

Established Hepatic Stellate Cell Lines in Hepatology Research

Ralf Weiskirchen *

Institute of Molecular Pathobiochemistry, Experimental Gene Therapy and Clinical Chemistry (IFMPEGKC),
RWTH University Hospital Aachen, D-52074 Aachen, Germany

* Corresponding author. E-mail: rweiskirchen@ukaachen.de (R.W.)

Received: 6 February 2023; Accepted: 17 March 2023; Available online: 21 March 2023

ABSTRACT: Hepatic stellate cells comprise a minor cell population in the liver, playing a key role in the pathogenesis of hepatic fibrosis. In chronic liver damage, these cells undergo a transition from a quiescent to a highly proliferative phenotype with the capacity to synthesize large quantities of extracellular matrix compounds such as collagens. Because of their pivotal role in liver disease pathogenesis, this hepatic cell population has become the focus of liver research for many years. However, the isolation of these cells is time consuming and requires the trained laboratory personnel. In addition, working with primary cells requires the following of ethical and legal standards and potentially needs the approval from respective authorities. Therefore, continuous growing hepatic stellate cells have become very popular in research laboratories because they are widely available and easy to handle, and allow a continuous supply of materials, and further reduction of lab animal use in biomedical research. This communication provides some general information about immortalized hepatic stellate cell lines from mouse, rats and humans.

Keywords: Cell culture; Cell lines; Hepatic stellate cells; Myofibroblasts; Liver; Fibrosis; In vitro; Translational research



© 2023 The authors. This is an open access article under the Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Hepatic fibrosis is the result of an imbalance between production and degradation of extracellular matrix. It can be caused by various causes, such as chronic viral hepatitis infection, alcohol abuse, genetic disorders, drug intoxication, cholestasis, metabolic disorders, parasitic infections, and cryptogenic causes [1]. The most relevant profibrogenic cells operating as cellular key drivers in the pathogenesis of liver fibrosis are hepatic stellate cells (HSCs). In the normal liver, HSC reside in the perisinusoidal space between hepatocytes and the sinusoids and exhibit a quiescent phenotype with the main function of storing vitamin A. During chronic hepatic disease, HSC progressively lose their intracellular vitamin A content, and become activated and transdifferentiate into fibrogenic myofibroblasts (MFBs) that drive the fibrogenic process by expressing large quantities of extracellular matrix compounds such as collagens. The activation process is further marked by increased expression of α -smooth muscle actin (α -SMA) and other typical markers including collagen type I (Col I), platelet-derived growth factor receptor type β (PDGFR β), Vimentin, cysteine- and glycine-rich protein 2 (CSRP2), Fibulin, tissue inhibitor of metalloproteinase 1 (TIMP1), secreted protein acidic and rich in cysteine (SPARC), transgelin (TAGLN), and many others [1].

Much of our current knowledge of HSC/MFB biology has been gained through primary culture studies. When HSC are cultured on the uncoated plastic, they undergo a spontaneous activation process that strongly resembles those observed in the injured liver. However, the isolation and purification of highly pure primary HSC is rather complex and the limited supply and ever-increasing demand for these cells, combined with the additional tightening of formal standards in animal welfare policy and other ethical issues, make working with these cells increasingly difficult [2].

Moreover, challenges resulting from the demand to implement the 3R principle of “Replacement”, “Reduction”, and “Refinement” originally proposed by Russell and Burch in 1959 are additional hurdles, which oppose the use of primary cells in biomedical research [3]. Therefore, scientists have generated more than 30 infinite HSC cell lines originating from mouse, rat, or human HSC (Table 1).

Table 1. Established hepatic stellate cell lines used in biomedical research studies.

Origin	Cell Line	Citations *	Search Criteria/Reference	Establishment	Most Important Characteristics
Mouse (<i>Mus musculus</i>)	GRX	66	“GRX cells” or “GRX and hepatic”	Spontaneous after <i>Schistosoma mansoni</i> infection	STR, expression of Col I, producer of retrovirus
	SV68c-IS	1	“SV68c-IS”	SV40T	Expression of SV40T, α -SMA, desmin
	A640-IS (33 °C)/ A640-IS (37 °C)	1	“A640-IS”	tsSV40T	Expression of SV40T (33 °C), α -SMA, Col I
	M1-4HSC/M-HT	1	“M1-4HSC”	Spontaneous from p19 ^{ARF} mouse	Expression of GFAP, α -SMA, Col I, desmin
	A7	4	“A7” and “hepatic stellate cell”	H-2K ^b - <i>ts</i> -A58 mice carrying tsSV40T	Grows only under permissive condition (33 °C) and not under non-permissive conditions (37 °C)
	Col-GFP HSC	3	“Col GFP HSC” not “GFP-Col-HSC”	SV40T	STR, Expression of SV40T, GFP, GFAP, α -SMA, Col I
	IMS/MT(-) and IMS/N	2	“IMS/MT”	Spontaneous from WT mice (IMS/N) or MT null mice (IMS/MT(-))	Expression of SV40T, α -SMA, desmin
	JS1 (C57/BI6 WT) JS2 (C57/BI6 TLR4 ^{-/-}) JS3 (C57/BI6 MyoD88 ^{-/-})	34	“JS1” or “JS-1” and “hepatic stellate”	SV40T	Expression of SV40T
	CFSC-2G, CFSC-3H, CFSC-5H, CFSC-8H, NFSC	>90	[4]	Spontaneous	Expression of Col I (to different content)
	HSC-T6	>600	“HSC-T6” [5]	SV40T	STR, Expression of SV40T, GFAP, α -SMA
Rat (<i>Rattus norvegicus</i>)	PAV-1	3	“PAV-1” and “hepatic”	Spontaneous	STR (unpublished), expression of α -SMA, Col I, desmin
	HSC-PQ	1	“HSC-PQ”	UV light	Expression of α -SMA, Col I, desmin
	BSC	2	“BSC” and “HSC line”	Spontaneous from rats subjected to BDL	Expression of GFAP, Col I, desmin
	MFBY2	1	“MFBY2”	Spontaneous from rats subjected to CCl ₄	Expression of GFAP, α -SMA, Col I
	T-HSC/Cl-6	21	“T-HSC/Cl-6”	SV40T	Expression of GFAP, α -SMA, Col I, desmin
	LSC-1	2	“LSC-1” and “hepatic stellate”	Spontaneous	Expression of GFAP, α -SMA, Col I, desmin
	RGF and RGF-N2	2	“RGF” and “hepatic stellate”	SV40T	Expression of GFAP, α -SMA
	LX-1	6	“LX-1 or LX1” and “hepatic stellate”	SV40T	Expression of SV40T, GFAP, α -SMA, Col I
	LX-2	1044	“LX-2 or LX2” and “hepatic stellate”	SV40T	STR, expression of GFAP, α -SMA, Col I
	LX-2 (Cas9)	0	[6,7]	SV40T + Cas9	Expression of endonuclease Cas9
Human (<i>Homo sapiens</i>)	LI90	49	“LI90” and “hepatic or liver”	Outgrow from epithelioid hemangioendothelioma	Expression of Col I, desmin
	TWNT-4	12	“TWNT-4”	hTERT/GFP	Expression of GFP, Col I
	TWNT-1	5	“TWNT-1”	hTERT/GFP	Expression of GFP, Col I
	GREF-X **	1	“GREF-X”	SV40T	Expression of Col I
	hTERT-HSC	5	“hTERT-HSC”	hTERT	Expression of GFAP, α -SMA, Col I
	HSC 180	1	[8]	Spontaneous derived from α -SMA positive cells of cirrhotic liver	Strongly positive for α -SMA; high producers of urokinase plasminogen activator during first 24 hours of culturing
	NPC-hTERT	1	“NPC-hTERT”	hTERT	Expression of α -SMA

