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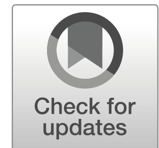

In vitro efficacy of liver microenvironment in bone marrow mesenchymal stem cell differentiation

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Abstract

Bone marrow–derived mesenchymal stem cells (BM-MSCs) represent an interesting alternative to liver or hepatocyte transplantation to treat liver injuries. Many studies have reported that MSCs can treat several diseases, including liver damage, just by injection into the bloodstream, without evidence of differentiation. The improvements were attributed to the organotrophic factors, low immunogenicity, immunomodulatory, and anti-inflammatory effects of MSCs, rather than their differentiation. The aim of the present study was to answer the question of whether the presence of BM-MSCs in the hepatic microenvironment will lead to their differentiation to functional hepatocyte-like cells. The hepatic microenvironment was mimicked *in vitro* by culture for 21 d with liver extract. The resulted cells expressed marker genes of the hepatic lineage including *AFP*, *CK18*, and *Hnf4a*. Functionally, they were able to detoxify ammonia into urea, to store glycogen as observed by PAS staining, and to synthesize glucose from pyruvate/lactate mixture. Phenotypically, the expression of MSC surface markers CD90 and CD105 decreased by differentiation. This evidenced differentiation into hepatocyte-like cells was accompanied by a downregulation of the stem cell marker genes *sox2* and *Nanog* and the cell cycle regulatory genes *ANAPC2*, *CDC2*, *Cyclin A1*, and *ABL1*. The present results suggest a clear differentiation of BM-MSCs into functional hepatocyte-like cells by the extracted liver microenvironment. This differentiation is confirmed by a decrease in the stemness and mitotic activities. Tracking transplanted BM-MSCs and proving their *in vivo* differentiation remains to be elucidated.

Keywords Bone marrow mesenchymal stem cells · Local microenvironment · Differentiation · Hepatic lineage · Gene expression · Hepatocyte functions

Introduction

Liver damage and chronic liver diseases represent a significant and severe health care problem that affects millions of patients all over the world. In end-stage liver disease, liver transplantation is mostly the first therapeutic option. However, it bears several major obstacles such as increasing shortage of suitable donor organs that markedly compromises liver transplantation, patients require lifelong immunosuppressive medication and hence medical supervision, and the procedure involves major surgery and is very costly (Hyder *et al*. 2018). An attractive approach to overcome the problems of whole organ transplantation is the transplantation of liver cells. Cell transplantation could help to alleviate donor organ shortage and, if proven effective, would be simpler, safer, and less expensive than a whole organ transplant. However, the lack of methodology to maintain hepatocytes in long-term *in vitro* culture retards the research development (Hyder *et al*. 2012). Other adult multipotent mesenchymal stem and reprogrammable cells still represent a promising alternative in regenerative medicine and in hepatic tissue engineering (Ungefroren *et al*. 2015; Ungefroren *et al*. 2016).

There are many studies that report the treatment of several diseases just by injection of mesenchymal stem cells (MSCs). MSCs were reported to repair liver fibrosis (Stutchfield *et al.* 2010; Ali *et al*. 2012; Yu *et al*. 2017), improve the outcome of islet grafts in diabetes (Kerby 2013), provide immunosuppressive effects in rheumatologic diseases (Sun *et al*. 2010; Keyszer 2011; Wang *et al*. 2011; Xu 2012; Liu *et al*. 2015a; Li *et al*. 2015), repair and improve cartilage quality in osteoarthritis and other osteochondral defects (Orozco *et al*. 2013; Harada 2015), prevent cell death in ischemic brain, recover motor and sensory function, and cause significant

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improvements in Parkinson, Alzheimer and other neurologic diseases (Okazaki 2008; Venkataramana *et al.* 2010; Honmou 2011; Liu *et al.* 2015b), and treat many cardiac dysfunctions (Ammar 2015; Kawamura 2015; de Morais *et al.* 2015; Roura 2015; Wang *et al.* 2015). However, all of these reports did not prove that MSCs have been differentiated into the target cells to replace the damaged tissue. Many mechanisms of action have been suggested for these MSCs, but none of them has suggested that MSCs have been differentiated *in vivo*, and little studies have even traced MSCs after their injection.

