

Cellular plasticity of trans- and dedifferentiation markers in human hepatoma cells *in vitro* and *in vivo*

SAMIR JABARI¹, MATTHIAS MEISSNITZER^{2*}, KARL QUINT¹, SUSANNE GAHR¹,
TILL WISSNIOWSKI¹, ECKHART G. HAHN¹, DANIEL NEUREITER^{2*} and MATTHIAS OCKER¹

¹Department of Medicine 1, University Hospital Erlangen, Erlangen, Germany;

²Institute of Pathology, Salzburger Landeskliniken, Paracelsus Private Medical University, Salzburg, Austria

Received February 4, 2009; Accepted March 30, 2009

DOI: 10.3892/ijo_00000314

Abstract. Tumor cells have the capability to trans- and to dedifferentiate, for example by reactivating embryonic development genes and stem cell characteristics. The aim of our study was to show the differential expression of stem- and progenitor cell markers in human hepatocellular carcinoma cell lines (HCC). Different human HCC cell lines (HUH7, HUH7 5-15, HUH7 pcDNA3.1, Hep3B and HepG2) were cultured under standard conditions *in vitro* or implanted subcutaneously (5×10^6 cells) in male NMRI mice. Specimens were characterized by quantitative real-time PCR, Western blotting, methylation-specific PCR and immunohistochemistry for markers of differentiation (cytokeratins, vimentin), embryonic development or stem cells (PTC, PDX-1, SHH, Thy1, c-kit, CD34, β -catenin, Ki-67). The investigated HCC cell lines showed different patterns of marker expression allowing to distinguish four distinct groups: the classical cholangiocellular type (Huh-7, Huh-7 pcDNA3.1, Hep3B) with expression of CK7/19, β -catenin and CD34; a dedifferentiated mesenchymal-proliferative type (Huh-7 5-15) characterized by CK19, Vimentin and Ki-67; a dedifferentiated embryonic-development type (Hep3B implanted in matrigel) with expression of CK19, β -catenin and PTC and a classical HCC type (HepG2) showing CK18/19 and β -catenin expression. HCC cell lines showed significantly different expression patterns of differentiation markers in a xenograft model. Furthermore, direct association of some markers was observed. The groups differ from each other in expression patterns, but also show that environmental factors play an important role in the behaviour of cells.

Introduction

The liver is an epithelial organ which consists mainly of two distinct differentiated cell types, hepatocytes and bile-duct cells (1,2). Bile duct cells are limited to the periportal region of the liver, while hepatocytes are present throughout the whole liver. Although the phenotype of these two cell types is different, they are thought to derive from one single stem cell, the oval cell, which is located at the periportal region (3-5). Oval cells are conceived to be the progenitor cells of both cell types, streaming out from the periportal region to repopulate, for example, injured liver tissue. It is still unclear which role this stem cell plays in postnatal human liver and whether it gives rise to both cell types or not (6-13). Other data show that division of hepatocytes may be the leading mechanism of hepatocellular renewal (11,14), while a third theory indicates that migrating bone marrow cells give rise to progenitor and terminated liver cell types (3-5).

Trans- and dedifferentiation programs as well as putative tumor stem cells have been identified in different human gastrointestinal cancers (15) and we have shown previously that pancreatic cancer cell lines show a differential expression of embryonic regulatory genes dependent on their environment (16,17).

Although some hepatocellular carcinoma cells (HCC) morphologically resemble oval cells as well as other hepatocytes, it is still uncertain whether hepatoma cells re-acquire embryonic pathways and therefore resemble stem cells or are really of stem cell origin (2,5). Different markers have been found to distinguish between progenitor cells (CK19, THY1) and differentiated (CK8, CK18) hepatocytes and we therefore investigated the epigenetic regulation of embryonic differentiation genes (PTC, SHH, THY1, c-kit and PDX-1) by methylation-specific PCR (MSP), quantitative real-time RT-PCR, immunoblotting and immunohistochemistry from typical HCC cell lines and xenografts established from these cells.

Materials and methods

Cell culture. HepG2 cells were cultured in RPMI-1640 medium containing 10% (v/v) fetal bovine serum, penicillin (10^6 U/l), streptomycin (10 mg/l) and 0.5% (v/v) gentamycin. Hep3B and HUH7 hepatoma cells were cultured in Dulbecco's

Correspondence to: Dr Matthias Ocker, Department of Medicine 1, University Hospital Erlangen, Ulmenweg 18, D-91054 Erlangen, Germany
E-mail: matthias.ocker@uk-erlangen.de

*Contributed equally

Key words: hepatocellular carcinoma, embryonic marker genes, trans- and dedifferentiation

minimal essential medium (DMEM) with the same supplements. HUH7pcDNA3.1 and the HCV replicon cell line HUH7 5-15, which stably expresses the non-structural hepatitis C virus genes NS3 through NS5B, were a gift of R. Bartenschlager (Department of Virology, University Heidelberg, Germany) (18,19) and were cultured in DMEM medium as described with 2% (v/v) G418. HUH7 5-15 was selected to investigate the influence of viral genes on cellular differentiation in HCC tumorigenesis and to compare the results with the parental cell line HUH7 and the transfection control HUH7pcDNA3.1. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. All cell culture media and supplements were obtained from Biochrom, Berlin, Germany.

Xenograft model. HCC cell lines were harvested and resuspended in sterile physiologic NaCl₂ solution. Cells (5×10⁶) were injected subcutaneously into the flank of 4- to 6-week old male NMRI mice (Harlan Winkelmann GmbH, Borcheln, Germany). For each cell line, three animals were used. Animals were kept in a light- and temperature-controlled environment and provided with food and water *ad libitum*. After 25 to 60 days, animals were sacrificed by cervical dislocation and specimens of tumors were either fixed in 10% phosphate-buffered formalin or snap-frozen in liquid nitrogen. Ethical approval was achieved before the beginning of the experiments.

RNA isolation and reverse transcription. Total cellular RNA was extracted by the use of peqGOLD RNAPure (PEQLAB Biotechnologie GmbH, Erlangen, Germany), as described in the manual. RNA was pooled from all cell lines and animals of one group. First-strand cDNA was synthesized using Superscript II RNase H-Reverse Transcriptase (Life Technologies, Karlsruhe, Germany). RNA (1 µg) of elute was added to 2 µl of Oligo Mix and then filled up with DEPC-water to a total volume of 12 µl. After annealing for 10 min at 70°C and immediate cooling on ice, 5X first-strand buffer (Life Technologies), 10 mM DTT (Life Technologies), 0.5 µl enzyme (100 U) and 0.5 mM deoxynucleotide triphosphate (Carl Roth GmbH, Karlsruhe, Germany) were added to get a total reaction volume of 21 µl. The reaction was allowed to proceed for 50 min at 42°C, 30 min at 52°C, 25 min at 60°C followed by 15 min at 70°C to inactivate the enzyme. cDNA was stored at -20°C until use.

Quantitative real-time RT-PCR. Primers were designed by use of Primer express software Version 2.0.0 (Perkin-Elmer Applied Biosystem, Foster City, CA, USA) and FastPCR Version 4.0.27 (Ruslan Kalendar 'PCR Team', Helsinki, Finland). The sequences of species-specific primers are listed in Table I. Primers were obtained from MWG Biotech, Martinsried, Germany. The 20 µl of total PCR volume consisted of 2 µl cDNA, 2 µl master mix SYBR-Green I (Applied Biosystems, Martinsried, Germany), 3.2 µl MgCl₂, 8.8 µl water and 4 µl primer in the following concentration: 800 nM each for GAPDH and 400 nM each for the residual genes. After denaturing and activation of the hotstart enzyme for 10 min at 95°C, the PCR assays were carried out for 45 cycles, with denaturation at 95°C for 10 sec, annealing and extension at 72°C for 1 min. To monitor amplification of

possible contaminated DNA, distilled water served as a negative control. Fluorometric PCR was performed with the Roche Lightcycler system (Roche Applied Science, Mannheim, Germany). Threshold cycle was set when emission intensity of measurable fluorescence calibrated to starting level became equal to 10 standard deviations of the baseline. The RT-PCR results were expressed as the ratio of molecules of target gene per 1000 molecules of the housekeeping gene (GAPDH). Melting curve analysis was performed after each PCR run. After denaturation at 95°C for 10 sec, a temperature ramp from 65 to 95°C in 20 min was run with simultaneous acquisition of fluorescence signals. Using negative first-deviation plots, we checked each sample for primer dimers and unspecific products due to mispriming.