* The number of citations was estimated by a PubMed search (conducted on 8 March 2023) using the given search terms; ** this cell line was shown to be of rat origin and listed by the International Cell Line Authentication Committee (ICLAC) under Registration ID: ICLAC-00123 as a misidentified cell line [9]. Abbreviations used are: α -SMA, α -smooth muscle actin (*Acta2*); BDL, bile duct ligation; Cas9, bacterial RNA-guided endonuclease (CRISPR-associated protein 9); CCl₄, carbon tetrachloride; Col I, collagen type I; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; hTERT, human telomerase reverse transcriptase; MT, metallothionein; STR, short tandem repeat; SV40T, large T-antigen from simian virus 40; tsSV40T, temperature sensitive SV40T; UV, ultraviolet; WT, wild type.

All these cell lines have specific cellular and morphological characteristics that are similar to those of primary HSC/MFB. They grow as adherent cells, express typical HSC/MFB markers, respond and transmit profibrogenic signals, and have the capacity to take up and store vitamin A. Therefore, these established HSC lines have been already important tools in investigations studying diverse aspects of HSC/MFB biology. Particularly, several of these lines have been extensively used in studies investigating aspects of profibrogenic signaling, extracellular matrix synthesis, retinoid metabolism, cellular toxicity, proliferation control, cell adhesion, and cell migration. In particular, murine cell line GRX [10,11], rat cell line HSC-T6 [5,12], different derivatives of rat cell line CFSC [4,13,14], and human LX-2 cell line [15,16] are used in many laboratories worldwide (cf. Table 1). The phenotype of most of these continuous growing lines determined by their overall biochemical and morphological characteristics suggests that most of these lines have to be classified as “activated HSC” or “fully transdifferentiated MFB” (Figure 1).