Little is known about the differentiation into hepatocyte-like cells after transplantation (Sato *et al.* 2005; Chamberlain *et al.* 2007). For stem cell transplantation to treat liver diseases, it is important to determine that MSCs will undergo differentiation after transplantation. Otherwise, the differentiation step into hepatocytes should be first undertaken *in vitro* before transplantation. Leaving the continuously dividing MSCs without differentiation *in vivo* has many risks including malignant transformation (Casiraghi *et al.* 2013). The interaction between stem cells and the environment surrounding them regulates differentiation or stem cell self-renewal so that a particular culture condition is required for differentiation potential towards hepatocytes; otherwise, stem cells will not be differentiated. To mimic the *in vivo* target tissue microenvironment, tissue homogenates have been used in several systems to induce *in vitro* stem cell differentiation into functional cells (Perán *et al.* 2010; Wang *et al.* 2012; Xue *et al.* 2016). Liver homogenate contains about 643 proteins that undertake many functions including providing high range of substances necessary for the growth of liver cells (Chu *et al.* 2004; Nhung *et al.* 2015). It is also likely to include all necessary stimulating factors and nutrients for hepatocytic differentiation (Fan *et al.* 2015; Kedarisetty *et al.* 2014), which are sufficient for liver regeneration after damage or partial removal (Michalopoulos 2014). Also, decellularized cell-deposited extracellular matrix was suggested to facilitate the hepatic maturation and promote stem cell–based liver generation (He *et al.* 2013). Liver homogenate was shown earlier to differentiate other kinds of stem cells as human umbilical cord stem cells (Xue *et al.* 2016) and placental amniotic membrane stem cells (Sarvandi *et al.* 2015) into hepatic linage. Here, we used different concentrations of liver homogenate to answer the question of whether the presence of bone marrow–derived MSCs in this hepatic environment will be sufficient for their differentiation into functional hepatocyte-like cells.

Material and Methods

Mesenchymal stem cell isolation and culture Rat bone marrow was isolated by flushing femurs and tibias by DMEM as described by Zhang and Chan (Zhang and Chan 2010). All bone marrow cells were cultured for 4 d, and the plastic adhered cells were washed several times and cultured until reached confluence. BM-MSCs in passages 3, 4, and 5 were used in this study.

Liver extract preparation and treatment Livers were excised from 7- to 12-d-old rats, washed many times with sterile PBS, cut into small segments in 10 ml sterile DMEM, and homogenized for 30 s at low speed on wet ice. The homogenate was centrifuged at 15000 rpm for 20 min at 4°C. The supernatant was sterile filtered (0.45 μm) then stored at −20°C until needed.

BM-MSCs in passages 3, 4, and 5 at 70% confluence were cultured in DMEM-F12 supplied with 5% FCS and different concentrations of liver extract (0 (control), 6, 18, 30, and 60 μg/ml) for 21 d. The culture medium was changed every 3rd day. Cells of different cultures were then characterized by flow cytometry using anti-CD90, anti-CD105, and anti-CD34.

Periodic acid-Schiff staining The differentiated hepatocyte-like cells should be capable of glycogen storage as hepatocytes do. Glycogen storage has been tested by periodic-acid Schiff (PAS) stain (Hui *et al.* 2017) at day 21. The medium was removed from the flasks; cells rinsed with PBS three times and fixed with 4% paraformaldehyde for 20 min at room temperature, washed 3 times with PBS for 2 to 3 min, oxidized for 15 min with 1% periodic acid, and washed 3× with deionized water. Cells were then stained with Schiff's reagent for 30 min and washed 3 times with PBS prior to microscopic examination and imaging.

Hepatocyte metabolic function testing Differentiated cells have been tested for their ability to detoxify ammonia into urea and to synthesize glucose from pyruvate/lactate. The experimental details were published in our previous reports (Hyder *et al.* 2018, 2012).

