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Proteomic Analysis Of The "Side Population" (SP) Cells From Murine Bone Marrow

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Abstract

Side population (SP) cells are a specialized cell type present at very low numbers in murine bone marrow and other tissues. These cells are considered to be multipotent stem cells capable of differentiating into several cell lineages in vivo and ex vivo. In this report we have isolated SP cells from suspensions of bone marrow of Balb-c mice by using a fluorescence-activated cell sorter (FACS) and we have done the comparative analysis of proteins expressed by the total bone marrow (TBM) and SP cells, by using standard procedures of 2D gel electrophoresis (2DE) followed by MALDI-MS and/or MS/MS. Immunofluorescence analysis of the expression of cytoskeletal proteins actin and vimentin and a multi drug resistance (MDR) family protein ABCG2 were done to further validate the biochemical proteomic data. The classification of the identified proteins into their functional categories indicated that SP cells over express stress proteins, cytoskeletal proteins and enzymes of the glycolytic metabolism. These results could be significant in understanding the physiology of stem cells in the bone marrow.

Keywords: Stem cells; Cell differentiation; Haematopoiesis; Flow cytometry

Abbrevations: TBM: Total Bone Marrow; SP: Side Population; FACS: Fluorescence Activated Cell Sorting

Introduction

Proteomic analysis of stem cells, such as human or mouse embryonic stem (ES) cells, human umblical cord blood cells, stem cells of neuronal origin etc. have been reported in previous studies (Baharvand, 2006 a, b; Van Hoof et al., 2006; Wang and Gao, 2005; Yin et al., 2005; Nagano et al., 2005; Elliott et al., 2004; Feldmann et al., 2005; Hoffrogge et al., 2006). More recently protein profiles of stem cells derived from adult tissues such as skin epithelia (Epstein, 2005), adipocytes, retina and primary or cultured mesenchymal bone marrow cells have been reported (Lee et al., 2006; Cavusoglu et al., 2003; Chen et al., 2005; Ye, 2006; Seshi, 2006). The comparison of the proteomes of undifferentiated stem cells with cells that are committed to lineage specific differentiation has been very useful to identify specific markers involved in the regulation of stem cell biology and in controlling the potential of stem cells for multi-lineage differentiation (Baharvand, 2006a; Unwin et al., 2003; Hayman, 2004; Kurosaki, 2007; Baharvand, 2007). A comparative assessment of proteins commonly expressed in stem cells derived from various origins has been reported in a recent review (Hoffrogge, 2007).

Over the past 10 years, several workers have studied a more specialized stem cell population, that is seen in both adult and embryonic tissues whose identity is exclusively based upon flow cytometric analysis (Goodell et al., 1997; Goodell et al., 1996). These cells are referred to as SP cells and they exhibit the MDR class of molecular pump activity in their plasma membrane-therefore when studied by flow cytometry they show a unique pattern of Hoechst dye efflux that defines their name (Zhou et al., 2001; Scharenberg et al., 2002; Jonker, 2005; Zhou et al., 2002). The properties of SP cells have been mainly studied from the mouse bone marrow however, they have been identified in many other tissues also (Kawanabe et al., 2006). SP cells have been shown to possess the potential for multi-lineage differentiation and are hence considered to be similar to primitive stem cells, however their stem cell like behaviour is not universally seen (Parmar et al., 2003; Macpherson et al., 2005; Triel et al., 2004). SP cells have also been associated with the malignant properties of certain cells where they have been projected as the so-called cancer stem cells (Hadnagya et al., 2006).

Considering the specialized behavior and differentiation potential of SP cells it has become necessary to know their protein profiles so that the regulatory proteins in these cells can be identified and a more rational explanation for their behaviour can be enumerated. At present there are no reports on the proteomic profile of murine SP cells.

In this paper we have compared the protein profiles of SP cells derived from the bone marrow of the laboratory mouse. We have found that mouse SP cells express approximately only 50% of the proteins that were seen in TBM cells between the molecular weights of 19 KDa and 200 KDa (112 spots vs 233 spots). Our analysis of 24 spots out of 112 spots in the SP and 44 spots out of 233 spots seen in TBM cells showed that cytoskeletal and stress proteins were among most significant proteins expressed in SP cells. Our immunofluorescence data also confirm the up-regulation of these cytoskeletal proteins such as actin and vimentin in SP cells as compared to TBMCs both in terms of staining per cell and the number of cells stained. Actin

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in TBMCs also showed a different localization in comparison to SP cells.

Experimental Section

Cell Source

All experiments were done with bone marrow cells obtained from Balb-c mice, males or females, aged between 6 to 8 weeks. The animals were obtained from the inbred colonies of these animals maintained at the Animal Facility of CCMB.

Bone Marrow Preparation

Bone marrow was flushed from murine femurs and tibias and a single cell suspension was prepared by passing the bone marrow through a 21-guage needle after which the cells were pelleted by centrifugation and re-suspended at 10^6 cells/mL in HBSS (Hanks Balanced Salt Solution) that contained 2% fetal calf serum (FCS) and 10 mM HEPES. The sample was centrifuged at 1500 rpm for 5 min at 4 °C. The nucleated cells were counted after staining with crystal violet containing 2% acetic acid.

SP Cell Isolation

SP cells were isolated from the bone marrow according to published methods (Lin and Goodell, 2006). Briefly, bone marrow was re-suspended at 10^6 cells/mL in prewarmed HBSS as outlined above. Hoechst 33342 (bis-Benzimide) (Sigma Aldrich, Dorset, UK) was added to the cells at a final concentration of 5 µg/mL and the cells incubated at 37 °C for 90 min. After this time, the cells were pelleted and resuspended in ice cold HBSS containing 2% FCS and 10 mM HEPES and maintained at 4 °C for analysis by flow cytometry.

Flow Cytometry

For sorting SP cells FACS Vantage SE (BD Biosciences) equipped with 488 nm primary laser and an ultraviolet laser at 350 nm as second laser (Coherent Innova 305/ 309) was used to excite the Propidium iodide and Hoechst dye respectively. Fluorescence from the Hoechst dye was measured with a 450/20 BP filter (Hoechst Blue) and a 675LP optical filter (Hoechst Red). A 610 DM SP filter was used to separate the two emission wavelengths. Propidium iodide (PI) fluorescence was also measured through the 675LP. Hoechst Blue vs. Red profile was displayed, with Hoechst blue (450 BP filter) on the Y- axis and Hoechst red (675 LP) on the X- axis. The detectors for both the channels were in linear mode, the voltages were adjusted so that dead cells line up in the lower left corner on a vertical line to the far right. SP cells exclude Hoechst 33342 and consequently have lower blue and red fluorescence emissions.

