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Transcriptomic fingerprinting of bone marrow-derived hepatic β_2m^-/Thy-1$^+$ stem cells

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Abstract

The aim of the present study was to determine if the bone marrow (BM) β_2m^-/Thy-1$^+$ stem cells isolated from common bile duct ligated (CBDL) rats possess hepatocyte-like characteristics in their global gene expression profiles. The Affymetrix RG U34A arrays were used to conduct transcriptomic profiling on BM β_2m^-/Thy-1$^+$ stem cells isolated from CBDL and control rats as well as primary hepatocytes. Forty-one probe sets were up-regulated more than 2-fold in CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells compared to control BM stem cells. Twenty-seven probe sets were present in both CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells and control hepatocytes but absent in control β_2m^-/Thy-1$^+$ BM stem cells, including Tcf1 and Dbp. Compared to the control β_2m^-/Thy-1$^+$ BM stem cells, CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells shared more commonly expressed genes with hepatocytes. Overall, CBDL-derived β_2m^-/Thy-1$^+$ stem cells displayed a different transcriptomic fingerprint compared with β_2m^-/Thy-1$^+$ BM stem cells isolated from control rats; and CBDL-derived β_2m^-/Thy-1$^+$ stem cells started to express some hepatocyte-like genes.

Keywords: Bone marrow-derived hepatic stem cells; DNA microarray; Transcriptomic profiling

Bone marrow-derived hepatic stem cells (BMDHSCs) that are able to proliferate and differentiate into mature hepatocytes could potentially provide a means of treating acute and end-stage chronic liver insufficiency in the future. It has been found that oval cells in the liver are the progenitor cells that can be induced to proliferate following hepatic injury, when growth of mature hepatocytes is suppressed [1]. For many years, oval cells were believed to originate from cells present in the canals of Herring [2] or from blast-like cells located near the bile ducts [3]. Recently, results of cross-sex and cross-strain BM and whole liver transplantation experiments indicated that the oval cells are derived from bone marrow stem cells (BMSCs), and that the BM is the ultimate source of hepatic progenitor cells [4–7]. This finding is further supported by a recent study showing that transplantation of adult bone marrow cells into fumarylacetoacetate hydrolase (FAH)-deficient mice, animals with a fatal congenital tyrosinemia type I, rescued the mice and restored their liver biochemical functions [8]. Although recent publications demonstrating that the transplanted BMSCs repopulated the liver primarily by cell fusion have raised serious question on the role of BMDHSCs in liver regeneration [9,10], the potential benefits derived from BMDHSCs certainly demand more studies in this area [11,12].

Recently, our laboratory isolated a sub-population of stem cells with specific hematopoietic markers, β_2m^-/Thy-1$^+$, from human and rat BM that can differentiate

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into mature functioning hepatocytes [13]. It was found that transplantation of β_2m^-/Thy-1^+ BMSCs into rats undergoing rejection resulted in a significant level of liver repopulation [14]. Furthermore, rat bone marrow-derived hepatic β_2m^-/Thy-1^+ stem cells could be induced to differentiate into functional hepatocytes metabolizing ammonia into urea by adding cholestatic serum into an in vitro culture system developed in our laboratory [13]. Interestingly, rat bone marrow β_2m^-/Thy-1^+ stem cells derived from common bile duct ligated (CBDL) animals, without in vitro co-culturing, expressed some liver function-related genes such as albumin (RT-PCR and immunohistochemical staining) [13]. However, it is unknown how similar these rat bone marrow β_2m^-/Thy-1^+ stem cells are to primary hepatocytes in their global gene expression profiles. To test the hypothesis that rat bone marrow-derived β_2m^-/Thy-1^+ stem cells possess hepatocyte-like characteristics (e.g., liver-specific gene expression) even without in vitro co-culturing with primary hepatocytes and cholestatic serum, the Affymetrix high-density oligonucleotide microarrays, Rat Genome U34A arrays, were used to conduct transcriptomic profiling. Our aims were (1) to determine the gene expression profile of rat β_2m^-/Thy-1^+ BM stem cells derived from CBDL rats; and (2) to compare the gene expression profiles among CBDL, sham control BM β_2m^-/Thy-1^+ stem cells, and primary rat hepatocytes. Our results showed that there were significant differences in gene expression profiles between CBDL and sham control β_2m^-/Thy-1^+ BM stem cells, and that CBDL β_2m^-/Thy-1^+ BM stem cells started to express certain liver-specific genes. Nevertheless, the CBDL-derived β_2m^-/Thy-1^+ BM stem cells still displayed a strikingly different gene expression profile from the primary hepatocytes.

Materials and methods

Chemicals and reagents. Chemicals for hepatocyte and BMSC isolations as well as magnetic bead cell sorting (MACS) were purchased from Sigma Chemical (St. Louis, MO). Antibodies against Thy-1 and β_2m were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Qiagen RNeasy mini kit was purchased from Qiagen (Valencia, CA). MessageAmp aRNA kits and oligo$(dT)_{24}$ anchored T7 primers were purchased from Ambion (Austin, TX). Biotin-11-CTP and biotin-16-UTP were purchased from PerKinElmer Life Science (Boston, MA). Streptavidin–phycoerythrin was purchased from Molecular Probes (Eugen, OR). Biotinylated anti-streptavidin was obtained from Vector Laboratories (Burlingame, CA). All PCR primers were obtained from Invitrogen (Carlsbad, CA). TaqMan reverse transcription reagents and SYBR Green PCR master mix were obtained from Applied Biosystems (Foster City, CA).

