Characterization of Pro-Fibrotic Signaling Pathways using Human Hepatic Organoids

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Financial support: Y.G. and G.P. were supported by awards from the NIDDK (1R56DK12930901A1). P.K.J., S.C.H., and A.E were supported by awards from the National Science Foundation (CBET 2033302).

The authors have declared that no conflict of interest exists.

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Abstract

Due to the limitations of available *in vitro* systems and animal models, we lack a detailed understanding of the pathogenetic mechanisms and have minimal treatment options for liver fibrosis. To overcome this barrier, we engineered a live cell imaging system that identifies collagen producing cells in a human multi-lineage hepatic organoid. This system was adapted for use as a microwell-based platform (i.e., microHOs) where exposure to PDGF or TGFβ1 induced the formation of thick collagen fibers. Transcriptomic analysis revealed that TGFβ1 exposure converted mesenchymal cells into myofibroblast-like cells, which contribute to the development of liver fibrosis. When pro-fibrotic intracellular signaling pathways were examined using pharmacological probes, the anti-fibrotic effect of receptor-specific tyrosine kinase inhibitors was limited to the fibrosis induced by the corresponding growth factor, which indicates that their anti-fibrotic efficacy would be limited to fibrotic diseases that were solely mediated by that growth factor. In contrast, GSK3β or p38 MAPK inhibitors could prevent TGFβ1- or PDGF-induced fibrosis in microHOs because they block intracellular signaling pathways that are commonly utilized by the TGFβ1 and PDGF receptors. Hence, these studies identified GSK3β and p38 MAPK inhibitors as potential new broad-spectrum therapies for liver fibrosis, and it is likely that other new therapies could subsequently be identified using this microHO system.

Word count: 220

Abbreviations: ARPKD, Autosomal Recessive Polycystic Kidney Disease; ECM, extra-cellular matrix; HCS, high content screening; HO, hepatic organoid; HSC, hepatic stellate cells; iPSC, induced pluripotent stem cells; MAPK, Mitogen Activated Protein Kinase; NASH, non-alcoholic steatohepatitis; SHG, Second Harmonic Generation.

Liver fibrosis is a pathological condition caused by the accumulation of extracellular matrix (**ECM**) that develops in response to chronic liver injury [1, 2]. It results from an interaction between parenchymal and nonparenchymal liver cells and involves endogenous and liver infiltrating immune cells [3, 4, 5]. The key non-parenchymal cell is the myofibroblast, which is generated when a fibrogenic stimulus induced by liver injury causes hepatic stellate cells (**HSC**) to transdifferentiate into myofibroblasts that produce fibril-forming collagens and other ECM components [5, 6, 7, 8, 9]. Before organoid methodology was available, *in vitro* models often did not have the spectrum of cell types that mediate fibrogenesis, which limited our ability to explore fibrogenic mechanisms. Commonly used rodent models require an injury inducing agent (carbon tetrachloride, bile duct ligation, or dietary modulation); are very time consuming and expensive to run; and any conclusions drawn from them are limited by concerns about their fidelity with the processes mediating the commonly occurring forms of human liver fibrosis [10]. However, the pathogenesis of liver fibrosis can best be studied using a multi-lineage human hepatic organoid (**HO**) that has the cellular components and the geometry of hepatic lobules, which enables the effect that biochemical signals have on fibrosis to be quantitatively characterized. We previously developed an *in vitro* model system that differentiates human induced pluripotent stem cells (**iPSCs**) into a multi-lineage HO that has hepatocytes, cholangiocytes, HSCs, and cells of other lineages (fibroblasts, macrophages, and endothelial cells); and bile ducts and other structures found in the liver lobule are formed in these HOs [11, 12]. HOs engineered to express a causative mutation for Autosomal Recessive Polycystic Kidney Disease (**ARPKD**) developed an extensive fibrosis [12] that was characterized by a marked increase in thick collagen fiber formation, which resembled that in liver tissue obtained from ARPKD patients. The myofibroblasts in ARPKD organoids resembled those in the livers of patients with viral-induced cirrhosis or advanced non-alcoholic steatohepatitis (**NASH**) [12]. Here, we engineered a live cell imaging system for identification of the collagen producing cells in hepatic organoids that was adapted for use in a microwell-based platform. This system was used to characterize the signals driving liver fibrosis at the whole organoid, single cell, and molecular levels. Moreover, when the interaction between the intracellular signaling pathways activated by two pro-fibrotic factors were characterized using pharmacological agents, two potential new approaches for treating liver fibrosis were identified.

Results

A live cell imaging system for hepatic fibrosis. Collagen is a triple helical protein that is composed of two COL1A1 (α1) and one COL1A2 (α2) chains, and increased collagen synthesis

by activated myofibroblasts is a major contributor to liver fibrosis [2]. To characterize fibrogenic mechanisms, we developed a live cell imaging system, which enabled collagen producing cells in HOs to be identified and quantitatively analyzed. A CRISPR-assisted insertion tagging system (CRISPaint) [13] was used to insert a Clover expression cassette (***COL1A1*-P2A Clover**) at the COOH terminus of the endogenous *COL1A1* gene in a control iPSC line. Since the insert has a self-cleaving P2A peptide, *COL1A1* mRNA expressing cells in the HOs produced from *COL1A1*-P2A Clover iPSCs were labelled with a fluorescent intracellular protein (**Fig 1A-B**). The Clover$^+$ cell population increased in HO cultures at the hepatoblast stage (day 9) (**Fig. 1C-D**). Immunostaining and flow cytometry revealed that Clover$^+$ cells: were markedly increased in day 21 HOs that were treated with TGFβ1 or PDGFβ (on day 13); co-expressed PDGFRβ and COL1A1; were present throughout the organoid; and were distinct from hepatocytes or cholangiocytes (**Figs. 1E-G, S1; Tables S1-S2**). Flow cytometry results confirm that Clover fluorescence is cell based (Figs. 1D). The pro-fibrotic effect of TGFβ and PDGF was consistent with the temporal pattern of expression of the mRNAs encoding their receptors (*PDGFRA, PDGFRB, TGFBR1, TGFBR2, TGFBR3, BMPR1B*) and intracellular signaling proteins (STATs, SMADs), which were expressed at the hepatoblast stage (day 9) (i.e., prior to when microHOs are exposed to PDGF or TFGβ on day 13) and in mature HOs (**Fig. S2**).

The spatial distribution and morphology of the collagen fibers in HOs were examined using Second Harmonic Generation (**SHG**) microscopy [14, 15], which has previously been used to quantitatively analyze liver fibrosis in human patients [16, 17] and to monitor fibrosis in ARPKD HOs [12]. Of importance, SHG measures thick collagen fiber formation; which is a complex biochemical pathway that requires the coordinated activity of multiple liver enzymes that include proteases and other post-translational processing enzymes that act on collagen, and subsequently the activity of lysyl hydroxylases and lysyl oxidases to generate thick collagen fibers. [18]. SHG images revealed that control HOs had sporadic networks of cross-linked collagen fibers surrounding the cells in some isolated regions, while the TGFβ1 or PDGF-treated organoids had more extensive networks of thick collagen fibers throughout the entire HO. A quantitative comparison indicated that TGFβ1 ($p < 0.05$) or PDGF ($p < 0.01$) exposure induced statistically significant increases in total collagen abundance and in the abundance of thick collagen fibers (i.e., > 3 um diameter) formed in HOs ($p<0.01$) (**Fig. 1H**). The SHG and immunostaining results indicate that PDGF or TGFβ1 increases the numbers of collagen synthesizing cells and the amount of thick collagen fibers formed in HOs.

*A high content screening system (**HCS**) for serially monitoring the effect of biochemical signals on liver fibrosis*. Culture conditions were modified to enable HOs to reproducibly grow to a standard size in microwells, which we refer to as '*microHOs*.' HCS was performed using a confocal fluorescence microplate imager with an automated program that we developed for analyzing multiple Z-stacked confocal images (**Fig. 2A-B**). TGFβ1 addition on day 13 induced a sustained increase in COL1A1^{+} cells in microHOs that was blocked by co-administration of TGFRβ1 tyrosine kinase inhibitors (10 uM SB431542 [19, 20] or A83-01 [21]) (**Fig. 2C-D**). PDGFβ addition on day 13 also increased the number of COL1A1^{+} cells in microHOs, which was blocked by addition of a PDGFRβ tyrosine kinase inhibitor (10 uM Imatinib [22, 23]) (**Fig. 3, top**). The microHO system was also used to examine the effect of five cytokines (IL-4 [24], IL-6 [25], IL-13, IL-33 [26, 27], TNFα [28]) and three growth factors (IGF1 [29], VEGF [30], CCL3 [31]) that had been directly or indirectly implicated in the pathogenesis of NASH or liver fibrosis. None of these agents induced fibrosis in microHOs (**Fig. S3**). Analysis of the temporal pattern of receptor expression in differentiating HO cultures using previously obtained transcriptomic data [12] revealed that the mRNAs encoding the IGF-1 (*IGF1R*), IL13 (*IL13RA1*), and VEGF (*VEGFR-1* (*FLT1*); *VEGFR-2 (KDR*)) receptors were expressed in day 9 hepatoblasts and in mature (day 21) organoids. In contrast, the mRNAs encoding the receptors for IL33 (*IL1RL1 or ST2*), IL4 (*IL4R*), or for the common gamma chain that is used for intra-cellular signaling by multiple cytokine receptors (*IL2RG*) were not expressed in day 9 hepatoblasts (**Fig. S4A**). Of note, the mRNAs encoding most of these receptors are abundantly expressed in mature HOs (**Fig. S4B**). Hence, the lack of a profibrotic effect of IGF-1 and VEGF is not due to the absence of receptor expression on day 9, which is prior to the time when microHOs were exposed to these agents. However, the lack of a profibrotic effect of IL-4 and IL-13 could result from the absence of functional receptors on day 9, which is just before the time of exposure to those cytokines.

Since activation of the hedgehog signaling pathway has been shown to increase *COL1A1* mRNA expression in myofibroblasts [32, 33] and to promote myofibroblast accumulation and liver fibrosis [34], we examined whether a hedgehog pathway activator would induce fibrosis in microHOs. Smoothened (**SMO**) is a seven transmembrane protein [35, 36] that accumulates in the primary cilium, and transduces a Hedgehog pathway signal intracellularly. However, addition of a SMO agonist (SAG), which activates Hedgehog signaling [37], did not induce fibrosis in microHO cultures (Fig. S3).

We next examined whether addition of seven free fatty acids (at 0 to 400 uM concentrations), which included their saturated and corresponding cis-9 (or trans-9) monounsaturated forms, on day 13 to microHO cultures had a pro-fibrotic effect. Previously obtained transcriptomic data from developing and mature HO cultures revealed that the mRNAs encoding proteins required for free fatty acid uptake (i.e., *SLC27A1, SCC27A5, FABP5, and Acyl-CoA Binding protein* (*ACBP*)) are expressed in day 9 HO cultures (**Fig. S5**). Even when the free fatty acids were added at concentrations below those previously reported to induce fibrosis in other types of HOs [38], the cells within microHOs abundantly accumulated fatty acids from the medium and there was evidence of lipotoxicity (**Fig. S6**). Somewhat unexpectedly based upon the results presented in [38], free fatty acid addition did not have a pro-fibrotic effect in microHOs (**Fig. S7**). The disparate findings may result from their using organoid stiffness [38] (rather than an increase in collagen thick fibers) as the criteria for fibrosis. Our results, which are consistent with recent findings by others [39], indicate that free fatty acid addition causes steatosis and lipotoxicity, but does not cause the fibrosis that develops in the advanced clinical stages of NASH. Altered differentiation conditions, adding other factors, or co-culture with other cell types may be required to enable HOs to model NASH-associated fibrosis.

Transcriptomic analysis. To molecularly characterize the pro-fibrotic effects of PDGFβ and TGFβ1, scRNA-Seq data was generated from day 21 control (22,211 cells), TGFβ1-treated (17,554 cells) or PDGFβ-treated (18,013 cells) microHOs. Eleven cell clusters were identified by analysis of the combined scRNA-Seq data (**Fig. 4A-B, Table S3**) and the cells in the PDGF- or TGFβ1-treated microHOs were reproducibly different from those in control microHOs (**Fig. S8A-C**). The differentially expressed genes in each cluster were analyzed to identify the cell type of each cluster (**Tables S4-S5**). Based upon expression of canonical mRNAs and by the level of concordance between the cell cluster transcriptomes and the cell types in human liver, the microHOs have cholangiocyte (Cho1-3), hepatocyte (Hep), mesenchymal cell (Mes1-4) and myofibroblast (MyoF_T1-2, MyoF_P) clusters. One myofibroblast cluster (MyoF_T1) was far more abundant in TGFβ1-treated microHOs than in control or PDGF-treated microHOs (37.5% of total vs <2%), while another myofibroblast cluster (MyoF_P) was more abundant in PDGF-treated microHOs than in control or TGFβ1-treated microHOs (20% of total vs <2%). A one-way ANOVA analysis comparing the cell type composition for each of the 5-independent biological replicates of the different types of microHOs analyzed confirmed that the percentages of MyoF_T1 ($p<0.015$) and Myo_P ($p<0.05$) were significantly increased in TGFβ- or PDGF-treated microHOs (vs control) (**Table S6**). Transcriptomic comparisons indicated that the

MyoF_T1 and MyoF_P clusters were most like that of myofibroblasts present in cirrhotic human liver (**Fig. 4C)**. Of importance, *COL1A1* and *Clover* mRNAs have an identical pattern of expression in the microHOs: both mRNAs are expressed in myofibroblasts and mesenchymal cells. In contrast, *EPCAM* mRNA (a hepatoblast and cholangiocyte marker) was not expressed in the *Clover* mRNA$^+$ cells (**Fig. 4D-E).** The mRNAs found in cholangiocytes (*KRT19*, *KRT7* and *CFTR)* or in hepatocytes and cholangiocytes (*HNF4A, AFP, CDH1, ALDHA1, APOB, FGB*) were not expressed in the *Clover* mRNA$^+$ cells in the microHOs (**Fig. S9**). The level of expression of myofibroblast marker mRNAs (*ACTA2, PDGFRA, FAP*) was very low in control microHOs, but these mRNAs were predominantly expressed in the myofibroblast (MyoF_T1/2, MyoF_P) clusters in PDGF- or TGFβ-treated microHOs (**Fig. S10**). Consistent with the profile of *FAP* mRNA expression in microHOs (Fig. S10), FAP is absent in most tissues under basal conditions but is highly expressed on activated myofibroblasts [40, 41]; and it is a well-established marker for activated fibroblasts [42]. The serine protease activity of FAP promotes liver fibrosis by activating HSC and promoting macrophage infiltration after liver injury [43]. Also, the pattern of expression of 9 mesenchyme and myofibroblast-specific genes in mesenchymal cells and myofibroblasts in human liver is retained in Mes2 and MyoF_T1 cells in microHOs (**Fig. S11**). Taken together, the transcriptomic results indicate that TGFβ1 or PDGF exposure converted the mesenchymal cell populations into myofibroblast-like cells in microHOs.

Pharmacologic characterization of pro-fibrotic signaling pathways. PDGF and TGFβ1 are well established drivers of liver fibrosis [44]. TGFRB activation leads to activation of SMAD signaling pathways [45, 46] and PDGFR cross-linking activates the STAT3 pathway [47 48], and both of these pathways have well-known roles in promoting hepatic fibrosis [48]. Since PDGFRβ and TGFβR1 inhibitors could block the PDGFβ and TGFβ1-induced increase in COL1A1$^+$ cells, respectively; the interaction between these intracellular signaling pathways was examined in microHOs. The TGFRβ inhibitor did not block the PDGFβ-induced increase, and the PDGFRβ inhibitor did not block the TGFβ1-induced increase in COL1A1$^+$ cells (Fig. 3). Also, addition of the combination of PDGFβ and TGFβ1 did not significantly increase the number of COL1A1$^+$ cells in microHOs relative to that caused by either factor alone (Fig. 3, bottom).

Although the anti-fibrotic effect of the receptor tyrosine kinase inhibitors was growth factor dependent, other signaling pathways that are jointly activated by the TGFβ1 and PDGF receptors could be essential for fibrosis. Both TGFRB [45, 46, 49] and PDGFR [50, 51] activate the p38

Mitogen Activated Protein Kinase (MAPK) and PI3K–Akt–mTOR intracellular signaling pathways, which are also pro-fibrotic. Therefore, we examined whether co-administration of PI3K-Akt-mTOR (rapamycin) or MAPK pathway (SB202190) inhibitors with PDGF or TGFβ1 on day 13 could reduce fibrosis. Co-administration of 10 uM SB202190 – an ATP pocket binding and highly selective p38α and β kinase inhibitor [52] – inhibited both TGFβ1 and PDGF-induced fibrosis in microHOs (**Fig. 5A-C**). Thus, p38 MAPK pathway activity is essential for either PDGF- or TGFβ1-driven fibrosis. Rapamycin partially inhibited PDGF-induced fibrosis but did not inhibit TGFβ1-induced fibrosis (**Fig. 5D-F**). The lack of inhibition of TGFβ1-induced fibrosis by rapamycin indicated that the downstream part of the PI3K-Akt-mTOR pathway was not essential for TGFβ1-induced fibrosis, but it may be required for PDGF-induced fibrosis.

