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Comparative Proteomic Analysis of Primary Mouse Liver c-Kit^{-}(CD45/TER119)$^{-}$ Stem/Progenitor Cells

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Abstract Liver stem/progenitor cells play a key role in liver development and maybe also in liver cancer development. In our previous study a population of c-Kit^{-}(CD45/TER119)$^{-}$ liver stem/progenitor cells in mouse fetal liver, was successfully sorted with large amount (10^6–10^7) by using immuno-magnetic microbeads. In this study, the sorted liver stem/progenitor cells were used for proteomic study. Proteins of the sorted liver stem/progenitor cells and unsorted fetal liver cells were investigated using two-dimensional electrophoresis. A two-dimensional proteome map of liver stem/progenitor cells was obtained for the first time. Proteins that exhibited significantly upregulation in liver stem/progenitor cells were identified by peptide mass fingerprinting and peptide sequencing. Nineteen protein spots corresponding to 12 different proteins were identified as showing significant upregulation in liver stem/progenitor cells and seem to play important roles in such cells in cell metabolism, cell cycle regulation, and stress. An interesting finding is that most of the upregulated proteins were overexpressed in various cancers (11 of 12, including 6 in human hepatocellular carcinoma (HCC)) and involved in cancer development as reported in previous studies. Some of the identified proteins were validated by real-time PCR, Western blotting, and immunostaining. Taken together, the data presented provide a significant new protein-level insight into the biology of liver stem/progenitor cells, a key population of cells that might be also involved in liver cancer development. J. Cell. Biochem. 102: 936–946, 2007.

Key words: liver stem/progenitor cells; immuno-magnetic microbeads; two-dimensional electrophoresis; liver cancer; cancer stem cell

Stem cell is a very small population of cells in various tissues. It is a self-renewing, typically multipotent progenitor with the broadest developmental potential in a particular tissue at a particular time [Pardal et al., 2003]. Stem cell is believed associated with cancer development. There are solid proofs that many cancers are derived from cancer stem cells, which share many similar properties with normal stem cells [Reya et al., 2001; Scadden, 2004; Bjerkvig et al., 2005; Huntly and Gilliland, 2005].

The liver is the central organ of nutrient digestion and processing, where most of the individual's metabolism occurs. It is believed that in the developing liver both hepatocytes and cholangiocytes differentiate from a common progenitor, or so-called liver stem cell [Suzuki et al., 2002; Shafritz et al., 2006]. However, little is known about the molecular events in liver stem cell that might govern the developing process of the liver.

Separation of stem cells is typically done based on surface marker expression. In the liver, however, few liver stem cell-specific markers are available until now, and therefore only recently liver stem/progenitor cells have been isolated based on some non-specific cell surface markers (c-Kit, CD45, TER119, c-Met, etc.) and identified [Suzuki et al., 2000, 2002; Strick-Marchand and Weiss, 2002; Strick-Marchand et al., 2004; Minguet et al., 2003; Tanimizu et al., 2003; Shafritz et al., 2006].

Abbreviations used: Actin, β-actin; AFP, α-fetoprotein; ALB, albumin; FL, full fetal liver cells; GPA, glycophorin A; LSC, liver stem/progenitor cells; MCS, MS-compatible silver stain; TS, traditional standard silver staining

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Proteomics is the study of the entire complement of proteins expressed by a cell, organism, or tissue type at a given time or under certain environmental conditions [Smith, 2000]. Proteomic study of stem cell would give much information to the knowledge of stem cell. To date, however, little proteomic information of liver stem/progenitor cell is available because of two main reasons. One is that liver stem/progenitor cell has been isolated and identified just recently, the other is that stem/progenitor cell is a very rare population of cells that little protein sample could be obtained. As for large-scale proteomic study of stem cells, such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS) analysis, it is required relatively large amounts of protein sample extracted from the cells [Unwin et al., 2003]. Thus, for the low-abundance samples such as stem cells whose culture in vitro may induce alterations in protein expression patterns, the key to such study relies on the successful obtaining of enough amounts of primary stem/progenitor cells with high purity. In our previous study, we successfully sorted large amounts (10^6–10^7) of liver c-Kit$^-$(CD45/TER119)$^-$ stem/progenitor cells with high purity using the method of immuno-magnetic microbeads and identified those cells as stem/progenitor cells with self-renewing capability and multilineage differentiation potential [He et al., 2006]. Based on this method, we performed the proteomic analysis of liver stem/progenitor cells in the present study. We obtained, for the first time, a 2D proteome map of primary liver stem/progenitor cells and identified a set of highly expressed cancer-related proteins in these cells.

MATERIALS AND METHODS

Mice and Cells

Animal experiments involving mice were approved by the institute's Animal Care and Use Committee. BALB/c mice were maintained under pathogen-free conditions. Day 0 of gestation was taken to be detection of a vaginal plug after overnight mating. Embryos were dissected and mixed and fetal liver cell suspensions were prepared by mechanical dissociation and then passed through a 30 μm nylon mesh to remove clumps.