Animal model-CBDL. Three Lewis male rats (200–250 g), purchased from Lab Animal (Harlan, Indianapolis, IN), underwent common bile duct ligation to generate cholestasis and in the control group, three Lewis male rats (200–250 g) underwent a sham operation. A standard operative technique was used as described previously [13,14]. All rats were housed in a climate-controlled (21 °C) room under a 12-h light–dark cycle. Animals were fed Rodent Chow 5001 (Ralston Purina, St. Louis, MO) and given tap water ad libitum. BM was harvested 10 days later to isolate β_2m^-/Thy-1^+ stem cells using a two-step MACS technique. Primary hepatocytes were also harvested from three sham control rats to compare their mRNA expression profile with that of β_2m^-/Thy-1^+ stem cells. The animals received humane care according to the guidelines prepared by the National Institute of Health, USA. All operations were performed under general anesthesia (methoxyfluorane) using sterile surgical technique.

Isolation of primary hepatocytes and BM β_2m^-/Thy-1^+ stem cells. Primary hepatocytes were isolated using an in situ two-step EDTA/collagenase portal vein perfusion technique as previously described [13,15]. After enrichment through Percoll density, the viability of cells was determined by the Trypan blue exclusion test. Rat BM cells were obtained by flushing femurs. The femurs were accessed through laparotomy to avoid contamination and to increase cell yield. Cells were suspended in DMEM/10% FBS and maintained at 4 °C until further use. Rat BM β_2m^-/Thy-1^+ stem cells were isolated using a two-step magnetic bead cell sorting technique as described previously [13].

DNA microarray. The Affymetrix gene chips (Rat Genome U34A arrays) were used for mRNA expression profiling. Experimental procedures for gene chips were performed according to the Affymetrix Gene Chip Expression Analysis Technical Manual. Following is a brief description. RNA was isolated from rat bone marrow stem cells and primary hepatocytes using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA). RNA quality was checked using Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE) before cDNA synthesis. Double-stranded cDNA was synthesized using the MessageAmp aRNA kit and an oligo$(dT)_{24}$ anchored T7 primer (Ambion, Austin, TX). Two samples (duplicate) of 2.5 μg of total RNA pooled from three different animals in each group (sham control β_2m^-/Thy-1^+ BM stem cells, CBDL β_2m^-/Thy-1^+ BM stem cells, and sham control rat primary hepatocytes) were used to start cDNA synthesis. Biotinylated aRNA was synthesized using biotin-11-CTP and biotin-16-UTP (PerKinElmer Life Sciences, Boston, MA) and reagents from the MessageAmp aRNA kit (Ambion, Austin, TX). Biotinylated cRNA products were purified using Ambion filter cartridges and fragmented to a size of 30–200 nt. Ten micrograms of biotinylated fragmented cRNA was then hybridized with Affymetrix GeneChip (RG U34A). After washing, the arrays were stained with streptavidin–phycoerythrin (Molecular Probes, Eugen, OR), signal amplified by biotinylated anti-streptavidin (Vector Laboratories, Burlingame, CA), and then scanned on an Agilent Genearray scanner. The intensity for each feature of the array was captured with Affymetrix GeneChip Software (MAS 5.0), according to standard Affymetrix procedures. The mRNA abundance was determined based on the average of the differences between perfect match and intentional mismatch intensities for each probe set. Gene induction or repression was determined using appropriate statistical packages (such as Silicon Genetics' GeneSpring 6.1, Affymetrix DMT 3.0).

Quantitative RT-PCR. Selected genes from Affymetrix GeneChip analysis were further verified using the ABI Prism 7700 Sequence Detection System. PCR primers were designed using Primer Express V1.5 software (Applied Biosystems, Foster City, CA) based on genes selected and the sequences downloaded from GenBank and NetAffx web site (http://www.affymetrix.com/analysis/index.affx) containing sequence information for all probes on GeneChips. PCR primers were purchased from Invitrogen (Carlsbad, CA). TaqMan reverse transcription reagents and SYBR Green PCR master mix were purchased from Applied Biosystems.

Real-time PCR was performed in a two-step process. In the first step, sample RNA (2 μg) or reference RNA was reverse transcribed in a volume of 20 μl containing TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM of each dNTP, 2.5 μM random hexamers, 0.4 U/μl RNase inhibitor, and 1.25 U/μl MultiScribe Reverse Transcriptase at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. In the second step,

