during fibrogenic liver injury. This response was evaluated by qRT-PCR that was used to evaluate gene expression. When co-cultured with hepatocytes, either in 2D plated plates or 3D spheroid cultures, HSCs stabilized cultures of primary human hepatocytes and elicited responses observed during liver fibrosis that included significantly upregulated collagen deposition and a-SMA expression. In conclusion, we have generated primary human quiescent and activated hepatic stellate cells with high purity and function. Those cells can be co-cultured in 2D plated system and 3D spheroid system to model the liver fibrogenic response. Our data indicates involvement of HSC produced growth factors, cytokines and chemokines in function of hepatocytes with implications in modeling fibrosis for drug metabolism and toxicity testing.

MATERIALS AND METHODS

Cell Isolation:
Hepatic stellate cells were isolated from whole liver human tissue. Briefly, tissue were digested with a three step EGTA, Pronase and Collagenase solution followed by mechanical digestion and purification of non-parenchymal cells using density gradient. Primary hepatocytes were selected from existing inventory and validated for ability to form 3D spheroids. These cells were then used for all 2D and 3D experiments.

Co-culture of Hepatic stellate cells with human primary hepatocytes: For 2D co-cultures, human primary hepatocytes were co-cultured along with activated hepatic stellate cells at 1:4 ratio of stellate cells/hepatocytes in 6 well plates and Gibco™ Collagen Coated 24-well plates (A1142802). Adv. DMEM media supplemented with 10% FBS for plating and 0% FBS for maintenance after initial 24hr. Additional GlutaMAX and P/S were used as needed. There was no overlay used for this experiment.

For 3D spheroids, ratio of 1:3 was used for co-cultures and approximately 500 stellate cells were used for every 1500 hepatocytes. Hepatic spheroids were made using 1500 hepatocytes. Nunclon™ Sphera™ super low attachment U-bottom 96-well Microplates (174925) were applied as an easy to use method for generation of 3D spheroids. Briefly, after cells were added in 200ul of plating media (Adv. DMEM supplemented with 10% FBS, GlutaMAX and P/S) to each well, plates were spun at 200g for 2 min and placed in the incubator for 5 days. At that point half of the media was replaced with fresh maintenance medium (Adv. DMEM media supplemented with GlutaMAX, P/S and 0% FBS) for 2 more days. Experimental treatment started at 7 days after initiation of co-cultures. At that time, half of the media was removed and replaced with maintenance medium. This step was repeated 2 times to dilute existing medium containing FBS. At that point, half media was removed and replaced with equal volume of maintenance medium containing either TGFβ (100ng/mL) or Methotrexate (200mM). In both cases this concentration was 2x of the final to which spheroids were exposed (TGFβ at 50ng/ml and Methotrexate at 100mM at final concentration). Similarly to this, media change with addition of fresh TGFβ and Methotrexate was repeated every other day. TGFβ (Gibco™, PHG9204), Methotrexate (Sigma, A6770-100MG)

Gene Expression analysis: Hepatic and co-culture spheroids were lysed using Trizol® followed by mRNA isolation. Following RT, gene expression was assessed using TaqMan® primers for Collagen 1 Alpha 1 (COL1A1) – HS00164004-M1, Collagen 4 Alpha 1 (COL4A1) – HS00266237-M1, Fibronectin (FN1) – HS00415006-M1; Actin, alpha 2, smooth muscle (ACTA2-aSMA) –HS00909449-M1. GAPDH was used as internal control.