Real-time PCR The expression of some genes characterizing hepatic cells, cell cycle genes, and stem cell marker genes has been tested. The expression of these genes has been analyzed by quantitative RT-PCR as described elsewhere (Hyder 2019). The forward and reverse primers for the selected genes are mentioned in Table 1.

Statistical analysis Data are presented as mean ± SEM. All experiments were carried out only in MSC culture passages 3, 4, and 5 ($N = 3$ in all cases). Experiments were repeated 3 times within each tested passage, but the average value was considered $N = 1$. Analysis of variance (ANOVA) was applied for the statistical analysis followed by t test as a post hoc test. A $p < 0.05$ was considered significant in all cases.

Table 1. Primer sequences used for qRT-PCR

Gene	Accession no	PF	PR	Product size (bp)
AFP	NM_012493.2	GCTTTCTGAGGGACGAAACC	AGGCCAGAGAAATCAGCAGT	193
Krt18 (CK18)	NM_053976.1	CTGGGGCCACTACTTCAAGA	GCGGAGTCCATGAATGTCAC	184
Hnf4a	NM_022180.2	GTGTGTCCATTCGCATCCTC	CATACTGCCGGTCGTTGATG	198
Nanog	NM_1100781.1	ATGCGGACTGTGTTCTCTCA	GCCACCTCTTGCACTTCATT	169
Sox2	NM_1109181.1	CGTCAAGAGGCCCATGAATG	TAATCCGGGTGCTCCTTCAT	204
Anapc2	NM_1100532.1	CTCCTCGTGTCCCTCAAAGT	GTTAGCCCAGCCACAATCTG	209
Cdk1 (cdc2)	NM_019296.1	CATATTTGCAGAGCTGGCGA	GTCCAAGCCGTTTTCATCCA	211
cyclin A1	NM_1011949.1	ACAGACCCAAGGCTCACTAC	CGACAAGCTGCAATTTCCCT	192
Abl1	NM_1100850.1	GGGACCAAAGAAGGCCTAGT	AAGGTTGGGGTCATTTTCGC	224
Actin beta	NM_031144.3	TGTGTTGTCCCTGTATGCCT	AATGTCACGCACGATTTCCC	223

Results

Expression of hepatocyte lineage-specific marker genes We first sought to determine whether cells stemming from BM-MSCs and differentiated using liver homogenate display mature characteristics of the hepatic lineage; we examined by PCR the gene expression patterns of various early hepatic marker genes. RNA was collected after the differentiation process and subsequently analyzed using RT-qPCR for the lineage-specific markers, namely hepatocyte nuclear factor 4 (*Hnf4a*), alpha-fetoprotein (*AFP*), and cytokeratin 18 (*CK18*, also known as *KRT18*). As shown in Fig. 1, the 3 genes were upregulated (ANOVA $p = 0.001$, 0.009, and 0.0013 for *Hnf4a*, *AFP*, and *CK18*, respectively). However, this upregulation was significant only in cells differentiated in the higher concentrations (30 and 60 μg/ml) of liver extracts (Student's *t* test $p < 0.05$), as compared with the corresponding mRNA expression of cells cultured with 0 μg/ml of liver extract.

Hepatocellular functional aspects in cells differentiated using liver extract Next, we examined the hepatic-related functions in cells derived from BM-MSCs and differentiated using liver homogenate. Ideally, the differentiation procedure should not only upregulate the hepatocyte-specific marker genes but also improve features of the newly differentiated cells in a way that they become functionally more hepatocyte-like. We therefore tested whether the used differentiation medium (liver extract) would alter functional parameters of the differentiated cells. Bone marrow–derived mesenchymal stem cells were allowed to differentiate for 3 wk in different concentrations of liver extract and at the end of this period were analyzed for hepatocyte-specific functions (Fig. 2).

All cell groups, regardless of treatment, formed and secreted urea in a quantity ranged from 28 to 30 mg/dl under basic conditions. Incubation with NH_4Cl increased urea formation significantly in all settings formerly differentiated using any concentration of liver extract (Fig. 2A). This reflects the ability of these differentiated cells to detoxify ammonia into urea, which is a proper hepatocellular function.

