

Cell differentiation mediated by co-culture of human umbilical cord blood stem cells with murine hepatic cells

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Abstract In the present study, purified human cord blood stem cells were co-cultivated with murine hepatic alpha mouse liver 12 (AML12) cells to compare the effect on endodermal stem cell differentiation by either direct cell-cell interaction or by soluble factors in conditioned hepatic cell medium. With that approach, we want to mimic in vitro the situation of preclinical transplantation experiments using human cells in mice. Cord blood stem cells, cultivated with hepatic conditioned medium, showed a low endodermal differentiation but an increased connexin 32 (Cx32) and Cx43, and cytokeratin 8 (CK8) and CK19 expression was monitored by reverse transcription polymerase chain reaction (RT-PCR). Microarray profiling indicated that in cultivated cord blood cells, 604 genes were upregulated 2-fold, with the highest expression for epithelial CK19 and epithelial cadherin (E-cadherin). On ultrastructural level, there were no major changes in the cellular morphology, except a higher presence of phago(ly)some-like structures observed. Direct co-culture of AML12 cells with cord blood cells led to less incisive differentiation with increased sex-determining region Y-box 17 (SOX17), Cx32 and Cx43, as well as epithelial CK8 and CK19 expressions. On ultrastructural level, tight cell contacts along the plasma membranes were revealed. FACS analysis in co-cultivated cells

quantified dye exchange on low level, as also proved by time relapse video-imaging of labelled cells. Modulators of gap junction formation influenced dye transfer between the co-cultured cells, whereby retinoic acid increased and 3-heptanol reduced the dye transfer. The study indicated that the cell-co-cultured model of human umbilical cord blood cells and murine AML12 cells may be a suitable approach to study some aspects of endodermal/hepatic cell differentiation induction.

Keywords Co-culture · Murine hepatic cells (AML12) · Umbilical cord blood cells (UCBC) · Cell differentiation

Introduction

Stem cells vary in their differentiation potential. Adult human umbilical cord blood stem cells (UCBC) showed preferably a lineage-specific haematopoietic differentiation (Chularojmontri and Wattanapitayakul 2009), but some reports described a differentiation potential towards other cell types in vitro and in vivo, including models for tissue regeneration (Lagasse *et al.* 2000; Jang *et al.* 2004; Saez-Lara *et al.* 2006; Minamiguchi *et al.* 2008). However, the results remain controversial, depending on the target cell and the methods applied (Saez-Lara *et al.* 2006; Chen *et al.* 2008; Hombach-Klonisch *et al.* 2008).

Endodermal/hepatic differentiation in vitro is a multistep process. Each step is controlled by different cytokines. Differentiated cells are characterized by specific marker profiles (Lavon and Benvenisty 2005; Dong *et al.* 2009; Song *et al.* 2010). In most of the published protocols, soluble factors were used to induce a non-haematopoietic differentiation of haematopoietic stem cells (Chen and Zeng 2011). For hepatic differentiation in vitro, UCBC were treated for instance with hepatocyte growth factor (HGF) or epidermal growth factor and oncostatin M (Wang *et al.* 2003; Yan *et al.* 2009).

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Differentiation was analyzed mainly by the induction of endodermal/hepatic marker expression, morphology and functional properties. For hepatic differentiation of UCBC, conditioned medium from injured murine hepatocytes in transwell chambers was found to be efficient to induce differentiation measured by liver-specific phenotype and functional changes (Jang *et al.* 2004).

Direct co-culture of UCBC with stromal feeder cells was found to be optimal for haematopoiesis and superior to cytokine treatment (Zhang *et al.* 2006). In vivo, transplanted cord blood cells into liver-injured mice were described to be functionally integrated into the organ, as measured by albumin production (Kakinuma *et al.* 2003; Fujino *et al.* 2007). Further adult stem cells isolated from cord blood and human foetal liver were intrahepatically transplanted in irradiated NOD scid gamma (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl/SzJ}) immunodeficient mice strain (NSG) mice and lead to an efficient development of human haematopoietic and human hepatocyte-like cells in vivo (Chen *et al.* 2013).