Flow Cytometry (FC) Analysis

Dissociated liver cells were incubated at 4°C for 10 min with biotinylated anti-CD45.2, TER119 monoclonal antibodies (mAbs) (eBioscience, San Diego, CA). After three washes with staining medium (PBS containing 3% FBS), cells were incubated with FITC-conjugated anti-c-Kit mAb (eBioscience), and streptavidin-labeled phycoerythrin (eBioscience) at 4°C for 10 min. Finally, cells were washed three times and resuspended in staining medium. The labeled cells were analyzed with FACSAria (Becton Dickinson). Gating was implemented based on negative control staining profiles.

Immuno-Magnetic Sorting

Dissociated fetal liver cells were first labeled with biotinylated anti-CD45.2, TER119, and c-Kit mAbs at 4°C for 10 min. Cells were washed twice with PBS containing 0.5% BSA and 2 mM EDTA. Enrichment of target cells by magnetic activated cell sorting (MACS) kit (Milteny Biotec, Bergisch Gladbach, Germany) was carried out according to the manufacturer's protocol. Briefly, cells were resuspended in 80 μl of same buffer per 10^7 total cells and incubated with anti-biotin microbeads for 15 min at 6–12°C. Cells were washed twice and finally resuspended in 500 μl of buffer per 10^7 total cells. A pre-moistened MS-Column (Milteny Biotec) was placed in the magnetic field of a suitable MACS separator (Milteny Biotec). The cell suspension was applied onto the column and was washed three times. Effluents were collected as the negative fraction. Magnetically labeled cells as the positive fraction were collected by flushing the column with buffer after the column was placed outside of the magnetic field. The sorted cells were then incubated at 4°C for 10 min with phycoerythrin-streptavidin and analyzed with FACSCalibur (Becton Dickinson), or frozen at −80°C prior to protein extraction and 2D electrophoresis.

RT-PCR and Real-time PCR

Detection of gene expression by RT-PCR was conducted as described [He et al., 2004c]. Briefly, equal amounts of RNAs were reverse-transcribed and the cDNAs were amplified by the PCR at 94°C for denaturing, 54°C for annealing, and 72°C for extension. β-actin mRNA was used as an internal control.

Previous experiments determined 30 cycles to be optimal. The gene-specific intron-spanning primers used were as follows: α-fetoprotein (amplification of 821 bp), sense 5′-TCACACCCGCTTCCCTCATCCT-3′°C, antisense 5′-CATCCTGCAGACACTCCAG-3′; albumin (amplification of 705 bp), sense 5′-AGAAGACACCCTGATTACTCT-3′°Cantisense 5′-TCGAGAAGCAGGTGTCCTTGT-3′; c-Kit (amplification of 412 bp), sense 5′-GAACCTTCTGCACTCAACGGAG-3′, antisense 5′-GAAAATGCTCTCTGGTGCCATCC-3′; CD45(amplification of 411 bp), sense 5′-CATATGTACTCCACTGTGAGC-3′, antisense 5′-CATAGGCAAGTAGGGACACTTC-3′; glycophorin A (amplification of 485 bp), sense 5′-CTGACAGACACTCCCAGTATG-3′, antisense 5′-GAACTCAAAGGCACACTGTTG-3′; β-actin (amplification of 390 bp), sense 5′-GTCATCACTATTGGCAACGAGCG-3′, antisense 5′-CTAGAAGCACTTGCGGTGCACG-3′. The amplified products were analyzed by electrophoresis on 1% agarose gels and stained with ethidium bromide.

Relative quantitative real-time PCR was performed using the SYBR Green I DNA binding dye on the iQ5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) to confirm the results from 2-DE, following the manufacturer's instructions. The primer sequences were as follows: HSP60, sense 5′-GAACTGCCTTACTGGATGCTGCTG-3′, antisense 5′-CCTGCCTTGAGCTTCCTGTCAC-3′; CK8: sense 5′-GCATTCATACGAAGACCACCAGC-3′, antisense 5′-CACGACATCAGAAGACTCGGAC-3; TPI: sense 5′-GGTTCTGTGACTGGAGCAACCTG-3′, antisense 5′-CAACCTAGTCCATGCTATGCAGG-3′; enolase 1: sense 5′-GATGACCTCACAGTGACCAACC-3′, antisense 5′-CAGTCTTGATCTGCCCAGTGCAG-3; SOD1: sense 5′-GGTTCCACGTCCATCAGTATGG-3′, antisense 5′-GGAATGCTCTCCTGAGAGTGAG-3′; Pdia3: sense 5′-CCTGTCAAGGTTGTGGTAGCAG-3′, antisense 5′-GCACATCATTGGCTGTGGCATCC-3′; β-actin: sense 5′-CACAGGCATTGTGATGGACTCC-3′, antisense 5′-CATCTCCTGCTCGAAGTCTAGAG-3′. The PCR conditions were 10 min at 95°C, followed by 40 cycles of 20 s at 94°C, 30 s at 57°C and 30 s at 72°C. Fluorescent data were specified for collection for 15 s at 80°C according to the melt curve analysis. The PCR products were specific for each gene as confirmed by agarose gel electrophoresis. Each gene was normalized to the housekeeping gene (β-actin) from the same sample before fold change was calculated to account for variations between different samples. All PCR assays were performed in triplicate and results are represented by the mean values ± SD.