Protein isolation and Western blotting. Proteins were isolated after centrifuging hepatoma cells at 1,000 rpm for 10 min, discarding the liquid phase and adding 50 µl of Jie's protein lysis buffer, consisting of 10 mM NaCl₂, 0.5% NP₄O, 20 mM Tris-HCl pH 7.4, 5 mM MgCl₂, 10 µg/ml Prot-I, 1 mM PMSF. After 30 min cooling on ice with intermediate vortexing, the suspension was divided into two portions, ready to use and stored at -80°C.

Samples were subjected to electrophoresis on precast Novex Tris-Glycine gels (Invitrogen) and then blotted to nitrocellulose membranes. Membranes were blocked with 5% low fat milk at 4°C overnight and then incubated with primary antibodies as described in Table II. Signals were retrieved using enhanced chemiluminescence on Kodak X-Omat LS film. Membranes were glycine shocked and re-probed with an antibody against β-actin as an internal standard. Densitometry was performed with GelScan 5 software (BioSciTec, Frankfurt, Germany) by normalizing density values to β-actin values of each sample.

DNA isolation and methylation-specific PCR (MSP). Cellular DNA was extracted using the QIamp DNA mini kit (Qiagen, Hilden, Germany) for each cell line and from snap-frozen tumor samples according to the manufacturer's instructions.

Bisulphate modification of the DNA was accomplished by the use of the EZ Methylation-Gold kit (Zymo Research, Orange, USA) as described in the manual. DNA elements with methylated cytosins remain unmodified after bisulfite treatment while unmethylated cytosins will be completely converted into uracil nucleosides and further to thymine after PCR, thus giving different primer recognition sequences for MSP.

PCR-mix (2X) (12.5 µl) (Fermentas, St. Leon-Rot, Germany) were added to 2 µl of bisulfonated/unmodified DNA sample followed by 1 µl of forward and reverse primers (400 nM each). Finally, 8.5 µl of water was added. PCR reaction was run at a temperature of 94°C for 2 min followed by 35 cycles (94°C for 10 sec, 60°C to 70°C according to annealing temperature of the primer for 20 sec, 72°C for 20 sec) and terminated by 72°C for 4 min and immediate cooling down to 4°C. Primer sequences and annealing temperatures are given in Table III.

PCR products were subjected to 2% (w/v) agarose gel electrophoresis after staining with 6x Loading Dye Solution (Fermentas). GeneRuler 100 bp DNA Ladder Plus was used as a length standard (Fermentas GmbH, St. Leon Roth, Germany).

Table I. Sequences of primers used for real-time PCR experiments.

	NCBI reference sequence no.	Amplicon (bp)	Primer
CK7	X13320	75	Forward (exon 4): 5'-TGAATGATGAGATCAACTTCCTCAG-3' Reverse (exon 5): 3'-TGTCGGAGATCTGGGACTGC-5'
CK8	BC000654	101	Forward (exon 7): 5'-CTGGGATGCAGAACATGAGTATTC-3' Reverse (exon 8): 3'-GTAGCTGAGGCCGGCTTGT-5'
CK18	BC000180	86	Forward (exon 5): 5'-GAGACGTACAGTCCAGTCCTTGG-3' Reverse (exon 6): 3'-CCACCTCCCTCAGGCTGTT-5'
CK19	NM_002276	1377	Forward (exon 1): 5'-CGGGCATAAAAGGCGCCAGGT-3' Reverse (exon 6): 3'-GAGACCCCGAAGACGACAGGAAAC-5'
CK20	X73502	105	Forward (exon 5): 3'-TCCCAGAGCCTTGAGATAGAACTC-5' Reverse (exon 6): 5'-GTTGGCTAACTGGCTGCTGTAAC-3'
Vim	003380	208	Forward primer (exon 5): 5'-TGAGGCTGCCAACCGGAAACA-3' Reverse primer (exon 6): 5'-TTGGCCTGCAGGATGAG-3'
PTC	NM_000264	139	Forward (exon 3): 5'-CCAATCTTCTGGCGAGTATAATTTAAT-3' Reverse (exon 2): 3'-TGGTTGTGGGCCTCCTCAT-5'
SHH	X13320	117	Forward (exon 3): 5'-GGAAGCAGCCTCCCGATT-3' Reverse (exon 2): 3'-CGAGTCCAAGGCACATATCCA-5'
PDX-1	NM_008814	64	Forward (exon 1): 3'-TTCCCATGGATGAAGTCTACCAA-5' Reverse (exon 1+2): 5'-CGTAGGCGCCGCCTG-3'
THY-1	NM_006288	235	Forward (exon 4): 5'-GACCCGTGAGACAAAGAAGC-3' Reverse (exon 4): 3'-TTGACCAGTTCACACTCCCG-5'
C-KIT	NM_000222	3998	Forward (exon 1): 5'-TCGCTGCACTTGGGCGAGAGCT-3' Reverse (exon 21): 5'-TTTTCCCACTCCTGTGGGAGC-3'
GAPDH	NM_002046	226	Forward (exon 4): 5'-GAAGGTGAAGGTCGGAGTC-3' Reverse (exon 5): 3'-GAAGATGGTGATGGGATTTC-5'

Immunohistochemistry. Immunohistochemical staining procedures were applied, as described previously (16,17). In short: 5- μ m sections were deparaffinized using graded alcohols. After antigen retrieval with microwave (MW) (10 mmol/l citrate buffer, pH 6.0; 8 min at 560 W and 8 min at 800 W) or trypsin, the sections were incubated overnight using specific antibodies (Table II). Next, depending on the primary antibody, biotinylated rabbit anti-goat or goat anti-rabbit (Dako, Denmark) antibodies were applied on the sections. Detection was done using the Dako streptavidin-biotin-complex and alkaline phosphatase system, following the user's manual (Dako). Development was done using DAB (Roche Molecular Biochemicals, Mannheim, Germany). Nuclei were counterstained with hematoxylin.

Hematoxylin-eosin staining was used to evaluate basic histomorphology of the specimens. The epithelial mucopolysaccharides were detected by the PAS reaction. The stained sections were independently analyzed (by DN and MM). Representative images were taken from 4 high power fields (x400 magnification) in each following scheme: negative (-), weak (+), moderate (++), strong (+++), differing focal expression being indicated in round brackets. Additionally, the percentage of positive cells per high power field was

determined using ImageAccess Enterprise 5 software (Imagic Bildverarbeitung, Glattbrugg, Switzerland). For further statistical analysis the calculated product of intensity and extensity was used ranging from 0 (negative) to a maximum of 300 (3 staining intensity and 100% positive cells) section.

Statistics. Statistical analysis was performed with SPSS 14.0 using analysis of variances (ANOVA) in order to compare the different groups of xenografts. LSD and Tukey-HSD methods were applied as *post hoc* tests. Correlation coefficients were calculated according to Pearson. For detecting homogeneous clusters of cases based on measured characteristics hierarchical cluster analysis (average linkage method) were carried out. Additionally, factor analysis with varimax method were used to condense the relevant variable in the clusters. All tests were two tailed. A P-value of <0.05 was considered statistically significant.

Results

Analysis of mRNA expression. Expression of cytokeratin (CK) 7, 8, 18, 19 and 20 as well as of vimentin (vim) was analysed

Table II. Primary antibodies and antigen retrieval methods used for Western blotting or immunohistochemical analysis.