In this paper, we applied both a co-culture approach and hepatic conditioned medium of murine alpha mouse liver 12 (AML12) cells for an in vivo investigation of the endodermal/hepatic differentiation potential of adult UCBC. We used the murine hepatic AML12 cells for co-culturing because they express a high level of messenger RNA (mRNA) for serum and gap junction proteins like albumin, alpha-1 antitrypsin, transferrin and connexin 32 (Cx32). Alpha-1 antitrypsin stimulates the HGF. Transferrin is essential for cell growth and differentiation of a variety of cells in vitro. With that approach, we wanted to simulate a mouse-like micro-environment for potential preclinical in vivo studies. Differentiation was evaluated by gene expression, using real-time reverse transcription polymerase chain reaction (RT-PCR), microarray analysis and on the ultrastructural level by electron microscopy. As a main result, we found that direct co-culture but not conditioned medium induced an increase of cytoskeleton markers like cytokeratin 8 (CK8) and CK19, which could be an evidence for a transition from mesenchymal to epithelial cell type as described in literature.

Materials and Methods

Isolation and purification of human umbilical cord blood stem cells—Cluster of differentiation 34⁺ (CD34⁺) cells. UCBC were isolated with the MACS cell isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany) according to the manufacturer's instruction. Fluorescence-activated cell sorting (FACS) analysis with anti-CD34 phycoerythrin (PE) antibody (Becton Dickinson, Heidelberg, Germany) was performed to determine the percentage of the positive fraction. The freshly isolated cells were cultured according to in-house developed in vitro protocols (Wulf-Goldenberg *et al.* 2008).

Cell culture. All cell culture experiments were performed in a 5% CO₂ incubator at 37°C.

Murine AML12 cell culture and production of hepatic conditioned medium. Murine AML12 cells (ATCC-Nr.CRL-2254) were cultured according to the manufacturer's instruction (www.atcc.org). For production of hepatic conditioned medium, serum-free Sautin medium (Sautin *et al.* 2001) (Biochrom, Berlin, Germany) was used. Confluent AML12 cells were cultivated with Sautin medium for 1, 3 or 7 d to produce hepatic conditioned medium. Medium supernatant was collected and sterile filtrated.

Culture of UCBC with hepatic conditioned medium. 5 × 10⁴ cells/cm² UCBC were seeded in Matrigel-coated wells and were cultured with 1 d, 3 d or 7 d hepatic conditioned medium supplemented with 100 ng/ml stem cell factor (SCF) and 20 ng/ml HGF. Cells were cultured for 7 d. After 4 d, half of the medium was changed. Samples were taken at day 1 and 7 for gene expression analysis.

Co-culture of murine AML12 cells with UCBC. For co-culture, 5 × 10⁴ cells/cm² UCBC were seeded on a confluent AML12 cell layer in Sautin medium supplemented with 100 ng/ml SCF and 20 ng/ml HGF. Medium was changed after 4 d. Samples were taken at day 1 and 7 for gene expression analysis. UCBC cells were displaced from the AML12 cell layer by carefully rinsing with PBS. Efficiency was microscopically controlled, and the rinsing step was repeated until all UCBC were detached from the AML12 cell layer. Collected UCBC were counted and analyzed with human-specific primers. Murine AML12 cells were used as negative control.

Reverse transcription and real-time polymerase chain reaction. Total RNA was prepared from UCBC and AML12 cells using the RNeasy Micro and Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantification of RNA was performed by spectrophotometry using Nanodrop device (PeqLab, Erlangen, Germany).

RT-PCR of RNA (200 ng) was realized using TaqMan RT reagent (Applied Biosystems, Darmstadt, Germany), real-time PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany) and human specific primer assays (Applied Biosystems, Darmstadt, Germany), which are listed as well as the PCR conditions in Table S1 (supplementary data).

Relative quantification (ΔC_T method) was used to analyze the gene expression level. Cycle threshold (C_T) value was normalized to those of housekeeping gene glyceraldehyd-3-phosphat dehydrogenase (GAPDH). The following scoring system was used:

ΔC_T	Score	Expression level
>16.0	–	No detectable expression
12.1–16.0	+	Low expression
8.1–12.0	++	Moderate expression
4.1–8.0	+++	High expression
0–4.0	++++	Very high expression

Electron microscopy. Freshly isolated UCBC and cells treated with hepatic conditioned medium were pelleted and fixed with 2% formaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer for 2 h.

Murine AML12 cells as adherent cells and co-culture of AML12 cells with human stem cells were performed on lumox™ dishes (Greiner Bio-one, Frickenhausen, Germany) as described above, washed two times with phosphate buffer and fixed like the UCBC. All samples were stained with 1% Osmiumtetroxid (O_5O_4) for 2 h, dehydrated in a graded ethanol series and propylene oxide and embedded in Poly/Bed^R 812 (Polysciences, Inc., Eppelheim, Germany). Ultrathin sections were contrasted with uranyl acetate and lead citrate.

