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iPSC-/ESC-derived hepatic stellate cells in 2D and 3D

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Abstract

Liver Fibrosis is a major concern for the world as it causes chronic inflammation which again induces an unhealthy and abnormal healing response. Most common causes to liver fibrosis are alcohol consumption, non-alcoholic steatohepatitis (NASH), hepatitis B(HBV), autoimmune hepatitis, non-alcoholic fatty liver disease (NAFLD) and cholestatic liver diseases. Continuous fibrosis in the liver can lead to losing normal function of the liver and eventually end with liver failure. As of now in vitro models of hepatic stellate cells (HSCs) are needed to eventually be able to produce models that can be used to test treatments against fibrosis.

By differentiating HSCs from iPSCs and hESC the derived cells were tested with both immunostaining and RT-qPCR which checked gene expression to conclude if the cells had differentiated into HSC-like cells.

The iHSCs were then used to construct two types of 3D cultures where the one culture called HSC in GelTrex. GelTrex matrix were selected to mimic ECM of space of Disse, which is a mixture of reduced growth factor (RGF) and basement membrane extract (BME). The GelTrex samples were also treated with TGF-β1 to see if this would increase expression of fibrosis-associated genes and to investigate presence of fibrosis-associated proteins. The GelTrex samples showed none reproduceable results as the construction of the GelTrex droplet was unsuccessful, due to air bubbles effecting the 3D culture. The RNA isolation was performed with poor technique at the RNA extraction step, which resulted in a deviation throughout the results.

The other 3D culture was called for spheroid models, which were made with co-culturing of HepG2 and iHSC to mimic the cell-cell interaction. Construction of the spheroids were done by a mixture of HepG2 and HSC with a 5:1 ratio. The cells were separated into control and cells treated with TGF-β1. The cells were sorted from the spheroid and analyzed for changed in gene expression. Sorting showed proliferation of HepG2 in spheroids, which elevated the ratio, and there for the ratio should be tweaked to lower for construction on spheroids. RT-qPCR showed error in RNA extraction in RNA isolation together with the time span on sorting cells, which might have been too low.

Abbreviation

α-SMA	Alpha smooth muscle actin
A-SMA	Alpha smooth muscle actin
ALP	Alkaline phosphate
ALT	Alanine transaminase
ASA	Acetylsalicylic acid
AST	Aspartate transaminase
BME	Basement membrane extract
BMP4	Bone morphogenetic protein 4
CaHSC	Central vein-associated hepatic stellate state
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acic
ET-1	Endothelin-1
DPBS	Dulbecco's phosphate-buffered saline
FACS	Fluorescence activated cell sorting
FGF2	Fibroblast growth factor 2
Gfap, quiescent state	Glial fibrilliary acidic protein
GGT	Gamma-glutamyl transferase
GT	GelTrex
HBV	Hepatitis B
hESC	Human embryonic stem cell
HSC	Hepatic stellate cells
iPSC	Induced pluripotent stem cell
ITS	Insulin-Transferring-Selenium
LFT	Liver function test
Lrat	Lecithin-retinol acetyltransferase
LSEC	Liver sinusoidal endothelial cells
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepathis
NFκB	Nuclear factor kappa-light-chain-enchancer of activated B cells
NO	Nitric Oxide
PA	Palmitic acid
PaHSC	Portal vein-associated hepatic stellate cells
PBS	Phosphate Buffered Saline
PSC	Pluripotent stem cell
PT	Serum bilirubin rothrombin time
RGF	Reduced growth factor
Ri	Rock inhibitor
ROS	Reactive oxygen species
TF	Transcription factor
TGF	Transforming growth factor
TGFβ	Transforming growth factor beta
TGF-β1	Transforming growth factor beta 1
TNFα	Tumor necrosis factor alpha

Contents

Introduction

Liver

The liver is the largest internal organ in the human body, which has a critical role in different processes such as blood volume regulation, immune system support and cholesterol homeostasis (Trefts et al., 2017). The liver is supplied with nutrients and blood through the portal vein and the hepatic artery. The portal vein carries oxygenated blood with nutrients from veins of the spleen, stomach, and intestine (Trefts et al., 2017). The hepatic artery carries oxygenated blood from the heart. The liver is supplied with a volume of 25-30% of the total amount of blood through the resting cardiac output, where 70-75% of this blood is supplied through the portal vein while the rest flows through the hepatic artery (Flavio G. Rocha, 2012).

In *Figure 1* (A) one can see the representation of the liver as a hepatic lobule, a hexagonal structure which display a functional unit of liver tissue. Hepatic lobule is divided for three zones, placed between portal triad and central vein. The portal triad consists of a hepatic artery, a portal vein, and a bile duct. The oxygenated blood from the hepatic artery is mixed with the nutrient-rich deoxygenated blood from the portal vein and travels through the lobule and towards the central vein. Throughout the flow from the portal triad to the central vein the lobule is graded into different zones as the cells in the liver are zonated since each zone has a different degree of nutrients. The closer the cells are to the portal triads the higher grade of nutrients is found, but towards the central vein it will decrease.

The periportal Zone is often referred to as Zone 1, while the pericentral Zone is called Zone 3. As blood flows through to the central vein, the hepatocytes constructs a gradient of nutrients along the periportal-pericentral axis as the hepatocytes uptake oxygen, metabolic hormones and other nutrients (Cunningham & Porat-Shliom, 2021). The gradient has a crucial part in driving the different cells in the lobule to have a differential gene expression, which makes it capable to separate cells from each zone (Cunningham & Porat-Shliom, 2021).

In figure 1 (B) one can see how the sinusoids in the liver has been illustrated and how the blood flows through the sinusoid and to the central vein. In the sinusoid lies several different cells such as hepatocytes, endothelial, Kupffer and stellate cells (Trefts et al., 2017).

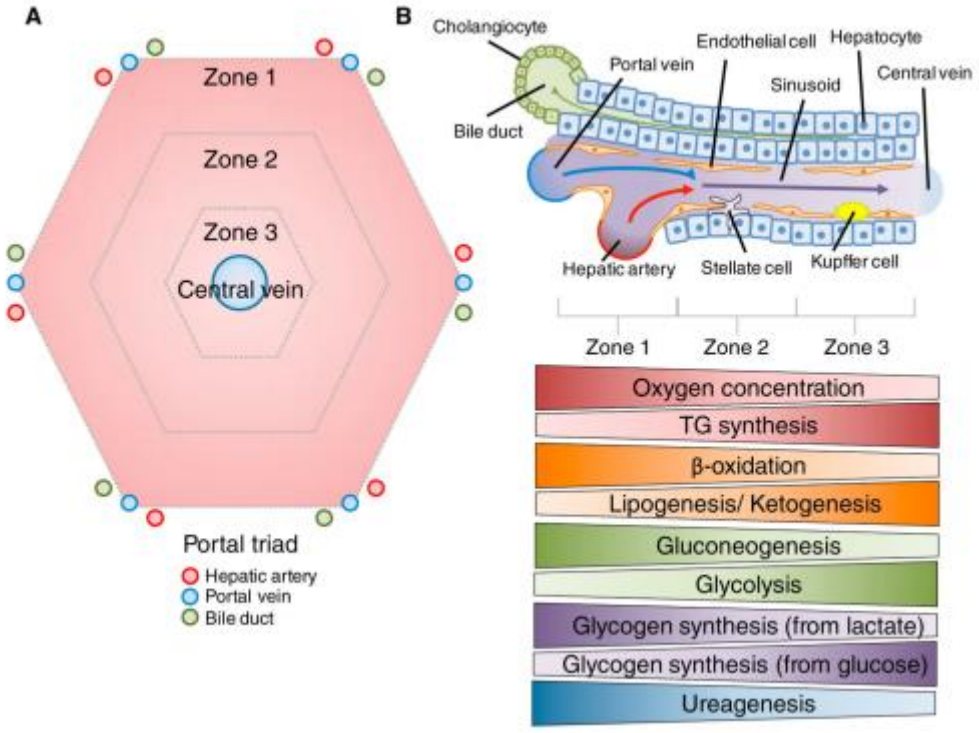

Figure 1:(A) Representation of the liver as a hepatic lobule. (B) A representation of a sinusoid within the liver with corresponding zonation of metabolic processes. (Trefts et al., 2017)

Sinusoid

Figure 1 (B) illustrates how the liver sinusoidal endothelial cells (LSEC) align the sinusoid with spaces between them and the hepatocytes, this space is called the space of Disse. Space of Disse consists of extracellular matrix and separate layer of LSEC from layer of hepatocytes. Endothelial cells in the liver are fenestrated meaning that they contain fenestrations which are ovoid transcellular holes enabling transport. LSEC secrete different factor regulating liver homeostasis and vascular tension, including nitric oxide (NO) and endothelin-1 (ET-1). Nitric oxide relaxes smooth muscle cells and establish vessel patency. Endothelin-1 is a hormone which can be secreted from the endothelial cells and into the sinusoid to enhance expression of adhesion of specific surface adhering molecules. The

secretion of ET-1 into the sinusoid can result in inducing leucocytes and platelets to migrate into the endothelial wall, which could result in blood coagulation (Haller, 1997; Satchell & Braet, 2009).

Hepatocytes are liver epithelial cells and have a variety of different functions which are crucial for the body homeostasis. Hepatocytes are metabolically active and produce and secrete plasma proteins into the bloodstream. The cells have an ability to convert amino acids into glucose, which then can be stored in glycogen and triglycerides (Mescher, 2021; Mohammed, 2019). Hepatocytes are enforced into apoptosis or triggered to compensatory regeneration when affected by chronic liver diseases (Mohammed, 2019). Hypoxic hepatocytes are a source of Transforming growth factor beta 1(TGF-β1) within cirrhosis or the last fibrotic stage. Damage hepatocytes have an ability to induce activation of Hepatic stellate cell (HSC) and stimulate fibrotic actions of myofibroblasts, due to the releasing of reactive oxygen species (ROS) (Mohammed, 2019).

Kupffer cells are found within the outer layer of the sinusoid, which is illustrated in figure 1 (B). The Kupffer cells play a big part in the immune response, as they are responsible for phagocytose and partially degrading bacterial antigens before transporting them to the hepatocytes for excretion into the bile duct.

Hepatic stellate cells (HSC) are found in the perisinusoidal space, also known as the space of Disse between hepatocytes and LSECs, and represent 10% of all liver cells. (Tsuchida & Friedman, 2017) (Saxena, 2018). In the healthy liver, HSCs are in their quiescent state and are responsible for the storage of vitamin-A (retinol) and lipids (Senoo, 2004). The HSCs also have other properties such as regulation the sinusoidal circulation and production of a range of different growth factors and other mediators such as hepatocyte growth factor and vascular endothelial growth factor (Kitto & Henderson, 2021). HSCs can be activated by inflammatory mediators, which will induce their differentiation into myofibroblasts (Kitto & Henderson, 2021). The activation of the HSCs tends to occur during inflammation caused by infection, drugs, alcohol, toxic substances, or autoimmune hepatitis (Aydin & Akcali, 2018). Due to the variety of different markers that the HSCs expresses it's possible to distinguish them from the other liver cells. The different markers which have been used to distinguish HSC are lecithin-retinol acetyltransferase (Lrat), alpha smooth muscle action (α-SMA, activated state), glial fibrillary acidic protein (Gfap, quiescent state) and desmin (Kitto & Henderson, 2021). In *Figure 2* illustrates the effect of HSC activation in liver and the functions and features of HSC in quiescent, activated, and inactivated state.

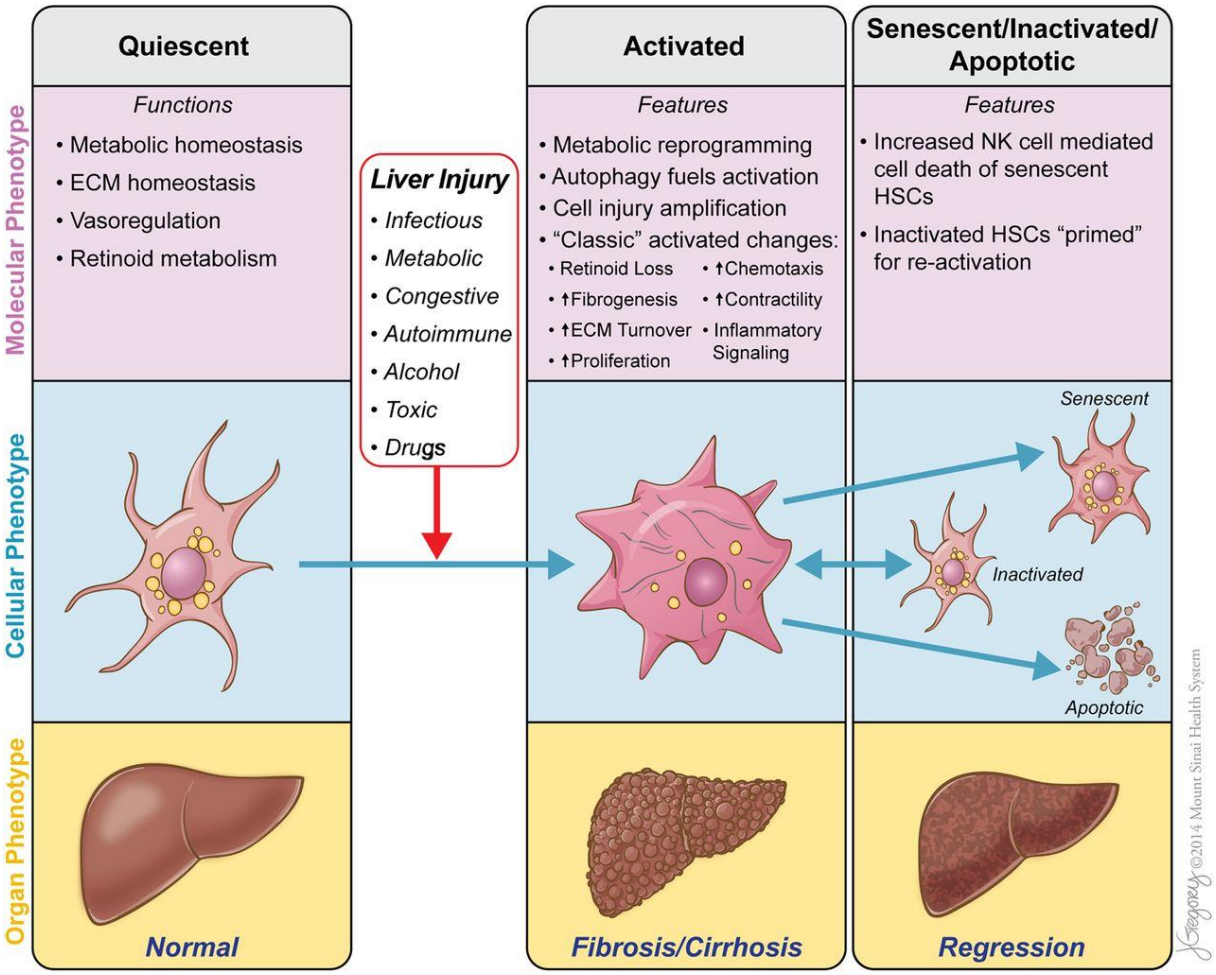

Figure 2: shows the development of quiescent HSC to activated HSC and how the liver reacts to the activation, due to consequences of the HSC activation (Lee et al., 2015).

Functions of the liver

The liver is responsible for a set of different function to support the body such as detoxifying several different metabolites, producing digestive enzymes, and synthesizing proteins. When a liver function test (LFT) is done to map if an individual has any disease. One is then reviewing the different concentrations of proteins within the blood, due to the liver secreting the protein into the blood. The proteins which are analyzed is often alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphate (ALP), gamma-glutamyl transferase (GGT), serum bilirubin prothrombin time (PT) and albumin (Lala et al., 2021; Stoll et al., 1992). The LFTs of a normal functioning liver is shown in *Table 1*, uneven values to this table will indicate liver function problems.

Table 1: This table shows the normal values of the LFT references. Elevated or decreased levels from these references would indicate as problems in liver function.

LFT references	**Amount in the bloodstream (IU/L)**
Alanine transaminase	0-45 (IU/L)
Aspartate transaminase	0-35 (IU/L)
Alkaline phosphatase	30-120 (IU/L)
Gamma-glutamyl transferase	0-30 (IU/L)
Bilirubin	2-17 micromoles/L
Prothrombin time	10,9-12,5
Albumin	40-60 g/L

Fibrosis

Fibrosis is a pathological process characterized by excessive deposition of connective tissue in response to injuries or diseases. Organs highly exposed to fibrosis will lead to malfunction, due to excessive production of connective tissue that replaces the space where functional cells can exist and operate. This will result in a higher chance of mortality (Wynn & Ramalingam, 2012). The fibrous scar formation is led by accumulation of extracellular matrix(ECM) (Kitto & Henderson, 2021).

While there are many different injuries and diseases on the liver that can lead fibrosis, some of the most common causes, as stated in (Aydin & Akcali, 2018), are alcohol consumption, non-alcoholic steatohepathis (NASH), hepatitis B (HBV) and hepatitis C, autoimmune hepatits, non-alchoholic fatty liver disease (NAFLD), and cholestatic liver diseases. All these injuries and diseases can lead to wounds on the liver that causes chronic inflammation, which again induces an unnormal healing response, where HSCs gets activated, becoming myofibroblasts that enhances the production of ECM. The enhanced production eventually leads to accumulations of ECM, which again forms fibrous scar formations.

Once fibrous scars have formed, the liver will struggle to maintain normal function, leading to hepatocyte loss, and eventually liver failure. Further progression of liver fibrosis leads to cirrhosis, although it is a reversible process that can be helped by eliminating fibrotic response-causing agents, if the cirrhosis has not advanced too far **(Aydin & Akcali**, **2018).**

As mentioned quiescent HSCs can be activated by liver injuries, where one can divide the different mechanisms, referred to as pathways, into "core" and "regulatory" ones (Tsuchida & Friedman, 2017). Pathways that lead to fibrosis across both tissue and disease, are classified as core pathways, while pathways that are mainly restricted to tissue are defined as regulatory (Tsuchida & Friedman, 2017). The cells behavior changes when they are exposed to stimuli over a period of time, which will result in fibrogenesis, matrix degradation, chemotaxis, retinoid loss, cytokine release and which leads to accumulation of ECM (Aydin & Akcali, 2018). The myofibroblasts will then secrete extracellular matrix proteins and matrix metalloproteinases which eventually could end up in cirrhosis, illustrated in *Figure 2* (Aydin & Akcali, 2018). Studies have shown how functional zonation between HSC cells which have been activated in the portal vein and the central vein. The central vein-associated HSCs (CaHSCs) which lies in the Pericentral Zone has shown to lack nutrients that results in a preponderant quantity of fibrosis, compares to the fibrosis in the Periportal Zone where the portal vein associated HSCs (PaHSCs) lies. Patterns of fibrosis-associated periportalization was observed in mice models, which led to complete resistance to hepatotoxic doses of acetaminophen (Ghallab et al., 2019). Several inflammatory pathways were identified such as, Transforming growth factor beta (TGFβ), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and Tumor necrosis factor alpha (TNFα) as most active. The pathways resulted in Transcription factor (TF) activities that were inferred and dominated by TF with an increased activity that mediated inflammation, cell stress and hypoxia response (Ghallab et al., 2019). This shows that fibrosis can affect the lobular zonation and amplify the fibrosis.