Antigen specificity	Type	Dilution	Antigen retrieval	Vendor
CK7	Mouse monoclonal	1:200	TRS	NovaCastra
CK8/CK18	Mouse monoclonal	1:400	TRS	NovaCastra
CK19	Mouse monoclonal	1:100	TRS	Dako
CK20	Mouse monoclonal	1:50	TRS	NovaCastra
Vimentin	Mouse monoclonal ^a Goat polyclonal	1:2000	TRS	Dako ^a SantaCruz
β-catenin	Mouse monoclonal	1:200	TRS	Dako
Ki-67	Mouse monoclonal	1:500	TRS	Dako
pdx-1	Mouse monoclonal polyclonal	1:20 ^a 1:5000	TRS	R&D ^a Chemicon
shh	Goat polyclonal	1:100 ^a 1:200	MW, TRS	SantaCruz
ptc	Goat polyclonal	1:50 ^a 1:200	MW, TRS	SantaCruz
CD34	Mouse monoclonal	1:200	TRS	Dako
c-kit	Mouse monoclonal	1:200	TRS	NovaCastra
Thy-1	Goat polyclonal	1:50	MW, TRS	SantaCruz
β-actin	Mouse monoclonal	1:5000		Sigma

MW, microwave (10 mmol/l citrate buffer, pH 6.0; 10 min at 800 W and 10 min at 560 W); TRS, target retrieval solution (pH 9.0; Dako, Germany) used instead of citrate buffer. ^aImmunohistochemistry.

to determine the differentiation status of HCC xenografts (Fig. 1A). Expression of CK7 was low in Hep3B, Huh-7 and Huh-7 pcDNA3.1 with a slight increase in Huh-7 5-15. HepG2 xenografts showed a 5-fold higher expression of CK7. In contrast, CK8 was highly expressed in Hep3B and Huh-7 but was reduced to low levels in the other cell lines. CK18 was evenly expressed in all investigated cell lines, while only low levels of CK19 were detected. Expression of CK20 was high in HepG2 and low to moderate in the other cell lines. All cell lines showed also a low to moderate expression of vimentin.

In vitro, expression of CK7 was low to moderate in all cell lines except Huh-7 pcDNA3.1 which showed a 40-fold higher expression (data not shown). CK8 was expressed at very low levels, while CK18 expression was homogeneous and moderate in all investigated cell lines. Expression of CK19 and CK20 was moderate in all cell lines. Except for Huh-7 5-15 cells, where a 2-fold induction was observed, the mesenchymal marker vimentin was expressed at low levels only in the other cell lines.

We then investigated the expression of embryonic differentiation and stem cell marker genes in the xenograft model (Fig. 1B). Hep3B and Huh-7 showed a similar pattern of high expression of SHH and c-kit, intermediate expression of PDX-1 and absence or low expression of PTC and Thy-1. HepG2 cells, in contrast, did not express SHH and showed only low levels of Thy-1 and c-kit, while PDX-1 was highly

expressed here. In Huh-7 5-15 cells, only Thy-1 and c-kit were detectable by quantitative real-time RT-PCR. Interestingly, these markers were expressed at very low levels in the control cell line Huh-7 pcDNA3.1. This cell line also expressed SHH, PTC and PDX-1 at moderate levels.

The analysis of these markers under *in vitro* conditions showed a differential pattern (Fig. 1C). SHH was homogeneously expressed in all cell lines except HepG2, where a marked suppression of this gene was observed. PTC was not detectable under *in vitro* conditions, while PDX-1 showed a constant expression in all cell lines. Thy-1 was expressed at moderate to high levels, while c-kit was highly expressed in Hep3B, Huh-7 and Huh-7 pcDNA3.1 but was not detectable in HepG2 and was strongly suppressed in Huh-7 5-15.

Comparison of in vitro and in vivo mRNA analysis. The comparison of the two experimental setups revealed differences in the expression of the investigated markers. While no changes were observed for CK7, except for a strong upregulation in Huh-7 pcDNA3.1 xenografts, CK8 was induced in Hep3B and Huh-7 samples from nude mice. The hepatocellular marker CK18 was unaffected by the change of environment, but CK19 was lost in all investigated xenografts compared to cell culture conditions. The glandular/intestinal marker CK20 was downregulated in Huh-7 and its derivatives *in vivo* while it was unchanged in Hep3B and even induced in HepG2. The

Table III. Sequences of primers used for methylation-specific PCR analysis.

	NCBI reference sequence no.	Amplicon (bp)	Primer
C-KIT	NT_022853	160	Forward: 5'-AGCAAGAGTTAACGATTCCGGAG-3' Reverse: 3'-CCAGAAAGGTGTAAGTGCCTCCT-5'
C-KIT MSP	NT_022853	164	Forward: 5'-GTTTGTAGGTTTTTCGTCGC-3' Reverse: 5'-CGCAACCTTCGATTAATCTC-3'
PDX-1	NT_024524	100	Forward: 5'-GGGTTTTTTTTTGAGGGAGTT-3' Reverse: 5'-CTTTCCACACTAACTTTAAT-3'
PDX-1 MSP	NT_024524100	100	Forward: 5'-GTTTTTTTTCGGAGGGAGTC-3' Reverse: 5'-TTTCCACGCGTAACTTTAA-3'
PTC	NT_008470	110	Forward: 5'-AGTAGTGGTTGGTTTGTTAATT-3' Reverse: 3'-CACTACTACCACTACTACAAAC-5'
PTC MSP	NT_008470	110	Forward: 5'-TAGCGGTTGGTTTGTTAATC-3' Reverse: 5'-CGCTACTACCGCTACTACGA-3'
SHH	NC_000007	117	Forward: 5'-ACCGAGGGCTGGGACGAAGATGGC-3' Reverse: 5'-GCGAGCCAGCATGCCGTACTTGCTG-3'
SHH MSP	NC_000007	120	Forward: 5'-TAGTTAGCGAGGGAGAGAGC-3' Reverse: 3'-GAACTATCCCCGTACGAATC-5'
THY-1	NC_000011	120	Forward: 5'-TTATTTTTATATTAATGTGGGATTGT-3' Reverse: 5'-TCCAATTACTACACCCAACCTCAA-3'
THY-1 MSP	NC_000011	124	Forward: 5'-TATTTTTATATTAATGCGGGATCGT-3' Reverse: 5'-CGATTACTACACCCAACCTCGAA-3'

MSP, methylation-specific primer.

expression of the mesenchymal marker vimentin decreased in all xenograft specimens except for Huh-7 5-15 samples, where a moderate expression was established.

The *in vitro* expression pattern of SHH was also reflected *in vivo*, except for Huh-7 5-15 where a strong induction was observed. PTC was not detectable under cell culture conditions and only Huh-7 pcDNA3.1 xenografts showed moderate levels of this receptor. PDX-1, Thy-1 and c-kit tended to be downregulated *in vivo*, except for an increased expression of PDX-1 in HepG2 and Huh-7 pcDNA3.1 xenograft samples and a slight increase of c-kit in HepG2.

These findings indicate a differential expression of differentiation markers and genes regulating embryonic development dependent on the tumor cell environment.

Western blot analysis in vitro/vivo (Fig. 2). On the protein level, SHH was expressed in all cell lines *in vitro* and *in vivo*, with a slight decrease in Huh-7 xenografts, while its expression was increased in Huh-7 5-15 specimens. Thy-1 was expressed in all samples but was downregulated in the xenografts compared to cell culture material, which parallels the mRNA results described above. No expression was observed in Hep3B xenografts. PTC also showed a constant expression in all investigated samples with an increase in signal intensity in HepG2 xenografts and decreased expression in Huh-7 pcDNA3.1 xenografts. Corroborating the mRNA findings, no

expression of the mesenchymal marker vimentin was observed, while cognate bands were detectable *in vitro*. Interestingly, PDX-1 was not detectable from HepG2 cell culture samples (although high mRNA levels were observed). All other cell lines, except Huh-7 pcDNA3.1 *in vivo*, expressed PDX-1 under both conditions.

Methylation-specific PCR analysis in vitro and in vivo (Fig. 3 and Table IV). MSP analysis revealed a methylated DNA status of Thy-1 in all cell lines and under both investigated conditions, while all other gene loci were not methylated *in vitro* or *in vivo*. Only PDX-1 showed a methylation signal in HepG2 *in vitro*, which was lost in xenograft specimens.