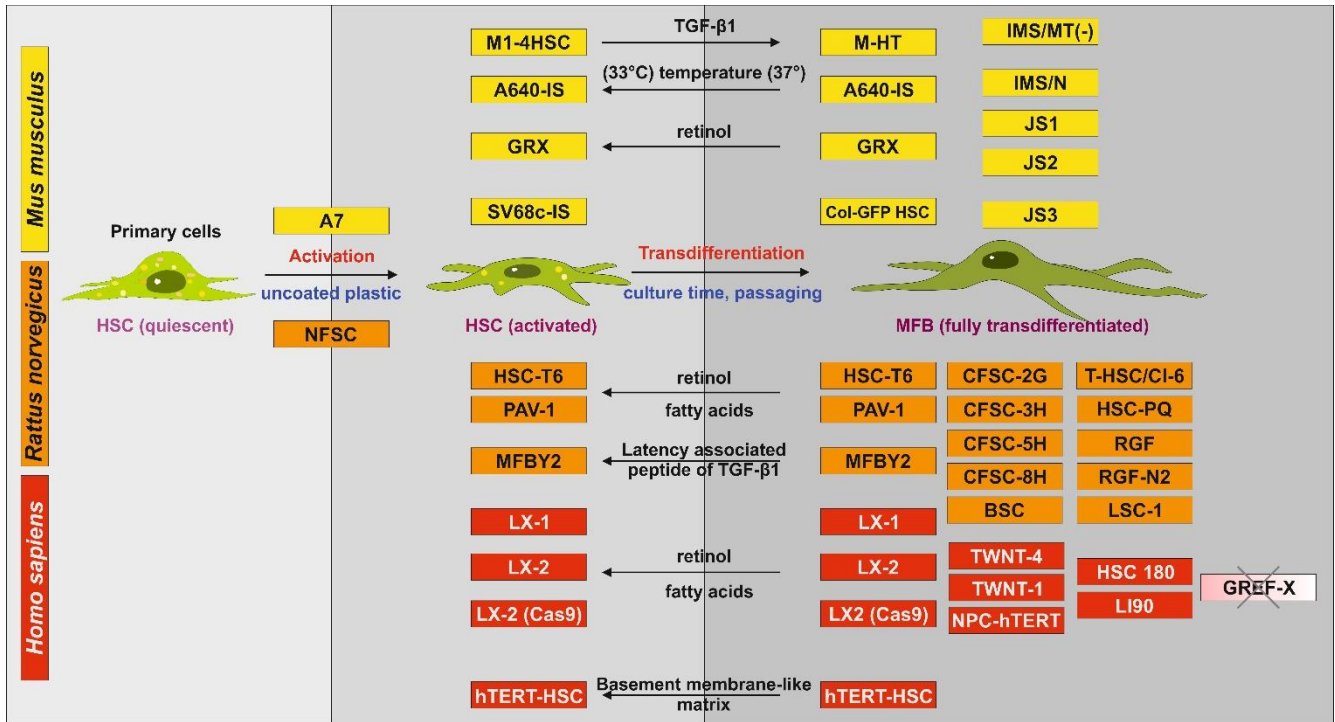


Figure 1. Classification of hepatic stellate cell lines according to their activation status. Most murine (yellow), rat (orange) and human (red) cell lines represent an activated or transdifferentiated phenotype. However, some of the lines (e.g., GRX, A640-IS, HSC-T6, PAV-1, MFBY2, LX-1, LX-2, hTERT-HSC) can be partly reverted into an activated phenotype by treatment with retinol, fatty acids, or the latency-associated peptide of TGF-β1. Other HSC cell lines lose some myofibroblastic features when cultured on special matrixes (e.g., hTERT-HSC) or when the temperature-sensitive large T antigen used for immortalization is inactivated by shifting the cells from the permissive to the non-permissive temperature (e.g., A640-IS). There are only a few cell lines that are not fully transdifferentiated (e.g., A7 does not proliferate under non-permissive condition; NFSC isolated from normal liver is less activated than the CFSC derivatives isolated from cirrhotic livers). Please note that the cell line GREF-X is listed in the repository of the International Cell Line Authentication Committee (ICLAC) as a misidentified cell line (registration ID: ICLAC-00123) [9].

Although some of these cell lines can partly revert into the “activated” phenotype by treatment with high concentrations of retinol, fatty acids, latency-associated peptide, or by culturing on special basement membrane-like matrix components, none is capable to fully acquire back a quiescent cell state. In contrast to these immortalized cell lines, it has been shown that primary MFB isolated from human cirrhotic livers changed to an α-SMA negative, lipid droplet-containing quiescent morphology when re-cultured on a basement membrane-like substrate (i.e., Matrigel) [17]. Moreover, this treatment further reduced the expression of Col I, while the expression of matrix metalloproteinase-1 (MMP-1) was induced, which underpins the reversal to an activated phenotype. Another study previously demonstrated that embedding the cells in a three-dimensionally distributed extracellular matrix prepared of rat tail type I collagen is suitable to revert their morphology, proliferation rate and functions [18].

Each cell line has some characteristics that can be used for cell authentication. The most important one is the short tandem repeat (STR) profile that is established for some continuous growing HSC lines (e.g., GRX, Col-GFP HSC, HSC-T6, CFSC-2G, LX-2), which allows to confirm cell line identity and to identify misidentification or cross-contamination. In addition, the detection of typical HSC/MFB markers such as α-SMA, Col I, desmin, and glial fibrillary acidic protein (GFAP) might be useful for identification. Other cell lines express the green fluorescent protein (GFP), Simian virus 40 large T-antigen (SV40T), or human telomerase reverse transcriptase (hTERT) that were stably introduced into these cells during the immortalization process. Moreover, some HSC lines have special growth properties, morphologies, or other characteristic features that are useful for cell identification.

Nowadays, several of these HSC cell lines have become in particular attractive experimental tools in studies testing compounds for their anti-fibrotic activity. Palmitic acid for instance induced growth arrest and decreased α -SMA and Col I expression in the rat HSC line PAV-1 [19]. Likewise, glycyrrhizic acid attenuated liver fibrosis in human cell line LX-2 and carbon tetrachloride (CCl₄)-induced liver fibrosis, showing that results obtained in continuous growing HSC lines can provide valuable hints for the *in vivo* situation [20]. Anti-fibrotic effects of extracellular vesicles derived from tea leaves were also reported for both HSC line LX-2 and a mouse model of CCl₄-induced liver fibrosis [21]. In the same models, a derivative of ferulic acid termed FA11 showed excellent anti-fibrotic activity as assessed by reduced HSC activation and alleviated collagen expression [22].

Moreover, the anti-parasitic drug Ivermectin was recently shown to block activation in cultured CFSC cells and in mouse repeatedly subjected to CCl₄ [23]. In all these and many other studies, the efficacy of the drugs was majorly measured by their activity to suppress expression of genes associated with HSC activation and/or transdifferentiation (e.g., α -SMA, Col I), which underpins the key function of HSC in promoting fibrosis in response to liver injury. Unfortunately, most of the results of these preclinical studies were not translated to the clinic yet [24].

Nevertheless, all these *in vitro* studies conducted in established HSC lines show that anti-fibrotic acting agents are majorly effective in counteracting intracellular reactive oxygen species (ROS) formation, pro-inflammatory and pro-fibrotic signaling, and extracellular matrix generation. Therefore, these studies provide the basis to develop effective drugs for treatment of human liver disease.

2. Advantages and Disadvantages for Working with Continuous HSC/MFB Lines

There are only a few HSC/MFB lines available that were spontaneously immortalized in culture without further treatment, while most of them were derived from primary HSC cultures that are either transformed with the Simian virus 40 large T-antigen (SV40T), immortalized by introducing telomerase reverse transcriptase (TERT) activity, or by exposure to intensive radiation with ultraviolet (UV) light [6]. Consequently, respective cell lines in which genes have been artificially altered or incorporated in some way have to be classified as genetically modified cells and require a biosafety risk assessment. Nevertheless, although the primary HSC biology cannot be always accurately replicated, working with continuous growing HSC lines offers several fundamental advantages. They are cost effective, easy to handle, allow bypassing ethical concerns associated with the use of primary animal and human materials, and provide an unlimited supply of biological materials (Table 2).

Table 2. Advantages and disadvantages of working with continuous growing HSC lines versus primary cells.

