Biochemical analysis and quantification of hematopoietic stem cells by infrared spectroscopy

Udi Zelig
Ben-Gurion University of the Negev
Department of Biomedical Engineering
Beer-Sheva 84105 Israel

Ziv Dror
Soroka University Medical Center
Department of Pediatric Hemato-Oncology
Beer-Sheva 84101 Israel

Svetlana Iskovich
Schneider Children's Medical Center
Department of Pediatric Hematology-Oncology
Center for Stem Cell Research
Petach Tikva 49202 Israel

Amir Zwielly
Ben-Gurion University of the Negev
Department of Physics
Beer-Sheva 84105 Israel

Miri Ben-Harush
Soroka University Medical Center
Department of Pediatric Hemato-Oncology
Beer-Sheva 84101 Israel

Ilana Nathan
Ben-Gurion University of the Negev
Department of Clinical Biochemistry
Beer-Sheva 84105 Israel
   and
Soroka University Medical Center
Institute of Hematology
Beer-Sheva 84101 Israel

Shaul Mordechai
Ben-Gurion University of the Negev
Department of Physics
   and
Cancer Research Center
Beer-Sheva 84105 Israel

Joseph Kapelushnik
Soroka University Medical Center
Department of Pediatric Hemato-Oncology
Beer-Sheva 84101 Israel

Abstract. Identification of hematopoietic stem cells (HSCs) in different stages of maturation is one of the major issues in stem cell research and bone marrow (BM) transplantation. Each stage of maturation of HSCs is characterized by a series of distinct glycoproteins present on the cell plasma membrane surface, named a cluster of differentiation (CD). Currently, complicated and expensive procedures based on CD expression are needed for identification and isolation of HSCs. This method is under dispute, since the correct markers' composition is not strictly clear, thus there is need for a better method for stem cell characterization. In the present study, Fourier transform infrared (FTIR) spectroscopy is employed as a novel optical method for identification and characterization of HSCs based on their entire biochemical features. FTIR spectral analysis of isolated mice HSCs reveals several spectral markers related to lipids, nucleic acids, and carbohydrates, which distinguish HSCs from BM cells. The unique "open" conformation of HSC DNA as identified by FTIR is exploited for HSCs quantification in the BM. The proposed method of FTIR spectroscopy for HSC identification and quantification can contribute to stem cell research and BM transplantation. © *2010 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.3442728]

1 Introduction

Hematopoietic stem cells (HSCs) are characterized by the ability to self-renew, differentiate into various lineage-committed cells, and sustain hematopoiesis over the lifetime of an animal.[1] In the stem cell research discipline, accuracy in characterizing HSCs or other kinds of stem cells is a primary goal and has immense implications on experiment analysis and interpretations.

Traditionally, isolation of human hematopoietic stem cells for therapeutic and research purposes is based mainly on expression of cell surface markers such as CD34.[2–4] However, both subsets of human CD34$^+$ and CD34$^-$ cells have demon-

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Address all correspondence to: Shaul Mordechai, Dept. of Physics and the Cancer Research Center, Ben Gurion University, Beer Sheva, 84105, Israel. Tel: +972-8-646 1749; Fax: +972-8-647 2924. E-mail: shaulm@bgu.ac.il

strated long-term repopulation ability *in vivo*.[5–7] Moreover, the expression of CD34 appeared to be reversible (from CD34$^+$ to CD34$^-$) in murine and human cells.[8,9] The heterogeneity of cell phenotypes possessing repopulating potential,[6,7,10,11] the variability of cell surface markers between species, and the dissociation of cell phenotype and reconstituting capacity impose difficulties in identification of the primitive bone marrow stem cells.[12,13] The alternative approach to isolation of the bone marrow-derived stem cell subset is based on the functional and physical properties of the cells.[11,14,15]

There are several common assays to evaluate stem cell capabilities for self-renewal and differentiation following stem cell isolation—repopulation in *in vivo* experiments in mice, xenogeneic transplantation using severe combined immunodeficient mice for identifying human engrafting cells,[16] long-term culture initiating cell (LTC-IC) assay for quantification of long-term culture initiating cells,[17] myeloid-lymphoid initiation cell assay for assessment of multipotent of human progenitors, etc.[18] All these methods for identification, verification, and quantification of HSCs as described before are complicated, time consuming, expensive, and demand highly skilled manpower. Thus, there is need for a simple biochemical method that can identify and quantify stem cells with a high degree of certainty for research and clinical use.