RESULTS

Isolation and Characterization of Human Myofibroblastic Stellate Cells (MF-HSC)

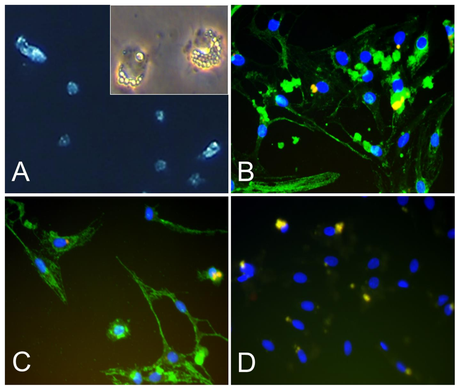


Figure 1. Morphological and Immuno-characterization of quiescent human hepatic stellate Cells. Cells were isolated from human liver tissue and Immunostained for stellate cell markers. (A) Vitamin A autofluorescence. Inset figure shows magnified view of stellate cells in phase contrast. (B) GFAP (C) Desmin (D) α-SMA.

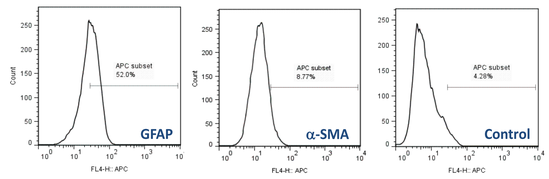


Figure 2. Representative flow cytometry analysis of isolated human quiescent stellate cells. Cells were isolated and labeled with anti-GFAP antibody (stellate cell marker) and α-SMA (activated stellate cell marker) followed by detection with alexa fluor 633 labeled secondary antibody.

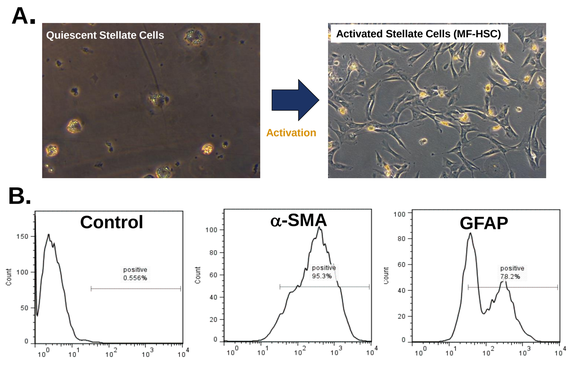
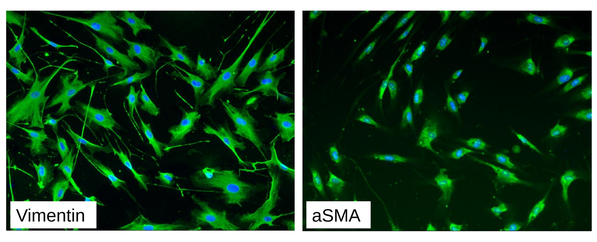


Figure 3. Generation of activated HSCs from quiescent cells. (A). Quiescent hepatic stellate cells were grown in uncoated tissue culture plates. After 7-10 days in culture cells activate to Myofibroblastic Hepatic Stellate Cells (MF-HSC) and start proliferating. **(B).** After activation and proliferation, 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 (Data not shown).



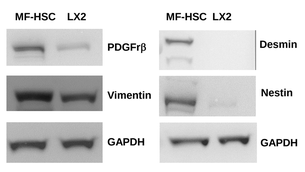


Figure 4. Characterization pf MF-HSC by IHC and Western blot. Vimentin and aSMA was used to identify MF-HSC and to determine their purity. Additional Western blot analysis of MF-HSC and LX2 (commercially available human hepatic stellate cell line) demonstrated presence of Nestin, PDGFrβ, Vimentin and Desmin in primary MF-HSC but only PDGFrβ and Vimentin could be detected in LX2. This supports true identity of our primary MF-HSC and their functionality.

2D Co-Cultures of Hepatocytes and MF-HSC

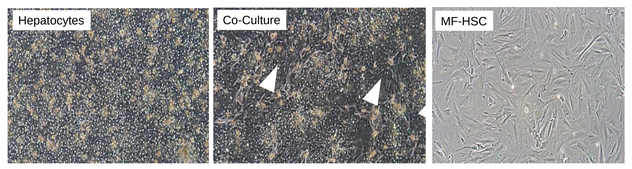


Figure 5. Morphological assessment of monocultures and co-culture of MF-HSC with primary human hepatocytes. Note that in each case, hepatocyte and MF-HSC monocultures, and combined hepatocytes and MF-HSC co-cultures formed well developed monolayers at approximately 95-100% confluence. Arrowheads indicate MF-HSC mixed between hepatocytes. Note that the addition of MF-HSC did not extend lifespan of hepatocytes and after 7 days cultures started to decline.