2D Electrophoresis

Cell pellets containing at least 2×10^6 cells were lysed in solubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 20 mM Tris, 1 mM PMSF, 2% pharmalyte 3–10) and stored at −80°C until use. Protein samples were diluted to 125 μl with a rehydration solution containing 8 M urea, 2% CHAPS, 0.5% IPG buffer pH 3–10 NL, 0.2% DTT and loaded on 7 cm pH 3–10 NL IPG strips (Amersham Biosciences). The total Vhs was 25,000–400,000. The first dimensional IEF was performed according to the manufacturer's protocol (Amersham Biosciences). Focused gels were stored at −80°C prior to SDS–PAGE. For SDS–PAGE, IPG strips were incubated for 15 min in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% w/v glycerol, 2% w/v SDS) supplemented with 10 mg/ml DTT, followed by a 15 min incubation in equilibration buffer supplemented with 25 mg/ml iodoacetamide, then rinsed once with SDS–PAGE buffer. The second dimension electrophoresis was carried out on 12.5% polyacrylamide gels at 15 mA/gel constant current, room temperature, and terminated when the bromophenol dye front had migrated to the lower end of the gels.

Gel Staining and Image Analysis

The 2D gels were subjected to Coomassie Brilliant Blue staining or silver staining. Silver staining was performed according to standard protocols or MS-compatible protocol as described [Shevchenko et al., 1996], with slight modification. The stained gels were scanned using an ImageScanner (Amersham Pharmacia) and analyzed with ImageMaster software (Amersham Pharmacia). Three separate gels visualized by either standard or MS-compatible silver staining were analyzed in order to minimize the contribution of experimental variations and the spots displaying the same pattern were selected for further analysis. The normalized relative intensities of spots were used for comparison between liver stem cells and full fetal liver cells (each three gels using a same protein sample), and only those spots with significantly increased intensity in

gels of liver stem cells (Student's t-test, $P < 0.05$, all with an 1.4-fold increase or more) were selected for analysis by MS.

In-Gel Digestion

Protein spots were excised and destained twice with 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (1:1 v/v) and then equilibrated in 50 mM NH_4HCO_3 to pH 8.0. After dehydrating with ACN and drying in N_2 at 37°C for 20 min, the gel pieces were rehydrated in 15 ml trypsin solution (10 mg/ml in 25 mM NH_4HCO_3) at 4°C for 30 min and then incubated at 37°C overnight. Peptides were then extracted twice using 0.1% TFA in 50% CAN and dried with N_2.

MALDI–TOF–MS/MS Identification and Database Search

Each sample was re-suspended with 0.7 ml matrix solution (CHCA in ACN/water, 1:1, acidified with 0.1% TFA). The mixture was immediately spotted on the MALDI target and allowed to dry and crystallize. The analysis was performed on a 4,700 Proteomics Analyzer (Applied Biosystems). The instrument operated in the positive ion reflection mode at 20 kV accelerating voltage and batch mode acquisition control. Reflector spectra were obtained over a mass of 700–3500 Da. The first five precursor ions with highest intensity were selected for fragmentation. The spectra were internally calibrated using two trypsin autolysis peaks at m/z 842.510 and 2211.105. The proteins were identified by PMF and MS/MS using the program MASCOT (Matrix Science, London, UK) against an NCBInr database with GPS explorer software (Applied Biosystems). The searching was carried out in mouse species. A maximum of one missed cleavage per peptide was allowed, a mass tolerance of 0.3 Da, and MS/MS tolerance of 0.4 Da were used. Tryptic autolytic fragments and contamination were removed from the data set used for database search. Proteins with protein score more than 59 or best ion score (MS/MS) more than 30 were significant.

Western Blotting

For immunoblotting analysis, equal amounts of total proteins (10 μg) were run in SDS–PAGE and transferred onto polyvinylidene fluoride membranes, which was blocked with a blocking buffer containing Tris-buffered saline, 0.1% Tween 20 (TBST), and 5% nonfat dry milk. Membranes were washed with TBST and incubated with the primary antibody. After washing again with TBST, the membranes were blotted with a secondary antibody conjugated with horseradish peroxidase, and then detected with the ECL detection system (Amersham Biosciences, Piscataway, NJ). The immunoblotting results were scanned with an Image-Scanner (Amersham Pharmacia). The relative intensity of protein expression for each sample was normalized to GAPDH before fold change was calculated to account for variations between different samples.

