Stem Cells and Cancer Stem Cells 2
Therapeutic Applications in Disease and Injury

M.A. Hayat
Editor

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"Although touched by technology, surgical pathology always has been, and remains, an art. Surgical pathologists, like all artists, depict in their artwork (surgical pathology reports) their interactions with nature: emotions, observations, and knowledge are all integrated. The resulting artwork is a poor record of complex phenomena."

Richard J. Reed MD

Preface and Introduction

It is recognized that scientific journals and books not only provide current information but also facilitate exchange of information, resulting in rapid progress in the medical field. In this endeavor, the main role of scientific books is to present current information in more detail after careful additional evaluation of the investigational results, especially those of new or relatively new therapeutic methods and their potential toxic side-effects.

Although subjects of diagnosis, cancer recurrence, resistance to chemotherapy, assessment of treatment effectiveness, including cell therapy and side-effects of a treatment are scattered in a vast number of journals and books, there is need of combining these subjects in single volumes. An attempt will be made to accomplish this goal in the projected seven-volume series of Handbooks.

In the era of cost-effectiveness, my opinion may be minority perspective, but it needs to be recognized that the potential for false-positive or false-negative interpretation on the basis of a single laboratory test in clinical pathology does exist. Interobserver or intraobserver variability in the interpretation of results in pathology is not uncommon. Interpretative differences often are related to the relative importance of the criteria being used.

Generally, no test always performs perfectly. Although there is no perfect remedy to this problem, standardized classifications with written definitions and guidelines will help. Standardization of methods to achieve objectivity is imperative in this effort. The validity of a test should be based on the careful, objective interpretation of the tomographic images, photomicrographs, and other tests. The interpretation of the results should be explicit rather than implicit. To achieve accurate diagnosis and correct prognosis, the use of molecular criteria and targeted medicine is important. Equally important are the translation of molecular genetics into clinical practice and evidence-based therapy. Translation of medicine from the laboratory to clinical application needs to be carefully expedited. Indeed, molecular medicine has arrived.

Although current cancer treatment methods have had an important impact on cancer-related morbidity and mortality, the cure rates are modest. On the other hand, cell-based therapy has the potential to treat human conditions not treatable with available pharmaceutical agents, radiation, surgery, chemotherapy or hormonal therapy. Stem cells present important opportunity to elucidate manifold aspects of molecular biology and potential therapeutic strategies, especially in the areas of cancer and tissue/organ injuries. In other words, stem cell field has tremendous potential in deciphering the molecular pathways involved in human diseases. Some stem cell therapies already are being clinically used routinely; for example in leukemic therapy. Human

stem cells also have the potential for application in regenerative medicine, tissue engineering, and in vitro applications in drug discovery and toxicity testing. Stem cells represent populations of primal cells found in all multicellular organisms, which have the capacity to form a variety of different cell types.

A brief statement on the difference between tissue specific stem cells and embryonic stem cells is in order. Tissue specific stem cells (adult or somatic stem cell) can be isolated from a range of organs and tissues from fetal or adult organisms. These cells have a limited life span, senescence during in vitro propagation, and are multipotent; thus, can be differentiated into a limited number of specialized cells. Embryonic stem cells, on the other hand, are isolated from the inner cell mass of a fertilized egg that has been cultured in vitro to match the blastocyst stage (5–7 days post-fertilization). These cells possess infinite capacity to proliferate in vitro provided maintained in an appropriate condition. The advantage of these cells is that they are pluripotent and can give rise to any fetal or adult cell type.

This is volume 2 of the seven-volume series, *Stem Cells and Cancer Stem Cells: Therapeutic Applications in Disease and Injury*. A stem cell is defined as a cell that can self-renew and differentiate into one or more specialized cell types. A stem cell may be pluripotent, which is able to give rise to the endodermal, ectodermal, and mesodermal lineages; an example is embryonic stem cells. A stem cell may be multipotent, which is able to give rise to all cells in a particular lineage; examples are hematopoietic stem cells and neural stem cells. A stem cell may be unipotent, which is able to give rise to only one cell type; an example is keratinocytes. These types of stem cells are discussed in this volume.

A cancer stem cell is a cell type within a tumor that possesses the capacity of self-renewal and can give rise to the heterogeneous lineages of cancer cells that comprise the tumor. In other words, a cancer stem cell is a tumor initiating cell. A unique feature of a cancer stem cell is that although conventional chemotherapy will kill most cells in a tumor; cancer stem cells remain intact, resulting in the development of resistance of therapy.