Cell Lysis and Protein Preparation

Sorted SP cells from each batch were processed for protein extraction for which the sorted cells were initially collected in HBSS and centrifuged. The pelleted cells were suspended in a very small volume (~10 µl) of lysis buffer and stored at -70 °C. In order to maintain the consistency and reliability of our results in case of TBMC we ran 4 gels of each biological replicate a total of 3 replicates were studied and for SP cells 1 gel each from 3 biological replicates, each biological replicate contained cells obtained by pooling sorted cells from different batches. Silver staining of the gels was done under identical conditions so that reliable quantitative comparison of the gel spots could be done.

Proteins from TBM and sorted SP cells were extracted by the following method. Cells were washed using ice-cold HBSS buffer and centrifuged at 1500 rpm for 5 min. The supernatant was discarded by careful pipetting. The cells were re-supended in lysis buffer, (8 M urea, 0.2% ampholytes, 2% CHAPS, and 50 mM DTT) and mixed well by repeated pipeting. Samples were allowed to stand at 4 ºC for about 1 h with occasional sonication, transferred to Beckman thick wall tubes and centrifuged at 55,000 rpm in a Ultracentrifuge (Beckman TL-100) for 1h at 4 °C. The final cell free supernatants were quantified by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples were aliquoted and stored at -70 °C.

Two-dimensional Gel Electrophoresis

Total and SP cell lysate of 50 µg each was suspended in rehydration buffer containing 8 M urea, 50mM DTT, 2% CHAPS, 0.2% carrier ampholytes. IPG Strips (11cm, pH 4–7; Bio-Rad, Hercules, CA, USA) were rehydrated with samples overnight at room temperature. The first dimension IEF was performed with Bio-Rad electrophoresis unit at 20 °C. Before the second dimension separation, each focused IPG strip was incubated first in equilibration buffer (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol) containing 2% DTT and then in same equilibration buffer containing 2.5% Iodoacetamide for 45 min each. Incubations were carried out at room temperature with gentle shaking. The second dimension SDS PAGE was carried out using Hoefer Maxi gel electrophoresis unit. The equilibrated IPG Strips were loaded and run on 10% gels at a constant current of 10 mA per gel. Both the gels were run simultaneously and under identical conditions to gain the best reproducibility. Proteins on the gels were visualized using modified silver staining protocol (Gharahdaghi et al., 1999).

Gel Image Analysis

Gels were scanned using the Flour - S MultiImager (Bio-Rad) and analyzed with PD quest image analysis software (Bio-Rad). All images were taken under uniform settings, spot intensities were determined and normalized for small variations in staining or protein loads, using total density of the protein spots. Spot detections were carried out automatically by running 'Spot Detection Wizard', followed by the manual editing of each image to remove artifacts such as streaks and splotches. Each spot was checked and edited if required, by filtering, drawing, erasing and splitting, etc. A comparison of TBM and SP gel protein profiles was carried out for differentially expressed spots with more than 2-fold difference.

Protein Identification by MALDI-TOF-TOF-MS Analysis

Protein spots were excised from TBM silver-stained gels, destained and in-gel digested with proteomic grade trypsin (Sigma) overnight at 37 °C, (as described in the handout given by Applied Biosystems Inc., USA - the manufacturers of the MALDI-TOF/TOF 4800 instrument). Peptides from the gel were extracted using 50% Acetonitrile and 0.1% Tri fluoroacetic acid in water, lyophilized and stored at –20 °C till further analysis. The resulting peptides were desalted using ZipTips containing C18 resin, mixed with the matrix solution containing 5mg/ml

CHCA (α-cyano-4-hydroxycinnamic acid), and analyzed using a MALDI 4800 TOF-TOF analyzer (ABI, USA). The machine was calibrated to <10-ppm accuracy using calibration mixture of known standard synthetic peptides into mass range 800-4000 m/z. The Peptide Mass Fingerprinting (PMF) data were obtained and protein identification was carried out on PMF data user GPS software (ABI, USA) with Mascot (Matrix Science, London, UK) search engine and NCBI database with the following settings: taxonomy (*Mus musculus*), fixed modification (oxidation), variable modification (carbamidomethylation), charge state (+1) and precursor tolerance (50 ppm). The proteins identified were validated based on Mascot rank, number of peptides matched, sequence coverage and match of M_r/pI. The proteins identified were confirmed further by MS/MS of chosen peptides based on PMF. These peptides were fragmented in a CID chamber by laser and the resulting MS/MS ions were searched against database for protein ids with restrictions placed on peak density filter of 10 and maximal 50 peaks; minimum S/N filter of 10 and MS/MS fragment tolerance 0.25 Da. Contaminant peaks like polymer, keratin and trypsin peaks were filtered from PMF before submission for data base search. Only those proteins identified by PMF and/or MS/MS searches were listed.

Immunofluorescence Analysis

Indirect Immuno-fluorescence of cytoskeletal and stress proteins was performed on 95% ethanol fixed Cytospin preparations of TBM and SP cells to confirm the over expression of specific proteins. Primary antibodies used for this purpose were: anti-mouse vimentin antibody (Sigma, USA) and biotin-conjugated-anti-human ABCG2 antibody (Stem Cell Technologies); secondary antibodies used were Cy3 linked anti-mouse IgG (Sigma USA) and FITC conjugated streptavidin. In order to stain F actin, Alexa-fluor 488 tagged phalloidin (Invitrogen USA) was used. All incubations and washings were performed as per the manufacturer's instructions. Fluorescence visualization was done in a Ziess 210 Meta confocal microscope by using appropriate excitation and emission filters.

Results and Discussion

Sorting of SP Cells and Protein Extraction

SP cells from TBM cell suspension of Balb-c mice were sorted as described in Materials and Methods. In a typical cell sorting experiment cell suspension was prepared from 2 femur bones and ~4X10^7 cells, were stained with Hoechst dye (5 µg/mL) and the SP cell population was displayed on the flow cytometer. One typical display of mouse SP cells is shown in Figure 1A where 0.16% cells of the total bone marrow can be identified as SP cells. In order to confirm that these cells indeed represented the SP phenotype, the bone marrow cells were treated with Verapamil (2 µg/mL) before staining; in these cells only 0.01% SP cells could be identified (Figure 1B). Three separately prepared protein preparations were analysed to check the reproducibility from both the cell types. In our experimental conditions the yield of SP cells in the TBM cell suspension varied between 0.05-0.2% from experiment to experiment. These cell percentages correspond to the figures reported by other workers (Scharenberg et al., 2002, Jonker et al., 2005).

Two-DE Analysis and Protein Identification

Fifty µg proteins per gel from mouse SP and TBM cells were analyzed by 2DE as described. In our conditions proteins having molecular weights between 19 kDa and 110 kDa, and isoelectric points between 4 and 7 were well resolved. The results of the silver stained protein spots seen in TBM and SP cells are shown in Figure 2A and Figure 2B respectively. The same spots represented by green and red are shown in a template form in Figure 3A and Figure 3B where green and red colors represent spots from TBM and SP cells respectively. Proteins from TBM cells were analysed by 2DE four times and those from SP cells 3 times and identical spot pattern was seen in each experiment.