Property/Feature	Primary HSC	Immortalized HSC
Research relevance	High	Low
Ease-of-use	Needs optimized culture conditions	Standard culture conditions
Costs	High (requires trained personnel)	Cost-effective
Consistency	Medium	High
Cell growth potential	Low (contact inhibited, low multiplication)	High (potentially not contact inhibited, robust growth, high cell density)
Life span	Limited/finite	Unlimited/infinite
Origin	Isolated from donor organism	Derived spontaneously or by manipulation (e.g., with hTERT, SV40T, UV), mostly uniform clonal cell type)
Transfectability	Low	High (easy to transfect/manipulate)
Handling	Complex	Robust and easy to handle
Cell characteristics	May change with each passage	Stable (if not over-passaged)
Ethical objections/ Animal welfare	Needs votes from respective authorities	Not necessary or negligible
Cellular integrity	High	Low (e.g., genetic drift)
<i>In vivo</i> model	Yes (or close to)	No
Cryoconservation	Not easy and often not profitable or possible	Very easy
Susceptibility for cytokines and chemokines	High	Can be restricted
Gene expression	(Supposed) to express typical HSC gene profiles	In some cases they express unique gene patterns or abnormal traits
Genetics	Similar chromosome number as parent tissue but considerable variation in populations between preparations possible	Potential alterations in genotype during prolonged culturing
Risk of contamination	High	Low
Cell misidentification	Should be impossible	Possible
Cell authentication	Not necessary	STR profiling should be done regularly

Abbreviations used: hTERT, human telomerase reverse transcriptase; STR, short tandem repeat; SV40T, large T-antigen of Simian virus 40; UV, ultraviolet light.

Based on their clonal origin, they provide a consistent cellular source, allowing the establishment of highly reproducible results. Nevertheless, genetic drift occurring in over-passaged cultures and the immortalized phenotype *per se* could have strong impact on normal cell functions such as cell proliferation, cell cycle control, and many other native functions of HSC/MFB. In particular, the

indefinite replicative capacity, escape from cell growth crisis, and potentially altered responsiveness to external and internal stimuli might provide experimental pitfalls that provoke the experimental findings other than those obtained in primary cells. Consequently, it will be of fundamental importance to reproduce and verify key experimental findings in primary cells or other suitable models. Last but not the least, it is not possible to translate findings obtained in cell lines directly to human disease. There are a multitude of factors that influence the data generated in cell culture experiments and cell lines do not fully represent what is occurring *in vivo*. Therefore, in some cases primary HSC are the better choice for more accurately reproducing cellular features of HSC in human liver disease.

3. HSC/MFB Marker Gene Expression

A common feature of the activated HSC during chronic liver disease is the expression and upregulation of typical HSC/MFB marker genes such as α -SMA, SPARC, Vimentin, and many other liver fibrosis-related core genes [1] (Figure 2).

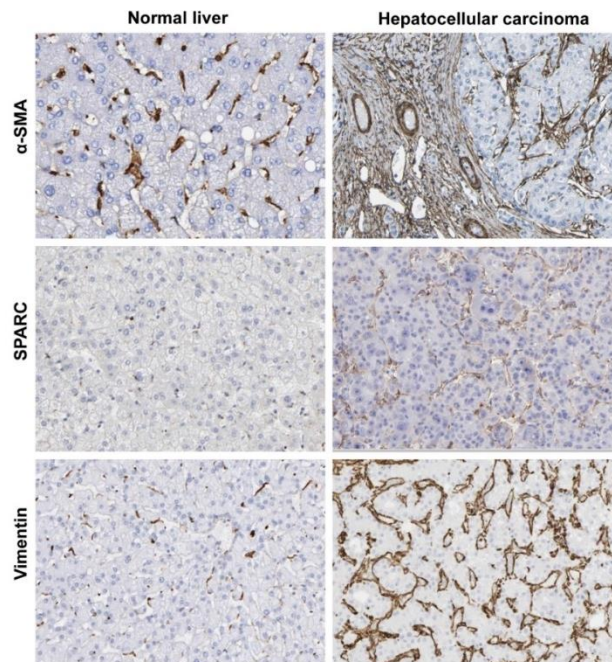


Figure 2. Immunohistochemistry of healthy and diseased human liver tissues. The tissues were stained for α -smooth muscle actin (α -SMA), secreted protein acidic and rich in cysteine (SPARC), and Vimentin representing three classical HSC/MFB markers that become increasingly expressed during hepatic insult. All images were taken from the Human Protein Atlas database [25].

Several of these markers are positively correlated with the degree of liver fibrosis, while others such as the glial fibrillary acidic protein (GFAP) show the reduced expression in HSC during fibrosis progression. Therefore, the expression of these markers is often used to estimate the activation status of HSC/MFB or therapeutic potential of novel anti-fibrotic therapies. We have recently analyzed the transcriptomes of human cell line LX-2 (unpublished) and rat cell lines HSC-T6 [7], CFSC-2G [10], and PAV-1 (unpublished) by mRNA-Seq whole transcriptome analysis. Importantly, all these cell lines were shown to express typical HSC/MFB gene expression traits that include α -SMA (*ACTA2*), biglycan (*BGN*), Caldesmon 1 (*CALD1*), *COL1A1*, *COL3A3*, *SPARC*, Fibronectin (*FBN1*), Vimentin (*VIM*), and many others (Table 3). In addition, desmin representing a characteristic HSC/MFB marker in rodents was expressed in all rat HSC lines, while the expression was only rather low in human cell line LX-2. Noteworthy, several HSC/MFB markers (e.g., *PDGFRB*, Cellular communication network factor 2 (*CCN2/CTGF*), Retinol binding protein 1 (*RBPI*), Heart- and neural crest derivatives-expressed 2 (*HAND2*), Transgelin (*TAGLN*), and Lumican (*LUM*)) were expressed in significant different quantities in the three rat HSC lines, suggesting that each cell lines is more or less suitable for studies in which aspects of respective functions of these genes should be investigated.