As stated above, given that human embryonic stem cells possess the potential to produce unlimited quantities of any human cell type; considerable focus has been placed on their therapeutic potential. Because of the pluripotency of embryonic stem cells, they have been used in various applications such as tissue engineering, regenerative medicine, pharmacological and toxicological studies, and fundamental studies of cell differentiation. The formation of embryoid bodies, which are three-dimensional aggregates of embryonic stem cells, is the initial step in the differentiation of these cells. Embryonic stem cells can differentiate into derivatives of three germ layers: the endoderm, mesoderm, and ectoderm. Therefore, embryoid body culture has been widely used as a trigger for the in vitro differentiation of embryonic stem cells.

Support and development of the stem cell field, especially the application of human embryonic stem cells, mesenchymal stem cells, hematopoietic stem cells, gliosarcoma stem cells, intestinal stem cells, thyroid stem cells, and cancer stem cells, in cancer and other diseases and tissue/organ repair (regeneration), are described. The damage or injury of living tissues is a major challenge during adult life in humans. Enhancing the regenerative potential of cells devoted to tissue repair (the stem cells) either endogenous or supplied from outside, is one of the most important challenges and developments in the medical field. This aspect of therapy is discussed in detail in this volume.

Ischemia is one of the diseases discussed in this volume. Ischemic heart diseases represent one of the major causes of morbidity and death worldwide. Cell based therapies are useful for cardiac regeneration following ischemic heart disease. The finding that heart contains a reservoir of resident stem and progenitor cells, has opened new perspectives in the biology of cardiac regeneration, suggesting the exploration of experimental procedures aimed at in vitro expansion of cardiac stem cells for in vivo transplantation. Hematopoietic stem cells, mesenchymal stem cells, or neural stem cells have been successfully used for the treatment of experimental stroke. Human marrow stem cells show promise as a potential therapy for restoration of function after ischemic stroke. Some of these procedures are detailed in this volume.

Another example of the therapy for a disease discussed in this volume is repairing retina using transplantation. Other examples of therapies using stem cells detailed in this volume include bone defects and acute myocarditis. Methods for the isolation of bone marrow stromal cells from bone marrow, induced pluripotent stem cells, human embryonic stem cells, and cancer stem cells are presented. The rational for transplantation of normal stem cells is included.

Hematopoietic stem cell transplantation is increasingly being performed in patients with malignancies, non-malignant hematological disorders, and autoimmune diseases. Renal injury is a common complication after such treatment, and is associated with high morbidity and mortality. Renal insufficiency and proteinuria are the symptoms of this injury. Both acute kidney injury and chronic kidney disease can occur after hematopoietic stem cell transplantation. Such renal injury is thought to be caused by antibody-mediated endothelial cell injury in chronic graft-versus-host disease. Chronic graft-versus-host disease, a frequent complication of bone marrow transplantation, occurs among 30–50% of bone marrow recipients. This disease is characterized by skin, gut, and liver involvement. Treatment of this disease using allogenic mesenchymal stem cells is described in this volume.

A new promising medical scenario has been discovered by using nanotechnology that provides unique opportunities of building and/or modifying biomaterials and scaffolds with specific, functional molecules and/or drugs carried by nanoscale fibers or particles. This technology facilitates delivery, for example of drugs at the desired sites in precise amounts.

By bringing together a large number of experts (oncologists, neurosurgeons, physicians, research scientists, and pathologists) in various aspects of this medical field, it is my hope that substantial progress will be made against terrible human disease and injury. It is difficult for a single author to discuss effectively the complexity of diagnosis, therapy, including tissue regeneration. Another advantage of involving more than one author is to present different points of view on a specific controversial aspect of cancer cure and tissue regeneration. I hope these goals will be fulfilled in this and other volumes of the series. This volume was written by 116 contributors representing 15 countries. I am grateful to them for their promptness in accepting my suggestions. Their practical experience highlights their writings, which should build and further the endeavors of the readers in this important area of disease. I respect and appreciate the hard work and exceptional insight into the nature of cancer and tissue injury provided by these contributors. The contents of the volume are divided into four subheadings: Stem Cells, Cancer Stem Cells, Diseases, and Tissue Repair (Regeneration) for the convenience of the reader.