PD Quest 2DE analysis of the gels, which aids in the com-

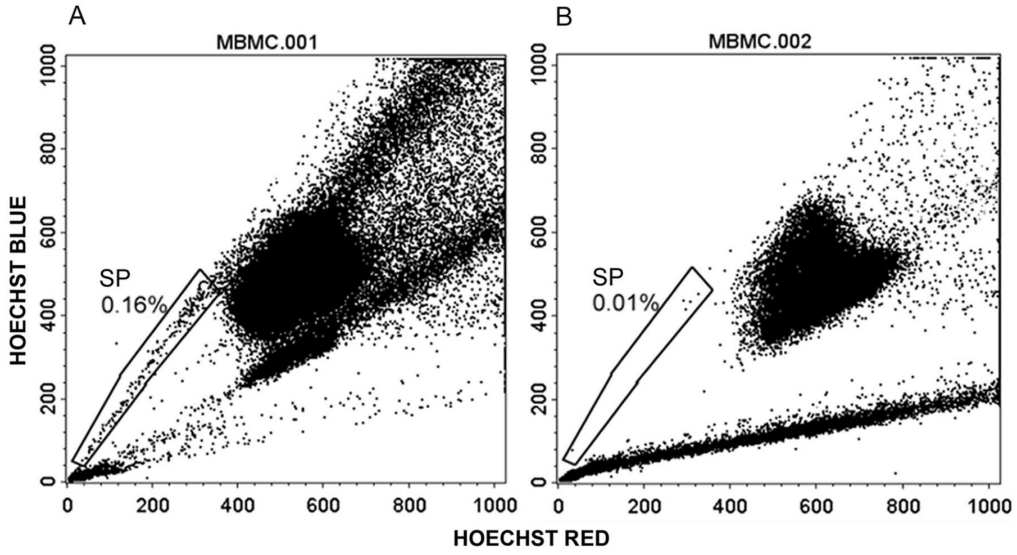

Figure 1: 2D dot plots of the flow cytometric analysis of SP cells from the bone marrow of adult mice. Hoechst dye efflux in TBM without (panel A) and with verapamil (panel B) was assayed by flow cytometry as described in the text. The percentages of SP cells in the gated area are indicated.

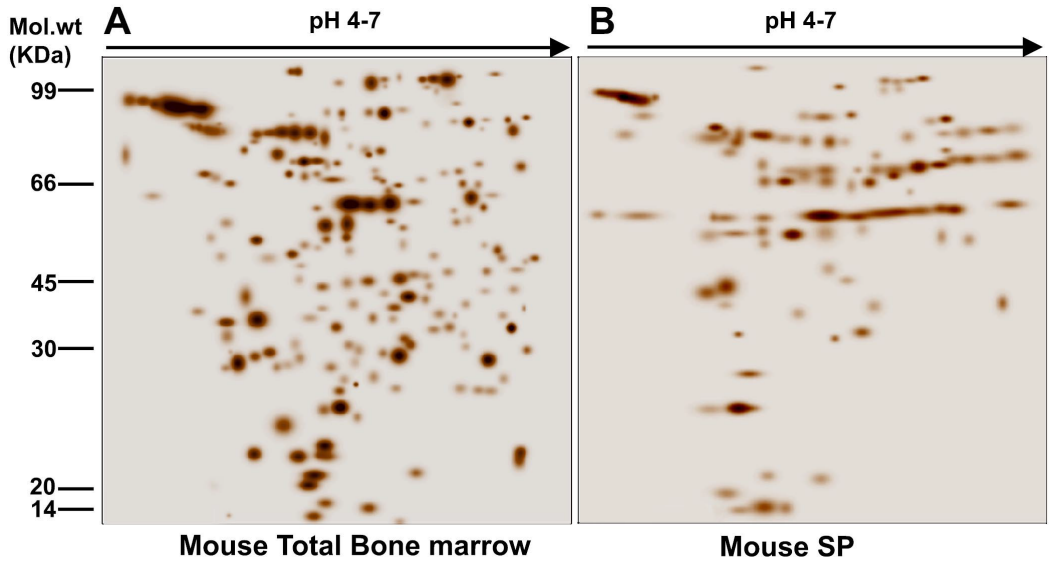

Figure 2: Silver stained 2-D gel images of TBM and SP cells of mouse bone marrow. 50 µg protein from cell lysates of TBM (A) and SP (B) cells were subjected to 2-DE followed by silver staining and image analysis. IEF (pH 4-7 linear gradient) is in the horizontal direction and SDS-PAGE (10% gel) is in the vertical direction.