WNT/β-catenin signaling contributes to the pathogenesis of liver fibrosis and NASH [53, 54], which led to the suggestion that it could be targeted by anti-fibrotic therapies [55]. Since glycogen synthase kinase 3β (GSK3β)-mediated phosphorylation of β-catenin targets it for ubiquitin-dependent proteasomal degradation, GSK3β inhibitors will stabilize β-catenin and promote the transcription of β-catenin target mRNAs [56, 57]. Therefore, we examined the effect that co-administration of a Wnt/β-catenin pathway activator (Wnt3a) or of a GSK3β enzyme inhibitor (10 uM CHIR99021), which has been shown to activate the Wnt/β-catenin pathway [58], had on fibrosis in microHOs. Wnt3a co-administration had a minimal effect on the extent of PDGF- or TGFβ1-driven fibrosis (Fig. S3). The GSK3β inhibitor by itself did not increase COL1A1^{+} cells in the organoids (Fig. S3). However, co-administration of the GSK3β inhibitor with either PDGF or TGFβ1 blocked fibrosis in microHOs (Fig. 3). Of importance, cell viability in microHOs was not reduced by addition of TGFβ1, nor by adding the GSK3β or TGFRβ inhibitors **(Fig. S12).** Our results indicate that p38 MAPK or GSK3β kinase inhibitors can block liver fibrosis irrespective of whether it is driven by either PDGF or TGFβ1.

This microHO system could also be used to test the potential anti-fibrotic efficacy of candidate medications for liver fibrosis. For example, nintedanib and pirfenidone are the only two FDA-approved drugs for treatment of pulmonary fibrosis [59]. Both inhibit multiple tyrosine kinases and have been shown to reduce lung fibrosis in animal models [60, 61, 62] and in patients with interstitial lung fibrosis [63, 64, 65,66, 67]. Although the mechanism for their anti-fibrotic effect is unknown, it has been suggested that they could also be used to treat liver fibrosis [48]. However, co-administration of 10 uM pirfenidone or 10 uM nintedanib did not inhibit the TGFβ1-induced fibrosis in

microHOs. While pirfenidone did not inhibit PDGF-induced fibrosis, nintedanib delayed the onset of PDGF-induced fibrosis in microHOs (**Fig. 5D-F**). Of note, the tested concentrations were >10-fold above the nintedanib concentration that inhibited PDGF-stimulated cellular proliferation (IC_{50} 64 nM) and PDGFR auto-phosphorylation (IC_{50} 22-39 nM) [68] and of the pirfenidone concentration (1 uM) that inhibited proliferation and TGFβ mRNA expression [69] in cultured human fibroblasts. The partial inhibitory effect of nintedanib of PDGF-induced fibrosis is consistent with its inhibition of the PDGF receptor tyrosine kinase activity (IC_{50} 60 nM) [70].

Discussion

We demonstrate that microHOs and the live cell imaging system developed here can be used to characterize the biochemical signals and intracellular signaling pathways that drive liver fibrosis and for assessing potential anti-fibrotic therapies. Of the twenty agents tested using microHOs – which included cytokines, growth factors, fatty acids, a hedgehog agonist, and Wnt ligands – only PDGF and TGFβ1 had a strong pro-fibrotic effect. For several of the cytokines, growth factors and fatty acids tested, the absence of a pro-fibrotic effect in the organoid could be due to the absence of an immune system and/or of other cell types that could be required for a pro-fibrotic effect. However, a key finding emerging from this study is that the anti-fibrotic efficacy of PDGFR and TGFRβ tyrosine kinase inhibitors was dependent upon the factor driving the fibrosis, and this could explain why it has been so difficult to develop therapies for liver fibrosis. When fibrosis is driven by PDGFRβ-STAT3 pathway activation – as occurs in ARPKD liver fibrosis [12] or in murine models of lung and bone marrow fibrosis [71] – PDGFR inhibitors will exhibit anti-fibrotic efficacy; but they will be less effective when the fibrosis is driven by other mechanisms. There are organ-specific and species-specific differences in the mechanisms mediating tissue fibrosis [10] and different types of liver injury produces different patterns of liver fibrosis in humans [72]. Consistent with differences in the factors that drive different types of fibrosis, microHO data indicates that at least one (and possibly both) of the drugs that were FDA approved for treatment of lung fibrosis may not be effective when used for liver fibrosis. As another example, a combination treatment with PDGFR (imatinib) and TGFRβ (galunisertib [73]) inhibitors was more effective than blockade of either pathway alone in a murine radiation-induced lung fibrosis model [74]. To optimally treat liver fibrosis, therapies may have to be adjusted based upon the pathogenic driving factors, which may vary in different patients or in response to different inciting causes. There is already evidence that Diabetes patients can be subdivided into distinct sub-groups based upon clinical features and biomarker results [75], and

efforts are underway to optimize treatment selection for the different types of diabetes patients [76]. Just as in other diseases, application of the principles of '*precision medicine'* (i.e., using genetic or genomic information to optimize treatment selection [77]) could enable therapies for liver fibrosis to be successfully developed.

Another key finding was that GSK3β and p38 MAPK inhibitors potently blocked the fibrosis induced by TGFβ1 or PDGF. The GSK3β inhibitor effect was unexpected since modulating Wnt/β-catenin signaling has had variable effects on liver fibrosis in mouse models [78, 79]; and in a murine bile duct ligation model, GSK3β inhibitor administration produced an increase the extent of liver fibrosis [80]. As discussed in supplemental note 1, there are multiple nodes where the TGFβ, Wnt/β-catenin and the p38 MAPK pathways interact. These interactions could explain how a GSK3β inhibitor could block TGFβ-induced fibrosis; but the mechanism for the GSK3β inhibitor effect on PDGF-induced fibrosis is less clear. Nevertheless, our results indicate that the p38 MAPK pathway, which is activated by both TGFβ1 and PDGF receptors, plays a key role in fibrosis. Although there was no prior data demonstrating that it had an effect on liver fibrosis, the ability of SB202190 to inhibit fibrosis in microHOs is consistent with prior studies showing that this agent inhibited the development of renal interstitial [81] and corneal [82] fibrosis in animal models, and the conversion of human corneal fibroblasts into myofibroblasts *in vitro* [83]. However, additional studies will have to be performed to characterize the mechanism(s) by which the Wnt/β-catenin and p38 MAPK pathways, along with the SMAD and STAT3 pathways that are also activated by TGFβ1 and PDGF receptors, jointly contribute to liver fibrosis. In summary, live cell imaging of human microHOs has identified GSK3β and p38 MAPK inhibitors as potential new therapies for liver fibrosis, and it is likely that other new therapies could subsequently be identified using this system.

Data availability. All raw and processed single cell RNA-seq data were deposited in the Gene Expression Omnibus (GEO) and are available under accession GSE228214.

Author Contributions. Y.G, S.R., A.H., P.K.J generated experimental data; Z.F., Y.G., P.K.J., A.E., M.W., S.C.H. and G.P. analyzed data; The paper was written by Y.G. and G.P with input from all authors.

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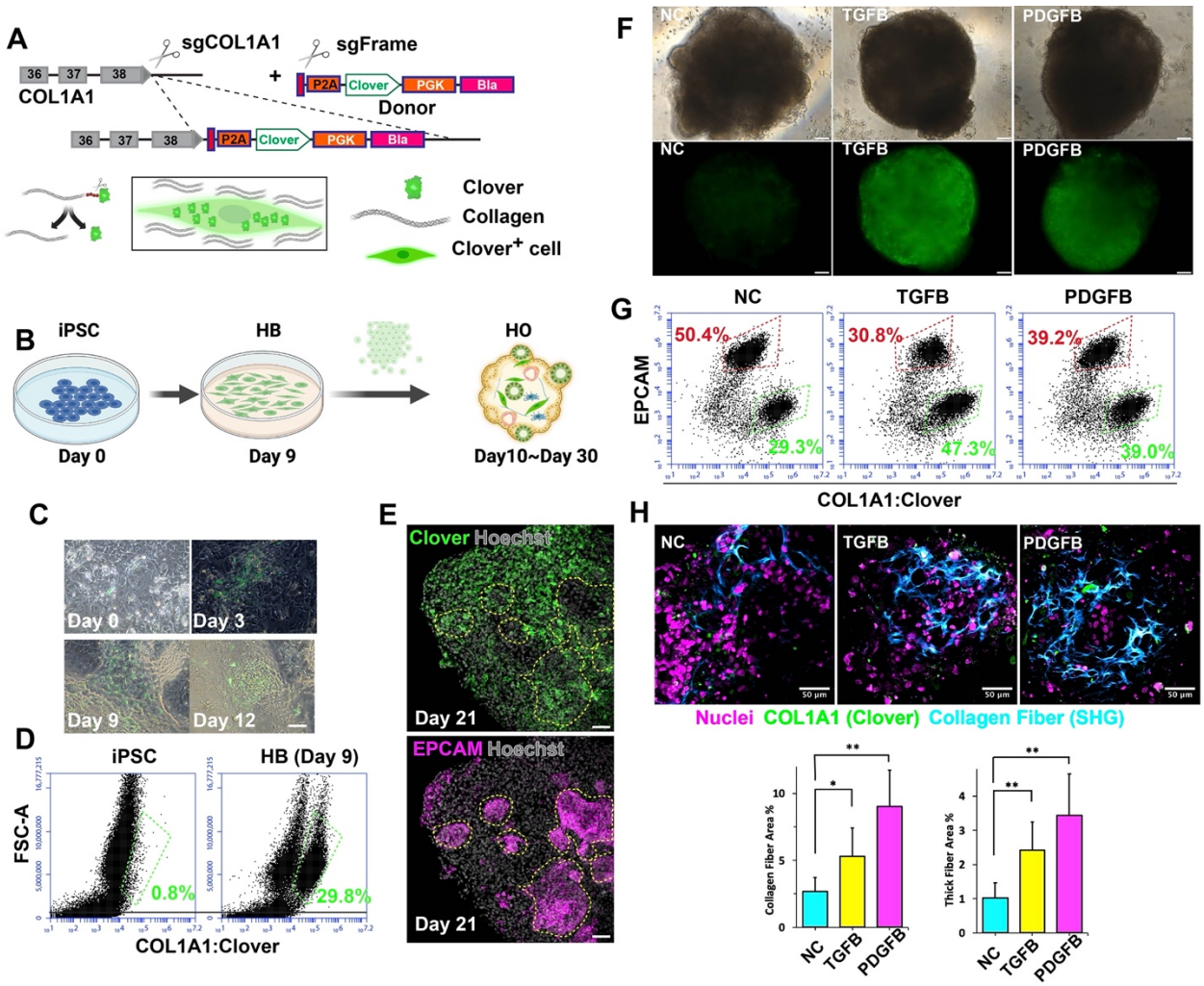


Figure 1. (A) *Top:* A diagram of the CRISPaint system used to insert P2A-Clover at the 3' end of *COL1A1* in an iPSC line. After blasticidin selection, cells were cloned to generate the COL1A1-P2A-Clover iPSC line. *Bottom*: The self-cleaving P2A peptide enables *COL1A1* expressing cells to be labelled with a fluorescent intracellular protein (Clover). (**B**) The collagen producing cells in a HO produced from COL1A1-P2A-Clover iPSCs are labelled with an intracellular fluorescent Clover protein. (**C**) An overlay of bright field and fluorescent images of differentiating COL1A1-P2A Clover HO cultures reveals that the number of Clover$^+$ cells increase between days 3 and 12. Scale bar: 50 μm. (**D**) Flow cytometry scatter plots show that the Clover$^+$ cell population is absent in iPSC (day 0) but appears in (day 9) hepatoblasts in differentiating *COL1A1*-P2A Clover HO cultures. (**E**) Stacked confocal images show the location of antibody-stained EPCAM$^+$ (bottom) or fluorescent Clover$^+$ (top) cells in day 20 COL1A1-P2A-Clover organoids. The yellow dashed circles (bottom) indicate

areas with EPCAM$^+$ (i.e., hepatocytes or cholangiocytes) cells; and Clover$^+$ cells are not found within those areas but are present in other areas of the HO. Scale bar: 50 μm. (**F**) Bright field (top) and fluorescence (bottom) images of day 21 *COL1A1*-P2A Clover HOs treated on day 13 with no addition (NC), 50 ng/ml TGFβ1 or 50 ng/ml PDGFβ. Both of these growth factors induced a marked increase in COL1A1$^+$ cells. Scale bars, 50 μm. (**G**) Flow cytometry scatter plots show that Clover$^+$ cells are increased in mature day 21 *COL1A1*-P2A Clover HOs after TGFβ or PDGF exposure. Also, the Clover$^+$ cells are distinct from the EPCAM$^+$ (hepatocytes or cholangiocytes) cells in the HOs. (**H**) SHG analysis of the collagen fibers formed in human HOs. *Top:* Depth color-coded projections of collagen fibers within day 21 control organoids, or day 21 organoids that were treated with 50 ng/ml TGFβ1 or 50 ng/ml PDGF on day 13. Control organoids (left) have isolated regions with relatively thin collagen fibers (shown in blue). In contrast, the TGFβ1 or PDGF-treated organoids form a network of thick collagen fibers that extend throughout the entire organoid. Collagen producing cells (green) can also be seen in these images. Scale bars, 50 μ. *Bottom:* A quantitative comparison of collagen fiber area in SHG images was performed for control, TGFβ1- or PDGF-treated hepatic organoids ($n = 5$ per measurement) on day 21. There is a statistically significant increase in total collagen abundance (collagen fiber area=total area with collagen fibers/area of the imaged organoids) in the organoids after exposure to TGFβ1 (*, $p < 0.05$) or PDGF (**, $p < 0.01$, Welch t-test). There was also a statistically significant increase in the abundance of thick collagen fibers (i.e., those fibers > 3 um diameter) in the TGFβ1 and PDGF-treated hepatic organoids.

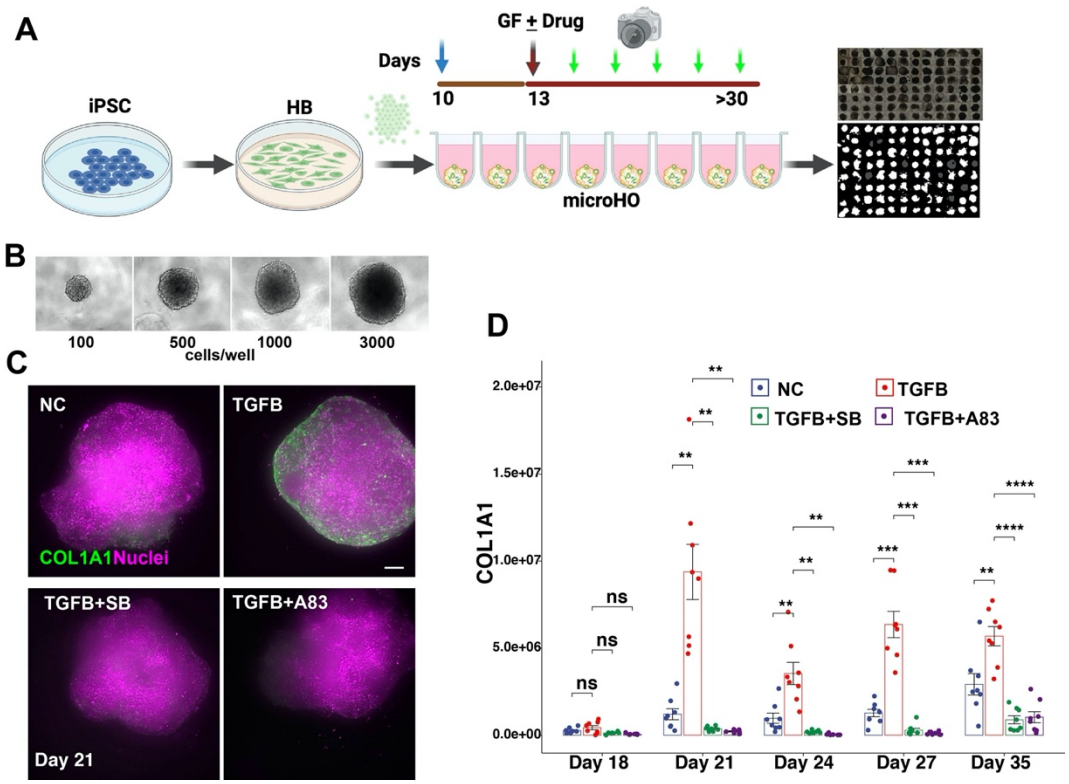


Figure 2. (**A**) A diagram of the HCS platform used for analysis of microHOs. COL1A1-P2A iPSCs are differentiated into hepatoblasts (HB), and 10,000 HBs are placed in each microwell on day 10. Growth factors (GF) and/or drugs are added on day 13, and the cultures are differentiated into hepatic organoids. Culture fluorescence is serially assessed to measure the number of COL1A1-producing cells in the microHO. Bright field (top) and fluorescence (bottom) images of a microwell plate with microHOs is shown on the right. (**B**) A cell number titration experiment was performed by adding the indicated numbers of HBs to microwells and the cells were differentiated through day 12. Bright field images on day 12 reveal that microHOs have a uniform shape and their size is dependent upon the number of input cells. (**C, D**) TGFβ1-induced fibrosis in microHOs is blocked by TGFRβ1 inhibitors. COL1A1-P2A Clover HOs were formed by adding 10,000 HBs to each microwell. Then, either nothing (NC), or 50 ng/ml TGFβ1 ± 10 uM TGFRβ1 inhibitors (SB431542 (SB) or A83-01 (A83)) was added to each microwell on day 13. (C) Maximum intensity projection (MIP) images of z stack sections obtained from the indicated type of microHOs on day 21. Scale bar 100 um. Clover expression is green, and nuclei stained with Hoechst 33342 are purple. (D) The amount of COL1A1^{+} cells within a microHO were serially measured on days 18 through 35. Each dot represents a measurement made on one microHO, and 8 microHOs per treatment were assessed per condition. The significance indicators are ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001 or ****,

$p<0.0001$ for measurements in TGFβ1 treated vs NC microHOs; and for microHOs that were treated with TGFβ1 and drug, the p-values are calculated relative to the TGFβ1 treated microHOs. A two-way ANOVA indicates that drug treatment and time are two variables that have a significant interaction on the fluorescence measurements ($p=1.66 \times 10^{-15}$) (**Table S7**).

