Identification, isolation, quantification and systems approach towards CD34, a biomarker present in the progenitor/stem cells from diverse lineages

Sudheer Shenoy P. *,1, Bipasha Bose *,1

Department of Stem Cell and Regenerative Medicine, Yenepoya Research Center, Yenepoya University, University Road, Mangalore 575018, Karnataka, India

Abstract

Mesenchymal stem cells (MSCs) constitute the diverse progenitor populations in almost every tissue and are of immense importance in the field of regenerative medicine. CD34 is a cell surface glycoprotein identified first as a marker for the MSCs of hematopoietic origin. CD34 is now known to be expressed in cells of diverse lineages (tissues of non-hematopoietic origin) such as ectoderm, mesoderm and endoderm and is considered as a general marker for progenitor cells. Here, we present detailed protocols to obtain pure populations of MSCs from three diverse lineages such as skeletal muscle, skin, and liver from mouse tissues. We also present here the protocol for systems biology approach (proteomic analysis) of these purified cells. This proteomic approach can elucidate key signalling pathways and proteins utilized by these CD34 positive cells in undifferentiated and differentiated conditions. Furthermore in-depth proteomic analysis can also identify the altered proteome which is responsible for their function during non-clinical and clinical conditions.

Keywords:
Mesenchymal stem cells
Skin
Skeletal muscle
Liver
CD34+ cells
Isolation
Identification
Quantification
Proteomics
Mass spectrometry

© 2017 Elsevier Inc. All rights reserved.

1. Introduction

Mesenchymal stem cells (MSCs) are derived exclusively from mesenchyme, the embryonic connective tissue of mesodermal origin [1,2]. However, the name MSCs has also been interchangeably used for stromal cells of mesodermal origin. Such stromal cells primarily support the epithelial tubes, sacs or tissues. Diverse cell types given rise by the mesenchyme include cartilage, smooth muscle, pericytes, mesothelium, and fibroblasts. Bone marrow-derived MSC (BM-MSC) is a classical example [3]. BM-MSCs are currently also being used for treating serious problems like spinal cord injury [4]. Other types of MSCs, which have gained impetus in research and, up to a limited extent in clinical trials/applications are adipose tissue-derived MSC [5,6], Wharton jelly MSCs from cord blood [7], umbilical cord-derived MSCs [8] dental pulp MSC [9], MSC from nasal polyps [10], skeletal muscle-derived MSCs [11], amniotic fluid MSCs [12]. MSCs have been broadly identified by cell surface marker expression profile and have been known for simultaneous expression of the markers like CD34, CD44, CD105, CD90, CD73 [13,14].

One of the important markers expressed on various kinds of MSCs is CD34 [13,15]. CD34 is a transmembrane glycoprotein. Clinically, CD34 is associated with the selection and enrichment of MSCs from bone marrow commonly offered as a therapy for leukaemia patients [16,17]. Due to these historical and clinical associations, it is a common misconception that CD34+ cells in non-hematopoietic samples represent hematopoietic contamination. Regarding the tissue distribution, cells expressing CD34 are normally found in the umbilical cord and bone marrow as hematopoietic cells, a subset of mesenchymal stem cells, endothelial progenitor cells, endothelial cells of blood vessels but not lymphatic’s (except pleural lymphatics), mast cells, a sub-population dendritic cells (which are factor XIIIa-negative) in the interstitium and around the adnexa of dermis of skin, as well as cells in soft tissue tumors like dermatofibrosarcoma protuberans (DFSP), gastrointestinal stromal tumor (GIST), solitary fibrous tumor (SFT), hemangiopericytoma (HPC), and to some degree in Malignant peripheral nerve sheath tumor MPNSTs, cells of haematological malignancies [18,19] and leukemic stem cells [20].

* Corresponding authors.
E-mail addresses: shenoy@yeneponya.edu.in, shenoy2000@yahoo.com (P. Sudheer Shenoy), Bipasha.bose@yeneponya.edu.in, Bipasha.bose@gmail.com (B. Bose).

