Stem Cell Culture and Its Applications

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Abstract

Cells are the structural and functional unit of life. The dynamics of life encompass the continuous interaction of the cells with the environment and the energy balance that it maintains for sustenance. Eukaryotic organisms' cells have evolved with specific functional commitments and are at the terminal stage of differentiation. With a better understanding of cellular development and differentiation mechanism, it is now appreciated that cells in general have a certain degree of plasticity and can alter with changes in the internal and external

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T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_12-1

environment. Furthermore, a population of uncommitted cells is also found in eukaryotes. These cells can differentiate into various lineages as and when necessary and are denoted as **stem cells**. The stem cells are not only found in a rapidly dividing fertilized embryo (**embryonic stem cells**) but are also found in specific niches of adult organs (**adult stem cells**) and provide a repository of uncommitted cells with a high degree of plasticity. The primary function of these pluripotent stem cells is to maintain homeostasis with the ability to replenish lost or defunct cells. Stem cell research has made immense progress in the past decade and holds great promise in future regenerative medicine. In today's scenario, one of the most widespread uses of stem cells is bone marrow transplantation, and it has revolutionized the treatment of some cancers. Although stem cell therapy is yet to be introduced in many other clinical practices, it is progressively being evaluated as a treatment option for many present-day incurable diseases.

Keywords

Stem cells · Differentiation · Pluripotent · Reprogramming · Bone marrow

1 Introduction

Stem cells are an integral component of our system and continue to be so from the early stages of development to the end of life (Andrews et al. 2005). Essentially, stem cells are undifferentiated cells that have the unique property to develop into the specialized and committed cells of the organism's body (Kumar et al. 2010). Thus, stem cells play a vital role in the development, growth, maintenance, and repair of cells of various organs of the body. Stem cells provide foundational support to every organ and tissue in the human body. The highly specialized cells present in various tissue types originate from an initial pool of stem cells generated immediately after fertilization (Siminovitch et al. 1963). The living body continues to rely on stem cells to replace injured tissues and cells that are lost every day. Stem cells have two fundamental properties: 1) the ability to self-renew, dividing in a way that makes copies of themselves, and 2) the ability to differentiate, giving rise to the mature types of cells that make up our organ and tissues.

Stem cell research holds tremendous potential for the development of novel therapies for many serious diseases and injuries. Embryonic stem cells provide the platform for developing an in vitro model of mammalian development and represent a putative new source of differentiated cell types for cell replacement therapy (Keller 2005). Stem cell-based treatments have been established as a clinical standard of care for some conditions, such as hematopoietic stem cell transplants for leukemia and epithelial stem cell-based treatments for burns and corneal disorders, thus expanding the possibility of stem cell-based therapies (Daley 2012). In the recent past, scientists have gained sufficient experience with stem cell research to consider the possibilities of growing them outside the body for long periods of time. With such advances,

rigorous experiments can be conducted in such a way that specific tissues and organs can be grown with therapeutic competence.

Cell proliferation within a developing multicellular organism is an important characteristic that is followed by the process of differentiation and specialization. Differentiation takes place in steps. At each successive cell division and differentiation, the range of possible future identities for that cell lineage is progressively narrowed, until it culminates in a single cell type. Once a cell lineage has differentiated in the direction of a particular specialized cell type, all progeny cells are committed to becoming similar in all identities. Notably, a few groups of cells remain undifferentiated, and these are called stem cells. Embryonic stem cells have the potential to generate every cell type found in the body. Just as importantly, these cells can, under the right conditions, be grown and expanded indefinitely. Hence, these cells can serve as an important tool for researchers to learn about early developmental processes that are otherwise difficult to access. Such studies would help in establishing strategies that could lead to the development of therapies designed to replace or restore damaged tissues. Stem cells thus have the ability to recreate functional tissues.

Like all other types of stem cells, the adult stem cells (ASC) have in common two important characteristics. First, they are geared towards making identical copies of themselves for very long periods of time which usually spans throughout the life of the individual; this ability to proliferate is referred to as long-term self-renewal. Second, they give rise to mature cell types that have characteristic morphologies (shapes) from the organ they reside on and are capable of performing tissue-specific specialized functions. Commonly, an intermediate cell type or types are generated prior to fully differentiated stem cells and are termed as a precursor or progenitor cell. The partially differentiated progenitor cells found in adult tissues are often defined as pluripotent ASC that can divide and give rise to organ/tissue-specific differentiated cells. These pluripotent adult stem cells are termed “committed” cells that are already destined to differentiate along particular cellular pathways, although in rare cases some alterations in commitment can be observed and such changes need to be defined in a different cellular environment.

2 Types of Stem Cells

Stem cells are pluripotent cells found in all multicellular organisms. These cells retain the ability to renew themselves through mitotic cell division and can differentiate into a diverse range of specialized cell types (Becker et al. 1963). Two major categories of mammalian stem cells are (a) embryonic cells found in blastocysts of a developing embryo and (b) adult stem cells (ASC) found in adult tissues for constant replenishment of damaged tissues throughout the lifetime of a living being. In a developing embryo, stem cells can differentiate into specialized embryonic tissues, which differentiate into the specific lineage of cells with specialized cellular activities. Cellular repair and regeneration is an important process in adult organisms, which is accomplished by progenitor stem cells to maintain the turnover of damaged

and dying cells. This phenomenon is commonly observed in rapidly turned-over tissues like blood, skin, or intestine (Kumar et al. 2010). ASCs are in general undifferentiated cells found living within specific differentiated tissues in our bodies to generate new cells that can come to rescue to replenish any dead or damaged cells. The term "somatic stem cell" is sometimes used to refer to adult stem cells. ASCs are typically scarce in native tissues which has rendered them difficult to study and extract for research purposes. Resident in most tissues of the human body, discrete populations of ASCs generated cells to replace those that are lost through normal repair, disease, or injury. ASCs are found throughout the lifetime of an individual in tissues such as the umbilical cord, placenta, bone marrow, muscle, brain, fat tissue, skin, gut, etc. Adult stem cells were isolated and employed for blood products in the middle of the twentieth century, precisely in 1948. Twenty years later in 1968, this important technique was further expanded when purified ASC were prepared and used for clinical purposes, particularly for blood-related diseases.

Studies proving the specificity of developing ASCs are controversial; Some data specifically indicates that the adult stem cells can differentiate only to the cells of the tissue of origin while others have shown that the ASCs are more versatile and retain the power to differentiate into any tissue type. It is too early to accept either of the proposition with certainty and more experiments are needed for concrete confirmation. Stem cells are unique in their inherent characteristics to be transformed into specialized cells that are consistent with cells of various tissues such as muscles or nerves through cell culture, their use in medical therapies has been recognized and investigated. Interestingly when a stem cell divides, it can either remain a stem cell or turn into a differentiated cell, such as a muscle cell or a red blood cell, or other such types of cells. Thus, the populations of undifferentiated stem cells remain more or less constant during a major part of an individual's life.

Another subset of stem cells that has received considerable attention and exploration is cancer stem cells (CSC). Even when the cancer is diagnosed and treated at an earlier stage, some residual cells still remain and may cause tumor recurrence later with metastasis and aggressiveness. Growing evidence has implicated that these residual cells, which could be found during any stage of cancer progression that is responsible for causing the therapeutic resistance, possess stem-like properties/functions known as cancer stem cells. Hence, this population of cells represents the critical subset within the tumor mass in perpetuating the tumor, even after what seems to be effective therapy and leads to tumor aggression. In recent decades, the CSC theory generates much attention and excitement, whereby scientists believed this theory will revolutionize our understanding of the cellular and molecular events during cancer progression contributing to therapy resistance, recurrence, and metastasis. The CSC theory of cancer progression presents a tumor as a hierarchically organized tissue with a CSC population at the top rank in the hierarchy, which then generates the more differentiated bulk of the tumor cells with lower or limited proliferative potentials. Normal stem cells and CSCs have several commonalities that include self-renewing characteristics including heterogeneity and aberrant cell growth that may lead to benign and neoplastic tumors.

Cancer stem cells are generally characterized by the expression of stem cell-associated surface markers such as CD133, CD44, CD90, and side population cells (SP) by which they can be isolated and enriched in vitro and in vivo, although no single marker can be used to define the CSC populations. It is imperative that better understanding of CSCs characteristics, which alters in accordance with their micro-environment, often can be correlated to the different stages of cancer progression. It is thus very important to understand at the molecular level the strategies adopted by the CSC to remain re-renewable in an effort to better devise effective therapeutic strategies for harnessing the CSC population.

2.1 Embryonic Stem Cells

Embryonic stem cells (ESCs) are derived from the undifferentiated inner mass cells of a human embryo. These cells are pluripotent, with the ability to divide and differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. It essentially means that the embryonic stem cells have the ability to mature into each of the more than 220 cell types found in the adult body as long as and when they are specified to do so. The time-bound activity of the specific signaling molecules is the major determinant in guiding the differentiation and growth of ESCs. Thus, embryonic stem cells are distinguished by two distinctive properties: their pluripotency, and their ability to replicate indefinitely. Pluripotency of the embryonic stem cells can be distinguished from adult stem cells that are primarily multipotent and can produce only a limited number of cell types. Embryonic stem cells can thus be utilized as useful tools for both research and regenerative medicine as these cells can divide limitlessly for a continuous supply of cells for research and clinical use.

2.1.1 Culture of Embryonic Stem Cells

Embryonic stem cells originate from human embryos that are 3–5 days old. In vitro fertilization is often employed to create and harvest these cells. Thus, the process involves fertilizing an embryo in the test tube within the laboratory settings instead of implantation inside the female uterus. Often these embryonic stem cells are also termed pluripotent stem cells. These cells are equipped with the ability to differentiate into virtually any other type of cell that is present within the body of the organism. One of the primary intentions of this investigation is to understand the exact mechanism of the process of differentiation from an embryonic stem cell to a fully differentiated cell of specific tissues and organs.

Scientists have been interested in determining the controlling factors of the process of differentiation. Many techniques have been developed by researchers to manipulate and direct the stem cells to a definitive lineage – a process termed directed differentiation. One of the pioneering efforts and accomplishments of isolating human embryonic stem cells (hESCs) from the inner cell mass (ICM) of human blastocysts was achieved by Ariff Bongso's group in 1994 (Bongso et al. 1994) (Fig. 1).