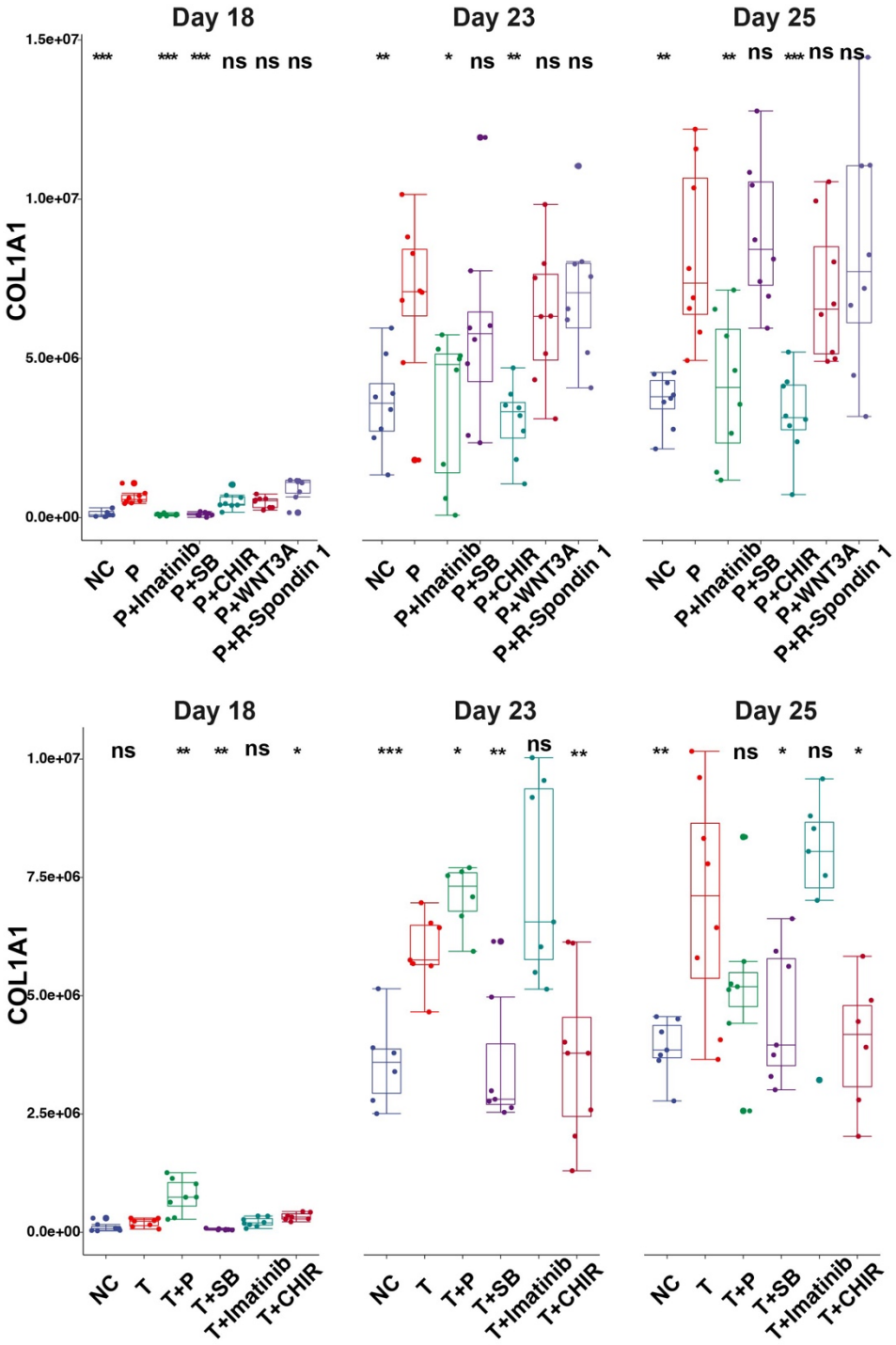


Figure 3. *Top:* The PDGFβ-induced increase in COL1A1^{+} cells is blocked by PDGFRβ and GSK3β inhibitors. *COL1A1*-P2A Clover HOs were formed by adding 10,000 cells from day 9 organoid cultures to each microwell. Then, either nothing (NC), 50 ng/ml PDGFβ (P) or 50 ng/ml PDGFβ with 10 μM imatinib, 10 uM SB431542 (SB), 10 uM CHIR99021 (CHIR), 100 ng/ml

Wnt3a or 100 ng/ml R-spondin 1 was added to each microHO on day 13. *Bottom:* The TGFβ1 induced increase in COL1A1$^+$ cells was blocked by TGFRβ and GSK3β inhibitors. Either nothing (NC), 50 ng/ml TGFβ1 (T), 50 ng/ml TGFβ1 with 50 ng/ml PDGF (T+P), or 50 ng/ml TGFβ1 and either 10 μM imatinib, 10 uM SB431542 (SB), or 10 uM CHIR99021 (CHIR) was added to each microHO on day 13. microHO fluorescence, which indicates the amount of COL1A1$^+$ cells, was serially measured on days 18 through 25. Each dot represents a measurement made on a microHO, the thick line is the median for 8 microHOs that were assessed, and the box plot shows the 25 to 75% range for the measurements. In the panels: ns, not significant; *, $p<0.05$; or **, $p<0.01$, ***, $p<0.001$ for the measurement relative to the value in the NC; or when growth factors or inhibitors were added, the p-values were calculated relative to that of the PDGFβ or TGFβ1 treated cultures. A two-way ANOVA indicates that drug treatment and time are two variables that have a significant interaction on the fluorescence measurements (Table S7).

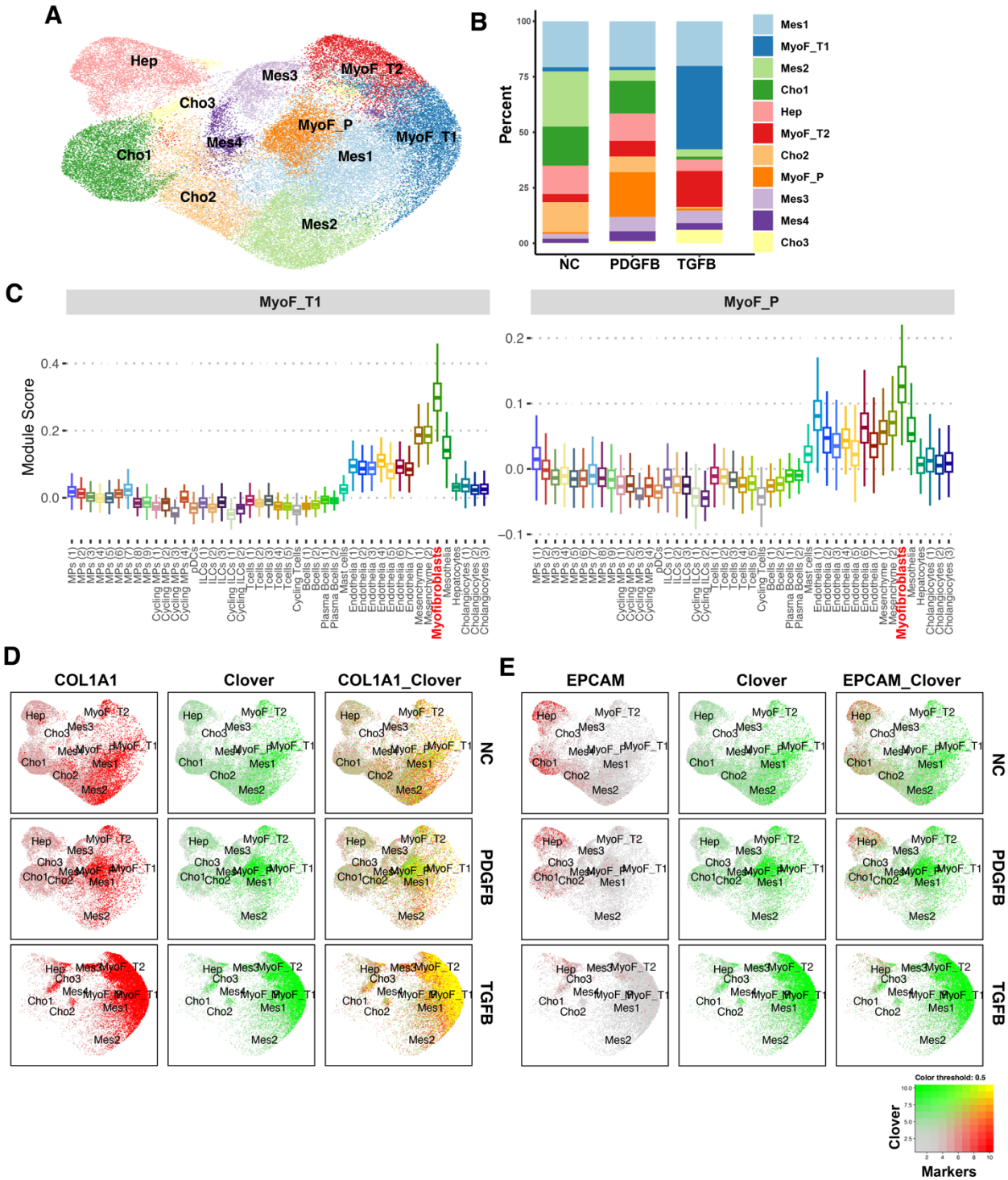
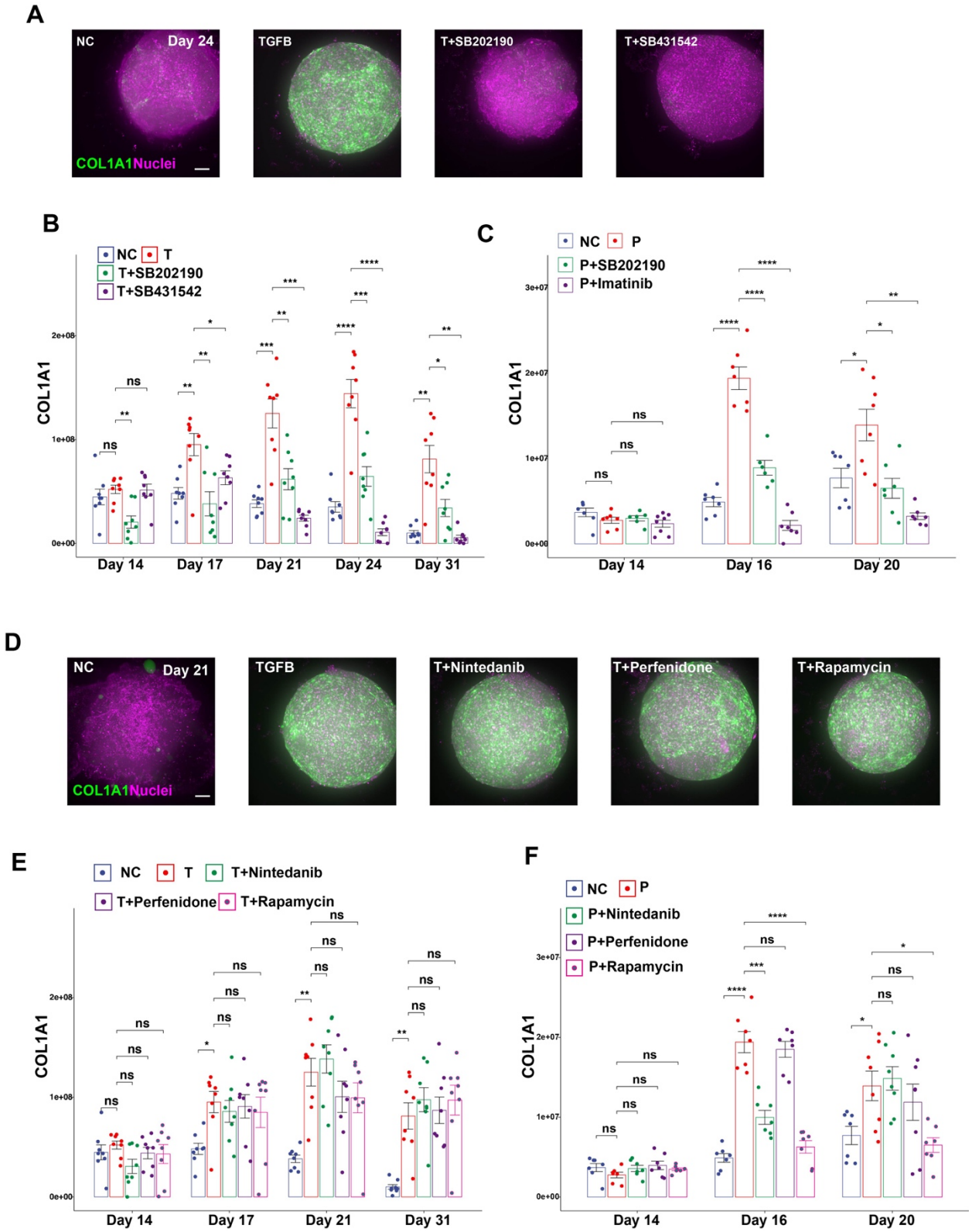


Figure 4. (**A**) A combined sample u-MAP plot shows scRNA-seq data obtained from the cells in day 21 control (NC), PDGFβ- (P) or TGFβ1 (T)-treated microHO cultures. (**B**) The percentages of the 11 different clusters identified by K-nearest neighbor analysis of the scRNA-Seq data shown in (A) are graphed. Each cluster is indicated by a different color and the cell types for

each cluster were annotated based upon the genes shown in Table S2. The hepatocyte (Hep) and cholangiocyte (Cho1-3) clusters are indicated. There are four mesenchymal clusters (Mesen1-4): Mes1 is abundant in all three types of organoids, while Mes2 abundance is increased in PDGFβ-treated microHOs. There are three myofibroblast-like clusters (MyoF_T1, MyoF_T2, MyoF_P), and the abundance of MyoF_T1 and MyoF_T2 cells are markedly increased in TGFβ1-treated microHOs, while the abundance of MyoF_P cells is increased in PDGFβ1-treated microHOs. (**C**) Among the cells in cirrhotic human liver, the MyoF_T1 and MyoF_P transcriptomes are most similar to that of myofibroblasts. The MyoF_T1 or MyoF_P gene signatures were compared with the transcriptomes of the various cell types in cirrhotic human liver tissue (GSE136103 [84]), which include macrophages (MP) and innate lymphoid cells (ILC). The boxplots show that the MyoF_T1 and MyoF_P signatures are most enriched in the myofibroblasts present in cirrhotic human liver tissue. There was a statistically significant difference in the module scores obtained for the various cell types as determined by one-way ANOVA ($p < 0.0001$). The dots represent data points located 1.5-fold above (Q3 + 1.5 * IQR) or 1.5-fold below (Q1 – 1.5 * IQR) the interquartile (IQR) range. (**D, E**) Feature plots show the level of expression of *COL1A1* and *Clover* mRNAs (D), or *EPCAM* and *Clover* mRNAs (E) in the u-MAP plot shown in (A). As shown in the color threshold diagram, dot colors represent level of expression of the mRNAs. *COL1A1 and Clover* mRNAs have an overlapping expression pattern; they are predominantly expressed in myofibroblasts, and in mesenchymal cells. In contrast, *EPCAM* mRNA is expressed in hepatocyte and cholangiocyte clusters; and *EPCAM* mRNA expression does not overlap with that of *Clover* mRNA.


A
NC
Day 24
COL1A1Nuclei
TGFB
T+SB202190
T+SB431542
B
NC
T
T+SB202190
T+SB431542
COL1A1
Day 14
Day 17
Day 21
Day 24
Day 31
C
NC
P
P+SB202190
P+Imatinib
COL1A1
Day 14
Day 16
Day 20
D
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Day 21
COL1A1Nuclei
TGFB
T+Nintedanib
T+Perfenidone
T+Rapamycin
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Day 20

Figure 5. TGFβ1- or PDGF-induced fibrosis in microHOs is inhibited by a p38 MAPK (SB202190) inhibitor (A-C); but only the PDGF-induced fibrosis is inhibited by pirfenidone or rapamycin (D-F). *COL1A1*-P2A Clover microHOs were formed by adding 10,000 HBs to each microwell. Then, either nothing (NC), 50 ng/ml TGFβ1 (T) or 50 ng/ml (PDGF) was added on day 13 to each microwell in the presence or absence of 10 uM of the following drugs: SB431542 (TGFRβ inhibitor), SB202190 (p38 α inhibitor), nintedanib, pirfenidone or rapamycin. (A, D) Maximum intensity projection (MIP) images of z stack sections obtained from microHOs treated with no addition (NC), or 50 ng/ml TGFβ ± 10 uM of the indicated drugs. Scale bar 100 um. Clover expression is green, and nuclei stained with Hoechst 33342 are purple. (B-C, E-F) The amount of $COL1A1^+$ cells within a microHO was serially measured on days 14 through 31. Each dot represents a measurement made on a microHO, the thick line is the median of 8 microHOs that were assessed, and the box plot shows the 25 to 75% range for all measurements per condition. When only growth factors were added, the p-values were relative to the NC, and the significance indicators are ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ or ****, $p<0.0001$. When growth factors and inhibitors were added, the p-values were calculated relative to the PDGFβ or TGFβ1 treated microHOs (without added drug). A two-way ANOVA analysis indicates that drug treatment and time are two variables that have a significant interaction on the fluorescence measurements (Table S7).