To measure the ability of these cells to perform gluconeogenesis, after the 3-wk differentiation period, they were incubated for 24 h with a buffer supplemented with Na-pyruvate and Na-L-lactate. Stimulation with pyruvate/lactate induced higher glucose secretion compared with non-stimulated cultures. As observed for urea, the effect on gluconeogenesis from lactate/pyruvate was significantly higher ($p < 0.05$, *t* test) in cells differentiated with any concentration of liver extract than in the corresponding control (Fig. 2B).

To observe the ability of liver extract-differentiated cells to store glycogen, after the 3-wk differentiation period, they were subjected to PAS staining. Figure 2C shows representative micrographs of stained cells after incubation for the 21 d without (upper panel) or with liver extract (60 mg/ml, lower panel). PAS-positive cells could be observed after differentiation using any of the applied liver extract concentrations. Regarding cell morphology, it should be mentioned that most differentiated cells kept their fibroblast-like spindle-shaped morphology, although they looked bigger or more round. However, the characteristic polygonal structure of hepatocytes could not be observed after differentiation with any liver extract concentration.

Stem cell markers in cells differentiated from BM-MSCs by liver extract The differentiation of BM-MSC into hepatocyte-like cells using liver extract should be accompanied by inhibition of stemness markers. We have examined the gene expression of 2 pluripotency marker genes, namely *sox2* and *Nanog*, and analyzed some phenotypic cell surface markers of MSCs.

Cell surface marker proteins of MSCs were examined by flow cytometry after differentiation for 21 d using different concentrations (0–60 μg/ml) of liver extract. Both undifferentiated and differentiated cells were positive for the cell surface markers CD90, and CD105 while being negative for CD34

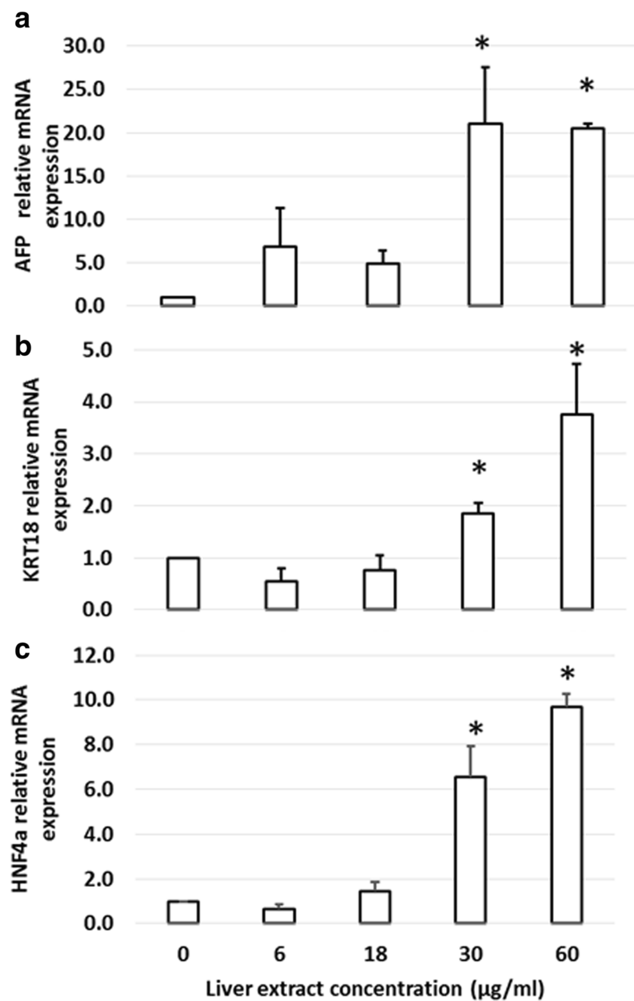

Figure 1. Expression of the hepatic lineage marker genes α-fetoprotein (*A*), cytokeratin-18 (*B*), and hepatocyte nuclear factor 4 alpha (*C*) in BM-MSCs differentiated for 21 d by different concentrations of liver homogenate. Data are presented as mean ± SEM of $N = 3$ as described in the "Material and Methods" section. Statistical analyses: ANOVA $p < 0.05$ in all cases, *asterisk* denotes significantly higher value than that of the control (post hoc *t* test $p < 0.05$).