1 Equally contributing.
non-hematopoietic cell types including muscle satellite cells [21], muscle-derived stem cells [22], adipose tissue [23], corneal keratocytes [24], interstitial cells [25], epithelial progenitors [26], and vascular endothelial progenitors [27] also express CD34. The presence of CD34 on non-hematopoietic cells in various tissues has been linked to progenitor and adult stem cell phenotypes [28]. Another marker CD45, classically known as leucocyte common antigen (LCA) is a protein tyrosine phosphatase receptor type C. CD45 is associated with the surface of all differentiated hematopoietic cells including HSCs, leucocytes and osteoclasts of hematopoietic origin [29,30]. MSCs, as adult stem cells have gained applications in biotechnology and medicine because of their ease in harvesting, expansion in culture without any loss of stemness and tissue acceptance, due to the lack of expression of HLA-DR [31]. Accordingly, ethical issues pertaining the research and use of MSCs are considerably less.

Systems biology or proteomics approach is the study of proteins in large scale which involves mass spectrometers (MS). This MS based proteomics has gained popularity since last ten years due to its speed, sensitivity and ability to simultaneously detect multiple targets/interacting proteins. The new generation MS can identify and quantify thousands of proteins which include low expressing, along with their post translational modifications and relevant signalling pathways accurately. It has now become easy to identify common protein markers expressed by MSCs as a large scale screening tool (mass spectrometry) and also to understand protein dynamics in these MSCs. Proteomic analysis of MSCs will give an insight to a set of proteins such as surface markers or receptors, signalling molecules, extracellular matrix, secreted growth factors, expression of genes (transcription factors) of undifferentiated and differentiated cells and proteins involved in cellular stress and mitochondrial damage [32]. Moreover, conventional molecular approaches such as qRT-PCR gene expression analyses, western blot and immunofluorescence are mandatory to validate the proteomics data before reaching any conclusion.

Till now, as per the best of our knowledge, there are no reports of isolation of CD34+ cells from tissues of ectodermal, mesoderm and endodermal origins. Accordingly, here we describe a protocol to isolate and characterise mouse CD34+ cells from all three germ layers. We have designed this protocol in our laboratory that is being currently used in an ongoing study encompassing the cellular, molecular and functional similarities/differences between CD34+ cells from all three germ lines.

2. Materials

2.1. Cell line, media and supplements

- C57BL/6j mice
- Surgical instruments
- Anesthesia ketamine and xylazine/CO₂ inhalation.
- Electrical shaver.
- 1 mL syringe with 25G needle (BD 305903).
- Matrigel coated plates (Corning 354234).
- Myogenic cells/Liver cells in sterile conditions Refer to [33,34] (Bose et al., 2016 and Gharabeh et al., 2008 for primary mouse cell isolation)
- Culture Dish (100 mm, Nunc 172958).
- Phosphate buffer saline (Thermo Fisher Scientific, 10010-023).
- 0.25% Trypsin-EDTA (Thermo Fisher Scientific, 25200-056).
- Collagenase type-1(Sigma C0130).
- Dispase (Sigma D4693).
- Gentamycin (Thermo Fisher Scientific, 15750-060).

2.2. Growth medium (GM) cells from skeletal muscle and liver

- DMEM high-glucose (Thermo Fisher Scientific, 11995-040) 500 mL.
- 10% Fetal bovine serum (FBS) (Hyclone SH30071.03) 50 mL.
- 10% Horse serum (HS) (Thermo Fisher Scientific, 26050-088) 50 mL.
- 1% L-Glutamine (Thermo Fisher Scientific, 25030-081) 5 mL.
- 1% Penicillin-Streptomycin (P/S) (Thermo Fisher Scientific, 15140-122) 5 mL.
- 0.5% Chicken embryo extract (CEE) (US Biologicals, C3999) 2.5 mL.
- mM β-Mercaptoethanol (Sigma–Aldrich, M6250) 500 μL.