Sections were examined with a FEI Morgagni electron microscope, and digital images were taken with a Morada CCD camera and the iTEM software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Dye transfer. UCBC were labelled with pH26 (Sigma-Aldrich, Munich, Germany) and murine AML12 cells with Calcein-AM (Sigma-Aldrich, Munich, Germany) according to the manufacturer's instructions. The cells were co-cultured for at least 48 h, and the uptake of calcein acetoxymethylester (Calcein-AM) in co-cultured Paul Karl Horan-26 (pKH26)-labelled UCBC was measured by FACS. Influences on dye transfer were investigated by using 10 μ M retinoic acid (Sigma-Aldrich, Munich, Germany) on the one hand and 1 mM 3-heptanol (Sigma-Aldrich, Munich, Germany) on the other hand (microarray analysis (supplementary data); video-imaging (supplementary data)).

Results

UCBC cultivated with hepatic conditioned medium. Hepatic conditioned medium from murine AML12 cells was harvested on day 1, 3 and 7 and added to UCBC. After 7 d of cultivation with hepatic conditioned medium, the cell number of UCBC cells was 1.2-fold increased with 90% cell viability and a more adherent appearance, compared to cells cultivated in stem cell medium only (data not shown).

Electron microscopy. The cultivated cells were analyzed by electron microscopy for morphological changes. As shown in Fig. 1, untreated UCBC are characterized by high nucleus-to-plasma ratio, low microvilli numbers on cell surface and spare cell-to-cell contacts. In contrast, in most of the UCBC, cultivated for 7 d with hepatic conditioned medium, large numbers of microvilli per cell and sporadically cell protrusions with characteristic features of kinocilia or stereocilia are visible. Tight cell-to-cell contacts and in some cases membrane fusion were detectable. At both time points, electron-dense gap junction (GJ) structures were not found. In addition, in about 50% of the cells, structures with phagolysosomal appearance are detectable, associated occasionally with degradation of cell organelles. The percentage of phagolysosomal structures in treated cells was higher in comparison to cultivation with 1 d and 3 d hepatic conditioned medium (data not shown), suggesting few apoptotic processes during longer conditioning time.

Real-time RT-PCR. Expression pattern of UCBC was characterized by real-time RT-PCR using a panel of human specific primer sets of early endodermal and hepatic markers, CK, Cx and epithelial cadherin (E-cadherin). Untreated UCBC showed a high level of CD34 transcripts, with moderate CCAAT/enhancer binding protein alpha (C/EBP α) and E-cadherin expression (Table 1). Typical endodermal and hepatic markers (alpha fetoprotein (AFP), albumin (ALB) or GATA binding protein to DNA sequence 5'-(A/T)GATA(A/G)-3' (GATA4)) were determined at low levels or not detectable. Cx43 and Cx32 were low expressed in UCBC, a result which is in agreement with immunohistochemical staining (data not shown). Expression pattern of hepatic conditioned medium treated UCBC was analyzed after a cultivation time of 1 d and 7 d, respectively. Due to cultivation, the CD34 and E-cadherin expression is reduced, but still detectable. CK8, CK19 and Cx32 and Cx43 expression increased to a moderate or high level, whereas the endodermal marker AFP and hepatic markers ALB and hepatocyte nuclear factor 4alpha (HNF4 α) remained unchanged.

However, cultivation of UCBC in stem cell medium supplemented with cytokines revealed no comparable significant changes in expression levels. The growth factor addition of HGF and SCF into stem cell media had no effect on the CK19 expression.

Microarray analysis. Whole genome microarray profiling was performed to confirm and analyze additional genes, involved in cellular changes induced by hepatic conditioned medium. Compared to untreated cells, 604 genes were upregulated more than 2-fold and 1,126 genes were downregulated in UCBC cultivated with hepatic conditioned medium for 10 d. Genes involved in endodermal and hepatic differentiation were diversely upregulated, E-cadherin (CDH1) with the