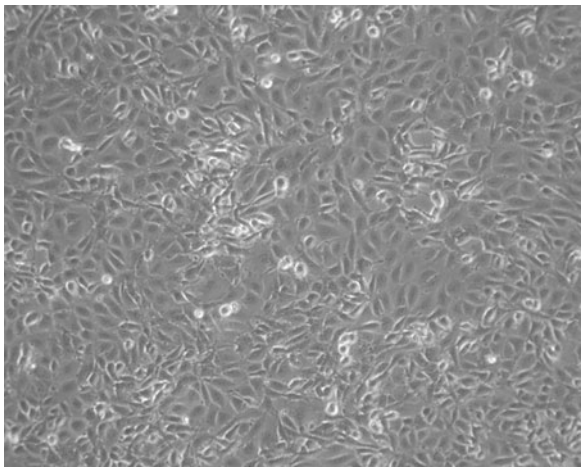

Fig. 1 Human embryonic stem cells in culture: Radhashree Maitra (Montefiore Medical Center, Bronx, NY)

2.1.2 Culturing Embryonic Stem Cells (Adopted from Philip H. Schwartz et al. (2011))

Reagents

- Dulbecco's modified Eagle medium: Ham's F12 (DMEM/ F12 + GlutaMAX. Invitrogen, #10565).
- Dulbecco's phosphate buffered saline (DPBS−−) without Mg^{2+} and Ca^{2+}.
- Dulbecco's phosphate buffered saline (DPBS++) with Mg^{2+} and Ca^{2+}.
- KnockOut™ Serum Replacement (KSR) (Invitrogen, #108280–028).
- 2-Mercaptoethanol, 55 mM (1000×) in PBS (such as Invitrogen, #21985–023).
- GlutaMax (100×) (Invitrogen, #35050).
- Human basic fibroblast growth factor (bFGF) (Stemgent, #03–0002).
- MEM-Nonessential amino acids (NEAA) 100× (10 mM) (Hyclone, #SH30238.01).
- Hybri-Max dimethyl sulfoxide (Sigma-Aldrich, #D2650).
- Water for embryo transfer (Sigma, #W1503, see Note 3).
- Fetal bovine serum (FBS) (Hyclone, #SH30070.03).
- Pen-strep 100× (optional) (Invitrogen, #15070–063).
- TrypeLE-Express (Invitrogen, #12604).
- Collagenase IV (20,000 U, Invitrogen, #17104–019).
- CF-1 mouse embryonic fibroblasts (ATCC, #SCRC-1040).
- Nikon Object Marker, catalog # MBW10020 (optional).
- 6-well vacuum gas plasma-treated tissue culture dishes (such as BD Falcon, #353046).
- Sterile nylon membrane syringe filter (Pall Life Sciences, #PN 4433).
- Nalgene freezing container (containing isopropanol).
- 20 μL pipette tips (Eppendorf and others).
- 150 mm Tissue culture dishes (TPP, #93150).

Media and Stock Solutions

- Human Basic FGF (bFGF) (10 μg/ml, 1 ml)
- Dissolve 10 μg human bFGF in 1 ml KSR.
- Aliquot in 50 μl samples.
- Store thawed aliquots at 4 °C for up to 2 weeks.
- Store frozen aliquots at −20 °C or − 80 °C for 6 months.
- Collagenase IV (200 U/ml, 100 ml).
- Dissolve 20,000 U of collagenase IV in 100 mL of DMEM/F12 + GlutaMax. This is usually ~1 mg/ml final concentration.
- Add to a 250 ml, 0.2 μm filter unit and filter sterilize.
- Aliquot in 5–10 ml in sterile tubes and store at −20 °C until use.
- KnockOut™ Serum Replacement.
- Aliquot as follows:
- Thaw 500 mL bottle at 4 °C and aliquot into sterile 50 mL tubes and store at −20 °C.
- Mix thoroughly when thawed, both for the initial aliquotting and when for use in media preparation.
- MEF Medium (500 ml):
  - Combine 440 mL DMEM/F12 + GultaMax, 50 mL FBS, 5 mL GlutaMax, 5 ml NEAA.
  - Sterile filter 2 μm. Store at 4 °C.
  - Warm to room temperature in the hood before use, discard unused medium after 2 weeks.
- Human Stem Cell (PSC) Medium (100 ml):
- Combine, in order, 78.8 mL DMEM/F12 + GlutaMax with 20 mL KSR, 1.0 ml, 100× NEAA, 100 μL bFGF stock solution, and 100 μL of 1000× 2-mercaptoethanol.
- Filter using 2 μm PES filter.
- Store at 4 °C when not in use and discard any unused medium after 2 weeks.
- Human PSC Cryopreservation Medium (10 ml, 2×).
- Combine 2 ml of human PSC medium, 6 mL of FBS, and 2 ml of DMSO.
- Mix thoroughly.
- Sterile filter using a syringe filter approved for use with DMSO (e.g., nylon membrane).
- Keep cold and use immediately. This is a 2× solution.

Procedure

These methods presume that all PSC culture is carried out in 6-well plates.

Preparation of Feeder Cell Stocks

- The traditional feeder cells are mitotically inactivated, low-passage mouse embryonic.
- fibroblasts, usually from CF-1 strain mice. These MEFs are seeded at a wide range of densities depending on the different PSC cell lines being grown. For

example, the original "H" series lines from WiCell were grown in the presence of MEFs seeded at 75,000 cells/cm^2. Many of the lines and others have been successfully grown on denser feeder layers. It will take some trial and error in your laboratory to determine the optimum density. Human-derived fibroblasts of various origins have also been successfully used as a feeder cell layer. Although not conclusively agreed upon, there is not much of an advantage of human versus mouse feeder cells. In general, some trial-and-error steps are necessary to determine the optimal feeder layer concentration.

A cryopreserved vial of mouse embryonic fibroblast has to be thawed quickly using a 37 °C water bath taking care that the cap is not submerged, followed by washing with 70% alcohol prior to moving it to the laminar flow hood. The contents are then carefully removed to a 15 ml centrifuge tube. 10 ml of warm MEF medium should then be added slowly and dropwise, while the tube is gently shaken.

- Aspirate the supernatant post-centrifugation for 5 mins at 200 × g followed by resuspension of the pellet in 5 ml of MEF medium.
- Add an additional 15 ml of MEF media after seeding onto a 150 mm tissue culture dish (TPP) with 0.1% gelatin coating. Rock the plate gently sidewise and back and forth in an incubator for even cell distribution.
- The cells have to be monitored daily. It usually divides extremely fast. Confluency can be reached in just 24 h.
- Once confluent the cells should be split at a 1:2 ratio using TrypLE Express. The media has to be aspirated out and the plate rinsed with 5 ml DPBS and add 10 ml of RT or 37 °C TrypLE Express.
- The cells start to lift off the plate within 24–48 h, the enzyme is next inactivated by adding 10 ml of MEF medium which has to be previously pre-warmed.
- Cells are next collected in a sterile conical tube and centrifuged for 5 min at 200 × g. Post-aspiration the cells are resuspended in 40 mL of medium and reseeded into 2–150 mm dishes. This step has termed passage I.
- The process is repeated with continuous monitoring and replating using MEFs until passage 5 is reached. In passage 5, the cells are lifted to irradiate them @ 3000 rads for the purpose of inactivation.
- At this stage, the cells can be frozen in DMEM supplemented with 30% FBS and 10% DMSO. Depending on the PSC line, the freezing density can be determined. Typically, feeder cells can be frozen in a single vial (~3.5 × 10^6/vial) to seed an entire 6-well plate.

Preparation of a Feeder Layer

- Coating the plate with 0.1%gelatin for 2–24 h before plating MEFs is necessary for the PSCs to attach. Plate the feeder cells 24 h before starting a PSC culture.
- A vial of irradiated MEFs cells is plated and seeded onto the plate in a MEF medium.
- MEFs are attached by overnight so the MEF medium can be aspirated out and rinsed with DPBS++. This should be followed by the addition of 1 ml/well PSC

medium. At least an hour is necessary for conditioning MEFs prior to seeding with the PSCs.

2.1.3 Thawing of Cryopreserved Pluripotent Stem Cells

- Often a growth lag is observed after thawing and plating PSCs, and may lead to a waiting period of several days before the colonies can be seen. Cultures during the lag period can be monitored microscopically under 4× magnification 24 h post-thawing and plating, but the medium should only be changed at least after 48 h. Usually floating debris and dead cells are observed upon thawing the cells, which is quite normal.
- The cells are thawed quickly, but gently shaking the vial and placing it in a 37 °C water bath and waiting until all ice has melted (about 60 s). Next, the vial is sprayed with 70% alcohol and dried with a Kimwipe.
- Remove the cells slowly and aseptically from the vial and place them in 15 ml conical tube. The entire process should be performed in a biosafety cabinet. Next, add 10 ml of room temperature PSC medium and tap gently.
- Centrifuge at 200 × g for 5 min.
- Aspirate out the supernatant.
- 3 ml of PSC medium is next added to the centrifuge tube, gently triturated, and transferred to one well of a 6-well dish that has been previously prepared with inactivated MEFs.
- The 6-well plate is then placed into the 37 °C incubator.
- 3–7 days have to be waited for the cells to attach. During this time, replace half of the medium every other day being careful not to aspirate out and lose the cells.
- The medium should be replaced every day starting from day 4 to day 7 since thawing the cells, most of the cells should appear to have been attached.

2.1.4 Passaging of Pluripotent Stem Cells

Dissociation with a single cell population is traditionally unhealthy for human PSCs. Thus, the most reliable method for passaging undifferentiated PSC cultures has been the manual breakup of the colonies. This method although exhausting is still recommended for beginners unless familiarity with the cell culture techniques is developed and can easily recognize the differentiation of cells in the cultures.

2.1.5 Mechanical Dissociation

There are various tools that can be used for mechanical colony disruption and are often an individual preference. It has been observed that needles and pipette tips are commonly used because of their low cost and easy availability.

- The culture should be evaluated daily under the phase-contrast microscope at 10X magnification.
- Split the cells onto 3–6 plates of the same size as the original culture, taking into account the density of the original culture. If desired, the cells can be plated onto different-sized plates or dishes, and in such case calculate the volume of media to be added based on the surface area of the plates.