It is my hope that subsequent volumes of the series will join the first two volumes in more complete understanding of this medical field. There exists a tremendous, urgent

demand by the public and the scientific community to address to cancer diagnosis, treatment, cure, and hopefully prevention and therapy for tissue injuries. In the light of existing cancer calamity and disabilities, government funding must give priority to eradicating deadly malignancies over military superiority.

I am thankful to Dr. Dawood Farahi and Dr. Kristie Reilly for recognizing the importance of medical research and publishing through an institution of higher education.

Union, New Jersey M.A. Hayat
April 2011

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Part I
Stem Cells

Chapter 1

Isolation of Bone Marrow Stromal Cells from Bone Marrow by Using a Filtering Device (Method)

Tomoki Aoyama and Junya Toguchida

Abstract Bone marrow stromal cells (BMSCs) include cells with multi-directional differentiation potential, such as the mesenchymal stem cells (MSCs). For clinical use, it is important to develop safe and efficient methods of isolating BMSCs from the bone marrow. A new concept is to use a filtering device that selectively traps BMSCs from bone marrow aspirates based on its affinity to the filter material. The cells are then recovered by a retrograde flow in a closed system. This method is more efficient, faster, and easier to use than the density gradient method. Because this method is performed in a closed system without centrifugation, no biologically clean area is required, giving this method a great advantage in clinical applications.

Keywords Bone marrow stromal cells · Mesenchymal stem cells · Multipotent adult progenitor cells · Mononuclear cells · Red blood cells · Ethylenediaminetetraacetic acid

Introduction

Bone marrow stromal cells (BMSCs) contain cells with multi-directional differentiation potential, which are designated as mesenchymal stem cells (MSC) (Caplan, 1991), multipotent adult progenitor cells (MAPC) (Jiang et al., 2002), marrow-isolated adult multilineage inducible (MIAMI) cells (D'Ippolito et al., 2004), or multilineage-differentiating stress-enduring (Muse) cells (Kuroda et al., 2010). It is not yet clear whether these cells are distinct, overlapping, or even identical. In spite of such ambiguity, BMSC-derived multipotent cells have been used in various fields of regenerative medicine, because they can be isolated and propagated without difficulty. However, there are some points of concern, especially when these cells are used for clinical applications. Several methods of isolating BMSCs from bone marrow have been reported, most of which consist of 2 steps. The first step is to separate mononuclear cells (MNCs) from red blood cells (RBCs), which otherwise prevent the initial growth of MNCs upon ex vivo culturing (Horn et al., 2008). In the second step, BMSCs are separated from the hematopoietic MNCs based on their property to adhere to plastic dishes. The most popular method for the first step is density-gradient centrifugation by using sucrose gradients (Bøyum, 1964; Peterson and Evans, 1967). The aqueous solution containing sucrose, commercially available as Ficoll-Parque^{TM} ($\rho = 1.077$ g/mL), is sterile, has low endotoxin content, and is guaranteed to maintain high viability of the separated cells (>90%). The density gradient methods, however, require skill and time. Alternatively, RBCs can be burst by treatment with ammonium chloride, potassium bicarbonate, or ethylenediaminetetraacetic acid (EDTA) (Horn et al., 2008). The number of MNCs obtained by this method has been shown to be higher than those obtained by the density gradient centrifugation methods (Horn et al., 2008). Both methods need chemicals to separate BMSCs. Finally, centrifugation with low gravity can also separate MNCs from RBCs (Caterson et al., 2002). While this method does not use chemical solutions, it does employ centrifugation.

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We have developed a new method to isolate MNCs that uses neither chemical solutions nor centrifugation (Ito et al., 2010). A cell separation-by-filtering method has been used in various fields. For example, nonwoven fabrics are used to trap leukocytes (Takenaka, 1996). Because pure mechanical trapping based on cell size will damage cell membranes, we were interested in developing a filter that will trap BMSCs by their affinity to the material. BMSCs attach to plastic materials (Pittenger et al., 1999) and have an affinity for hydrophilic materials (Kim et al., 2007). In this chapter, we describe the methods to isolate BMSCs from bone marrow by using filtering devices.