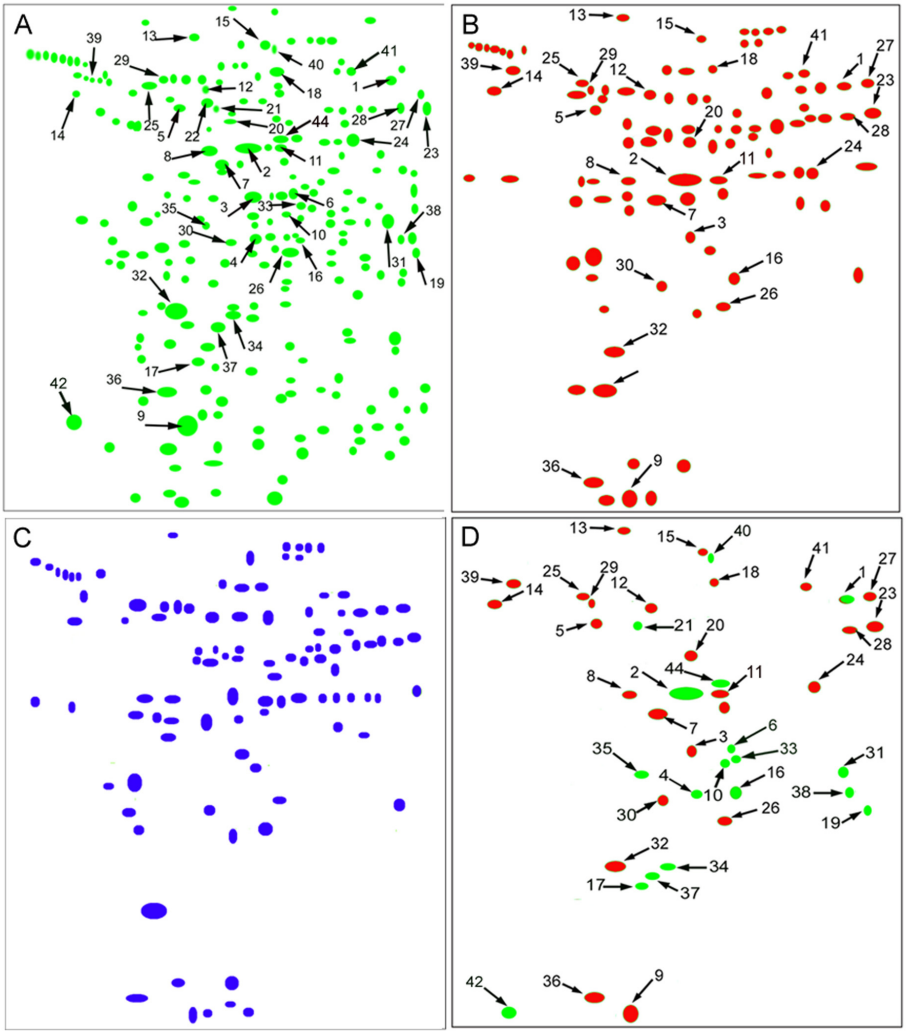

Figure 3: Templates of the spots prepared from 2-DE gels of TBM and SP cells of the mouse. All spots shown in panels A (green) and B (red) respectively correspond to the silver stained proteins seen in Figs 2A & 2B. In panel 3C, spots common to both the cell types as determined by PD Quest analysis (overlapping spots in Fig 2A & 2B) are shown in blue color. Panel 3D shows proteins expressed exclusively in TBM (green) or SP cells (red). Spots marked by an arrow were excised from the TBM gels and digested with trypsin and the peptides were used for MS/ MS analysis

parative identification and quantification of proteins isolated in 2DE, was used to evaluate the positions and protein expression in the 2DE gels. This analysis showed 233 spots seen in TBM and only 112 spots in SP cells. All spots seen in the 2DE of SP cells were also seen in TBM cells indicating that among the total proteins of TBM cells approximately 50% were also present for the proteome of SP cells. A comparison of these 112 common proteins expressed in both SP and TBM cells showed that 29 proteins were over expressed and 17 proteins were under expressed in SP cells.

Protein identification of the gel spots was done from the TBM gels. 150 spots were subjected to trypsin digestion and analyzed using MALDI TOF-TOF. Forty-four Proteins with confirmed identities could be classified into 8 categories in accordance with their function as documented in NCBI Gene Ontology database and ExPasy (http://www.expasy.org/sprot/). These were cytoskeletal proteins, stress response proteins, metabolic proteins, signal transduction membrane proteins, transcriptional regulators, immune response proteins and others. Specific protein identities could be established from 44 spots of which 24 were also seen in SP cells. The names and other details of these proteins are given in Table 1 (included as a supplementary information). In some cases, different spots in the 2DE gels represent spots with the same MS/MS ID this can be accounted for post-translational modifications, which could change the location of the spot positions for same protein. Due to this overlap the identities of many different spots in the Table 1 are either similar or same.

Comparative Analysis of TBM and SP Cell Proteins With Other Studies

To date there is no report available on the proteomic profile of mouse TBM cells. Previous reports have studied bone marrow proteins before and after chemical or radiation damage and their derivations. For instance Chen et al. have studied the proteome of the "plasma" obtained from mouse bone marrow in response to radiation damage and identified 21 proteins in the pH range of 4-7 (Chen et al., 2005). A comparison of their data with the present study revealed β-actin, γ-actin and HSP 71 (cognate) as common proteins expressed in the SP cell proteome. Similarly, the study of Kim et al., (2005), who reported 11 differentially expressed proteins from PTH treated mouse bone marrow cells in the same pI range (4-7), shows aldehyde dehydrogenase (Aldh2), calreticulin and vimentin as common proteins seen in the mouse TBM and SP cells. It is important to note that all these proteins are also expressed in many other kinds of stem cells, as described in a recent review on stem cell proteomics (Hoffrogge 2007).

Besides these two reports there are no other reports on the TBM proteome of mouse hence, in this study we have attempted to get a better resolution of proteins in a narrowly defined region of pI (4-7) and molecular weight (14-99 KDa), and identify as many proteins in that region as possible. Our second objective was to compare the proteins of SP cells in the same range with the proteins reported to be expressed in stem cells so that the functional relationship between SP cells and stem cells can be determined.

Functional Significance of SP Cell Specific Proteins

After confirming the identity of 24 proteins from SP cells, we classified the proteins on the basis of comparative levels with the TBM cells and their biological significance. Among the 24 spots for which we could obtain confirmed IDs by MALDI TOF-TOF, 4 proteins (beta-actin, gamma actin, Vimentin and M2 pyruvate kinase) were over expressed (>2 folds) in SP cells. Simiarly another set of 3 proteins (HSP8, HSP1, ATP synthase) were down regulated. In general it can be said that most cytoskeletal proteins (in particular different type of actins) were up regulated in SP cells as compared to TBM cells.

Up Regulated Proteins

(a) Pyruvate Kinase: Over expression of genes of the glycolytic pathway in bone marrow SP cells was shown by Liadaki et al., (2005) using transcriptomic analysis (Liadaki et al., 2005). Another study, performed by Unwin et al., (2006), showed that long term reconstituting HSCs ($Lin^{-}Sca^{+}Kit^{+}$; LSK^{+}) over express glycolytic proteins. These results are in concordance with our observation where in the glycolytic enzyme pyruvate kinase (spot 24) is upregulated. Pyruvate kinase is present as four different isozymes (L, R, M1, M2), the expression of which differs from one tissue to another. Hematopoietic stem cells and embryonic stem cells express M2-PK, which is characteristic of tissues with high rates of nucleic acid synthesis, and the isozyme switches from M2-PK to R-PK during differentiation into erythroid lineage (Mazurek, 2005; Takegawa, 1983). It is also a molecular marker for hepatic oval cells, which have some phenotypic traits that are typical of BM stem cells, an evidence suggesting that cells in the adult organism have a remarkable degree of plasticity (Petersen, 1999).

b) Vimentin: Expression of cytoskeletal proteins in stem cells has been reported earlier. We observed the overexpression of vimentin (spot No. 5) in SP cells. Vimentin is a member of the intermediate filament family of proteins is responsible for maintaining cell shape, integrity of cytoplasm and stabilizing cytoskeletal interactions. It is characterized as a mesenchymal stem cell marker and its enrichment in thyroid SP (Hoshi et al., 2007), lung SP (Summer, 2003), adult liver stem cells (Herrara et al., 2006) and the down regulation during differentiation (Hoffroge et al., 2006; Seshi, 2006) throws light on stem cell like properties. Changes in its expression and filament organization have been reported in response to stresses such as heat shock, and oxidative stress suggesting a role for the vimentin network under conditions of strain.

c) Actins: The cytoskeleton is a dynamic structure capable of reorganization as required by the cell type, specification, stage of development and environmental conditions. Several mechanical parameters like structure and dynamics of stem cells appear to be regulated by the actin cytoskeleton and Yourek et al., (2007) have shown stem cells to alter their cytoskeletal components during differentiation. The expression of beta actin (spot 9) and gamma actin (spot 11) increased in SP cells in comparison to TBM cells.