- Remove the overtly differentiated colonies so as not to disturb these during the dissociation process.
- The medium from the dish is discarded and replaced with fresh PSC medium.
- The colonies are next dissected by hand, either under a low-power dissecting microscope or in a laminar flow hood.
- Manual passaging of human PSCs in culture can be achieved using a sterile needle or pipette or sterile scalpel for slicing the colonies into about 100 s pieces.
- Each colony should be broken and moved into suspension by moving the tip around and across each colony in a crosshatch or a spiral motion. Pipette tips are generally a better tool for this disruption than the needles due to their larger bore. As the colonies are relatively large at the time of passage, it is generally easy to observe individual colonies on the plate, and, with practice; the passaging of an entire dish can be achieved in less than 20 min.
- Post-colony disruption a 5 ml pipette can be used to transfer the culture medium containing the dissected colonies to a 15 ml or 50 ml conical tube. The plate is rinsed with 1 ml PSC media and then circulate the medium sequentially from well to well before adding it back to the same 15 ml tube.
- The volume of PSC media is brought up appropriately and cells in the tube are made ready for seeding new plates.
- The cell clumps are gently triturated with a sterile 10 ml pipette and divide the cell suspension into the prepared culture dishes on top of the feeder layer. Trituration should be performed gently. Avoid overtrituration as the goal is to achieve a relatively uniform distribution of the cell clumps without creating single cells.
- The newly seeded plates are placed in the incubator. The plates should be vigorously moved back and forth, side to side, and forward and backward to ensure even dispersion while being careful not to splash any medium onto the cover of the culture dish.

2.1.6 Collagenase Dissociation

Enzyme-mediated dissociation techniques vary between laboratories and generally follow various modifications of the original protocol. Exceptional care has to be exercised when using cultures that have been maintained by manual passaging. The cells cannot be passaged by enzymatic dissociation unless it is allowed to adapt to the new set of conditions. If proper care is taken, enzymatic passaging can provide a convenient and efficient way of maintaining PSC culture stocks.

- The culture medium is discarded.
- Followed by washing the culture with DPBS++.
- The cells are then treated with 2 ml per well 200 U per mL collagenase IV solution for 10 min at 37 °C. The culture must be checked under the microscope to observe the curling of the cells when the collagenase treatment can be terminated.
- The collagenase is next removed and replaced with 2 ml per well of PSC medium.
- A 5 ml pipette can be used next to gently dislodge the “good” colonies from the plate and transfer them to a 15 ml centrifuge tube. Alternatively, the differentiated

colonies can be carefully removed prior to treating the culture plate with collagenase.
- The cells are then gently triturated the clumps with a sterile 10 ml pipette and subsequently plated on a feeder layer of MEFs. Formation of single-cell suspension should be avoided and relatively uniform suspension of cell clumps should be achieved each containing several hundred cells.
- Finally, the cells can be divided into three to six dishes of the same size as the original culture, depending on the starting cell density. If different-sized dishes are used for plating, the appropriate cell dilution should be calculated based on the surface area.

2.1.7 Cryopreservation

To stabilize cultures with specific genetic characteristics at specific points in time, cryopreservation is essential. If cells are not cryopreserved, then it has to be continuously subcultured which will pose a serious threat. With each passaging and continuous subculturing, the cells may accumulate genetic changes and become heterogeneous. It is thus very important to use validated stock vials to initiate new experiments to maximize the long-term use of cell lines and minimize any experimental variation and ensure reproducibility.

The traditional method of cryopreservation of PSCs involves freezing the cells in large clusters with a medium containing FBS and DMSO. For PSCs, this technique has proven to be extremely almost inefficient with little or no cell recovery. If cryopreserved with media containing FBS and DMSO, the time from thawing the vial to having cultures ready for experimentation takes weeks to months. Scientists have now developed much more efficient techniques in conjunction with alternative culture systems.

- Cells are prepared for cryopreservation after it has reached the same stage at which it would be normally passaged.
- The culture media must be changed just before harvesting the cells.
- 1.8 ml cryogenic vials are first labeled with cell line name, date, and passage number.
- 2× stock cryopreservation medium is first prepared and stored on ice.
- The cells are next dislodged from the plate, mechanically, by using a sterile pipette tip. Some researchers may also prefer to treat the cell culture with 2 mL per well 200 U per ml collagenase IV in DMEM/F12 for 10 min at 37 °C.
- If the collagenase method is used, then the collagenase is removed and replaced with PSC medium (3 mL for each well of a 6-well dish).
- For each well of a 6-well dish, the cells are collected preferably in a 3 mL PSC medium and transferred to a 15 mL conical centrifuge tube.
- The collected cell suspension must then be centrifuged for 5 min at 200 × g. The supernatant is aspirated out, leaving a small volume of media just to cover the pellet.

- The cell pellet should then be gently resuspended in a conditioned PSC medium (usually 1.5 ml for each well of a 6-well dish or one-half of the final freezing volume). A 5 ml pipette may be used to gently triturate the clumps.
- An equivalent volume of ice-cold 2× cryopreservation medium is then added and mixed constantly by tapping the sides of the tube.
- 1.0 ml cell mixture is next added to each cryogenic vial (i.e., about three vials per well).
- Finally, the cell suspension is then rapidly transferred to the precooled (4 °C) Nalgene freezing container (containing isopropanol), and placed immediately in a freezer at −70 °C to −80 °C. The next day, the cryovials are transferred to liquid nitrogen for long-term storage.

2.1.8 Markers of Embryonic Stem Cells

Embryonic stem cells (ESCs) retain pluripotency and self-renewing ability due to both their inherent properties and the culture conditions in which they are propagated (Zhao et al. 2012). The expression of ESC markers is specific to their stages and differentiation process. In recent years, a wide range of cell surface markers has been identified as markers of undifferentiated ESCs, especially for the human species. Proteins involved in the cell signal pathways are also known to have important contributions to cell fate decisions. Unique gene expression patterns in ESCs can be used as the identification to distinguish a specific cell type from others. There are many molecules that can affect the pluripotency and self-renewal of ESCs. Identification, characterization, and categorization of these molecules will provide useful tools for the identification and isolation of the ESCs and subsequent ESC studies. A serious challenge is the overlap of ESC markers with those of tumor stem cells, making it difficult to use these markers for ESC identification and isolation. In addition, understanding the mechanisms that regulate the pluripotency of human ESCs (hESCs) remains a major challenge, as recent studies have shown that human and mouse ESCs differ in these mechanisms despite their similar embryonic origins (Prowse et al. 2007). Detailed knowledge of the mechanism of function of these markers is needed for the proper uses of ESCs and elucidation of the mechanisms governing the pluripotency and self-renewal of ESCs. The most common marker of ESCs that has been well studied by different laboratories includes **Oct 4, Nanog, SOX2, SSEA4,** and **Tra-1-60**. Antibodies to these proteins are commercially available.

- **Oct-4** (octamer-binding transcription factor 4) is involved in the self-renewal and replenishment of undifferentiated embryonic stem cells and is coded by the Pou5f1 gene. This gene is an important marker for undifferentiated cells. Oct-4 expression is required to be closely regulated; little or excess expression may cause unwanted differentiation of the cells (Niwa et al. 2000).
- **Nanog** is a homeobox gene coded by the NANOG1 gene. It is an important transcriptional factor that maintains the pluripotency of embryonic stem cells (ESCs) by actively suppressing the expression of the cell determination factors (Heurtier et al. 2019).

- **SOX2** (SRY (sex determining region Y)-box 2) belongs to the high mobility group box (HMG) gene and belongs to an important family of transcription factors that is critical in maintaining self-renewal, or pluripotency, of undifferentiated embryonic stem cells. (Rizzino 2009).
- **SSEA4** or stage-specific embryonic antigen-4 (SSEA-4) is encoded by the FUT4 (Fructosyltransferase 4) gene and is essentially a glycosphingolipid. SSEA4 is often expressed by ESCs. It is also expressed in certain cancers and used as a prognostic marker (Truong et al. 2011).
- **TRA-1-60** or T cell receptor alpha locus is expressed by ESC. This protein is expressed on the cell surface and plays an important role in cell differentiation. It is alternatively known as podocalyxin and is encoded by PODXL Gene (Schopperle and DeWolf 2007). It has been found to be expressed in embryonic cancers.

2.1.9 Usage of Embryonic Stem Cell

Research with embryonic stem cells reveals the possibility of implementing treatments of life-threatening human ailments and alleviating the cost and disease burden. Diseases like as type I diabetes, Parkinson's disease, heart disorders, and spinal cord damages are worth mentioning. The dual characteristics of the embryonic stem cells to proliferate indefinitely and to differentiate to mature tissue types can serve as a source of limitless supply of tissue-specific cells that can be therapeutically employed for cell therapy procedures with the goal to regenerate functional organs. However, there are several hurdles to be overcome for the successful clinical utilization of these cells. Furthermore, they can be employed for synthesis of organoids or development of organs like liver which can be used for transplantation. The ESC holds great promise with immense clinical application in the future. There are ethical issues and skepticism which has been the main hindrance in the stem cell-related research (Leventhal et al. 2012).

2.2 Neural Stem Cells

Neural stem cells (**NSCs**) are self-renewing tissue-specific stem cells found in both the fetal and adult nervous systems. NSCs were first generated from radial glial cells. Neural stem cells exist in the adult nervous system of all mammals as well as in the developing mammalian nervous system. Primitive embryonic stem cells can serve as a good source to generate neural stem cells. NSCs primarily differentiate into neurons, astrocytes, and oligodendrocytes and can be used to study the nervous system. Some neural progenitor stem cells persist within and are confined to specific regions in the brain. Neural stem cells are found to be present in the brain for the entire life. Their proliferation and differentiation are strictly balanced by intrinsic and extrinsic regulators (Jobe et al. 2012). The capability of culturing neural stem cells that are derived from embryonic stem cells, especially from induced pluripotent stem cells in vitro, makes it possible to provide an unlimited source for generating neurons, astrocytes, and oligodendrocytes, which can be used in neurological

disease modeling, neurotoxicological studies, and in cell replacement therapies for neurological diseases *(Yang et al. in Principles of Regenerative Medicine, 2008)*. Experimental evidence indicates that the mammalian central nervous system development undergoes both asymmetric divisions to give rise to progenitors (IPCs) as well as symmetric divisions to expand the NSC pool. The neurons, astrocytes, and oligodendrocytes differentiate from the neuronal stem cell in a temporal sequence.