Immunostaining

Cells were resuspended in PBS, smeared on glass slides, and air-dried. The cells were then fixed on the slides with 4% paraformaldehyde. The slides were incubated for 3 h at room temperature with a mouse anti-CK8 mAb (Chemicon International, Temecula, CA), followed by a secondary Ab. Avidin-biotin/3,3′-diaminobenzidine (Ventana Medical Systems, Tucson, AZ) was used for detection, and hematoxylin was used for counterstaining.

RESULTS

Immuno-Magnetic Sorting of Mouse Fetal Liver Stem/Progenitor Cells

Embryo fetal liver contains lymphohematopoietic progenitors were characterized by the CD45 (common leukocyte marker) and TER119 (erythroid cell) antigens. Double staining of embryonic day 12.5 fetal mouse liver cells with anti-mAbs (TER119 + CD45) and anti-c-Kit (also CD117) mAb revealed four cell populations (Fig. 1A). The c-Kit$^-$(CD45/TER119)$^-$ cell population, which was considered to be liver stem cells or containing liver stem cells [Suzuki et al., 2000, 2002; Minguet et al., 2003], was successfully sorted with the negative surface markers c-Kit$^-$(CD45/TER119)$^-$ by using immuno-magnetic microbeads and identified as liver stem/progenitor cells in our previous study [He et al., 2006]. The sorted liver stem/progenitor cell population contains always more than 97.9% of c-Kit$^-$(CD45/TER119)$^-$ cells as identified by FC analysis (Fig. 1B) and the cell viability exceeded 98% as determined by trypan blue dye exclusion (data not shown). Before given to proteomic analysis, the sorted liver stem/progenitor cells were further qualified for the

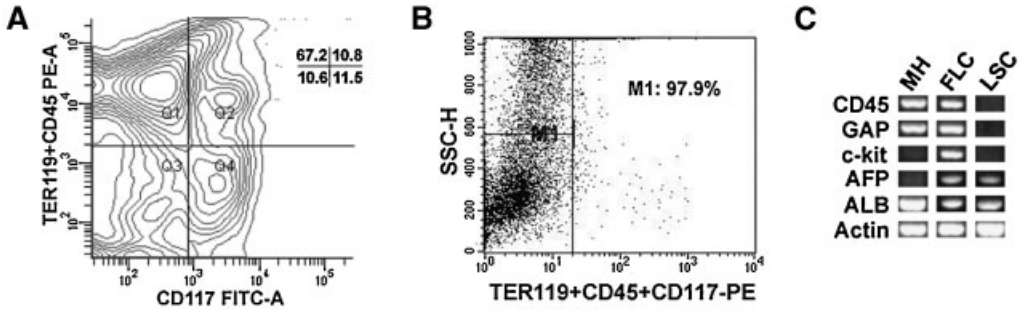

Fig. 1. Flow cytometric fractionation and analysis of sorted fetal mouse liver cells. **A**: Two-color FC analysis of embryonic day 12.5 mouse fetal liver cells by FACSAria. A mixture of biotinylated anti-CD45.2 and anti-TER119 mAb versus FITC anti-CD117 mAb defined the four populations of the contour plot. **B**: Cells of c-Kit$^-$(CD45/TER119)$^-$ were sorted by immunomagnetic microbeads and analyzed by FACSCalibur. **C**: RT-PCR was conducted on total RNA from mouse hepatocytes (MH), full fetal liver cells (FLC), and sorted primary liver stem/progenitor cells (LSC). GPA, glycophorin A; AFP, α-fetoprotein; ALB, albumin; Actin, β-actin.

marker gene expression by RT-PCR. As shown in Figure 1C, the sorted cells did not express *CD45*, *glycophorin A* (erythroid lineage marker), and *c-Kit* genes, but expressed the genes coding for α-fetoprotein and albumin, the markers for hepatocytes.

2D Protein Expression Profile of Liver Stem/Progenitor Cells and Identification of Differentially Expressed Proteins

Protein separation was performed in nonlinear 7 cm 2D gels with pI ranges of 3–10. For limited protein sample of liver stem/progenitor cells, we used only 60 μg of sample for the preliminary gel visualized by Coomassie Brilliant Blue staining, which resulted in limited protein spots displayed. Figure 2A shows one representative pair of proteome profilings for liver stem/progenitor cells versus full fetal liver cells. More protein spots were displayed when using silver staining, even less protein sample used (40 μg, data not shown). For the advantage of protein identification by MS, we also used MS-compatible silver staining, and about 400 spots were detected on the map of liver stem/progenitor cells (Fig. 2B) with the ImageMaster-2D analysis software.