Over the last decade, Fourier transform infrared (FTIR) spectroscopy has been established as a simple and rapid clinical diagnostic method, since it offers important clues regarding changes in macromolecules, such as lipids, proteins, carbohydrates and nucleic acids, in a sample.[19] Each cell type can be recognized based on its individual spectral signature, which is derived from its characteristic biochemical composition and structure. In the present research, we investigated the biochemical characteristics of HSCs using FTIR spectral analysis. In addition, we evaluated its applicability for quantifying HSCs in bone marrow (BM) following isolation according to a novel approach based on HSC functional and physical properties (the smallest population of murine nucleated bone marrow cells Fr25lin$^-$).

2 Materials and Methods

2.1 Animals

Mice used in this study were C57BL/6J and B.6SJL/Boy strains purchased from Jackson Laboratory (Bar Harbor, Maine). All experimental animals were housed in a conventional clean facility using barrier mouse housing facilities [Tecniplast (Exton, Pennsylvania) individually ventilated chambers], and given *ad-libitum* access to autoclaved mouse chow and acidified water.

2.2 Isolation of Fr25lin$^-$ Cells

Whole bone marrow cells were harvested by flushing medullar cavities of femurs, tibias, and iliac bones. Single cell suspensions of 5 to 10×10^8 wBMC from ten mice were loaded into the chamber of a counterflow centrifuge (Beckman Instruments, Palo Alto, California) at a flow rate of 15 ml/min with a constant rotor speed of 3000 rpm at RT. The smallest subset of nucleated cells (Fr25) were collected at a rate of 25 ml/min in 200 ml. Lineage-positive cells, including T and B lymphocytes, macrophages, granulocytes, and

erythroid cells, were immunomagnetically depleted. The Fr25 cells were incubated for 30 min at 4 °C with rat antimouse monoclonal antibodies (mAb) against GR-1 (RB6-8C5), Mac-1 (M1/70), B220 (RA3-3A1/6.1), and CD-5 (53-7.3) extracted from hybridoma cell lines (ATCC, Rockville, Maryland), and purified Ter-119 (eBioscience Incorporated, San Diego, California). Antibody-coated cells were incubated with sheep antirat magnetic beads ($4 \times 10^7/10^7$ cells) (Dynal Biotech ASA, Oslo, Norway) at 4 °C for 15 min. Rosetted cells were precipitated by exposure to a magnetic field, and the supernatant containing lineage-negative cells was collected. The isolated cells were evaluated for viability by the trypan blue exclusion test and evaluated for size characteristics and expression of hematopoietic lineage markers using a Vantage SE flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey).

2.3 Fourier Transform Infrared Microscopy Measurements and Data Analysis

1.5 μl of packed cells were deposited on a zinc selenide (ZnSe) slide to form an approximate monolayer of cells, and then air dried for 15 min under laminar flow to remove water. Measurements of selected microscopic sites with an aperture of 100 μm and similar ADC rates were performed using the FTIR microscope IR scope 2 (Bruker Optik GmbH, Ettlingen, Germany) in transmission mode as described previously.[20] FTIR microscopy (MSP) was chosen as a favorable methodology for this study, since other systems such as FTIR-attended total reflectance (ATR) which can measure cells in their native culture media, are limited in their sensitivity due to water interference, restricted penetration depth of the evanescent waves into the sample (about 3 to 4 μm), and higher contribution from scattering effects compared to FTIR-MSP.[21]

The spectra were baseline corrected using a polynomial rubber band with 64 points (OPUS software Bruker Optik GmbH, Ettlingen, Germany) and were vector normalized in the region 700 to 4000 cm^{-1} or min-max normalized in the region of 700 to 1800 cm^{-1}. To obtain precise absorbance values at a given wave number with minimal background interference, the second derivative spectra were used to determine intensities of the biomolecules of interest. The second derivative method is highly susceptible to changes in full width at half maximum (FWHM) of the IR bands. However, in the case of biological samples, all cells from the same type are composed of similar basic components, which give relatively broad bands. Thus, there is a possibility of neglecting the changes in the band FWHM.[22]