Supplemental Information

Characterization of Pro-Fibrotic Signaling Pathways in Human Hepatic Organoids

Supplemental note 1: *Interactions between the TGFβ, Wnt/β-catenin and p38 MAPK pathways*. The TGFβ1 and Wnt/β-catenin pathways have several interaction points. (i) TGF-β significantly enhances hepatic WNT-5A expression *in vivo* and in myofibroblasts *in vitro* [1]. (ii) The interdomain regions of SMAD transcription factors have two residues that are phosphorylated by GSK3β in the nucleus, and their phosphorylation state regulates SMAD transcriptional activity and turnover [2]. Hence, the ability of the GSK3β inhibitor to block TGFβ1-induced fibrosis in microHOs could (at least in part) be mediated by altering the phosphorylation state of these two sites, which could alter SMAD transcription factor activity. (iii) During development, receptor tyrosine kinase and Wnt/β-catenin pathway signals were shown to be integrated by the level of SMAD phosphorylation [3].

The Wnt/β-catenin and p38 MAPK pathways also interact at several nodes. (i) Wnt ligands have been shown to activate p38 MAPK activity [4]. (ii) GSK3β phosphorylation by p38 MAPK regulates its kinase activity. MAPK kinases have a Thr–Gly–Tyr dual phosphorylation motif within their kinase activation loop, and both sites must be phosphorylated to fully activate the kinase. The MAP2K kinases MKK3 and MKK6 are the major upstream kinases for p38 activation [5]. However, phosphorylation on Ser^{389} on GSK3β is a major mechanism for downregulating its kinase activity and p38α was shown to inactivate GSK3β by this mechanism: p38-mediated phosphorylation of Ser^{389} on GSK3β was shown to cause β-catenin accumulation and activation of the Wnt signaling pathway [4, 6]. By this mechanism, p38 MAPK inhibitors could reduce Wnt pathway activation, which could prevent the development of a fibrosis that was dependent upon Wnt signaling.

Materials and Methods

Chemicals. The growth factors, drugs, antibodies, and other staining reagents are shown in Tables S6-S7.

iPSCs and HO generation. The human iPSC line (C3) used in this study was prepared as previously described [7]. The biopsy sample used to generate this iPSC line was obtained according to a protocol (number 10368) approved by the Institutional Review Board at Stanford. The CRISPR-assisted insertion tagging system (CRISPaint) [8] plasmid (pCRISPR-HOT-Clover-

BlastR) was obtained from Addgene (Plasmid #138569; http://n2t.net/addgene:138569). A clover expression cassette (**P2A-Clover**) was inserted at the COOH terminus of the endogenous *COL1A1* gene of the iPSC line without a STOP code using a sgRNA (TTGGGATGGAGGGAGTTTAC). After Blasticidin selection, colonies obtained from single cells were picked and genotyped for the correct in-frame insertion using forward (actcccacgtggtaatgccc); and reverse (cttcagggtcagcttgccg) primers. iPSCs were differentiated into hepatic organoids (HOs) via culture in a series of media containing different growth factors using previously described methods [7].

Second Harmonic Generation (SHG) microscopy. SHG (collagen fibers) images from day 21 control, PDGF-treated and TGFβ1-treated HOs were collected on a multimodal nonlinear optical microscope setup, consisting of a modified inverted confocal microscope (Nikon, Ti2-E with C2 scanner and 60X water immersion objective, NA=1.27) and a picosecond-pulsed laser source (APE picoEmerald S, 2 ps pulse width, 80 MHz repetition rate, and 10 cm^{-1} bandwidth). As previously described[7], SHG data collection was made in the epi-direction with two narrow bandpass filters (Semrock FF01-400/12, Thorlabs MF390-18) and one shortpass filter (Thorlabs FESH0500). For the quantitative analyses, the area of the collagen fibers (identified by Otsu thresholding) was calculated for each SHG image and divided by the total sample area, forming the collagen area fraction (in %). To determine the diameter of the collagen fibers, the local thickness of the fibers was evaluated by applying the ImageJ Local Thickness routine (see the Analyze menu in Fiji) [9] to the SHG data. The volume fraction of thick collagen fibers (diameter ≥ 3 μm) was estimated by dividing the number of pixels represented by local thickness values ≥ 3 μm by the total number of pixels of the stack. To test whether the volume fractions obtained for control and TGFβ1- or PDGF-treated organoids were significantly different, the Welch's t-test was applied (* $p<0.05$ and ** $p<0.01$).

microHO generation. To produce microHOs, 50~60% confluent iPSC cultures were switched to an endoderm differentiation medium that consisted of DMEM/F12 + ITS (GIBCO) supplemented with 0.1 mM nonessential amino acids, 1 mM pyruvate and 2 mM L-alanyl-L-glutamine dipeptide (GlutaMAX). On days 1 to 2, 100 ng/ml Activin-A (Peprotech, Rocky Hill, NJ), 10 ng/ml BMP4 (Peprotech), 100 ng/ml bFGF (Peprotech), 3 mM CHIR99021 (Selleckchem, Houston, TX) and 10 uM LY294002 (Selleckchem) were added this medium. On day 3, 100 ng/ml Activin-A and 100 ng/ml bFGF were added the definitive endoderm differentiation medium. From days 4 to 9, 20 ng/ml FGF10 (Peprotech) and 20 ng/ml BMP4

were added to the hepatoblast medium, which consisted of which consisted of Advanced RPMI 1640 Medium (Gibco) that was supplemented with GlutaMAX and ITS. After day 9, the hepatoblasts were dissociated to single cells in Accutase (Invitrogen) medium with 10 uM Y-27632 (Santa Cruz Biotechnology, Dallas, Texas). Then, 1000 to 5000 cells/well were re-aggregated in low-cell-adhesion Nunclon™ Sphera™ 96 well Microplates (ThermoFisher) that contained serum-free HO growth and differentiation medium, which consisted of William's E medium supplemented with 0.1% Polyvinyl alcohol (Sigma-Aldrich), 0.1 mM nonessential amino acids, 1 mM pyruvate, 2 mM L-alanyl-L-glutamine dipeptide (GlutaMAX), 10 mM Y-27632, 100 ng/ml EGF, 10 ng/ml HGF, 10 μM Dexamethasone and 10 μM Hydrocortisone (Sigma, St. Louis MO). On day 13, 50 ng/ml of TGFβ1 (Peprotech, 100-21; or Sinobiological, 10804-HNAC) or 50 ng/ml of PDGFB (Miltenyibiotec, 130-108-163; or Peprotech, 100-14B) was added to each microwell.

microHO analysis. For COL1A1^{+} and Hoechst 33342 (Invitrogen, H3570) fluorescence measurements, 3D stacked images were captured from day 14 to day 31 cultures using a Molecular Devices ImageXpress Micro Confocal system. For confocal imaging20 to 40 planes crossing the whole organoid at 20 um intervals were captured using a 10X 0.45 Plan Apo 4mm WD objective with a 60-um pinhole. For each 10X confocal z-stack field (enough to cover one whole organoid), maximum intensity projections (MIPs) were generated from all of the acquired z-planes (the number planes ranged from 20 to 40 depending on the batch size). MIPs from each channel belonging to the same well were used for organoid segmentation and feature extraction by applying the machine learning based 'Trainable Weka Segmentation' [10] plugin from FIJI [11]. Each individual organoid edge was automatically segmented based on the nuclei signal (Hoechst 33342). Cell numbers were determined using the 'Find Maxima' function in defined area of an organoid after segmentation. COL1A1-P2A-Clover^{+} cells were segmented based on fluorescence. For training purposes, each analyzed element in every image (total 12 images) were used to train the classifiers by manual labelling all positive spots. Then, for experimental image analysis, the saved classifier was used to generate the probability map for each image. Segmented areas were then isolated, thresholded and binarized, and the Integrated Density was calculated from segmented area. An unpaired Student's t-test was used to test whether the measurements were significantly different between each comparison group. The macro script used for this analysis is available upon request.

Immunohistochemistry. Fresh organoids were harvested and fixed in 4% paraformaldehyde for 30 minutes. All organoids were either stained directly or embedded in low melting point agarose (IBI Scientific, Dubuque, Iowa). The primary antibodies used for staining are listed in Table S5. The organoids were stained with the primary antibody, and permeabilized by incubation with 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and 10% chicken serum (Jackson ImmunoResearch, Bar Harbor, MA) overnight. Then, secondary antibody-staining was performed using an Alexa Fluor labeled chicken anti IgG (H+L), which was cross-adsorbed with a secondary antibody in 10% chicken serum (Invitrogen, Pleasanton, CA).

scRNA-Sequencing. Control, PDGFB- or TGFβ1-induced microHOs (n>30 per group) were harvested from multiple independently prepared sets of cultures, and single cell suspensions were prepared by protease digestion as described [7]. The single cell suspensions were visually inspected under a microscope, cells were counted using a Scepter™ 2.0 Handheld Automated Cell Counter (EMD Millipore, Burlington, MA), and then resuspended in PBS with 0.01% BSA. scRNA-seq libraries were prepared by using the spit-pool based scRNA-Seq method (Evercode™ WT Mini v2 from Parse biosciences) according to the manufacturer's instructions. In brief, ~5,000 cells from each group were split into 3 wells for the first round of sample barcoding, and the final pooled cells were divided into two equal libraries for sequencing. The expression matrix was generated using the 'Parse Biosciences analysis pipeline.' A total of 38,716 features that were generated from 13,160 cells were generated from four experimental batches, and 57, 7778 cells were recovered that passed quality control for processing of the scRNA-Seq data.

Flow cytometry iPSCs were dissociated into single cells by incubation with 5 μM ETDA (Invitrogen) in PBS; and hepatoblasts were dissociated using Accutase (Gibco). Single cell suspensions were prepared from microHOs by protease digestion as described [7]. Flow cytometry was performed using at a BD Accuri C6 flow cytometer using the conjugated antibodies listed in Table S2, and the data was analyzed using FCS Express (denovosoftware.com) software. A density plot of forward scatter height (FSC-H) vs. forward scatter area (FSC-A) was used to exclude doublets.

scRNA-Seq data analysis. The scRNA-Seq data was imported into 'Seurat' [12] for the subsequent analysis steps. (i) Cells with unique gene counts <200 or where the percentage of mitochondrial mRNAs was >10% were removed. (ii) A total of 2000 variable genes were

identified using the default settings. Four batches of data were integrated by standard processing methods using the Seurat integration function. Unwanted sources of variation were removed by regression analysis, which was performed to mitigate the effect of signals caused by mRNAs with unique molecular identifiers and mitochondrial expression. (iii) Ten principal components were used to construct the shared nearest neighbor (SNN) graph, and the parameter regulating the resolution of the 'Find Clusters' program, was set to 0.5. This resulted in the identification of 11 unique clusters for all 57,778 cells. To identify the cell type of each cluster, differentially expressed genes for the pre-defined cell types were computed using the 'FindAllMarkers' function within the Seurat Package with the following parameters: only.pos = TRUE, min.pct = 0.25, logfc.threshold = 0.25. Seurat then identifies the differentially expressed genes using the non-parametric Wilcoxon rank sum test.

Calculation of the Module Score. The module score was calculated to assess the relationship between microHO clusters and the different types of cells in human cirrhotic liver tissue using scRNA-Seq data that was obtained from cirrhotic human liver tissue (GSE136103) [13]. Differentially expressed genes were generated for each cell cluster in microHOs using the Wilcoxauc function of the R package presto (https://github.com/immunogenomics/presto). The top 200 upregulated genes were used as the gene signature, which was then used to calculate the cluster's module score using the AddModuleScore function of the Seurat package.

2-way analysis of variance (ANOVA) calculations. The 2-way ANOVA was used to compare the means of the COL1A1:Clover signal (calculated as area * mean intensity (IntDen)) between the different drug treatment groups and time points, while also assessing interaction effects between the treatment groups and time. The two-way ANOVA was performed using the 'aov' function of the 'R\stats' package, and both groups and time points were analyzed as categorical independent variables. The null hypothesis was that there is no difference in the means of the different treatment groups or timepoints, and that the treatment groups and time points do not interact in any way. Since the two-way ANOVA of the data shown in Figures 2D, 3A, 3B, 5B, and 5C use separate datasets, a correction for multiple testing is not needed.

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Table S1. Drugs and chemicals used in these studies.

Reagent	Company	Catalog #
palmic acid	SigmaAldrich	P5585
Oleic acid	SigmaAldrich	O1383
Stearic acid	SigmaAldrich	S4751
Myristic Acid	Caymanchem	13351
Myristoleic Acid	Caymanchem	9002461
Palmitoleic Acid	Caymanchem	10009871
Elaidic Acid	Caymanchem	90250
CDCA	SigmaAldrich	C9377
Activin A Protein	Sinobiological	10429-HNAH
Animal-Free Recombinant Human EGF	Peprotech	AF-100-15
EGF	Sinobiological	10605-HNAE
FGF4	Peprotech	100-31
HGF	Sinobiological	10463-HNAS
FGF10	Sinobiological	10573-HNAE
FGF2 Protein, Human, Recombinant	Sinobiological	10014-HNAE
FGF10	Peprotech	100-26
Human PDGF-BB IS	Miltenyibiotec	130-108-163
Human VEGF (165) IS	Miltenyibiotec	130-109-383
Recombinant Human PDGF-BB (carrier-free)	Biolegend	577302
Recombinant Human PDGF-BB	Peprotech	100-14B
Recombinant Human PDGF-BB Protein, CF	rndsystems	220-BB-010In Stock
TGF beta 1 Protein, Human, Rhesus, Cynomolgus, Canine, Recombinant	Sinobiological	10804-HNAC
Recombinant Human BMP-4	Peprotech	120-05
Recombinant Human BMP-4 (carrier-free)	Biolegend	795606
Recombinant Human/Mouse/Rat Activin A	R&D	338-AC-010
Recombinant Human FGF-Basic (146 a.a.)	Peprotech	AF-100-18C
Wnt3a	Peprotech	315-20
OSM	Peprotech	300-10
IL3 Protein, Human, Recombinant (His Tag)	Sinobiological	11858-H08H
IL6	R&D	7270-IL-010/CF
IL-13 Protein, Human, Recombinant	Sinobiological	10369-HNAC
IL-33 Protein, Human, Recombinant	Sinobiological	10368-HNAE
IGF1	R&D	291-G1-200
R-Spondin 1 Protein	R&D	4645-RS
JAG-1 protein	StemRD	Cat#: JAG-1-pep-100
SB202190 (FHPI)	Selleckchem	S1077
SB431542	Selleck	S1067
Sirolimus (Rapamycin)	Medkoo	100766
Imatinib mesylate	Medkoo	100470
DAPT (GSI-IX)	Selleckchem	S2215
LDN-193189	Selleckchem	S2618
SAG	Selleckchem	S7779
IWR1-en	Selleckchem	S7086
A-83-01	Santa Cruz Biotechnology	sc-203791
PI-103	Tocris	2930
PD0325901	Santa Cruz Biotechnology	sc-205427
CHIR99021	Tocris	4423
Wnt-C59	Tocris	5148
BIO	Selleckchem	S7198
Thiazovivin	Medchemexpress	HY-13257
Nintedanib	Tocris	7049
Pirfenidone	Tocris	1093
polyvinyl alcohol	SigmaAldrich	P8136
Matrigel	BD	354234

Table S2. Antibodies and staining reagents used in these studies.

Name	Company	Catalog#	Dilution
CK8	abcam	ab53280	1/100 - 1/250
CK8	DSHB	TROMA-I	200
COL1	ABCAM	ab34710	1:100
GFP Tag Polyclonal Antibody	thermofisher	A-11122	200-2000
GFP Antibody (B-2)	scbt	sc-9996	50
Human Albumin Antibody	Bethyl	A80-129A	1:200 – 1:2,000
PDGFRB	eBioscienc	14-1402-82	100
PDGFRB	ABCAM	ab32570	100
PDGFRB	R&D	AF385-SP	100
PDGFRB	ll Signaling Technolo	3169T	100
LipidSpot™ Lipid Droplet Stains	biotium	#70069-T	
MitoSox Red	Invitrogen	M36008	
Alexa Fluor® 647 anti-human CD326 (EpCAM) Antibody	biolegend	324212	1:20

Cluster	Cell Type	NC (%)	PDGF (%)	TGFβ (%)
0	Mes1	20.7	20.5	20.2
1	MyoF_T1	1.9	1.5	37.5
2	Mes2	24.9	4.8	3.3
3	Cho1	17.6	14.7	1.3
4	Hep	12.8	12.3	5.2
5	MyoF_T2	3.6	7.07	16.2
6	Cho2	13.5	7.1	0.7
7	MyoF_P	0.8	20.1	1.0
8	Mes3	2.2	6.5	5.7
9	Mes4	1.9	4.4	3.0
10	Cho3	0.1	1.0	6.0

Table S3. The cell types and the percentage of the total number of cells in the 11 cell clusters identified by analysis of scRNA-Seq data in day 21 control, PDGF-, and TGFβ1-treated microHOs are shown. The transcriptomes of the 10 other cell clusters in the day 21 microHOs were defined using canonical markers and by comparison with the cells in control and cirrhotic human livers. Based upon the expression of canonical mRNAs and level of concordance between the cell cluster transcriptomes and the cell types identified in human liver, the microHOs have cholangiocytes (Cho1-3), hepatocyte (Hep), mesenchymal cell (Mes1-4) and myofibroblast (MyoF_T1-2, MyoF_P) clusters. A myofibroblast cluster (MyoF_T1) was far more abundant in the TGFβ1-treated microHOs than in control or PDGF-treated microHOs (37.5% of total vs <2%), while another myofibroblast cluster (MyoF_P) was more abundant in the PDGF-treated microHOs than in control or TGFβ1-treated microHOs (20% vs <2% of the total number of cells).