Harvesting Bone Marrow

Bone marrow can be harvested from the pelvic bones of human, dog, goat, and pig. It is important to note that the volume of bone marrow aspirated from 1 portal should remain within the set limit to avoid contamination of the peripheral blood (in case of humans, it is 15–20 mL). To obtain more samples, either additional portals or portals on contralateral sites should be used. When using smaller mammals such as rabbit, rat, and mouse, the bone marrow is harvested from long bones. In this chapter, the method of obtaining bone marrow from the human iliac crest is described.

Materials

Reagents:

- Heparin (10,000 U/mL)
- Lidocaine (for local anesthesia)

Supplies:

- Bone marrow harvesting needle (Medical Device Technologies Inc., USA)
- Sterile plastic disposable syringe: 20 mL (for local anesthesia) and 30 mL
- Sterile needle: 23G (for local anesthesia)
- Disinfectant
- Sterile drape

Equipment:

- Operating table

Space:

- Operating room

Methods

Have the patient or volunteer lie face down on the operating table.
Disinfect the area surrounding the posterior iliac crest with disinfectants.
Drape the area surrounding the posterior iliac crest.
Apply a local anesthesia to the posterior iliac crest.
Prepare a 30-mL syringe with 1 mL heparin.
Insert the bone marrow harvesting needle into the iliac crest, and attach the heparinized syringe.
Aspirate 10–20 mL of bone marrow by rapid pulling. Note that slow pulling can contaminate the sample with peripheral blood.
To prevent clotting, agitate the syringe immediately.

Filtering Device 1 (BASIC-SET)

Bone Marrow MSC Separation Device/BASIC-set contains minimum supplies. The sample is processed in open air. Therefore, an operating room or biohazard cabinet is needed for processing. Processing is quite easy for the BASIC-set. Thus, the BASIC-set enables on-demand processing at the operating table.

Materials

Reagents:

- Sterilized physiological saline (100 mL) for priming and washing
- Sterilized physiological saline (50 mL) for cell harvesting

Supplies:

- Bone marrow MSC separation device BASIC-set (KANEKA CO., Osaka, Japan)
- Sterilized filter (pore size, 70 μm)
- 4 sterile plastic disposable syringes (Luer-Slip or Luer-Lok type; 50 mL)
- Priming syringe (Luer-Slip or Luer-Lok type; 50 mL)

Bone marrow fluid syringe (Luer-Slip or Luer-Lok type; 50 mL)
Washing solution syringe (Luer-Slip or Luer-Lok type; 50 mL)
Cell harvest solution syringe (ONLY Luer-Slip; 50 mL)
Waste solution container (150 mL)
Cotton alcohol pads

Equipment:

Syringe pump

Space:

Operating room or biohazard cabinet

Methods

Preparing the syringes (see Note 8.1)

Priming solution: Load the priming syringe with 50 mL of physiological saline solution.
Bone marrow fluid: Filter the bone marrow fluid through the sterilized filter, and load the bone marrow fluid syringe with that fluid; *see* Note 8.2.
Washing solution: Load the washing solution syringe with 30 mL of physiological saline solution.
Cell harvest solution: Load the accompanying Luer-Lok syringe with 50 mL of physiological saline solution; *see* Note 8.3.

Set-up (Fig. 1.1a)

Detach the cap on the column inlet, and connect the accompanying Luer-Lok joint to the nozzle on the column inlet.
Detach the cap on the column outlet, connect the accompanying 3-way cock to the nozzle on the column outlet, and detach the cap at the male-taper end of the 3-way cock.
Remove the cap located on the downstream port of 3-way cock.

Priming (Fig. 1.1b)

Connect the priming syringe to the Luer-Lok joint, and open the flow channel of the 3-way cock (Fig. 1.1b).
Tilt the assembled syringe and column, and gently push the syringe to deliver the solution, while removing air from within the column (Fig. 1.1b); *see* Note 9.4.
After removing all the air, position the assembled syringe and column horizontally, and deliver the remaining priming solution. Collect the fluid passed through the column into the waste solution container.
Close the flow channel for the 3-way cock, while tilting the assembled syringe and column.
With the Luer-Lok joint filled with physiological saline, remove only the syringe.

Processing the bone marrow fluid (Fig. 1.1c)

Orient the column vertically (point the column outlet down). Connect the bone marrow fluid syringe to the Luer-Lok joint, and allow it to stand for 3 min; *see* Notes 8.5 and 8.6.
Open the flow channel on the 3-way cock, and process the bone marrow fluid through the column at a rate of 6 mL/min, equivalent to 2 drops/s; *see* Note 8.7.
Collect the solution passed through the column into the waste solution container. After processing the bone marrow fluid, close the flow channel of the 3-way cock.
With the Luer-Lok joint filled with bone marrow fluid, remove only the syringe.