Table 1
Primers used for qRT-PCR

Gene	GenBank ID	Affy probe ID	Forward	Reverse
Alb	NM_134326	rc_AA860062	5′-CAAAACTAACTGTGAGCTTTACGAGAA-3′	5′-GGCGTTTTGGAATCCATACTCT-3′
Afp	X02361	X02361	5′-AGGCTCTGCATCACCAGCTT-3′	5′-AATGGTGGAGGGACGTAGGTT-3′
Tcf1	J03170	J03170	5′-CACTCAAACAACAACGCACTGA-3′	5′-TTTGCCTCCTGCGTAGTTACAA-3′
Hnf-4α	X57133	X57133	5′-AGAGCGCCTGGGTGTAACTTAG-3′	5′-TCCCTCTTGTCACATCCTCTTTG-3′
Met	U65007	U65007	5′-CTGTTGCAAGGCAGAAGACTCTT-3′	5′-ACAAGGCGTCTGGACAGTACTCT-3′
Kit	D12524	D12524	5′-ACTGTAAACGGAAGGCCTCATG-3′	5′-CGTTGCACCCTACCCTTCCT-3′
Cxcr4	U90610	U90610	5′-GCCTGTGGATGGTGGTGTTC-3′	5′-GGATGAGACCCACCATGATGT-3′
Hp	K01933	K01933	5′-TGGTATGCAGCTGGGATCCT-3′	5′-CTCAGCTACGGCACAACTCTTG-3′
Tf	D38380	D38380	5′-ACTGCTGCATGGGCTAAGGA-3′	5′-GCACAGCAGCTGGAAGTCTTC-3′

Note. Alb, albumin; Afp, α-fetoprotein; Tcf1, transcription factor 1 and previously named as hepatocyte nuclear factor-1 (HNF-1); Hnf-4α, hepatocyte nuclear factor-4 α; Met, met proto-oncogene (HGF receptor); Kit, c-kit receptor tyrosine kinase (stem cell factor receptor); Cxcr4, chemokine receptor (LCR1); Hp, haptoglobin; and Tf, transferrin.

real-time PCR was carried out in a MicroAmp Optical 96-well plate using SYBR Green PCR master mix reagents (Applied Biosystems). Each well contained 5–50 ng (depending on genes) of reverse-transcribed cDNA, SYBR Green PCR buffer, which included SYBR Green 1 dye and passive reference 1, 5.5 mM $MgCl_2$, 200 μM each of dATP/dCTP/dGTP, 400 μM dUTP, 300 nM each of forward and reverse primers, 0.01 U/μl AmpErase UNG, and 0.03 U/μl AmpliTaq Gold DNA polymerase in a total volume of 25 μl. The thermal cycling conditions for real-time PCR were: (a) 50 °C for 2 min, (b) 95 °C for 10 min, and (c) 40 cycles of melting (95 °C, 15 s) and annealing/extension (60 °C, 60 s). PCRs were monitored in real time using the ABI PRISM 7700 Sequence Detector (Applied Biosystems). GAPDH was also determined along with each target gene in all samples, and GAPDH was used as an internal control to normalize target gene expression level. A comparative threshold cycle (C_t) method ($\Delta\Delta C_t$) was used to calculate the relative gene expression (fold change) between test and reference sample for genes whose primers passed an efficiency test (ABI User Bulletin #2). Otherwise, a standard curve for each target gene as well as housekeeping gene (GAPDH) was generated with reference RNA. Relative quantitation of gene expression was determined using the standard curve method as described in ABI's User Bulletin #2.

Primer sequences for quantitative real-time RT-PCR (qRT-PCR). All primer sequences are listed in Table 1.

Statistical analysis. Data were analyzed with GeneSpring 6.1 software (Silicon Genetics) [16,17], Affymetrix Microarray Suite 5.0, and Data Mining Tool 3.0. Only those transcripts detected as present or absent in duplicate arrays were reported.

Results

Genes present in CBDL β_2m^-/Thy-1$^+$ BM stem cells and rat primary hepatocytes but absent in sham control-derived β_2m^-/Thy-1$^+$ BM stem cells

There were 27 probe sets (genes) present in both CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells and primary hepatocytes but absent in sham control-derived β_2m^-/Thy-1$^+$ BM stem cells (Table 2 and Fig. 1). This list included many expression sequence tags (ESTs) and some important liver functional genes such as CYP2B15, D-site albumin promoter-binding protein, and Kruppel-like factor 9, a cDNA isolated from rat liver cDNA library with binding activity in the promoter region of CYP1A1 [18]. In addition, a GST gene (GST13) was turned on in the CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells, but was not expressed in the sham control BM stem cells. The induction of this detoxification enzyme may be a response to CBDL.

CBDL β_2m^-/Thy-1$^+$ BM stem cells share more commonly expressed genes with rat primary hepatocytes than sham control β_2m^-/Thy-1$^+$ BM stem cells

Fig. 2 is a Venn diagram showing the shared and distinct gene expression among different BM β_2m^-/Thy-1$^+$ stem cells and hepatocytes. In Fig. 2, gene lists used for Venn diagram were the probe sets that were present on two arrays in each group (CBDL BMSC, 2695 probe sets; sham control BMSC, 2243 probe sets; and primary hepatocytes group, 2388 probe sets). It was shown that the CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells shared more genes with rat primary hepatocytes than sham control rat β_2m^-/Thy-1$^+$ BM stem cells (see Fig. 2 Venn diagram: CBDL β_2m^-/Thy-1$^+$ BMSCs, 189/2695 = 7%; sham control β_2m^-/Thy-1$^+$ BMSCs, 24/2243 = 1%; excluding the genes expressed in all three groups).