Table S4. The cell types for the 11 cell clusters were determined by analysis of the differentially expressed genes (shown in table S5) identified for each cluster. Selected mRNAs that are representative of the identified cell type for each cluster are shown.

Cluster	Marker mRNAs
Mes1	*COL14A1, ADAMTS1, DLK1, PDGFA, DPP, LUM*
MyoF_T1	*ACTA2, CLIP, TIMP3, ITGA11, COL1A1, TIMP1, ATCB2*
Mes2	*CNTN5, COL11A1, KDR*
Cho1	*KRR19, KRT7, SOX9, EPCAM*
Hep	*AFP, FGB, APOB, CKB, CFTR, CDH17, ALB, SERPINA1*
MyoF_T2	*COL1A1, ACTA2, C3, KLF3, FLNC*
Cho2	*KRR19, KRT7, SOX9, EPCAM*
MyoF_P	*PAPPA, COL1A1, ACTA2*
Mes3	*NCAM1, VIM, PDGFRA, PDGFRB, CXCL14*
Mes4	*NCAM1, VIM, PDGFRA, PDGFRB, MKI67*
Cho3	*KRR19, KRT7, SOX9, EPCAM*

Table S5 is provided at the end of this file.

Table S6. The results of 1-way ANOVA analyses of the cell percentages calculated by analysis of the scRNA-Seq data for the five biological replicates from the control, PDGF- and TGFβ-treated microHO preps shown in Figure S8. The degrees of freedom (DF), the sum of the squares (Sum Sq) and mean squared (mean Sq) of the variance, calculated F-value, and the probability Pr(>F) from the ANOVA are shown. A Pr(>F) < 0.05 indicates that there was a significant effect of the indicated variable. There were significant differences in the % of MyoF_T1, Mes2, MyoF_P and Cho3 cells as indicated by the p-value: ***; 0.001, **; 0.01, *; 0.05.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Mes1	2	5	2.6	0.009	0.991	
Residuals	12	3599	299.9			
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
MyoF_T1	2	1259	629.7	6.046	**0.0153**	*
Residuals	12	1250	104.1			
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Mes2	2	747.4	373.7	8.941	**0.00419**	**
Residuals	12	501.6	41.8			
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Cho1	2	339.8	169.92	1.946	0.185	
Residuals	12	1047.8	87.32			
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Hep	2	134.6	67.28	0.974	0.405	
Residuals	12	828.7	69.05			
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
MyoF_T2	2	145.5	72.73	2.739	0.105	
Residuals	12	318.7	26.56			
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Cho2	2	169.3	84.63	2.754	0.104	
Residuals	12	368.7	30.73			
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
MyoF_P	2	425.9	213.0	3.988	**0.047**	*
Residuals	12	640.8	53.4			
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Mes3	2	61.31	30.66	2.503	0.123	
Residuals	12	146.99	12.25			
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Mes4	2	22.8	11.40	1.045	0.382	
Residuals	12	130.9	10.91			
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Cho3	2	90.6	45.30	4.242	**0.0404**	*
Residuals	12	128.1	10.68			

Table S7. The results of 2-way ANOVA analyses of the data shown in the indicated figures are shown. For these analyses, time and treatment group was treated as categorical variables. The degrees of freedom (DF), the sum of the squares (Sum Sq) and mean squared (mean Sq) of the variance, calculated F-value, and the probability (Pr(>F) from the 2-way ANOVA are shown for the treatment group, time, and treatment-by-time analyses. A Pr(>F) < 0.01 indicates that there was a significant effect of the indicated variable.

Figure 2D	**DF**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Treatment	3	6.11E+14	2.04E+14	110.79	<2.00E-16
Time	4	1.47E+16	3.67E+14	19.98	4.92E-13
Treatment by Time	12	2.48E+14	2.07E+13	11.23	1.66E-15
Residuals	140	2.57E+14	1.84E+12		

Figure 3A	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Treatment	6	2.96E+14	4.93E+13	14.087	1.24E-12
Time	2	1.08E+15	5.38E+14	153.846	<2.00E-16
Treatment by Time	12	1.44E+14	1.20E+13	3.431	0.000185
Residuals	147	5.14E+14	3.50E+12		

Figure 3B	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Treatment	5	1.15E+14	2.30E+13	13.919	1.63E-10
Time	2	7.34E+14	3.67E+14	222.459	<2.00E-16
Treatment by Time	10	7.65E+13	7.65E+12	4.637	1.69E-05
Residuals	109	1.80E+14	1.65E+12		

Figure 5B	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Treatment	3	1.22E+17	4.06E+16	75.096	<2.00E-16
Time	4	2.54E+16	6.36E+15	11.75	2.95E-08
Treatment by Time	12	5.57E+16	4.64E+15	8.578	4.56E-12
Residuals	140	7.57E+16	5.41E+14		

Figure 5C	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Treatment	3	1.06E+15	3.52E+14	54.89	<2.00E-16
Time	2	5.33E+14	2.66E+14	41.51	1.02E-12
Treatment by Time	6	6.49E+14	1.08E+14	16.86	4.78E-12
Residuals	72	4.62E+14	6.42E+12		

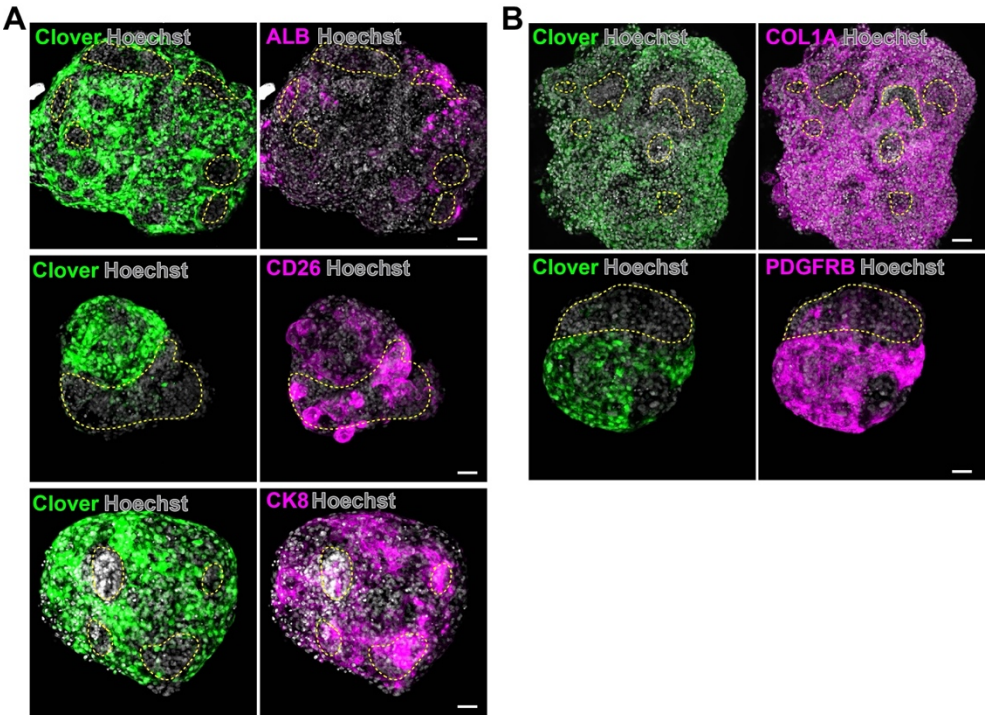

Figure S1. Immunofluorescent images of day 20 *COL1A1*-P2A Clover HOs stained with antibodies to Clover, Albumin (Alb), CD26 and CK8 (**A**); or antibodies to Clover, COL1A and PDGFRB (**B**). As seen in the merged images, the Clover$^+$ cells are distinct from the ALB$^+$ and CD26$^+$ and/or CK8$^+$ hepatocytes and cholangiocytes; while PDGFRB and COL1A are co-expressed in the Clover$^+$ cells. The dotted lines surround areas that are not stained with Clover and the Hoechst staining identifies nuclei. Scale bars, 50 μm.

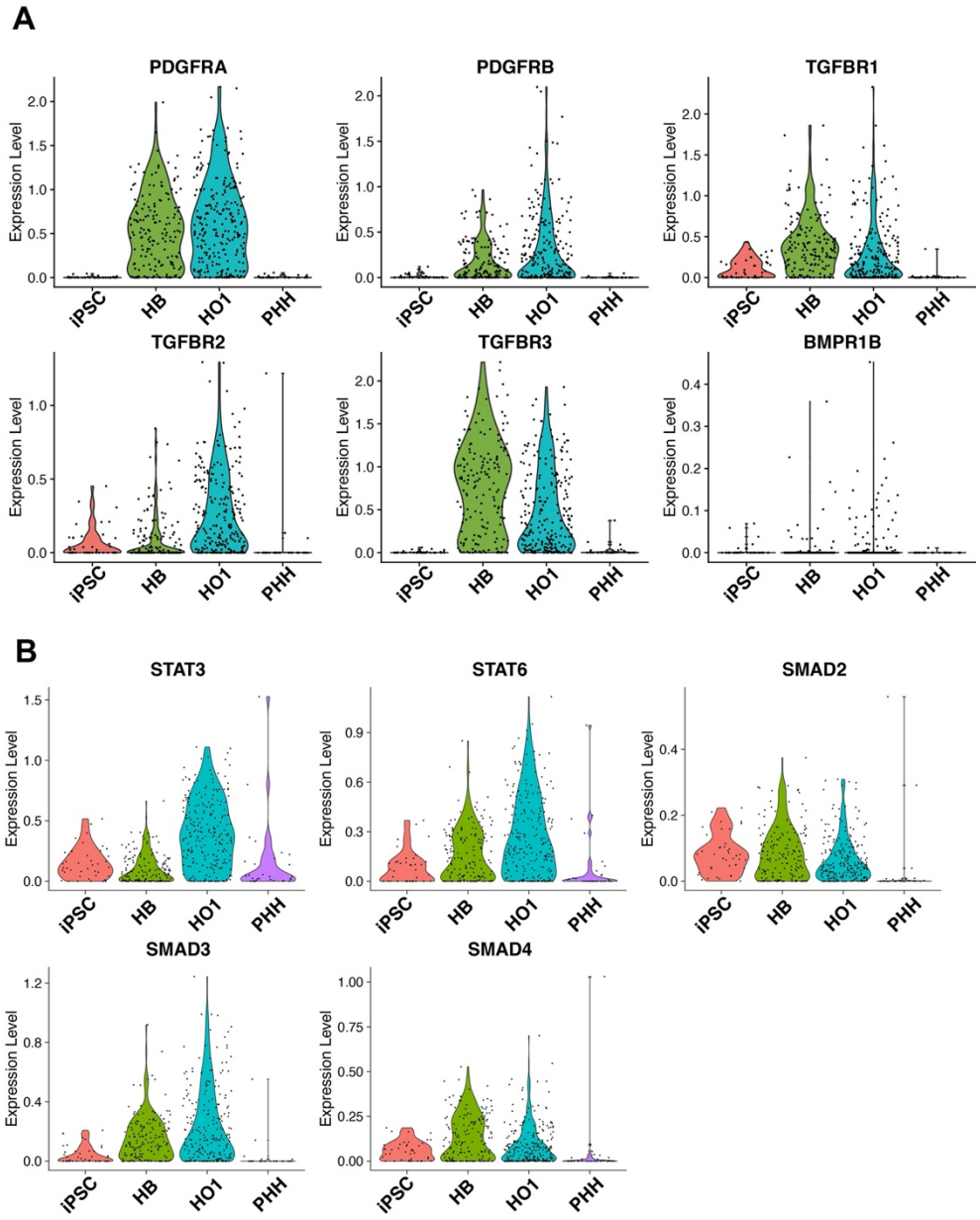

Figure S2. (**A, B**) Violin plots showing the level of expression of mRNAs encoding the receptors for PDGF (*PDGFRA, PDGFRB*) and TGFβ1 (*TGFBR1, TGFBR2, TGFBR3, BMPR1B*); and of *STAT3*, *STAT6*, and *SMAD2-4* mRNAs in developing and mature HO cultures. Previously obtained scRNA-Seq data [14] was generated from iPSC (day 0), day 9 hepatoblast (HB) and day 21 mature organoid cultures. For comparison purposes, scRNA-Seq data obtained from primary human hepatocytes (PHH) is also shown.

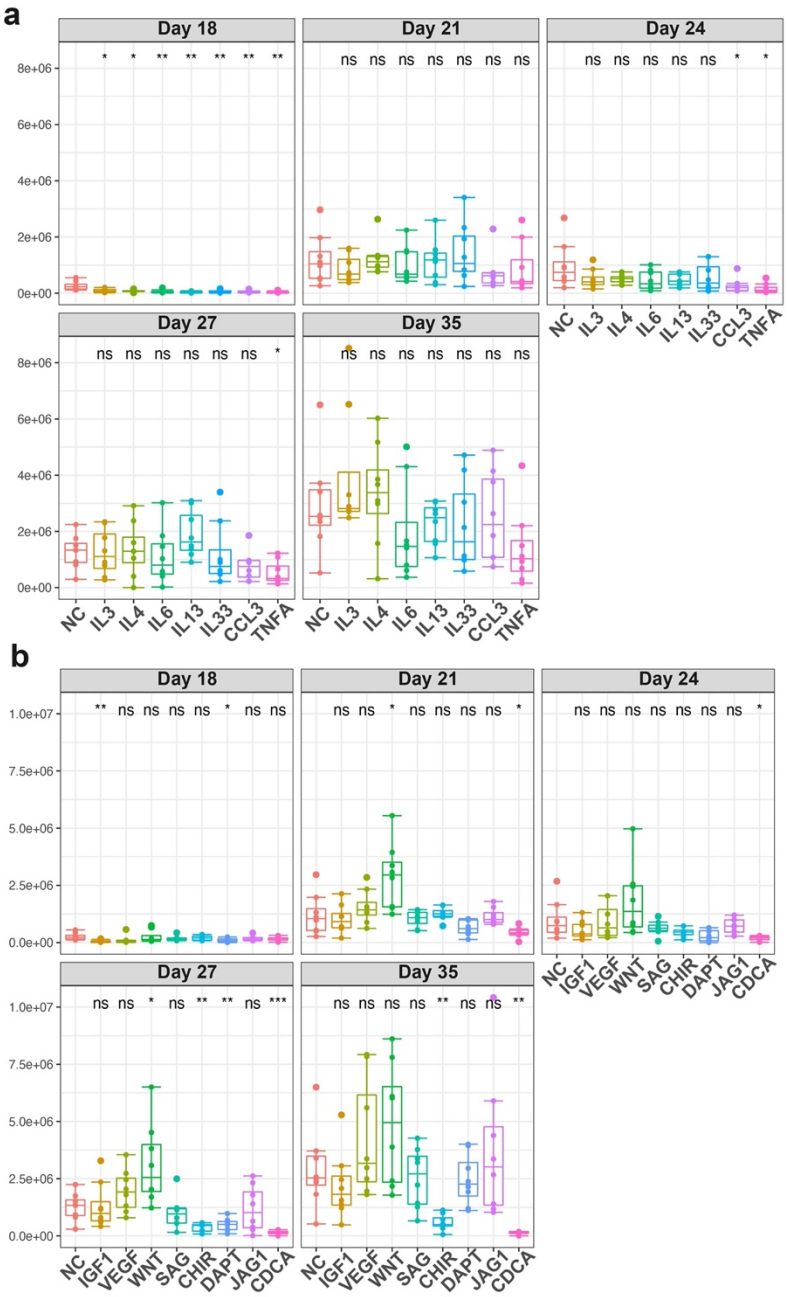

Figure S3. *COL1A1*-P2A Clover HOs were incubated with (**A**) 100 ng/ml IL-3, 100 ng/ml IL-4, 100 ng/ml IL-6, 100 ng/ml IL13, 100 ng/ml IL-33, 100 ng/ml CCL3, 50 ng/ml TNFa; or (**B**) 100 ng/ml IGF1, 50 ng/ml VEGF, 100 ng/ml Wnt3a, 10 μM SAG (Smoothened agonist), 3 μM CHIR99021, 10 μM DAPT (Gamma-Secretase Inhibitor), 100 ng/ml JAG1, 10 μM chenodeoxycholic acid (CDCA), or no addition (NC). Culture fluorescence, which indicates the amount of COL1A1^{+} cells in the HOs, was serially measured on days 18 through 35. Each dot

represents a measurement made on one HO, the thick line is the median of 8 organoids analyzed per condition, and the box plot shows the 25 to 75% range for all measurements per condition. With the possible exception of Wnt3a, none of these added agent caused a significant and or a sustained increase in $COL1A1^+$ cells in any of the organoid cultures. CDCA induced a decrease in $COL1A1^+$ cells.