Fibrotic liver models

By constructing fibrosis liver models one can expose the cells to different factors, which can be analyzed by measuring the gene expression or staining the cells. Constructing in vitro models with mono-layer cultures of freshly isolated HSCs from differentiation of IPSC or human embryonic stem cell (hESC) gives difficulties holding the HSCs quiescent. Spontaneous HSC activations in 2D culturing restrict the possibility to analyze the initial stages of induced fibrosis. One factor that limits the in vitro modelling of fibrosis in HSCs is the absence of hepatocytes. A more suited modelling is to make a multicellular system that includes both hepatocytes and HSCs. A multicellular system would give a more suitable

environment for analyzing the interplay between hepatocytes and HSCs in the fibrotic development (Mohammed, 2019; van Grunsven, 2017).

Several types of techniques have been used to form different types of 3D fibrotic liver models. The 3D spheroid hepatic model has been constructed by using micro molds to hold the cell in micro wells that are made with the help of centrifugal force. Another type of 3D model is by making cultures in a medium with hydrogel that are placed on a surface as a droplet. Even though this hydrogel will gimmick the ECM environment, this will not be a specific ECM environment, which means that the models are limited regarding cell viability and functionality (Pati et al., 2016) (Mohammed, 2019).

Materials & Methods

Materials

Product	Company	Catalog number
Coating of plates with GelTrex		
Gibco™ GelTrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	Thermo Fischer	A1413302
Gibco™ DMEM/F-12	Fisher scientific	11520396
Nunc™ Cell-Culture Treated Multidishes	Thermo Fisher	150628
Nunc™ Non-Treated Multidishes Plating and splitting cells for differentiation	Thermo Fisher	144530
Essential 8™ Medium	ThermoFisher	A1517001
mTeSR™1 cGMP, feeder-free maintenance medium for human ES and iPS cells	StemCell technologies	85850
Rock inhibitor Y-27632 (Dihydrochloride)	StemCell technologies	72304
Accutase® solution	Merc	A6964
iHSC differentiation		
mTeSR™1 cGMP, feeder-free maintenance medium for human ES and iPS cells	StemCell technologies	85850
DMEM/F-12, GlutaMAX™ Supplement, 10x 500 ml	Life Technologies AS	31331-093
MEM Non-Essential Amino Acids Solution (100X)	Thermo Fisher	11140035
B-27™ Supplement (50X), serum free	Thermo Fisher	17504-044
Recombinant Human/Murine/Rat Activin A (CHO derived)	Peprotech	120-14P
CHIR 99021	TOCRIS	4423
Recombinant Human BMP-4 (HeLa derived)	Peprotech	120-05
Dexamethasone	Sigma	D4902-100 mg
Insulin-Transferrin-Selenium (ITS -G) (100X)	ThermoFisher	41400045
2-Phospho-L-ascorbic acid trisodium salt	SIGMA-Merck	49752-10G
Recombinant Human FGF-acidic	Peprotech	100-17A
Recombinant Human FGF-3 Protein	R&D	1206-F3- 025
Retinol, synthetic, ≥95% (HPLC), crystalline, 100 mg	Sigma	R7632-100MG
Palmic acid	Sigma	P5585-10G
RNA isolation		
TRIzol™ Reagent	Thermo fisher scientific	15596018
Chloroform	SigmaAldrich	372978
Fisher Chemical 2.5LT Isopropanol, Molecular Biology Grade	Thermo fisher scientific	17150576
Etanol, GPR RECTAPUR® 96% 1L	VWR	20824.296
RNase-free water		
Glycogen, RNA grade	ThermoFisher Scientific	R0551

UltraPure™ DNase/RNase-Free Distilled Water	Thermo Fisher Scientific	10977049
CDNA synthesis		
High-Capacity cDNA Reverse Transcription Kit	ThermoFisher Scientific	4368813
RT-qPCR		
TaqMan™ TaqMan™ Gene Expression Master Mix	Fisher Scientific	10005754
RNase-free water	QIAGEN	129114
Probes		
Hs02786624_g1 GAPDH	Life Technologies AS	4351370
Hs01019589_m1 PDGFRB	Life Technologies AS	4331182
Hs00977641_m1 ALCAM	Life Technologies AS	4331182
Hs00157258_m1 TaqMan probe DES	Thermo fischer scientific	4453320
Immunofluorescence		
DPBS, no calsium, no magnesium	Thermo Fisher	14190-169
95% PFA powder	Sigma-Aldrich	158127- 500G
Donkey Serum, 100 ml	Sigma-Aldrich	S30-100ML
Triton-x100	Sigma-Aldrich	
PBS	Thermo Fisher scientific	AM9625
Human PDGFR beta Antibody produced in goat	R&D	AF385
Monoclonal Anti-Fibronectin antibody produced in mouse	Sigma Aldrich	F0916-.2ML
Anti-Vimentin antibody [RV202] - Cytoskeleton Marker (ab8978)	Abcam	ab8978
Anti-T antibody produced in rabbit	Sigma Aldrich	HPA003322-25UL
Anti-VE Cadherin antibody - Intercellular Junction Marker (ab33168)	Abcam	ab33168
Alexa Fluor® 488 AffiniPure Donkey Anti-Goat IgG (H+L)	Jackson ImmunoResearch	705-545- 147
Alexa Fluor® 647 AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	715-605- 150
Cy™3 AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	711-165- 152
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Thermo Fisher	D1306

HSC culturing

iPSC cell culture

One calculated the amount of media each well in the different well plates required and then thawed GelTrex(GT, Thermo Fisher, A1413302) fast with 500 µl DMEM-F12(Fisher

scientific, 15520396) medium the GT aliquoted tube until the GT was thawed. Afterwards the coating medium was distributed with appropriate amounts into each wells using a serological pipette. The coated plates were incubated at 37 °C for minimum 30 minutes. The plates could then be stored at 4°C for 1-2 weeks.

iPSCs (WTC-11) were cultured in Essential 8 medium(E8) and hESCs in mTeSR medium. The plates were coated with 1% Geltrex and stored in an incubator at 37 °C with 5 % CO_2. 0.5mM EDTA in DPBS was used to detach the cells from the plates, for then to be replated at a dilution of 1:3-1:5.

HSC differentiation

HSCs were generated from iPSC and hESC by following a differentiation protocol. The stem cells were plated on day 0 as the start of the differentiation process. The differentiation process lasted for 12 days. Cell samples were collected at day 0, 3, 6 and 12. The day 0 and 12 cells were used in the later experiments connected to 3D and 2D culturing. The cells were exposed to a variety of different growth factors from day 0-12, which is shown in *Table 2*. The stem cells were seeded into the GT-coated plated with a confluency of 60-70%.

Table 2: Shows the basal media with growth factor for each day of feeding in the differentiation of hESC/iPSC to HSC.

	Day 1	
Media	DMEM/F12 with Glutamax + 1% NEAA + 1% B27	
	[STOCK]	**[Final volume for 10 mL]**
Activing A	100 µg/mL(1000x)	100 ng/mL (10µl)
CHIR99021	100 mM	3 µM
Bone morphogenetic protein 4 (BMP4)	100 µg/mL (5000x)	20 ng/mL (2µl)
	Day 2	
Media	DMEM/F12 with Glutamax + 1% NEAA + 1% B27	
	[STOCK]	**[Final volume for 10 mL]**
Dexamethasone	1 mM	2.5 µM (25 µl)
Insulin-Transferring-Selenium (ITS)	1x	0.25x (2.5 µl)
Acetylsalicylic acid (ASA)	100 mM	100 µM (10 µl)
Bone morphogenetic protein 4 (BMP4)	100 µg/mL (5000x)	20 ng/ml (2 µl)
	Day 4	
Media	DMEM/F12 with Glutamax + 1% NEAA + 1% B27	
	[STOCK]	**[Final volume for 10 mL]**
Dexamethasone	1 mM	2.5 µM (25 µl)
Insulin-Transferring-Selenium (ITS)	1x	0.25x (2.5 µl)

Acetylsalicylic acid (ASA)	100 mM	100 µM (10 µl)
Bone morphogenetic protein 4 (BMP4)	100 µg/mL (5000x)	20 ng/ml (2 µl)
Fibroblast Growth factor 2 (FGF2)	100 µg/mL	20 ng/mL (2 µl)

	Day 6	
Media	DMEM/F12 with Glutamax + 1% NEAA + 1% B27	
	[STOCK]	**[Final volume for 10 mL]**
Dexamethasone	1 mM	2.5 µM (25 µl)
Insulin-Transferring-Selenium (ITS)	1x	0.25x (2.5 µl)
Acetylsalicylic acid (ASA)	100 mM	100 µM (10 µl)
Bone morphogenetic protein 4 (BMP4)	100 µg/mL (5000x)	20 ng/ml (2 µl)
Fibroblast Growth factor 2 (FGF2)	100 µg/mL	20 ng/mL (2 µl)

	Day 7, 8, 10, 12	
Media	DMEM/F12 with Glutamax + 1% NEAA + 1% FBS	
	[STOCK]	**[Final volume for 10 mL]**
Dexamethasone	1 mM	2.5 µM (25 µl)
Insulin-Transferring-Selenium (ITS)	1x	0.25x (2.5 µl)
Acetylsalicylic acid (ASA)	100 mM	100 µM (10 µl)
Fibroblast Growth factor 2 (FGF2)	100 µg/mL	20 ng/mL (2 µl)
Retinol	10 mM	5 µM (5 µl)
Palmitic acid (PA)	100 mM	100 µM (10µl)

At day 0 basal media was prepared with Rock inhibitor (Ri) to ensure the survival of the stem cells. The precoated GT-plates were aspirated and the Ri-medium were applied to the wells and left at room temperature until the cells were ready to be added. The cells were washed with PBS three times, before being incubated with EDTA for 3 minutes at 37 °C. After the 3 minutes the cells were detached from the plates and were pipetted several times up and down gently to create a single-cell suspension with a 1 ml pipette. The single-cell suspension was transferred to the remaining Ri-medium. The GT-plates were aspirated for medium and the Ri-medium with suspension were added to the GT-plated wells.

At day 6 the cells were ready for splitting. Accutase was used to detach the cells instead of EDTA due to the solution being more gently with the cells than EDTA, before incubating for 3 minutes at 37 °C with 5% CO_2. After the incubation the Accutase was aspirated and a

blocking medium consisting of DMEM/F-12 + 1 % FBS was applied. The cells were then transferred with the blocking medium into a 15 mL tube and centrifuged for 3 minutes at 100 x g. The supernatant was discarded, and the remaining pellet was resuspended in Ri-medium that was prepared. The resuspended cells were evenly distributed into prepared plates, before being incubated at 37 °C with 5% CO_2 while the remaining cells were centrifuged again, to store the cells for RNA isolation in a -80 °C freezer.

Immunostaining 2D culture of HSC

Day 1

Culture medium was aspirated, and the slides were washed with DPBS and fixed with by applying 0.5 mL of 4% PFA into each well, followed by an incubation for 10-20 minutes at room temperature. The PFA was aspirated after the incubation before the slides were washed twice with PBS with 3 minutes incubation at room temperature between each wash. After the washing steps the slides could be stored at 4 °C for up to 4 months. The slides were permeabilized and blocked for 1 hour by applying 500 µL of a solution which consisted of 10% FBS + 0.1% Triton-X100 solution in PBS to each slide at room temperature. The blockings solution was aspirated before the slides were washed with PBS with 3 minutes incubation at room temperature between each wash. Blocking solution with 100 µL primary antibodies were applied to each slide and stained overnight at 4 °C. The primary antibodies used for this staining were PDGFR-β (dilution factor 1:50), Vimentin (dilution factor 1:400).

Day 2

The staining solution was aspirated, before the slides were washed with PBS 3 times where the slides were incubated at room temperature for 5 min each wash. The slides were then stained with 100 µL of the secondary antibodies diluted in 1:1000 (vol/vol) in PBS for 1 hour at room temperature. The secondary antibodies used for the second staining were Alexa Fluor 488 (dilution factor 1:300) and Alexa Fluor 647 (dilution factor 1:400). After the incubation the solution was aspirated and a solution of DAPI (1:500) in PBS was applied to each slide and incubated for 10 minutes at room temperature. Afterwards the solution was aspirated, and the slides were washed 3 times with PBS with incubation for 10 minutes each time the slides were washed. After the washing the cover slips were dried and mounted on glass slides, placing them face down on the mounting media which was preheated to 78 °C. The slides were marked and stored at -20°C until imaging.

Immunofluorescence imaging was done where low light intensity was used to rather correct the brightness and contrast later. The pictures were taken analyzing only one stain at the time.

Harvesting cells for RNA isolation

The medium was aspirated before the wells were applied with PFA-solution and put into incubation at 37 °C. The cells were collected by pipetting the PFA-solution up and down. The pipetting was done several times to make sure all the cells were collected into Eppendorf tubes. The collected cells in the Eppendorf tube were then centrifuged at 200g for 2 minutes. The supernatant was discarded with a suction pump and a 1000µl pipette to precisely discarding as much medium as possible without discarding the material. Soon after the supernatant was discarded the pellet was resuspended with permeabilizing medium before the Eppendorf tubes were centrifuged again at 200g for 2 minutes. After the centrifuging the supernatant was discarded, and the pellet was then stored into small Eppendorf tubes at -80 °C.

iHSC RNA isolation

The samples were stored at -80 °C were added 0.75 ml TriZol™ Reagent per 0.25 ml of sample (5-10 x 10^6) to dissolve the pellets. When applying the TriZol™ Reagent the samples were pipetted up and down several times for homogenization. The lysates were then centrifuged for 5 minutes at 12 000 x g 4-10 °C, continued with an extraction of the supernatants which were transferred into new tubes. The new tubes were incubated for 5 minutes to permit complete dissociation of the nucleoprotein's complexes. The samples new tubes were applied with 0.2 chloroform per 1 ml of TriZol™ Reagent that was used for the lysis of the samples and incubated for 2-3 minutes. The samples were then centrifuged for 15 minutes at 12 000 x g at 4°C right after shaking the tubes. After the centrifuging the samples ended up with 3 aqueous phases. From the top to the bottom was DNA, RNA, and protein. The RNA phases were extracted from each tube and transferred into new tubes.

The RNA samples were applied 7.5 µl of RNase-free glycogen as a carrier to the aqueous phase, followed by 0.5 ml of isopropanol per 1 mL of TriZol™ Reagent used for the lysis. The samples were then incubated for 10 minutes, before centrifugation for 10 minutes at 12

000 x g at 4 °C. The supernatant was discarded without discarding the white gel-like pellet that was formed on the bottom of the tube, due to the total RNA precipitation.

The pellets were resuspended in 1 ml of 75% ethanol per mL of TriZol™ Reagent used for the lysis. The samples were then vortexed briefly before centrifugation for 5 minutes at 7500 x g at 4 °C. The supernatants were discarded, and the washing step was repeated 2 times more. The RNA pellets were air dried for 5-10 minutes after washing for the third time.

The RNA pellets were applied with 20-50 µL of RNase-free water, this was done with pipetting up and down. At last, the samples were incubated in a water bath or heat block for 10-15 minutes at 55-60 °C and stored at -70 °C.

cDNA-synthesis from cultured iHSC

The RNA samples were used to synthesize cDNA for the Reverse Transcriptase qPCR analysis. Alle the samples and reagents were kept on ice between the steps. The RNA samples were diluted to a concentration of 20ng/µL, which could be found out by checking the RNA concentration of the samples with the Nanodrop analysis. A master mix (MM) was prepared where 10 µL of the MM was used for each RNA sample. Each 10 µL of MM contained 2 µL 10x RT buffer, 0.8 µL 25x dNTP, 2 µL 10x Primer, 1 µL Transcriptase and 4.2 RNase-free water. New tubes were marked and applied 10 µL Master mix and 10 µL of RNA sample (which consisted of 200ng RNA), before they were incubated in four steps. Step 1 was 10 minutes at 25 °C, step 2 was for 120 minutes at 37 °C, step 3 which was 5 minutes at 85 °C, at last came the fourth step which was 15-20 minutes at 4 °C.

qPCR-analysis of cDNA from cultured iHSC

The cDNA samples were collected and put on ice, before they were mixed with probe mixes that contained different probes to detect specific genetic markers. This was done to measure the expression of genes with the RT-qPCR. The probe mixture for each reaction contained 5 µL TaqMan master mix, 0.5 µL of the desired probe, and 3.5 µL of Rnase-free water, which would result in a total volume of 9 µL. 1 µL cDNA sample and 9 µL probe mix were added to each well in the RT-qPCR plate and was seal and centrifuged at 1000 x g for 2 min in room temperature before starting the analysis on the instrument. The setup used for the analysis was ViiA7 system, with a 96-well plate, with comparative Ct, TaqMan reagents and standard properties. The cycling setup ran for approximately 1.5 hours with cycle count of 40 cycles. In Table 3 one can see the temperature stages of the cycle.

Table 3: Shows the temperature stages for the RT-qPCR setup.

Stage	Step	Temperature (°C)	Time
Hold	RT (if applicable)	50	02:00
	Activation	95	10:00
Cycling (40 cycles)	Denaturing	95	00:15
	Anneal/Extension	60	01:00

After receiving the results from the RT-qPCR test, one used the values to do a quantitative ΔΔCT calculation to get RQ values, which one could construct graphs with. The ΔΔCT method is a formula which is used to calculate the relative fold gene expression of samples when performing a qPCR. The ΔΔCT method shows the difference between ΔCT of a treated sample and control sample that's untreated, hence the ΔCT is the difference of the CT value of the gene of interest and a housekeeping gene, the gene of interest is normalized by both the housekeeping gene and the control sample.

HepG2 cells

Thawing of HepG2

Vials from the storage removed and was kept in dry ice until start of thawing in water bath. The vials were quickly thawed in a 37 °C water bath until a small ice pellet remained in the vial. All the cells were carefully transferred from the cryovial to a 15 ml tube immediately after thawing. 5mL of complete DMEM + 10%FBS + 1% P/S medium were then applied into the tube using a 5mL serological pipette and adding the medium drop by drop (this was done to avoid perforation of the cells, due to osmosis). The cells were then spun at a relative centrifuge force of 200 x g for 4 min at room temperature. The supernatant was discarded, and the pellet was resuspended with 10 mL DMEM + 10% FBS + 1% P/S medium. The cells were seeded in prepared 75 cm^2 flasks and incubated at 37 °C with 5% CO_2.