Utilizing neural stem cells as a model for developmental neurotoxicity assessment has recently received broad attention, and the applications have been growing. Extensive knowledge regarding the characteristics of neural stem cells, specifically the controls of their proliferation and differentiation is necessary before the full potential of neural stem cells can be utilized. Importantly, the differentiation pattern that is available to the daughter cells has to be delineated. The development of a profiling pattern of the neural stem is urgently needed to establish a standardized protocol of testing for the assessment of possible neurotoxicity. The regulatory mechanisms determining the process of self-renewal and fate specification developmental features of NSCs have contributed tremendously to our understanding of neuron-related developmental diseases as well as human brain development in general. Additionally, this information has guided scientists to refine protocols for pluripotent stem cell differentiation into specific nervous system-related cell types for disease modeling as well as to modulate clinical approaches.

2.2.1 Preparation of Neural Stem Cells

Starting from Human Pluripotent Stem Cells (Adopted: Pistollato et al. 2017)
Generation of embryoid bodies (EBs) (Days 0 → 1) The procedure requires significant manual command and precision. Equal-sized fragments of the HiPSC colony are essential to obtain homogenous embryoid bodies (EBs) in the next steps. Colonies with large cytoplasmic fractions and small nucleoli that are not morphologically identical should be discarded.

- The hiPSC medium (3 ml/60-mm Petri dish) should be replenished before cutting the undifferentiated hiPSC colonies to about 1 mm in diameter under sterile conditions.
- 1 ml syringe with a 30G needle can be used to cut the undifferentiated colonies into fragments of approximately 200 μm x 200 μm. A dissecting microscope at 4X magnification is used within a laminar flow cabinet at room temperature during the operation.
- A 200 μl pipette is employed to detach the colony fragments from the dish surface by gently pipetting the medium underneath to lift the pieces of the cell colony.
- All of the detached fragments along with the media are transferred into a 15-ml tube using an appropriately sized pipette.
- The culture plate is then rinsed with 2 ml complete hiPSC medium to collect all fragments.
- The collection is followed by centrifugation at 112 x g for 1 min.

- Complete iPSC EB media is aspirated onto the supernatant to gently resuspend the fragments.
- The colony fragments are next plated in a 60 mm ultralow attachment Petri dish and incubated overnight at 37 °C and 5% CO_2.
- Next day (Day 1), the EBs and the culture media must be transferred into a 15 ml tube.
- The EBs collected in the 15 ml tube should be centrifuged at 112 x g for 1 min.
- The supernatant is aspirated out with care not to lose any EBs which should then be gently resuspended in a complete hiPSC EB medium.
- The EBs are then replated onto a new 60-mm ultralow attachment Petri dish (5 ml/60 mm Petri dish).
- The Petri dish should be next incubated overnight at 37 °C and 5% CO_2.

On Day 1, the dishes are coated with basement membrane matrix (e.g., matrigel, referred to hereafter as "standard matrix") or any other suitable protein substrate (e.g., laminin).

- The standard matrix is stored at −80 °C in 200 µl aliquots using cold 1.5 ml tubes and cold 10 ml pipettes.
- 200 µl standard matrix is thawed on ice.
- 200 µl standard matrix is diluted in 20 ml DMEM/F12 medium (1:100 dilution).
- 60 mm Petri dishes are coated with the standard matrix solution typically 5 ml per dish.
- The coated dishes are then incubated at 37 °C overnight. Note: These dishes will be used to plate the EBs (about 50 EBs/dish) and to generate the neuroepithelial aggregates (rosettes).

Generation of neuroepithelial aggregates (rosettes) (Days 2 → 7)

- **On Day 2,** the standard matrix coating solution should be removed from the 60-mm Petri dishes and refilled with 5 ml/dish of complete neuroepithelial induction medium (NRI) without any prior rinsing off the plate.
- The floating EBs are next transferred to the coated dishes (~50 EBs/dish) using a 200 µl pipette under 4x magnification (microscope) in a laminar flow hood.
- It is extremely important to select homogenous, medium-sized EBs (~200–300 µm in diameter). Very small EBs will not survive during neuroectodermal differentiation, while very large ones tend to undergo core necrosis.
- The dishes are then incubated at 37 °C and 5% CO_2.
- **On Day 3**, the dishes should be observed under the microscope at 10x magnification to ensure that the EBs are attached properly.
- Complete NRI medium is used to perform a total medium change. The process should be done carefully and gently.
- Every alternate day, the NRI culture media is changed for up to Day 7. At this point, the neuroepithelial aggregates (rosettes) should become visible.

- **On Day 7**, the standard matrix or laminin should be coated onto the experimental plate or 96-well plates (100 μl/well), 24-well plates (250 μl/well), 12-well plates (500 μl/well), 60 mm Petri dishes (4 mL/dish).
- The matrix-coated plates/dishes are incubated for at least 2 h at 37 °C and 5% CO_2.

Rosette dissociation and neuronal differentiation (Days 8 → 28) Caution: This procedure will require good manual skills and precision. To avoid collecting mesodermal and endodermal cells, only ectoderm rosette-like structures should be dissociated and collected.

- **On Day 8, t**he rosette-like structures are cut into fragments using a stereoscopic microscope at 10X magnification in sterile conditions. 1 ml syringe with a 30G needle should be used. The rosettes tend to loosen from the culture dish when touched with the needle; hence, caution should be exercised.
- The detachment of the rosette fragments is completed using a 200-μL pipette.
- The dish is transferred under the laminar flow hood and the rosette fragments are collected employing a 5 ml pipette along with the culture NRI medium into a 15 ml conical centrifuge tube. The dish should be rinsed with 2 ml NRI medium for complete recovery of all fragments.
- The rosette fragments are next centrifuged at 112 x g for 2 min and the supernatant is aspirated out.
- Next the pellets are gently resuspended in 1 ml, 1x DPBS (without calcium and magnesium), the rosette fragments are aspirated up and down using a 1000-μl pipette to ensure partial dissociation.
- About 4 ml of complete NRI media should be added followed by cell counting using trypan blue on a hemocytometer or a digital cell counter slide. 20 μl of cell suspension is added to 20 μl Trypan blue to prepare a 1:1 dilution before counting. This step will not be of any relevance if the cells cannot be brought to a single-cell suspension. If the rosette fragments are not observed to be completely dissociated, the rosette fragments derived from about 50 EBs/60-mm dish should be resuspended in 50 ml complete NRI media.
- The standard matrix (or laminin) coating solution is aspirated out of the Petri dishes, plates, and/or MEA chips. Caution should be taken to not let them dry.
- Next the cells are plated in a complete NRI medium at a concentration of about 15,000 cells/cm^2.
- The plates are incubated overnight at 37 °C and 5% CO_2.
- **On Day 10**, a total media change is performed and replenished with a complete neuronal differentiation medium (ND).
- The complete ND medium is changed and replenished twice a week until Day 28.

2.2.2 Expansion and Differentiation into Mixed Neurons and Glia

NOTE: The procedure described below is employed for expanding and maintaining NSCs derived from rosette fragments. This technique allows for incrementing the number of cells for differentiation and chemical testing.

- 60 mm Petri dish (or a T-25 flask) is coated with 5 mL of standard matrix DMEM/F12 coating solution and incubated for at least 2 h at 37 °C and 5% CO_2
- The rosette fragments that were previously derived are placed in a conical 15 ml tube and centrifuged at 112 x g for 2 min.
- Next the pellet is gently resuspended in a 5 ml neural induction medium (NIM).
- The cells are next transferred onto a standard matrix-coated 60 mm Petri dish/flask culture rosette-derived NSCs in the presence of NIM, refreshing the medium every other day until the cells reach confluence.
- After confluency is achieved, the NSCs are passaged as detailed in the following steps.
- The NSCs should be passaged at least once a week using freshly coated dishes, flasks, or plates.
- The complete NIM media is aspirated out, and the NSCs are gently rinsed with DPBS (without calcium and magnesium).
- 1.5 ml of 0.05% trypsin-EDTA pre-warmed to 37 °C should be added to the 60 mm Petri dish (or T- 25 flask) containing the cells and placed in the incubator for 1 min only.
- The flask is tapped on the sides gently to detach the cells.
- 1.5 ml trypsin inhibitor pre-warmed to 37 °C is added and the cells are transferred to a 15-ml centrifuge tube.
- An equal volume of NIM is added to the Petri dish or the flask to rinse the cells.
- The cells are next collected in a 15-mL tube and centrifuged at 130 x g for 3 min.
- The supernatant is removed and the cells are gently resuspended in 1 ml complete NIM using a 1 ml pipette.
- The cell suspension can be further diluted (10 to 100 times as necessary) in 3 or 4 ml complete NIM and the cells are counted after trypan blue staining and counted on an automated cell counter.
- The NSCs are next plated onto the 60-mm Petri dish or flask usually at a density of about 50,000 cells/cm^2.
- A total media change must be given every 48 h with complete NIM media.

2.2.3 Cryopreservation and Thawing NSCs

This is an important step to be followed carefully if frozen cells are re-thawed.

- The NSCs cells at the time of passaging are collected and centrifuged at 130 x g for 3 min.
- The NSCs are very gently resuspended in 3 x 10^6/ml of freezing medium.
- The cells are then aliquoted in suitable vials for cryopreservation (about 0.5 mL = 1.5 x 10^6/vial).
- The vials are placed in a container filled with isopropanol and stored at −80 °C for a minimum of 2 h and a maximum of up to 2 weeks.
- The vials should then be transferred to the vapor phase of a liquid nitrogen tank.
- When cell culture revival is necessary, the frozen vial is quickly thawed in a water bath at 37 °C.

- Using a 1000-μL pipette the cells are gently collected in a 7 ml pre-warmed complete NIM in a 15-ml tube.
- The 15 ml tube is next centrifuged at 130 x g for 3 min to collect the cell pellet.
- The supernatant must be removed and the cell pellet is gently resuspended in 1 ml complete NIM with the help of a 1000 μl pipette.
- The cells are resuspended in 3 or 4 ml complete NIM and quantified by counting the cells using trypan blue and an automated cell counter.
- To initiate a fresh culture. The NSCs are plated in a coated 60 mm culture dish or flask at a density of about 50,000 cells/cm^2.