Washing (Fig. 1.1d)

Connect the washing solution syringe to the Luer-Lok joint, and open the flow channel of the 3-way cock.
Deliver the washing solution at a rate of 6 mL/min, equivalent to 2 drops/s, to wash out the unwanted cells within the column (Fig. 1.1d). Collect the fluid passed through the column into the waste solution container.
Close the flow channel on the 3-way cock (by rotating it 90° clockwise) after all the washing solution has been delivered.
Remove only the syringe.
Turn the column upside down, remove the air filter on the accompanying cell harvest bag, and connect it to the Luer-Lok joint.

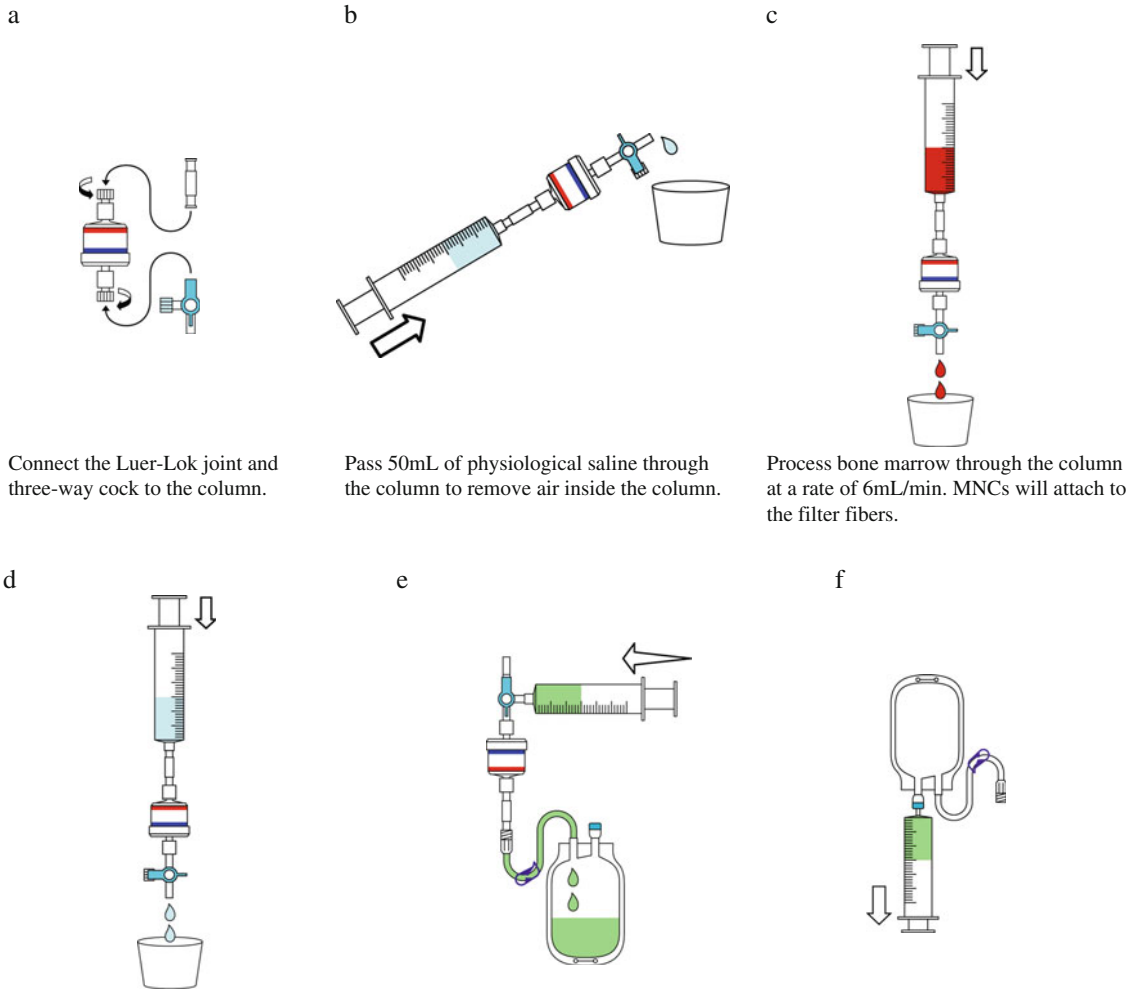

Fig. 1.1 Processing protocol for the filtering device (BASIC-Set)