By comparing protein expression levels between c-Kit$^-$(CD45/TER119)$^-$ primary liver stem/progenitor cells and full fetal liver cells, we focused on the proteins upregulated in liver stem/progenitor cells. Nineteen protein spots corresponding to 12 different proteins were identified using 2D-PAGE followed by MALDI-MS or peptide sequencing. These proteins were numbered on Fig. 2B and all significantly increased in liver stem/progenitor cells (an 1.4–12.7-fold increase, compared by Student's *t*-test and $P < 0.05$), as analyzed from the gels visualized by either traditional or MS-compatible silver staining. Most upregulated proteins correspond to enzymes involved in cell metabolism (glutamate dehydrogenase 1, enolase 1, triosephosphate isomerase, and lactate dehydrogenase 1), detoxication and cell protection (Glutathione S-transferase/mu 1 and Cu/Zn superoxide dismutase), or protein degradation and cell cycle regulation (proteasome subunit P42 homolog, proteasome 28 subunit/alpha and Ran protein), others are chaperones (protein disulfide isomerase associated 3 and heat shock protein 1) or cytoskeleton protein (cytokeratin endo A). More detailed information of these proteins is given in Table I.

The Association of the Upregulated Proteins in Liver Stem/Progenitor Cells and Cancer

A remarkable finding is that most of the upregulated proteins in liver stem/progenitor cells (11 of 12) were overexpressed in various kinds of cancers, as reported in previous studies (Table I). Also we noted that six of these proteins were overexpressed in HCC. The cropped images of the protein spots of these six proteins are shown in Figure 3. Among these proteins, three are enzymes and the other three are chaperones or cytoskeleton protein.

Validation of Differentially Expressed Proteins

In order to examine whether the changes in those six differentially expressed proteins in Figure 3 were due to transcriptional or other regulatory mechanisms, we performed real-time PCR on the genes of these proteins. We

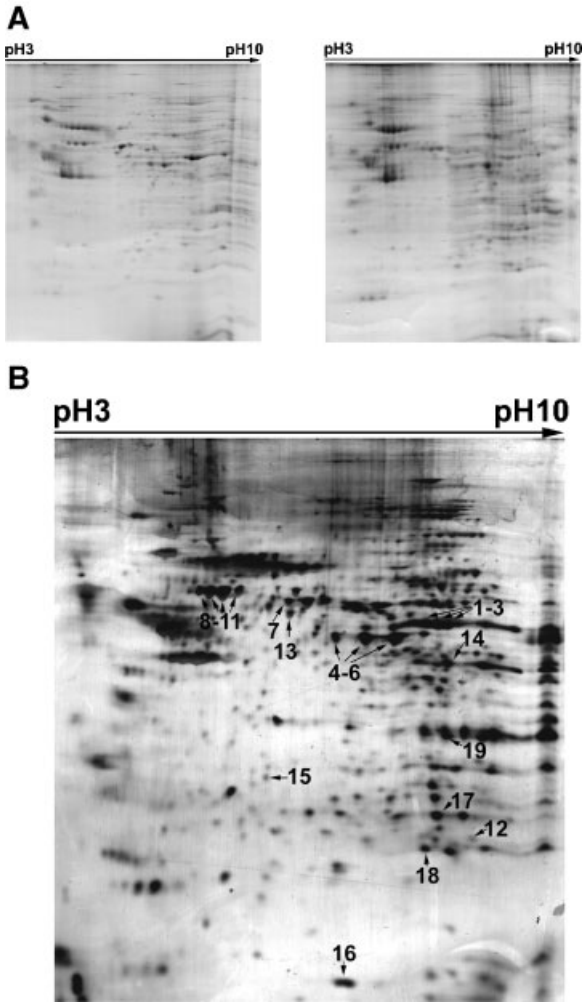

Fig. 2. Representative 2D map of proteins of primary liver stem/progenitor cells. **A**: Detected by Coomassie Brilliant Blue staining (60 μg of protein sample for liver stem/progenitor cells (**left**) and 175 μg of protein sample for full fetal liver cells (**right**)). **B**: Detected by MS-compatible silver staining (40 μg of protein sample of liver stem/progenitor cells). The identified proteins corresponding to each numbered spot are shown in Table I. Each gel shown was representative of three gels performed.

found direct correlations between the expression level of mRNA and protein in four genes (*HSP60*, *CK8*, *TPI*, and *SOD1*)(Fig. 4A). Although the mRNA level of *enolase* 1 was not significantly upregulated, Western blotting indicated that enolase 1 was upregulated by 1.6-fold in liver stem/progenitor cells (Fig. 4B), consistent with the result from 2-DE (Table I, Fig. 3).

To further verify the unusual high expression of CK8, immunostaining was performed to compare the CK8 protein expression in liver stem/progenitor cells and fetal liver cells depleted of liver stem/progenitor cells (that is, the positive fraction after MACS sorting). All liver stem/progenitor cells were strongly positive for CK8 expression, whereas nearly no CK8 expression was observed in fetal liver cells depleted of liver stem/progenitor cells (Fig. 5), indicating that CK8 expression is relatively specific for liver stem/progenitor cells.