2.3. Growth medium (GM) cells from skin

- DMEM high-glucose (Thermo Fisher Scientific, 11995-040) 500 mL.
- 10% Fetal bovine serum (FBS) (Hyclone SH30071.03) 50 mL.
- 1% L-Glutamine (Thermo Fisher Scientific, 25030-081) 5 mL.
- 1% Penicillin-Streptomycin (P/S) (Thermo Fisher Scientific, 15140-122) 5 mL.
- mM β-Mercaptoethanol (Sigma–Aldrich, M6250) 500 μL.

2.4. Immunohistochemistry (IHC)

10% Neutral Buffered formalin for fixation of the tissues.
- Paraffin block for embedding tissues.
- Microtome (Leica, RM2245).
- Ethanol Grades (100, 95, 80, and 50%, respectively).
- Citrate buffer (Sigma W302600) for antigen retrieval.
- Blocking buffer: 1% BSA or 5% Normal Donkey Serum (Sigma) in PBS.
- Permeabilization Buffer: 0.5% Triton X-100.
- Primary antibody and secondary antibodies are given in Tables 1.
- Mounting media with DAPI (Sigma F6057).
- Fluorescence microscope (Zeiss).

2.5. Flowcytometry

FC Fixation Buffer: 4% (v/v) PFA in PBS.
- FACS Buffer: PBS with 2% (w/v) fetal bovine serum (Hyclone).
- Triton X-100 (Sigma T9284) 0.2% (v/v).
- Primary and secondary antibodies with recommended dilution have given in Table 1.

2.6. Systems biology approach

2.6.1. Sample preparation for total Proteomics:

- Protein estimation kit (Bradford Bio-Rad)
- Protease inhibitor cocktail solution (Roche Diagnostics, Germany).
- Precast SDS-PAGE Gels (Bio-Rad 161-0993).
- Trypsin solution: Sequencing grade trypsin (20 μg/ml).
- Dissolve one vial containing 20 μg of trypsin (sequence grade) in 1 mL of 0.1 M ammonium bicarbonate solution (dissolve 395
All tissue biopsies should be obtained under the relevant Institutional Animal Ethics Committee guidelines and regulations. Freshly isolated biopsies are recommended.

Regarding the tissue biopsies; it is recommended that the biopsies should be collected from younger animals for superior cells. Younger animals will give more proliferating cells when compared to older animals. Skeletal muscle cells can be harvested from hind limb as well as fore limbs and can be combined to get more number of cells. Skin needs to be shaved and then cleaned to avoid contamination in culture. Liver biopsies need to be processed quickly.

### 3.2. Isolation of cells from skin

#### 3.2.1. Collection of skin biopsy

Skin biopsies were collected from 5-week old mouse C57BL/6J after sacrificing the mouse with CO2 inhalation. The skin was shaved using a shaver before dissection and sterilized with an antibiotic-antimycotic solution [35,36]. The skin biopsy was collected in vials containing DMEM high glucose (Thermo Scientific, USA), 10% FBS (HyClone, USA), antibiotic-antimycotic solution (Sigma, USA), and gentamycin (Thermo Scientific). The skin biopsies can be stored at 4 °C for a maximum of 1 week.

#### 3.2.2. Isolation of cells from skin biopsy

Cells were isolated and cultured as described by [35,36] with certain modifications. Briefly, the biopsies were trimmed of excess fat, washed serially in 70% alcohol, povidone-iodine (Mundipharma, Switzerland), and 1×, 5× and 10× antibiotic-antimycotic (AB/AM) solution (Thermo scientific) (Fig. 1).

Depending on the thickness of the dermis, the biopsies were incubated in 0.2–0.4% dispase (Sigma) for 1–18 h overnight at 4 °C. After dispase digestion, the epidermis was separated from the dermis.