For example, the highest expression of *TAGLN* was found in HSC-T6 (~764 TPM) and CFSC-2G (~655 TPM), while the mRNA content was about 39–45 fold lower in PAV-1 cells (~17 TPM). In liver, *TAGLN* is a typical marker of smooth muscle cells (i.e., such as HSC), correlating to the expression of *COL3A1*, *FBN1*, *LUM*, and *ACTA2* (Figures 3 and 4), suggesting that studies aiming to address functions of *TAGLN* biology should be best performed in HSC-T6 or CFSC-2G.

Contrarily, the expressions of the cellular network factors 2 (CCN2) and 3 (CCN3) as well as *PDGFRB* show higher expression in PAV-1 cells compared to HSC-T6 or CFSC-2G, suggesting that gene effects of CCN2, CCN3 or *PDGFRB* should be best analyzed in PAV-1 cells. This cell line was originally established in year 2002 as a new rat HSC line with capacity to convert retinol into retinoic acid [26]. Matching to this, the expression of *RBPI* is highest in cell line HSC-T6. Moreover, this is in agreement with the previous reports showing that this cell line expresses all necessary compounds such as $RAR\alpha$, $RAR\beta$, $RAR\gamma$, $RXR\alpha$, $RXR\beta$,

RXR γ , CRBP1, and ARAT, making this cell line particularly useful for studies of retinoid metabolism [5,12]. Certainly, these suggestions are only rough indications that need to be independently confirmed before conducting extensive studies.

Table 3. Expression of canonical HSC/MFB markers in representative immortalized human and rat HSC/MFB lines.

Gene/Protein Product	Human		Rat	
	LX-2 *	HSC-T6 *	CFSC-2G *	PAV-1 *
<i>ACTA2</i> / α -SMA	96.0886541	2.377	10.9245	58.2526
<i>BGN</i> /Biglycan	2.73012	841.461	1687.81	3509.01
<i>CALDI</i> /Caldesmon 1	507.400728	227.555	297.848	578.118
<i>CCL2</i> /Chemokine CC motif ligand 2	14.3178076	55.5073	35.1433	123.678
<i>COL1A1</i> /Collagen type I α 1	693.60197	2033.74	1408.42	2470.3
<i>COL3A1</i> /Collagen type III α 1	167.732678	2128.9	64.4932	423.594
<i>COLEC11</i> /Collectin 11	1.118629	0.0744481	3.84107	1.29906
<i>CCN2</i> /Cellular communication network factor 2 (CTGF)	256.742	45.756531	353.12398	1365.66302
<i>CCN1</i> /Cellular communication network factor 1 (CYR61)	937.72148	24.9337	151.881	192.761
<i>DCN</i> /decorin	1.4164593	73.9243	0.272873	44.9865
<i>HGF</i> /hepatocyte growth factor	0.3430743	0.547905	0.109268	8.39761
<i>IGFBP3</i> /Insulin-like growth factor-binding protein 3	33.709325	0.599421	0.469016	138.786
<i>IGFBP6</i> /Insulin-like growth factor-binding protein 6	152.83748	529.325	215.876	406.343
<i>IGFBP7</i> /Insulin-like growth factor-binding protein 7	248.148175	14.7997	72.1664	383.173
<i>MYL9</i> /Myosin light chain 9	1395.217	2.44449	1.44567	1.13264
<i>OLFML3</i> /Olfactomedin-like 3	20.165811	178.359	128.959	549.215
<i>RBPI</i> /Retinol binding protein 1	0.398955	25.3842	0	1.28709
<i>SPARC</i> /Secreted protein acidic and rich in cysteine	1179.48348	2188.34	1040.23	2414.09
<i>TAGLN</i> /Transgelin	835.88305	764.439414	654.744194	17.3826
<i>TPM2</i> /Tropomyosin	624.13319	388.3356	677.053	687.2863
<i>TIMP1</i> /Tissue inhibitor of metalloproteinase 1	376.35223	5518.57	714.162	646.539
<i>CSR2</i> /Cysteine- and glycine-rich protein 2	62.755821	199.67237	63.86065	280.13174
<i>DES</i> /Desmin **	0.1318046	5.26991	21.9033	45.9847
<i>GFAP</i> /Glial fibrillary acidic protein	1.82362715	0.60103	0.108417	0.0115619
<i>VIM</i> /Vimentin	3380.30598	2054.81	3853.69	7038.67
<i>LRAT</i> /Lecithin retinol acyltransferase	0.0876739	0.0125274	0.0883818	0.0160815
<i>PDGFRB</i> /Platelet-derived growth factor receptor β	24.108819	15.385941	0.1721456	108.38523
<i>HAND2</i> /Heart- and neural crest derivatives-expressed 2	5.159146	6.28394	24.6465	203.225
<i>CYGB</i> /Cytoglobin	1.25045	1.25666	0.290752	0.619563
<i>SYNM</i> /Synemin	13.3249814	1.16676	48.209828	22.70161
<i>NGFR</i> /Nerve growth factor receptor	3.09171	0.433394	4.02414	0.135868
<i>FBNI</i> /Fibronectin	89.8374679	16.5948	2.29356	27.8874
<i>LUM</i> /Lumican	4.884931	12.8412	0.694259	86.2333
<i>GAPDH</i> /Glyceraldehyde-3-phosphate dehydrogenase	4846.55905	8842.06	6186.63	3994.18

* All expression values are given in transcripts per million (TPM). ** HSC/MFB marker in rodents (not always expressed in human livers). Expression data for HSC-T6 and CFSC-2G were taken from previous studies [4,5], while data for LX-2 and PAV-1 was taken from unpublished data.