Making single suspension of HepG2

The cells were in a flask filled with conditioning medium, which was first aspirated. After the aspiration the cells in the flask were rinsed with 3 ml PBS. Afterwards the wells were applied with 1 ml Trypsin which would detach the cells from each other, due to the protein cleaving between the cells. The trypsin was aspirated before reapplying 1 ml trypsin again for then to incubate the plates in 37 C for 3 min. When the incubation was complete the cells were then observed in the brightfield microscope to check if the cells were a bit detached or not after shaking the flask gently. 3 ml of medium (DMEM-F12+10% FBS+1% P/S) was transferred to the flask splashing it into the flask side where most of the cells were. The medium was pipetted up and splashed several times before it extracted from the flask and transferred into a 15ml tube. The splashing was done to detach the cells from each other to make the suspension a single suspension. The tube was centrifuged at 200x G for 3 minutes. A new flask was prepared with 7 ml medium, after the centrifuging was done the supernatant in the tube was aspirated. 2 ml medium was added to the tube and pipetted up and down to homogenize the suspension to then transfer it into the prepared flask with 7 ml medium. At last, the flask was incubated in 37 °C.

3D culturing in spheroids

Making the 3D microwells

For the formation of 3D spheroids, agarose microwells were used. Agarose microwells were fabricated using in-house made 3D-printed stamps. First low melting agarose was diluted in PBS (0,2% w/v), melted in the microwave to obtain homogeneous solution. Afterwards 2,5 ml of the agarose gel was applied into each well of 6wells-plate. 3D printed microwell stamps were placed on top of the agarose gel. After the agarose solidified the wells were rinsed with 2 ml of PBS (phosphate buffered saline, Thermo fisher, AM9625) into the wells, for so to be aspirated with a suction pump. 2ml of cell culture medium was then applied into the well, then the plates were incubated for minimum 40 minutes in 37 degrees Celsius before using the micro wells.

Making spheroids

The plate with microwells containing medium was aspirated, they were set ready to be filled with a D12 medium containing the cells. After thawing the cells, the suspension had a known

number of cells per ml, due to the counting done with a brightfield microscope and a Bürker counting chamber. When making the spheroids both HepG2 and HSC cells were used with a ratio of 5:1. When making 4 wells with spheroids one would make a 2ml suspension with 500 000 HSC cells and 2,5 million HepG2 cells to distribute 125 000 HSC cells and 1,3 million HepG2 cells into each well. After applying 0,5 ml of the mixed cells suspension of HSC and HepG2 cells the plates were then centrifuged at 100g for 2 min. After the centrifuging the medium from each well was aspirated. After aspiration of the wells a d12 medium with rock inhibitor was added to each well, and then the wells were checked under the brightfield microscope to conclude if the spheroid formation was successful. After the observation the wells were incubated overnight at 37 °C. The cocultured spheroids were then feed for approximately 4 days until they were harvested.

Harvesting spheroids

The medium was aspirated before the wells were applied with PFA-solution and put into incubation at 37 °C. The cells were collected by pipetting the PFA-solution up and down carefully until one could see the spheroids detaching the microwells. The pipetting was done several times to make sure all the spheroids was collected into an Eppendorf tube. The collected spheroids in the Eppendorf tube were then centrifuged at 200g for 2 minutes. The supernatant was discarded with a suction pump and a 1000µl pipette to precisely discarding as much medium as possible without discarding the material. Soon after the supernatant was discarded the pellet was resuspended with (permeabilizing medium I guess) before the Eppendorf tube was centrifuged again at 200g for 2 minutes. After the centrifuging the supernatant was discarded, and the pellet was stored into small Eppendorf tubes at -80 °C

Sorting cells from spheroids

Spheroids were collected in 1.5 mL Eppendorf tubes with the medium. After the spheroids had sedimented the cell culture medium was discarded without disturbing the spheroids. 1 mL of PBS was applied to the tube and the spheroids were let once again to sediment. The PBS was carefully discarded without disturbing the spheroids. 0.5 mL PBS was then added and quickly spun down, for so to discard the PBS. 300 µl of cell recovery solution were applied in the tube before incubation on at shaking platform where the tube was place horizontally for 15 minutes at -4 °C temperature. The spheroids were then pipetted up and down carefully to see if the spheroids became “lose”. The spheroids and cells were spun down, before the recovery solution were removed. 500 µL accutase was applied and the tube was incubated on a shaking

platform for 7 minutes at room temperature. The spheroids were then pipetted up and down to achieve a single cell suspension. The cells were then centrifuged at 200 x g for 2 minutes before the accutase was aspirated. The cells were applied 500 µL of analysis solution, which consisted of 4.5 mL DPBS with calcium and magnesium, 450 µL FBS and 5µL Rock inhibitor. The tube with cells were then kept on ice until cell sorting. The cells were sorted by a nanocellect wolf G2 machine which separated the HSCs and HepG2 cells, which had been cultures inside the spheroids. After collection of the cells the cells were collected in Eppendorf tubes and centrifuged. The supernatant was removed, and the cell pellets were immediately put in dry ice until storing them at -80°C

Immunostaining spheroids

Day 1

Spheroids were transferred to Eppendorf tubes, where the spheroids got time to sink to the bottom of the tubes. The process of waiting until the spheroids had sunken to the bottom of each Eppendorf tube before aspirating the medium was done for every step with aspiration. After all the spheroids were accumulated in the bottom the medium was aspirated carefully. The spheroids were then washed 1 time with PBS and the PBS was then aspirated. Afterwards 1ml 4% PFA was applied to each tube and incubated the tubes for 20 minutes. After the 20 minutes the PFA was aspirated, and the spheroids were washed with PBS twice. After the last washing step with PBS the spheroids could be stored at 4°C for up to two months. For the immunostaining the spheroids were permeabilized and blocked with a solution containing PBS with 10% FBS, 0,2% Triton-X100 solution and 1% DMSO for 2 hours at room temperature in a horizontal position. Every incubation in this method was done with the Eppendorf tubes lying in a horizontal position on a rotating platform at a slow speed. After 2 hours the medium was aspirated, and the Eppendorf tubes were washed twice with PBS with a 5-minute incubation each time. After the washing and aspiration, the Eppendorf tubes were applied primary antibodies (PDGFR-b, a-SMA and Col1a1) with a solution of 1% FBS and 0,1% TritonX-100 in PBS before letting the spheroids stain overnight.

Day 2

The primary antibody solution was then aspirated before the spheroids were washed 3 times with PBS with a 15-minute incubation each time. After the last washing step, the spheroids

were incubated for 1 hour in room temperature with secondary antibodies (Alexa Fluor 647, Alexa Fluor 488, CyTM3) which were diluted 1:1000 (vol/vol) in a solution of 1% FBS and 0.1% TritonX-100 in PBS. After the incubation the secondary antibody solution was aspirated and a new solution with Hoechst 33258 1:10000(vol/vol) in PBS was applied, incubated for 10 minutes at room temperature and aspirated. Thereafter the spheroids were washed 3 times with PBS with a 20 min incubation time for each wash. At last, the spheroids were store in the tubes with PBS at -20 °C (for temporary storage, 6 months and more).

RNA-isolation of spheroids

The samples were stored at -80 C° were added 0.75 ml TriZol™ Reagent per 0.25 ml of sample (5-10 x 10^6) to dissolve the pellets. When applying the TriZol™ Reagent the samples were pipetted up and down several times for homogenization. The lysates were then centrifuged for 5 minutes at 12 000 x g 4-10 °C, continued with an extraction of the supernatants which were transferred into new tubes. The new tubes were incubated for 5 minutes to permit complete dissociation of the nucleoprotein's complexes. The samples new tubes were applied with 0.2 chloroform per 1 ml of TriZol™ Reagent that was used for the lysis of the samples and incubated for 2-3 minutes. The samples were then centrifuged for 15 minutes at 12 000 x g at 4°C right after shaking the tubes. After the centrifuging the samples ended up with 3 aqueous phases. From the top to the bottom was DNA, RNA, and protein. The RNA phases were extracted from each tube and transferred into new tubes.

The RNA samples were applied 7.5 µl of RNase-free glycogen as a carrier to the aqueous phase, followed by 0.5 ml of isopropanol per 1 mL of TriZol™ Reagent used for the lysis. The samples were then incubated for 10 minutes, before centrifugation for 10 minutes at 12 000 x g at 4 °C. The supernatant was discarded without discarding the white gel-like pellet that was formed on the bottom of the tube, due to the total RNA precipitation.

The pellets were resuspended in 1 ml of 75% ethanol per mL of TriZol™ Reagent used for the lysis. The samples were then vortexed briefly before centrifugation for 5 minutes at 7500 x g at 4 °C. The supernatants were discarded, and the washing step was repeated 2 times more. The RNA pellets were air dried for 5-10 minutes after washing for the third time.

The RNA pellets were applied with 20-50 µL of RNase-free water, this was done with pipetting up and down. At last, the samples were incubated in a water bath or heat block for 10-15 minutes at 55-60 °C and stored at -70 °C.

cDNA-synthesis of RNA from spheroids

The RNA samples were used to synthesize cDNA for the Reverse Transcriptase qPCR analysis. Alle the samples and reagents were kept on ice between the steps. The RNA samples were diluted to a concentration of 20ng/µL, which could be found out by checking the RNA concentration of the samples with the Nanodrop analysis. A master mix (MM) was prepared where 10 µL of the MM was used for each RNA sample. Each 10 µL of MM contained 2 µL 10x RT buffer, 0.8 µL 25x dNTP, 2 µL 10x Primer, 1 µL Transcriptase and 4.2 RNase-free water. New tubes were marked and applied 10 µL Master mix and 10 µL of RNA sample (which consisted of 200ng RNA), before they were incubated in four steps. Step 1 was 10 minutes at 25 °C, step 2 was for 120 minutes at 37 °C, step 3 which was 5 minutes at 85 °C, at last came the fourth step which was 15-20 minutes at 4 °C.

qPCR-analysis of cDNA from spheroids

The cDNA samples were collected and put on ice, before they were mixed with probe mixes that contained different probes to detect specific genetic markers. This was done to measure the expression of genes with the RT-qPCR. The probe mixture for each reaction contained 5 µL TaqMan master mix, 0.5 µL of the desired probe, and 3.5 µL of Rnase-free water, which would result in a total volume of 9 µL. 1 µL cDNA sample and 9 µL probe mix were added to each well in the RT-qPCR plate and was seal and centrifuged at 1000 x g for 2 min in room temperature before starting the analysis on the instrument. The setup used for the analysis was ViiA7 system, with a 96-well plate, with comparative Ct, TaqMan reagents and standard properties. The cycling setup ran for approximately 1.5 hours with cycle count of 40 cycles. In Table 3 one can see the temperature stages of the cycle.

After receiving the results from the RT-qPCR test, one used the values to do a quantitative ΔΔCT calculation. The ΔΔCT method is a formula which is used to calculate the relative fold gene expression of samples when performing a qPCR. The ΔΔCT method shows the difference between ΔCT of a treated sample and control sample that's untreated, hence the

ΔCT is the difference of the CT value of the gene of interest and a housekeeping gene, the gene of interest is normalized by both the housekeeping gene and the control sample.

3D culturing in hydrogel

3D culture in hydrogel (evt endre litt på navnet)

50 µl of HSC single cells suspension in concentration 1 mln/ml was transferred to an Eppendorf tube. 100 µl Geltrex was added to the Eppendorf tube with cells suspension and carefully mixed. 25 µl of cell suspension with hydrogel was transferred in the middle of well in 24-wells plate, and incubated for 3-5 min at 37ºC. After hydrogel polymerized 0,5 ml medium (DMEM-F12+10% FBS+1% P/S) was added to each well with the hydrogel droplets. For the 3 last droplets the same medium was applied but was treated the next day same medium after adding 0,5 µl TGF-β (100 000ng/ml) which would induce fibrosis from the cells. After filling each droplet-well with medium the droplets were put into incubation at 37°C, in CO2 atmosphere.

Immunostaining 3D culture in hydrogel

Day 1

The medium in each well for each sample was discarded carefully and washed with PBS 1 time. After aspirating the PBS the wells were applied with 0.5 ml 4% PFA per well, which were incubated for 20 minutes at room temperature. The PFA was aspirated, and the organoids were washed with PBS twice with an incubation time of 10 minutes at room temperature each time. The fixed organoids were then permeabilized and blocked for 2 hours with a 10% FBS, 0,2% (vol/vol) Triston-X100 solution, 1% DMSO in PBS at room temperature. The solution was aspirated from each well, followed by washing the organoids twice with PBS with a 5-minute incubation between each wash. The organoids were then stained overnight at 4 ℃ with primary antibody diluted in a solution of 1% (vol/vol) donkey serum and 0.1% TritonX-100 in PBS. The wells were applied with 250 µL of the solution and the primary antibodies used in this solution were PDGFR-β (dil factor 50), α-SMA(dil factor 400) and COL1a1(dil factor 400).

Day 2

The medium in the wells were aspirated and the organoids were washed 3 times with PBS which was done with a 15-minute incubation between each time. After the washing the organoids were stained for 1 hour at room temperature with secondary antibodies and diluted

1:1000 (vol/vol) in a solution of 1% donkey serum and 0.1% TritionX-100 in PBS. The secondary antibodies used were Alexa Fluor 647(dil factor 400), Alexa Fluor 488(dil factor 300) and CyTM3(dil factor 400). The solutions were then aspirated, followed by applying a new solution of Hoechst 33258(vol/vol, dil factor 100) in PBS which incubated for 10 min at room temperature. Solutions were aspirated before washing the organoids 3 times with PBS with an incubation time of 20 minutes between each wash. The organoids were then stored at 4 °C covered in PBS.

Immunofluorescence imaging was done where low light intensity was used to rather correct the brightness and contrast later. The pictures were taken analyzing only one stain at the time.

Harvesting cells from 3D culture in hydrogel for RNA isolation

The medium was aspirated before the wells were applied with PFA-solution and put into incubation at 37 °C. The cells were collected by pipetting the PFA-solution up and down carefully until the geltrex droplets fully dissolved. The pipetting was done several times to make sure all the cells were collected into Eppendorf tubes. The collected cells in the Eppendorf tube were then centrifuged at 200g for 2 minutes. The supernatant was discarded with a suction pump and a 1000µl pipette to precisely discarding as much medium as possible without discarding the material. Soon after the supernatant was discarded the pellet was resuspended with (permabilizing medium I guess) before the Eppendorf tubes were centrifuged again at 200g for 2 minutes. After the centrifuging the supernatant was discarded, and the pellet was then stored into small Eppendorf tubes at -80 °C

RNA-isolation of 3D culture in hydrogel

The samples were stored at -80 C° were added 0.75 ml TriZol™ Reagent per 0.25 ml of sample (5-10 x 10^6) to dissolve the pellets. When applying the TriZol™ Reagent the samples were pipetted up and down several times for homogenization. The lysates were then centrifuged for 5 minutes at 12 000 x g 4-10 °C, continued with an extraction of the supernatants which were transferred into new tubes. The new tubes were incubated for 5

minutes to permit complete dissociation of the nucleoprotein's complexes. The samples new tubes were applied with 0.2 chloroform per 1 ml of TriZol™ Reagent that was used for the lysis of the samples and incubated for 2-3 minutes. The samples were then centrifuged for 15 minutes at 12 000 x g at 4°C right after shaking the tubes. After the centrifuging the samples ended up with 3 aqueous phases. From the top to the bottom was DNA, RNA, and protein. The RNA phases were extracted from each tube and transferred into new tubes.

The RNA samples were applied 7.5 µl of RNase-free glycogen as a carrier to the aqueous phase, followed by 0.5 ml of isopropanol per 1 mL of TriZol™ Reagent used for the lysis. The samples were then incubated for 10 minutes, before centrifugation for 10 minutes at 12 000 x g at 4 °C. The supernatant was discarded without discarding the white gel-like pellet that was formed on the bottom of the tube, due to the total RNA precipitation.

The pellets were resuspended in 1 ml of 75% ethanol per mL of TriZol™ Reagent used for the lysis. The samples were then vortexed briefly before centrifugation for 5 minutes at 7500 x g at 4 °C. The supernatants were discarded, and the washing step was repeated 2 times more. The RNA pellets were air dried for 5-10 minutes after washing for the third time.

The RNA pellets were applied with 20-50 µL of RNase-free water, this was done with pipetting up and down. At last, the samples were incubated in a water bath or heat block for 10-15 minutes at 55-60 °C and stored at -70 °C.

cDNA-Sythesis of RNA-isolation from 3D culture in hydrogel

The RNA samples were used to synthesize cDNA for the Reverse Transcriptase qPCR analysis. Alle the samples and reagents were kept on ice between the steps. The RNA samples were diluted to a concentration of 20ng/µL, which could be found out by checking the RNA concentration of the samples with the Nanodrop analysis. A master mix (MM) was prepared where 10 µL of the MM was used for each RNA sample. Each 10 µL of MM contained 2 µL 10x RT buffer, 0.8 µL 25x dNTP, 2 µL 10x Primer, 1 µL Transcriptase and 4.2 RNase-free water. New tubes were marked and applied 10 µL Master mix and 10 µL of RNA sample

(which consisted of 200ng RNA), before they were incubated in four steps. Step 1 was 10 minutes at 25 °C, step 2 was for 120 minutes at 37 °C, step 3 which was 5 minutes at 85 °C, at last came the fourth step which was 15-20 minutes at 4 °C.

qPCR-analysis of cDNA from 3D culture in hydrogel

The cDNA samples were collected and put on ice, before they were mixed with probe mixes that contained different probes to detect specific genetic markers. This was done to measure the expression of genes with the RT-qPCR. The probe mixture for each reaction contained 5 µL TaqMan master mix, 0.5 µL of the desired probe, and 3.5 µL of Rnase-free water, which would result in a total volume of 9 µL. 1 µL cDNA sample and 9 µL probe mix were added to each well in the RT-qPCR plate and was seal and centrifuged at 1000 x g for 2 min in room temperature before starting the analysis on the instrument. The setup used for the analysis was ViiA7 system, with a 96-well plate, with comparative Ct, TaqMan reagents and standard properties. The cycling setup ran for approximately 1.5 hours with cycle count of 40 cycles. In Table 3 one can see the temperature stages of the cycle.

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Statistical methods

Gene expression experiments were done on a RT-qPCR machine. The data received from the RT-qPCR machine were CT values for each genetic marker and were used in a quantitative ΔΔCT method. The ΔΔCT method is a formula which is used to calculate the relative fold gene expression of samples when performing a qPCR. The ΔΔCT method shows the difference between ΔCT of a treated sample and control sample that's untreated, hence the ΔCT is the difference of the CT value of the gene of interest and a housekeeping gene, the gene of interest is normalized by both the housekeeping gene and the control sample.