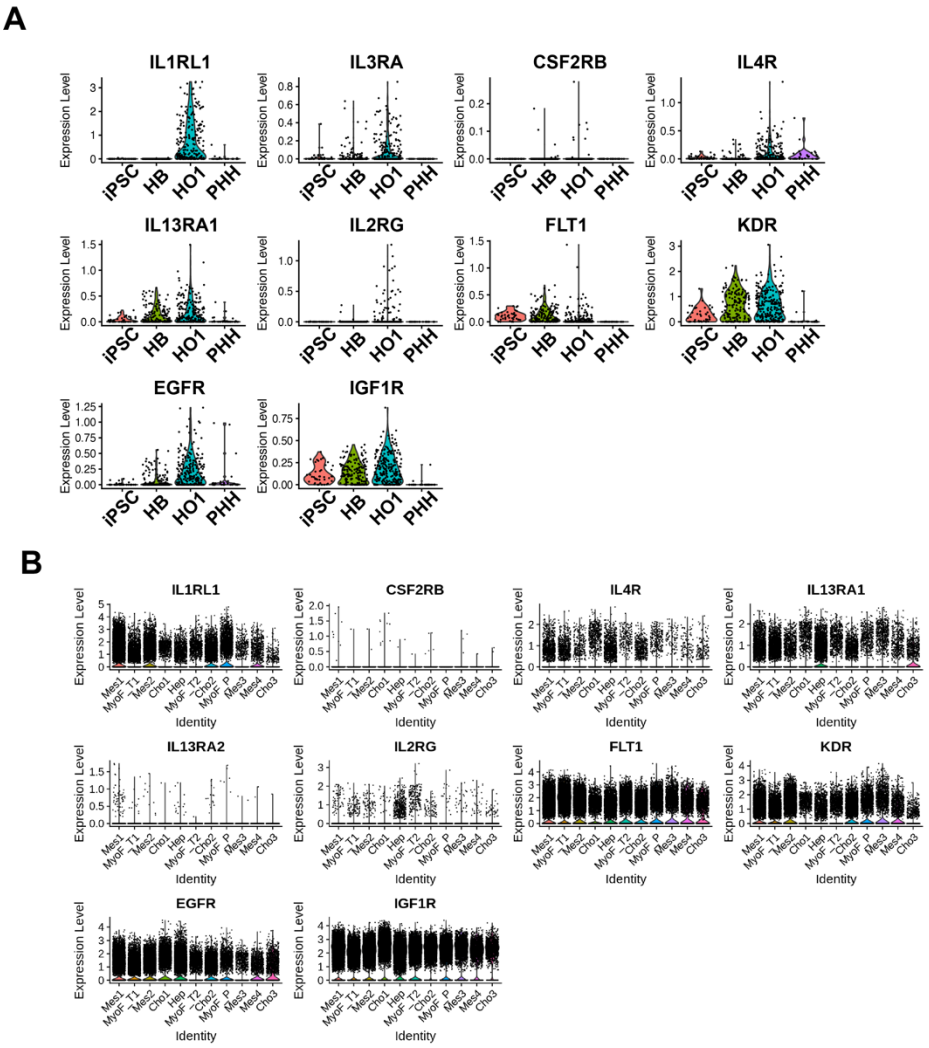

Figure S4. (**A**) Violin plots showing the level of expression of mRNAs for the following receptors during HO development: *IL1RL1, IL4R, IL13RA1, IL2RG, FLT1, KDR,* and *IGF1R*. scRNA-Seq data was generated from iPSC (day 0), day 9 hepatoblast (HB) and day 21 mature organoid (HO1) cultures. For comparison purposes, scRNA-Seq obtained from primary human hepatocytes (PHH) is also shown. This scRNA-Seq dataset was obtained from [14]. (**B**) Violin plots showing the level of mRNA expression for the receptors shown in (A) in the 11 cell clusters identified in day 21 control, PDGF- and TGFβ-treated microHOs using the scRNA-Seq data generated in this paper.

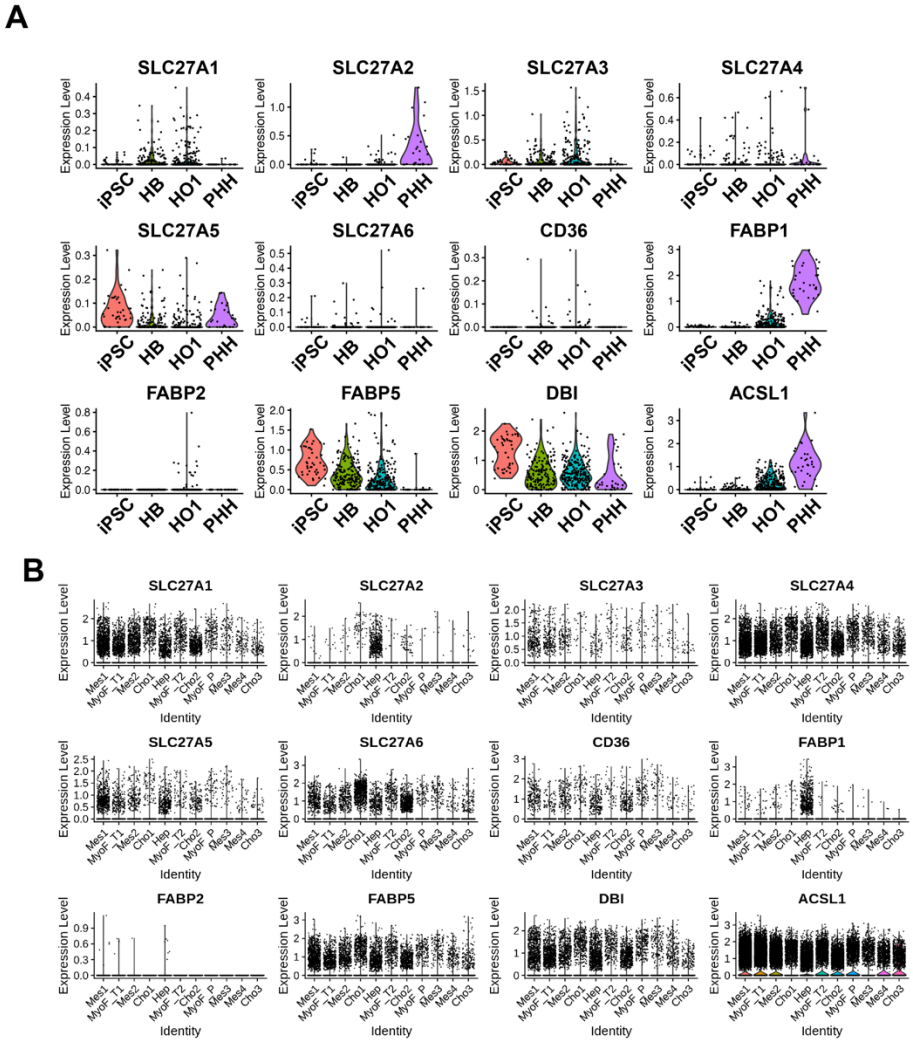

Figure S5. (**A**) Violin plots showing the level of expression of mRNAs for the following fatty acid transport proteins during HO development: *SLC27A1-6, CD36, FABP1-2, FABP5, DBI* and *ACSLI.* The previously obtained scRNA-Seq data [14] was generated from iPSC (day 0), day 9 hepatoblast (HB) and day 21 mature organoid cultures. For comparison purposes, scRNA-Seq obtained from primary human hepatocytes (PHH) is also shown. (**B**) Violin plots showing the level of mRNA expression for the for the fatty acid transport proteins shown in (A) in the eleven cell clusters identified in day 21 control, PDGF- and TGFβ-treated microHOs using the scRNA-Seq data generated in this paper.

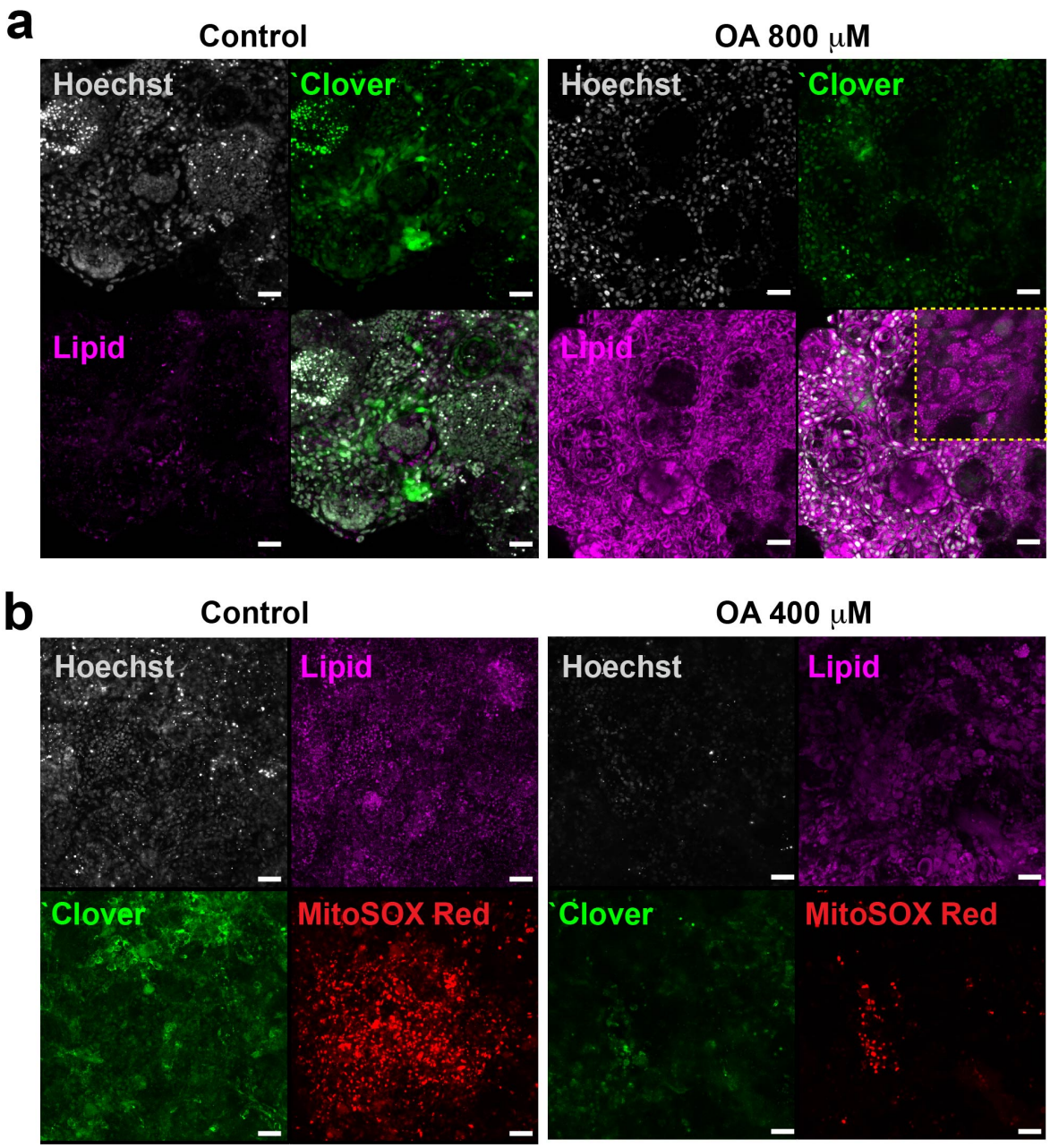

Figure S6. Oleic acid (OA) induces toxicity in HOs. Either 800 (**A**) or 400 (**B**) uM OA was added to HO cultures on day 13, and the HOs were analyzed by the HCS system on day 24. Although there was a marked increase in intracellular fatty acids within the HOs that were incubated with OA (LipidSpot), OA addition did not increase in COL1A1 expression (Clover). Moreover, there was a marked decrease in cell viability within the HOs that were incubated with OA, as shown by the MitoSox Red analysis.

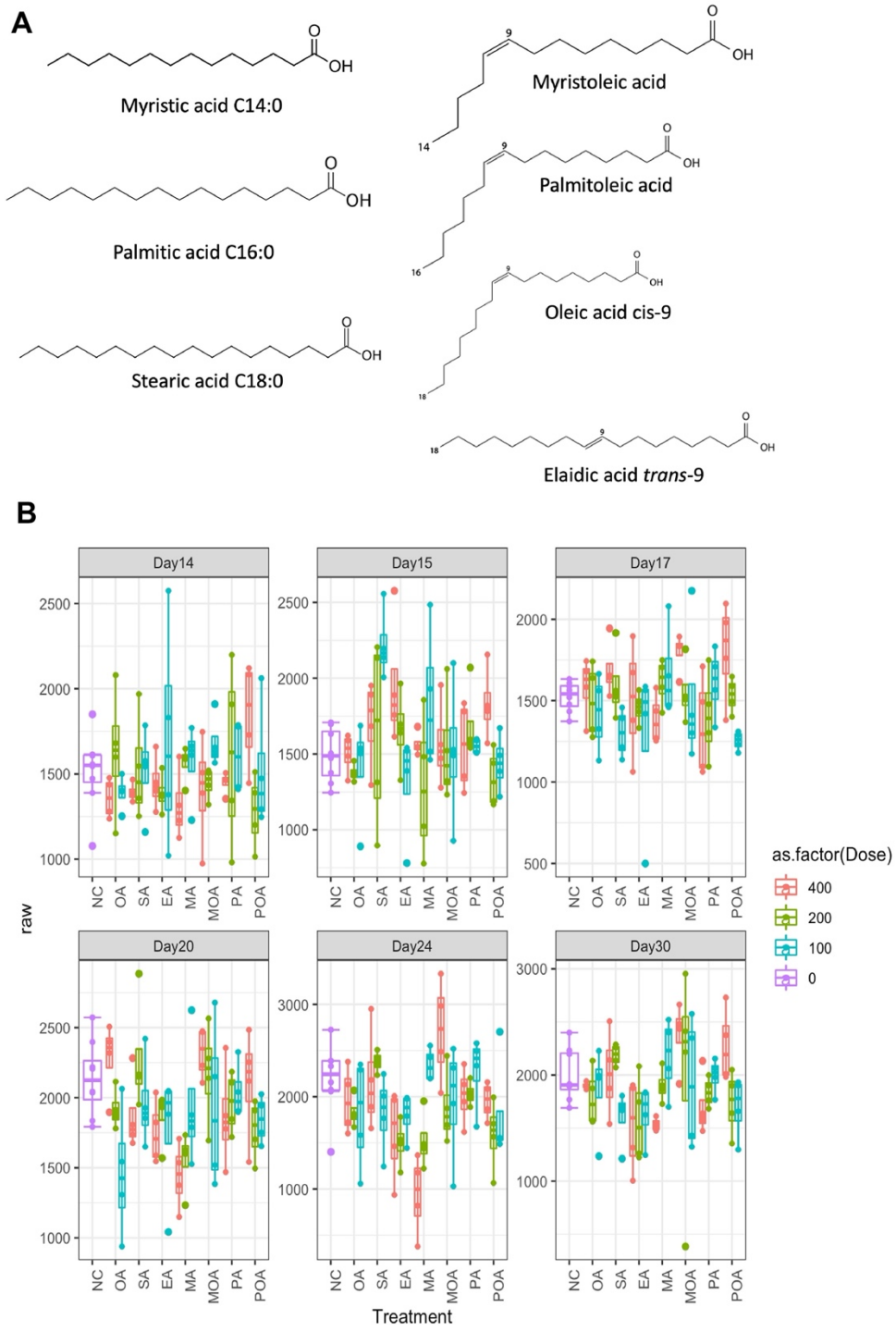

Figure S7. Free fatty acids do not increase collagen expression in HOs. (**A**) The structures of the free fatty acids whose effect on collagen expression in HOs was analyzed. Unsaturated FFAs (myristic (MA), palmitic (PA) and stearic (SA)) and their corresponding FFA with cis-9 (Myristoleic (MOA), palmitoleic (POA), and oleic (OA)) or trans-9 (elaidic (EA)) double bonds were analyzed. (**B**) *COL1A1*-P2A Clover HOs were incubated with 0, 100, 200 or 400 uM of the

indicated free fatty acid on day 13. The fluorescence in these cultures, which indicates the amount of COL1A1^{+} cells in the HOs, was serially measured on days 14 through 30. Each dot represents a measurement made on one HO, the thick line is the median of 8 organoids analyzed per condition, and the box plot shows the 25 to 75% range for all measurements per condition. The free fatty acids did not induce a significant increase in COL1A1^{+} in any of the cultures.