The upregulation of these two cytoskeletal proteins Actin and Vimentin was confirmed by immunostaining and confocal microscopy (Figure 4). As can be seen in the TBMC panel (Figure 4 A), the staining of cells for both actin (panel b) and vimentin (panel c) is less or totally absent in comparison to the staining

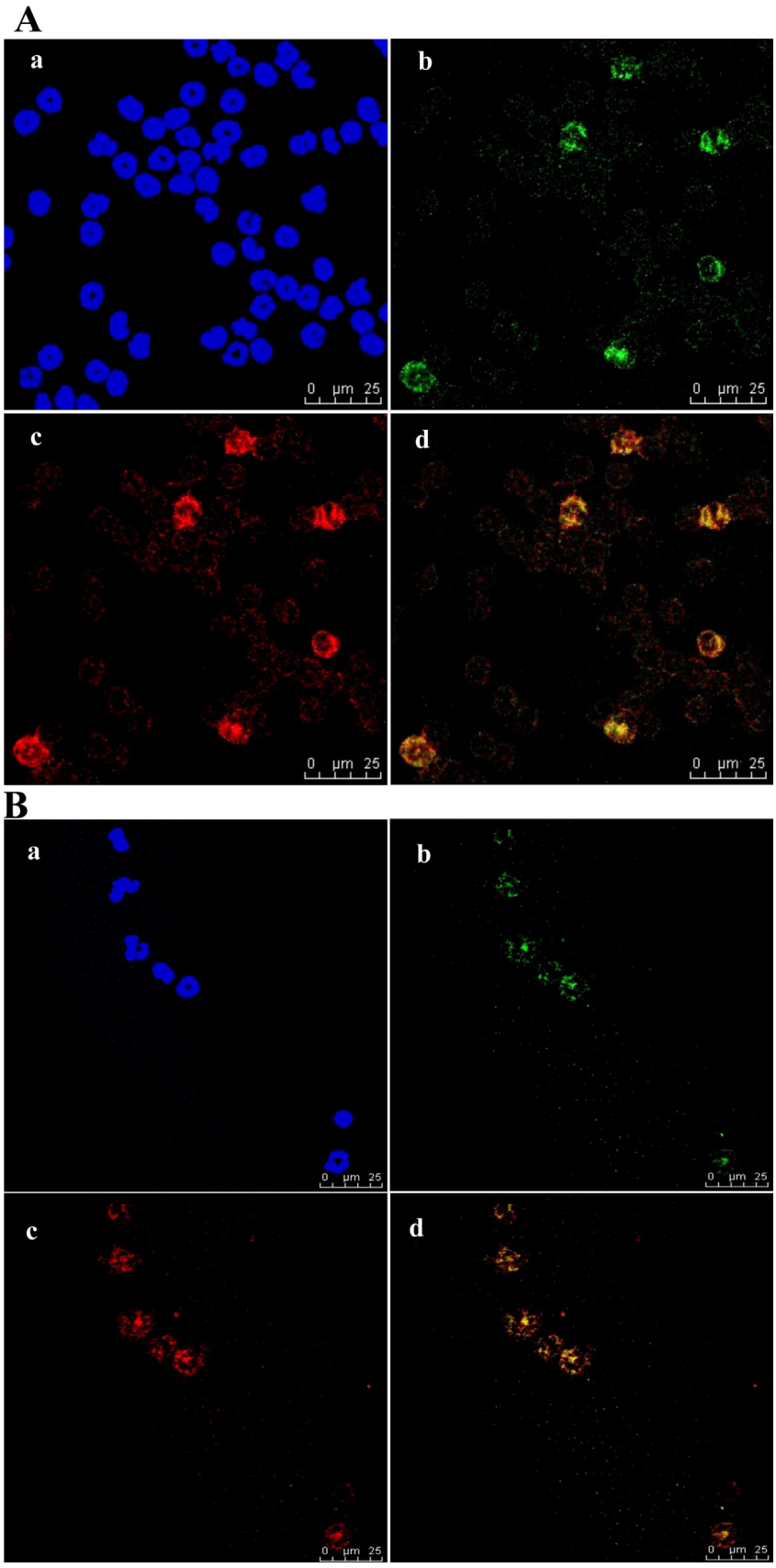

Figure 4: Expression profile of Actin and Vimentin in the TBM (A) and SP cells (B). Cells were stained for DAPI, rhodamine labeled phalloidin (for actin) and anti-vimentin antibodies as described in Materials and Methods, Sub-panels 'a,b and c' show DAPI, actin and vimentin staining respectively and sub-panel d shows merged fluorescence of actin and vimentin.

seen in the SP cells (corresponding panels in Figure 4B). The size of the SP cells is also smaller.

Down Regulated Proteins

a) Stress response proteins: Heat shock proteins and chaperonins, including members of the 60-kDa and 70-kDa heat shock protein families expressed in human $CD34^+$ stem/progenitor (Tao et al., 2005) and mouse embryonic stem (Nagano et al., 2005) cells, were also observed in the SP proteome. This indicates that SP cells possess the characteristics of cells under stress and their response to various environmental stress conditions. Son et al., (2005) have found HSPA8 protein to be markedly downregulated upon differentiation and proposed it as a novel cell-surface marker for undifferentiated human embryonic stem cells (Son et al., 2005). This was consistent withour findings of down regulation of HSP 8 (spot 15) and HSP 1 (spot 18) in SP proteome of mouse and to re-emphasize on stem cell like characteristics of SP cells.

b) ATP Synthase: Mitochondrial H^+-ATP synthase is required for cellular energy provision and for efficient execution of apoptosis. Its contribution in apoptosis could be mediated via ROS, produced during mitochondrial electron transport. The generation of ROS is a physiological process that determines the life span of cells and organisms (Feng et al., 2001). Mitochondria H^+-ATP synthase β subunit protein expression is down regulated in the SP cells. In this regard, and because of the coupling between mitochondrial respiration and oxidative phosphorylation, the down-regulation of the H^+-ATP synthase in SP cells would limit the flux of electrons down the respiratory chain, and therefore, the generation of the superoxide radical, a promoter of DNA damage and likely a signal for induction of the mitochondrial cell-death pathway. These findings have led us to suggest that repression of the bioenergetic function of mitochondria is a special strategy of the SP cells in order to ensure their perpetuation.

Similarity of Proteins Between SP cells and Other Stem Cells

In a recent proteomic study, the first of its kind, on rat mesenchymal stem cell differentiation induced by 5-azacytidine, 34 proteins in the pH range of 5-8 were identified (Ye et al., 2006) and that data is strikingly similar to the proteins seen in our analysis of mouse SP cells. Four proteins reported in this study namely vimentin, glucose regulated protein (GRP), Eno-1 and M2 pyruvate kinase are also seen in the mouse SP proteome. Some other proteins like ATP synthase β chain and calreticulin are expressed commonly in mouse SP cells and other types of stem cells. The similarity of proteins expressed in mouse SP cells and other stem cell differentiation associated proteins, is seen in various stem cell types such as embryonic stem cells (Nagano et al., 2005, Elliott et al., 2004, Van Hoof et al., 2006) hematopoietic stem cells (Tao et al., 2004) and neural stem cells (Hoffrogge et al., 2006). These studies support the possibility that SP cells could have stem cell like properties.