The dermis was chopped into small pieces and incubated in 0.35% collagenase (Sigma) for 30 min at 37 °C. The tissue suspension was filtered through a 70 μm pore size cell strainers and centrifuged for 5 min at 1000 rpm. The cell pellet is resuspended in growth medium for skin cells. The medium was replaced every alternate day. Cells were passaged on reaching 70–80% confluency.

#### 3.3. Isolation of cells from skeletal muscle

Dissect out the hind limb skeletal muscles of the mouse in sterile conditions. Wash the biopsy three times with sterile DPBS or HBSS to remove any debris that may have adhered to the tissue during the dissection [34]. Remove the connective and adipose tissue from the tissue sample using forceps and scissors. Mince the remaining tissue very finely with scissors.

Transfer the minced tissue slurry into a 15-ml tube and centrifuge at 3000 rpm at 4 °C for 5 min. Remove the supernatant and start the digestion of the muscle slurry by adding 10 ml of pre-warmed 0.2% collagenase-type-1.

Incubate in a shaker incubator for 1 h at 37 °C or, alternatively, shake by hand every 10 min. Centrifuge the tissue–enzyme solution at 3000 rpm at 4 °C for 5 min, discard the supernatant and resuspend the cell pellet in 10 ml of GM. Pass the resuspended pellet through a 70 μm cell strainer (BD).

Plate the solution containing cells in a 100 mm dish and observe under the microscope.

Cell suspension should contain large numbers of cells with refractive nuclei as well as tissue debris. Incubate at 37 °C in a humidified, 5% CO2 incubator for 3 h.

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Catalog no.</th>
<th>Concentration (μg/ml)</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45-APC</td>
<td>BD Pharmingen</td>
<td>559864</td>
<td>2 μg/ml for FACS</td>
<td>Rat IgG2b</td>
</tr>
<tr>
<td>CD34-PE</td>
<td>BD Pharmingen</td>
<td>551387</td>
<td>2 μg/ml for FACS</td>
<td>Rat IgG2a</td>
</tr>
<tr>
<td>Sca-1</td>
<td>R &amp; D systems</td>
<td>AF1226</td>
<td>2 μg/ml for FACS</td>
<td>Goat IgG</td>
</tr>
</tbody>
</table>

### 2.7. Characterization of CD34+ cells

Mouse mesenchymal stem cell functional identification and characterization kit (R&D Systems, Minneapolis, MD: Cat No SC010)

3. Methods

All animal experiments were performed after obtaining prior approval from institutional animal ethical committee bearing approval number YU-IAEC 10/04.08.2015

#### 3.1. Dissection of animals and isolation of cells

All reagents and material used must be sterile.

Tissue biopsies: freshly harvested skeletal muscle/skin/liver biopsies obtained from the hind limb/clean shaven skin/liver of 5 week old wild-type mice C57BL/6J.
Three hours after plating, fast adhering cells will attach to the bottom of the dish; label this cell population as PP1 (Fig. 2).

Transfer the supernatant containing slow adherent cells into a Matrigel coated 100 mm dish and label PP2 (preplate 2). Add some fresh media (GM) to the PP1 dish.

After 24 h, transfer the supernatant/cell suspension of the PP2 dish into a new matrigel coated 100 mm dish and label as PP3. Add some fresh media (GM) to the PP2 flask and return to the incubator. Repeat this step every 24 h until population PP6 is reached (Fig. 2).

Observe under the inverted microscope after 24 h of plating most of the slowly adhering cells typically attach by PP6. At this stage, cells appear small, round, shiny, sparse and will take at least four days to attain a good morphology.

3.4. Isolation of cells from liver

Intact livers were obtained from 5-week old C57BL/6J mice [37,38,39]. Briefly, the livers were collected, washed in cold PBS, minced and digested at 37 °C in 0.2% collagenase type 1 A (Sigma Aldrich) for 20 min and filtered through 70 μm pore size cell strainers (BD). The cell suspensions were washed two times by centrifugation at 1000 rpm for 10 min. Cell suspensions were depleted of RBCs by adding RBC lysis buffer for 10 min at room temp.