(Fig. 3A). The CD90 expression decreased from 92.7 to 56.3%, while the expression of CD105 decreased from 96.1 to 61.9% after differentiation using 60 µg/ml of liver extract.

We have also examined the effect of MSC differentiation by different concentrations of the liver extract on the expression of stemness genes *Nanog* and *sox2* by qRT-PCR (Fig. 3B). The results revealed a significant transcriptional downregulation of both genes, even in response to the lowest applied liver extract concentration. These findings suggest that differentiation of BM-MSCs into hepatocyte-like cells is accompanied by the downregulation of stem cell characters.

The differentiation process is accompanied by transcriptional downregulation of genes involved in cell cycle regulation
Stem cells are characterized by continuous division, a feature that is inhibited by differentiation. Since the inhibition of mitotic activity involves transcriptional changes in key cell cycle regulators, we examined by qPCR analysis whether the differentiation of BM-MSCs by liver extract into hepatocyte-like cells was associated with the regulation of genes that have regulatory roles in different stages of the cell cycle. As seen in Table 2, the 21-d differentiation period using liver extract significantly downregulated the mRNA expression of the *ANAPC2* and *CDC2* significantly, and *ABL-1* and *cyclinA1* insignificantly. These transcriptional effects on cell cycle genes are likely mediated by a differentiation process.

Discussion

Direct injection/transfusion of MSCs has been reported to improve the function of different damaged tissues and systems (Okazaki 2008; Stutchfield *et al*. 2010; Sun *et al*. 2010; Venkataramana *et al*. 2010; Honmou 2011; Keyszer 2011; Wang *et al*. 2011; Ali *et al*. 2012; Xu 2012; Kerby 2013; Orozco *et al*. 2013; Ammar 2015; de Morais *et al*. 2015; Harada 2015; Li *et al*. 2015; Kawamura 2015; Liu *et al*. 2015a; Liu *et al*. 2015b; Roura 2015; Wang *et al*. 2015; Yu *et al*. 2017). Mechanisms of action are not clearly elucidated, but suggested to be due to the stem cell–derived organotrophic factors (Schuppan and Pinzani 2012; Liu *et al*. 2015c) and exosomes (Rong *et al*. 2019) that treat and protect cells from damage or activate endogenous mechanisms to restore the injured tissue, and to the low immunogenicity, immunomodulatory, and anti-inflammatory effects of MSCs (Meier *et al*. 2013; Jang *et al*. 2014; Zekri *et al*. 2015). The aim of the present study was to elucidate whether the presence of MSCs in the hepatic microenvironment will lead to their differentiation to functional hepatocyte-like cells. This is crucial because of the multifunctional nature of hepatocytes and to avoid the risk resulted from the presence of undifferentiated continuously dividing cells inside the liver tissue.

There are 3 conditions to consider the differentiation process of these BM-MSCs into hepatocyte-like cells successful. The first condition is that differentiated cells must have less expression of stemness markers. In the present study, a transcriptional downregulation was observed in both pluripotency genes *sox2* and *Nanog*. The characteristic MSC surface marker proteins decreased. However, despite the apparent differentiation, the resulted cells still expressed the mesenchymal stem cell markers CD90 and CD105, suggesting, in agreement with previous studies (Campard *et al*. 2008; Bao *et al*. 2016), incomplete differentiation.

The second condition to consider the BM-MSC differentiation into hepatocyte-like cells successful is that differentiated cells must have inhibited cell division and cell cycle parallel to the differentiation process. The present data showed downregulation at the mRNA level of cell cycle regulatory genes *ANAPC2*, *CDC2*, *cyclin A1*, and *ABL-1*. ANAPC2 controls