2.4 Statistics

Data are presented as the mean $\pm$ standard error (SEM) derived from at least three independent experiments. Statistical analysis was performed using the student t-test. *P*-values <0.05 were considered significant. Linear regressions were done by the least-squares method, and the data analysis package (Origin, Micro-Cal, Incorporated, Northampton, Massachusetts) was used when comparing a series of datasets. Data reduction by principal component analysis (PCA) was implemented as previously described.[23]
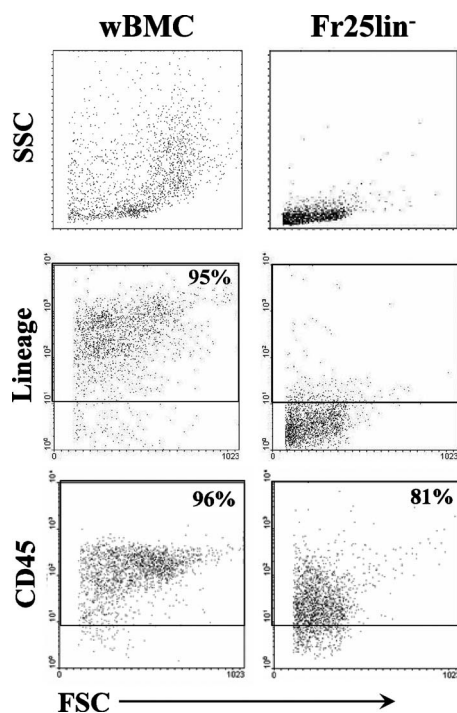
wBMC **Fr25lin⁻**

Fig. 1 Evaluation of cell size and expression of hematopoietic markers in wBMC and Fr25lin⁻ cells. Whole bone marrow (wBMC) and elutriated lineage-depleted Fr25 (Fr25lin⁻) cells were stained with a cocktail of monoclonal antibodies against hematopoietic lineage markers (GR-1, Mac-1, B220, Ter-119, and CD5) and CD45.

3 Results

3.1 Characterization of Hematopoietic Stem Cells by Flow Cytometry

To obtain fractions of bone marrow cells enriched in primitive stem cells, we adopted a technique of counterflow centrifugal elutriation.[24] The elutriated fraction collected at a flow rate of 25 ml/min was immunomagnetically depleted of cells expressing hematopoietic lineage markers using monoclonal antibodies against granulocytes, macrophages, T and B lymphocytes, and erythroid cell-specific antigens. The size characteristics of wBMC and Fr25lin⁻ cells as determined by flow cytometric analysis of side and forward scatter parameters are demonstrated in Fig. 1. Fr25lin⁻ cells represented the smallest fraction of the unfractionated wBMC population. Bone marrow is a heterogeneous population containing both hematopoietic and nonhematopoietic cells. To evaluate the hematopoietic contents of the Fr25lin⁻ subset, expression of the pan-hematopoietic marker CD45 was examined and compared to whole BMC. Both BMC and Fr25lin⁻ cells were composed of a major hematopoietic population (96 and 81%, respectively).

3.2 Fourier Transform Infrared Microscopy Analysis of Hematopoietic Stem Cells

Biochemical analysis of extracted HSCs and comparison to wBMC was conducted using FTIR-MSP. FTIR-MSP spectra of wBMC and HSCs in the regions 3000 to 2800 cm⁻¹ and 1800 to 700 cm⁻¹ after baseline correction and min-max normalization to amide II are shown in Fig. 2. The spectra exhibit significant differences between wBMC cells and HSCs at several wave numbers; each corresponds to specific functional groups related to fundamental cellular components such as lipids, proteins, and carbohydrates/nucleic acids.

The region 3000 to 2800 cm⁻¹ contains absorbance bands due to symmetric and asymmetric CH₃ and CH₂ stretching vibrations related mainly to proteins and lipids, respectively.[25] The absorbance ratio of methylene to methyl bands quantified by the bands integrated absorbance at 2960 and 2873 cm⁻¹ divided by 2922 and 2852 cm⁻¹ was higher for the HSCs than for BM ($p < 0.05$). Although the parameter of lipids/proteins is significant for characterizing HSCs, the difference between HSCs and BM cells is quite small, thus, there is a need for a better biochemical parameter.