Expression pattern of markers in HCC-xenografts (Fig. 4 and Table V). i) The expression of intermediate filaments (CK7, CK8/18, CK19, CK20 and vimentin) was significantly differential in all xenografts (ANOVA test, $p < 0.001$). Expression of CK8/18 was lowest in Huh-7 derived cell lines whereas highest expression was found in native Hep3B or Hep3B implanted to nude mice with matrigel (Hep3B-MG) and especially in HepG2 xenografts. In contrast, the expression of CK7 was mainly found in Huh-7 5-15. This was associated with high expression of vimentin. Interestingly, CK19 was expressed in Hep3B as well as in HepG2 xenografts. CK20 expression levels were low in all investigated HCC xenografts.

Table IV. Methylation-specific PCR analysis.

	Hep3B		HepG2		Huh-7		Huh-7 pcDNA3.1		Huh-7 5-15	
	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>
SHH	-	-	-	-	-	-	-	-	-	-
Thy-1	+	+	+	+	+	+	+	+	+	+
c-kit	-	-	-	-	-	-	-	-	-	-
PDX-1	-	-	-	+	-	-	-	-	-	-
PTC	-	-	-	-	-	-	-	-	-	-

Results of MSP analysis from cell culture and xenograft specimens. +, methylation and -, no methylation.

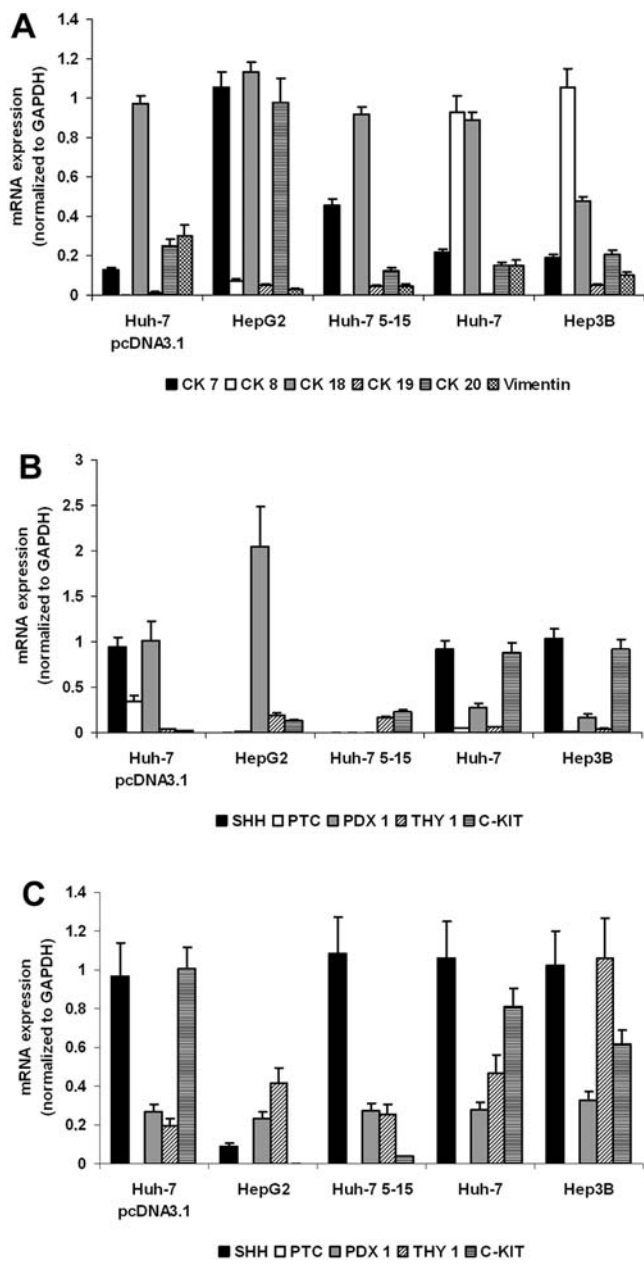


Figure 1. Quantitative real-time RT-PCR analysis of markers associated with differentiation and embryonic development in HCC cell lines and xenografts. mRNA levels were normalized to GAPDH expression. Analysis of differentiation markers (A) and embryonic development genes (B) from xenograft specimens and cell culture samples (C). Shown are mean \pm SEM of three independent specimens.

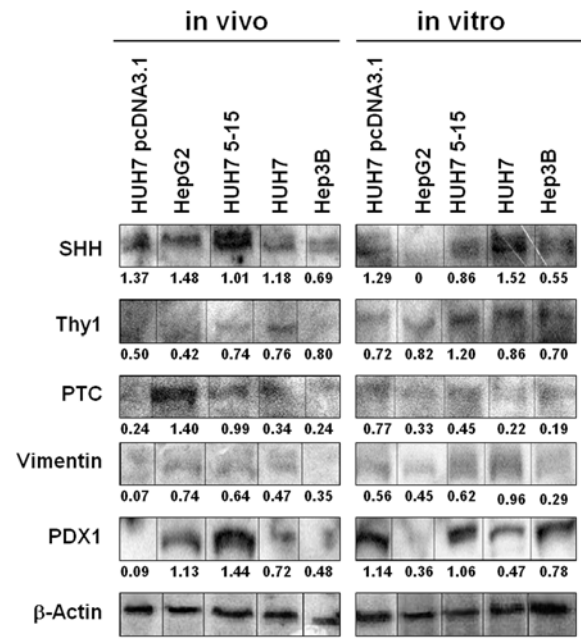


Figure 2. Representative Western blotting results comparing the expression of Sonic hedgehog (SHH), Thy-1, Patched (PTC), vimentin (VIM) and the pancreatic duodenal homeobox gene 1 (PDX-1) from HCC xenograft samples (left) and under standard cell culture conditions (right). β -actin bands were used to show equal loading of lanes. Numbers depict densitometric values normalized to β -actin values of each sample.

ii) Throughout all HCC xenografts, analysis of progenitor factors (c-kit, CD34, Thy-1) showed comparatively low expression levels. Peak expression levels of CD34 were observed in HepG2, THY-1 expression was highest in Hep3B-MG xenografts (ANOVA test, $p < 0.001$). iii) Finally, analysis of embryonic/developmental markers showed heterogeneous expression levels. Membranous β -catenin expression was mainly found in Huh-7, Hep3B and Hep3B-MG, whereas cytoplasmatic β -catenin was especially high in HepG2 cells. In addition, PDX1 was at highest levels in HepG2 xenografts (ANOVA test, $p < 0.001$). Expression of SHH was low in all xenografts, whereas expression of PTC was heterogeneous and peak PTC expression levels were found in Huh-7 pcDNA3.1 and Hep3B-MG (ANOVA test, $p < 0.001$).

Correlation analysis of mRNA and protein expression in vitro and in vivo. Comparing mRNA and protein expression of the