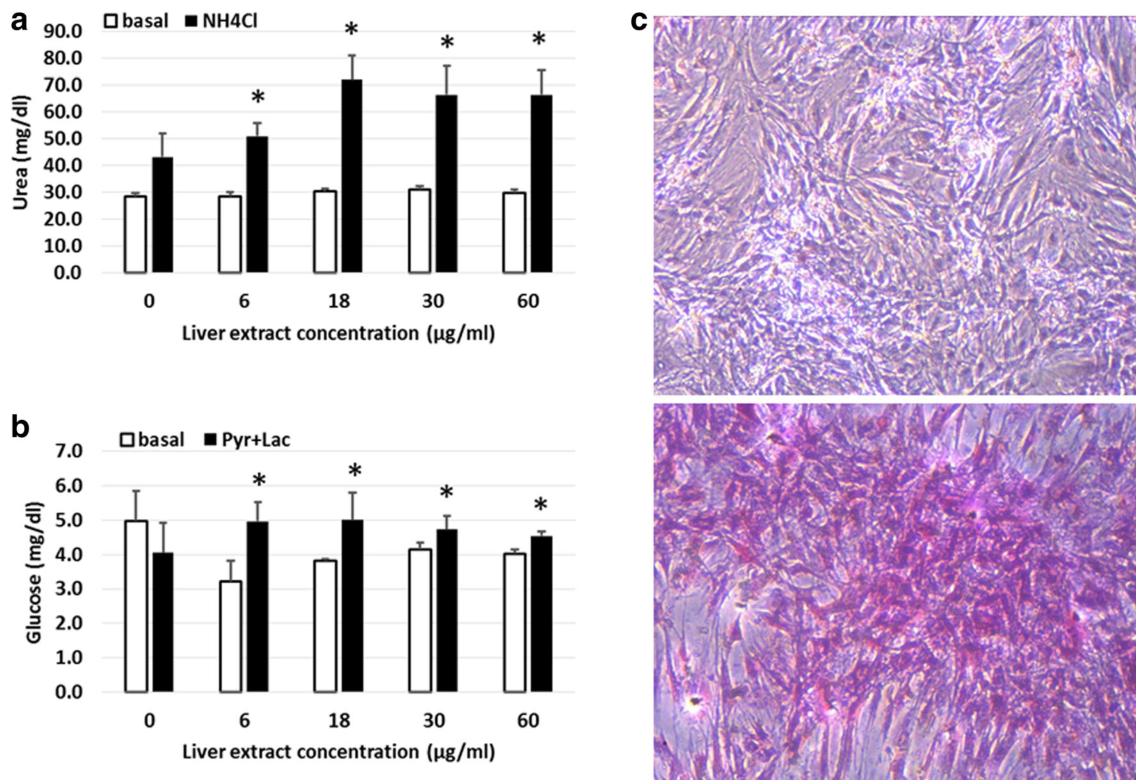

Figure 2. Metabolic functions of hepatocyte-like cells differentiated from BM-MSCs by incubation with different concentrations of liver extract for 21 d. (*A*) Detoxification: differentiated cells are able to detoxify ammonia to urea. Cells were incubated with or without 5 mM NH_4Cl for 24 h before the estimation of urea. (*B*) Gluconeogenesis: differentiated cells were incubated with a mixture of lactate and pyruvate for 24 h before the estimation of formed glucose. (*C*) Glycogen storage: MSCs incubated in either 0 (*upper photo*) or 60 mg/ml (*lower photo*) of liver extract for 21 d were subjected to PAS staining. Data are presented in (A) and (B) as mean ± SEM. Statistical analyses: *asterisk* denotes significantly higher value of the stimulated cells than that of the basal value (*t* test $p < 0.05$).

the regulation of the G1/S and G2/M transitions, CDC2 takes part in M-phase regulation, ABL-1 regulates the S-phase and DNA replication, and cyclin A defines control points of the cell cycle by binding CDK2 and CDC2 resulting in 2 distinct cyclin A kinase activities, one appearing in S phase and the other one in G2 phase. Inhibition of ANAPC2 and CDC2 has been reported to induce cell cycle arrest at G2/M (Pagano *et al*. 1992; Heilman *et al*. 2005; Enserink and Kolodner 2010; Hyder *et al*. 2012, 2018). As shown above, the results showed that differentiation was associated also with a decrease in pluripotency, which is known to be associated with self-renewal and proliferation (Hyder *et al*. 2018). The down-regulation of *sox2*, for example, can exert exiting of the cell cycle (Bylund *et al*. 2003) which is in line with the inhibited cell cycle regulating gene activity in the present study.