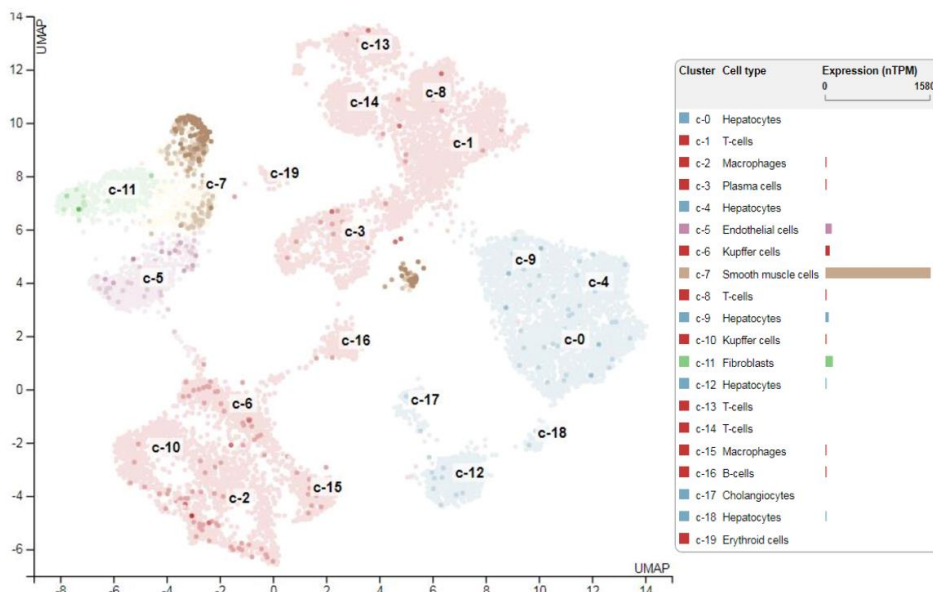


Figure 3. Transgelin expression in liver. Single cell mRNA sequencing of various liver cell subpopulations demonstrates the highest expression of transgelin in the smooth muscle cell fraction (i.e., HSC). Data was taken from the Human Protein Atlas database [25].

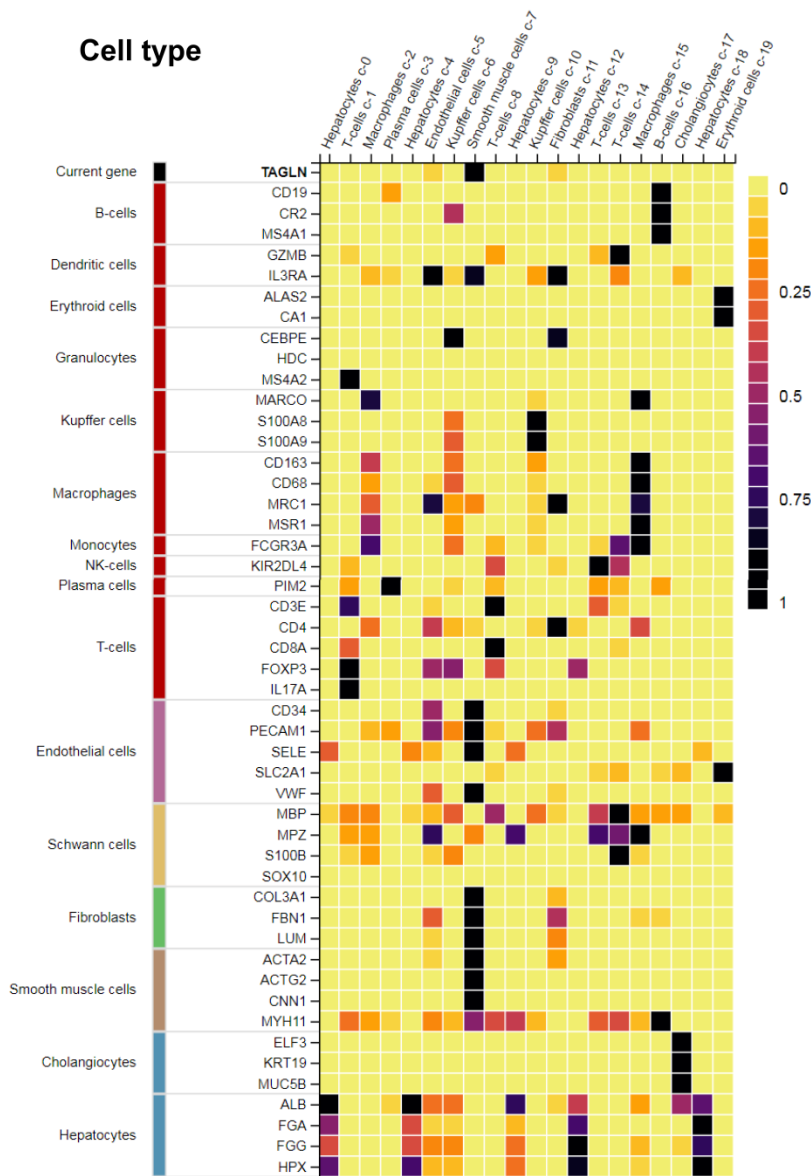


Figure 4. Correlation of Transgelin (*TAGLN*) expression to other genes. Transgelin shows the highest expression in smooth muscle cells. The expression of this smooth muscle cell-specific gene correlates with the expression of Collagen type III $\alpha 1$ (*COL3A1*), Fibronectin 1 (*FBN1*), Lumican (*LUM*), and α -smooth muscle actin (*ACTA2*). Data was taken from the Human Protein Atlas database [25].

4. Functional Aspects of Immortalized HSC Lines

As discussed, it is well-accepted that HSC lines are appropriate models to study general cellular and biochemical aspects of HSC biology. They express almost all relevant key markers of HSC/MFB and exhibit many features that are hallmarks of HSC, confirming that they originate from primary HSC. Nevertheless, it is obvious that all these cell lines also have pronounced differences when comparing them to primary HSC/MFB occurring in the *in vivo* situation. First, primary HSC have an overall low doubling time and can be passaged only two or three times before they enter replicative senescence and die, while established HSC lines grow significantly faster and represent unlimited self-replicating sources that can be continuously passaged. Of course, the infinite growth character of cell lines is caused by immortalized agents (e.g., SV40T, hTERT) that override the cell cycle and remove the biological brakes on proliferative control. This may *per se* modulate the general biology of the cells and affect biochemical pathways that might be relevant to HSC biology. Second, it is obvious that a clonal cell line encompasses a static phenotype, while primary HSC in culture undergo a transition from a quiescent to an activated and transdifferentiated phenotype associated with complex cellular alterations. Third, the expression repertoire of HSC lines might differ from other HSC lines and their primary counterparts. There are also some general biochemical differences between continuous growing HSC lines and primary HSC. Exemplarily, it is well-accepted that primary HSC are hardly transfectable [27], while most of the lines are accessible for the uptake of foreign DNA. Similarly, continuous HSC lines can show divergent responsiveness towards growth factors. For instance, PDGF, representing the most effective mitogen for primary HSC [28,29], has only marginal stimulatory effects on proliferation on some immortal HSC lines [8]. Moreover, single-cell RNA sequencing, cell tracing experiments, and genetic ablation