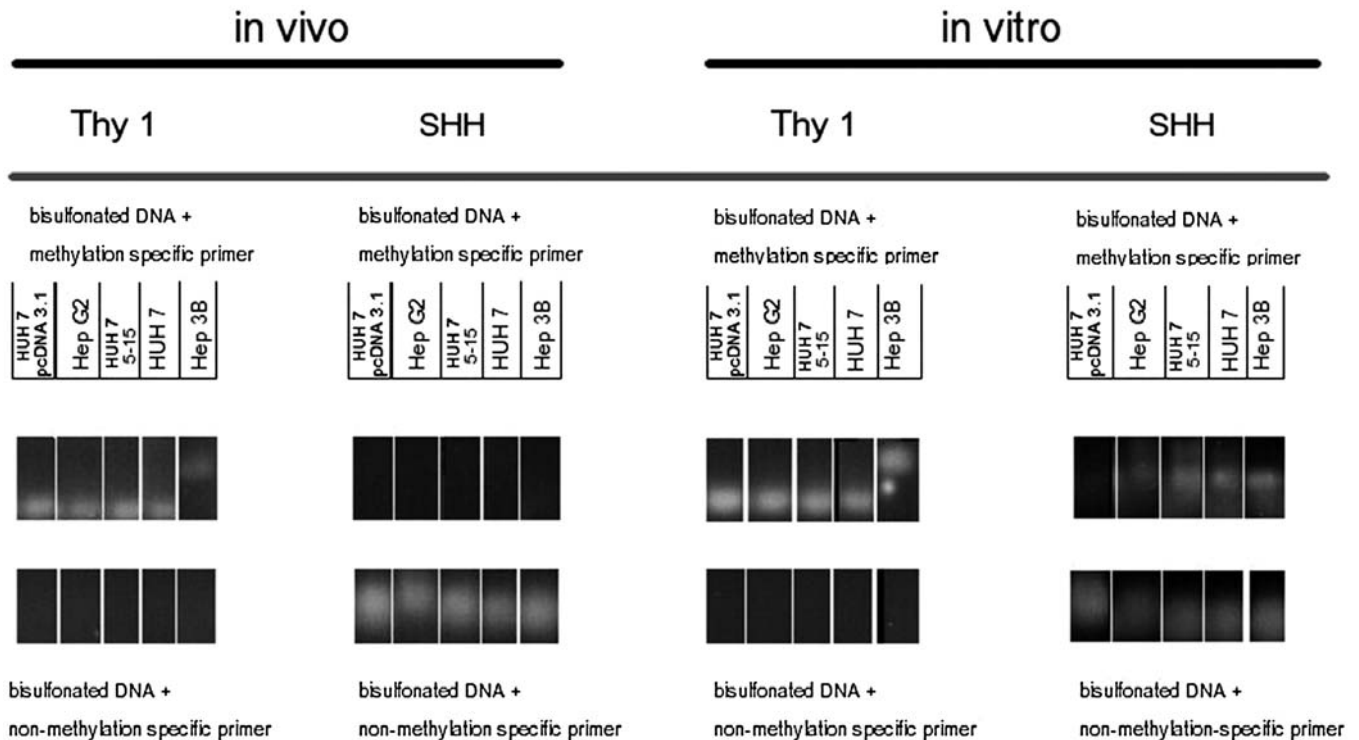


Figure 3. Methylation-specific PCR analyses *in vitro* and *in vivo* indicate a methylated DNA status of Thy-1 in all cell lines under both experimental conditions. With the exception of PDX-1 in HepG2 *in vitro*, all other gene loci were not methylated *in vitro* or *in vivo*.

investigated markers revealed great differences between the *in vitro* and the *in vivo* situation: The majority of used markers showed a tendentious negative association between mRNA expression *in vitro* and protein expression in the xenografts with a significant association for PDX-1 ($r=-0.599$, $p=0.005$) and PTC ($r=0.521$, $p=0.019$). Looking at mRNA and protein expression *in vivo* revealed completely different results. With the exception of CK7 the expression of mRNA and protein were positively linked for all the other markers used at significance levels for PDX-1 ($r=0.691$, $p=0.001$) and PTC ($r=0.678$, $p=0.001$).

Correlation analysis of markers in HCC-xenografts (Table VI). Correlation analysis showed heterogeneous, significantly positive and negative associations: The ductal marker CK7 was positively correlated with the glandular/intestinal marker CK20. Interestingly, the hepatocellular marker CK8/18 was positively associated with the known ductal marker CK19 and the progenitor marker Thy-1 but negatively linked to the mesenchymal marker vimentin. CK19, on the other hand, showed a significantly positive correlation with embryonic/developmental markers such as cytoplasmatic β -catenin and PDX-1 as well as a negative association with PTC. Finally, the mesenchymal marker vimentin was negatively associated with the progenitor markers CD34 and cytoplasmatic β -catenin.

Looking at the progenitor marker CD34, a positive correlation to the embryonic/developmental markers PDX-1 and cytoplasmatic β -catenin as well as vimentin was observed. Thy-1 showed a significantly positive association with CK8/18 and membranous β -catenin. No significant association between c-kit and any other investigated marker was found.

Finally, the embryonic/developmental markers showed heterogeneous associations with i) progenitor markers such as membranous β -catenin and Thy-1 as well as cytoplasmatic β -catenin with CD34 or PDX-1; ii) with other embryonic markers such as cytoplasmatic β -catenin and PDX-1 and finally iii) with intermediate filaments such as cytoplasmatic β -catenin with vimentin, PDX-1 with CK19 or PTC with CK19.

Hierarchical cluster and factor analysis. After normal standardization of the variable values being statistically different in the ANOVA test (Ki-67, CK7, CK8/18, CK19, vimentin, CD34, Thy-1, β -catenin, PDX1 and PTC) the hierarchical cluster analysis revealed four groups with following relevant factors using factor analysis: Group 1 (Huh-7, Huh-7 pcDNA3.1 and Hep3B): CK7, CK19, β -catenin and CD34 (classical CCC type); group 2 (Huh-7 5-15): CK19, vimentin, Ki-67 (dedifferentiated mesenchymal-proliferative type); group 3 (Hep3B-MG): CK19, β -catenin and PTC (dedifferentiated embryonic-development type); group 4 (HepG2): CK18, CK19, β -catenin (classical HCC type).

Discussion

Our analysis of differentiation markers in human hepatic cancer cell lines in a xenograft animal model revealed the following major results: i) Different hepatic cancer cell lines showed significantly different expression patterns of the differentiation markers in a xenograft animal model. Direct association of some markers was observed. ii) In comparison, *in vivo* and *in vitro* protein expression were significantly different from the mRNA expression *in vitro*, but were mainly in line with the mRNA expression *in vivo*. iii) Cluster

Table V. Expression levels inside different HCC xenografts.

	Score (mean \pm standard deviation of mean) intensity (mean)/extensity levels (mean) ^a					
	HUH7	HUH7 PC	HUH7 5-15	Heb3b	Heb3b MG	HepG2
Ki 67 ^b	26.2 \pm 2.6	40.0 \pm 2.1	60.0 \pm 2.1	22.7 \pm 1.1	52.2 \pm 2.6	49.7 \pm 2.3
Intermediate filaments						
CK7	1.9 \pm 0.9 0.6/2.2	0.06 \pm 0.06 0.1/0.1	8.8 \pm 4.1 1.3/5.6	4.0 \pm 1.9 0.8/4.0	4.8 \pm 1.8 1.1/4.0	0.3 \pm 0.2 0.5/0.5
CK8/18	4.1 \pm 0.6 1.0/4.1	1.6 \pm 0.3 1.0/1.6	8.1 \pm 3.3 1.2/5.8	27.3 \pm 14.3 1.2/18.6	136.3 \pm 10.7 2.1/64.0	107.3 \pm 29.1 2.3/43.5
CK19	2.4 \pm 1.2 1.1 \pm 1.7	neg. neg./neg.	3.7 \pm 0.6 1.0 / 3.7	9.6 \pm 5.1 1.2/6.5	2.8 \pm 0.7 0.8/3.0	65.3 \pm 19.9 1.7/35.3
CK20	neg. neg./neg.	2.6 \pm 0.6 1.0/2.6	4.6 \pm 2.8 1.1/3.1	3.1 \pm 0.3 1.0/3.1	2.3 \pm 0.7 0.8/2.5	2.7 \pm 1.0 0.7/2.7
Vim	59.5 \pm 12.6 2.3/24.3	58.2 \pm 5.9 2.0/29.1	15.2 \pm 2.6 1.7/8.6	27.7 \pm 11.8 1.5/15.8	16.8 \pm 6.4 1.5/10.0	4.8 \pm 2.0 1.1/3.7
Progenitor markers						
C-kit	1.7 \pm 0.6 0.8/1.8	neg. neg./neg.	2.3 \pm 1.0 0.7/2.5	1.0 \pm 0.6 0.5/1.0	0.8 \pm 0.5 0.5/1.0	1.1 \pm 0.5 0.7/1.3
CD34	1.8 \pm 1.4 0.7/1.1	0.3 \pm 0.2 0.5/0.5	3.1 \pm 0.4 1.0/3.1	2.0 \pm 0.7 0.8/2.1	0.9 \pm 0.2 0.8/1.0	4.0 \pm 0.7 1.0/4.0
Thy-1	4.7 \pm 1.0 1.0/4.7	3.5 \pm 0.6 1.0/3.5	18.7 \pm 3.1 1.2/14.7	14.1 \pm 1.4 1.1/12.7	45.1 \pm 13.7 2.1/20.0	6.7 \pm 2.7 1.2/4.8
Embryonic/ Development markers						
β -cat	85.1 \pm 8.5	23.7 \pm 2.7	3.8 \pm 2.1	105.9 \pm 39.2	150.3 \pm 45.9	13.1 \pm 1.9
mb ^c	1.7/49.0	1.0/23.7	0.7/4.1	1.8/52.0	1.8/75.2	1.0/13.1
β -cat	7.2 \pm 3.0	1.6 \pm 0.9	9.8 \pm 2.8	2.8 \pm 1.1	3.1 \pm 1.0	42.6 \pm 11.0
cp ^c	1.5/4.1	0.5/1.6	1.2/7.3	0.7/2.8	0.8/3.2	1.5/27.2
PDX-1	2.6 \pm 0.5 1.0/2.6	2.0 \pm 0.6 0.8/2.1	3.5 \pm 1.9 1.0/2.5	1.5 \pm 0.2 1.0/1.5	0.8 \pm 0.5 0.5/1.0	13.5 \pm 3.5 1.7/7.1
SHH	0.1 \pm 0.1 0.1/0.2	0.8 \pm 0.5 0.5/0.8	0.06 \pm 0.06 0.1/0.1	0.1 \pm 0.07 0.2/0.02	0.06 \pm 0.06 0.1/0.1	0.2 \pm 0.2 0.2/0.2
PTC	5.7 \pm 2.1 1.1/4.7	11.0 \pm 1.9 1.2/9.0	5.1 \pm 2.2 1.5/2.8	1.2 \pm 0.4 0.7/1.2	8.6 \pm 3.9 1.2/6.1	0.2 \pm 0.2 0.2/0.2