The cell pellet is resuspended in growth medium used for muscle cells. The preplate method is followed here till PP2. The medium was replaced every alternate day.

Cells were passaged on reaching 70–80% confluency.

3.5. Identification of CD34+ cells in diverse lineages-

From tissues of various lineages, ectoderm (skin), mesoderm (skeletal muscle) and endoderm (liver).

3.5.1. Sample preparation

Formalin-fixed samples are placed in paraffin infiltrated tissues in a mould with a small volume of liquid paraffin. Cool briefly to immobilize the tissues. Place the base of a cassette on top of the mould. Fill with liquid paraffin, and then cool.

Cut thin slices (10 μm) on a microtome, and float sections in a water bath. Mount sections on to charged slides and dry overnight. Using charged slides helps the section to adhere to the slide.

3.5.2. Deparaffinization/rehydration

To remove paraffin wax, place sections in three containers of xylene for 5 min each. Fresh xylene should be used as incomplete deparaffinization can also lead to inconsistent staining.

To start rehydration, place sections in two containers of 100% ethanol for 10 min each. Place sections in containers of 95%, 80% and 50% ethanol for 10 min each.
To complete the rehydration process, wash sections two times in dH₂O for 5 min each.

3.5.3. Antigen unmasking/retrieval

For antigen unmasking or antigen retrieval following method is used with citrate buffer

Preparation of citrate buffer: Prepare 10 mM sodium citrate in PBS and adjust the pH 6.0
Bring slides to a boil in citrate buffer, pH 6.0; maintain at just below boiling temperature for 10 min. Cool slides on bench top for 30 min.
To heat the sections, use a microwave. (Alternative method to heat sections is pressure cooker). It is important not to over or under heat samples as this can cause inconsistent staining results.

Since equipment differs, the heating protocol must be optimized for each laboratory's make and model of microwave using blank slides.

3.5.4. Staining

After antigen retrieval wash the slides with PBS 3 x 5 min each. Permeabilize for 15 min with 0.5% Triton X-100 in PBS.
Block the sections with 5% normal donkey serum or 5% FBS in PBS for 30 min.
Use a hydrophobic pen to draw a large circle around the sample, taking care not to touch the sample. (This creates a hydrophobic boundary so that a smaller volume of antibody solution can be used and, if desired, allows multiple sections on one slide to be stained with different antibodies).

---

Fig. 3. Representative sorting plot of CD34⁺/CD45⁻ cells from mouse skin/skeletal muscle/liver. Sorting of CD34⁺/CD45⁻ cells from young mice (5 weeks) by fluorescence activated cell sorting (FACS). After exclusion of cell debris and cell doublets by applying a forward and side-scatter gates, gate P4 was applied to sort for the CD34⁺ and CD45⁻ population. For each fluorochrome, isotype controls were used.
In the case of tissue sections, blocking can be carried out using the blocking reagent supplied in the M.O.M. (mouse-on-mouse) kit, (Vector Laboratories) as per manufacturer’s instructions followed by overnight incubation with anti-mouse CD34 primary antibody (Table 1 for antibody).

Wash the section with PBS 3 × 5 min each. Incubate for one hour with secondary antibody (goat anti-mouse FITC). Incubation was followed by mounting the tissue sections using Prolong Gold antifade reagent with DAPI (Thermo Fisher Scientific). Cells were visualized using Leica Fluorescence microscope and images were captured using Image-Pro software.

3.6. Purification of CD34+ cells from diverse lineages

Flow cytometry based isolation of CD34+ cells from various lineages, ectoderm (skin), mesoderm (skeletal muscle) and endoderm (liver).

Cells derived from C57BL/6J WT mice from skeletal muscle, liver and skin were FACS sorted for CD34+ antibody as per the method described by Bose et al. [33] using Bio-Rad S3e cell sorter (Bio-Rad USA)/BD FACS Aria flow cytometer (BD Biosciences, San Jose, CA).