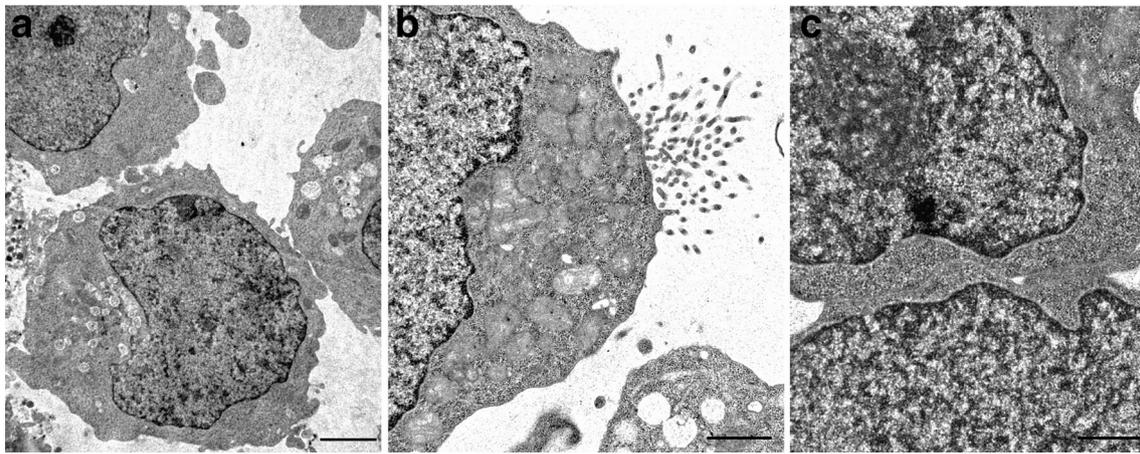


Fig. 1 Electron micrographs of UCBC (CD34⁺ stem cells). Morphological comparison between (a) freshly isolated UCBC and (b, c) after cultivation with conditioned medium for 7 d. Untreated UCBC show no distinct microvilli formation and hardly cell-to-cell contacts, whereas (b)

treated stem cells developed few cell protrusions with characteristic features of kinocilia or stereocilia and (c) membrane fusions. Scale bars at (a) 1 μ m and (b, c) 500 nm.

highest increase up to 16-fold and CK19 up to 5-fold. Gene expression data are summarized in Table 2. Besides the selected endodermal hepatic genes, genes of the erythroid differentiation (NUSAP1, MAFB) and for blood vessel formation (ANPEP, DHCR7, JAG1, PSEN1) were upregulated. In contrast, transcripts for Cx43 (GJA1) and CD34⁺ were expressed to a lesser extent.

UCBC in co-culture with AML12 cells. UCBC were seeded on a confluent AML 12 cells layer and co-cultivated for 7 d.

Dye transfer and live cell imaging. To quantify the cell-cell communication, UCBC were labelled with pH26, murine AML12 cells with Calcein-AM and then co-cultured. After a co-cultivation time of 48 h, the number of pH26/Calcein-

AM-positive UCBC was estimated by FACS analysis using quadrant statistic analysis (Fig. 2). As demonstrated, in the absence of AML12 cells, pH26-labelled cells showed a homogenous cell population of 98% positive cells with a middle fluorescence intensity (mFI) of 800 (Fig. 2a, b). Forty-eight hours after co-cultivation, about 7.18% of the UCBC were found to be Calcein-AM-positive (Fig. 2c). Modulators of gap junction formation and interference with connexins were tested in the UCBC/AML12 co-culture model. For that, cells were cultured in the presence of retinoic acid and 3-heptanol for 48 h. Retinoic acid increased the number of pH26/Calcein-AM-positive UCBC cells to 15.32% (Fig. 2d), whereas the inhibitor 3-heptanol decreased the number to 1.19% (Fig. 2e). The results demonstrated the functionality of the applied dye transfer method for measuring

Table 1 Effects of hepatic conditioned medium on umbilical cord blood stem cells (CD34⁺ stem cells)

Marker	Cultivation with conditioned medium			Cultivation in stem cell medium	
	Day 0	Day 1	Day 7	Day 1	Day 7
CD34	+++/++++	+++	+/+++	++++	+++
SOX17	±	±	+	–	–
FOXA2	–	–	–	–	–
AFP	–	–	–	–	–
C/EBP α	+++	+++	+++/++++	+++	+++
HNF4 α	±	±	–	–	–
ALB	–	±	+	+	–
CK8	+	+	++	++	–
CK18	++	+++	++	n.d.	n.d.
GATA4	–	–	–	n.d.	n.d.
CK19	++	++	+++	+	+
Cx32	±	±	++	–	–
Cx43	+	±	++	+	–
E-Cadherin	+++	+++	++	n.d.	n.d.

Expression levels of human specific markers were determined by real-time RT-PCR. For relative quantification, we used the ΔC_T method as follows: $\Delta C_T < 0-4$ (++++) very high expression, $\Delta C_T 4.1-8$ (+++) high expression, $\Delta C_T 8.1-12$ (++) moderate expression, $\Delta C_T 12.1-16$ (+) low expression, $\Delta C_T > 16$ (–) no expression and n.d. not determined

cell-cell communication and suggest the participation of connexins.