Firstly, the CT values were used to find an average CT value for each sample's replications. The average CT value from the housekeeping gene was used to calculate ΔCt for the target genes. This was done by subtracting the average CT value of the housekeeping gene from the average CT value of the target's genes. Afterwards the average ΔCT values were calculated before subtracting them from the ΔCT value chosen to be the reference, which often is the control sample. The result of this was the ΔΔCT value which was used to calculate RQ. This was done by calculating $2^{-\Delta\Delta CT}$.

Excel was used to calculate standard deviation, variance, t-value, and p-value to evaluate the significance of the data.

Results

Generation and characterization of iPSC/hESC-derived HSC

Pluripotent stem (PS) cells were differentiated and investigated in both gene and protein expression to evaluate if one could produce HSC-like cells, that could be suitable for later 3D culturing. Figure 3 illustrate the differentiation process from day 0 to 12 of PS cells.

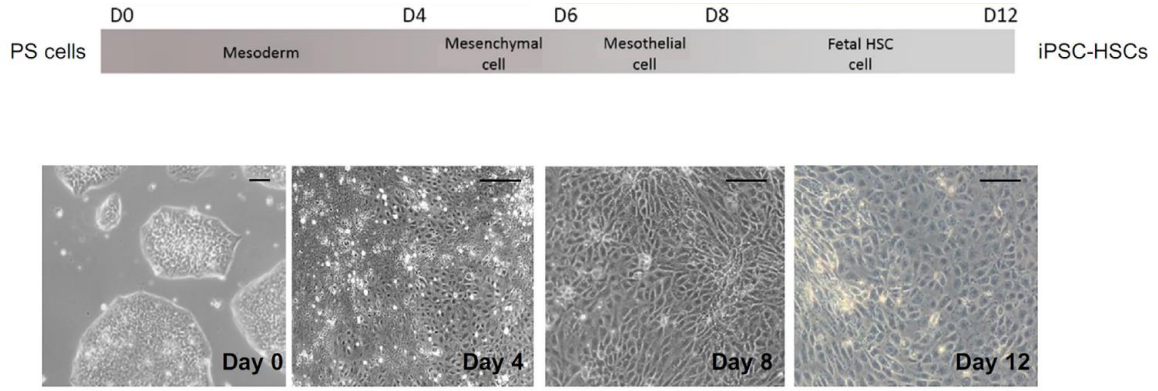

Figure 3:shows the differentiation of Pluripotent stem cells from day 0-12.

iPSC-derived HSCs

Directed differentiation is a sensitive process highly dependent on different factors. The iPSCs were nurtured with a base medium with different growth factors depending on which day of the differentiation it was. By following *Table 2* the differentiation of the WTC cell line was done, and pictures were taken on each day to document the differentiation of the cells.

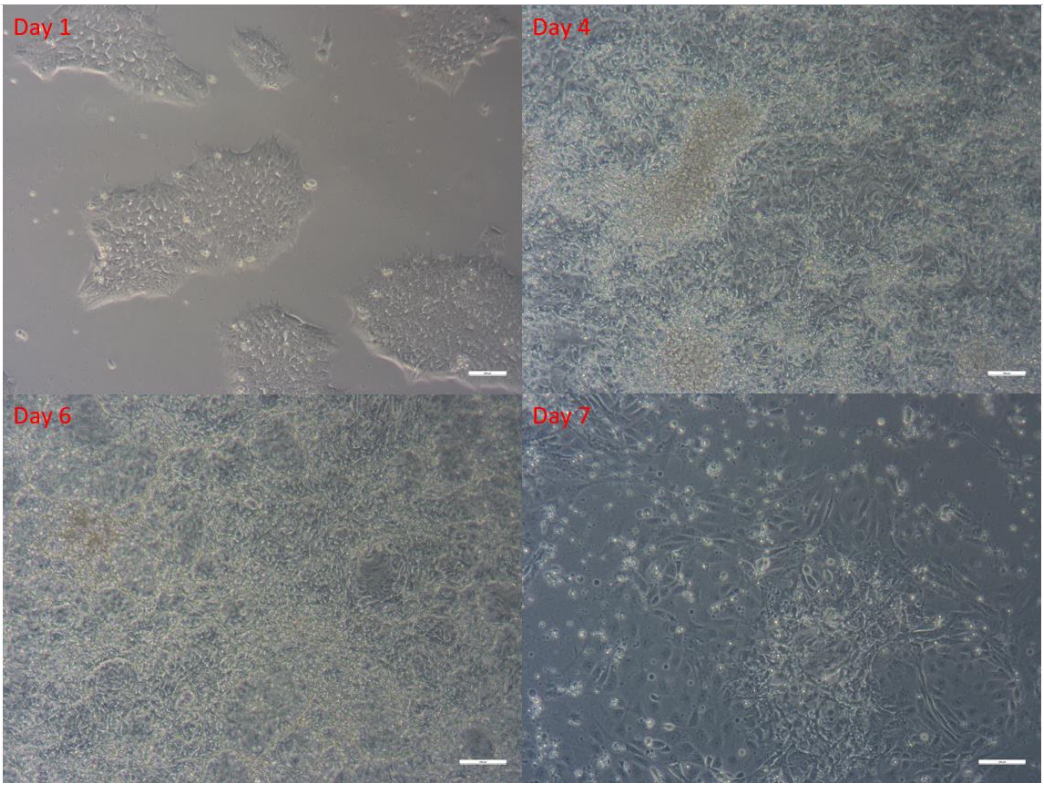

Figure 4: illustrates day 1, 4, 6 and 7 of the differentiation procedure for iPSC to iHSC.

Figure 4 shows Day 1, 4, 6 and 7 of the differentiation of WTC-11. Day 1 shows the iPSCs growing in colonies at an approximate 30% confluency. Within 3 days one can see the confluency has increased simultaneously as the morphology has evolved as the cells now

grows outwards from the colonies and starts scattering. This indicates that between day 1-4 the cells had gone into a mesoderm state (Coll et al., 2018). The cells were clustered some places, but one could see how the cells were spreading out and how the confluency had risen. Day 4 and day 6 has a similar morphology with an increase in confluency. The cells were split, and one could see after the split that the cells had expanded. Day 4-6 shows how the cells have gone from a mesoderm state and become mesenchymal cells, which have a stretched morphology and appears in clusters (Coll et al., 2018). After the split from day 6 one can see that the cells now had begun to develop into cells which can be identified as mesothelial cells at day 7, as the cells had started to acquire the HSC morphology. Mesothelial cells often appear uniform but can be present in cluster which naturally would be the case as the cells proliferate. The splitting was necessary to avoid overgrowth and to expand the cells.

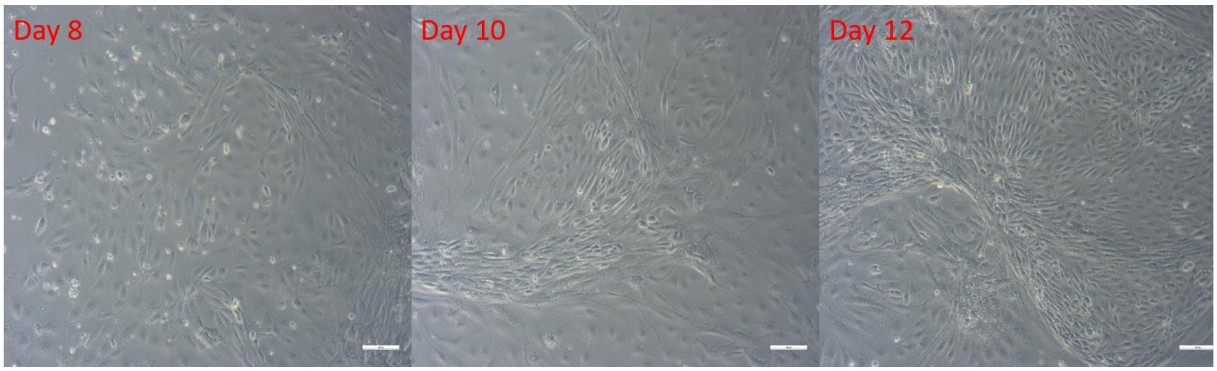

Figure 5: illustrates day 8, 10 and 12 of the differentiation procedure for iPSCs to iHSC.

The picture illustrates day 8, 10 and 12 of the differentiation procedure for iPSCs to iHSC

Figure 5 Shows Day 8, 10 and 12 of the differentiation of WTC-11. Throughout day 8-12 there was an accumulation of cells where the cells clustering looks like a web. The cells at day 8 shows cells which have differentiated from mesenchymal cells to mesothelial cells. By day 10 the confluency has risen, and the cells are differentiating to HSCs. The HSC-like cells are stars shaped at a quiescent state, however in an activated state the cells tend to get a rounder shape than the quiescent HSCs.

hESC-derived HSCs

By following *Table 2* the differentiation of the H1 cell line was done. All the steps Pictures were taken for each day of the differentiation to document how the cells appeared during the differentiation.

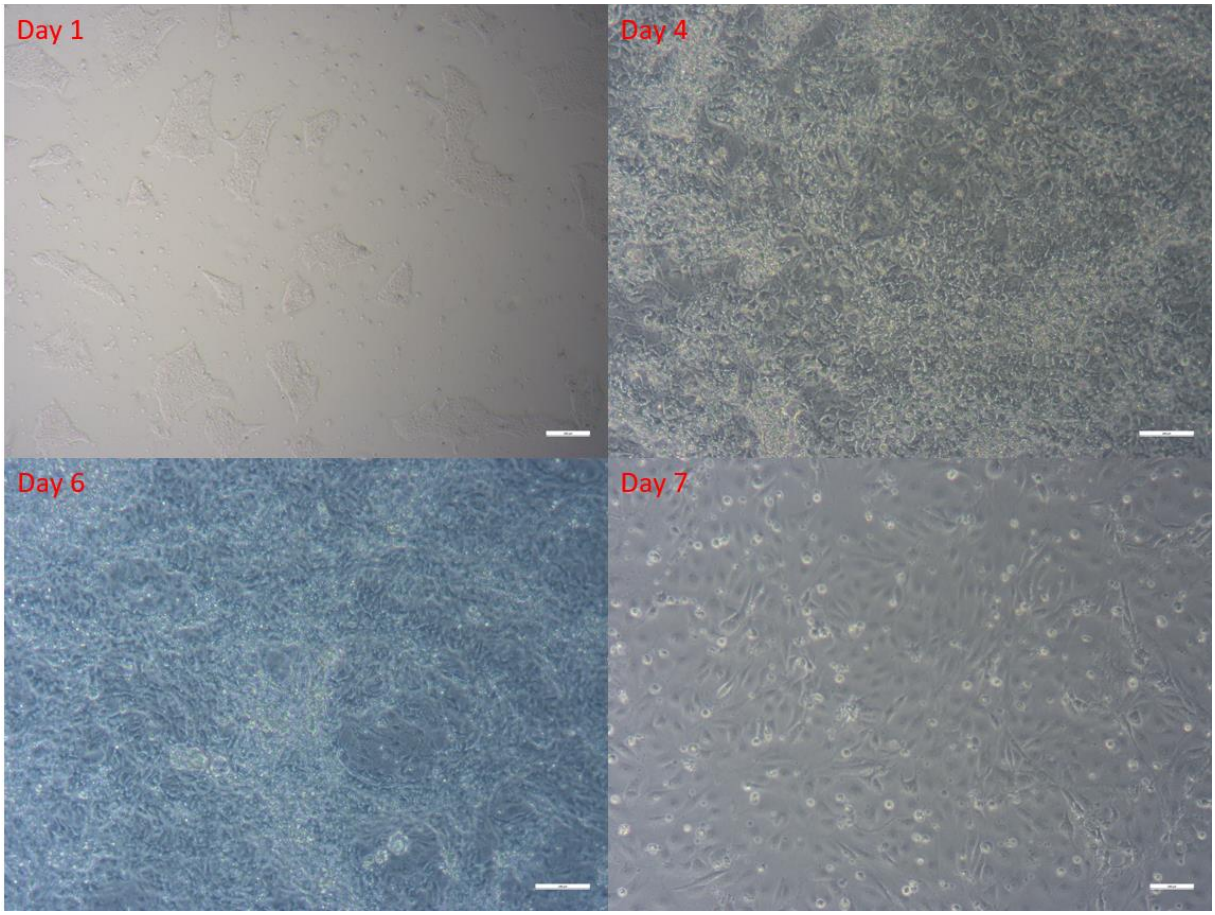

Figure 6: illustrates day 1, 4, 6 and 7 of the differentiation procedure for hESC to iHSC.

Figure 6 shows day 1, 4, 6, 7 from the differentiation of H1. Day 1 shows how the hESCs are growing in colonies at an approximately 20% confluency. Within 3 days the morphology evolved, and the cells were starting to grow outwards from the colonies and started scattering. This change indicates that the cells had gone into a mesoderm state between day 1-4. The cells had proliferated from day 4 to 6 and the cells had begun to get a stretched morphology and appeared in cluster, which could indicate that the cells had gone from a mesoderm state to a mesenchymal state. After the split at day 6 the cells had acquired an HSC morphology which indicates that the cells had started to differentiate from a mesenchymal state to a mesothelial state, due to the cells appearing uniform and getting even more stretched.

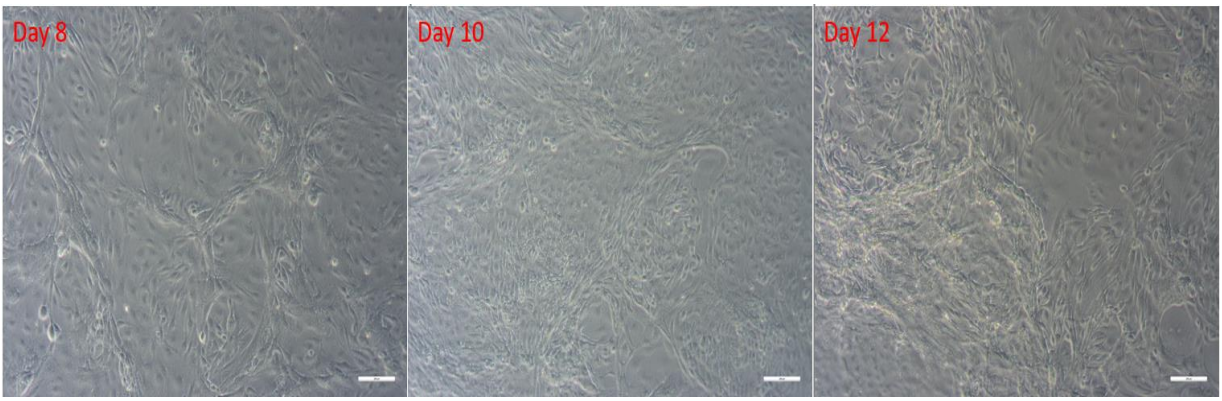

Figure 7: illustrates day 8, 10 and 12 of the differentiation procedure for hESC to iHSC

Figure 7 Shows Day 8, 10 and 12 of the differentiation of H1. Throughout day 8-12 the cells exceeded to proliferate, and the cell clustering looked like a web. Day 8 shows an increase in mesothelial cells as more cells have become more stretched. Now the cells are starting the form these web shaped formations. By day 10 the confluency has risen, and the cells are differentiating to HSCs as the HSC-like cells have a star shapes form at the quiescent state, however in activated state the cells tend to get a rounder shape than the quiescent ones(Coll et al., 2018).

Expression of HSC-spesific genes in iPSC-derived HSCs

A RT-qPCR analysis was done to investigate the gene expression of the genes PDGFR-β, ALCAM, and DESMIN with GAPDH as a housekeeping gene. The analysis was done after one had extracted RNA from the cells and synthesized cDNA from the RNA that was isolated. Both WTC-11 and H1 were analyzed with samples from day 0 and day 12 for each cell line. The RQ values from *Appendix table 1-4* were used undergo a t-test. The results of the t-test were shown in *Appendix table 20*. *Appendix table 20* shows a significant increase in gene expression of PDGFR-β from the differentiation of the cell line WTC-11 from day 0 to day 12. For the H1 cell line it shows a statistically significant increase in gene expression of PDGFR-β. The results indicated that there was no statistically significant increase in the gene expression of ALCAM in neither of the cell lines. However, for the expression of DESMIN the WTC-11 cell line didn't have any statistically significant increase in expression, while the H1 cell line had a significant increase in expression.

From *Figure 8-10* the RQ-values are represented in the graphs with the mean RQ values for each cell line and day of differentiation, where the error bars represent the standard deviation of the values. The figures have been marked with ns and *, where ns stands no statistically significant increase in expression, while * represent significant increase in expression. *Figure 8-10* was constructed by using *Appendix table 5-7*.

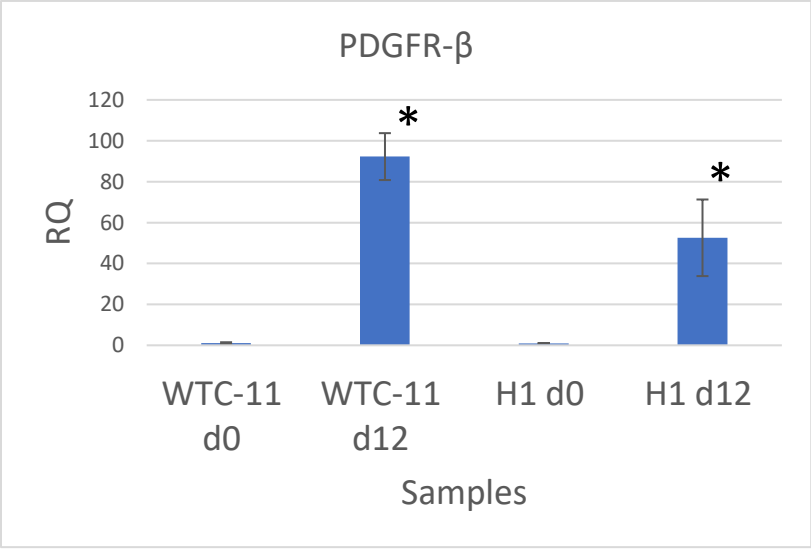

Figure 8: Illustrates gene expression of PDGFR-β, where y-axis represent relative expression while x-axis represents samples day 0(d0) and day 12(d12) for both iPSC (WTC-11) and hESC (H1). The graphs have error bars which represents the standard deviation.

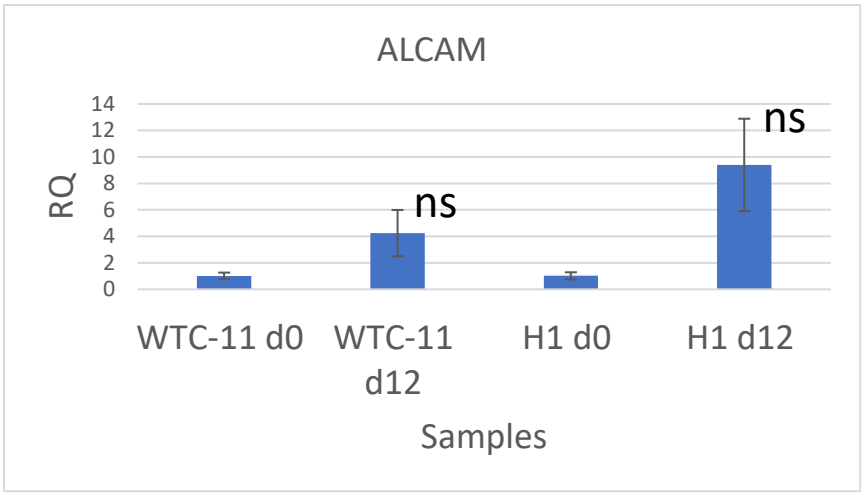

Figure 9: Illustrates gene expression of ALCAM, where y-axis represent relative expression while x-axis represents samples day 0(d0) and day 12(d12) for both iPSC (WTC-11) and hESC (H1). The graphs have error bars which represents the standard deviation.