^aScore as well as intensity and extensity levels (mean \pm standard deviation). ^bFor Ki-67 only extensity levels were included. ^cExpression of β -catenin was differentially evaluated: mb, membranous and cp, cytoplasmatic. Neg, negative.

and factor analysis could extract major groups of differentiation within the analyzed hepatic cancer cell lines.

Overall, the investigated differentiation markers showed an association with the hepatic cancer cell lines: Huh7 and its derivatives were associated with a high expression of CK7 and vimentin but with a low expression of CK8/18. Hep3B and HepG2, in contrast, were mainly correlated with expression of

CK8/18 and CK19. CK8/18 is usually expressed in normal hepatocytes, while CK7/19 is additionally found in cholangiocytes and especially in hepatic progenitor cells (20,21). The overall very low expression of CK20 suggests that gastro-intestinal glandular differentiation does not play an important role in HCC cell lines as was previously demonstrated for human HCC (22,23). In addition, the

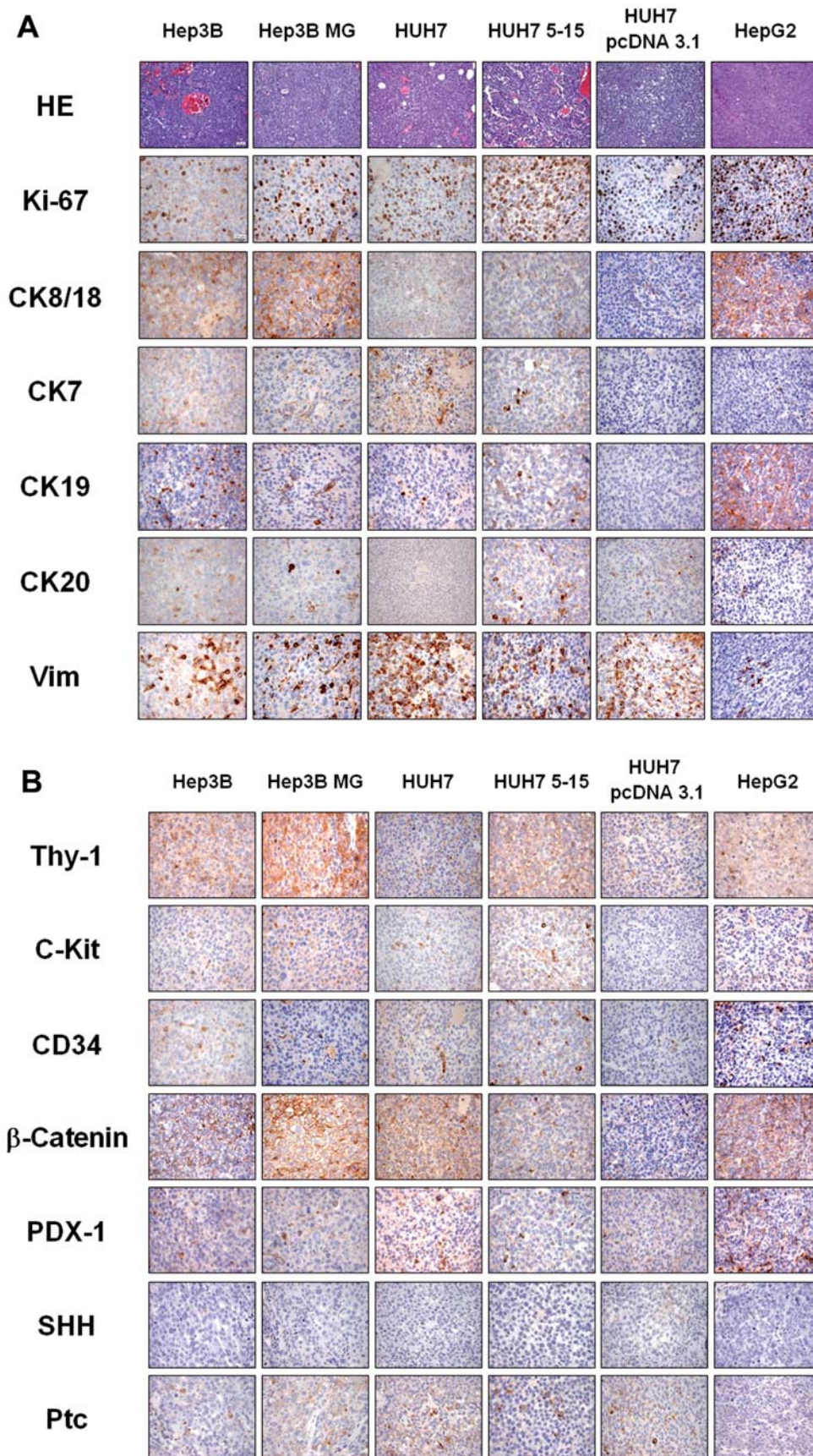


Figure 4. Typical expression pattern of trans- and dedifferentiation inside HCC tumor cell line implants in nude mice as determined by immunohistochemistry (magnification: HE-staining $\times 200$) (bar $50 \mu\text{m}$), all others $\times 400$ objective (bar $20 \mu\text{m}$). The expression of CK7, CK8/18, CK19, CK20 and vimentin were used as markers for intermediate filaments differentiation, expression of C-kit, CD34 and Thy-1 as markers for progenitor differentiation and finally β -catenin, PDX-1, SHH and PTC as markers for embryonic/development differentiation. Based on these protein expression levels the hierarchical cluster and factor analysis identified the following predominant differentiation: Group 1 (Huh-7, Huh-7 pcDNA3.1 and Hep3B): CK7, CK19, β -catenin and CD34 (classical CCC type); group 2 (Huh-7 5-15): CK19, vimentin, Ki-67 (dedifferentiated mesenchymal-proliferative type); group 3 (Hep3B-MG): CK19, β -catenin and PTC (dedifferentiated embryonic-development type); group 4 (HepG2): CK18, CK19, β -catenin (classical HCC type).

Table VI. Correlation analysis giving significant associations between the applied biomarkers.