PBS-washed cell suspensions were incubated in the blocking buffer (mouse FcR blocker; BD Biosciences, San Jose, CA) and followed by antibody staining (Antibody details are provided in Table 1.) Controls included unstained cells and isotype controls please see (Table 1) for the details of the isotype controls used.

The Analysis was done using BD FACS-Diva software/FCS 6 Express.

After gating the cells by applying forward and side scatter and exclusion of cell debris and doublets by applying side scatter-area versus side scatter-width gate, a third gate was applied to first sort the CD34+ and CD45− populations. Hence, the final population of our interest was obtained as CD34+ and CD45− (Fig. 3).

3.7. Quantification of CD34+ cells in diverse lineages

Analytical flow cytometry for analysis of cells from tissues of various lineages ectoderm (skin), mesoderm (skeletal muscle) and endoderm (liver)

After deriving and culturing cells from tissues of all the three lineages, cells were trypsinized and collected as a single cell suspension. Two washes in PBS were given, and cells were centrifuged at 1000 rpm for 5 min to collect the cell pellet.

The cell pellet was fixed in 4% PFA for 20 min at 4°C and processed immediately (1 mL of PFA was used to fix 3 × 10^6 cells).

Alternatively, the PFA fixed cells were stored at 4°C and analyzed within 15 days of harvesting.

For analysis, the PFA fixed cells were centrifuged, PFA decanted out, and the cell pellet washed twice with PBS.

The cell pellet was then resuspended in 2 mL FACS buffer. For staining, 0.1 × 10^6 cells were aliquoted in 1.5 mL microfuge tubes.

Fig. 4. (A) Phase contrast images of MSCs after sorting. (B) Flow cytometric analysis of cultured MSCs for bonafide MSC markers CD34 and Sca-1. Passage 4 cultures of MSCs were analyzed for the expression of CD34 and Sca-1. Gate P2 was used to discriminate the negative populations (green scatter plots), as obtained, by isotype controls, and positively stained cells are displayed as dark blue scatter plots in gate P3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Please cite this article in press as: P. Sudheer Shenoy, B. Bose, Methods (2017), http://dx.doi.org/10.1016/j.ymeth.2017.06.035
Cells were then centrifuged, supernatant aspirated out, and the cell pellet containing 0.1 million cells was resuspended in 100 μL FACS buffer.

0.1 Million cells each of unstained control, isotype control and the respective primary antibody were processed in an identical manner in three separate microfuge tubes.

Primary antibodies were added to each cell suspension of 0.1 million cells and mixed well by pipetting. Primary antibody incubation was carried out at room temperature for 1 h.

The Concentration of the isotype control was kept same as that of the respective primary antibody. Each of the samples was washed twice after the primary antibody incubation using FACS buffer.

Again 100 μL of cell suspension was left at the bottom of the tube.

Respective secondary antibodies were added to the cell suspension of 100 μL from previous step and incubation was carried out for 30–45 min at 4 °C in the dark.

Post-secondary antibody incubation, each sample was washed with FACS buffer, centrifuged; supernatant aspirated leaving the stained cell suspension in 100 μL volume.

Each sample was stained in approximately 300 μL of FACS buffer, transferred to FACS tube and kept on ice until analysis.

Samples were analyzed on a BD FACS Canto or any flow cytometer capable of excitation at 488 and 630 nm wavelengths.

After excluding the dead cells and debris by applying forward scatter vs. side scatter gates, appropriate positive populations were obtained.

Isotype controls were used for compensation controls and appropriate gating.

Gated populations enabled quantitative comparisons of marker expression in samples collected from various steps of differentiation.

The data was analyzed using the FACS Diva software (BD Biosciences).

10,000 events were acquired per sample and results were represented in the form of a histogram or dot plots (Fig. 4.)