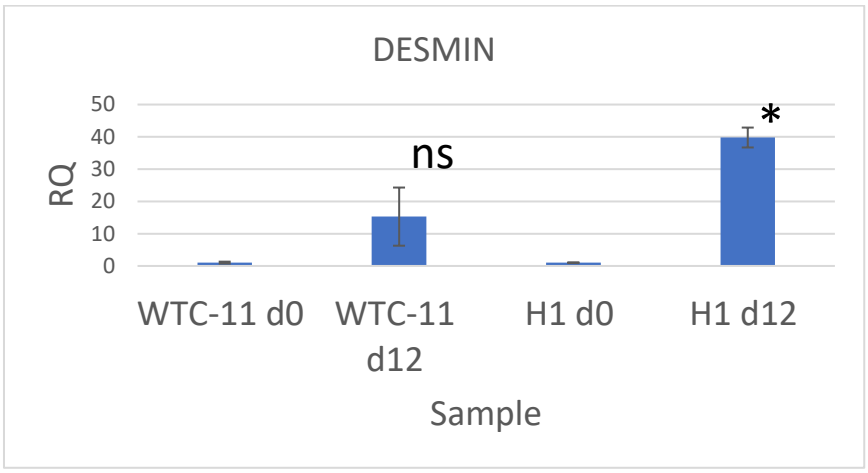

Figure 10: Illustrates gene expression of Desmin, where y-axis represent relative expression while x-axis represents samples day 0(d0) and day 12(d12) for both iPSC (WTC-11) and hESC (H1). The graphs have error bars which represents the standard deviation.

Expression of HSC-specific marker on proteins

To analyze phenotype of generated cells, one immunostained the cells to investigate the protein expression and distribution within the cells. By confirming the presence of the different proteins, one could track if the differentiation was successful.

The cell lines were cultured on coverslips and were immunostained for immunofluorescence microscopy. The coverslips were fixated at day 12 of the differentiation and were analyzed to investigate the protein expression of selected marker in the cells. *Figure 11* shows DAPI fluorescent stain which is illustrated with blue. DAPI binds to nucleic acid and colors the nucleus in the cells. *Figure 12* shows PDGFR-β, which is illustrated in green. PDGFR-β binds to platelet-derived growth factor receptor β which again is present in myofibroblasts that can be tracked to hepatic stellate cells (Coll et al., 2018). In *Figure 13* Vimentin is illustrated with the red color. Vimentin is the major intermediate filament protein, which is present in mesenchymal state of the differentiation. With all the staining and results indicates that successful staining of DAPI and Vimentin that there are cells which have differentiated into the mesenchymal state. By observing the PDGFR-β staining indicates that HSC-like cells were successfully differentiated, due to the PDGFR-β. *Figure 14* shows a merged picture of all the staining in one picture.

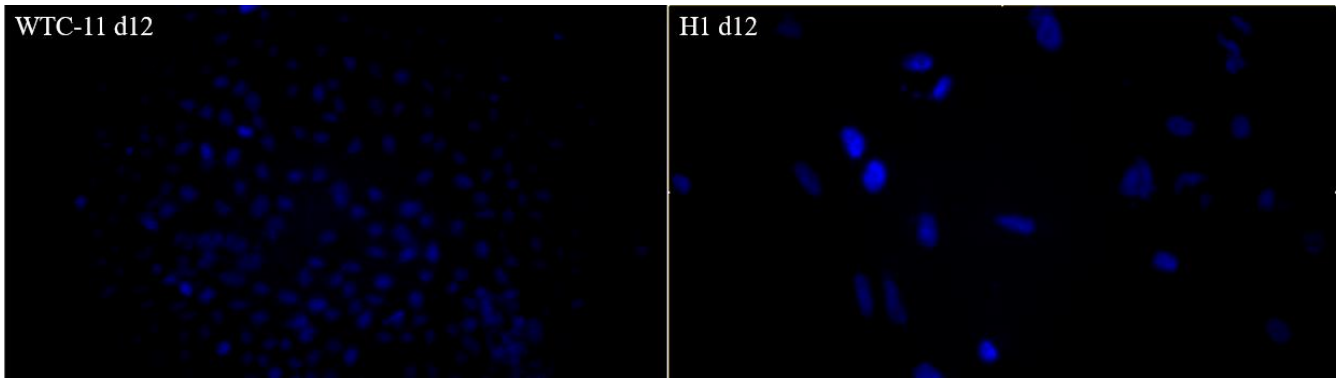

Figure 11: Images of both WTC-11 day 12(d12) and H1 day12(d12) after staining of DAPI.

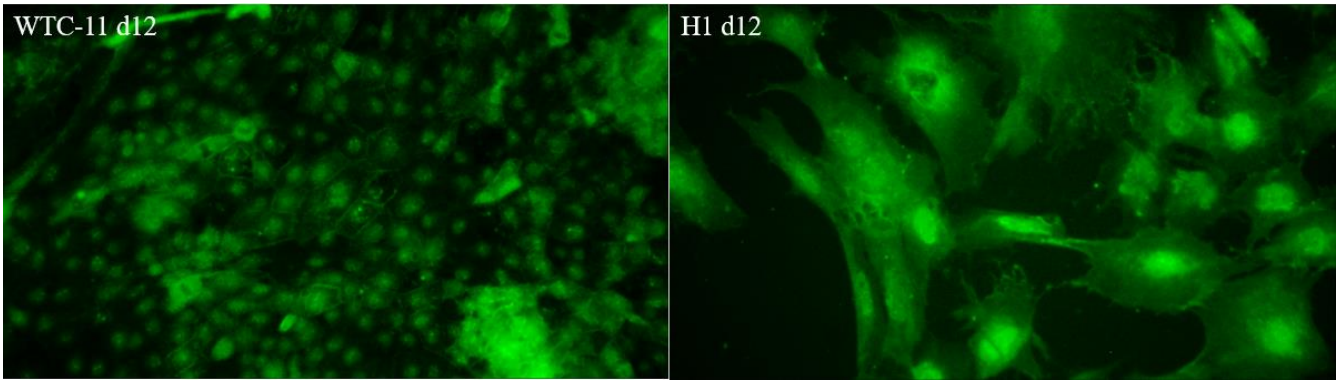

Figure 12: Images of both WTC-11 day 12(d12) and H1 day12(d12) after staining of PDGFR-β.

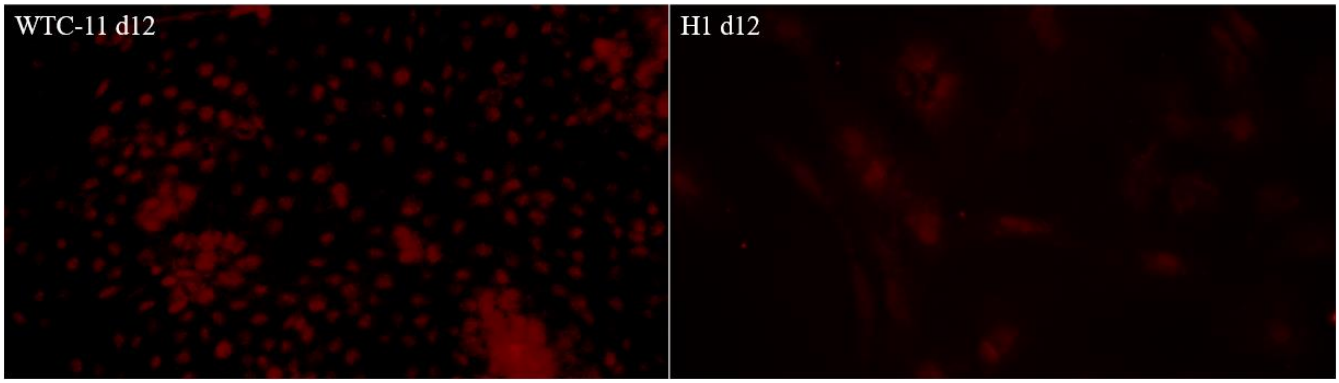

Figure 13: Images of both WTC-11 day 12(d12) and H1 day12(d12) after staining of Vimentin.

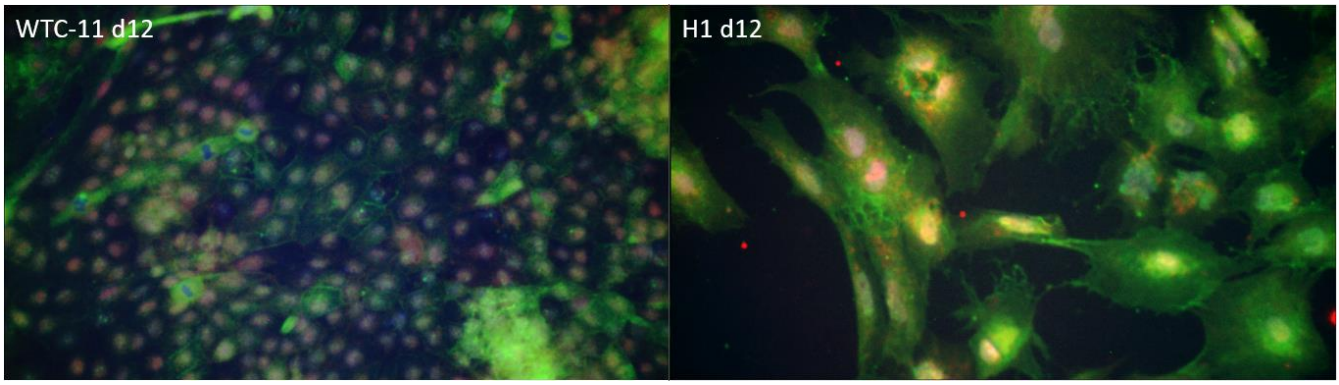

Figure 14: Images merged for both WTC-11 day 12(d12) and H1 day12(d12) of all stainings.

3D culture of iHSC

In vivo HSC exist in the 3D microenvironment, and therefore experience different physical cues, than in 2D artificial cell culture in vitro. To mimic 3D microenvironment and have more physiologically correct in vitro system, we established 3D culture of iHSC. We investigated cell behavior in two types of 3D culture: in monoculture of single cells embedded in extracellular matrix (ECM), and in co-culture with HepG2 cells as spheroids. For this we assessed TGFb-induced activation of iHSC in 3D culture and compared it to the 2D culture.

Establishing 3D culture of iHSC

To mimic ECM of space of Disse we selected Geltrex matrix, representing mixture of reduced growth factor (RGF) and basement membrane extract (BME). This soluble medium was then mixed with the day 12 iHSC to construct a 3D culture which could represent HSCs in the space of Disse environment in vitro. iHSC was resuspended to a concentration of 1mln/mL and 50 µL was transferred to a tube and mixed with 100 µL Geltrex. 25µL were then evenly distributed to each well that was going to contain a hydrogel droplet.

The HSCs were differentiated until day12 and then used to make 3D cultures in Geltrex. *Figure 15-18* illustrates how the 3D cultures looked through the brightfield microscope during the 3 days of culturing, before the cells were harvested for RNA isolation or for immunostaining. Day 1 appear to have the clearest picture of cells as it seems like the other days have more clusters of cells on top of each other. At day 2 a relative increase in round shaped cells occurs while the number of stretched cells has decreased. At day 3 one can see how the TGF- cells are more uniform, while the TGF+ treated cells have more of the round shaped cells that are stacking upon each other.

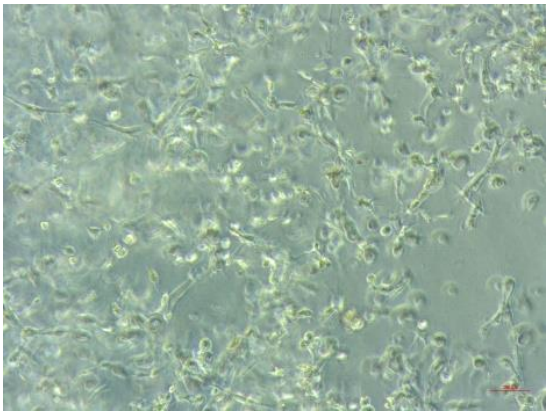

Figure 15: Representative image of 3D culture of HSCs in Geltrex at day 1, Scale bar 100 µm

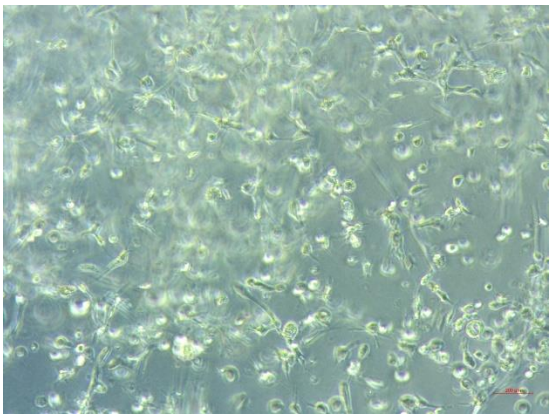

Figure 16: Representative image of 3D culture of HSCs in GelTrex at day 2, Scale bar 100 µm.

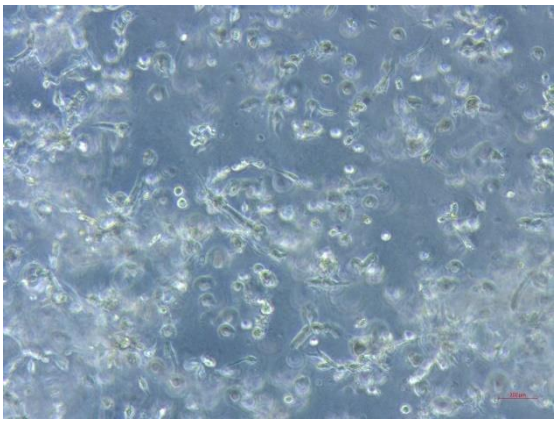

Figure 17: Representative image of 3D culture of HSCs in GelTrex at day 3, Scale bar 100 µm.

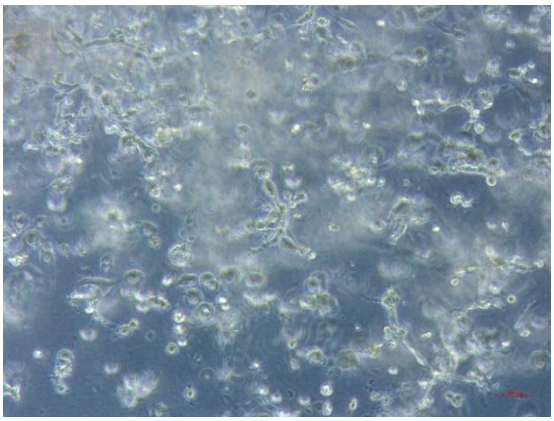

Figure 18: Representative image of 3D culture of HSCs in GelTrex treated with TGF-β1 at day 3 before harvesting the cells, Scale bar 100 µm

.

3D fibrotic liver tissue models were constructed with HepG2 and HSCs to mimic the cell-cell interaction HSCs would experience *in vivo*. By constructing these spheroids the goal was to

try to see if there was any difference in expression of genes and phenotypes as the HSCs would be cultured in this environment and treated with TGF-β1.

The spheroids were constructed with HepG2 and HSC cells where the ratio between the cell lines were a ratio of 5:1 with 2.5 mln HepG2 cells and 0.5 mln HSCs. Spheroids were made and fed every day to maintain the spheroids. *Figure 19-20* display how the spheroids looked through the microscope from the day they were constructed to the day they were harvested.

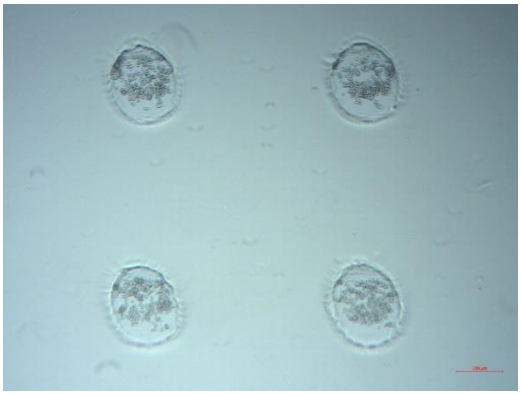

Figure 19: Bright field image of HepG2 and iHSC in the agarose microwells at the start of spheroids formation. Scale bar 200 µm.

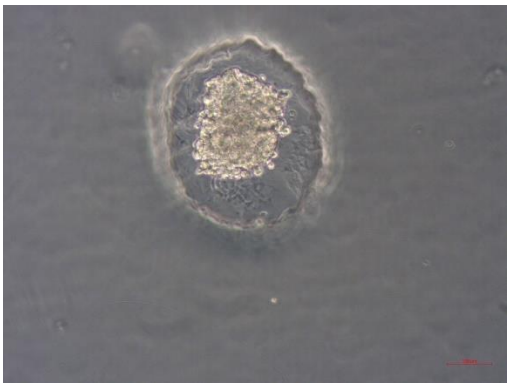

Figure 20: Bright field image of spheroid in microwell after 4 days of culture. Scale bar 100 µm.

Gene expression from 3D culture of HSC in spheroids

Next, we evaluated expression of HSC-specific genes in iHSC in 3D culture. For the iHSC in 3D Geltrex culture RNA was isolated directly from the culture, using standard methods. To evaluate gene expression in iHSC in spheroids 3D culture, we applied fluorescencs activated cell sorting (FACS) to separate EGFP-positive population of iHSC, from not labelled HepG2.

FACS indicated that only 1920 ± 0.8 % of cells in spheroids were EGFP positive *Fig. 21*. This dramatic changes in the cell ratio (initially iHSC represented about 20 % of all cells based on cell counting), most probably could be caused by HepG2 proliferation and therefore increased number of HepG2, but not iHSC.

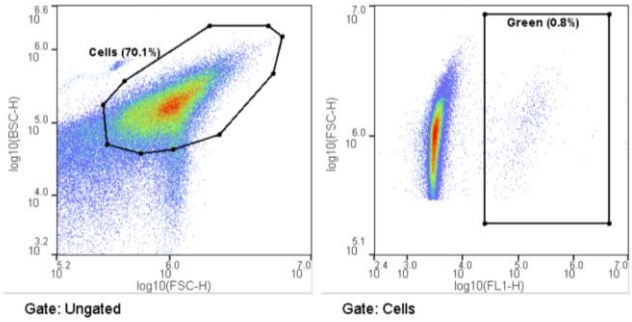

Figure 21: Left – Density plot showing the cells population from organoids. Right – Density plot showing a region of EGFP positive cells.