Proteins of SP Cells that Reflect their Stem Cell Like Properties

The stem cell niche consists of a specialized microenvironment that nurtures and regulates the stem cell pool. Recent studies have shown that oxygen can directly regulate the differentiation of stem/precursor cells, and may participate in the maintenance of stem cells in the stem cell niche. Notably, hematopoietic stem cells are concentrated in hypoxic areas and can be envisaged as a specialized cell-type, well adapted to an apparently hostile milieu where, nevertheless, they undergo self-renewal. Under hypoxic conditions, the differentiation of stem cells as well as precursor cells is inhibited and is shown to play a critical role in the maintenance and multi-lineage ability of the stem cell phenotype. As an adaptation to hypoxia, cells express increased amounts of glycolytic enzymes so as to adjust to the anaerobic respiration. Recent quantitative proteomic analysis of isolated bone marrow population based on cell surface markers Lin^- Sca^+Kit^+ (LSK) indicates that these cells express high levels of glycolytic proteins (Unwin et al., 2006).

Aldehyde dehydrogenase, Aldh2 (spot 27) a novel marker for stem cells, plays a critical role in their protection from alkylating agents. It is expressed highly in hematopoietic stem and progenitor cells (Pearce and Bonnet 2007, Storms et al., 1999). This enzyme plays a significant role in Retinoic acid metabolism known to be involved in diverse biological processes such as embryogenesis, growth and differentiation. This is known to be upregulated in various stem cell types but the expression levels of it in SP cells are not significantly high. The expression of this protein in SP cells re-emphasizes its stem cell like properties.

Enolase 1, alpha is a glycolytic enzyme that catalyzes conversion of 2-phosphoglyceric acid to phosphoenolpyruvic acid. Migration and differentiation of cells are associated with increased rates of glycolysis and glucose transport. It is a biochemical marker for epithelial stem cells and daughter cells. Eno1 was reported to be present in progenitor cells (Yin et al., 2005) and mouse ES cells (Nagano et al., 2005).

Rho GDP dissociation inhibitor (GDI) alpha (spot 32) is a negative regulator of Rho GTPases, which play important role in cell proliferation. Rho-GDI was shown to be down regulated at the onset of murine HSC differentiation (Eckfeldt et al., 2005) and its non-regulated expression in mouse ES cells (Nagano et al., 2005; Elliott et al., 2004) can be ascertained to progenitor cell phenotype.

ATP Binding Cassette transporter P-glycoprotein 2 (ABCG2) a MDR 1 family member, has been associated with normal and cancer stem cells in several reports (Seo et al., 2007; Gulati et al., 2008; Pascal et al., 2007). Although we did not find the ABCG2 protein specific spot in our gel analysis we did the immunocytochemical localization of this important stem cell marker on mouse TBM and SP cells. The comparison of the staining of sorted SP cells and TBMCs is shown in Figure 5. As can be clearly seen SP cells showed a stronger staining for the transporter protein than TBMCs indicating that SP cells in the mouse bone marrow overexpress this protein.

This, in addition to the observation that SP cells like stem cells modulate the production of ROS by up regulating the proteins in glycolytic pathway, is significant evidence for supporting the contention that SP cells have stem cell like properties.

Conclusions

In this study we have described the proteome of SP cells obtained from murine bone marrow and we have identified some

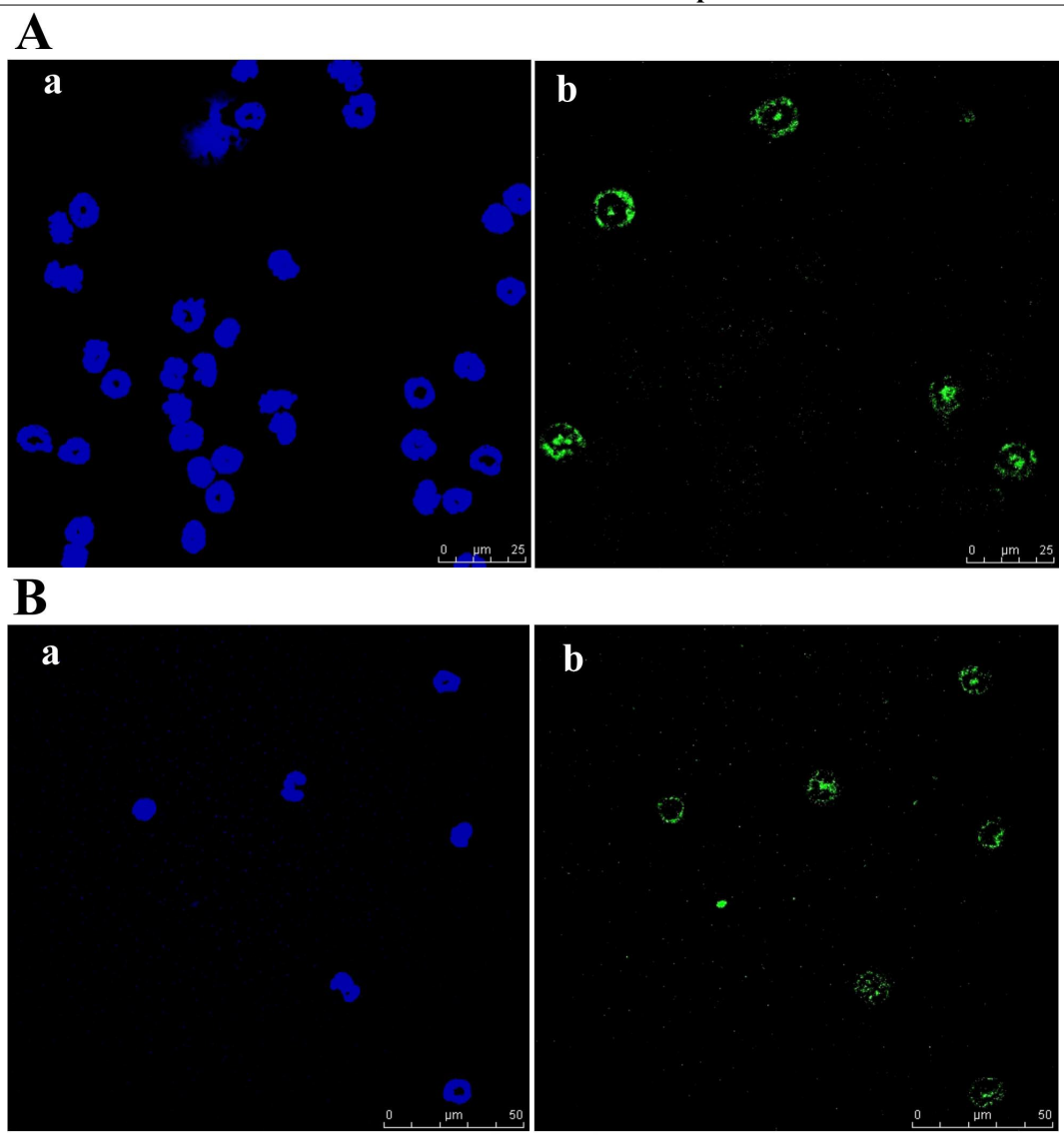

Figure 5: Expression of the transporter protein ABCG2 in the TBM (A) SP cells (B) Sub-panels 'a' and 'b' show DAPI and ABCG2 staining respectively.