Genes with more than 2-fold up-regulation in CBDL β_2m^-/Thy-1$^+$ BM stem cells compared to sham control β_2m^-/Thy-1$^+$ BM stem cells

In addition to the above-mentioned genes that were only present in the CBDL β_2m^-/Thy-1$^+$ BM stem cells but were absent in the sham control β_2m^-/Thy-1$^+$ BM stem cells, there were many genes (41 probe sets) that had more than 2-fold up-regulation in CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells compared with genes in the sham control β_2m^-/Thy-1$^+$ BM stem cells (Table 3). This list of up-regulated genes included those genes present in all three different cells as well as genes expressed only in two different BM stem cells. Acute phase protein haptoglobin (2.2-fold), IL-1 β-converting enzyme (2.1-fold), as well as transferrin (2.2-fold), the

Table 2
Genes present in CBDL β_2m^-/Thy-1$^+$ BMSCs and primary hepatocytes but absent in sham control β_2m^-/Thy-1$^+$ BMSCs

Probe ID	Gene	CBDL[a] BMSC	Control[b] BMSC	Control[c] hepatocyte	Description
M35300	Atpi	P	A	P	ATPase inhibitor
J02827	Bckdha	P	A	P	Branched chain keto acid dehydrogenase subunit E1, α polypeptide
rc_AA892382	Cml1	P	A	P	Camello-like 1
D17349	CYP2B15	P	A	P	Cytochrome P450 2B15
J03179	Dbp	P	A	P	D-site albumin promoter binding protein
X15958	Echs1	P	A	P	Enoyl Coenzyme A hydratase, short chain 1
L38482	EST	P	A	P	Similar to serine protease gene
rc_AA894321	EST	P	A	P	Similar to WD repeat and phosphoinositide-binding protein SR1
rc_AA893192	EST	P	A	P	Transcribed sequences
rc_AA892754	EST	P	A	P	Transcribed sequences
rc_AA891108	EST	P	A	P	*Rattus norvegicus* transcribed sequences
rc_AA891851	EST	P	A	P	Transcribed sequence with weak similarity to protein ref: NP_062558.1
rc_AA894193	EST	P	A	P	Transcribed sequence
rc_AA859848	EST	P	A	P	Similar to RIKEN cDNA 0610038L10 gene (LOC311241)
rc_AA892828	EST	P	A	P	Similar to human pyruvate dehydrogenase E1 component β subunit
S83436	GSTK1	P	A	P	Glutathione *S*-transferase subunit 13, rGSTK1
D00913	Icam1	P	A	P	ICAM-1; intercellular adhesion molecule-1
M86235	Khk	P	A	P	Ketohexokinase
D12769	Klf9	P	A	P	Kruppel-like factor 9, mediates the expression of growth-associated genes
rc_AA874784	Lipa	P	A	P	Lipase A, lysosomal acid
D30649	LOC54410	P	A	P	Alkaline phosphodiesterase
Z11995	Lrpap1	P	A	P	Low density lipoprotein receptor-related protein associated protein 1
rc_AI105463	Mtpn	P	A	P	Myotropin
rc_AA800120	Slc25a20	P	A	P	Solute carrier family 25 (carnitine/acylcarnitine translocase), member 20
J03170	Tcf1	P	A	P	Transcription factor 1 (previously named as HNF1); plays a role in the regulation of liver-specific genes
U91561	U91561	P	A	P	Pyridoxine 5-phosphate oxidase, rate-limiting enzyme in PLP biosynthesis
rc_AA859954	Vmp1	P	A	P	Vacuole membrane protein 1

P, present; A, absent. Results were derived from two arrays for each group.
[a] CBDL BMSC, CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells.
[b] Control BMSC, sham control-derived β_2m^-/Thy-1$^+$ BM stem cells.
[c] Primary hepatocytes were isolated from sham control rats.

gene involved in iron metabolism, were up-regulated more than 2-fold in the CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells compared with that of sham control BM β_2m^-/Thy-1$^+$ BM stem cells. An un-annotated sequence (probe set ID: U31866) was found significantly up-regulated in the CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells (2.4-fold vs. sham control β_2m^-/Thy-1$^+$ BM stem cells). This sequence was also abundantly expressed in rat primary hepatocytes (expression signal: 28800, these signal intensity data not shown in Table 3). A BLAST searching (translated query vs. protein database) indicated that its translated amino acid fragment shared 100% identity with a recently cloned rat liver regeneration-related protein LRRG03 (GenBank Accession No. AAP97736). Interestingly, its translated amino acid fragment also had 100% similarity with rat transferrin.

CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells expressed certain liver-specific and liver-enriched genes

Comparison of certain liver-specific and liver abundant genes among three different groups are shown in Table 4. Although no significant differences were observed in many liver-specific and liver-enriched genes such as albumin and CCAAT/enhancer-binding protein between CBDL and sham control-derived β_2m^-/Thy-1$^+$ BM stem cells in our microarray analysis, significant different gene expressions (>2-fold up-regulation) were shown in transferrin and GSTm1. In addition, the following genes such as apolipoprotein C-III, carnitine palmitoyltransferase, transcription factor 1 (previously named as HNF1), and D-site albumin promoter-binding protein were present in CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells, but were not detectable in sham control β_2m^-/Thy-1$^+$ BM stem cells (Table 4).