2.2.4 Characterization of Neuronal and Glial Cells

Differentiated neuronal and glial derivatives can be characterized using various techniques, such as quantitative real-time PCR and/or immunohistochemistry and high content imaging.

2.2.5 Quantitative Real-Time PCR Analyses (Schmittgen and Livak 2008)

- The hiPSC colony fragments, EBs, and/or NSCs are centrifuged at 130 × g for 3 min.
- The cell pellet is then resuspended in 100 μl, cold RNA lysis buffer usually provided in the kit for RNA extraction.
- The process can be alternatively achieved by collecting the neuronal/glial derivatives directly from the plates by aspiration of the medium and adding the cold RNA lysis buffer directly to the wells to collect the lysed cells.
- The RNA isolation is then performed following the kit manufacturer's instructions.
- The RNA is quantified by nanodrop measurements or other measurement techniques.
- 500 ng of total RNA is used to prepare cDNA by reverse transcription.
- A qPCR reaction mix is prepared in duplicate using the appropriate master mix and primer sets.
- The mean fluorescence is recorded in real time: Typically, 40–45 cycles are run with primers annealing temperature set at 60 °C.
- The relative expression of RNA transcripts is determined by normalization to the expression of the housekeeping gene which might be GAPDH or β-actin as a reference gene. Finally, the expression of the desired gene is determined by ΔΔCt method. Alternatively, another suitable method for quantifying gene expression can be used.

2.2.6 Immunocytochemistry and High-Content Imaging (Obernier and Alvarez-Buylla 2019)

- All the colonies including hiPSC, NSCs, and/or neuronal/glial derivatives should be first fixed for 15 min at room temperature with cold 4% paraformaldehyde.
- The cells are then carefully washed with 1X PBS and the plates can be stored at 4 °C for up to 1 month.

- Just prior to staining, the fixed cells have to be permeabilized in permeabilization buffer (1x DPBS containing 0.1% triton-X-100 and 3% BSA) for 15 min at room temperature.
- The permeabilization buffer is aspirated out and the cells are incubated in blocking buffer (3% BSA/1X DPBS) for 15 min at room temperature, a step important to prevent the nonspecific binding of antibodies.
- After 15 min, the blocking buffer is removed by quick centrifugation, and the cells are incubated with primary antibody diluted in blocking buffer overnight at 4 °C.
- The next day, the antibody solution is removed and the stained cells are washed 3 times with cold 1x PBS at 4 °C by centrifugation.
- The cells are incubated at room temperature for 45 min in a blocking buffer containing fluorochrome-conjugated secondary antibodies, followed by counterstaining the nuclei with DAPI dye.

The mean fluorescence intensity should next be quantified, and the relative percentages of cell types should be determined utilizing a suitable high-content imaging platform: There is a necessity to incubate some cells with secondary antibodies alone to estimate the background level of the fluorescent intensity. The expression of PSC-specific markers, such as SSEA4, should be assessed by flow cytometric analysis of live undifferentiated hiPSCs. Further, some of the Undifferentiated hiPSC colonies can be analyzed for alkaline phosphatase activity with BCIP/NBT kits, following the manufacturers' instructions. Additionally, reverse phase protein array (RPPA) assays and analyses should be performed.

2.2.7 Electrophysiological Measurements (Vassallo et al. 2017)

- The dissociated rosette fragments are plated after seven divisions or NSCs derived from rosettes on coated multielectrode arrays are plated in a complete ND medium at a cell concentration of approximately ~1×10^5 cell.
- The cells are allowed to differentiate for three continuous weeks in a complete ND medium, with a routine media change twice a week.
- Once the differentiation is completed, the MEA chips are sealed with a semipermeable membrane under a laminar flow hood to keep the cultures sterile for repeated measurements.
- One of the electrodes is next replaced with one ground reference so that recordings from the remaining electrodes can be collected properly.
- The mean firing rate (MFR; the number of spikes/min) is recorded using an MEA amplifier with the integrated temperature process control that is adjusted to 37 °C and 5% CO_2.
- The relevant peaks are detected from the MEA raw data using a threshold limit of -4.7σ (σ represents the standard deviation of the basal noise).
- The recorded data can then be appropriately processed using suitable software.

2.2.8 Neural Stem Cell Marker

Marker-specific identification of neural stem cells is essential for studying the basic mechanisms of the generation of cellular diversity in the CNS, upon which therapeutic treatments for CNS injuries, degenerative diseases, and brain tumors may be based (Kaneko et al. 2000). Progress has been made in delineating the properties of adult NSCs, specifically the lineages they develop into as well as the signaling pathways that determines their behavior, but as of the date the extrinsic and/or intrinsic factors that promote quiescence/dormancy and activation of NSCs remain inconclusive and the detailed understanding of the mechanisms that sustain the NSC pool while ensuring life-long neurogenesis is still lacking. Furthermore, a significant hurdle is the heterogeneity of the population of primary progenitors/NSCs in the mammalian brain is yet to be overcome. The consequences of the heterogeneity in terms of affecting the NSC function are still obscure (Obernier and Alvarez-Buylla 2019). Most utilized neural stem cell markers include **Nestin, SOX2, HES 1, and Notch1** while many more are being investigated.

- **Nestin** (an acronym for neuroectodermal stem cell marker) is a type VI intermediate filament protein that in humans is encoded by the NES gene. Nestin functions in the development of neurons.
- **SOX2** (SRY (sex determining region Y)-box 2) belongs to the high-mobility group box (HMG) gene functions as a transcription factor that is essential for maintaining self-renewal or pluripotency and is expressed in multipotent neural stem cells at all stages of human life (Ellis et al. 2004).
- **HES1** (hairy and enhancer of split-1) is a transcription factor that is encoded by the Hes1 gene and is one of the seven members of the Hes gene family. Hes genes code nuclear proteins that suppress transcription (Andrews et al. 2005) It is expressed in both neuroepithelial cells and radial glial cells and is involved in maintaining but not in creating neural stem cells.
- **Notch 1** is a single-pass transmembrane receptor encoded in humans by the NOTCH1 gene. Several developmental processes controlling cell fate decisions are controlled by proteins that can be grouped as Notch family of proteins. Notch 1 promotes the differentiation of progenitor cells into astroglia (Tanigaki et al. 2001). Notch 1, when activated before birth, induces radial glia differentiation, but postnatally induces the differentiation into astrocytes (Chambers et al. 2001).

2.2.9 Usage of Neural Stem Cells

Neural stem cells have been shown to engage in the migration and replacement of dying neurons. Cell death is a characteristic of acute CNS disorders as well as neurodegenerative disease. The cell loss is further amplified by the lack of regenerative abilities for cell replacement and repair in the CNS. One way to circumvent this is to use cell replacement therapy via regenerative NSCs (Xu et al. 2011). Neuronal precursors (also called neuroblasts) divide and give rise to nerve cells (neurons) of various types. Glial precursors give rise to astrocytes or oligodendrocytes. Astrocytes are a kind of glial cells, which lend both mechanical and metabolic support to neurons; they make up 70–80% of the cells of the adult brain. Oligodendrocytes, on

the other hand, make myelin, the fatty material that forms the sheath of the neuronal axons and speeds nerve transmission. Under in vivo conditions, neuronal precursors do not give rise to glial cells and glial precursors do not give rise to neurons. In contrast, a fetal or adult CNS (central nervous system – the brain and spinal cord) stem cell may give rise to neurons, astrocytes, or oligodendrocytes, depending on the signals it receives and its three-dimensional environment within the brain tissue (Ellis et al. 2004). It is now widely accepted in the scientific world that the adult mammalian brain has a niche of neuronal stem cells. However, there is no consensus about how many populations of CNS stem cells exist, how they may be related, and how they function in vivo. Notably, the role of NSCs during diseases is now being elucidated by several research groups around the world. The responses during stroke, multiple sclerosis, and Parkinson's disease in animal models and humans are part of the current investigation. The results of this ongoing investigation may have future applications to treat human neurological diseases (Fig. 2).

The flow chart (above) details the process of differentiation of human pluripotent stem cells (hPSCs) to neuronal cells. The cell size at the various stages of development along with the type of growth media that facilitates the differentiation is depicted.

2.3 Mesenchymal Stem Cells

Human MSCs (hMSCs) are non-hematopoietic, multipotent stem cells with the capacity to differentiate into mesodermal lineages such as osteocytes, adipocytes, and chondrocytes as well ectodermal (neurocytes) and endodermal lineages (hepatocytes) (Ullah et al. 2015). Mesenchymal stem cells or MSC are often referred to as cells isolated from stroma, the connective tissue that surrounds other tissues and organs. These cells are often referred to as "stromal cells" which is a more accurate annotation. MSCs were first isolated from the bone marrow and were shown to be capable of making bone, cartilage, and fat cells. It is generally agreed that in an embryo a mesenchymal stem cell is a pluripotent progenitor cell that divides many times and whose progeny eventually gives rise to skeletal tissues: cartilage, bone, tendon, ligament, marrow stroma, and connective tissue (Caplan 1991). In 1959, a bone marrow (BM) transplant was applied for the first time in patients after confirming its therapeutic effects in dogs, as a hematopoietic stem cell-based therapy (Abdal Dayem et al. 2019). Alexander Friedenstein first identified MSCs as colony-forming unit fibroblast and osteoprogenitors with a fibroblast-like shape, that can grow in adherent cell colonies, and were distinctly different from the hematopoietic stem cell (HSC). In 2007, Sacchetti et al. documented the self-renewal potential of osteoprogenitors in BM sinusoids by showing their ability to organize specifically in the hematopoietic microenvironment (Sacchetti et al. 2007). In subsequent years, the MSCs were successfully grown from other tissue types, including adipocytes and cord blood cells. MSCs are proposed to have stem cells, with immunomodulatory properties, and are currently being tested as treatments for several types of disorders, although the benefits are yet to be established. Scientists do not fully understand

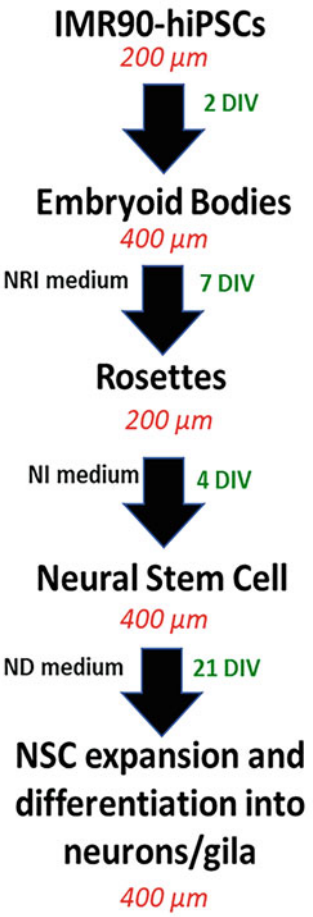

Fig. 2 Expansion and differentiation of hiPSC cells neurons/glia

whether these cells are stem cells or what types of cells they can generate. Scientists do agree that not all MSCs are identical and that their characteristics depend on where in the body they come from and how they are isolated and grown. MSCs do indeed possess a diverse range of therapeutic applications.