Cell-cell interaction of labelled UCBC and confluent AML12 cells was visualized by time relapsed video-imaging over a time period of 16 h (Fig. S1 supplementary data). As demonstrated on adherent, murine AML12 cells, UCBC possess high motility with a sporadic cell contact to the murine cells. However, most of the UCBC get no direct contact with AML12 cells for a longer time period. This result may explain the low level of communicating cells, measured as well by dye transfer.

Electron microscopy. Co-culture systems of murine AML12 and UCBC were investigated on ultrastructural level at days 1 and 7. As demonstrated in Fig. 3a, no distinct morphological changes of UCBC were detectable at day 1. Compared to freshly isolated UCBC (see Fig. 1a), they maintained their round morphology with high nucleus-to-plasma ratio, only few microvilli and no dense cell-to-cell contacts. At day 7 of co-culture, microvilli structures and a flattened cell phenotype appeared more frequently (Fig. 3b). Occasionally, tight UCBC-to-AML12 cell contacts along the plasma membrane are present (Fig. 3c).

Real-time RT-PCR. Expression pattern of UCBC in co-culture with murine AML12 cells was investigated at day 1 and 7 (Table 3) by real-time PCR. Besides a slight reduction of CD34 marker expression, seven different RNA transcripts were upregulated in a time-dependent manner. The transcripts for CK8, CK18 and CK19 were elevated to a moderate or high

level. The expressions of Cx32 and Cx43 were also upregulated from a low to moderate rate during cultivation. The higher expression of sex-determining region Y-box 17 (SOX17) in co-cultivated UCBC suggests an early endodermal differentiation, whereas forhead box A2 gene name (Hepatocyte nuclear factor 3beta (HNF3 β)) (FOXA2), AFP and ALB were not affected, suggesting no endodermal or hepatic differentiation. For comparison, human HepG2 cells are characterized by a high expression of these transcripts (Table 3).

Discussion

The aim of this study was to demonstrate the in vitro endodermal/hepatic differentiation potential of human UCBC with two different methods. The UCBC were freshly isolated, and an enrichment of CD34-positive cells was performed with common bead technology. We compared the differentiation induction potential of murine AML12 cells used either in direct co-culture with UCBC or as donor for conditioned medium. We intended to investigate the mechanism of cell-cell interaction for endodermal/hepatic induction of these adult stem cells.

The murine AML12 cells were used as the source for production of conditioned medium and effector cells in direct co-culture as they express high levels of mRNA for serum albumin, alpha 1 antitrypsin, transferrin and the gap junction proteins Cx26 and Cx32. We found an increased expression of epithelial markers like CK8 and CK19 as well as markers for gap junctions like Cx32 and Cx43 as revealed by real-time

Table 2 Gene expression profile of umbilical cord blood cells (CD34⁺ stem cells) after 10 d cultivation in conditioned medium

Endodermal/hepatic genes		Various genes			
Gene symbol	Fold change	Gene symbol	Fold change	Gene symbol	Fold change
SOX17	1.22	TDO2	1	CD34	0.03
FOXA2	1.05	WNT3	2.15	GJA1	0.31
AFP	1.67	WNT3A	1	CXCR4	2.86
GATA4	1.3	WNT11	1.71	CD38	3.19
CEBPA	1.67	IGFBP1	2.43	KIT	1.71
HNF4A	1.84	TERT	1.27		
ALB	0.72	T	1	NUSAPI	5.4
KRT18	0.93	APOF	1.31	MAFB	3.34
KRT8	1.63	TTR	1.85		
KRT19	5.56	PAX6	1.32	ANPEP	3.47
KRT7	1.39	GATA6	1.61	DHCR7	3.58
CDH1	16.68	CYP2C9	1.28	JAG1	2.62
GJB1	1.75	CYP2C19	1.37	PSEN1	2.37
MET	1.41	CYP3A4	1.35		
TF	1.39	CYP7A1	1.3	GFI1	2.83
SERPINA1	4.31	CYP2B6	1.2	NTRK1	3.5
TAT	1.44	CYP3A7	1.75		
HPRT1	1.03	CYP1A1	1.71		

Change in gene expression is compared to non-cultivated cells (fold change)