Validation of mRNA expression of selected genes by quantitative real-time RT-PCR

To confirm the microarray analysis results, a few genes including liver-specific and -enriched genes were further determined by real-time RT-PCR. The comparison of a validation study on these genes is presented in Table 5. There were many genes such as albumin, Afp, Hnf-4α, Met, and Kit that were not detected by the microarray analysis, all absent in CBDL and sham control-derived β_2m^-/Thy-1$^+$ BM stem cells, were detected

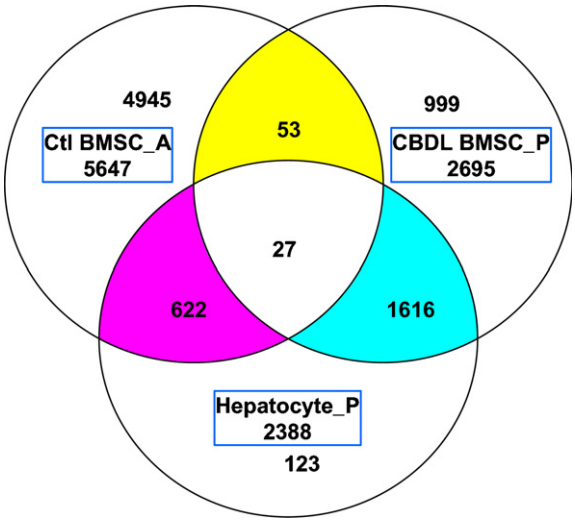

Fig. 1. Venn diagram illustrating genes present in CBDL BMSCs and hepatocytes but absent in sham control BMSCs. Ctl_BMSC_A, probe sets or genes absent in sham control-derived β_2m^-/Thy-1^+ BMSCs. CBDL_BMSC_P, probe sets or genes present in CBDL-derived β_2m^-/Thy-1^+ BMSCs. Hepatocyte_P, probe sets or genes present in control primary hepatocytes. Note that 27 probe sets (genes) were absent in sham control BMSCs, but present in both CBDL BMSCs and primary hepatocytes.

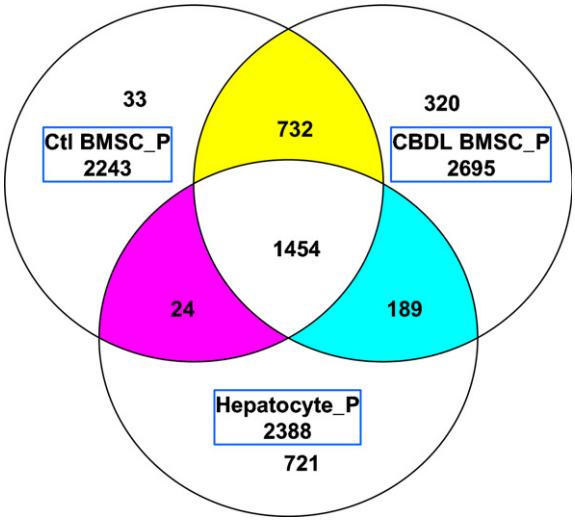

Fig. 2. Venn diagram detailing shared and distinct gene expression among different BMSCs and hepatocytes. Genes selected were the probe sets present on two arrays in each group. Ctl_BMSC_P, probe sets or genes present in sham control-derived β_2m^-/Thy-1^+ BMSCs. CBDL_BMSC_P, probe sets or genes present in CBDL-derived β_2m^-/Thy-1^+ BMSCs. Hepatocyte_P, probe sets or genes present in sham control primary hepatocytes. There were 189 commonly expressed genes (probe sets) between CBDL β_2m^-/Thy-1^+ BMSCs and primary hepatocytes, whereas there were only 24 commonly expressed genes (probe sets) between sham control β_2m^-/Thy-1^+ BMSCs and primary hepatocytes (excluding the genes present in all three different cells, 1454 probe sets).

by qRT-PCR, indicating that qRT-PCR was much sensitive than the microarray analysis. The threshold cycles (C_t) for these five genes were around 26.0 (Alb), 27.0 (Afp), 26.0 (Hnf-4α), 28.5 (Met), and 25.5 (Kit) in both CBDL and sham control-derived β_2m^-/Thy-1^+ BM stem cells. Consistent with what was found by the GeneChip analysis, no significant difference of mRNA expression was observed in Alb, Afp, Hnf-4α, Met, and Kit. In addition, qRT-PCR can detect the presence of Tcf1 in β_2m^-/Thy-1^+ BM stem cells and there was a 1.3-fold difference in Tcf1 mRNA expression between CBDL and sham control-derived β_2m^-/Thy-1^+ BM stem cells. For this gene (Tcf1), the GeneChip analysis made an "absent' call in the sham control-derived β_2m^-/Thy-1^+ BM stem cells, but a "present" call in the CBDL-derived β_2m^-/Thy-1^+ BM stem cells. There was a 2.3- and 1.9-fold up-regulation of transcript in two genes (Tf and Hp) by qRT-PCR analysis, and both genes had also shown 2.2-fold increases in mRNA expression in the CBDL-derived β_2m^-/Thy-1^+ BM stem cells in GeneChip analysis (Table 5), indicating excellent consistency between microarray and real-time RT-PCR results.