DISCUSSION

Stem cells represent currently one of the most promising areas in medical research and proteomics takes stem cell analysis to another level [Levchenko, 2005]. Limiting the proteomic research of stem cells, however, is the fact that it is difficult to obtain relatively large amounts of protein sample [Unwin et al., 2003]. Although some proteomic researches have been done on cultured stem cells (e.g., Feldmann et al., 2005; Yin et al., 2005; Lee et al., 2006), which could be expanded in vitro to provide sufficient protein sample required for proteomic analysis such as 2D electrophoresis and MS, few studies have been done on primary stem/progenitor cells. As a result, little information is available on the in vivo biochemical and developmental processes of primary stem/progenitor cells, including liver stem/progenitor cells. Two commonly used methodologies, magnetic activated cell sorting, and high-speed fluorescence-activated cell sorting, have been employed to obtain enriched populations of various stem/progenitor cells based on the cellular surface markers. In the latter case, however, there are some disadvantages. For example, a high fluid pressure might damage the biological activity of the sorting cells; for rare cells such as stem cells, to isolate large amounts of them is also time-consuming [Gangopadhyay et al., 2004; He et al., 2006]. To obtain enough protein sample, we developed a method sorting large amounts of primary liver stem/progenitor cells using immuno-magnetic microbeads [He et al., 2006]. Taking the advantage of this method, which offers opportunities for generating sufficient rare primary cells for proteomic analysis, we obtained in the present study, for the first time, a 2D proteome map of primary liver stem/progenitor cells.

We also identified a set of highly expressed proteins in liver stem/progenitor cells, which should shed light on the in vivo biochemical processes of this rare population as well as the role of liver stem/progenitor cells in liver

TABLE I. List of Highly Expressed Proteins Identified in Liver Stem/Progenitor Cells

Spot no.[a]	Protein name	Accession no.	MW (kD)/PI (nominal)[b]	No. of matched peptides	Protein score	Fold increase[c]	Major function	Overexpressed in cancers [references]
1–3	Glutamate dehydrogenase 1	gi|6680027	61.30/8.05	23	228	12.7	Nitrogen metabolism	Malignant gastrointestinal stromal tumor [Kang et al., 2006]
4–6	Enolase 1	gi|54673814	47.11/6.37	22	316	1.7[d]	Glycometabolism	HCC [Li et al., 2005; Takashima et al., 2005]; ovarian carcinoma [Wang et al., 2004]; esophageal squamous cell carcinoma [Qi et al., 2005]; gastric cancer [He et al., 2004b]; pancreatic adenocarcinoma [Shen et al., 2004]; colorectal cancer [Tomonaga et al., 2004]; lung squamous carcinoma [Li et al., 2006]
7	Protein disulfide isomerase associated 3 (Pdia3)	gi|23958822	56.64/5.88	25	202	4.7	Chaperones	HCC [Kim et al., 2002]; breast infiltrating ductal carcinoma [Somiari et al., 2003]; lung squamous carcinoma [Li et al., 2006]
8–11	Heat shock protein 60 (HSP60)	gi|16741093	60.92/5.91	27	361	1.4[d]	Chaperones	HCC [Lim et al., 2002; Blanc et al., 2005]; gastric cancer [He et al., 2004b]; oral tongue carcinoma [He et al., 2004a]; colorectal cancer [Tomonaga et al., 2004]
12	Glutathione S-transferase, mu 1	gi|61402231	25.95/7.71	19	245	1.8	Cellular detoxification	
13	Cytokeratin endo A (CK8)	gi|220392	54.53/5.7	31	313	11.6	Cytoskeleton protein	HCC and cholangiocarcinoma [Srisomsap et al., 2004]; ovarian carcinoma [Wang et al., 2004]; gastric cancer [He et al., 2004b]
14	Proteasome subunit P42 homolog (Psmc6 protein)	gi|34783985	43.33/7.25	12	73	7.4	Protein degradation	Ovarian carcinoma [Wang et al., 2004]
15	Proteasome 28 subunit, alpha	gi|6755212	28.66/5.73	14	155	1.4	Protein degradation	Breast infiltrating ductal carcinoma [Somiari et al., 2003]
16	Cu/Zn superoxide dismutase (SOD1)	gi|226471	15.75/6.03	7	102	1.8	Antioxidant	HCC [Ai et al., 2005]; ovarian carcinoma [Wang et al., 2004]
17	Triosephosphate isomerase (TPI)	gi|1864018	22.49/5.62	10	101	2.0	Glycometabolism	HCC [Takashima et al., 2003; Lee et al., 2005]; ovarian carcinoma [Wang et al., 2004]; gastric cancer [He et al., 2004b]; lung adenocarcinomas [Chen et al., 2002]; colorectal cancer [Tomonaga et al., 2004]; lung squamous carcinoma [Li et al., 2006]
18	Ran protein	gi|12846283	24.41/7.01	9	83	2.0	Cell cycle control	Gastric, colon, pancreas and lung cancers [Azuma et al., 2004]
19	Lactate dehydrogenase 1, A chain	gi|6754524	36.48/7.62	10	90	4.7	Lactate metabolism	Ovarian carcinoma [Wang et al., 2004]

[a]The spot numbers are those shown in Figure 2B.
[b]Nominal MW/PIs were obtained from NCBI database.
[c]Fold increases shown were calculated based on the image analysis of MS-compatible silver-stained gels of primary liver stem/progenitor cells and full fetal liver cells, compared by Student's t-test and $P < 0.05$.
[d]Fold increase was calculated based on the total volume of the density of spots of the several isoforms identified as the same protein.