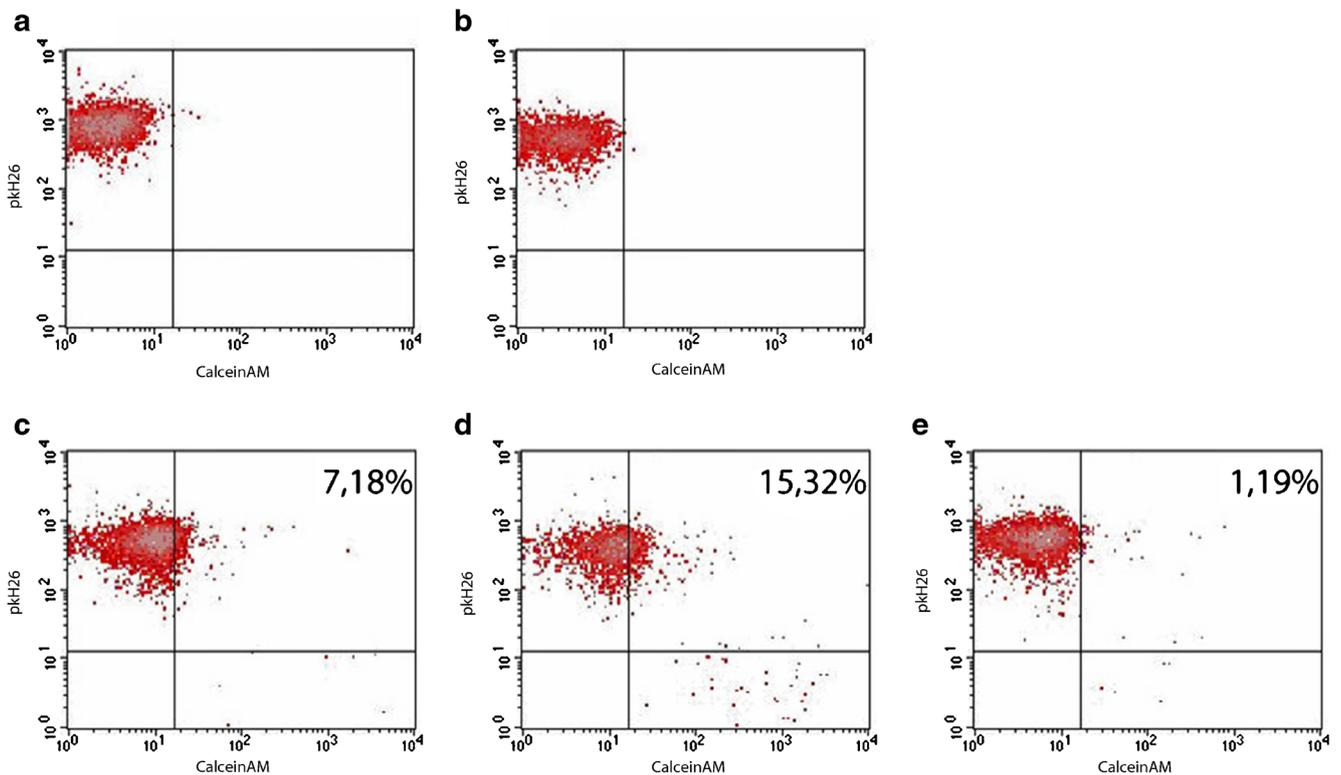


Fig. 2 Flow cytometry analysis of dye transfer between AML12 cells (calcein labelled) and UCBC (pkH26 labelled) in co-culture. At 0 h (a) and 48 h (b) after labelling of UCBC with pkH26, no distinct changes in Calcein-AM mediated fluorescence intensity measurable. (c) After 48 h in co-culture with Calcein-AM labelled AML12 cells, 7.18% of UCBC

took up Calcein-AM and were double-positive. (d) Treatment with retinoic acid increased uptake of Calcein-AM and double-positive cells to 15.32%. (e) In contrast, incubation with 3-heptanol for 48-h reduced uptake and double-positive cells to 1.19%.

PCR analysis in UCBC cultured with conditioned medium. The endodermal/hepatic marker expression profile of SOX17, FOXA2, AFP and HNF4 α was not significantly changed on cells cultivated with hepatic conditioned medium compared to untreated UCBC. The results of microarray analysis revealed the diverse upregulation from genes involved in endodermal differentiation, like E-cadherin up to an 16-fold increase and CK19- up to a 5-fold increase. Further, we determined changes in gene profile for blood vessel formation and erythroid differentiation. The results of both mRNA methods agree essentially. Protein expression was analyzed on cultured stem

cells exemplarily. The expression of CD34 and CD45 was considerably reduced on stem cells after cultivation with hepatic conditioned medium or in co-culture, respectively (data not shown). Slight changes on mRNA level of cultured stem cells could be noticed due to the high sensitivity and specificity of the used mRNA methods. These changes are not distinguishable on protein level. On ultrastructural level, some phenotypic changes appear, like large numbers of microvilli per cell and structures with phagolysosomal appearance.