Discussion

It was not known until recently whether stem cells and their progeny participate in the regenerative liver repair process or not. Now, it appears plausible that the restitutive response of liver to different injuries may involve proliferation of cells at different levels in the liver cell lineage [19–21]. There are three kinds of cells in the hepatocyte lineage that respond to injury: mature hepatocytes, hepatic progenitor cells or oval cells located within the intrahepatic biliary tree, and hematopoietic stem cells with hepatic potential residing in the BM [19–21]. Mature hepatocytes, which proliferate after normal liver tissue renewal, less severe liver damage, are numerous, unipotent, "committed," and respond rapidly to liver injury and liver cell loss by one or two cell cycles, but can only produce hepatocytes. Oval cells, which are activated to proliferate when liver damage is extensive and chronic, or if proliferation of hepatocytes is inhibited, are rare in liver, but have sustained proliferation potential and can give rise to biliary cells and hepatocytes [22,23]. However, recent studies indicate that oval cells are derived from bone marrow stem cells [4–7]. Bone marrow-derived hepatic stem cells contribute to the low renewal rate of hepatocytes, make a more significant contribution to regeneration [4,24–26], and even completely restore normal function in a murine model of hereditary tyrosinemia [8].

A number of genes have been implicated in liver regeneration following partial hepatectomy (PHx) [27–31]. Many genes have been investigated under conditions inducing oval cells to better understand the molecular mechanism of liver regeneration [4,32]. Our laboratory recently isolated a sub-population of stem

Table 3
Genes with more than 2-fold up-regulation between CBDL and sham control-derived β_2m^-/Thy-1^+ BMSCs

Probe ID	Gene	Fold_I[a]	CBDL[b] BMSC	Control[c] BMSC	Control[d] hepatocyte	Description
rc_AI070848	Actb	2.1	P	P	P	Actin, β
rc_AI171962	Anxa1	3.1	P	P	A	Annexin 1, plays a role in regulation of insulin secretion
S57478	Anxa1	5.9	P	P	A	Annexin 1, plays a role in regulation of insulin secretion
rc_AA818025	Cd59	2.0	P	P	P	CD59 antigen; may play a role in regulation of complement
X13044	Cd74	2.4	P	P	A	CD74 antigen (invariant polypeptide of MHC class II antigen-associated)
U23055	Ceacam1	2.9	P	P	A	Carcinoembryonic antigen-related cell adhesion molecule 1
rc_AI014091	Cited2	2.0	P	P	P	Cbp/p300-interacting transactivator, a transcription factor
rc_AA894029	Cybb	2.2	P	P	A	Endothelial type gp91-phox gene
E03358	EST	2.0	P	P	P	cDNA encoding rat polyfunctional protease component C3
K03039	EST	2.1	P	P	A	L-CA variant 4; rat mRNA for leucocyte-common antigen
rc_AI014135	EST	2.4	P	P	P	EST, similar to rat CDK103 mRNA
rc_AI639012	EST	2.1	P	P	P	EST, similar to cDNA sequence BC019776 (LOC316842)
U31866	EST	2.4	P	P	P	Nclone10 mRNA
X68782	EST	2.1	P	P	A	Rat Ig delta heavy chain constant region and 3′ ut
K01933	Hp	2.2	P	P	P	Haptoglobin; may play a role in inflammatory response
S79676	Ice	2.1	P	P	A	IL-1β-converting enzyme; ICE
rc_AI137583	Id2	2.6	P	P	P	Inhibitor of DNA binding 2, dominant negative helix–loop–helix protein
rc_AI230256	Id2	2.3	P	P	P	Inhibitor of DNA binding 2, dominant negative helix–loop–helix protein
X61381	Ifitm3l	2.1	P	P	P	Interferon induced transmembrane protein 3-like
D90211	Lamp2	2.6	P	P	P	Lysosomal membrane glycoprotein 2, intrinsic lysosomal membrane protein
rc_AA946503	Lcn2	3.1	P	P	A	Lipocalin 2
J02962	Lgals3	2.0	P	P	A	Lectin, galactose binding, soluble 3; may play a role in immune function
D88586	Loc192264	4.3	P	P	A	Eosinophil cationic protein, similar to human eosinophil-derived neurotoxin
U23056	LOC287009	2.3	P	P	A	C-CAM4 protein
rc_AA957923	Mcpt2	2.2	P	P	A	Mast cell protease 2
U67888	Mcpt4	2.4	P	P	A	Mast cell protease 4
U72143	Mcpt9	2.1	P	P	A	Mast cell protease 9
AJ007288	Mmp8	2.4	P	P	A	Neutrophil collagenase
U24441	Mmp9	2.7	P	P	A	Matrix metalloproteinase 9 (gelatinase B, 92-kDa type IV collagenase)
U17254	Nr4a1	2.7	P	P	A	Immediate early gene transcription factor NGFI-B
D90258	Psma3	2.1	P	P	P	Proteasome (prosome, macropain) subunit, α type 3
U77038	Ptph6	2.3	P	P	A	Protein tyrosine phosphatase, non-receptor type 6
rc_AA849648	Rpl21	2.1	P	P	P	Ribosomal protein L21
K02815	RT1-Ba	2.0	P	P	A	RT1 class II, locus Ba; may play a role in antigen presentation
X56596	RT1-Bb	2.1	P	P	P	RT1 class II, locus Bb; may play a role in antigen presentation
X53054	RT1-Db1	3.6	P	P	A	RT1 class II, locus Db1; may play a role in antigen presentation
rc_AI171966	RT1-DMb	2.1	P	P	A	RT1 class II, locus DMb
U31599	RT1-DMb	2.2	P	P	A	RT1 class II, locus DMb
J03627	S100a10	2.5	P	P	A	S-100-related protein, clone 42C
D38380	Tf	2.2	P	P	P	Transferrin
Z68145	Vpreb1	2.1	P	P	A	Pre-B lymphocyte gene 1

P, present; A, absent.