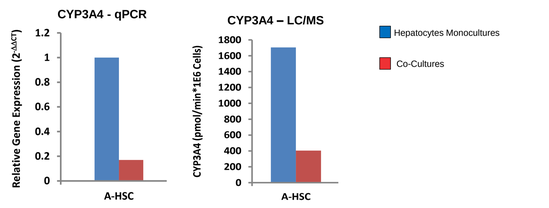


Figure 6. Co-culture of primary hepatocytes and MF-HSC resulted in declined CYP3A4 activity. As expected, MF-HSC impacted metabolic performance of primary hepatocytes by reducing CYP3A4 activity measured at day 4 by qRT-PCR gene expression analysis and by LC/MS. This was expected as when MF-HSC are plated on rigid plastic, they produce cytokines and growth factors that have been implicated in liver regeneration and repair, a process that often is characterized by decreased metabolism.

3D Spheroid Co-Cultures of Hepatocytes and MF-HSC

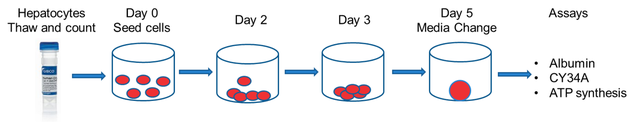


Figure 7. Workflow of Nunclon™ Sphera™ super low attachment U-bottom plate system that allows assembly of primary hepatocytes and co-cultures into 3D-spheroids.

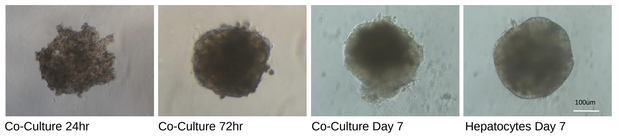


Figure 8. Representative images of 3D Spheroids of Co-cultures of Hepatocytes and MF-HSC during their formation using Nunclon™ Sphera™ super low attachment U-bottom plate. Approximately 1500 hepatocytes and 500 MF-HSC were used to make each spheroid. Note progression of cells going from cell aggregate (24hr) to more compacted 3D structure (72hr) and finally fully assembled spheroid (7 days). Note the morphological difference of outside surface of co-culture spheroid when compared to more defined and "smoother" hepatocyte spheroid.

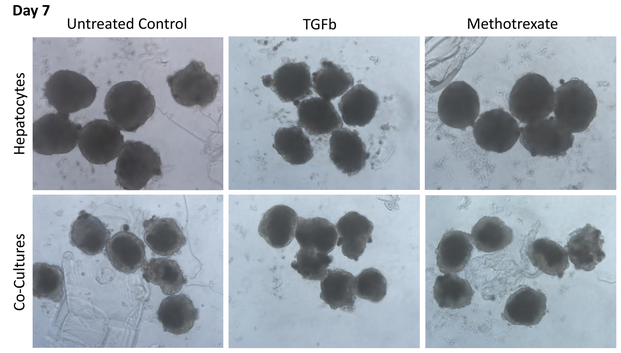


Figure 9. Images displaying morphology of 3D Spheroids of Co-cultures of Hepatocytes and MF-HSC, and Hepatocytes alone following treatment with TGFβ and Methotrexate for 7 days. Note that each spheroid was treated individually in each well and then each treatment group was combined for the imaging. Also note that phenotypic morphology shows differential density of hepatocyte spheroids that appear more uniform and denser than co-culture spheroids. Co-culture spheroids appear to be more transparent containing dense centers.