3.8. Characterization of isolated CD34+ cells from three diverse lineages

Immunophenotyping of MSCs isolated from three diverse lineages should be positive for CD34, CD44, CD29, CD90, CD73, CD105, CD146 and negative for CD45, CD31, CD56, CD14 and HLA-DR cell surface markers (Fig. 5).

Functional characterization was done by, inducing CD34+ cells to osteogenic, chondrogenic and adipogenic differentiation using the mouse mesenchymal stem cell functional identification kit as per the manufacturer’s protocol for 15–21 days in multiwell cell culture chamber slides (NUNC) (Fig. 6).

---

**Fig. 5.** Flow cytometric analysis (Immunophenotyping) of cultured MSCs from three diverse lineages for the expression of mesenchymal stem cell (MSC) markers. Passage 4 cultures of MSCs were analyzed for the cell surface expression of CD44, CD73 and CD105. Gate P2 was used to discriminate the negative populations (green scatter plots), as obtained, by isotype controls, and positively stained cells are displayed as dark blue scatter plots in gate P3. Percentage positive cells are mentioned in the respective plots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Fig. 6.** Microscopic images of multilineage differentiation of MSCs isolated from three diverse lineages. (Adipogenic, chondrogenic and osteogenic differentiation).
Differentiated cells were fixed with 4% PFA at the end of the protocol; adipocytes were stained with oil red O and simultaneously stained with goat anti–mouse FABP4 antibody. Chondrocyte sections can be stained with Safranin O or simultaneously stained with sheep anti-mouse collagen-2 antibody. Osteocytes can be stained with the von Kossa stain for calcium deposition [40,41] or simultaneously stained with goat anti-mouse Osteopontin antibody. Slides were also observed under a phase-contrast light microscope (Olympus) as wells as under fluorescence microscope (Leica). Images were captured using the Image-pro software.

![Image](image.png)

**Fig. 7.** Representative Proteomic comparison of CD34+ MSCs isolated from three diverse lineages. Total cell extracts were labeled with acrylamide isotopes and processed using LC-MS. (A) Illustrates the relative quantification of peptides obtained from three different cell types. (B) Venn diagram represents the possible evaluation of entire data set of proteins obtained a, b, c) represents proteins obtained and are unique to skin, skeletal muscle and liver d) represents proteins that are common to all the three lineages e) represents common proteins that are expressed by skin and liver f) represents common proteins that are expressed by liver and skeletal muscle g) represents common proteins that are expressed by skeletal muscle and skin. Liquid chromatography–mass spectrometry; m/z, mass-to-charge ratio.
3.9. Systems biology approach towards the possible applicability of CD34+ cells from diverse lineages as a biomarker for progenitor cells

The isolated and sorted CD34+ and CD45- cells were expanded and processed for proteomic studies.

3.9.1. Cell lysis and sample preparation for proteomics

Denaturation buffer, containing protease inhibitor cocktail (500 ul), was added to the cells in the dish. Scrape the dish using a cell scraper, mix the solution and keep it on the ice. Centrifuge the solution at 20,000 x g to remove cell debris. Store the solution at 4 °C until further use.

Protein concentration was estimated from cellular extract using the Bradford Assay kit according to the manufacturer’s instructions. Total cell extract (50 μg) was taken for protein reduction by adding 5 μl of reduction solution and maintain the reaction at 37 °C for 1 h. Protein alkylation was performed by adding 10 μl of the alkylation solution and maintained the reaction at room temperature for one additional hour.

Total cell extract was loaded onto SDS-PAGE in a 12% precast gel (to fractionate the complex protein mixtures) according to the manufacturer’s instructions, and the gels were stained with silver stain [42]. Each gel lane can be taken individually and can perform in-gel trypsin digestion of the gel fragments. Wash the gel fragments with 200 μl of 0.1 M ammonium bicarbonate (AB) and 50% acetonitrile (AN) solution 3 times. Discard the wash solution. Further dry gel fragments in a speed vac.