The phosphate bond absorption at ∼1237 cm⁻¹ (asymmetric vibration) and 1088 cm⁻¹ (symmetric vibration) is more prominent in the HSCs spectra than in the wBMC spectra, as presented in Figs. 2(a) and 2(b). These bands are related mainly to nucleic acids and carbohydrates.[25,26] Another band that is more prominent in HSCs is located at 966 cm⁻¹ and corresponds to C–C/C–O stretching of deoxyribose-ribose skeletal vibration of the DNA.[27]

3.3 Principal Component Analysis and Statistical Analysis of Fourier Transform Infrared Microscopy Spectra

To determine the main spectral characteristics of HSCs and BM cells, unbiased PCA analysis was conducted. The FTIR-MSP spectra are composed of 286 vector components corresponding to absorbance intensities. PCA analysis reduces the number of variables to six principle components (PCs).[24] The best separation between HSC and BM cell spectra was achieved by PC4 versus PC1, as presented in Fig. 3(a). PC1 adequately separates between the two groups of cells, while PC4 provides the heterogeneity of each one of the individual groups. According to PC4, HSCs have less heterogeneity than the total BM cell population. The loading of each PC reveals the main vibrational bands for distinction between HSCs and BM cell spectra [Fig. 3(b)]. As observed in Fig. 3(b), there are several major bands contained in the PC1 loadings such as 966, 1088, and 1240 cm⁻¹, which correspond to nucleic acids/carbohydrates, and the ∼1678 cm⁻¹ band, which corresponds to untiparallel β-sheets of proteins and to C=O stretching vibrations that are hydrogen bonded.[25] PC4 loadings show a different set of bands [Fig. 3(b)] as expected. The later contributes mainly to the heterogeneity of both HSCs and BM cells.

The bands that give the finest distinction according to the PCA analysis correspond mostly to the DNA. A student t-test was employed to determine the ability of these bands to serve as markers for identifying HSCs in the BM using FTIR-MSP, as presented in Fig. 3(b). To verify that these biomarkers correspond to DNA, an additional characteristic band of DNA at 780 cm⁻¹ was also tested and was found to be appropriate for characterizing HSCs [Figure 3(c)].[27]

3.4 Quantification of Hematopoietic Stem Cells by Fourier Transform Infrared Microscopy

To evaluate the utility of FTIR for quantifying the number of HSCs from wBMC (which also contain $0.3 \pm 0.1\%$ of
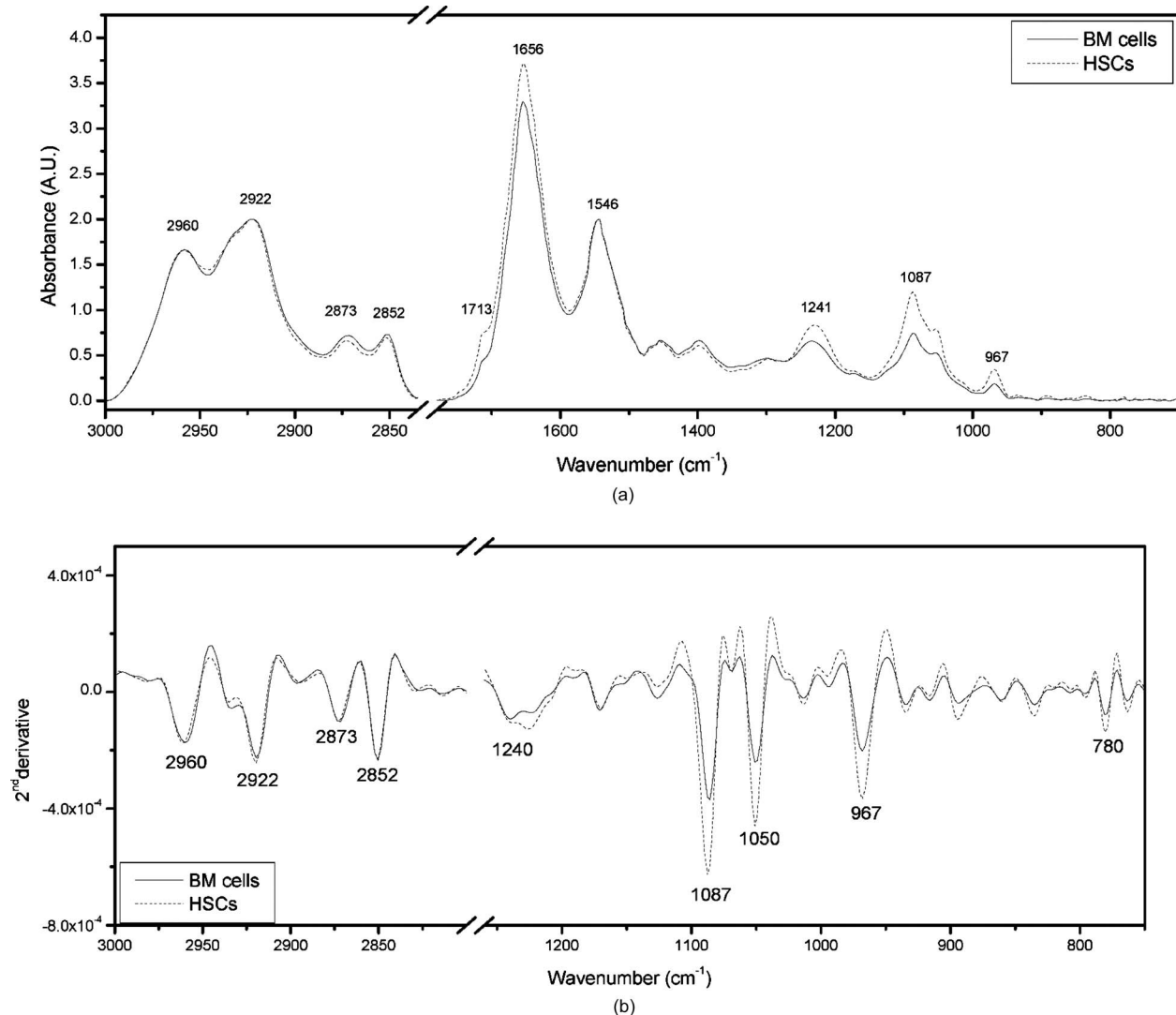
Fig. 2 Fourier transform infrared (FTIR) spectra of bone marrow (BM) cells and hematopoietic stem cells (HSCs). (a) Representative FTIR spectra exhibit significant differences between BM cells and HSCs after baseline correction and amide-II normalization. Each spectrum represents the average of five measurements at different sites for each sample. The main absorbance bands are marked. (b) Second derivative extended spectra of BM cells and HSCs after baseline correction and vector normalization.