To evaluate activation of iHSC in 3D culture, both Geltrex and spheroids 3D culture were incubated with TGFb during 24 h. Actiavtion of iHSC was evaluated by gene expression analysis and by immunofluorescence microscopy.

Importantly, we observed higher number of iHSC in spheroids treated with TGFb *Fig. 22*. It could be explained by the TGFb-induced proliferation of HSC.(Mohammed, 2019) However, we need further analysis of culture to prove this assumption.

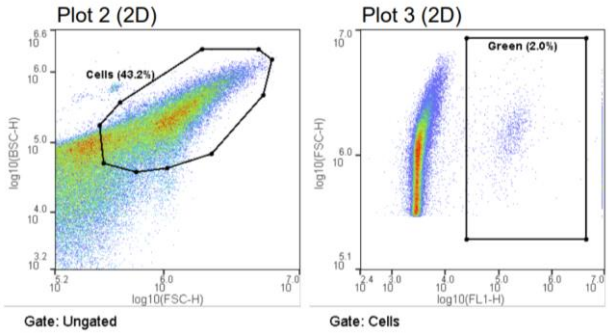

Figure 22: Left – Density plot showing the cells population from organoids. Right – Density plot showing a region of EGFP positive cells.

Gene expression of PDGFR-β, ALCAM and Desmin in the sorted spheroids were represented in *Figure 23-25* which were constructed by using data from *Appendix table 14-16*. The iPSC Babk samples from day 0, 6 and 12 were called babk0/6/12, while the sorted HSC cells both from control samples and TGF-β1 treated samples were called Sorted Cells Ctrl and Sorted Cells TGF. The graphs have error bars which represents the standard deviation. *Figure 23* shows a slight increase in expression for PDGFR-β with treatment compared to the control. Looking at the expression of ALCAM in *Figure 24*, one can see a significant increase in RQ value for the treated sample compared to the control sample. The difference in gene expression between the control and treated samples on Desmin in *Figure 25* indicated relatively no difference.

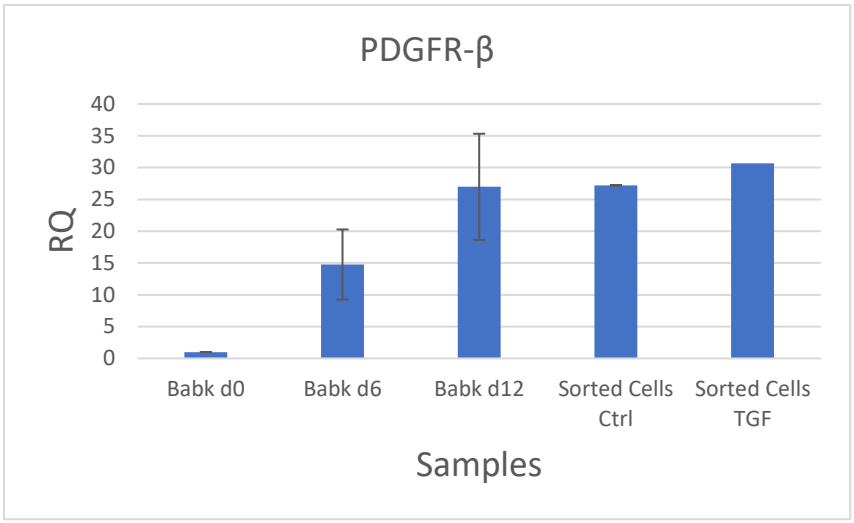

Figure 23: Illustrates gene expression of PDGFR-β, where y-axis represent relative expression while x-axis represents samples of the HSC from the different days of differentiation

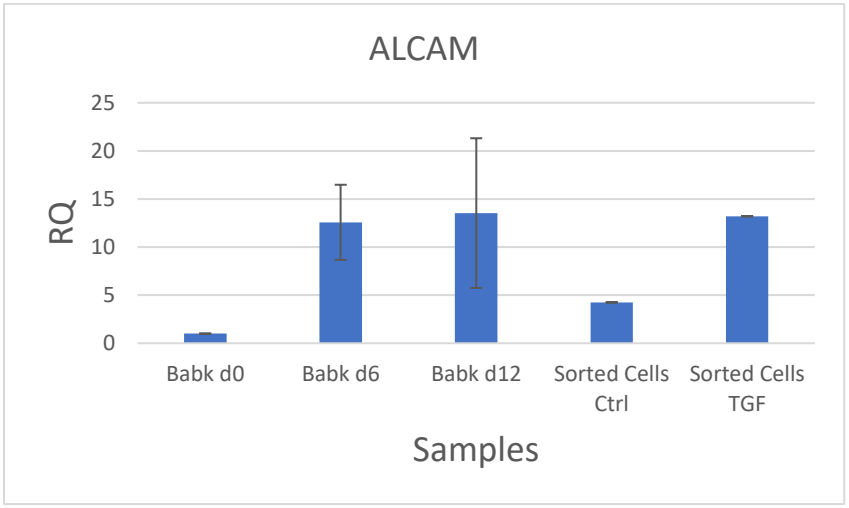

Figure 24: Illustrates gene expression of ALCAM, where y-axis represent relative expression while x-axis represents samples of the HSC from the different days of differentiation.

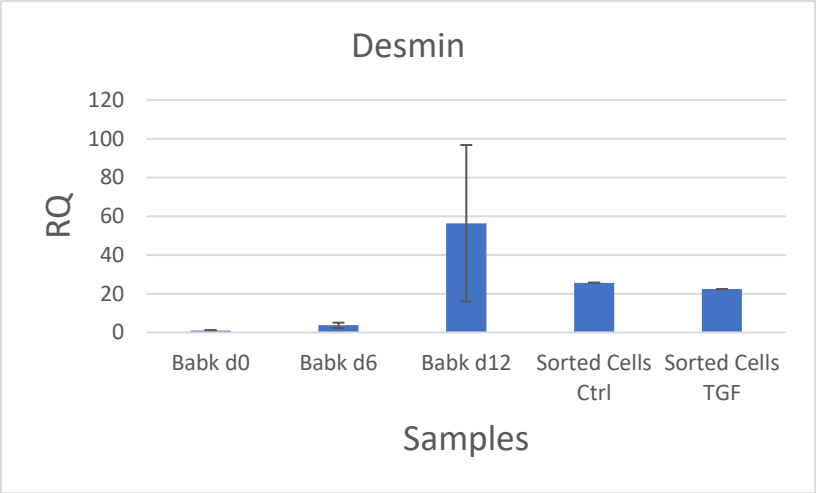

Figure 25: Illustrates gene expression of Desmin, where y-axis represent relative expression while x-axis represents samples of the HSC from the different days of differentiation.

Expression of PDGFR in iHSC in 2D and 3D culture was at the same level, indication preservation of cell phenotype in different culture conditions. unlike PDGFR expression of ALCAM and Desmin was lower in 3D culture.

Gene expression from 3D culture of HSC in geltrex

A RT-qPCR analysis was done to investigate the gene expression of the genes SMAD7, Col1a1, and Acta2 with GAPDH as a housekeeping gene to see if treatment of TGF-β1 would increase the gene expression. The samples are named Gel which indicates the samples from the 3D culturing of HSC in GelTrex. The Gel Ctrl samples are the controls while the Gel TGF are the samples which were treated with TGF-β1. The RQ values from *Appendix table 8-10* were used undergo a t-test. The results of the t-test were shown in *Appendix table 21*. It was not possible to execute a t-test, due to errors in the results for all the replicates of the Gel Ctrl. The t-tests only performed for the genes Col1a1 and Acta2, which is represented in *Appendix table 21*. *Appendix table 8-11* shows an increase in RQ mean from the Gel Ctrl compared to Gel TGF. Since the p-values for both Col1a1 and Acta2 are over 0.05 one could conclude that there lies no statistically significant difference between the results. Nevertheless, the RQ-mean values in Acta has an increase in the TGF-β1 treated GelTrex sample.

From *Figure 26-28* the RQ-values are represented in the graphs with the RQ values for Gel Ctrl and Gel TGF, where the error bars represent the standard deviation of the values. The figures are marked with ns (not significant) or *(significant) to indicate if there is a significant increase of expression between the control and TGF-β1 treated GelTrex sample. *Figure 26-28* was constructed by using *Appendix table 11-13.*

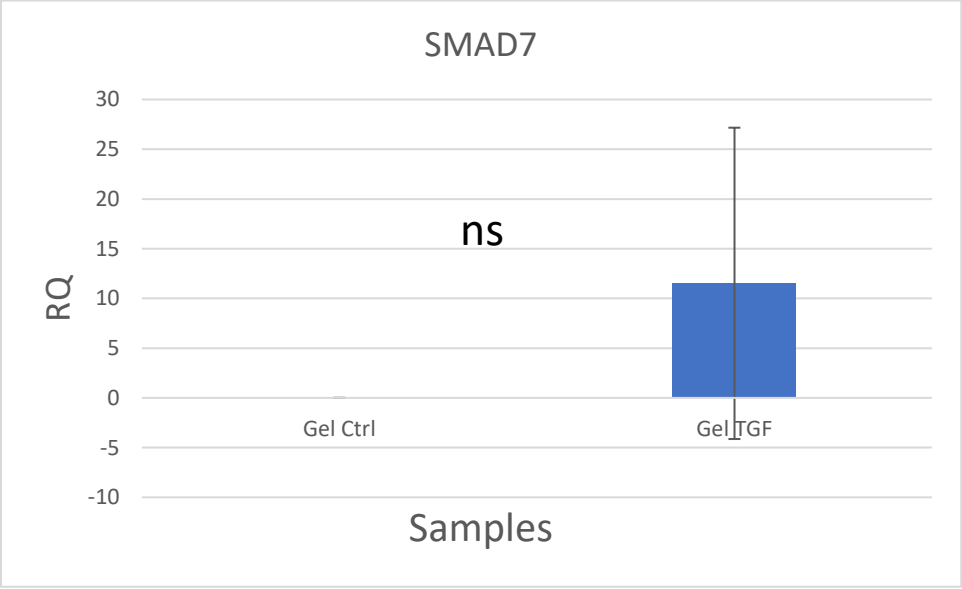

Figure 26: Illustrates gene expression of SMAD7, where y-axis represent relative expression while x-axis represents samples GelTrex control (Gel Ctrl) and GelTrex TGF-β1 treated (Gel TGF), which consisted of 3 replicates each.

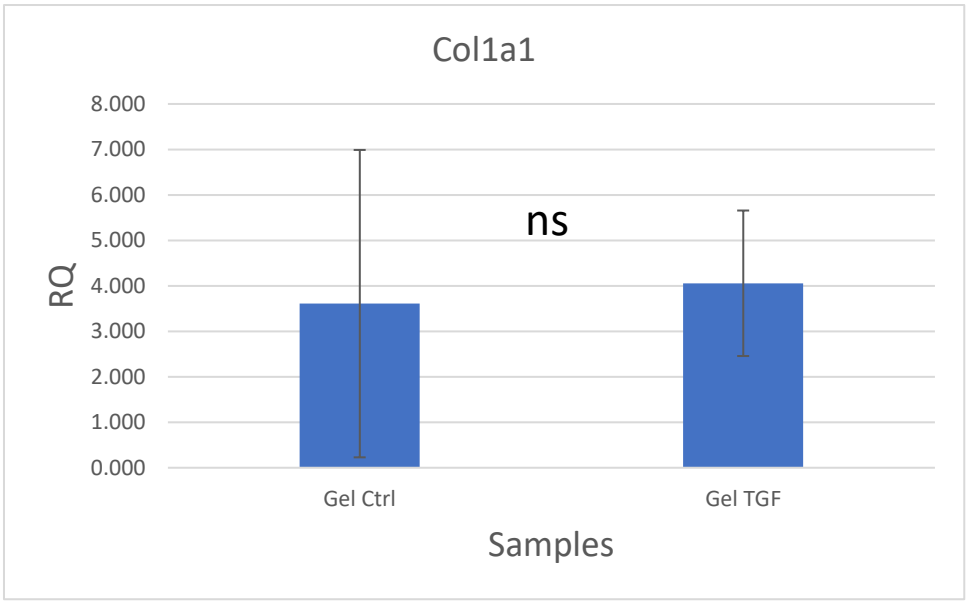

Figure 27: Illustrates gene expression of Col1a1, where y-axis represent relative expression while x-axis represents samples GelTrex control (Gel Ctrl) and GelTrex TGF-β1 treated (Gel TGF), which consisted of 3 replicates each.

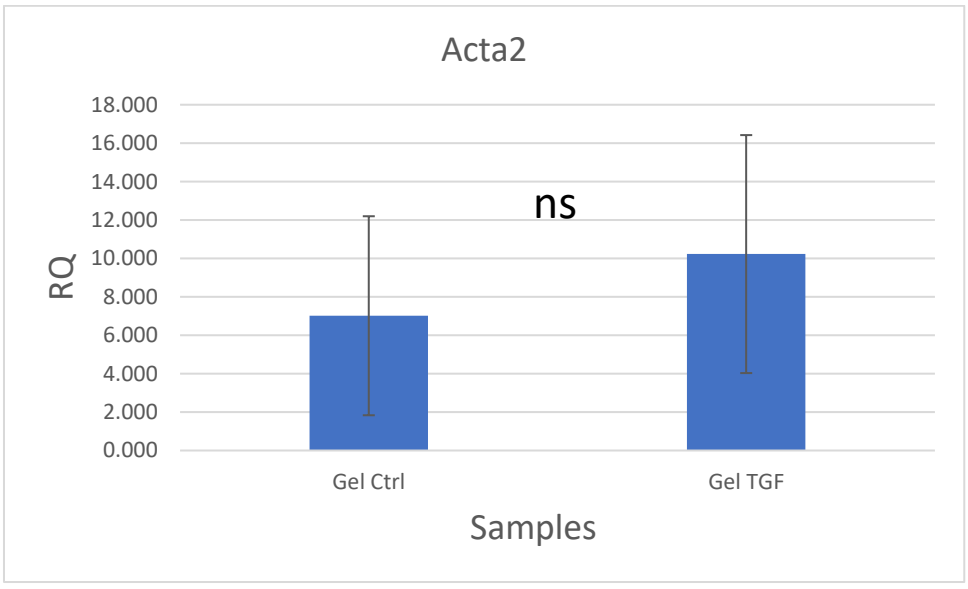

Figure 28: Illustrates gene expression of Acta2, where y-axis represent relative expression while x-axis represents samples GelTrex control (Gel Ctrl) and GelTrex TGF-β1 treated (Gel TGF), which consisted of 3 replicates each.

Phenotype of HSC in 3D culture

The fixated geltrex samples with HSCs were then immunostained for PDGFR-β, which bound to platelet-derived growth factor which indicates that there is HSCs in the culture. A-SMA (α-smooth muscle actin) was stained to mark that the myofibroblasts were activated. Collagen 1 a 3 was stained to target the collagen which is secreted from HSCs during liver fibrosis development. Hoecst was also stained to indicate the nucleus in each cell to see all the cells in the culture.

By using all these stain-markers the 3D cultures were analyzed under a fluorescence microscope to determine whether the cultures contained cells with the proteins each marker targeted. *Figure 29-32* represents the imaging of the both the control and TGF-β1 treated GelTrex cultures, where TGF-β1- represent the control and TGF-β1 + represent the GelTrex cultures that were treated with TGF-β1. One can see that there is expression of each marker in the TGF-β1 culture, which will indicate that there are HSCs present in the culture, and that activated HSCs are also present.

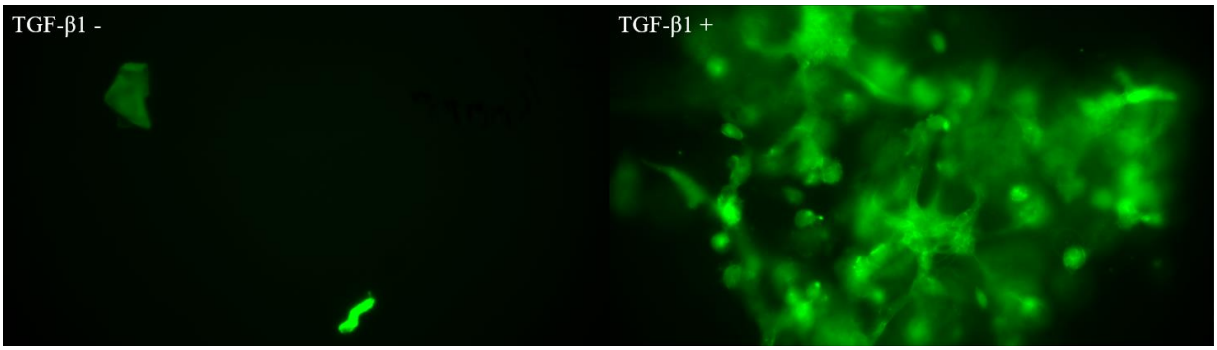

Figure 29: Illustrates PDGFR TGFR-β staining on 3D culture of HSC in ECM.

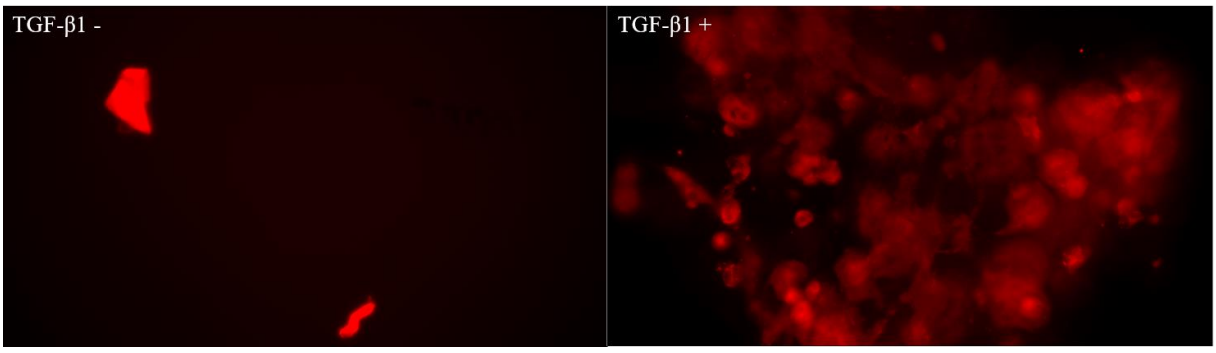

Figure 30: Illustrates A-SMA staining on 3D culture of HSC in ECM.