2.3.1 Preparation of Mesenchymal Stem Cells

MSCs are multipotent stem cells that can be easily isolated from various tissues and organs of the human body, such as bone, fat tissue, cartilage, hepatic tissue, blood, and muscle. Crisan et al. 2008 have reported that irrespective of the tissue origin, long-term cultured perivascular cells retained myogenic properties; exhibited at the clonal level osteogenic, chondrogenic, and adipogenic potentials; expressed MSC markers; and migrated in a culture model of chemotaxis. MSC markers were found to be expressed at the surface of native, perivascular cells. Thus, blood vessel walls

may harbor a reserve of progenitor cells that may be integral to the origin of the elusive MSCs and other related adult stem cells. MSCs can be autologous (same patient-derived) or allogeneic (derived from another patient), which differentially influences the clinical application of MSCs in the various appropriate clinical settings (Trounson & McDonald 2015).

2.3.2 Mesenchymal Stem Cell Isolation from Mouse Bone Marrow (Adopted: Soleimani and Nadri 2009)

Materials

Reagents

- DMEM with 2 mM L-glutamine and without ribonucleosides and ribonucleotides
- FBS
- 0.25% trypsin/1 mM ethylenediaminetetraacetic acid
- Hanks' balanced salt solution
- Streptomycin
- L-glutamine
- Penicillin
- $NaHCO_3$
- FBS

Procedure

- Mice (Balb/c, 6–8 weeks old) are euthanized by cervical dislocation to harvest the bone marrow. The animal skeleton is washed with 70% ethanol, an incision is made around the perimeter of the hind limbs at the point where it is attached to the trunk. Initially, the skin is removed by pulling toward the skin by foot, which is cut at the anklebone. Further contact of the hind limb with the animal's fur is thus eliminated, which can be a potential source of contaminating bacteria. The hind limbs are dissected from the trunk of the body by cutting along the spinal cord with care not to damage the femur under the laminar flow hood. The limbs are preserved on ice in DMEM supplemented with 1Xpenicillin/streptomycin while awaiting further dissection.
- The bone marrow (BM) is harvested in a hood using a proper sterile technique. The ends of the tibia and femur are incised just below the end of the marrow cavity using a pair of sharp blades. A 27-gauge needle attached to a 10 ml syringe containing complete media has to be inserted into the spongy bone by the removal of the growth plate. The marrow plug is flushed out through the cut end of the bone with 1 ml of complete media and collected in a 10 ml tube on ice.
- Strong flushing is necessary during marrow cell preparation.
- The cell suspension should be filtered through a 70-mm filter mesh to remove any bone pieces/spicules or muscle and cell clumps. The yield and viability of cells is determined by Trypan blue exclusion and counting on a hemocytometer. Typically, 70 x 10^6 BM cells are obtained from one donor.

- The freshly isolated BM cells are next cultured in 95-mm culture dishes using 1 ml complete medium and plated at a density of 25 x 10^6 cells per ml.
- The plates are incubated at 37 °C with 5% CO_2 in a humidified chamber avoiding any disturbances. The nonadherent cells that accumulate on the surface of the dish are removed after 3 h; the plate is replenished with a fresh aliquot complete medium.
- Second round of media change is performed after an additional 8 h of culture, and another aliquot of 1.5 ml fresh complete medium is added. This step is repeated every 8 h for up to 72 h of initial culture.
- It is important to exercise caution when changing the media which should be done very slowly and gently to avoid unwanted lifting of the MSCs growing in the culture media.
- The adherent cells (passage 0) should be washed with phosphate-buffered saline, and fresh media is replaced every 3 to 4 days for healthy growth. It is normal to observe individual cells as adherent spindle-shaped cells under phase-contrast microscopy which may appear around the third day of culture.
- The culture generally becomes (65–70)% confluent within 4 to 8 days, the culture achieves full confluence within 2 weeks. At this stage, the cultures typically exhibit two characteristics: first, plates may contain distinct colonies of fibroblastic cells that vary in size; and second may contain very small numbers of hematopoietic cells interspersed between or on the colonies.
- After 2 weeks of initiating culture, the cells should be washed with phosphate-buffered saline, and cells are lifted by incubation in 0.5 ml of 0.25% trypsin/1 mM ethylenediaminetetraacetic acid for 2 min at room temperature. The trypsin is neutralized after 2 mins by adding 1.5 ml complete medium, and all lifted cells are transferred to a 25-cm^2 flask. The non-lifted cells should be discarded.
- The time and temperature of passaging are very critical and should be very carefully monitored (if the time and temperature were higher than 2 min and 25 °C, respectively, non-MSCs together with mMSCs would be lifted from plastic culture dishes).
- The culture media should be replaced every 3 days (replacing with 6 ml medium each time). Typically, cell confluence should be achieved within 7 days.

Timing

- Day 1, Steps 1–5: harvesting, seeding, and change of media of bone marrow cells
- Day 2–3, Step 5: slowly change the media of culture.
- Day 4–14, Steps 6 and 7: change the medium every 3 to 4 days.
- Day 14, Step 8: subculturing adherent bone marrow cells.
- Day 15–21, Step 9: obtain a purified population of mesenchymal stem cells with spindle-shaped morphology that appears to gradually increase on days 4 to 10.
- 21 days of culturing marrow cells.

2.3.3 Markers of Mesenchymal Stem Cells

Flow cytometry analysis demonstrated that the cluster of differentiation markers currently used to define MSCs, such as CD105, CD166, CD90, CD44, CD29, CD73,

and CD9. Many of these markers are also expressed by fibroblasts. Recently, real-time polymerase chain reaction confirmed that expression of CD106, integrin α11, and insulin-like growth factor-2 in MSCs was at least tenfold higher than in fibroblasts, whereas expression of matrix metalloproteinase 1 and matrix metalloproteinase 3 was almost 100-fold lower. It is relatively easy to recognize MCSs from hematopoietic stem cells as the former does not express the CD45 cell surface protein marker. The MSCs also have an upregulation of CD106 as confirmed by immunostaining and live cell flow cytometry analysis and this can further help to distinguish itself from fibroblasts.

2.3.4 Use of Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a subset of adult stem cells that may be particularly useful for stem cell-based therapies for three reasons. **First**, MSCs have been isolated from a variety of mesenchymal tissues, including bone marrow, muscle, circulating blood, blood vessels, and fat, thus making them abundant and readily available (Deans and Moseley 2000; Zhang et al. 2009). **Second,** MSCs can differentiate into a wide array of cell types; including osteoblasts, chondrocytes, and adipocytes. This suggests that MSCs may have broader therapeutic applications compared to other adult stem cells. **Third,** MSCs exert potent paracrine effects enhancing the ability of injured tissue to repair itself. In fact, animal studies suggest that this may be the predominant mechanism by which MSCs promote tissue repair.

The mesenchymal stem cells are universal in their presence and are found throughout the body's organs. Those harboring the bone marrow can be differentiated to become bone, cartilage, fat, and muscles. Notably, the MSCs are found to be able to alter immune functions when studied in several experimental models. These abilities have created considerable interest in developing ways of using mesenchymal stem cells to treat a range of musculoskeletal abnormalities, cardiac diseases, and some immune abnormalities such as graft-versus-host disease following bone marrow transplant. Other stem cell treatment modalities are in the early stages of development and hold great promise for the future.

MSC-based therapy has been found to cause certain paracrine effects in angiogenesis, antiapoptotic, and immunomodulatory processes. MSCs are known to influence innate and specific immune cells. MSCs produce many molecules having immunomodulatory parameters. These include prostaglandin E2 (PGE2) (Spaggiari et al. 2009), nitric oxide (Ren et al. 2010), indoleamine 2,3-dioxygenase (IDO), IL-6, and other surface markers – FasL (Akiyama et al. 2012) PD-L1/2. MSCs insert an effect on macrophages, neutrophils, NK cells, mast cells, and dendritic cells in innate immunity. MSCs are also capable to migrate to the site of injury, where they polarize through PGE2 macrophages in the M2 phenotype which is characterized by an anti-inflammatory effect.

MSCs when cultured can secrete hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF) (Nagaya et al. 2005). In a rat model of myocardial ischemia, injection of human bone marrow-derived stem cells upregulated cardiac expression of VEGF, HGF, bFGF, angiopoietin-1 and angiopoietin-2, and PDGF (Yoon et al. 2005). In swine, injection

of bone marrow-derived mononuclear cells into the ischemic myocardium was shown to increase the expression of VEGF, enhance angiogenesis, and improve cardiac performance (Tse et al. 2007). Bone marrow-derived stem cells have also been used in several small clinical trials with conflicting results. In the largest of these trials (REPAIR-AMI), 204 patients with acute myocardial infarction were randomized to receive bone marrow-derived progenitor cells versus placebo 3–7 days after reperfusion. After 4 months, the patients that were infused with stem cells showed improvement in left ventricular function compared to control patients. At 1 year, the combined endpoint of recurrent ischemia, revascularization, or death was decreased in the group treated with stem cells (Leventhal et al. 2012).