HSCs)[11] based on the characteristic biomarkers of HSCs, we aimed to determine the minimal detectable percentage of HSCs in the BM. For this goal, HSCs were diluted with BM in decreasing percentages and measured by FTIR. Several wave numbers (1088, 966, and 780 cm^{-1}), which were found to be the most significant markers to characterize HSCs, were used in determining the maximal sensitivity of FTIR for HSCs quantification.

Figure 4 shows the characteristic spectra obtained with different concentrations of HSCs diluted in BM cells, and a correlation curve between HSC percentages in the BM and the respective absorbance at 966 cm^{-1} [Fig. 4(b)]. To evaluate the utility of quantifying HSCs in the BM, we calculated a linear regression curve for each single experiment, excluding errors due to the complicated cell extraction procedure. A good correlation was achieved ($R^2 > 0.98$). Using this spectral parameter, we were able to differentiate ($p < 0.05$) between samples diluted up to approximately 0.1% HSCs compared with pure BM samples. However, due to the complexity of this experiment, the exact sensitivity and accuracy limits of FTIR-MSP in detection of HSCs in BM should be further investigated.

4 Discussion

In the present study, we demonstrated the utility of FTIR spectroscopy for distinguishing HSCs from other BM cells according to differences in structure and quantity of several major biomolecules such as lipids, nucleic acids, and carbohydrates (Figs. 2 and 3). Based on these characteristic biochemical features of HSCs, we present FTIR as a practical tool for detection of HSCs in the BM (Fig. 4) for clinical and research applications.