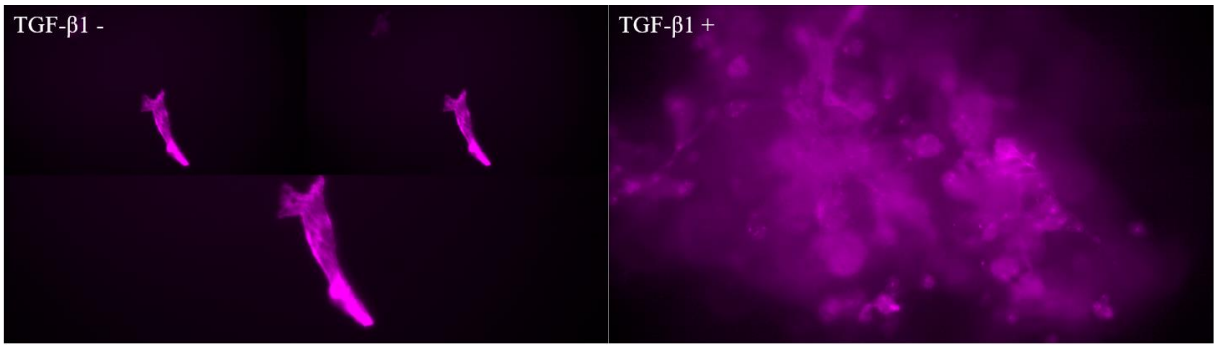

Figure 31: Illustrates Col-Cy3 staining on 3D culture of HSC in ECM.

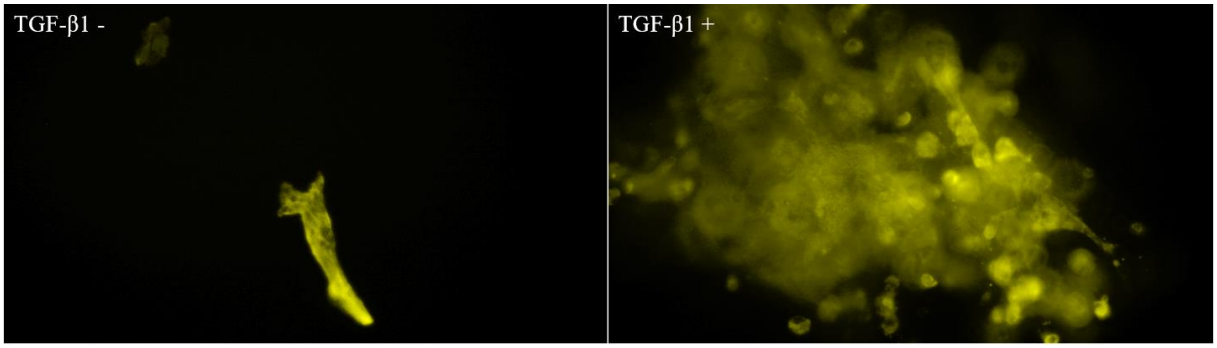

Figure 32: Illustrates Hoechst staining on 3D culture of HSC in ECM.

Testing of the effect of oxysterols on the activation of HSC

This experiment was done to evaluate the cumulative effect of Hydroxsterol within 24-27 hours of treatment, and with the presence of TGF-β1 to see the cumulative effect together with a profibrotic inducer.

HSCs were treated with hydroxsterols for 24, 25 and 27 hours to check if there was any significant upregulation of fibrosis-associated genes in response to the treatment plus treatment with and without TGFβ. 3 replicates were made for each treatment and a gene expression analysis was executed. The graphs in *Figure 33* were constructed by using the RQ-values from *appendix table 17-20*. *Figure 33* indicates no significant upregulation of fibrosis-associated genes in respone to the treatment with hydroxysterols (24 h) with or without TGFβ-treatment, but on the other hand there was a trend with the 25 hours samples which favored upregulation of SMAD and Akta genes.

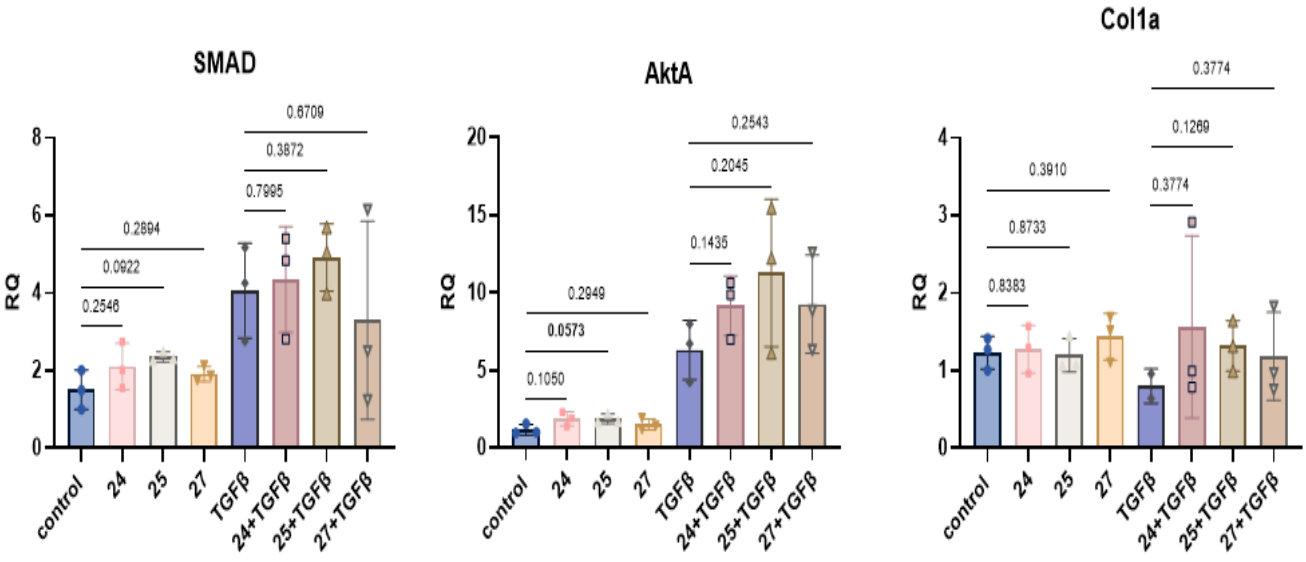

Figure 33: Shows the expression of SMAD, AktA and Col1a1 on the HSCs that had been treated with hydroxysterols and TGF-β.

Discussion

Generation and characterization of iPSC/hESC-derived HSC

iPSC/hESC derived to HSC

The reasoning for differentiation of both iPSC and hESC was to be able to follow a differential protocol which was previously published by (Coll et al., 2018) to produce HSC cultures that could be utilized to investigate both phenotypes of the cell and to measure the gene expression of genes with specific markers. The cells were also produced for culturing HSC in 3D models to investigate TGF-β1's induction of fibrosis.

The differentiation of both hESC and iPSC resulted in cells with characteristic phenotypes as HSC (Mohammed, 2019). By using the Bright field microscope for imaging, one could conclude that from day 0-12 gave a predicted morphology on the cells.

Expression of HSC-spesific genes in PSC derived HSC (cell line H1 and WTC-11) was done to investigate if the differentiated cells with similar phenotype as HSC in fact contained the genes which specific exists in HSC. RT-qPCR was used for this and the genes PDGFR-β, ALCAM and Desmin's expression were measured. PDGFR-β expression for both cell lines show a significantly increase from day 0 to day 12 of the differentiation, which will indicate the presence of platelet-derived growth factor receptor β which is found in HSC (Kitto & Henderson, 2021). ALCAM expression gave no significant increase from day 0 to day 12 for any of the cell lines. For Desmin expression the WTC-11 differentiation didn't give any significant increase in expression, due to differentiation, while the cell line H1 had a significant increase in expression. The reasoning for having these differences in the data might have been caused by poor technique in the pipetting work, when preparing the well plate for the RT-qPCR. There could be several other factors such as extraction of the RNA in the RNA isolation, or contamination during any of the procedures. Proceeding to analyze more parallels at the time could possibly give more stable data and prevent the data from having unesessary big gaps between each measurement as shown by the errorbars on *Figure 8-10* which shows the standard deviation of the RQ-mean values for the cell lines at the different days of the differentiation.

Expression of HSC-specific marker on proteins

To analyze the phenotype from the generated cells, the cells were immunostained and immunofluorescence imaging was done to investigate the presence of the specific proteins the cells were stained for. The cells showed both in the hESC and iPSC cultures that the presence of DAPI, PDGFR-β and Vimentin. DAPI indicates the cells containing the double stranded DNA and is used to locate the different cells. PDGFR-β was stained to indicate the presence of platelet-derived growth factor receptor β, which often is found in myofibroblasts that can be tracked to HSCs. Vimentin was stained to pinpoint if the cells have gone through a mesenchymal state. The H1 cells had less cells on the imaged compared to WTC-11. This might be a consequence of poor technique when mounting the cover slips on the glass slides with pinchers.

Establishing 3D culture of iHSC

3D culturing of iHSC were established to mimic the environment the HSC have in vivo. Spheroid models were made with co-culturing of HepG2 and iHSC to mimic the cell-cell interaction. Construction of the spheroids were done by a mixture of HepG2 and HSC with a 5:1 ratio. The spheroids were made without any problem and the spheroids were sorted after harvesting to sort out the HSC from the HepG2. By dividing the two cell types one could focus on analyzing gene expression of the HSCs between a control sample and a TGF-β1 treated sample. When sorting the cells *Figure 21* and *22* showed how the amounts of HepG2 were significantly higher than the HSCs, when looking at the ratio between the two types of cells. This may have been caused by a much higher proliferation of HepG2 compared to the HSCs.(Mohammed, 2019) The TGF-β1 treated spheroids had a higher ratio of HSCs than the control sample, which can indicate that the treatment exerted an increase of proliferation on HSCs. Even though these results seem like positive and shows that TGF-β1 treatment have given a desirable change, more parallels are needed to conclude anything.

When looking at results from the RT-qPCR on the sorted cells one can see how only PDGFR-β is the only gene that has increased in the gene expression compared to the 2D culturing. This can indicate that there has been some error in the RNA isolation, or that the sorting should have continued over a longer span of time. The ratio between HepG2 cells and HSCs could be tweaked, due to the exceeding proliferation of HepG2.

GelTrex matrix were selected to mimic ECM of space of Disse, which is a mixture of RGF and BME. By looking at *Figure 15-18* nothing indicates that the HSCs are proliferating to an extent that it is noticeable. Some of the GelTrex samples were immunostained while other were harvested for RNA isolation. The gene expression of the GelTrex samples shows no significant increase of expression between the control and treated samples, but an increase in RQ value for Acta2 was shown. There are many errors that may have occurred during this experiment, hence the standard deviation represented in the error bars in *Figure 26-28* shows that every sample varied a lot in result. This might have occurred, due to slow application of GelTrex to each well. When applying the GelTrex to the well plates some air bubbles were unfortunately applied to the GelTrex droplets, which could have been a factor for the unregular data. There could have been poor technique when extracting RNA during the RNA isolation, as well as unprecise pipetting at when preparing the qPCR plate.

The immunostained GelTrex samples showed in *Figure 29-32* that the staining was partly successful and that one could indicate that the TGF-β1 treated samples contained HSCs present in the culture with some activated HSCs as the imaging showed that α-SMA and Col-Cy3. The control samples didn't have that many cells in the imaging, which could be explained with the air bubbles that were discussed earlier for these GelTrex samples. A solution for the problem could be to make some extra medium before constructing the droplets, so there will be less option for error. More parallels should be run to reduce the error scale and have reproduceable results.

Testing the effect of oxysterols on the activation of HSC

HSCs were treated with hydroxsterols for 24, 25 and 27 hours to check if there was any significant upregulation of fibrosis-associated genes in response to the treatment plus treatment with and without TGFβ. *Figure 33* indicates no significant upregulation of fibrosis-associated genes in respone to the treatment with hydroxysterols (24 h) with or without TGFβ-treatment, but on the other hand there was a trend with the 25 hours samples which favoured upregulation of SMAD and Akta genes. When applying the cDNA samples and probe mixtures to every well, error might have occurred. When looking at the SMAD expression one can see that 27+TGFβ has a relative bigger error bar than the other samples, that represents the standard deviation. Same occurred for 24+TGFβ for the Col1a expression. The experiment should be repeated with 2 more cell lines to make a conclusion to be sure this applies generally for the PSC. The experiment could be repeated with a more prolonged treatment.

Conclusion

The differentiation and staining of both iPSCs and hESCs was successful, but the analysis of gene expression had its flaws. These flaws could be a result of poor technique during the RNA isolation, due to several different experiments having the same RNA isolation in the procedure and lacking the same reproductive results of the RT-qPCR analysis. To conclude that the RNA isolation was the main factor for the deviation of the results, the experiment must be repeated with caution during the extraction of RNA step.

Two types of 3D culturing of iHSC were established to mimic the cells environment in vivo. One of the 3D cultures were spheroids which were constructed with HepG2 and iHSC that had a 5:1 ratio between the HepG2 and iHSC cells. The spheroids through the cell sorting that the TGF-β1 gave an increase of cell sorted for the TGF-β1 treated sample compared to the control sample. Nevertheless, HepG2 had a proliferation, which made the ratio between HepG2, and HSC elevate. The ratio could be adjusted decreasing the amount of HepG2 when constructing the spheroids. In (Mohammed, 2019) it's written that a ratio of 2:1 had a greater potential in simulating liver fibrosis compared to 4:1. When looking at the RT-qPCR the sorted cells had increased expression in only PDGFR-β when comparing the expression to the 2D cultures. This understates the hypothesis of HepG2 and HSC ratio being immense.

Looking at the GelTrex samples that were established to mimic the ECM of space of Disse, the GelTrex samples had immense deviation in the results of the qPCR and staining. The Staining showed a successful staining for the TGF-β1 treated samples, but a small quantity of cells in the control samples, which could be explained by the air bubbles that accidently were added to the droplets. The RT-qPCR analysis of the GelTrex showed also low reproduceable for both control and TGF-β1 treated samples, that could also be explained with having a bad RNA extraction during the RNA isolation. The experiment should be repeated 3 times with more focus on constructing the 3D culture without air bubbles and extracting the RNA without contaminating the samples.

The effect test of oxysterols on activation of HSC was investigated by treating HSCs with hydroxsterols for 24, 25 and 27 to check if there were any significant upregulation of fibrosis-associated genes in response to the treatment plus treatment with and without TGFβ. no significant upregulation of fibrosis-associated genes in response to the treatment with hydroxysterols (24 h) with or without TGFβ-treatment, but on the other hand there was a trend with the 25 hours samples which favored upregulation of SMAD and Akta genes. The

experiment should be repeated with 2 more cell lines to make a conclusion to be sure this applies generally for the PSC. The experiment could be repeated with a more prolonged treatment.

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Appendix

Appendix table 1: Shows the CT values for the gene GAPDH, from the RT-qPCR of both the iPSC and hESC derived HSC.

GAPDH				
Sample	**Replication 1**	**Replication 2**	**Replication 3**	**Avg. CT**
WTC d0 #1	24.188	24.188	24.188	24.188
WTC d0 #2	25.129	25.129	25.129	25.129
WTC d0 #3	23.398	23.398	23.398	23.398
WTC d12 #1	29.678	29.678	29.678	29.678
WTC d12 #2	29.731	29.731	29.731	29.731
WTC d12 #3	33.430	33.430	33.430	33.430
H1 d0 #1	22.155	22.155	22.155	22.155
H1 d0 #2	21.395	21.395	21.395	21.395
H1 d0 #3	21.462	21.462	21.462	21.462
H1 d12 #1	22.559	22.559	22.559	22.559
H1 d12 #2	22.936	22.936	22.936	22.936
H1 d12 #3	23.242	23.242	23.242	23.242

Appendix table 2: Shows the CT values for the gene PDGFR, from the RT-qPCR of both the iPSC and hESC derived HSC.

PDGFR										
Sample	**Replication 1**	**Replication 2**	**Replication 3**	**Avg. CT**	**ΔCT**	**Avg. ΔCT**	**ΔΔCT**	**RQ**	**Avg.**	**Var.**
WTC d0 #1	32.746	32.746	32.746	32.746	8.558		-0.073	1.052		
WTC d0 #2	34.424	34.424	34.424	34.424	9.295	8.631	0.664	0.631	1.063	0.191572
WTC d0 #3	31.438	31.438	31.438	31.438	8.040		-0.591	1.506		
WTC d12 #1	31.998	31.998	31.998	31.998	2.320		-6.311	79.396		
WTC d12 #2	31.698	31.698	31.698	31.698	1.967		-6.664	101.406	92.268	131.5655
WTC d12 #3	35.476	35.476	35.476	35.476	2.046		-6.585	96.002		
H1 d0 #1	30.931	30.931	30.931	30.931	8.776		-0.149	1.109		
H1 d0 #2	30.418	30.418	30.418	30.418	9.023	8.925	0.098	0.935	1.003	0.008696
H1 d0 #3	30.439	30.439	30.439	30.439	8.977		0.052	0.965		
H1 d12 #1	26.057	26.057	26.057	26.057	3.498		-5.427	43.032		
H1 d12 #2	26.520	26.520	26.520	26.520	3.584		-5.341	40.542	52.574	350.6307
H1 d12 #3	25.955	25.955	25.955	25.955	2.713		-6.212	74.148		

Appendix table 3: Shows the CT values for the gene ALCAM, from the RT-qPCR of both the iPSC and hESC derived HSC.

ALCAM										
Sample	**Replication 1**	**Replication 2**	**Replication 3**	**Avg. CT**	**ΔCT**	**Avg. ΔCT**	**ΔΔCT**	**RQ**	**Avg.**	**Var.**
WTC d0 #1	29.558	29.558	29.558	29.558	5.370		0.064	0.957		
WTC d0 #2	30.739	30.739	30.739	30.739	5.610	5.306	0.304	0.810	1.019	0.0606592
WTC d0 #3	28.336	28.336	28.336	28.336	4.938		-0.368	1.291		
WTC d12 #1	33.707	33.707	33.707	33.707	4.029		-1.277	2.423		
WTC d12 #2	32.475	32.475	32.475	32.475	2.744		-2.562	5.905	4.248	3.0521794
WTC d12 #3	36.593	36.593	36.593	36.593	3.163		-2.143	4.417		
H1 d0 #1	28.129	28.129	28.129	28.129	5.974		-0.412	1.331		
H1 d0 #2	27.991	27.991	27.991	27.991	6.596	6.386	0.210	0.865	1.021	0.0716438
H1 d0 #3	28.050	28.050	28.050	28.050	6.588		0.202	0.869		
H1 d12 #1	26.122	26.122	26.122	26.122	3.563		-2.823	7.076		
H1 d12 #2	26.379	26.379	26.379	26.379	3.443		-2.943	7.690	9.39	12.192733
H1 d12 #3	25.883	25.883	25.883	25.883	2.641		-3.745	13.408		

Appendix table 4: Shows the CT values for the gene DESMIN, from the RT-qPCR of both the iPSC and hESC derived HSC.

