Class 5.

Harvesting of bone marrow tissue from murine bone and its further processing for fluorescent labelling

Introduction

Stem cells (SC) due to ability to self-renewal and wide differentiation potential (into cells targeted to specific developmental lines) represent the desired material from the viewpoint of regenerative medicine. One of the major challenges resulting from the use of SC in regenerative medicine is the development of an optimal protocols for their identification, isolation and expansion.

Recently, numerous reports have shown that adult murine as well as human specimens such as bone marrow, peripheral blood, solid organs and umbilical cord blood may contain primitive stem cell fractions with multi- and pluri- potent characteristics. Such SC populations include unrestricted somatic stromal cells (USSCs), multilineage-differentiating stress-enduring (Muse) cells, marrow-isolated adult multilineage inducible cells (MIAMI), multipotent adult progenitor cells (MAPCs), multipotent adult stem cells (MASCs) as well as a population of very small embryonic-like stem cells (VSELs). Murine VSELs defined representing small-sized cells expressing Sca-1 antigen but not expressing CD45 and hematopoietic lineages markers ($FSC^{low}/SSC^{low}/CD45^-/Lin^-/Sca-1^+$) have been initially identified in murine BM and subsequently found in several other adult murine organs as rare population of cells. Genetic analysis such as real-time RT-PCR in sorted murine $FCS^{low}/SSC^{low}/CD45^-/Lin^-/Sca-1^+$ cells has showed the increased levels of mRNA for embryonic stem cells markers such as SSEA-1, Oct-4, Nanog and Rex-1 that was also confirmed on protein level using immunofluorescent staining and ImageStream system imaging (ISS). In addition to listed above nonhematopoietic SC, bone marrow as well as other solid organs contain also hematopoietic SC.

Flow cytometry platform represent useful tool for identification and characterization of different stem cell populations. This technology uses highly specific monoclonal antibodies conjugated with colored fluorochromes. Inside a flow cytometer, cells in suspension are drawn into a stream created by a surrounding sheath of isotonic fluids that created laminar flow allowing the cells to pass individually through an interrogation point. At the interrogation point, a beam of monochromatic light, usually from a laser, intersects the cells. Emitted light is given off in all directions and is collected via optics that direct the light to a series of filters and dichroic mirrors that isolate particular wavelength bands. The light signals are detected by photomultiplier tubes and digitized for computer analysis.

Flow cytometry technique provides information about size (represented by FSC- *forward scatter*), granularity (represented by SSC- *side scatter*) of cells as well as fluorescence emitted by immunelabelled cells. Flow cytometry data are displayed as dot plots or histograms. Dot plot displays use two parameters to graph the data generated by flow analysis, with each dot representing the passage of one cell through the detector. The X- and Y-axes measure the different emissions, displaying a dot for each of the cells that show that particular emission. Histograms can also be used to display data from flow cytometry experiments. In these plots the X-axis shows the intensity of the detected signal and the Y-axis measures the number of events (cells) counted.

Aim of class:

Aim of the class is to identified different stem cell populations in murine bone marrow by employing multicolor flow cytometry.

Experimental protocols:

(Work in two people teams. Each team will receive one mice.)

Part I. Preparation of whole BM cells for identification of hematopoietic and non-hematopoietic SC by employing flow cytometry platform

1. Isolate tibias and femurs into Ø 60mm culture dish containing 5 ml of culture medium DMEM/F12.
2. Cut off the epiphysis of bones and flushed bones using 1ml syringe into new Ø 60mm culture dish containing 5 ml of culture medium DMEM/F12. Homogenize cell suspension using 1 ml syringe.
3. Filter cell suspension through Ø 40 µm strainer into 15 ml tube. Fulfill tube to the volume of 10 ml by culture medium DMEM/F12.
4. Centrifuge at 1800 rpm for 6 min at RT.
5. Discard supernatant and suspend cell pellet in 1 ml of Lysing Buffer (1x BD PharmLyse) to remove red blood cells. Incubate for 6 min at RT.
6. Fulfill tube to the volume of 5 ml by culture medium DMEM/F12 and centrifuge at 1800 rpm for 6 min at RT.
7. Discard supernatant and suspend cell pellet in 500 µl of staining buffer DMEM/F12+2%FBS.
8. Determine cell numer.

Part II. Staining of BM-derived total nucleated cells (TNCs) for flow cytometric analysis

1. Add 100 µl DMEM/F12+2%FBS and appropriate volume of cell suspension containing 1×10^6 cells into each of four 5 ml tubes (Compensation controls).
2. Add 100 µl DMEM/F12+2%FBS and appropriate volume of cell suspension containing 7×10^6 cells into 5 ml tubes (Stained sample).
3. Immunolabell TNCs with antibodies listed below according with scheme given in the table.

Sample	Number of cells	List of antibodies applied for immunolabelling
Stained	7×10^6 cells	1. Hematopoietic lineage markers (Lin): PE Rat anti- mouse Ly-6G and Ly-6C; PE Rat anti- mouse CC11b; PE Rat anti- mouse CD45R/B220; PE Rat anti- mouse Ter-119; PE Hamster anti- mouse γδ T- Cell Receptor; PE Hamster anti- mouse TCR β Chain <u>Add 1,5 µl of each = 9 µl of Lin mix per sample</u> 2. PE/Cy7 anti- mouse Ly-6A/E (Sca-1) = 5 µl per sample 3. FITC Rat anti-mouse CD45 = 7 µl per sample
Comp./ Empty	1×10^6 cells	Sample unstained
Comp./ PE	1×10^6 cells	Hematopoietic lineage markers (Lin) = 2 µl
Comp./ PE-Cy7	1×10^6 cells	PE/Cy7 anti- mouse Ly-6A/E (Sca-1) = 2 µl
Comp./ FITC	1×10^6 cells	FITC Rat anti-mouse CD45 = 2 µl

4. Stain for 20 min., on ice (protect samples from light).
5. Wash cells by adding 2 ml of DPBS (without Mg^{2+}, Ca^{2+} ions) and centrifuge at 1800 rpm for 6 min at RT.

6. Discard supernatant and resuspend pellets in 300 µl of DMEM/F12 + 2% FBS. Mix by pipetting.
7. Analyze by using BD FACS Aria III flow cytometry.

The scope of material that will be prepared to class:
1. General principles of flow cytometry.
2. Immunolabelling cells for analysis by fluorescent microscopy and flow cytometry techniques.
3. Features characteristic for stem cells.

Recommended materials:
1.http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Analysis/Flow-Cytometry/Flow-Cytometry-Technical-Resources.html?icid=fr-flow-4
2. Lin KK, Goodell MA. Detection of hematopoietic stem cells by flow cytometry. Methods Cell Biol. 2011;103:21-30.
3. Ratajczak MZ, Zuba-Surma EK, Machalinski B, Kucia M. Bone-marrow-derived stem cells--our key to longevity? J Appl Genet. 2007;48(4):307-19.
4. Zuba-Surma EK, Ratajczak MZ. Analytical capabilities of the ImageStream cytometer. Methods Cell Biol. 2011;102:207-30.

Acknowledgments