2.4 Hematopoietic Stem Cells

The first characterization of hematopoietic stem cells started when animals given lethal doses of irradiation suffered bone marrow failure, and this failure could be reversed by injection of nonirradiated bone marrow cells (Ford et al. 1956). The concept of multipotential hematopoietic progenitors derived from the first quantitative experiments on bone marrow restoration indicated that limiting numbers of bone marrow cells can give rise to clonal colonies of myeloid-erythroid cells. Essentially, these hematopoietic stem cells give rise to other blood cells through a process termed hematopoiesis. In embryonic development, this is derived from the mesoderm layer of the embryo. In adults, this process occurs in the red bone marrow. It is interesting that the generation of hematopoietic stem cells continues throughout life, and on average, a healthy individual produces more than 500 billion blood cells every day.

Various blood cell lineage differentiates from the HSCs. The two major lineages that HSCs differentiate into are myeloid and lymphoid. The myeloid cells include monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, and megakaryocytes to platelets. Lymphoid cells include T cells, B cells, natural killer cells, and innate lymphoid cells. The myeloid and lymphoid lineages both are involved in dendritic cell formation.

HSCs are generally distributed at a 1:10,000 (one is to ten thousand ratios) in myeloid tissue and constitutes cells with capabilities of long-term and short-term proliferation abilities and are generally committed to multipotent, oligopotent, and unipotent progenitors.

Adult bone marrow is a rich source of hematopoietic stem cells, especially in the pelvis, femur, and sternum. They are also found in umbilical cord blood and, in small numbers, in the peripheral circulation. Hematopoietic stem cells exist in a state of quiescence, or reversible growth arrest. The quiescent HCSs help the cells survive for extended periods of time in the hypoxic bone marrow environment (2016–2017). When provoked by cell death or damage, HSCs exit quiescence and begin actively dividing again. This process is under intricate regulation. The transition from dormancy to propagation and back is regulated by the cell signaling pathway, namely, MEK/ERK pathway and PI3K/AKT/mTOR pathway (Baumgartner et al. 2018).

Dysregulation of these signaling pathways can lead to stem cell exhaustion or the gradual loss of active hematopoietic stem cells in the blood system.

In the early stages of differentiation, HSC gives rise to different subtypes often termed as colony-forming units (CFU). There are seven well-characterized subtypes each giving rise to a definite cell lineage.

- Colony-forming unit-granulocyte-erythrocyte-monocyte-megakaryocyte (CFU-GEMM)
- Colony-forming unit-lymphocyte (CFU-L)
- Colony-forming unit-erythrocyte (CFU-E)
- Colony-forming unit-granulocyte-macrophage (CFU-GM)
- Colony-forming unit-megakaryocyte (CFU-Meg)
- Colony-forming unit-basophil (CFU-B)
- Colony-forming unit-eosinophil (CFU-Eos)

2.4.1 Isolation of Hematopoietic Stem Cells

It is difficult to isolate a pure population of hematopoietic stem cells. In general, HSCs are characterized by their small size and low staining with vital dyes such as rhodamine 123 (rhodamine lo) or Hoechst 33342 (side population). The development of Fluorescence-Activated Cell Sorting (FACS) has been crucial characterization and purification of HSCs. FACS is a well-established technique that can accurately recognize and quantify small numbers of cells in large mixed populations of live cells. More importantly, FACS-based cell sorting allows the preparations of HSCs to near 100% purity including rare cell populations. This capability enables the testing of these cells in various assays HSCs can be isolated by flow cytometry where the combination of several different cell surface markers is used to separate the rare HSCs from the surrounding blood cells. HSCs lack expression of mature blood cell markers and are thus called Lin- (Lineage minus). A combination of several positive cell-surface markers along with the lack of expression of lineage markers is used for the detection and isolation of HSCs.

2.4.2 Procedure

The best yield is with fresh samples. It is advisable to free the sample if not processed within 48 h of procurement. The preparation details might need adjustments depending on the specific tissue source. If samples cannot be processed within 48 h, they should be frozen.

Isolation of Human CD34+ Hematopoietic Progenitor Cells

- Samples are diluted in 1:1 in D-PBS without Mg^{2+} or Ca^{2+}.
- Next, 20 ml of Ficoll is poured into a 50-ml tube and layered slowly (tilting the tube and running the cells down the side of the tube), 25 ml of diluted blood or marrow is layered on top.
- The Ficoll-containing tube is then centrifuged at room temperature @1100 g for 20 min.
- Half of the top layer is removed and discarded.

- The "cloudy" interface layer (~10 ml) should be carefully pipetted off and transferred into a clean 50-ml tube. The cells are then washed with 50 mL PBS without Mg^{2+} or Ca^{2+}.
- The cells are next in media with serum or protein (D-PBS or Hanks' solution with 2–6% FBS or HSA).
- The cells are washed twice in D-PBS without Mg^{2+} and Ca^{2+} and centrifuged after each wash.
- RBCs are lysed and removed by resuspending in cold (0.1 M) NH_4Cl solution at 3–4 times the original sample volume followed by incubation on ice for 10 min each time.
- The cells are again washed twice in D-PBS without Mg^{2+} and Ca^{2+} and centrifuged after each wash. The cells are next resuspended in media with serum or protein (D-PBS or Hanks' solution with 2–6% FBS or HSA) at a cell concentration of 10^7/ml.
- The control cells are removed at this point (~10^5 cells/tube) following the technique.
- Unstained cells; irrelevant antibody controls for FITC, phycoerythrin, and Cy5; single-color positive controls for FITC, phycoerythrin, and Cy5.
- The remaining cells are next stained by adding the appropriate antibodies for the chosen procedure. Cells are incubated for 30 min at 4 °C for optimal antibody binding.
- The cells are washed twice and resuspended in Hanks' solution + FBS containing 2 μg/ml propidium iodide (PI) (P4170). Cells are now ready for sorting.
- Appropriate flow cytometer setting are used to sort the cells.

2.4.3 Markers of Hematopoietic Stem Cells

HSC assays, in combination with the ability to purify HSCs, have provided increasingly detailed insight into the cells and the early steps involved in the cell differentiation process. Several marker combinations have been developed that describe human HSCs, including clusters of differentiation markers such as CD34, CD38, Lin, CD90, CD133, and fluorescent substrates for the enzyme, aldehyde dehydrogenase. The use of highly purified human HSCs has been mainly experimental, and clinical use typically employs enrichment for one marker, usually CD34.

2.4.4 Use of Hematopoietic Stem Cells

The clinical use of stem cells holds great promise, although the application of most classes of adult stem cells is either currently untested or is in the earliest phases of clinical testing (Koc et al. 2000); hematopoietic stem cell research has made significant progress. The hematopoietic stem cells are currently the best-studied stem cells backed by more than 50 years of clinical research and investigation. In 2021, more than 1.5 million transplants were performed worldwide. Essentially, bone marrow transplant is a medical treatment that replaces your bone marrow with healthy cells. The replacement cells can either come from your own body or from a donor. A bone marrow transplant is alternatively termed a stem cell transplant or, more specifically, a hematopoietic stem cell transplant. This type of transplantation

can be used to treat certain types of cancer, such as leukemia, myeloma, lymphoma, and other blood and immune system diseases that affect the bone marrow. Thus, currently, the main indications for bone marrow transplantation are either hematopoietic cancers (leukemias and lymphomas) or the use of high-dose chemotherapy for non-hematopoietic malignancies (cancers in other organs). Other indications include diseases that involve genetic or acquired bone marrow failure, such as aplastic anemia, thalassemia sickle cell anemia, and increasingly, autoimmune diseases. The study of HSCs remains active and continues to advance very rapidly. Fueled by new basic research and clinical discoveries, HSCs hold promise for such indications as treating autoimmunity, generating tolerance for solid organ transplants, and directing cancer therapy. One of the major hurdles of HSC transplantation is the availability of matching grants for the diseased host. Currently, one of the major research goals is to achieve the optimal expansion of HSCs. Future developments in genomics and proteomics, as well as in gene therapy, have the potential to widen the horizon for clinical application of hematopoietic stem cells even further.

2.5 Adult Stem Cells

Embryos are not the solitary source of stem cells, rather they are also found in several adult tissues and are important for normal tissue function. Many cells do not live throughout the entire life of the organism and require replenishment. Some cells have a defined life period and eventually die and must be replaced by cell division and differentiation throughout the lifetime of the organism. **Adult stem cells,** also known as somatic stem cells or tissue stem cells, are incompletely differentiated cells residing in many tissues of adult organisms whose normal function is to divide and replace cells that are lost through routine physiological processes. These are termed stem cells and are located in the niche of several tissue regions where cells are continually being lost: skin, gut lining, uterine cervix, bone marrow, and many glands. An important characteristic of a stem cell is that when it divides normally, one daughter cell remains undifferentiated as a stem cell and the other differentiates and is, therefore, less likely to continue dividing. This pool of undifferentiated stem cells is continuously maintained within the tissue niche. Essentially, these processes are tightly coordinated in adult organisms, such that cells lost from specific tissues are replaced by the correct number and type of cells.

Bone marrow is one of the first tissue in the body in which adult stem cells were identified. The porous interior portions of the long bones are a continuous source of pluripotent stem cells throughout an individual's entire life and give rise to both red blood cells, which carry oxygen, and white blood cells, which are part of the immune system. Red blood cells (RBC/erythrocytes) have a life of about 120 days and are required to be produced to replace those that have reached the end of their life span or have been lost through injury. Both the types and numbers of new cells produced are tightly regulated. Deadly diseases like leukemia or myeloma, which are blood cell cancers that develop in the bone marrow, are often a consequence of the disregulation of this process of differentiation. Since the bone marrow contains

pluripotent stem cells, bone marrow transplants can reestablish the pluripotent blood cells and/or the different types of immune cells in individuals lacking them. Transplantation of bone marrow between two individuals requires the exact matching of a set of cell-surface marker proteins; otherwise, the recipient's body may reject the transplanted cells, which can lead to a life-threatening complication called graft-versus-host disease. In some cases, a person's own bone marrow cells can be removed and later transplanted back, for example, after the person's own immune cells have been killed by cancer therapy. This works very well because a person's own cells will not trigger graft-versus-host disease and would be immediately recognized as self.