of the proteins that could be involved in regulating the properties of self-renewal and directed differentiation of SP cells. This study also provides the first comprehensive analysis of the proteome of the mouse bone marrow. Thus, these data could enhance our understanding of the molecular mechanisms involved in the regulation of the stem cell like properties of SP cells in murine bone marrow.

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References

1. Baharvand H, Hajheidari M, Zonouzi R, Ashtiani SK, et al. (2006) Comparative proteomic analysis of mouse embryonic stem cells and neonatal-derived cardiomyocytes. Biochem Biophys Res Commun 349: 1041-1049.» CrossRef » Pubmed » Google Scholar
2. Baharvand H, Hajheidari M, Kazemi S, Ashtiani SK, Salekdeh GH (2006) Proteomic signature of human embryonic stem cells. Proteomics. 6: 3544-3549.» CrossRef » Pubmed
3. Baharvand H, Fathi A, van Hoof D, Salekdeh GH (2007) Concise review: trends in stem cell proteomics. Stem Cells 25: 1888-903.» CrossRef » Pubmed » Google Scholar
4. Cavusoglu N, Thierse D, Mohand-Said S, Chalmel F, Poch O, et al. (2003) Differential proteomic analysis of the mouse retina: the induction of crystallin proteins by retinal degeneration in the rd1 mouse. Mol Cell Proteomics 2: 494-505.» CrossRef » Pubmed » Google Scholar
5. Chen C, Lorimore SA, Evans CA, Whetton AD, Wright EG (2005) A proteomic analysis of murine bone marrow and its response to ionizing radiation. Proteomics 5: 4254-4263. » CrossRef » Pubmed » Google Scholar
6. Eckfeldt CE, Mendenhall EM, Flynn CM, Wang TF, Pickart MA (2005) Functional analysis of human hematopoietic stem cell gene expression using zebrafish. PLoS Biol 3: e254. » CrossRef » Pubmed » Google Scholar
7. Elliott ST, Crider DG, Garnham CP, Boheler KR, van Eyk JE (2004) Two-dimensional gel electrophoresis database of

murine R1 embryonic stem cells. Proteomics 4: 3813-3832. » CrossRef » Pubmed » Google Scholar

8. Epstein SP, Wolosin JM, Asbell PA (2005) P63 expression levels in side population and low light scattering ocular surface epithelial cells. Trans Am Ophthalmol Soc 103: 187-199. » CrossRef » Pubmed » Google Scholar

9. Feldmann Jr RE, Bieback K, Maurer MH, Kalenka A (2005) Stem cell proteomes: a profile of human mesenchymal stem cells derived from umbilical cord blood. Electrophoresis 26: 2749-2758. » CrossRef » Pubmed » Google Scholar

10. Feng J, Bussiere F, Hekimi S (2001) Mitochondrial electron transport is a key determinant of life span in *Caenorhabditis elegans*. Dev Cell 1: 633-644. » CrossRef » Pubmed » Google Scholar

11. Gharahdaghi F, Weinberg CR, Meagher DA, Imai BS, Mische SM (1999) Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. Electrophoresis. 20: 601-605. » CrossRef » Pubmed » Google Scholar

12. Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria M, et al. (1997) Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 3: 1337-1345. » CrossRef » Pubmed » Google Scholar

13. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183: 1797-1806. » CrossRef » Pubmed » Google Scholar

14. Gulati AS, Ochsner SA, Henning SJ (2008) Molecular properties of side population-sorted cells from mouse small intestine. Am. J Physiol Gastrointest Liver Physiol 294: 286-94. » CrossRef » Pubmed » Google Scholar

15. Hadnagya A, Gaboury L, Beaulieu R, Balicki D (2006) SP analysis may be used to identify cancer stem cell populations. Exp Cell Res 312: 3701-3710. » CrossRef » Pubmed » Google Scholar

16. Hayman MW, Przyborski SA (2004) Proteomic identification of biomarkers expressed by human pluripotent stem cells. Biochem Biophys Res Commun 316: 918-923. » CrossRef » Pubmed » Google Scholar

17. Hoffrogge R, Mikkat S, Scharf C, Beyer S, Christoph H, et al. (2006) 2-DE proteome analysis of a proliferating and differentiating human neuronal stem cell line (ReN cell VM). Proteomics 6: 1833-1847. » CrossRef » Pubmed » Google Scholar

18. Hoffrogge R, Beyer S, Hubner R, Mikkat S, Mix E, et al. (2007) 2-DE profiling of GDNF over expression-related proteome changes in differentiating ST14A rat progenitor cells. Proteomics 7: 33-46. » CrossRef » Pubmed » Google Scholar

19. Hoshi N, Kusakabe T, Taylor BJ, Kimura S (2007) Side population cells in the mouse thyroid exhibit stem/progenitor cell-like characteristics. Endocrinology148: 4251-4258. » CrossRef » Pubmed » Google Scholar

20. Herrera MB, Bruno S, Buttiglieri S, Tetta C, Gatti S, et al. (2006) Isolation and characterization of a stem cell population from adult human liver. Stem Cells 24: 2840-2850. » CrossRef » Pubmed » Google Scholar

21. Jonker JW, Freeman J, Bolscher E, Musters S, Alvi AJ, et al. (2005) Contribution of the ABC transporters Bcrp1 and Mdr1a/1b to the side population phenotype in mammary gland and bone marrow of mice. Stem Cells 23: 1059-1065. » CrossRef » Pubmed » Google Scholar

22. Kawanabe N, Murakami K, Takano-Yamamoto T (2006) The presence of ABCG2-dependent side population cells in human periodontal ligaments. Biochem. Biophys. Res Commun 344: 1278-1283. » CrossRef » Pubmed » Google Scholar

23. Kim SH, Jun S, Jang HS, Lim SK (2005) Identification of parathyroid hormone-regulated proteins in mouse bone marrow cells by proteomics. Biochem Biophys Res Commun 330: 423-429. » CrossRef » Pubmed » Google Scholar

24. Kurosaki H, Kazuki Y, Hiratsuka M, Inoue T (2007) A comparison study in the proteomic signatures of multipotent germline stem cells, embryonic stem cells, and germline stem cells. Biochem Biophys Res Commun 353: 259-267. » CrossRef » Pubmed » Google Scholar