[a] Fold_I, fold increase (CBDL BMSC vs. sham control BMSC). The fold change between two groups was calculated from the normalized average mRNA expression level (signal) in each group. The average signal of each gene was derived from duplicate chips hybridized with cRNA synthesized from pooled three mRNA samples in each group.

[b] CBDL BMSC, CBDL-derived β_2m^-/Thy-1^+ BM stem cells.

[c] Control BMSC, sham control-derived β_2m^-/Thy-1^+ BM stem cells.

[d] Primary hepatocytes were isolated from sham control rats.

cells, β_2m^-/Thy-1^+, from both human and rat BM utilizing a two-step magnetic bead cell-sorting procedure [13]. These cells, after co-culturing with cholestatic hepatocytes and serum, expressed hepatocyte-specific markers such as albumin. This was further confirmed by RT-PCR demonstrating that BM-derived hepatic stem cells expressed albumin and its transcription factor C/EBPα and interestingly, after co-culturing, the cells showed de novo expression of Tcf1 (Hnf-1) while losing expression of Hnf-4 [13].

Table 4
Comparison of mRNA expression of selective liver-enriched or liver-specific genes between CBDL and sham control-derived β_2m^-/Thy-1^+ BMSCs

Probe ID	Gene	CBDL BMSC[a]		Control BMSC[b]		Hepatocyte[c]		Description
		Flag	Signal[d]	Flag	Signal[d]	Flag	Signal[d]	
J02596	Apoc3	P	268	A		P	36957	Apolipoprotein C-III; VLDL that comprises a major component of the lipid transport system
rc_AA860062	Alb	A		A		P	32123	Albumin
X04979	Apoe	P	1188	P	1073	P	46623	Apolipoprotein E; plays a role in plasma lipoprotein transport
X60769	Cebpb	P	1010	P	927	P	912	CCAAT/enhancer-binding protein (C/EBP), β
L07736	Cpt1a	P	237	P	216	P	859	Carnitine palmitoyltransferase 1, liver
J05470	Cpt2	P	122	A		P	529	Carnitine palmitoyltransferase 2
U44948	Csrp2	P	293	P	241	P	83	Cysteine rich protein 2; may act as an adaptor molecule in the JAK/STAT signaling pathway
J03179	Dbp	P	138	A		P	263	D-site albumin promoter-binding protein; binds to the D-site of the albumin promoter
rc_AI169802	Fth1	P	6049	P	5323	P	6992	Ferritin, heavy polypeptide 1; overexpressed during hepatic tumor development
X12367	Gpx1	P	12868	P	10447	P	11271	Glutathione peroxidase I
X04229	Gstm1	P	856	P	463	P	14395	Glutathione *S*-transferase, μ 1
J03752	Mgst1	P	4620	P	3689	P	28978	Microsomal glutathione *S*-transferase 1
rc_AA892832	rELO1	P	1694	P	1256	P	4555	Fatty acid elongase 1
J03170	Tcf1	P	208	A		P	255	Transcription factor 1 (previously named as HNF1); plays a role in the regulation of liver-specific genes

P, present; A, absent.
[a] CBDL BMSC, CBDL-derived β_2m^-/Thy-1^+ BM stem cells.
[b] Control BMSC, sham control-derived β_2m^-/Thy-1^+ BM stem cells.
[c] Primary hepatocytes were isolated from sham control rats.
[d] The average signal of each gene was calculated from duplicate chips hybridized with cRNA synthesized from pooled 3 mRNA samples in each group.

Table 5
Microarray data validated by qRT-PCR-fold change of mRNA expression between CBDL and sham control β_2m^-/Thy-1^+ BM stem cells

Gene[a]	Microarray[b]			qRT-PCR (CBDL vs. control)[c]
	Control	CBDL	Fold change (M ± SD)	Fold change (M ± SD)
Alb	A	A	N/A	1.1 ± 0.11
Afp	A	A	N/A	1.0 ± 0.09
Tcf1	A	P	N/A	1.3 ± 0.14
Hnf-4α	A	A	N/A	1.2 ± 0.05
Met	A	A	N/A	0.9 ± 0.14
Kit	A	A	N/A	1.2 ± 0.15
Cxcr4	P	P	1.6 ± 0.38	1.3 ± 0.41
Tf	P	P	2.2 ± 0.69	2.3 ± 0.49
Hp	P	P	2.2 ± 0.37	1.9 ± 0.23

[a] Alb, albumin; Afp, α-fetoprotein; Tcf1, transcription factor 1 (previously named as HNF1); Hnf-4α, hepatocyte nuclear factor-4α; Met, met proto-oncogene (HGF receptor); Kit, c-kit receptor tyrosine kinase (stem cell factor receptor); Cxcr4, chemokine receptor (LCR1); Tf, transferrin; and Hp, haptoglobin.
[b] Microarray determined mRNA expression using RG U34A GeneChip: A, absent or not detectable; P, present. Fold change of mRNA expression between CBDL and sham control β_2m^-/Thy-1^+ BM stem cells was calculated from two chips in each group. N/A, not applicable.
[c] qRT-PCR: for each gene, mRNA expression level was measured by real-time RT-PCR for both sham control and CBDL β_2m^-/Thy-1^+ BM stem cells, three samples (animals) were obtained in each group, and PCR was done in triplicate for each sample.