Add trypsin 20 μl to the gel fragments to hydrate it for 15 min cover the band with approximately 100 μl of 0.1 M AB solution. Carry out the digestion for overnight at 37 °C. Collect the solution and save. Peptides extracted from the gel pieces after washes (200 μl of 0.1 M AB and 50% AN solution) were pooled and dried in a speed vac. Extracted peptide solutions were transferred to the mass spectrometer. High-throughput LC-MS/MS data was collected for each single fraction obtained from pooled cell extract. Sample analysis method depends upon mass spectrometer used (please see the mass spectrometer user guide for sample analysis).

LC-MS/MS files can be processed through data bank search, protein inference, and quantitative analysis [43,44] (Fig. 7).

4. Conclusion

In conclusion, CD34+ cells have found a broad range of applications in regenerative medicine, right from transplantation of CD34+ bone marrow stem cells to leukemic patients, spinal cord injury, liver cirrhosis and peripheral vascular disease. However, in most of the cases, the source of such CD34+ cells, for clinical applications, have been from bone marrow. Considering the invasive procedure of bone marrow collection, isolation of CD34+ cells from other sources like skin, skeletal muscle and liver will be indeed advantageous. Furthermore, these tissues may be a good source for the isolation CD34+ progenitor cells which can be used in regenerative therapies.

5. Discussion

CD34+/45- are unique cell types that have been classically reported in hematopoietic lineage. However, we have identified the presence of such cell types in tissues from all lineages of non-hematopoietic origin such as liver, skin and adipose (unpublished results). Although, CD34+/45- cells are present in the non-hematopoietic lineages, functions or developmental pathways of these cells are not elucidated. Hence, systems approach to characterise CD34+/45- from all the non-hematopoietic lineages is likely to provide strong clues regarding origin, migration of such cells during embryonic developmental stages. Moreover, the data obtained using systems approach would also be helpful in pathway curation which can, in turn, provide clues for using such cells for cell therapy applications.

6. Notes (trouble shooting)

1) All reagents and material used must be sterile
2) All tissue biopsies (skeletal muscle, skin and liver) should be obtained under the relevant Institutional animal ethics committee guidelines.
3) Age of the mouse is critical; younger animals will yield superior and more proliferative cells. Skeletal muscle biopsies must be collected freshly either from the both hind limbs or hind limbs and forelimbs combined from the wild-type mice (C57BL/6J). Skeletal muscle and liver biopsies collected should be kept in PBS at 4 °C/ice till further processing.
4) Isolated mesenchymal stem cells from all the three tissues can be expanded till passage 4 and stored in LN2 until further use. Reduce repeated trypsinization of isolated cells; use (0.05% trypsin-EDTA or TrypLE).
5) Isolated and sorted cells from skeletal muscle will require 7–8 days to get up to 60–70% confluence and additional 1–2 weeks for expansion and cryopreservation.
6) Isolated cells will be positive CD34, CD44, CD73, CD105, CD146 and negative for CD45, CD31, CD56, CD14.
7) Always prepare fresh dithiothreitol (DTT) and acrylamide solutions, storing these solutions and using them will cause non-specific labelling.

Acknowledgements

The project involving the isolation of CD34+/45- from all three germ layers: ectoderm, mesoderm and endoderm and studying their cellular, molecular and functional similarities/differences has been funded by the Yenepoya University Seed Grant No- YU/Seed Grant/2015-042 awarded to the Principal Investigator Dr Bipasha Bose and the Co-Principal Investigator Dr Sudheer Shenoy P. The authors earlier published the original research article that includes isolation of CD34+/45- cells from muscle corresponding to this Protocol chapter in Bose et al. [33], where the funding source was duly acknowledged.

References


...
学霸图书馆

www.xuebalib.com

本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。

图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：

图书馆首页 文献云下载 图书馆入口 外文数据库大全 疑难文献辅助工具