Injecting the cell-harvest solution (Fig. 1.1e)

Remove the cap located at the side-connection point for the syringe on the 3-way cock.
Connect the Luer-Lok syringe pre-filled with cell harvest solution.
Confirm that the clamp on the tube connecting the cell harvest bag is open.
Operate the 3-way cock.
Flush the cell harvest solution manually (inject 50 mL of cell harvest solution in about 3 s), and harvest the cells trapped within the column into the cell harvest bag (Fig. 1.1e); *see* Note 8.8.
Close the flow channel on the 3-way cock.
Close the clamp on the tube connecting the cell harvest bag circuit.
Remove the cell harvest bag from the Luer-Lok joint.

Collecting the cell harvest solution (Fig. 1.1f)

Sterilize the cell harvest port with a sterile alcohol pad, and insert the cell harvest syringe into the port; *see* Note 8.9.
Collect the cell suspension (Fig. 1.1f).
Remove the syringe after collecting the suspension.

Notes

Remove any air from within the syringes before loading them with liquid (introducing air into the column may disrupt the flow of bone marrow).
A volume of up to 30 mL of bone marrow fluid can be used.
Take up the cell harvest solution in the accompanying syringe or in a syringe of an equivalent volume (50 mL). Using a syringe of a different volume may damage the circuit and may expose the operator to bone marrow fluid.
During priming, remove as much air as possible from inside the column. Priming from the bottom up will make this easier.
To orient the column vertically, hold the syringe and the column with a stand, a clamp, a syringe pump, or by hand.
Arrange the apparatus so that the column is orientated below the syringe. Allow it to stand still for 3 min to separate the oil (which may obstruct the column) within the bone marrow fluid. After 3 min, begin processing the bone marrow fluid.
When processing bone marrow fluid using a syringe pump, do not continue the process if the column gets obstructed. Continued use may result in damage to the connections and may cause the fluid to leak from the connections, potentially exposing the operator to direct contact with the bone marrow fluid. For this reason, never force delivery when you encounter unusual resistance.
If you encounter difficulty when pressing the syringe piston to deliver the cell harvest solution, the column may be obstructed. Try proceeding at a slower rate (e.g., injecting 50 mL of cell harvest solution in 10 s). Avoid excessive force. Stop delivery if you continue to encounter unusual resistance. Applying excessive force during this procedure may result in damage to the circuit and column and may expose the operator to direct contact with bone marrow fluid.
Connect a Luer-Slip-type syringe directly to the cell harvest port (without a needle).

Filtering Device 2 (ADVANCED-SET)

Bone Marrow MSC Separation Device/ADVANCED-set consists of a closed-line system. Theoretically, processing is possible in any space, but a clean area such as an operating room is recommended.

Materials

Reagents:

Sterilized physiological saline (100 mL) for priming and washing
Sterilized physiological saline (50 mL) for cell harvesting

Supplies:

Bone marrow MSC separation device ADVANCED-set (KANEKA CO., Osaka, Japan). The set consists of a Circuit set A (bone marrow bag, cell harvest bag, circuit, etc.) and Circuit set B (waste solution bag, circuit, etc.).
4 sterile plastic disposable syringes (Luer-Slip or Luer-Lok type; 50 mL)
Priming syringe (Luer-Slip or Luer-Lok type; 50 mL)
Bone marrow fluid syringe (Luer-Slip or Luer-Lok type; 50 mL)
Washing solution syringe (Luer-Slip or Luer-Lok type; 50 mL)
Cell harvest solution syringe (ONLY Luer-Slip; 50 mL)
Disinfectants (alcohol)
Cotton alcohol pads

Equipment:

Infusion stand
Infusion pump

Space:

Clean area is recommended.

Methods

Preparing the syringes (*see* Note 9.1)

Bone marrow fluid: Load the bone marrow fluid syringe with bone marrow fluid; *see* Note 9.2.

Cell harvest solution: Load the accompanying Luer-Lok syringe with 50 mL of physiological saline solution; *see* Note 9.3.

Set-up (Fig. 1.2a and b)

Close the clamp and roller clamp.