Isolation of HSCs, as performed in this study, is based on multiple characteristics of the stem cells. Using physiological features of the cells, such as size and granularity, we separated the smallest population of nucleated bone marrow cells. The
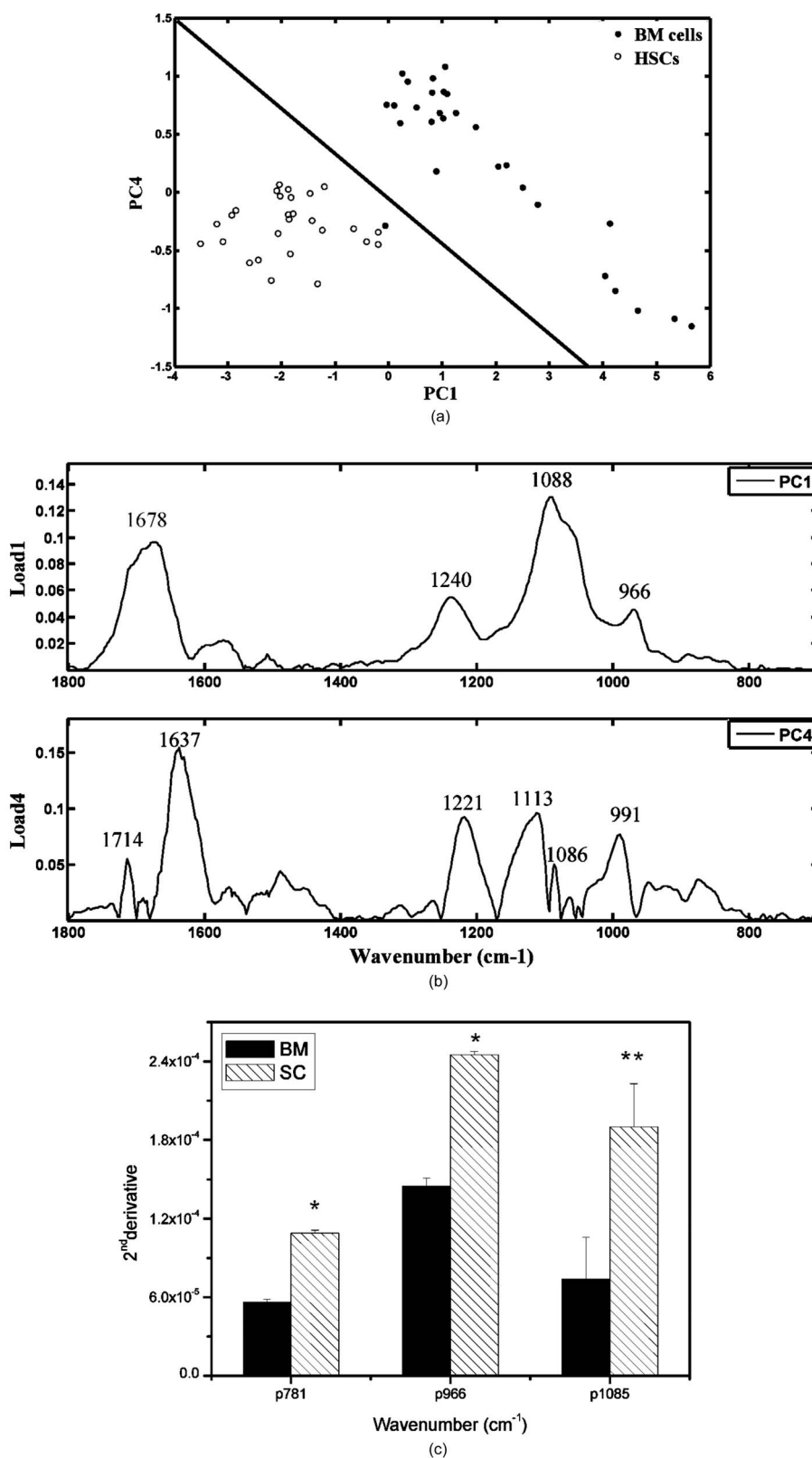
Fig. 3 Statistical analysis of FTIR-MSP spectra. (a) PCA was employed on FTIR-MSP spectra composed of 286 vector components, reducing it to six PCs, which describe 98.1% of the data variance. PC4 versus PC1 was found to give the best separation between HSCs and BM cell spectra. (b) Absolute loading values of PC1 and PC4 in the region 700 to 1800 cm^{-1}. (c) The absorbance bands at 781, 966, and 1085-cm^{-1}, which are mainly related