25. Lee HK, Lee BH, Park SA, Kim CW (2006) The proteomic analysis of an adipocyte differentiated from human mesenchymal stem cells using two-dimensional gel electrophoresis. Proteomics 6: 1223-1229. » CrossRef » Pubmed » Google Scholar

26. Liadaki K, Kho AT, Sanoudou D, Schienda J, Flint A, et al. (2005) Side population cells isolated from different tissues share transcriptome signatures and express tissue-specific markers. Exp Cell Res 303: 360-374. » CrossRef » Pubmed » Google Scholar

27. Lin KK, Goodell MA (2006) Purification of hematopoietic stem cells using the side population. Methods Enzymol 420: 255-264. » CrossRef » Pubmed » Google Scholar

28. Macpherson H, Keir P, Webb S, Samuel K, Boyle S, et al. (2005) Bone marrow-derived SP cells can contribute to the respiratory tract of mice in vivo. J Cell Sci 118: 2441-2450. » CrossRef » Pubmed » Google Scholar

29. Mazurek S, Boschek CB, Hugo F, Eigenbrodt E (2005) Pyruvate kinase type M2 and its role in tumor growth and spreading. Semin Cancer Biol 15: 300-308. » CrossRef » Pubmed » Google Scholar

30. Nagano K, Taoka M, Yamauchi Y, Itagaki C (2005) Large-scale identification of proteins expressed in mouse embryonic stem cells. Proteomics 5:1346-1361. » CrossRef » Pubmed

31. Parmar K, Sauk-Schubert C, Burdick D, Handley M, Mauch P (2003) $Sca^{+}CD34^{-}$ murine side population cells are highly enriched for primitive stem cells. Exp Hematol 31: 244-250. » CrossRef » Pubmed » Google Scholar

32. Pascal LE, Oudes AJ, Petersen TW, Goo YA, Walashek LS, et al. (2007) Molecular and cellular characterization of ABCG2 in the prostate. BMC Urol 10: 6. » CrossRef » Pubmed » Google Scholar

33. Pearce DJ, Bonnet D (2007) The combined use of Hoechst efflux ability and aldehyde dehydrogenase activity to identify murine and human hematopoietic stem cells. Exp Hematol 35: 1437-46. » CrossRef » Pubmed » Google Scholar

34. Petersen BE, Bowen WC, Patrene KD, Mars WM (1999) Bone marrow as a potential source of hepatic oval cells. Science 284: 1168-70. » CrossRef » Pubmed » Google Scholar

35. Scharenberg CW, Harkey MA, Torok-Storb B (2002) The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood 99: 507-512. » CrossRef » Pubmed » Google Scholar

36. Seo DC, Sung JM, Cho HJH, Seo KH, Choi IS, et al. (2007) Expression profiling of cancer stem cell in human lung adenocarcinoma A549 cells. Mol Cancer 22: 75. » CrossRef » Pubmed » Google Scholar

37. Seshi B (2006) An integrated approach to mapping the proteome of the human bone marrow stromal cell. Proteomics 6: 5169-5182.» CrossRef » Pubmed » Google Scholar

38. Son YS, Park JH, Kang YK, Park JS, Choi HS, et al. (2005) Heat shock 70-kDa protein 8 isoform 1 is expressed on the surface of human embryonic stem cells and downregulated upon differentiation. Stem Cells 23: 1502-1513. » CrossRef » Pubmed » Google Scholar

39.Storms RW, Trujillo AP, Springer JB, Shah L, Colvin OM, et al. (1999) Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc Natl Acad Sci USA 96: 9118-23.» CrossRef » Pubmed » Google Scholar

40. Summer R, Kotton DN, Sun X, Ma B, Fitzsimmons K, et al. (2003) Side population cells and Bcrp1 expression in lung. Am. J. Physiol. Lung Cell Mol Physiol 285: L97-L104. » CrossRef » Pubmed » Google Scholar

41. Takegawa T, Fujii H, Miwa S (1983) Change of pyruvate kinase isozymes from M2- to L-type during development of the red cell. Br J Haematol 54: 467-474.» CrossRef » Pubmed » Google Scholar

42.Tao W, Wang M, Voss ED, Cocklin RR, Smith JA, et al. (2004) Comparative proteomic analysis of human CD34+ stem/progenitor cells and mature CD15+ myeloid cells. Stem Cells 22: 1003-1014. » CrossRef » Pubmed » Google Scholar

43.Triel C, Vestergaard ME, Bolund L, Jensen TG, Jensen UB, et al. (2004) Side population cells in human and mouse epidermis lack stem cell characteristics. Exp Cell Res 295: 79-90.» CrossRef » Pubmed » Google Scholar

44. Unwin RD, Gaskell SJ, Evans CA, Whetton AD (2003) The potential for proteomic definition of stem cell populations. Exp Hematol 31: 1147-1159.» CrossRef » Pubmed » Google Scholar

45. Unwin RD, Smith DL, Blinco D, Wilson CL (2006) Quantitative proteomics reveals posttranslational control as a regulatory factor in primary hematopoietic stem cells. Blood 107: 4687-4694. » CrossRef » Pubmed » Google Scholar

46. Van Hoof D, Mummery CL, Heck AJ, Krijgsveld J (2006a) Embryonic stem cell proteomics. Expert Rev Proteomics 3: 427-437.

47. Van Hoof D, Passier R, Ward-Van Oostwaard D, Pinkse MW, et al. (2006b) A quest for human and mouse embryonic stem cell-specific proteins. Mol Cell Proteomics 5: 1261-73. » CrossRef » Pubmed » Google Scholar

48. Wang D, Gao L (2005) Proteomic analysis of neural differentiation of mouse embryonic stem cells. Proteomics 5: 4414-4426.» CrossRef » Pubmed » Google Scholar

49. Ye NS, Chen J, Luo GA, Zhang RL (2006) Proteomic profiling of rat bone marrow mesenchymal stem cells induced by 5-azacytidine. Stem Cells Dev 15: 665-676. » CrossRef » Pubmed » Google Scholar

50. Yin X, Mayr M, Xiao Q, Mayr U, et al. (2005) Proteomic dataset of Sca-1+ progenitor cells. Proteomics 5: 4533-4545. » CrossRef » Pubmed » Google Scholar

51. Yourek G, Hussain MA, Mao JJ (2007) Cytoskeletal changes of mesenchymal stem cells during differentiation. ASAIO J 53: 219-28.» CrossRef » Pubmed » Google Scholar

52. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, et al. (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 7: 1028-1034. » CrossRef » Pubmed » Google Scholar

53. Zhou S, Morris JJ, Barnes Y, Lan L, Schuett JD, et al. (2002) Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. Proc Natl Acad Sci USA 99: 12339-12344. » CrossRef » Pubmed » Google Scholar