Close all 3-way cocks (i.e., A (downwards), B (downwards), C (cell harvest bag), D (injection port for cell harvest solution)), and E (air filter)). Orient 3-way cocks as shown in Fig. 1.2a.

Remove the column caps, the circuit cap at the connection to the column, and the air filter.

Connect the column to the circuit so that it is oriented. (Connect the column to match the red and

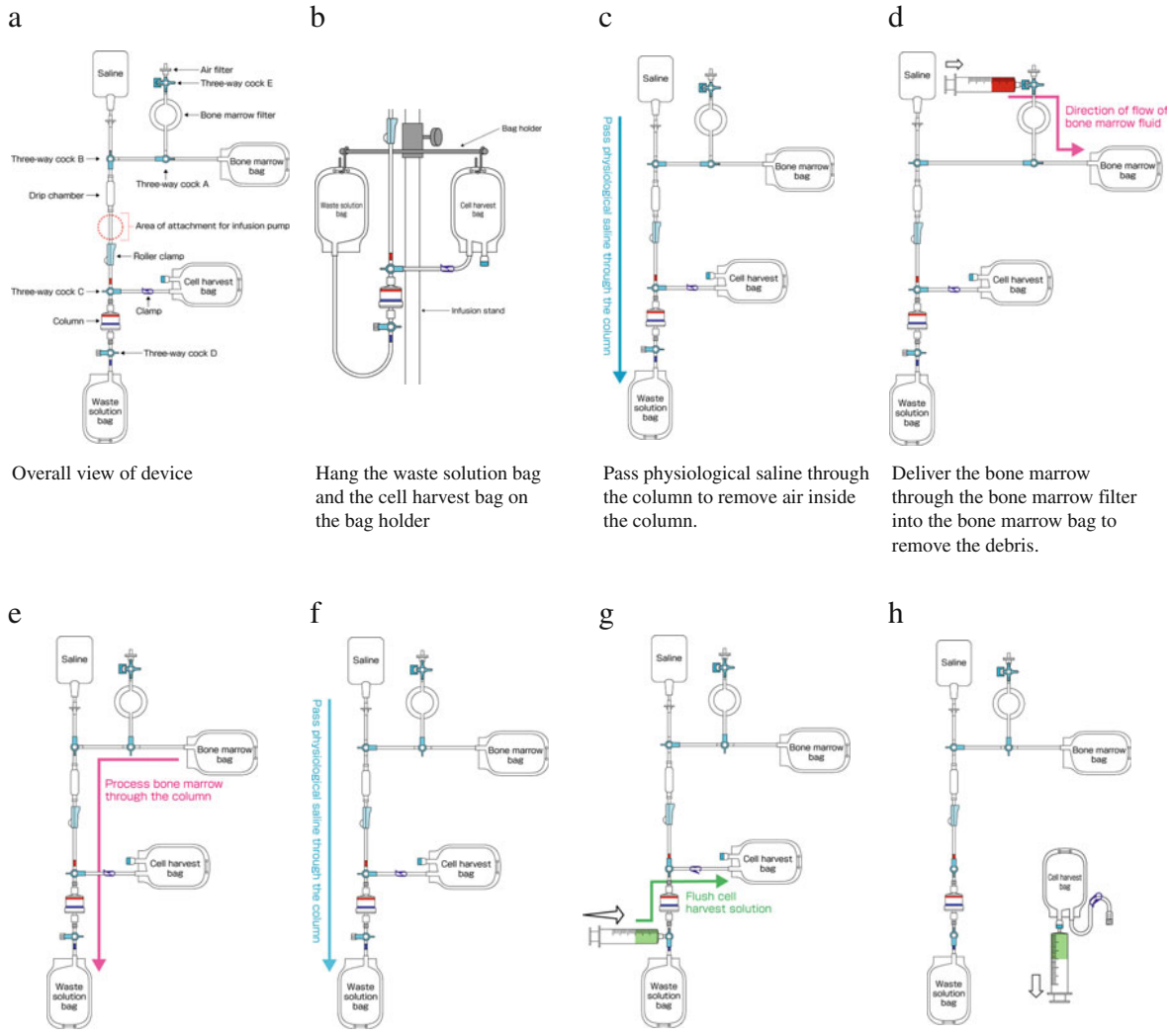

Fig. 1.2 Processing protocol for the filtering device (ADVANCED-Set)