to DNA, are more prominent in the HSCs spectra than in the BM spectra. Values plotted are the means ±SEM derived from at least three independent experiments. ($^{*}P < 0.001$, $^{**}P < 0.05$).
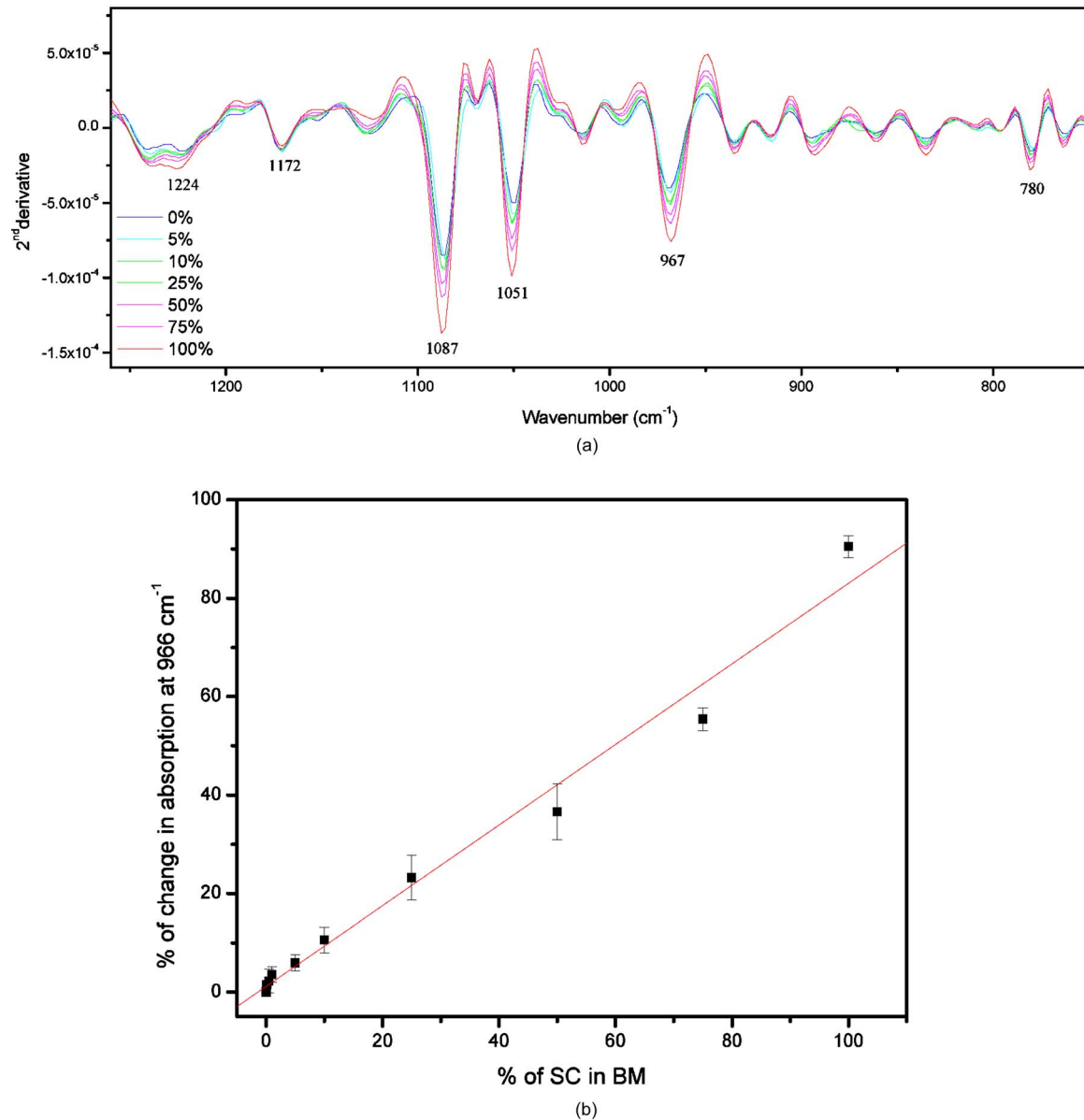
Fig. 4 Quantification of HSCs in whole BM cell populations by FTIR-MSP. Isolated HSCs were diluted (from 0 to 100% HSCs) in whole BM cell population. (a) Second derivative extended representative spectra of HSCs and BM cells after baseline correction and vector normalization. (b) A linear correlation between the absorbance band of the DNA at 967 cm^{-1} and the percentage of HSCs in BM cell population. Values plotted are the means ±SEM derived from at least three independent experiments.