	Positive (Correlation coefficient/p)	Negative (Correlation coefficient/p)
Ki-67	No correlation	Vim (-0.402/0.015)
CK7	CK20 (0.607/0.002)	
CK8/18	CK19 (0.430/0.036) Thy-1 (0.446/0.029)	Vim (-.475/0.019)
CK19	CK19 (0.430/0.036) β-cat cp (0.803/0.000) PDX-1 (0.802/0.000)	PTC (-0.395/0.05)
CK20	CK7 (0.607/0.002)	No correlation
Vim	No correlation	Ki-67 (-0.402/0.015) CK8/18 (-.475/0.019) CD34 (-0.493/0.014) β-cat cp (-0.409/0.047)
C-kit		No correlation
CD34	β-cat cp (0.407/0.048) PDX-1 (0.406/0.049)	Vim (-0.493/0.014)
Thy-1	Thy-1 (0.446/0.029) β-cat mb (0.462/0.023)	No correlation
β-Cat mb	Thy-1 (0.462/0.023)	No correlation
β-Cat cp	CK19 (0.803/0.000) CD34 (-0.409/0.047) PDX-1 (0.557/0.005)	Vim (-0.409/0.047)
PDX-1	CK19 (0.802/0.000) CD34 (0.406/0.049) β-cat cp (0.557/0.005)	No correlation
SHH		No correlation
PTC	No correlation	CK19 (-0.395, p=0.05)

Mb, membranous and cp, cytoplasmatic.

negative to very low expression levels of progenitor factors such as c-kit (CD117), CD34 or Thy-1 (CD90) underline that stem cell plasticity is not a very prominent phenomenon in the used setting, although higher expression levels of CD34 and Thy-1 were observed in HepG2 and Hep3B-MG xenografts, associated with higher proliferation activity in these cases as evidenced using Ki-67 staining. Recently, Thy-1 has been shown to be decisive for tumorigenicity of HCC cell lines, and although the overall expression of this marker is usually <1% (24) it was proposed as a novel marker for diagnosis and treatment of human HCC (25). Notably, expression of c-kit was linked to Hepatitis B virus infection, which is mirrored by the higher expression in Hep3B and Huh-7 in our setting, which were established on the basis of viral hepatitis (26,27).

In our experiments, embryonic/developmental markers play a more prominent role than progenitor markers. Literature on WNT-pathway gives evidence that a more membranous expression of β-catenin is associated with better grading and outcome of HCC (28-31). Membranous β-catenin expression was mainly found in Huh-7, Hep3B and Hep3B-MG, whereas cytoplasmatic β-catenin was especially high in HepG2 cells, which was also associated with another embryonic marker PDX-1. This marker is relevant in embryonic development of the pancreas and plays a decisive role also in pancreatic carcinogenesis (16,32). PDX-1 also induces a developmental redirection of liver cells to the pancreatic lineage and has been suggested to be involved in transdifferentiation process of the liver (33-35). Recently, it was shown that PDX-1 influences hepatic differentiation processes via repression of the key hepatic transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) (36), which itself is a member of the family of the liver enriched transcription factors that have been shown to regulate differentiation processes in hepatocellular carcinoma (37).

The Hedgehog (Hh) pathway is essentially involved in gastrointestinal and liver development as well as stem cell maintenance (38). While normal hepatocytes do not express Hh proteins, this pathway is commonly activated in hepatic stellate cells (39), HCC cell lines (38) and specimens (40,41) and biliary derived cells (42), usually accompanied by a high expression of the Hh receptor Patched (PTC) and its downstream transcription factors Gli. Consistent with this view, we found a high expression of the ligand SHH in all xenografts except HepG2 and Huh-7 5-15, whereas the receptor PTC showed high expression levels in Huh-7pcDNA3.1 and Hep3B-MG as demonstrated by immunohistochemistry.

Our comparison of the *in vitro* and the *in vivo* setting revealed differential expression patterns of the investigated markers and identifies the importance of matrix environment in tumor patterning. It is therefore interesting to note that under *in vitro* conditions, higher mRNA levels of the stem cell factors Thy-1 and c-kit were observed, while *in vivo* high levels of Shh were observed, indicating that differential pathways of growth control and regulation of differentiation can be reactivated dependent on the cellular environment. The tumor microenvironment is regarded as a key regulator of cellular survival, differentiation pathways and metastasis pathways (43,44), e.g. by soluble factors such as TGF-β (45) or by direct cell-cell or cell-matrix contacts (46). Dependent on the extracellular environment, a differential behaviour of tumor and stem cells has therefore been described (47). We are aware that our experimental approach using a subcutaneous xenograft model of HCC might not be representative when comparing our data with orthotopic models of liver cancer. Interestingly, the matrigel environment used for implantation of Hep3B cells to nude mice was already sufficient to influence the expression pattern of the investigated markers. Besides extracellular matrix, DNA methylation was another regulatory mechanism of selective gene activation showing heterogeneous methylation patterns between the used markers as well as the *in vitro/in vivo* situation. This is in line with recent investigations indicating the relevance of epigenetics in the tumorigenesis of hepatocellular carcinoma (48-50). Nevertheless, the relevance of these discrepant

methylation patterns must be investigated in further functional experiments.

The correlation analysis revealed interesting associations in the xenograft model: the ductal differentiation marker CK7 is associated with the gastrointestinal-glandular marker, whereas the hepatic differentiation marker CK8/18 is associated with the known ductal marker CK19 (23). In the liver CK19 is known to be a marker of dedifferentiation (51), which showed a positive correlation with other embryonic/development markers such as cytoplasmatic β -catenin, PDX-1 and PTC. Surprisingly, the mesenchymal marker vimentin was negatively associated with the progenitor markers CD34 and cytoplasmatic β -catenin, which was generally not the case in other studies (30,31). In line with the literature, and thereby indicating the association of progenitor potency and embryonic dedifferentiation, CD34 and Thy-1 were linked to the embryonic/developmental markers PDX-1 and β -catenin and *vice versa* (52-54).

In summary, our data show that different cell lines of human hepatocellular carcinoma have the capability to resort to different pathways of embryonic development or progenitor cell differentiation, dependent on the microenvironment. These findings highlight the importance of selecting appropriate experimental conditions, especially for investigating novel targeted therapies interfering with the growth factor or survival pathways that may be differentially expressed under varying experimental conditions.

Acknowledgements

We thank Gabriele Krumholz for support in animal care and experiments. The excellent technical assistance of Ursula Becker, Ines Grob-Achleitner, Astrid Taut and Isabel Zeitträger is gratefully acknowledged.