The last condition is that the differentiated cells must express some of the hepatic lineage marker genes and perform hepatic metabolic functions. The present study revealed a clear differentiation of BM-MSCs to hepatocyte-like cells. These cells expressed the hepatic lineage-specific genes *Hnf4a*, *AFP*, and *CK18*. They performed also some hepatocellular-specific functions: detoxification of ammonia into urea, storage of glycogen, and gluconeogenesis from pyruvate/lactate mixture. Thus, the applied hepatic tissue extract, which was suggested to mimic the liver microenvironment, was sufficient to differentiate BM-MSCs into hepatocyte-like cells.

Neonatal livers have been used for homogenization in the present study. Fetal and neonatal tissues are supposed to have higher levels of growth and differentiation factors than the adult tissue (Hyder *et al*. 2010). In fact, liver homogenate, if proven efficient, will represent a very cost-effective medium for the *in vitro* differentiation of bone marrow stem cells towards hepatic lineage. Therefore, some previous studies have applied it in different concentrations to promote the differentiation of other kinds of stem cells (Sarvandi *et al*. 2015; Xue *et al*. 2016). In one study (Sarvandi *et al*. 2015), 6 µg/ml concentration of liver extract was found the effective dose for induction of omentum tissue mesenchymal stem cell differentiation. In the other studies, higher concentrations of 50 mg/ml with decidua-derived stem cells (Bornstein *et al*. 2012) or 150 mg/ml (Xue *et al*. 2016) with umbilical cord stem cells have been applied. We applied concentrations ranging from 6 to 60 µg/ml and observed that the lowest concentration was sufficient to inhibit the stem cell and cell cycle gene expressions. However, hepatic cell functions and the