experiments have shown that primary HSC form a heterogeneous cell population *in vivo* [30–32]. It is obvious that this heterogeneity cannot be mimicked in a cell line of clonal origin. Finally, several cell lines need to be classified as the genetically modified, thereby providing potential biohazards. Therefore, the handling of those lines might require the approval from the responsible authorities and the specific institutional requirements that must be conform to legal requirements of the country in which experiments with respective cells are conducted.

Nonetheless, continuous HSC lines are key biological tools for a large abundance of studies aiming to address aspects of extracellular matrix synthesis/turnover, retinoid metabolism, fibrogenic signaling, cellular contractility/mobility, and drug efficacies. However, the above mentioned limitations in their usability and the fact that continuous cell lines are prone to genotypic and phenotypic drift limit their reasonable applicability in some studies.

5. Conclusions

Immortalized HSC/MFB lines have an unlimited life-span and stable phenotype. They are easy to handle and are widely available. Therefore, several continuous lines such as LX-2, GRX, HSC-T6 and diverse CFSC derivatives have become popular tools in biomedical research. They further allow replacing or avoiding animal use in experiments, thereby fostering the 3R principle (Replacement, Reduction and Refinement) launched over 60 years ago by Russell and Burch. Furthermore, they can be maintained in culture for an extended period of time, retaining their phenotype and function for many passages. Based on their clonal origin, continuous growing HSC/MFB cell lines provide a homogenous population, allowing the production of large quantities of uniform cells in a short period of time. Nevertheless, over-passaging, genetic drift, escape from cell growth crisis, altered responsiveness to external and internal stimuli might limit the use of these cells in some applications. Consequently, it has to be kept in mind that infinite HSC/MFB cell lines under some conditions do not behave identically equal to primary cells and can therefore not fully replace primary cells in hepatology research.

Acknowledgments

The author thanks Sabine Weiskirchen for preparing Figure 1 and the Graphical abstract of this Communication.

Ethics Statement

Not applicable.

Informed Consent Statement

Not applicable.

Funding

The laboratory of RW is supported by the German Research Foundation (grants WE2554/13-1, WE2554/15-1, WE2554/17-1) and the Interdisciplinary Centre for Clinical Research within the faculty of Medicine at the RWTH Aachen University (grant PTD 1-5). None of the funders had any role in the design of the study and decision to publish or preparation of the manuscript.

Declaration of Competing Interest

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Acharya P, Chouhan K, Weiskirchen S, Weiskirchen R. Cellular mechanisms of liver fibrosis. *Front. Pharmacol.* **2021**, *12*, 671640. doi:10.3389/fphar.2021.671640
2. Meurer SK, Weiskirchen S, Tag CG, Weiskirchen R. Isolation, purification, and culture of primary murine hepatic stellate cells: An update. *Methods Mol. Biol.* **2023**, *2669*, in press. doi:10.1007/978-1-0716-3207-9_1.
3. Russell WMS, Burch R. *The Principles of Humane Experimental Technique*; Methuen: London, UK, 1959.
4. Nanda I, Schröder SK, Steinlein C, Haaf T, Buhl EM, Grimm DG, et al. Rat hepatic stellate cell line CFSC-2G: Genetic markers and short tandem repeat profile useful for cell line authentication. *Cells* **2022**, *11*, 2900. doi:10.3390/cells11182900
5. Nanda I, Steinlein C, Haaf T, Buhl EM, Grimm DG, Friedman SL, et al. Genetic characterization of rat hepatic stellate cell line HSC-T6 for in vitro cell line authentication. *Cells* **2022**, *11*, 1783. doi:10.3390/cells11111783
6. Friedman SL, Weiskirchen R. Working with immortalized hepatic stellate cell lines. *Methods Mol. Biol.* **2023**, *2669*, in press. doi:10.1007/978-1-0716-3207-9_8.
7. Merck SCC613—LX-2 Cas9 Human Hepatic Stellate Cell Line. Available online: https://www.emdmillipore.com/US/en/product/LX-2-Cas9-Human-Hepatic-Stellate-Cell-Line,MM_NF-SCC613?ReferrerURL=https%3A%2F%2Fwww.google.com%2F (accessed on 17 March 2023).