next step of the isolation procedure relies on the exclusion of cells expressing characteristic lineage CDs rather than searching for CDs characterizing the HSCs themselves, as used in the common methods. As presented in Fig. 1, the isolated stem cell subset was characterized by their small size and primarily comprises the $CD45^+$ hematopoietic cells that did not express lineage markers. This cell population (Fr25lin$^-$) was previously shown to repopulate the hematopoietic system of myeloablative mice in the long term.[24]

Earlier FTIR spectroscopy studies on various types of stem cells reported a higher absorbance of nucleic acids in comparison to mature cells.[28,29] On the contrary, other groups reported an opposite trend of nucleic acid absorbance when immature leukemic cell lines were treated with all-transretinoic acid to promote differentiation.[30] These findings indicate a unique chromatin structure of stem cells related to their fundamental biological function.

Changes in chromatin structure are an important factor responsible for stem cell self-renewal, differentiation capability, and other functions associated with gene expression. Thus, the fate of a stem cell and the entire hematopoietic lineage is determined by chromatin remodeling.[31–33] DNA enzymes can influence HSC chromatin structure and histone modifications. For instance, the activity of DNA methyltransferase is known to be essential for HSCs self-renewal.[34] Recent research revealed that inhibition of histone deacetylase, which is responsible for "open" or "rigid" chromatin structure, enhances proliferation and self-renewal of HSCs.[35] Chromatin structure is

also affected by transcription activity, which is assumed to be elevated following an increase of mRNA found in HSCs.[36]

An increase in mRNA of embryonic stem cells following differentiation was found by Raman spectroscopy analysis, which indicates high transcription activity for stem cells.[37,38] In our results we did not find a significant difference between the absorption bands related to RNA in the spectra of HSCs and BM cells. Thus, it might indicate high mRNA levels of cells in both BM and HSCs. However, additional study is needed to confirm this issue using other standard biochemical methods.

The higher DNA absorbance in HSCs in comparison to BM cells, as revealed in our study, can imply abnormal cell cycle activity.[39] However, it is well known that most of the HSCs in the BM remain quiescent and only a limited number enter the cell cycle, thus they are unlikely to affect the FTIR spectra.[40] Other possible sources for this increase in IR "visible" DNA structure might designate less tight DNA conformation,[41] which can point for a highly and unique transcription activity of the HSCs, although most of them are in their quiescence state.[15,42,43] Thus, our results support the other findings, which indicted a unique chromatin conformation in HSCs.[31–33]

Additional absorption bands characteristic to HSCs over other BM cells are CH_3/CH_2, which correspond to total cellular lipids. Phospholipids, proteins, and other plasma membrane components participate in all cellular functions. HSCs can be recognized by specific glycoproteins (CDs) present on their membranes' surface, which affects plasma membrane characteristics. One such glycoprotein, for instance, is CD133 (Prominin-1), which is a common marker for HSCs as well as other SCs and cancer cells.[44,45] CD133 interacts with membrane cholesterol and is specific to cholesterol in membrane rafts. It is also assumed to affect membrane lipid composition and organization.[46] All these facts indicate an exclusive membrane composition and structure of HSCs, as confirmed by our results from FTIR spectral analysis. The higher CH_3/CH_2 ratio in HSCs relative to BM cells presented in this study was found by our group to indicate leukemia and other malignancies.[47] Thus we point out here a possible similarity between stemness and malignancy, which demands further study on membrane structure and composition.

In addition to the ability to characterize HSCs, FTIR can also be used for quantification of HSCs in the BM according to the individual biochemical profile as revealed earlier. The DNA spectral region was found by advanced, unbiased PCA statistical analysis (Fig. 3) as the most profound region suitable for basic quantification and achieved satisfying sensitivity. Our analysis indicates that one HSC per thousand BM cells can be identified (Fig. 4). Similar analysis was also carried out for the unique DNA absorbance band at 780 cm^{-1} and gave similar results (data not shown).

Thus, we suggest FTIR as a simple method for characterization of stem cells from the biochemistry level using a small amount of dried intact cells, in addition to the opportunity of quantification of stem cells in a sample (after calibration with 100% stem cells of the desired type). This test can be implemented as a supplementary tool to present existing standard methodologies for stem cell analysis for research and medical applications.

5 Conclusions

FTIR spectroscopy can supply valuable information regarding the unique biochemical composition and structure of HSCs. These spectral patterns of lipids, nucleic acids, and carbohydrates can arise from the HSC function in comparison to other cells in the BM. Furthermore, we show here a possible practical use of FTIR for identifying and quantifying HSCs in the BM prior to, or following, cell isolation, thus verifying the purity of cell extraction. Still, further research on other cell types in the hematopoietic lineage is needed to learn more about hematopoiesis from the biochemical viewpoint, and expand the applications of FTIR spectroscopy in biology and medicine.

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