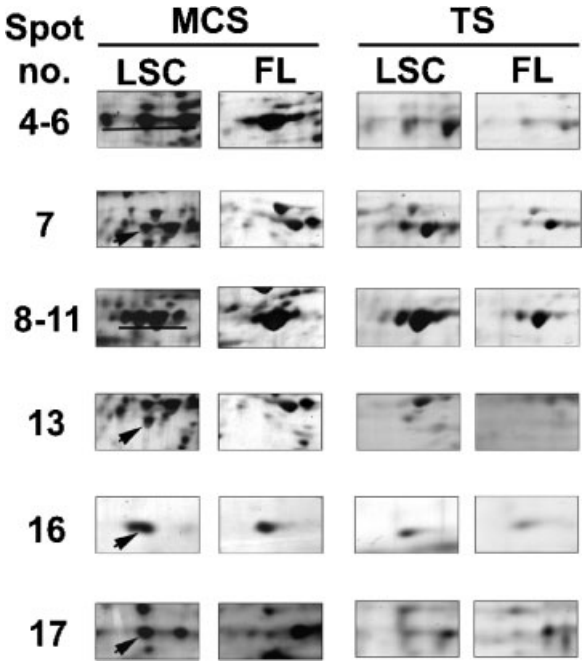

Fig. 3. Cropped images of six proteins highly expressed in primary liver stem/progenitor cells. These proteins were also overexpressed in HCC as reported in other studies. Images shown are representative of these spots in three gels of liver stem/progenitor cells or full fetal liver cells visualized either by MS-compatible or traditional standard silver staining. MCS, MS-compatible silver stain; TS, traditional standard silver staining; LSC, liver stem/progenitor cells; FL, full fetal liver cells.

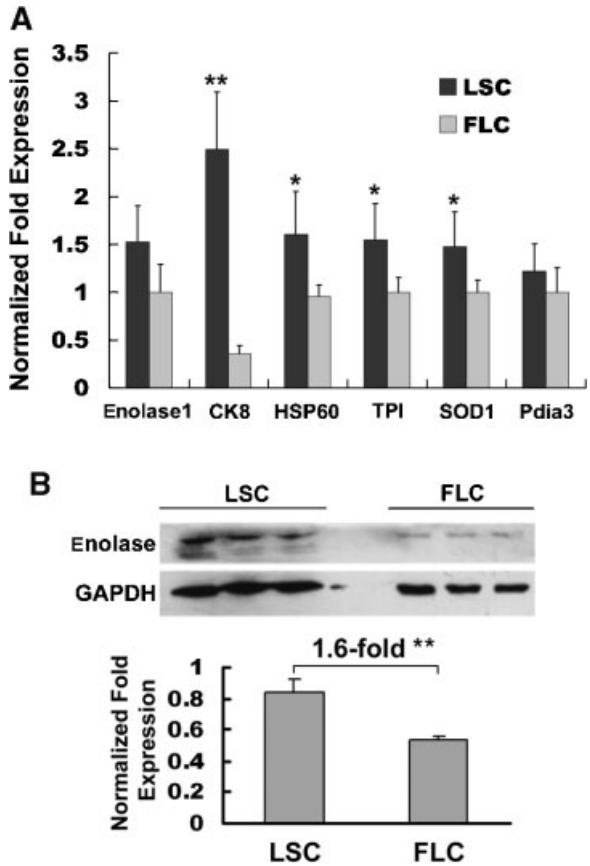

Fig. 4. Validation of differentially expressed proteins. **A**: Relative quantitative real-time PCR analysis of the mRNAs coding for the six differentially expressed proteins. β-actin was used as an internal control. Each RNA sample was analyzed in triplicate. **B**: Western blotting analysis of enolase 1 protein. Each protein sample was performed in triplicate. GAPDH was used as an internal control. Each bar represents the mean ± SD of relative intensity. Differences in expression in (A) and (B) were analyzed by the Student's *t*-test. *, $P < 0.05$, **, $P < 0.01$. LSC, liver stem/progenitor cells; FLC, full fetal liver cells. Data in (A) and (B) are representative of three individual experiments.

development. Many of these highly expressed proteins are enzymes involved in cell metabolism, indicating more active metabolism in some pathways in such a population of cells. In addition, more cell cycle control proteins were expressed in liver stem/progenitor cells. Ran protein, a small Ras-related GTPase, controls the cell cycle through the regulation of nucleocytoplasmic transport, mitotic spindle organization, and nuclear envelope formation [Azuma et al., 2004]. Psmc6 protein and proteasome 28 subunit/alpha are two subunits of proteasome, a multicatalytic proteinase complex responsible for the degradation of most intracellular proteins, including proteins crucial to cell cycle regulation, growth and development [Voorhees et al., 2003]. Furthermore, we found that the protein of cytokeratin endo A (CK8), whose mRNA begins to be detectable in eight-cell mouse embryo [Duprey et al., 1985], is obviously highly expressed in liver stem/progenitor cells of mid-term embryo (12.5 days), suggesting that it may play an important role in liver development.