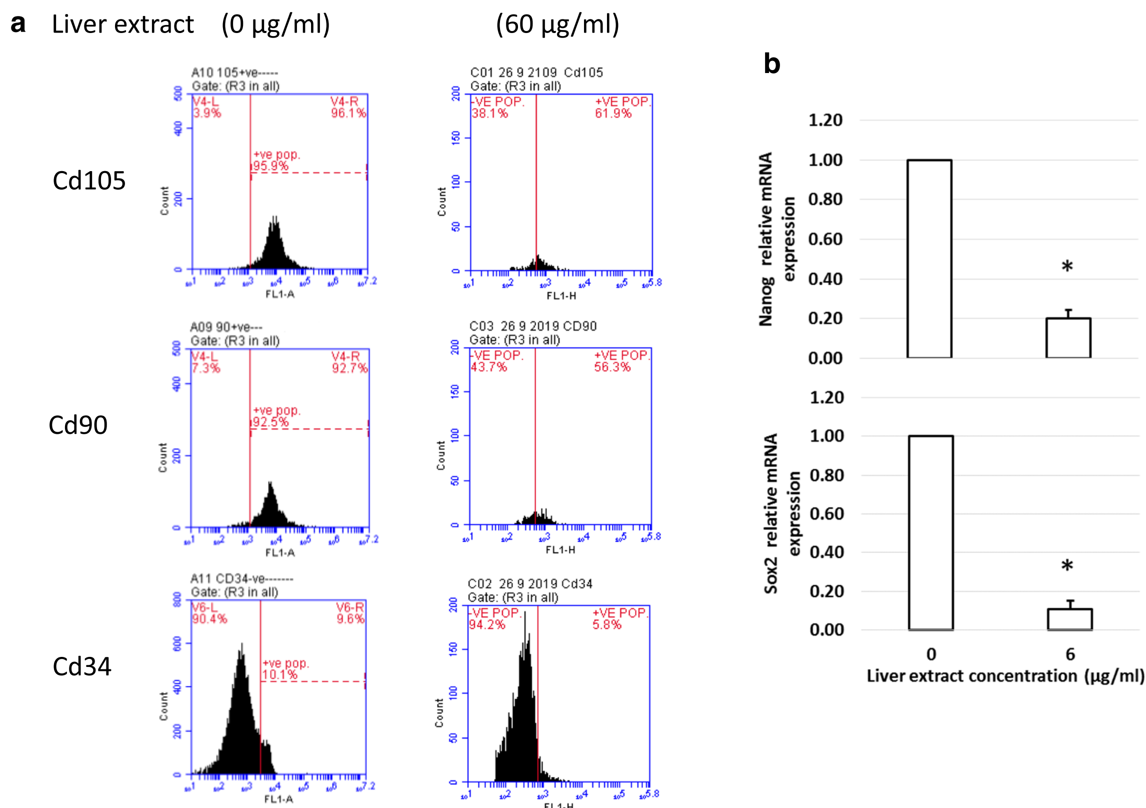

Figure 3. The differentiation process of BM-MSCs into hepatocyte-like cells by incubation with different concentrations of liver extract for 21 d is accompanied by a decrease of stem cell markers. (*A*) Cell surface marker proteins of MSCs were examined by flow cytometry. Cells were positive for CD90 and CD105 while being negative for CD34. Note the decrease in the expression of these markers after incubation with liver extract. (*B*) Incubation with liver extract decreases the expression of stem cell marker genes *Nanog* and *sox2*. Data are presented as mean ± SEM. Statistical analyses: *asterisk* denotes significantly lower value of differentiated cells than that of the control (*t* test $p < 0.05$).

expression levels of hepatic lineage genes were concentration dependent within the applied low range of concentrations. These results are in agreement with the previous reports of other stem cell types differentiated by liver homogenates.

Although they acquired some hepatocellular functions and became morphologically more round, the differentiated hepatocyte-like cells kept their original bipolar fibroblast-like structure. Also, as mentioned above, only a fraction of cells has lost the typical cell surface markers CD90 and CD105. Moreover, the downregulation of the cell cycle regulatory genes *cyclin A1* and *ABL1* was weak and not significant. All of these findings suggest that the differentiation of BM-MSCs into hepatocyte-like cells was partial. The differentiation of only a small fraction into hepatocytes is similar to the results of other *in vitro* studies using variable differentiation media as previously reported (Aurich *et al*. 2009; Lin *et al*. 2011; Piryaei *et al*. 2011), so that the entire functionality of the differentiated cell, regardless of the differentiation methodology, was only 8–23% of the native hepatocytes (Hengstler *et al*. 2005). This differentiation fraction may vary

Table 2. Effect of BM-MSCs incubation with different liver extract concentrations for 21 d on the relative expression of some cell cycle mRNA

Gene	Liver extract concentration (µg/ml)				
	0	6	18	30	60
ANAPC2	1.00 ± 0.00	0.42 ± 0.15*	0.46 ± 0.15*	0.43 ± 0.14*	0.69 ± 0.24
CDK1	1.00 ± 0.00	0.31 ± 0.19*	0.15 ± 0.07*		0.09 ± 0.02*
Cyclin A1	1.00 ± 0.00	0.59 ± 0.25	0.80 ± 0.34	0.79 ± 0.14	0.75 ± 0.24
ABL1	1.00 ± 0.00	1.04 ± 0.23	0.71 ± 0.22	0.94 ± 0.33	0.99 ± 0.21

Quantitative RT-PCR was performed using Sybr-green and calculations using the ΔΔCt method with β-actin as a house-keeping gene. Data are presented as mean ± SEM of $N = 3$ from 3 different culture passages. Statistical analyses: ANOVA $p < 0.05$ for *ANAPC2* and *CDK1* only, *asterisk* denotes significantly lower value than that of the control (post hoc *t* test $p < 0.05$)

in vivo, but this remains to be determined. Nevertheless, this limited fraction still raises the question about the efficacy and safety of direct MSC transplantation.

Conclusion

Bone marrow–derived mesenchymal stem cells can be differentiated *in vitro* into functional hepatocyte-like cells just by culturing in a hepatic microenvironment represented by liver homogenate. This differentiation was accompanied by a logic downregulation of cell division and stem cell markers. Labelling MSCs and persuading their landing and behavior in the liver after transplantation to prove this differentiation *in vivo* is still required.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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