DESMIN										
Sample	**Replication 1**	**Replication 2**	**Replication 3**	**Avg. CT**	**ΔCT**	**Avg. ΔCT**	**ΔΔCT**	**RQ**	**Avg.**	**Var.**
WTC d0 #1	34.186	34.186	34.186	34.186	9.998		0.228	0.854		
WTC d0 #2	35.136	35.136	35.136	35.136	10.007	9.770	0.237	0.849	1.028	0.0933
WTC d0 #3	32.703	32.703	32.703	32.703	9.305		-0.465	1.380		
WTC d12 #1	36.924	36.924	36.924	36.924	7.246		-2.524	5.752		
WTC d12 #2	35.461	35.461	35.461	35.461	5.730		-4.040	16.450	15.285	81.138
WTC d12 #3	38.636	38.636	38.636	38.636	5.206		-4.564	23.654		
H1 d0 #1	32.901	32.901	32.901	32.901	10.746		-0.196	1.145		
H1 d0 #2	32.484	32.484	32.484	32.484	11.089	10.942	0.147	0.903	1.005	0.0158
H1 d0 #3	32.452	32.452	32.452	32.452	10.990		0.048	0.967		
H1 d12 #1	28.198	28.198	28.198	28.198	5.639		-5.303	39.470		
H1 d12 #2	28.451	28.451	28.451	28.451	5.515		-5.427	43.012	39.786	9.4834
H1 d12 #3	28.979	28.979	28.979	28.979	5.737		-5.205	36.877		

Appendix table 5: Shows the RQ values from the expression of PDGFR.

PDGFR

Sample	Sample name	RQ	RQ mean	RQ SD	Sample name	RQ mean	RQ SD
WTC-11 d0	WTC d0 #1	1.051901779	1.063106087	0.4376898	WTC-11 d0	1.0631061	0.4376898
	WTC d0 #2	0.631126016			WTC-11 d12	92.268286	11.4702
	WTC d0 #3	1.506290467			H1 d0	1.0028071	0.0932521
WTC-11 d12	WTC d12 #1	79.39630705	92.26828606	11.4702	H1 d12	52.573798	18.725136
	WTC d12 #2	101.4060558					
	WTC d12 #3	96.00249531					
H1 d0	H1 d0 #1	1.109056861	1.002807063	0.0932521			
	H1 d0 #2	0.934543247					
	H1 d0 #3	0.96482108					
H1 d12	H1 d12 #1	43.03186114	52.57379757	18.725136			
	H1 d12 #2	40.54166215					
	H1 d12 #3	74.14786941					

Appendix table 6: Shows the RQ values from the expression of ALCAM.

ALCAM

Sample	Sample name	RQ	RQ mean	RQ SD	Sample name	RQ mean	RQ SD
WTC-11 d0	WTC d0 #1	0.956608158	1.019058041	0.246291	WTC-11 d0	1.019058	0.246
	WTC d0 #2	0.810003474			WTC-11 d12	4.2484661	1.747
	WTC d0 #3	1.29056249			H1 d0	1.0214703	0.268
WTC-11 d12	WTC d12 #1	2.423345321	4.248466111	1.747049	H1 d12	9.3914017	3.492
	WTC d12 #2	5.905257623					
	WTC d12 #3	4.416795389					
H1 d0	H1 d0 #1	1.330529041	1.021470277	0.267664			
	H1 d0 #2	0.864537231					
	H1 d0 #3	0.86934456					
H1 d12	H1 d12 #1	7.076323483	9.391401688	3.491809			
	H1 d12 #2	7.690087427					
	H1 d12 #3	13.40779415					

Appendix table 7: Shows the RQ values from the expression of DESMIN.

DESMIN

Sample	Sample name	RQ	RQ mean	RQ SD	Sample name	RQ mean	RQ SD
WTC-11 d0	WTC d0 #1	0.854	1.027547656	0.305519	WTC-11 d0	1.02754766	0.306
	WTC d0 #2	0.849			WTC-11 d12	15.2851221	9.008
	WTC d0 #3	1.380			H1 d0	1.00507466	0.126
WTC-11 d12	WTC d12 #1	5.752	15.28512209	9.007678	H1 d12	39.7863105	3.08
	WTC d12 #2	16.450					
	WTC d12 #3	23.654					
H1 d0	H1 d0 #1	1.145	1.005074664	0.125562			
	H1 d0 #2	0.903					
	H1 d0 #3	0.967					

H1 d12	H1 d12 #1	39.470	39.78631046	3.079515
	H1 d12 #2	43.012		
	H1 d12 #3	36.877		

Appendix table 8: Shows the CT values for the gene SMAD7, from the RT-qPCR of the HSCs from the 3D culture with geltrex.

SMAD7										
Sample	**Replication 1**	**Replication 2**	**Replication 3**	**Avg. CT**	**ΔCT**	**Avg. ΔCT**	**ΔΔCT**	**RQ**	**Avg.**	**Var.**
Day 12 #1	31.115	31.493	31.398	31.335	4.753	4.025	0.728	0.604	1.130	0.554
Day 12 #2	30.233	30.044	29.832	30.036	3.297		-0.728	1.656		
Gel Ctr #1	Undetermined	Undetermined	Undetermined	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Gel Ctr #2	Undetermined	Undetermined	Undetermined	#DIV/0!	#DIV/0!		#DIV/0!	#DIV/0!		
Gel Ctr #3	Undetermined	Undetermined	Undetermined	#DIV/0!	#DIV/0!		#DIV/0!	#DIV/0!		
Gel TGF#1	35.839	Undetermined	Undetermined	35.839	-0.131	2.386	-2.517	5.725	2.233	9.210
Gel TGF#2	Undetermined	36.541	Undetermined	36.541	2.830		0.444	0.735		
Gel TGF#3	37.068	Undetermined	Undetermined	37.068	4.460		2.074	0.238		

Appendix table 9: Shows the CT values for the gene Col1a1, from the RT-qPCR of the HSCs from the 3D culture with geltrex.

Col1a1										
Sample	**Replication 1**	**Replication 2**	**Replication 3**	**Avg. CT**	**ΔCT**	**Avg. ΔCT**	**ΔΔCT**	**RQ**	**Avg.**	**Var.**
Day 12 #1	24.604	24.302	23.987	24.298	-2.285	-2.607	0.322	0.800	1.025	0.101
Day 12 #2	23.952	23.922	23.557	23.810	-2.929		-0.322	1.250		
Gel Ctr #1	30.932	31.079	30.200	30.737	-2.570	-2.597	0.027	0.981	2.008	6.126
Gel Ctr #2	30.364	29.852	29.234	29.817	-4.869		-2.272	4.831		
Gel Ctr #3	39.266	36.017	34.874	36.719	-0.352		2.245	0.211		
Gel TGF#1	31.905	30.752	30.963	31.207	-4.764	-4.221	-0.543	1.457	1.060	0.175
Gel TGF#2	30.483	29.801	30.231	30.172	-3.540		0.681	0.624		
Gel TGF#3	28.486	28.331	27.933	28.250	-4.358		-0.137	1.100		

Appendix table 10: Shows the CT values for the gene Acta2, from the RT-qPCR of the HSCs from the 3D culture with geltrex.

Acta2										
Sample	**Replication 1**	**Replication 2**	**Replication 3**	**Avg. CT**	**ΔCT**	**Avg. ΔCT**	**ΔΔCT**	**RQ**	**Avg.**	**Var.**

Day 12 #1	29.927	30.085	29.732	29.914	3.332	2.597	0.734	0.601	1.132	0.564
Day 12 #2	28.635	28.389	28.782	28.602	1.863		-0.734	1.664		
Gel Ctr #1	33.780	36.293	34.606	34.893	1.586	0.751	0.835	0.560	1.172	0.749
Gel Ctr #2	34.831	34.531	34.442	34.601	-0.085		-0.835	1.784		
Gel Ctr #3	Undetermined	Undetermined	Undetermined	#DIV/0!	#DIV/0!		#DIV/0!	#DIV/0!		
Gel TGF#1	37.085	36.773	35.781	36.546	0.576	0.137	0.439	0.737	1.116	0.457
Gel TGF#2	33.829	33.861	35.308	34.333	0.621		0.485	0.715		
Gel TGF#3	31.853	31.618	31.991	31.821	-0.787		-0.924	1.897		

Appendix table 11: Shows the RQ values from the expression of PDGFR for HSCs in 3D culture with geltrex.

PDGFR							
Sample	**Sample name**	**RQ**	**RQ mean**	**RQ SD**	**Sample name**	**RQ mean**	**RQ SD**
Gel Ctrl	Gel Ctr #1	#DIV/0!	#DIV/0!	#DIV/0!	Gel Ctrl	#DIV/0!	#DIV/0!
	Gel Ctr #2	#DIV/0!			Gel TGF	11.514	15.650
	Gel Ctr #3	#DIV/0!					
Gel TGF	Gel TGF#1	29.524	11.514	15.650			
	Gel TGF#2	3.792					
	Gel TGF#3	1.225					

Appendix table 12: Shows the RQ values from the expression of Col1a1 for HSCs in 3D culture with geltrex.

Col1a1							
Sample	**Sample name**	**RQ**	**RQ mean**	**RQ SD**	**Sample name**	**RQ mean**	**RQ SD**
Gel Ctrl	Gel Ctr #1	1.219	3.609	3.380	Gel Ctrl	3.609	3.380
	Gel Ctr #2	5.999			Gel TGF	4.057	1.600
	Gel Ctr #3	0.262					
Gel TGF	Gel TGF#1	5.575	4.057	1.600			
	Gel TGF#2	2.387					
	Gel TGF#3	4.208					

Appendix table 13: Shows the RQ values from the expression of Acta2 for HSCs in 3D culture with geltrex.

Acta2							
Sample	**Sample name**	**RQ**	**RQ mean**	**RQ SD**	**Sample name**	**RQ mean**	**RQ SD**
Gel Ctrl	Gel Ctr #1	3.353	7.016	5.179	Gel Ctrl	7.016	5.179

	Gel Ctr #2	10.678			Gel TGF	10.225	6.194
	Gel Ctr #3	#DIV/0!					
Gel TGF	Gel TGF#1	6.754	10.225	6.194			
	Gel TGF#2	6.545					
	Gel TGF#3	17.376					

Appendix table 14: Shows the RQ values from the expression of PDGFR for HSCs from differentiation and cellsorting.

PDGFR

Sample	Sample name	RQ	RQ mean	RQ SD	Sample name	RQ mean	RQ SD
Babk d0	d0	1	1	0	Babk d0	1	0
Babk d6	d6 #1	18.655	14.756	5.514	Babk d6	14.756	5.514
	d6#2	10.857			Babk d12	26.971	8.344
Babk d12	d12#1	36.579	26.971	8.344	Sorted Cells Ctrl	27.198	0
	d12#2	22.787			Sorted Cells TGF	30.672	0
	d12#3	21.547					
	Sorted cells CTRL	27.198	27.198	0			
	Sorted cells TGF	30.672	30.672	0			

Appendix table 15: Shows the RQ values from the expression of ALCAM for HSCs from differentiation and cellsorting.

ALCAM

Sample	Sample name	RQ	RQ mean	RQ SD	Sample name	RQ mean	RQ SD
Babk d0	d0	1	1	0	Babk d0	1	0
Babk d6	d6 #1	15.336	12.576	3.903	Babk d6	12.576	3.903
	d6#2	9.816			Babk d12	13.534	7.779
Babk d12	d12#1	22.517	13.534	7.779	Sorted Cells Ctrl	4.246	0
	d12#2	8.955			Sorted Cells TGF	13.199	0
	d12#3	9.132					
	Sorted cells CTRL	4.246	4.246	0			
	Sorted cells TGF	13.199	13.199	0			

Appendix table 16: Shows the RQ values from the expression of DESMIN for HSCs from differentiation and cellsorting.

DESMIN

Sample	Sample name	RQ	RQ mean	RQ SD	Sample name	RQ mean	RQ SD
Babk d0	d0	1	1	0	Babk d0	1	0
Babk d6	d6 #1	4.642	3.665	1.383	Babk d6	3.665	1.383
	d6#2	2.687			Babk d12	56.356	40.387
Babk d12	d12#1	102.205	56.356	40.387	Sorted Cells Ctrl	25.627	0
	d12#2	26.047			Sorted Cells TGF	22.334	0
	d12#3	40.816					
	Sorted cells CTRL	25.627	25.627	0			
	Sorted cells TGF	22.334	22.334	0			

Appendix table 17: Shows the RQ values from the expression of SMAD for HSCs from hydroxysterols and TGF-β treatment.

SMAD

Sample	Sample name	RQ	RQ mean	RQ SD	Sample name	RQ mean	RQ SD
Ctrl	Ctrl1	1.000					
	Ctrl2	2.012		0.506	Ctrl	1.502	0.506
	Ctrl3	1.495	1.502				
24	24(1)	2.741					
	24(2)	2.018		0.597	24	2.105	0.597
	24(3)	1.557	2.105				
25	25(1)	2.311					
	25(2)	2.497		0.124	25	2.357	0.124
	25(3)	2.262	2.357				
	27(1)	1.755					
	27(2)	2.130		0.192		1.918	0.192
27	27(3)	1.869	1.918		27		
	TGF1	5.178					
	TGF2	2.762		1.219		4.066	1.219
TGF	TGF3	4.257	4.066		TGF		
	24+(1)	4.837					
	24+(2)	5.405		1.361		4.352	1.361
24+	24+(3)	2.815	4.352		24+		
	25+(1)	5.056					
	25+(2)	5.698		0.865		4.913	0.865
25+	25+(3)	3.986	4.913		25+		
	27+(1)	6.151					
	27+(2)	1.237		2.552		3.296	2.552
27+	27+(3)	2.500	3.296		27+		

Appendix table 18: Shows the RQ values from the expression of AktA for HSCs from hydroxysterols and TGF-β treatment.

AktA							
Sample	**Sample name**	**RQ**	**RQ mean**	**RQ SD**	**Sample name**	**RQ mean**	**RQ SD**
Ctrl	Ctrl1	1.000	1.174	0.335	Ctrl	1.174	0.335
	Ctrl2	1.560					
	Ctrl3	0.963					
24	24(1)	1.930	1.880	0.461	24	1.880	0.461
	24(2)	2.313					
	24(3)	1.396					
25	25(1)	2.226	1.871	0.308	25	1.871	0.308
	25(2)	1.676					
	25(3)	1.710					
27	27(1)	1.922	1.519	0.365	27	1.519	0.365
	27(2)	1.421					
	27(3)	1.213					
TGF	TGF1	6.731	6.322	1.908	TGF	6.322	1.908
	TGF2	4.242					
	TGF3	7.993					
24+	24+(1)	9.868	9.164	1.924	24+	9.164	1.924
	24+(2)	10.637					
	24+(3)	6.987					
25+	25+(1)	12.257	11.282	4.748	25+	11.282	4.748
	25+(2)	15.467					
	25+(3)	6.121					
27+	27+(1)	12.599	9.259	3.158	27+	9.259	3.158
	27+(2)	8.856					
	27+(3)	6.322					

Appendix table 19: Shows the RQ values from the expression of Col1a1 for HSCs from hydroxysterols and TGF-β treatment.

Col1a1							
Sample	**Sample name**	**RQ**	**RQ mean**	**RQ SD**	**Sample name**	**RQ mean**	**RQ SD**
Ctrl	Ctrl1	1.000	1.229	0.211	Ctrl	1.229	0.211
	Ctrl2	1.416					

	Ctrl3	1.271					
24	24(1)	1.571	1.276	0.305	24	1.276	0.305
	24(2)	1.295					
	24(3)	0.962					
25	25(1)	1.067	1.199	0.217	25	1.199	0.217
	25(2)	1.082					
	25(3)	1.449					
27	27(1)	1.515	1.436	0.302	27	1.436	0.302
	27(2)	1.690					
	27(3)	1.103					
TGF	TGF1	#DIV/0!	#DIV/0!	#DIV/0!	TGF	#DIV/0!	#DIV/0!
	TGF2	0.643					
	TGF3	0.957					
24+	24+(1)	2.915	1.564	1.175	24+	1.564	1.175
	24+(2)	0.994					
	24+(3)	0.784					
25+	25+(1)	1.317	1.318	0.326	25+	1.318	0.326
	25+(2)	1.645					
	25+(3)	0.994					
27+	27+(1)	1.827	1.182	0.569	27+	1.182	0.569
	27+(2)	0.964					
	27+(3)	0.755					

*Appendix table 20: Represent t-test of the RQ-mean values of the genes PDGFR, ALCAM and DESMIN for cell line WTC-11 and H1. The genes with significant expression were marked with ns for not significant increase in expression or * for significant increase in expression.*

	PDGFR-β		ALCAM		DESMIN	
	WTC-11 d0	*WTC-11 d12*	*WTC-11 d0*	*WTC-11 d12*	*WTC-11 d0*	*WTC-12 d12*
Mean	1.06	92.27	1.019	4.248	1.028	15.285
Variance	0.192	131.565	0.061	3.052	0.093	81.138
Observations	3	3	3	3	3	3
Hypothesized Mean Difference	0		0		0	
df	2		2		2	
t Stat	-13.762		-3.170		-2.740	
P(T<=t) one-tail	0.0026		0.0434		0.0557	
t Critical one-tail	2.920		2.920		2.920	
P(T<=t) two-tail	0.005 (*)		0.087 (ns)		0.111 (ns)	
t Critical two-tail	4.303		4.303		4.303	

	PDGFR-β		ALCAM		DESMIN	
	H1 d0	*H1 d12*	*H1 d0*	*H1 d12*	*H1 d0*	*H1 d12*
Mean	1.003	52.574	1.021	9.391	1.005	39.786
Variance	0.009	350.631	0.072	12.193	0.016	9.483
Observations	3	3	3	3	3	3

Hypothesized Mean Difference	0	0	0
df	2	2	2
t Stat	-4.770	-4.140	-21.794
P(T<=t) one-tail	0.0206	0.0268	0.0010
t Critical one-tail	2.920	2.920	2.920
P(T<=t) two-tail	0.041 (ns)	0.054 (ns)	0.002 (*)
t Critical two-tail	4.303	4.303	4.303

Appendix table 21: Represent the t-test of Col1a1 and Acta2 for both the Gel Ctrl and Gel TGF samples.

t-Test: Two-Sample Assuming Unequal Variances

	Col1a1		**Acta2**	
	Gel Ctrl	*Gel TGF*	*Gel Ctrl*	*Gel TGF*
Mean	2.00868	1.06035	1.17242	0.72609
Variance	6.12625	0.17481	0.74915	0.00025
Observations	3	3	2	2
Hypothesized Mean Difference	0		0	
df	2		1	
t Stat	0.654		0.729	
P(T<=t) one-tail	0.290		0.299	
t Critical one-tail	2.920		6.314	
P(T<=t) two-tail	0.580 (ns)		0.599 (ns)	
t Critical two-tail	4.303		12.706	

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