In direct co-culture approach of UCBC with murine cells, we found that the transcripts for CK8, CK18 and

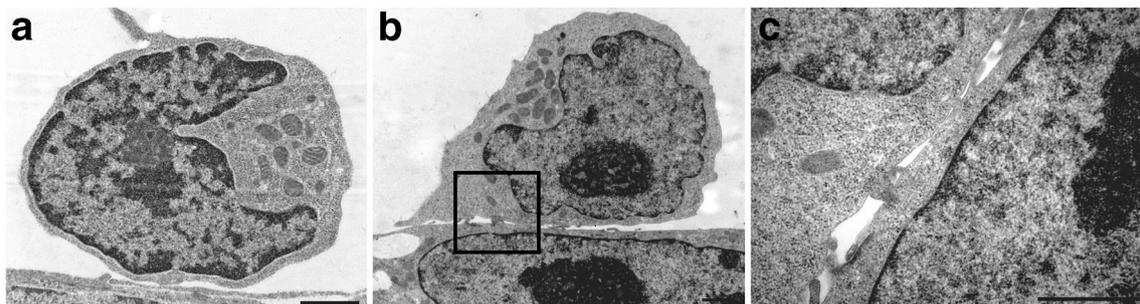


Fig. 3 Fine structure of co-culture between UCBC (CD34⁺ stem cells) and AML12 cells disclosed (a) 1 d after co-cultivation hardly any cell-to-cell contacts, and no morphological changes are visible. (b, c) After 7 d, a

flattened cell phenotype of UCBC is detectable, and close cell contacts between AML12 cells (down) and UCBC (above) occur. (c) Higher magnification of the region gated in (b). Scale bar at 1 μ m.

Table 3 Expression pattern of umbilical cord blood stem cells (CD34⁺ stem cells) after co-cultivation with murine AML12 cells

Marker	Day 0	Day 1	Day 7	HepG2
CD34	+++	+++	++/+++	-
SOX17	-	-	++	±
FOXA2	-	-	-	+++
AFP	-	-	-	++++
C/EBP α	+++	+++/++++	+++/++++	+++
HNF4 α	+	++	+	++++
ALB	-	-	-	++++
CK8	±	±	++	+++
CK18	++	++	++/+++	+++
GATA4	-	-	-	+++
CK19	++	++	+++	++
Cx32	-	++	++	++++
Cx43	±	+	++	+
E-Cadherin	+++	+++	++	++

Human specific markers were analyzed by real-time RT-PCR. For relative quantification, we used the ΔC_T method as follows: $\Delta C_T < 0-4$ (++++) very high expression, $\Delta C_T 4.1-8$ (+++) high expression, $\Delta C_T 8.1-12$ (++) moderate expression, $\Delta C_T 12.1-16$ (+) low expression and $\Delta C_T > 16$ (-) no expression. Human liver cancer (HepG2) cells were included for comparison

CK19 and connexins were upregulated to a moderate or high level. Further, the higher expression of SOX17 in co-cultivated UCBC suggests an early endodermal differentiation. In electron microscopy images taken after 7 d co-culturing, we found frequent microvilli structures and a flattened cell phenotype and occasionally tight UCBC-to-AML12 cell contacts along the plasma membrane. Cell-cell interaction of labelled UCBC and confluent AML12 cells was visualized by time-relapsed video-imaging. Here, we found a high motility of UCBC with a sporadic cell contact to the murine cells. Also, a low level of communicating cells was measured by dye transfer of UCBC co-cultured with murine AML12 cells.