The most interesting finding is that most of the identified highly expressed proteins in liver stem/progenitor cells were overexpressed in at least two kinds of cancers. These proteins include chaperones, cell cycle control protein, and enzymes (Table I). They may play an important role in cancer proliferation and development, and suggest some similarity between stem cells and cancer. One of them, enolase 1, a key glycolytic enzyme, was observed to be overexpressed in at least seven kinds of cancers, including liver caner, where expression of enolase 1 also correlated positively with tumor size and venous invasion [Takashima et al., 2005]. In addition, we found that six

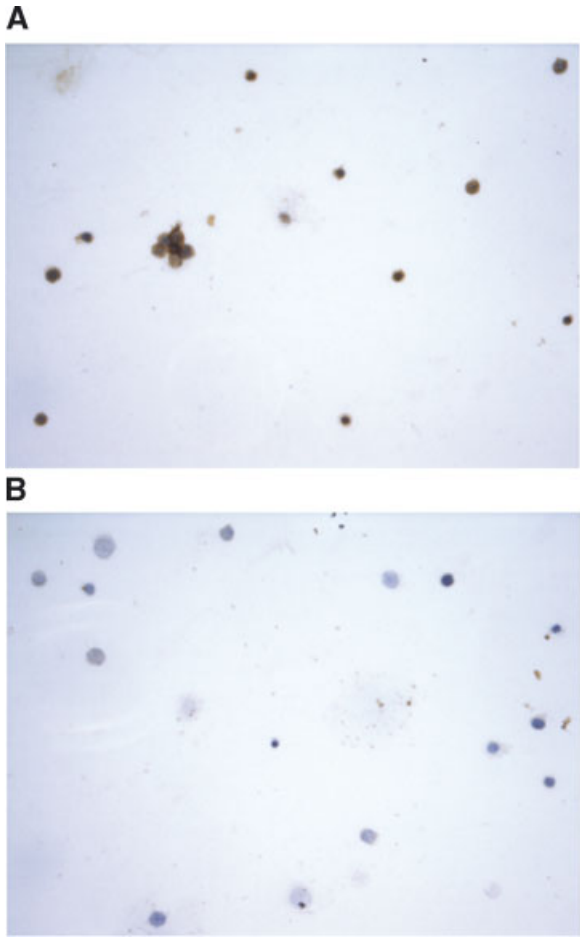

Fig. 5. Immunohistochemical staining for the detection of CK8 protein. **A**: Liver stem/progenitor cells; (**B**) fetal liver cells depleted of liver stem/progenitor cells. Images are representative of multiple microscopic fields observed. Original magnification: 400×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

proteins that highly expressed in liver stem/progenitor cells were also overexpressed in liver cancer, suggesting that there is a possible association between liver stem/progenitor cells and liver cancer.

It is now widely believed that most cancers are derived from cancer stem cells that parallel the normal stem cell compartment [Reya et al., 2001; Scadden, 2004; Bjerkvig et al., 2005; Huntly and Gilliland, 2005]. Cancer stem cells share the same properties of self-renewal and differentiation with normal stem cells, with the addition of similar phenotype of normal stem cells isolated from the same tissue. The existence of such cells has implications for current conceptualizations of malignant transformation and therapeutic approaches to cancer [Polyak and Hahn, 2006]. However, there is no direct evidence supporting the existence of liver cancer stem cells until now. In this study, our results showed that normal liver stem/progenitor cells share similar expression patterns of some metabolism enzymes and other proteins with liver cancer and other kinds of cancers, implying the possibility that liver cancer is derived from liver stem/progenitor cells (or liver cancer stem cells).

Of course, the solid proof supporting that liver cancer is derived from liver stem/progenitor cells is the existence of liver cancer stem cells. Although the data in this study are somewhat preliminary, it is of interest to perform further studies on the highly expressed proteins in liver stem/progenitor cells identified in this study that might be useful for the identification of the potential liver cancer stem cells. For example, CK8, whose expression was found in this study to be relatively specific for liver stem/progenitor cells, might be a useful marker for identification of liver cancer stem cells. However, identification of cancer stem cells is usually done based on the protein expression of cellular surface markers. Thus, isolating more proteins in the surface of liver stem/progenitor cells by an alternative protein extracting method could be useful for the identification of some specific protein markers shared by both normal liver stem/progenitor cells and liver cancer stem cells, thereby useful for the isolating and identification of the potential liver cancer stem cells.

In conclusion, in this study we successfully identified a set of proteins that were highly expressed in primary c-Kit^{-}(CD45/TER119)$^{-}$ liver stem/progenitor cells. The data presented provide a significant new protein-level insight into the biology of liver stem/progenitor cells. Also the results should shed light on the identification of the putative liver cancer stem cells.

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