We speculated that β_2m^-/Thy-1^+ BM stem cells may possess certain hepatocyte-like features in their gene expression prior to their migration to liver and induction into oval cells. Our previous immunohistochemical staining indicated that 100% of β_2m^-/Thy-1^+ stem cells isolated from CBDL rat bone marrow were positively stained with albumin [13]. Although albumin, Afp, Hnf-4α, and Met were not detected by our microarray analysis, they were detected by quantitative real-time RT-PCR in both CBDL and sham control-derived β_2m^-/Thy-1^+ stem cells. Nevertheless, no significant difference was found between CBDL and sham control-derived β_2m^-/Thy-1^+ stem cells in mRNA expression for albumin, Afp, and Met. Microarray analysis revealed that Tcf1 was absent in sham control-derived β_2m^-/Thy-1^+ stem cells, but was present in CBDL-derived

β_2m^-/Thy-1$^+$ stem cells. The real-time RT-PCR confirmed that Tcf1, Hnf-4α, and Kit were slightly increased in the CBDL-derived β_2m^-/Thy-1$^+$ stem cells as compared to sham control BM stem cells (1.3-, 1.2-, and 1.2-fold, respectively, Table 5). These data support our speculation that CBDL-derived β_2m^-/Thy-1$^+$ stem cells started to express certain hepatocyte-like genes.

Interestingly, we observed a significant up-regulation (more than 2-fold) of transferrin in the CBDL-derived β_2m^-/Thy-1$^+$ stem cells compared to the sham control-derived BM stem cells, whereas the transferrin receptor was not up-regulated in the CBDL-derived β_2m^-/Thy-1$^+$ stem cells. Transferrin is a single-chain glycoprotein of molecular weight of 80 kDa [33–35]. Uptake of iron by hepatic cells through the transferrin/transferrin receptor pathway is considered quantitatively important [35]. A recent study indicated that transferrin may be a critical factor involved in the activation of hepatic stellate cells [36]. It is known that the activation and production of stellate cells is one of the critical steps in liver regeneration involving oval cells [2,37]. This is the first study that demonstrated that transferrin is elevated in the BM β_2m^-/Thy-1$^+$ stem cells, and it is unclear if this phenomenon may have something to do with the migration, induction, and differentiation of BM-derived hepatic stem cells into hepatocytes. In addition, it is found that acute phase proteins such as haptoglobin and complement component C3 were elevated in the serum following injury including PHx [38–40]. They were significantly increased in the regenerating liver with oval cell induction [31]. We also observed an up-regulation of gene expression in acute phase proteins such as haptoglobin and IL-1 β-converting enzyme, in our CBDL-derived BM β_2m^-/Thy-1$^+$ stem cells. The up-regulation of haptoglobin and IL-1 β-converting enzyme is most likely a response to the elevation of IL1 and IL6 or other cytokines [31,39,41,42]. These acute phase proteins may play an important role in the migration of BM β_2m^-/Thy-1$^+$ stem cells into the injured liver.

In summary, in addition to the distinct difference from mature primary hepatocytes in their gene expression profiles (scatter plotting data not shown), the CBDL-derived β_2m^-/Thy-1$^+$ stem cells displayed significant difference in transcriptomic fingerprints compared to the sham control rat β_2m^-/Thy-1$^+$ stem cells. Forty-one probe sets (genes), including a few ESTs, were up-regulated more than 2-fold in CBDL-derived β_2m^-/Thy-1$^+$ stem cells (Table 3). Furthermore, 27 probe sets (genes) were present in both CBDL-derived β_2m^-/Thy-1$^+$ stem cells and primary hepatocytes, but were not expressed in the sham control rat β_2m^-/Thy-1$^+$ stem cells (Fig. 1 and Table 2). Certain liver-specific and -enriched genes such as Tcf1, Dbp, and Apoc3 were up-regulated in the CBDL β_2m^-/Thy-1$^+$ BM stem cells, whereas certain acute phase proteins such as haptoglobin and IL-1 β-converting enzyme were significantly increased in CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells. Overall, our gene array data indicated that (1) CBDL-derived β_2m^-/Thy-1$^+$ stem cells had a different transcriptomic fingerprint from the β_2m^-/Thy-1$^+$ BM stem cells isolated from sham control rats; and (2) CBDL-derived β_2m^-/Thy-1$^+$ stem cells showed some, if not significant, hepatocyte-like features in their gene expression. It would be of interest to see how similar the transcriptome of CBDL-derived β_2m^-/Thy-1$^+$ BM stem cells is to mature hepatocytes, after being co-cultured with cholestatic serum and hepatoctyes.

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