8. González-Cuevas J, Bueno-Topete M, Armendariz-Borunda J. Urokinase plasminogen activator stimulates function of active forms of stromelysin and gelatinases (MMP-2 and MMP-9) in cirrhotic tissue. *J. Gastroenterol. Hepatol.* **2006**, *21*, 1544–1554.
9. International Cell Line Authentication Committee (ICLAC) Register of Misidentified Cell Lines. Available online: <https://iclac.org/> (accessed on 17 March 2023).
10. Borojevic R, Monteiro AN, Vinhas SA, Domont GB, Mourão PA, Emonard H, et al. Establishment of a continuous cell line from fibrotic schistosomal granulomas in mice livers. *In Vitro Cell Dev. Biol.* **1985**, *21*, 382–390. doi:10.1007/BF02623469
11. Schröder SK, Schüler HM, Petersen KV, Tesaro C, Knudsen BR, Pedersen FS, et al. Genetic and molecular characterization of the immortalized murine hepatic stellate cell line GRX. *Cells* **2022**, *11*, 1504. doi:10.3390/cells11091504
12. Vogel S, Piantedosi R, Frank J, Lalazar A, Rockey DC, Friedman SL, et al. An immortalized rat liver stellate cell line (HSC-T6): a new cell model for the study of retinoid metabolism in vitro. *J. Lipid Res.* **2000**, *41*, 882–893. doi:10.1016/S0022-2275(20)32030-7
13. Greenwel P, Schwartz M, Rosas M, Peyrol S, Grimaud JA, Rojkind M. Characterization of fat-storing cell lines derived from normal and CCl₄-cirrhotic livers. Differences in the production of interleukin-6. *Lab. Invest.* **1991**, *65*, 644–653.
14. Greenwel P, Rubin J, Schwartz M, Hertzberg EL, Rojkind M. Liver fat-storing cell clones obtained from a CCl₄-cirrhotic rat are heterogeneous with regard to proliferation, expression of extracellular matrix components, interleukin-6, and connexin 43. *Lab. Invest.* **1993**, *69*, 210–216.
15. Xu L, Hui AY, Albanis E, Arthur MJ, O’Byrne SM, Blaner WS, et al. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* **2005**, *54*, 142–151. doi:10.1136/gut.2004.042127
16. Weiskirchen R, Weimer J, Meurer SK, Kron A, Seipel B, Vater I, et al. Genetic characteristics of the human hepatic stellate cell line LX-2. *PLoS ONE* **2013**, *8*, e75692. doi:10.1371/journal.pone.0075692
17. Sohara N, Znoyko I, Levy MT, Trojanowska M, Reuben A. Reversal of activation of human myofibroblast-like cells by culture on a basement membrane-like substrate. *J. Hepatol.* **2002**, *37*, 214–221. doi:10.1016/s0168-8278(02)00103-4
18. Senoo H, Imai K, Sato M, Kojima N, Miura M, Hata R. Three-dimensional structure of extracellular matrix reversibly regulates morphology, proliferation and collagen metabolism of perisinusoidal stellate cells (vitamin A-storing cells). *Cell Biol. Int.* **1996**, *20*, 501–512. doi:10.1006/cbir.1996.0065
19. Abergel A, Sapin V, Dif N, Chassard C, Darcha C, Marcand-Sauvant J, et al. Growth arrest and decrease of alpha-SMA and type I collagen expression by palmitic acid in the rat hepatic stellate cell line PAV-1. *Dig. Dis. Sci.* **2006**, *51*, 986–995. doi:10.1007/s10620-005-9031-y
20. Guo M, Wang Z, Dai J, Fan H, Yuan N, Gao L, et al. Glycyrrhizic acid alleviates liver fibrosis in vitro and in vivo via activating CUGBP1-mediated IFN- γ /STAT1/Smad7 pathway. *Phytomedicine* **2022**, *112*, 154587. doi:10.1016/j.phymed.2022.154587
21. Gong Q, Zeng Z, Jiang T, Bai X, Pu C, Hao Y, et al. Anti-fibrotic effect of extracellular vesicles derived from tea leaves in hepatic stellate cells and liver fibrosis mice. *Front. Nutr.* **2022**, *9*, 1009139. doi:10.3389/fnut.2022.1009139
22. Xue T, Yue L, Zhu G, Tan Z, Liu H, Gan C, et al. An oral phenylacrylic acid derivative suppressed hepatic stellate cell activation and ameliorated liver fibrosis by blocking TGF- β 1 signalling. *Liver Int.* **2023**, *43*, 718–732. doi:10.1111/liv.15488
23. Ying H, Li L, Zhao Y, Ni F. Ivermectin attenuates CCl₄-induced liver fibrosis in mice by suppressing hepatic stellate cell activation. *Int. J. Mol. Sci.* **2022**, *23*, 16043. doi:10.3390/ijms232416043
24. Weiskirchen R. Hepatoprotective and anti-fibrotic agents: It’s time to take the next step. *Front. Pharmacol.* **2016**, *6*, 303. doi:10.3389/fphar.2015.00303
25. The Human Protein Atlas. The Open Access Resource for Human Proteins. Available online: <https://www.proteinatlas.org/> (accessed on 17 March 2023).
26. Sauvant P, Sapin V, Abergel A, Schmidt CK, Blanchon L, Alexandre-Gouabau MC, et al. PAV-1, a new rat hepatic stellate cell line converts retinol into retinoic acid, a process altered by ethanol. *Int. J. Biochem. Cell Biol.* **2002**, *34*, 1017–1029.
27. Weiskirchen R, Kneifel J, Weiskirchen S, van de Leur E, Kunz D, Gressner AM. Comparative evaluation of gene delivery devices in primary cultures of rat hepatic stellate cells and rat myofibroblasts. *BMC Cell Biol.* **2000**, *1*, 4. doi:10.1186/1471-2121-1-4
28. Pinzani M, Gesualdo L, Sabbah GM, Abboud HE. Effects of platelet-derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat-storing cells. *J. Clin. Invest.* **1989**, *84*, 1786–1793. doi:10.1172/JCI114363
29. Borkham-Kamphorst E, van Roeyen CR, Ostendorf T, Floege J, Gressner AM, Weiskirchen R. Pro-fibrogenic potential of PDGF-D in liver fibrosis. *J. Hepatol.* **2007**, *46*, 1064–1074. doi:10.1016/j.jhep.2007.01.029
30. Magness ST, Bataller R, Yang L, Brenner DA. A dual reporter gene transgenic mouse demonstrates heterogeneity in hepatic fibrogenic cell populations. *Hepatology* **2004**, *40*, 1151–1159. doi:10.1002/hep.20427
31. Filliol A, Saito Y, Nair A, Dapito DH, Yu LX, Ravichandra A, et al. Opposing roles of hepatic stellate cell subpopulations in hepatocarcinogenesis. *Nature* **2022**, *610*, 356–365. doi:10.1038/s41586-022-05289-6
32. Krenkel O, Hundertmark J, Ritz TP, Weiskirchen R, Tacke F. Single cell RNA sequencing identifies subsets of hepatic stellate cells and myofibroblasts in liver fibrosis. *Cells* **2019**, *8*, 503. doi:10.3390/cells8050503