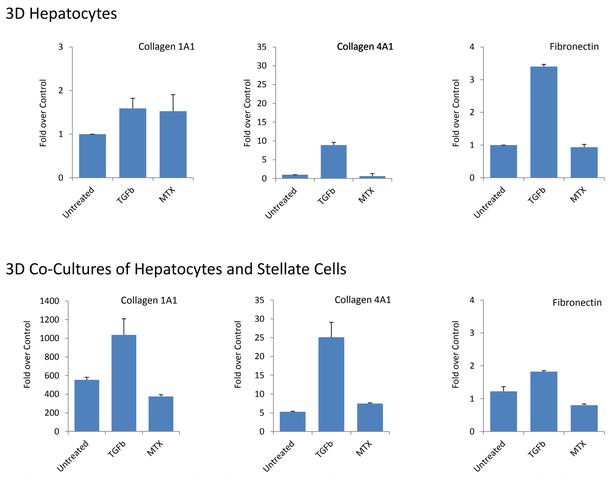


Figure 10. Expression of Collagens, and Fibronectin in 3D Co-culture of primary hepatocytes and MF-HSC and 3D Hepatocytes with and without treatment with TGFβ and Methotrexate. As expected, there is significant increase in basal expression of Collagen 1A1 in 3D co-cultures when compared with 3D hepatocytes. This is expected as activated MF-HSC should display strong expression of this fibrogenic collagen. Additional increase is observed following induction of fibrogenic response with TGFβ . Note that Methotrexate has not shown any visible change in gene expression after 7 day treatment suggesting that longer time may be needed to observe any change with this inducer of fibrosis.

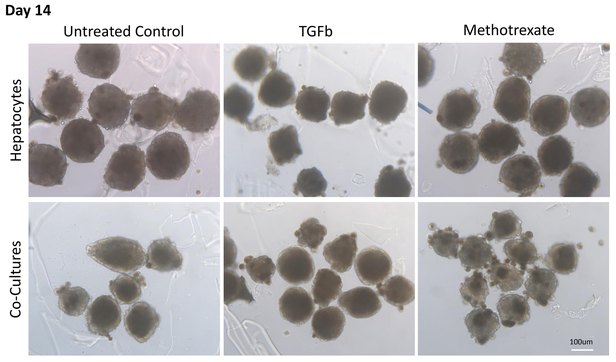


Figure 11. Images displaying morphology of 3D Spheroids of Co-cultures of Hepatocytes and MF-HSC, and Hepatocytes alone following treatment with TGFβ and Methotrexate for 14 days. Note that phenotypic morphology shows differential density of hepatocyte spheroids that appear more uniform and denser than co-culture spheroids much more than those at 7 days. Also note appearance of smaller "vesicle-like" structures that are predominantly visible in co-cultures following treatment with Methotrexate.

CONCLUSIONS AND FUTURE PLANS

- Our data indicates that we were able to isolate hepatic stellate cells from human liver tissue as indicated by positive staining for GFAP and Desmin. Those cells activate to Myofibroblastic Hepatic Stellate Cells (MF-HSC) and express Vimentin, Nestin, aSMA and Desmin.
- 2D co-culture of MF-HSC with hepatocytes showed decreased expression of CYP3A4. Similar decreased activity has been shown in NAFLD induced liver fibrosis (Woolsey SJ, et al. Drug Metab.Dispos. 2015 Oct;43(10):1484-90).
- 3D co-culture of primary hepatocytes and MF-HSC offer easy to assemble system to generate fibrotic cell model *in vitro*. The system easily allows culture for over 21 days and allows for significant expression of fibrogenic Collagens. This can be induced by TGFβ .
- Our preliminary data at 14 days treatment suggest that chronic treatment with Methotrexate may induce fibrogenic changes *in vitro*. This will be farther explored in our future studies.
- Future histological and IHC examination will be performed to fully characterize co-culture fibrotic model generated using Nunclon™ Sphera™ super low attachment U-bottom plate system and primary hepatocytes mixed with MF-HSC.

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