The endodermal/hepatic in vitro differentiation of adult UCBC (CD34⁺ stem cells) was described in several studies (Lysy *et al.* 2008; Esrefoglu 2013). Different in vitro protocols, mainly on the basis of combinations of chemicals, growth and differentiation factors, were used in order to induce multistep hepatic cell differentiation (Fiegel *et al.* 2003; Kakinuma *et al.* 2003; Teramoto *et al.* 2005; Ishii *et al.* 2008; Lavon 2010; Chen and Zeng 2011). For UCBC, combinations of growth and differentiation factors are known to contribute to the in vitro proliferation and differentiation of hepatic progenitor cells in the rodent and human liver (Fiegel *et al.* 2003). Further, a defined medium containing HGF induced albumin and CK19 expression which was accompanied by increased cell proliferation (Fiegel *et al.* 2003). In

another study, HGF in combination with SCF, leukaemia inhibitory factor (LIF), fibroblast growth factor-1 (FGF-1) and FGF-2 induced ALB⁺ cells with an expression of markers from hepatocyte lineage, like CK19, CK18 and AFP in human cord blood cells. These cells had the same phenotype as bipotential hepatic progenitor cells (Kakinuma *et al.* 2003). The addition of HGF and SCF into stem cell media had no effect on the CK19 expression, but both factors increased the CK8 and CK18 expression of cultured cells (Wulf-Goldenberg *et al.* 2008). We used in our study SCF and HGF for the induction of the endodermal differentiation. Our results demonstrated a slight early endodermal differentiation which was more intense after co-culture approach compared with the use of conditioned medium. The differences, compared to the results of literature reports, may be due to the use of other stem cell types and/or the inclusion of injured primary mouse liver cells as effector cells, suggesting an involvement of the injured microenvironment. In a published transwell co-culture system using bone marrow-derived cells and injured murine liver cells, epithelial CK18 expression, albumin, GATA4, HNF4 α and liver enzyme transcripts on protein and RNA level were found. These cells were converted into viable hepatocytes after transplantation into liver-injured mice (Jang *et al.* 2004). Coding proteins for cell-cell communication and GJ proteins like the connexins were discussed to play an important role during development and differentiation of stem cells (Elias and Kriegstein 2008; Kihara *et al.* 2008; Choi *et al.* 2010). However, their precise involvement and the contribution of connexins in these processes remain speculative. In our study, undifferentiated UCBC showed low Cx32 and Cx43 transcript expression. Both connexin transcripts were upregulated by cultivation with hepatic conditioned AML12 medium. GJ structures were not detectable by electron microscopy, but tight cell-cell contacts were visualized. Further, microvilli formation and sporadic cell protrusions with kinocilia- and stereocilia-like structures of cultivated cells could be observed. In the present study, this may suggest either low expression of connexins at protein level or a short half-life of the proteins, which is described at least for Cx43 (Simek *et al.* 2009; Solan and Lampe 2009). In haematopoietic stem cells, upregulation of Cx43 was discussed as a response to events that call for active division of stem cells, perhaps enabling them to divide (Rosendaal *et al.* 1994). In our study, upregulation of the connexins apparently was not clearly associated with induction of endodermal differentiation. Expression of hepatic cell-associated Cx32 in cultivated UCBC was only sparsely reported. In one study, cytokine-supported cultivation of umbilical cord stem cells led to the expression of Cx32 (Campard *et al.* 2008). This result is in comparison to our findings on co-culture with AML12 cells. Further, in direct co-culture of UCBC with AML12 cells, the expression of transcripts for CK8 and CK19, as well as for Cx32 and Cx43, was higher

compared to cells cultivated in hepatic conditioned medium. In addition, induction of SOX17 expression suggests an early endodermal cell differentiation obtained in direct co-culture. In hepatic differentiation protocols for stem cells, SOX17 expression was identified as an early marker of endodermal cell differentiation (Chen *et al.* 2006; Cho *et al.* 2008; Rolletschek *et al.* 2010). Mature hepatocytes and the human HepG2 cell line express SOX17 at low level (Matsui *et al.* 2006). The connexins process a pivotal function on the maintenance of the differentiated cell function, including albumin and bile secretion (Fladmark *et al.* 1997; Kojima *et al.* 2003; Naiki-Ito *et al.* 2010). Using dye transfer, the effects of stimulators (retinoic acid) and inhibitors (3-heptanol) of GJ-committed cell-cell interaction suggested in our experiments the participation of connexins. However, also, direct pharmacological drug effects cannot be excluded. Functional studies using small interfering ribonucleic acid (siRNA) inhibition are intended. No GJ formation was visible by electron microscopy in co-culture of UCBC with AML12 cells. But, tight cell-cell contact between neighbouring cells and infrequent cell fusions was detected. According to literature, the endodermal/hepatic differentiation potential of UCBC (CD34⁺ cells) is more restricted, compared to embryonic stem cells (Wagers *et al.* 2002; Wang *et al.* 2003; Masson *et al.* 2004; Agarwal *et al.* 2008; Basma *et al.* 2009; Esrefoglu 2013).

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