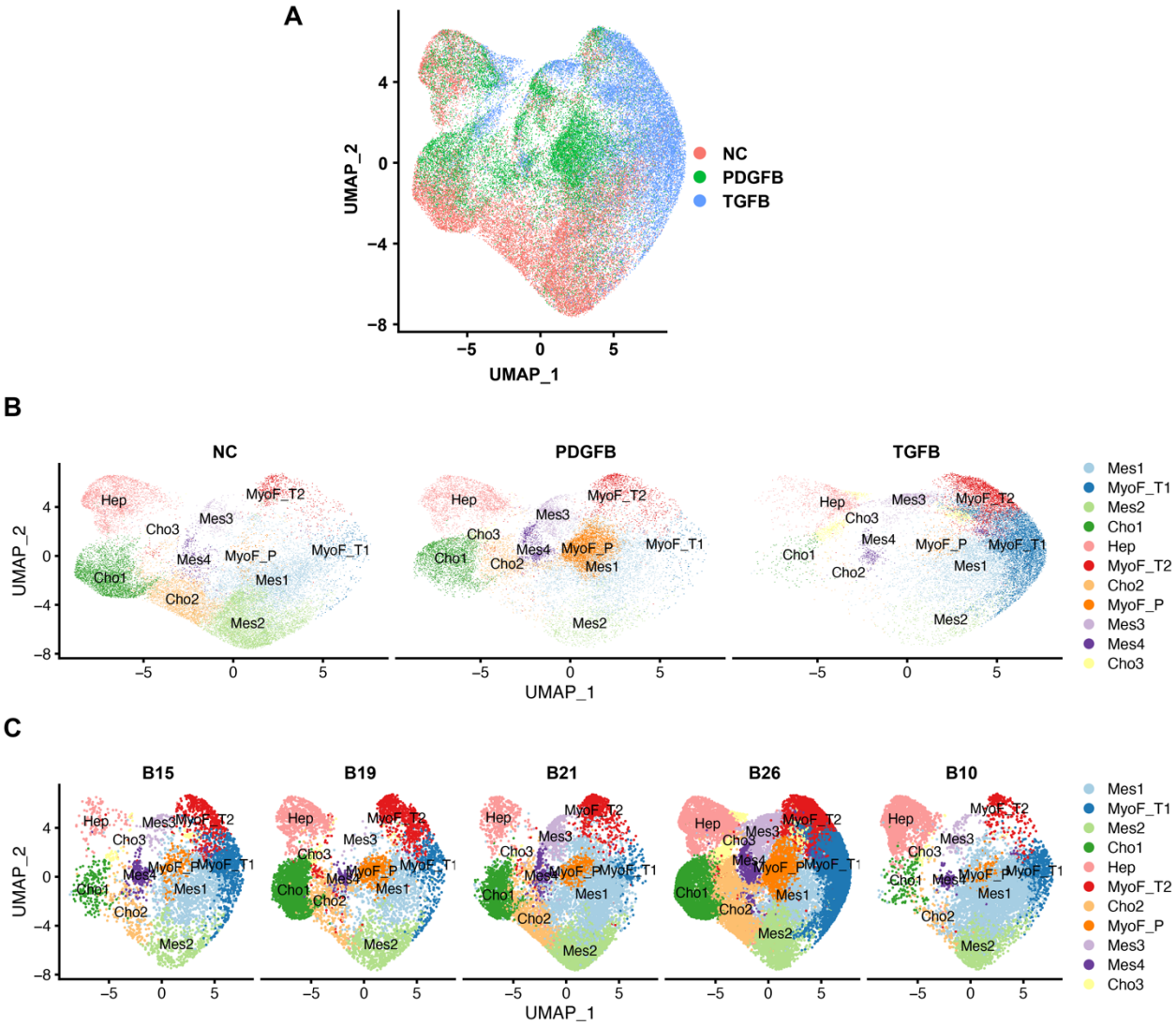

Figure S8. (**A**) A combined sample u-MAP plot shows the scRNA-seq data obtained from the cells in day 21 control (NC), PDGFβ-, or TGFβ1-treated microHO cultures. Each of the different type of microHO is indicated by the color shown on the right of the panel. (**B**) Separate u-MAP plots show the scRNA-seq data obtained from the cells in day 21 control (NC), PDGFβ-, or TGFβ1-treated microHO cultures. (**C**) The u-MAP plots for each of the five batches of microHOs that were separately analyzed by scRNA-Seq analysis. Each cluster is indicated by a different color as indicated on the right side of panels B and C.

HNF4A
Clover
HNF4A_Clover
SERPINA1
Clover
SERPINA1_Clover
CDH1
Clover
CDH1_Clover
AFP
Clover
AFP_Clover
KRT19
Clover
KRT19_Clover
KRT7
Clover
KRT7_Clover
rna_SOX9
Clover
rna_SOX9_Clover
CFTR
Clover
CFTR_Clover
ALDH1A1
Clover
ALDH1A1_Clover
APOB
Clover
APOB_Clover
FGB
Clover
FGB_Clover
NC
PDGFB
TGFB

Figure S9. Feature plots show the level of expression of each indicated mRNA with *Clover* mRNA in the u-MAP plots shown in Fig 8B. The uMAP plots for the normal control (NC), PDGF-, and TGFβ-treated microHOs are shown separately. The colors represent level of expression of the two mRNAs in each row as shown in the adjacent color threshold diagram. The mRNAs found in cholangiocytes (*KRT19*, *KRT7*, *CFTR),* hepatocytes *(SERPIN1A)* or hepatocytes and cholangiocytes (*HNF4A, AFP, CDH1, ALDHA1, APOB, SOX9, FGB*) were not expressed in the cells that expressed *Clover* mRNA in the microHOs.

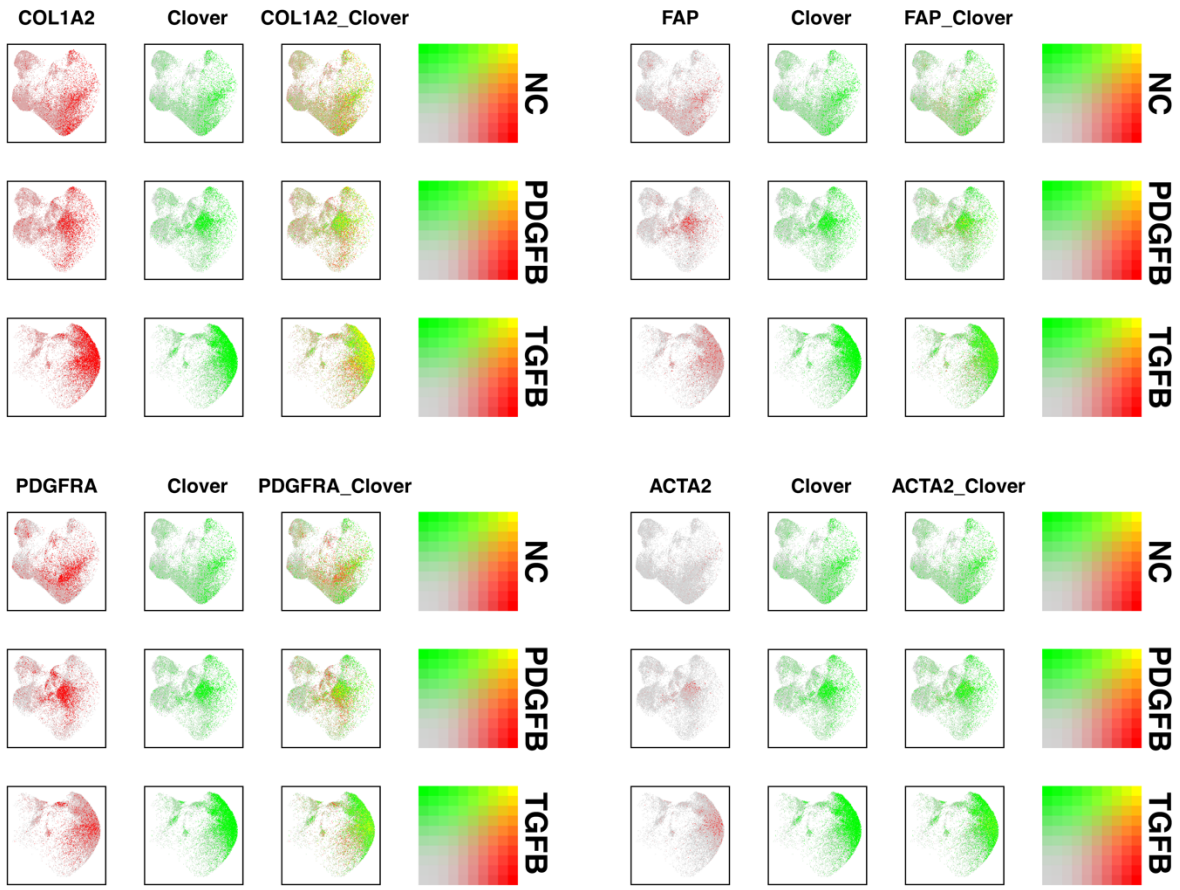

Figure S10. Feature plots show the level of expression of *COL1A2, PDGFRA, FAP or ACTA2* mRNAs with *Clover* mRNA in the u-MAP plots shown in Fig 8B. The u-MAP plots for the normal control (NC), PDGF-, and TGFβ-treated microHOs are shown separately. The colors represent level of expression of the two mRNAs in each row as shown in the adjacent color threshold diagram. *COL1A2 and Clover* mRNAs have an overlapping pattern of expression; they are expressed in myofibroblasts and in mesenchymal cells. In contrast, *ACTA2, PDGFRA* and *FAP* mRNAs are predominantly expressed in the myofibroblasts (MyoF-T1/2, MyoF_P) in PDGF- or TGFβ-treated microHOs.

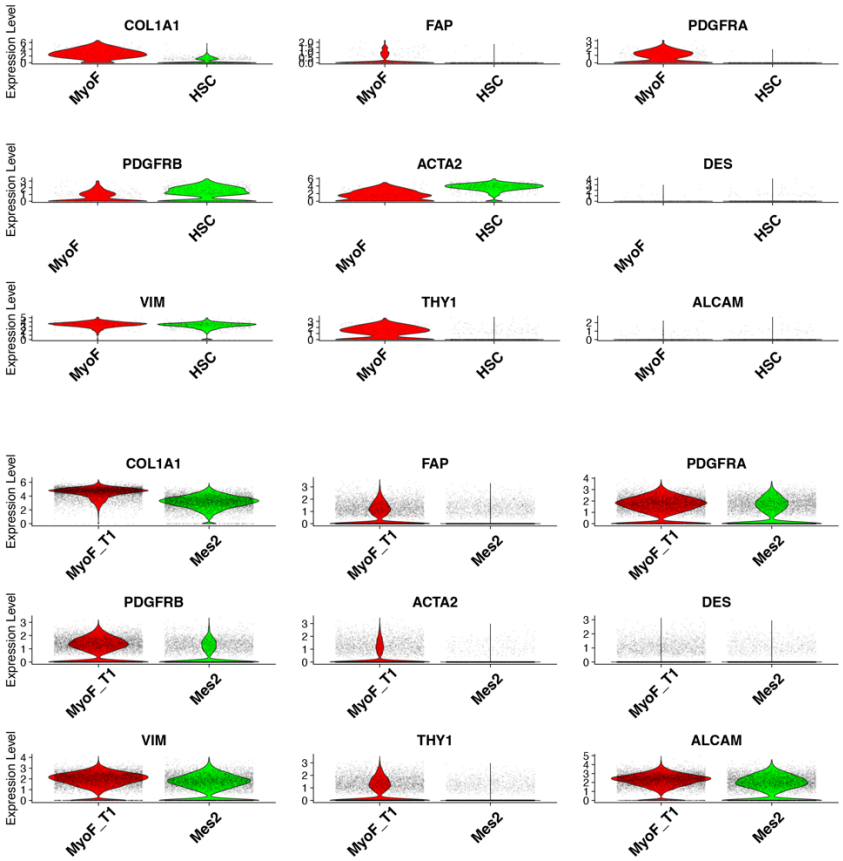

Figure S11. The pattern of expression of mesenchyme and MyoF-specific genes in hepatic stellate cells (HSC) and MyoF in human liver is mostly retained in Mes2 and MyoF_T1 cells in microHOs. scRNA-Seq data was analyzed to generate violin plots showing the level of expression of *COL1A1, FAP, PDGFRA, PDGFRB, ACTA2, DES, VIM, Thy1* and *ALCAM* mRNAs in HSC and MyoF in cirrhotic human liver (GSE136103; Top panel) and in Mes2 and MyoF_T1 cells in microHOs (Bottom panel). The cell cluster is indicated on the x-axis; and the y-axis shows the natural log transformed and normalized level of expression of each mRNA. Each dot shows the expression level in one cell.

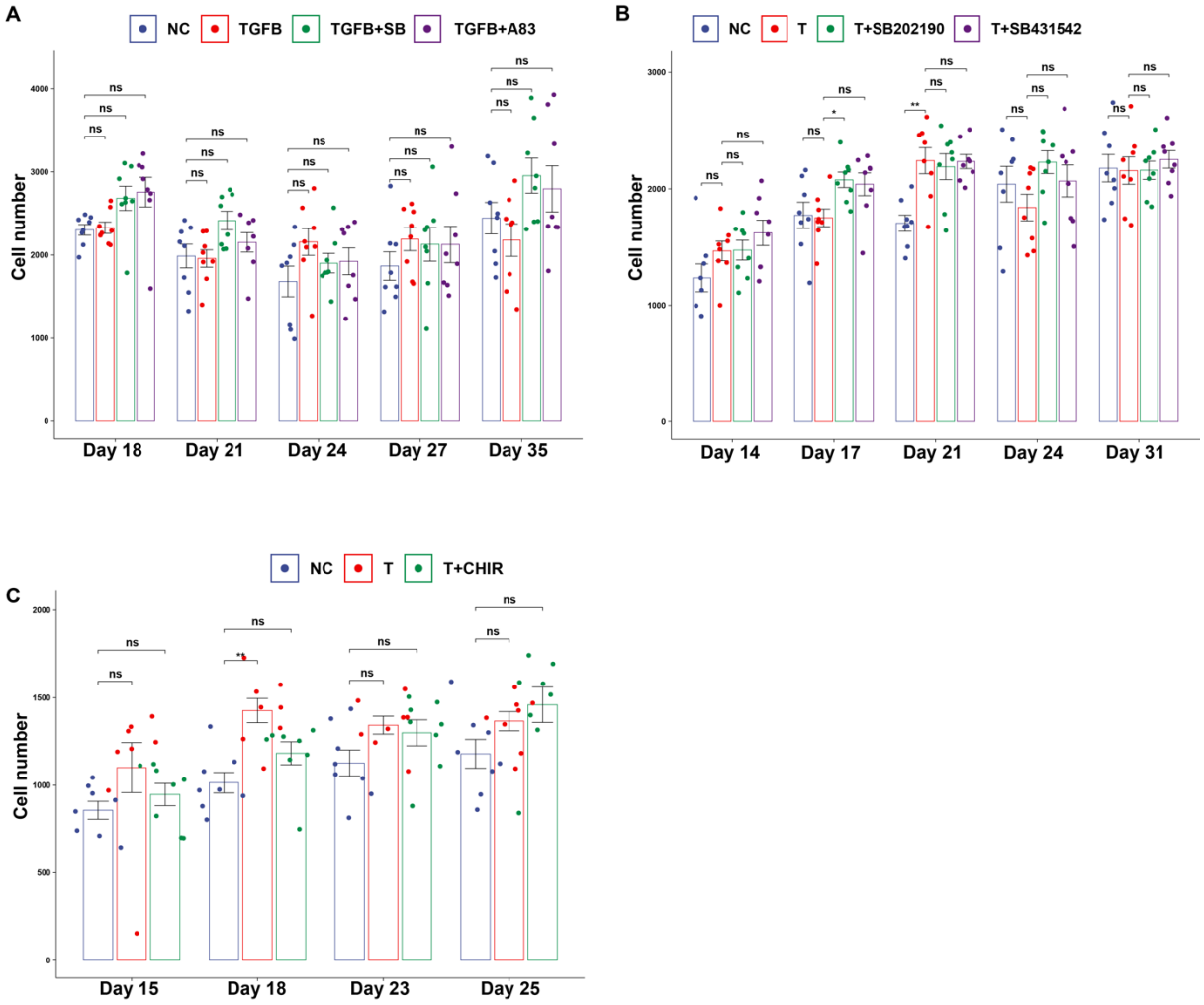

Figure S12. microHO viability was not affected by addition of TGFβ1, nor by any of the other tested drugs. microHOs were prepared and incubated with no addition (NC), 50 ng/ml TGFβ1 (T), or TGFβ1 ± the indicated inhibitor on day 13. The tested drugs were: (**A)** 10 uM TGFRβ1 tyrosine kinase inhibitors A83-01 or SB431542 (SB); (**B**) 10 uM TGFRβ1 inhibitor (SB431542) or 10 uM p38 α inhibitor (SB202190); and (**C**) 10 uM CHIR99021 (CHIR). The number of cells in each microHO was measured by counting the number of nuclei (stained with Hoechst 33342) on days 14 to 35. Each bar is the average of 8 individual measurements ± the SE, and individual datapoints are shown as dots. None of these additions caused a significant change (ns) in the number of cells in the treated microHOs relative to control microHOs.