References

- Thorgeirsson SS: Hepatic stem cells. *Am J Pathol* 142: 1331-1333, 1993.
- Ponder KP: Analysis of liver development, regeneration, and carcinogenesis by genetic marking studies. *FASEB J* 10: 673-682, 1996.
- Corcelle V, Stieger B, Gjinovci A, Wollheim CB and Gauthier BR: Characterization of two distinct liver progenitor cell subpopulations of hematopoietic and hepatic origins. *Exp Cell Res* 312: 2826-2836, 2006.
- Wu XZ and Yu XH: Bone marrow cells: the source of hepatocellular carcinoma? *Med Hypotheses* 69: 36-42, 2007.
- Thorgeirsson SS: Hepatic stem cells in liver regeneration. *FASEB J* 10: 1249-1256, 1996.
- Sirica AE, Mathis GA, Sano N and Elmore LW: Isolation, culture, and transplantation of intrahepatic biliary epithelial cells and oval cells. *Pathobiology* 58: 44-64, 1990.
- Aterman K: The stem cells of the liver - a selective review. *J Cancer Res Clin Oncol* 118: 87-115, 1992.
- Fausto N, Lemire JM and Shiojiri N: Cell lineages in hepatic development and the identification of progenitor cells in normal and injured liver. *Proc Soc Exp Biol Med* 204: 237-241, 1993.
- Sigal SH, Brill S, Fiorino AS and Reid LM: The liver as a stem cell and lineage system. *Am J Physiol* 263: G139-G148, 1992.
- Sell S: Liver stem cells. *Mod Pathol* 7: 105-112, 1994.
- Grisham JW: A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H3. *Cancer Res* 22: 842-849, 1962.
- Sell S: Cellular origin of cancer: dedifferentiation or stem cell maturation arrest? *Environ Health Perspect* 101 (Suppl 5): 15-26, 1993.
- Grisham JW: Migration of hepatocytes along hepatic plates and stem cell-fed hepatocyte lineages. *Am J Pathol* 144: 849-854, 1994.
- Zajicek G, Oren R and Weinreb M Jr: The streaming liver. *Liver* 5: 293-300, 1985.
- Neureiter D, Herold C and Ocker M: Gastrointestinal cancer - only a deregulation of stem cell differentiation? (Review). *Int J Mol Med* 17: 483-489, 2006.
- Neureiter D, Zopf S, Dimmler A, *et al*: Different capabilities of morphological pattern formation and its association with the expression of differentiation markers in a xenograft model of human pancreatic cancer cell lines. *Pancreatology* 5: 387-397, 2005.
- Quint K, Stintzing S, Alinger B, *et al*: The expression pattern of pdx-1, shh, patched and gli-1 is associated with pathological and clinical features in human pancreatic cancer. *Pancreatology* 9: 116-126, 2009.
- Lohmann V, Korner F, Koch J, Herian U, Theilmann L and Bartenschlager R: Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285: 110-113, 1999.
- Pietschmann T, Lohmann V, Rutter G, Kurpanek K and Bartenschlager R: Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. *J Virol* 75: 1252-1264, 2001.
- Lai YS, Thung SN, Gerber MA, Chen ML and Schaffner F: Expression of cytokeratins in normal and diseased livers and in primary liver carcinomas. *Arch Pathol Lab Med* 113: 134-138, 1989.
- Roskams T, De Vos R, Van Eyken P, Myazaki H, Van Damme B and Desmet V: Hepatic OV-6 expression in human liver disease and rat experiments: evidence for hepatic progenitor cells in man. *J Hepatol* 29: 455-463, 1998.
- Chu P, Wu E and Weiss LM: Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. *Mod Pathol* 13: 962-972, 2000.
- Yamamoto T, Uenishi T, Ogawa M, *et al*: Immunohistologic attempt to find carcinogenesis from hepatic progenitor cell in hepatocellular carcinoma. *Dig Surg* 22: 364-370, 2005.
- Yang ZF, Ho DW, Ng MN, *et al*: Significance of CD90⁺ cancer stem cells in human liver cancer. *Cancer Cell* 13: 153-166, 2008.
- Yang ZF, Ngai P, Ho DW, *et al*: Identification of local and circulating cancer stem cells in human liver cancer. *Hepatology* 47: 919-928, 2008.
- Lee ES, Han EM, Kim YS, *et al*: Occurrence of c-kit⁺ tumor cells in hepatitis B virus-associated hepatocellular carcinoma. *Am J Clin Pathol* 124: 31-36, 2005.
- Chung CY, Yeh KT, Hsu NC, *et al*: Expression of c-kit proto-oncogene in human hepatocellular carcinoma. *Cancer Lett* 217: 231-236, 2005.
- Inagawa S, Itabashi M, Adachi S, *et al*: Expression and prognostic roles of beta-catenin in hepatocellular carcinoma: correlation with tumor progression and postoperative survival. *Clin Cancer Res* 8: 450-456, 2002.
- Hsu HC, Jeng YM, Mao TL, Chu JS, Lai PL and Peng SY: Beta-catenin mutations are associated with a subset of low-stage hepatocellular carcinoma negative for hepatitis B virus and with favorable prognosis. *Am J Pathol* 157: 763-770, 2000.
- Tien LT, Ito M, Nakao M, *et al*: Expression of beta-catenin in hepatocellular carcinoma. *World J Gastroenterol* 11: 2398-2401, 2005.
- Suzuki T, Yano H, Nakashima Y, Nakashima O and Kojiro M: Beta-catenin expression in hepatocellular carcinoma: a possible participation of beta-catenin in the dedifferentiation process. *J Gastroenterol Hepatol* 17: 994-1000, 2002.
- Neureiter D, Zopf S, Leu T, *et al*: Apoptosis, proliferation and differentiation patterns are influenced by Zebularine and SAHA in pancreatic cancer models. *Scand J Gastroenterol* 42: 103-116, 2007.
- Sapir T, Shternhall K, Meivar-Levy I, *et al*: Cell-replacement therapy for diabetes: Generating functional insulin-producing tissue from adult human liver cells. *Proc Natl Acad Sci USA* 102: 7964-7969, 2005.
- Li WC, Horb ME, Tosh D and Slack JM: In vitro trans-differentiation of hepatoma cells into functional pancreatic cells. *Mech Dev* 122: 835-847, 2005.
- Horb ME, Shen CN, Tosh D and Slack JM: Experimental conversion of liver to pancreas. *Curr Biol* 13: 105-115, 2003.

36. Meivar-Levy I, Sapir T, Gefen-Halevi S, *et al*: Pancreatic and duodenal homeobox gene 1 induces hepatic dedifferentiation by suppressing the expression of CCAAT/enhancer-binding protein beta. *Hepatology* 46: 898-905, 2007.
37. Hayashi Y, Wang W, Ninomiya T, Nagano H, Ohta K and Itoh H: Liver enriched transcription factors and differentiation of hepatocellular carcinoma. *Mol Pathol* 52: 19-24, 1999.
38. Sicklick JK, Li YX, Jayaraman A, *et al*: Dysregulation of the Hedgehog pathway in human hepatocarcinogenesis. *Carcinogenesis* 27: 748-757, 2006.
39. Sicklick JK, Li YX, Choi SS, *et al*: Role for hedgehog signaling in hepatic stellate cell activation and viability. *Lab Invest* 85: 1368-1380, 2005.
40. Huang S, He J, Zhang X, *et al*: Activation of the hedgehog pathway in human hepatocellular carcinomas. *Carcinogenesis* 27: 1334-1340, 2006.
41. Patil MA, Zhang J, Ho C, Cheung ST, Fan ST and Chen X: Hedgehog signaling in human hepatocellular carcinoma. *Cancer Biol Ther* 5: 111-117, 2006.
42. Omenetti A, Porrello A, Jung Y, *et al*: Hedgehog signaling regulates epithelial-mesenchymal transition during biliary fibrosis in rodents and humans. *J Clin Invest* 118: 3331-3342, 2008.
43. Le NH, Franken P and Fodde R: Tumour-stroma interactions in colorectal cancer: converging on beta-catenin activation and cancer stemness. *Br J Cancer* 98: 1886-1893, 2008.
44. Witz IP: Tumor-microenvironment interactions: dangerous liaisons. *Adv Cancer Res* 100: 203-229, 2008.
45. Massague J: TGFbeta in Cancer. *Cell* 134: 215-230, 2008.
46. Schuppan D and Ocker M: Integrin-mediated control of cell growth. *Hepatology* 38: 289-291, 2003.
47. Marongiu F, Doratiotto S, Montisci S, Pani P and Laconi E: Liver repopulation and carcinogenesis: two sides of the same coin? *Am J Pathol* 172: 857-864, 2008.
48. Tischoff I and Tannapfe A: DNA methylation in hepatocellular carcinoma. *World J Gastroenterol* 14: 1741-1748, 2008.
49. Herath NI, Leggett BA and MacDonald GA: Review of genetic and epigenetic alterations in hepatocarcinogenesis. *J Gastroenterol Hepatol* 21: 15-21, 2006.
50. De Zhu J: The altered DNA methylation pattern and its implications in liver cancer. *Cell Res* 15: 272-280, 2005.
51. Durnez A, Verslype C, Nevens F, *et al*: The clinicopathological and prognostic relevance of cytokeratin 7 and 19 expression in hepatocellular carcinoma. A possible progenitor cell origin. *Histopathology* 49: 138-151, 2006.
52. Gao X, Song L, Shen K, Wang H, Niu W and Qin X: Transplantation of bone marrow derived cells promotes pancreatic islet repair in diabetic mice. *Biochem Biophys Res Commun* 371: 132-137, 2008.
53. Li H, Li X, Lam KS, Tam S, Xiao W and Xu R: Adeno-associated virus-mediated pancreatic and duodenal homeobox gene-1 expression enhanced differentiation of hepatic oval stem cells to insulin-producing cells in diabetic rats. *J Biomed Sci* 15: 487-497, 2008.
54. Loo CK and Wu XJ: Origin of stellate cells from submesothelial cells in a developing human liver. *Liver Int* (In press).