Table S5. Twenty mRNAs that are differentially expressed in each of the 11 cell clusters identified in microHOs. The gene symbol, p-value, adjusted p-value, and fold differential expression (log_2) for each differentially expressed gene are shown. Pct.1 and Pct.2 indicates the contribution of that feature to defining the indicated cluster based upon principal component analysis.

	cluster	gene	p_val	avg_log2FC	pct.1	pct.2	p_val_adj
1	0	*COL14A1*	0.00	0.74	0.77	0.55	0
2	0	*ADAMTS1*	0.00	0.69	0.87	0.6	0
3	0	*KCND2*	0.00	0.65	0.75	0.53	1.41073166685289E-295
4	0	*DLK1*	0.00	0.64	0.97	0.88	0
5	0	*IL1RL1*	0.00	0.63	0.6	0.4	6.07272463504603E-113
6	0	*COL6A3*	0.00	0.58	0.99	0.93	0
7	0	*IL6ST*	0.00	0.57	0.99	0.95	0
8	0	*ITGA1*	0.00	0.56	0.93	0.74	0
9	0	*ZNF804A*	0.00	0.55	0.85	0.66	9.89305658045823E-202
10	0	*CLMP*	0.00	0.55	0.92	0.68	0
11	0	*PDGFRA*	0.00	0.54	0.91	0.76	0
12	0	*SOX5*	0.00	0.54	1	0.99	0
13	0	*DCN*	0.00	0.54	0.98	0.89	0
14	0	*LAMA2*	0.00	0.53	0.96	0.8	0
15	0	*LUM*	0.00	0.51	0.96	0.82	0
16	0	*SERPINE2*	0.00	0.49	0.99	0.93	0
17	0	*DPP4*	0.00	0.49	0.97	0.85	0
18	0	*COL1A2*	0.00	0.48	1	0.99	2.39332526066761E-224
19	0	*RSPO2*	0.00	0.47	0.9	0.69	0
20	0	*TGFBR3*	0.00	0.45	0.88	0.65	0
21	1	*CILP*	0.00	2.11	0.98	0.8	0
22	1	*TIMP3*	0.00	1.99	1	0.96	0
23	1	*COMP*	0.00	1.96	0.87	0.67	0
24	1	*ITGA11*	0.00	1.52	0.88	0.64	0
25	1	*COL1A1*	0.00	1.49	1	0.98	0

26	1	*AC097487.1*	0.00	1.48	0.65	0.59	0
27	1	*TRABD2B*	0.00	1.43	0.9	0.64	0
28	1	*MXRA5*	0.00	1.37	0.96	0.79	0
29	1	*TIMP1*	0.00	1.32	1	0.97	0
30	1	*CCN2*	0.00	1.32	0.86	0.67	0
31	1	*ABTB2*	0.00	1.32	0.98	0.76	0
32	1	*LTBP2*	0.00	1.30	0.91	0.67	0
33	1	*NOX4*	0.00	1.27	0.93	0.76	0
34	1	*EGFP*	0.00	1.27	0.96	0.82	0
35	1	*SMYD3*	0.00	1.27	0.99	0.93	0
36	1	*AF165147.1*	0.00	1.23	0.81	0.71	0
37	1	*SPON1*	0.00	1.22	0.75	0.55	0
38	1	*SERPINE2*	0.00	1.22	1	0.94	0
39	1	*MICAL2*	0.00	1.17	0.96	0.79	0
40	1	*CLU*	0.00	1.17	0.92	0.73	0
41	2	*CNTN5*	0.00	2.15	0.88	0.64	0
42	2	*LINC01088*	0.00	2.05	0.97	0.71	0
43	2	*LINC02388*	0.00	2.05	0.93	0.66	0
44	2	*POSTN*	0.00	1.70	0.95	0.74	0
45	2	*NPNT*	0.00	1.67	0.92	0.73	0
46	2	*ARHGAP6*	0.00	1.63	0.88	0.59	0
47	2	*SLIT3*	0.00	1.58	0.91	0.78	0
48	2	*DIRC3*	0.00	1.48	0.91	0.68	0
49	2	*PKHD1L1*	0.00	1.44	0.76	0.51	0
50	2	*CLSTN2*	0.00	1.35	0.82	0.62	0
51	2	*BCO2*	0.00	1.35	0.94	0.69	0
52	2	*PDPN*	0.00	1.29	0.98	0.87	0
53	2	*CPS1*	0.00	1.28	0.94	0.7	0
54	2	*KCNMA1*	0.00	1.26	0.94	0.8	0
55	2	*LINC00894*	0.00	1.26	0.92	0.68	0
56	2	*PWWP3B*	0.00	1.25	0.87	0.59	0

57	2	*COL11A1*	0.00	1.25	0.98	0.87	0
58	2	*KDR*	0.00	1.24	0.87	0.59	0
59	2	*PRLR*	0.00	1.24	0.96	0.77	0
60	2	*GHR*	0.00	1.23	0.88	0.68	0
61	3	*UNC5C*	0.00	2.45	0.96	0.62	0
62	3	*GRHL2*	0.00	2.36	0.91	0.64	0
63	3	*ITGB6*	0.00	2.32	0.96	0.7	0
64	3	*GABRP*	0.00	2.29	0.86	0.58	0
65	3	*PDE4D*	0.00	2.20	0.96	0.81	0
66	3	*ARHGAP29*	0.00	2.11	0.89	0.51	0
67	3	*PLD5*	0.00	2.08	0.86	0.67	0
68	3	*ROR1*	0.00	2.08	0.86	0.56	0
69	3	*AC015522.1*	0.00	2.00	0.98	0.77	0
70	3	*PRTG*	0.00	1.93	0.83	0.51	0
71	3	*NRXN3*	0.00	1.88	0.89	0.66	0
72	3	*ST6GALNAC3*	0.00	1.85	0.89	0.66	0
73	3	*NEBL*	0.00	1.85	0.91	0.75	0
74	3	*ANKS1A*	0.00	1.81	0.88	0.77	0
75	3	*EYA1*	0.00	1.76	0.67	0.53	0
76	3	*NAALADL2*	0.00	1.75	0.84	0.65	0
77	3	*SEMA5A*	0.00	1.74	0.95	0.73	0
78	3	*PTN*	0.00	1.67	0.8	0.55	0
79	3	*THSD4*	0.00	1.65	0.84	0.56	0
80	3	*PATJ*	0.00	1.64	0.82	0.63	0
81	4	*AFP*	0.00	3.58	0.72	0.53	0
82	4	*CHST9*	0.00	1.90	0.77	0.39	0
83	4	*APOB*	0.00	1.82	0.51	0.46	1.57013013564819E-97
84	4	*CEACAM6*	0.00	1.79	0.65	0.44	0
85	4	*FGB*	0.00	1.71	0.55	0.45	7.57761739439617E-259
86	4	*LINC00511*	0.00	1.68	0.87	0.54	0
87	4	*NTN4*	0.00	1.68	0.71	0.55	0

88	4	*SERPINA1*	0.00	1.58	0.53	0.43	3.26816794412982E-195
89	4	*DNAJC15*	0.00	1.47	0.91	0.73	0
90	4	*CDH17*	0.00	1.44	0.58	0.44	0
91	4	*CDH2*	0.00	1.38	0.95	0.79	0
92	4	*FOXP2*	0.00	1.37	0.73	0.55	0
93	4	*EPCAM*	0.00	1.26	0.75	0.52	0
94	4	*APOA1*	0.00	1.25	0.62	0.37	0
95	4	*MARCHF1*	0.00	1.23	0.64	0.44	0
96	4	*CDH1*	0.00	1.23	0.77	0.55	0
97	4	*CCSER1*	0.00	1.21	0.8	0.54	0
98	4	*MAML3*	0.00	1.20	0.9	0.75	0
99	4	*LINC00278*	0.00	1.20	0.78	0.59	0
100	4	*LCP1*	0.00	1.16	0.76	0.55	0
101	5	*PI16*	0.00	2.48	0.92	0.74	0
102	5	*C3*	0.00	2.00	0.74	0.59	0
103	5	*IGFBP6*	0.00	1.93	0.81	0.67	0
104	5	*HTRA3*	0.00	1.86	0.77	0.63	0
105	5	*GABRB3*	0.00	1.69	0.4	0.47	0.645287107
106	5	*SLC24A2*	0.00	1.49	0.75	0.65	0
107	5	*KRT14*	0.00	1.32	0.65	0.58	0
108	5	*PRSS23*	0.00	1.30	0.83	0.69	0
109	5	*TIMP3*	0.00	1.21	1	0.96	0
110	5	*ATP10A*	0.00	1.19	0.78	0.69	0
111	5	*ANK2*	0.00	1.19	0.85	0.73	0
112	5	*TNS1*	0.00	1.17	0.87	0.73	0
113	5	*CSPG4*	0.00	1.16	0.7	0.59	0
114	5	*CD55*	0.00	1.14	0.72	0.67	4.49192035504069E-284
115	5	*KLF3*	0.00	1.10	0.78	0.71	0
116	5	*CPAMD8*	0.00	1.07	0.58	0.42	0
117	5	*AC099520.1*	0.00	1.05	0.5	0.5	2.92243869181999E-49
118	5	*ABI3BP*	0.00	1.04	0.76	0.67	0

119	5	*ZBTB7C*	0.00	1.03	0.57	0.58	3.96295910582397E-99
120	5	*FLNC*	0.00	1.02	0.73	0.61	0
121	6	*UNC5C*	0.00	0.84	0.94	0.63	0
122	6	*GABRP*	0.00	0.78	0.79	0.6	0
123	6	*ST6GALNAC3*	0.00	0.78	0.9	0.67	0
124	6	*GRHL2*	0.00	0.77	0.89	0.65	0
125	6	*PRTG*	0.00	0.71	0.82	0.53	0
126	6	*PDE4D*	0.00	0.69	0.96	0.82	0
127	6	*ROR1*	0.00	0.69	0.85	0.57	0
128	6	*ARHGAP29*	0.00	0.68	0.83	0.54	0
129	6	*ANKS1A*	0.00	0.65	0.91	0.77	0
130	6	*ITGB6*	0.00	0.65	0.93	0.71	0
131	6	*PLD5*	0.00	0.62	0.84	0.68	0
132	6	*LINC02388*	0.00	0.61	0.74	0.69	3.14703394653217E-146
133	6	*EYA1*	0.00	0.60	0.67	0.54	1.6275168304562E-266
134	6	*DIAPH3*	0.00	0.60	0.68	0.55	1.51594497149573E-217
135	6	*NRXN3*	0.00	0.59	0.88	0.67	0
136	6	*FREM2*	0.00	0.59	0.66	0.52	1.11716953189223E-242
137	6	*KCNQ5*	0.00	0.58	0.73	0.57	5.17999574470969E-237
138	6	*AC015522.1*	0.00	0.58	0.97	0.78	0
139	6	*PTN*	0.00	0.57	0.71	0.57	6.4862311734237E-293
140	6	*SEMA5A*	0.00	0.57	0.94	0.74	0
141	7	*TFPI2*	0.00	2.00	0.62	0.46	0
142	7	*PAPPA*	0.00	1.65	0.98	0.89	0
143	7	*PDE5A*	0.00	1.55	0.73	0.57	0
144	7	*ADAMTS1*	0.00	1.53	0.8	0.65	0
145	7	*AREG*	0.00	1.35	0.69	0.64	5.25882782978605E-270
146	7	*ITGA1*	0.00	1.26	0.88	0.77	0
147	7	*ABCC4*	0.00	1.23	0.71	0.64	0
148	7	*MSC-AS1*	0.00	1.20	0.55	0.51	1.60206265130991E-185
149	7	*PIEZO2*	0.00	1.13	0.62	0.48	3.62254977687614E-291

150	7	*SUCLG2-AS1*	0.00	1.12	0.73	0.65	0
151	7	*CLMP*	0.00	1.11	0.79	0.72	0
152	7	*KYNU*	0.00	1.07	0.45	0.5	1.09972579275846E-41
153	7	*IGFBP5*	0.00	1.06	0.86	0.8	0
154	7	*ROBO2*	0.00	1.05	0.84	0.71	0
155	7	*RORB*	0.00	1.05	0.64	0.65	1.71419981781713E-181
156	7	*MASP1*	0.00	0.98	0.6	0.52	2.14815931343417E-221
157	7	*AL691420.1*	0.00	0.94	0.69	0.74	1.90329965031377E-129
158	7	*KCND2*	0.00	0.94	0.6	0.58	1.12904013826135E-87
159	7	*PAMR1*	0.00	0.93	0.66	0.64	1.05279467805712E-135
160	7	*MMP16*	0.00	0.91	0.79	0.73	6.45407093018707E-251
161	8	*CXCL14*	0.00	2.54	0.82	0.61	0
162	8	*KIRREL3*	0.00	2.47	0.64	0.58	1.78087718527724E-275
163	8	*NRP2*	0.00	2.07	0.81	0.59	0
164	8	*GSN*	0.00	2.01	0.93	0.84	0
165	8	*AC002463.1*	0.00	1.95	0.66	0.56	0
166	8	*CRHBP*	0.00	1.89	0.53	0.52	2.68844774508409E-87
167	8	*ATRNL1*	0.00	1.84	0.67	0.58	7.4046528162926E-205
168	8	*NRG3*	0.00	1.81	0.78	0.51	0
169	8	*CDH13*	0.00	1.79	0.63	0.48	1.90104856701329E-243
170	8	*MIAT*	0.00	1.79	0.7	0.56	0
171	8	*NTM*	0.00	1.77	0.59	0.55	1.11151682293596E-121
172	8	*PLCB4*	0.00	1.76	0.74	0.59	0
173	8	*OXCT1*	0.00	1.76	0.61	0.56	1.22313326537527E-195
174	8	*NPAS2*	0.00	1.59	0.67	0.53	1.64759745644261E-255
175	8	*PI16*	0.00	1.56	0.8	0.76	2.48410109644837E-303
176	8	*CD109*	0.00	1.54	0.75	0.7	3.32247596107369E-263
177	8	*LINC01592*	0.00	1.53	0.54	0.55	4.53240260414655E-120
178	8	*TEK*	0.00	1.48	0.73	0.63	7.56466873318405E-289
179	8	*PARD3B*	0.00	1.45	0.76	0.7	1.08312332593575E-226
180	8	*PODXL*	0.00	1.43	0.76	0.7	2.30063348380999E-244

181	9	*MKI67*	0.00	2.57	0.94	0.59	0
182	9	*TOP2A*	0.00	2.44	0.89	0.56	0
183	9	*CENPF*	0.00	2.23	0.89	0.58	0
184	9	*ASPM*	0.00	2.09	0.86	0.53	0
185	9	*TPX2*	0.00	2.02	0.88	0.57	0
186	9	*CENPE*	0.00	1.62	0.78	0.44	0
187	9	*DIAPH3*	0.00	1.56	0.88	0.55	0
188	9	*ANLN*	0.00	1.56	0.82	0.49	0
189	9	*PRC1*	0.00	1.39	0.73	0.44	0
190	9	*KIF11*	0.00	1.37	0.77	0.49	0
191	9	*NUSAP1*	0.00	1.36	0.71	0.41	0
192	9	*CIT*	0.00	1.34	0.75	0.5	0
193	9	*KPNA2*	0.00	1.31	0.73	0.46	3.90947789658089E-290
194	9	*CDK1*	0.00	1.27	0.68	0.51	1.05324536288692E-221
195	9	*KIF4A*	0.00	1.26	0.71	0.49	0
196	9	*NCAPG*	0.00	1.19	0.7	0.5	6.08843872357421E-298
197	9	*CEP128*	0.00	1.18	0.79	0.55	3.67991367428791E-298
198	9	*KIF14*	0.00	1.17	0.66	0.45	1.75116932862194E-259
199	9	*CKAP2L*	0.00	1.16	0.69	0.51	7.57255848194669E-251
200	9	*MIR924HG*	0.00	1.10	0.72	0.57	6.10640620425961E-203
201	10	*MMP9*	0.00	2.77	0.67	0.53	8.08397127732943E-193
202	10	*LAMC2*	0.00	2.72	0.97	0.74	0
203	10	*KRT6A*	0.00	2.63	0.89	0.67	0
204	10	*LAMA3*	0.00	2.51	0.8	0.65	2.88170788687901E-275
205	10	*LAMB3*	0.00	1.81	0.8	0.62	3.27852024209837E-276
206	10	*KRT17*	0.00	1.79	0.49	0.51	1.15843945877946E-40
207	10	*COL17A1*	0.00	1.77	0.68	0.59	5.38500051358062E-140
208	10	*INPP4B*	0.00	1.35	0.81	0.63	2.04825297095848E-212
209	10	*KRT14*	0.00	1.22	0.51	0.59	3.23324533571871E-21
210	10	*TMEM132D*	0.00	1.22	0.62	0.56	1.35736286299835E-92
211	10	*SEMA3C*	0.00	1.15	0.95	0.8	1.78100630981075E-230

212	10	*THBS1*	0.00	1.13	0.88	0.7	1.06459363129595E-135
213	10	*DSC2*	0.00	1.13	0.85	0.68	9.22506907450692E-173
214	10	*ITGB4*	0.00	1.12	0.49	0.45	3.83789725369348E-66
215	10	*TNS4*	0.00	1.09	0.42	0.52	1.58597324817715E-08
216	10	*NTN4*	0.00	1.09	0.69	0.56	8.74503754757718E-152
217	10	*ITGA6*	0.00	1.08	0.66	0.63	4.50495301171499E-66
218	10	*LINC00511*	0.00	1.08	0.84	0.57	1.39860819563529E-216
219	10	*FRMD6*	0.00	1.06	0.78	0.61	8.84377502264119E-144
220	10	*CHST11*	0.00	1.05	0.83	0.67	5.58462409190851E-165