Adult stem cells are found in small numbers in most adult tissues, such as bone marrow or adipose tissue. Compared with embryonic stem cells, adult stem cells have somewhat limited ability to give rise to various cells of the body. Adult stem cells differ from embryonic stem cells as they are not as undifferentiated as embryonic stem cells; all the same they are still capable of forming many types of cells in addition to the type found in the tissue from which they were derived. However, emerging evidence suggests that adult stem cells may be able to create various types of cells. For instance, bone marrow stem cells may be able to create bone or liver cells. In tissue culture, bone marrow stem cells can develop into other types of cells, including muscle or nerves, given the right set of hormones, and signaling molecules. Transplants of these adult stem cells are being investigated for their future potential to regenerate other types of tissue in addition to blood cells. It appears that, in tissue culture, it may be possible to bring adult stem cells back to full potentiality, that is, the ability to differentiate into any and all kinds of cells. This is not yet known for certain, however.

In the current scientific era, adult stem cells are considered the gold standard for clinical applications and are being repeatedly tested and accepted for a growing number of conditions. The adult stem cells do not have any ethical taboo regarding their isolation, and their practical advantages over pluripotent stem cells have led to many current clinical trials, as well as some therapies approved through all phases of Food and Drug Administration (FDA) testing. There are sufficient peer-reviewed publications claiming successful outcome with adult stem cell therapies. Significant therapeutic benefit in clinical trials and progress toward fully tested and approved treatments have been documented in recent years. Phase I/II trials suggest potential cardiovascular benefits from bone marrow-derived adult stem cells as well as with umbilical cord blood-derived cells. Impressive results have been reported indicating prominent success in treating neurological conditions, including ischemic strokes. Another disease that has shown positive long-term progression-free outcomes is multiple sclerosis and often the disease outcome also included complete remission. Significant benefits in early trials for patients with type I diabetes mellitus and spinal cord injury have been reported. Thus, adult stem cells are starting to be used as vehicles for genetic therapies, such as for epidermolysis bullosa. Several published studies indicate that even in the aged adult human body (age over 60 years) there are multiple tissues that contain multipotent somatic stem cells.

2.5.1 Isolation of Adult (Somatic) Stem Cells

It is being increasingly accepted that stem cells are responsible for the provision of continuous homeostasis of our tissues and organs, which life span is partially determined by the stem cell pool quality, proportional distribution, and local micro-environmental factors. Several types of adult human tissues like bone marrow, adipose tissue, and skin (from different body locations) can serve as potential sources of multipotent somatic stem cells for application in regenerative medicine. Adult stem cells are currently being isolated from many tissues in the body. The methods of isolation and culture are dependent on the source and lineage. Many isolations and purification protocols involve flow cytometry and cell sorting. Positive and negative sorting for cell surface markers can quickly generate enriched populations.

2.5.2 Markers of Adult Stem Cells

Adult stem cells often resemble the mesenchymal stem cell markers and exhibit expression of cell surface markers including CD44, CD90, CD105, CD106, CD166, and Stro-1, lack of the expression of hematopoietic markers, no immunogenic effect, and replacement of damaged tissues. These properties led to the development of progressive methods to isolate and characterization of adult stem cells from various sources for therapeutic applications in regenerative medicine**.**

2.6 Induced Pluripotent Stem Cells

Stem cell research from nonembryonic stem cells has progressed more than embryonic stem cell research. Induced pluripotent stem (iPS) cells, with the same pluripotent characteristics as embryonic stem cells, have replaced embryonic stem cells in many research publications. The iPS cells have distinct advantages compared with embryonic stem cells because they can be sourced from almost any individual or tissue, healthy or diseased, more cheaply and efficiently than embryonic stem cells and without the ethical concerns about their process of creation and isolation. As these are patient-derived cells, there is a high possibility for the production of pluripotent-derived patient-matched cells that can be a therapeutic reintroduction to the patient at a later time. However, great caution is warranted because iPS cells, as with embryonic stem cells, also show genetic instability in culture and may thus show tumorigenic potential**.** The ethical issues regarding embryonic stem cells and the possible limitations of adult stem cells (including difficulties obtaining them from some adult tissues), scientists have sought ways to generate special cells that would exhibit stem-like properties. In 2006, Japanese biologist *Shinya Yamanaka* succeeded in "reprogramming" skin cells from adult mice, returning the cells to a pluripotent state by engineering them to express four transcription activator proteins. The following year, *Yamanaka and colleagues* repeated the accomplishment of using human skin cells. The reprogrammed cells are called induced pluripotent stem cells. Similar to embryonic stem cells, when iPS are exposed to appropriate signals, these induced stem cells will differentiate into a wide range of cell types. This is the

triumph of regenerative medicine with the major benefit of this strategy is that, in theory, any cell type needed could be generated from a patient's own stem cells, which can be easily obtained, and being "self-sourced" will not stimulate any immune reactions. In 2010, *Yamanaka* and British biologist *John Gurdon* were jointly awarded the Nobel Prize in Physiology or Medicine for their discoveries regarding the reversible nature of the differentiation process.

It is yet to be completely explored if induced pluripotential stem cells have a potentiality as broad as embryonic stem cells, as the signals required to specify all cell types have not yet been defined. However, in 2009, mice were born from induced pluripotential stem cells using a technique called tetraploid complementation. These embryos had a low rate of implantation in recipient mothers, and some had physical abnormalities, but some did survive and go on to reproduce, suggesting that the induced pluripotential stem cells were able to give rise to all necessary cell types. Such an experiment would not be considered ethical by humans. Differentiated cells, such as skin cells, can be reprogrammed back into a pluripotent stem cell state. Reprogramming can generally be achieved over several weeks by forced expression of genes that are known to be master regulators of pluripotency. At the end of this reprogramming process, these master regulators would remodel the expression of an entire network of genes. Those associated with the pluripotent state, essentially reversing the developmental process, will replace features of differentiated cells.

2.6.1 Isolation of Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are essentially the somatic cells that have been reprogrammed to become pluripotent. Thus, any somatic cell could be essentially and theoretically reprogrammed. Initially, somatic cell nuclear transfer (SCNT) technique was tested out for understanding the process of reprogramming. The first somatic cell nuclear transfer (SCNT) experiments were successful in demonstrating that somatic cells possess a full genome and opened the door to the reprogramming of somatic cells to pluripotent state. Although this technique played a crucial role in our understanding of pluripotency and remains one of the most efficient methods available to reprogram somatic cells, SCNT is not an ideal strategy for producing pluripotent cells because it requires the use of an unfertilized egg. The isolation and manipulation of unfertilized eggs can be only utilized in the setting of in vitro fertilization, but the scarcity of eggs in the research setting prohibits large-scale use of SCNT. Thus, new technology was necessary for the ethical and efficient generation of pluripotent stem cells. For many stem cells like blood cells, reprogramming have been relatively easy and straightforward while others like the cardiac cells are more technically challenging. At first, iPSCs were generated by the introduction of the transcription factors Oct3/4, Sox2, Klf4, and c-Myc in cells maintained in culture conditions. Other investigators have since used various combinations of these transcription factors. In 2006, the *Yamanaka group* overcame this ethical controversy when they described a protocol whereby somatic cells could be dedifferentiated into a pluripotent state following the transduction of a four previously mentioned transcription factor cocktail. Following this initial study, numerous

groups have described protocols to generate induced pluripotent stem cells (iPSCs). These protocols have simplified the reprogramming strategy by employing polycistronic reprogramming cassettes and flanking such polycistronic cassettes with loxP or piggyBac recognition sequences.

The introduction of relevant transcription factors cloned into plasmid expression vectors was utilized at very early-stage experiments, but the efficiency was low, and occasionally expression plasmids can integrate into genomic DNA. This was followed by the introduction of transcription factors cloned into retroviral and lentiviral vectors and since has been the most widely adopted technique for generation of the iPSCs. There were several concerns raised regarding the gene silencing epigenetic effects of the viral vectors. A major consideration for using retroviruses to generate iPSCs is that the viruses integrate into the host DNA. Depending on the integration site, integration can have deleterious effects on the cells, altering gene expression and increasing the risk of tumor formation. *Stadfield and associates* in 2008 used adenovirus as an alternative, and Sendai virus, an RNA virus, was contemporarily used by *Seki and colleagues*, as vectors to transduce transcription factors since these do not integrate into the genomic DNA of the cell. Furthermore, the retro/lenti-mediated introduction of transcription factors is often very inefficient. A more efficient method was found to be the combination of lentiviruses and microRNAs (miRNAs) to reprogram cells were reported by *Anokye-Danso and accomplices* in 2012. These small RNAs bind to mRNA and either inhibit translation or cause degradation of transcripts. miRNA clusters, including miR-290-295 and miR-302-367, have been shown to enhance reprogramming of somatic cells into iPSCs.

Another approach avoiding virus-mediated transduction of transcription factors was the use of miRNA mimics and the viral vector to enhance expression of transcription factors. miRNA mimics are double-stranded modified RNAs that mimic mature miRNAs (fully processed cellular miRNAs). miRNA mimics do not require a vector and are capable of being transfected directly into cells. *Judson and accomplices* in 2009 have shown that the combined use of transcription factors and miRNA mimics produces more homogeneous iPSC clones. An advantage of using miRNA mimics with transcription factors is that the transcription factor c-myc, an oncogene, is not required.

Apart from viral vectors and miRNA mimics, several chemical compounds that modulate enzymes controlling epigenetic modifications have been evaluated for increasing the efficiency of transduction by transcription factors. DNA methyltransferase and histone deacetylase (HDAC) inhibitors were shown to potentiate the efficacy of transduction. It has been reported that HDAC inhibitor valproic acid was the most effective, increasing reprogramming efficiency by 100-fold. With the miRNA mimics, the use of valproic acid eliminated the need to transduce the oncogene c-myc. A number of other inhibitors of kinases, such as the glycogen synthase kinase-3 (GSK3) inhibitor CHIR99021 and the MEK inhibitor PD0325901, and other enzymes that are in pathways involved in pluripotent induction also exhibited an enhanced efficiency in reprogramming.

2.6.2 Markers of Induced Pluripotent Stem Cells

Undifferentiated induced pluripotent stem (iPS) and embryonic stem (ES) cells share many common markers that can be functionally characterized by the ability to differentiate into cells of the three germ layers. In addition to the ability to give rise to all cell types, a number of molecular markers have been identified to verify the pluripotent status of stem cells. For example, human pluripotent stem cells express the cell surface proteins SSEA-4 and alkaline phosphatase and the transcription factors Oct-3/4 and Nanog. The ability to verify stem cell pluripotency using established markers at the start of an experiment helps ensure that downstream stem cell proliferation and differentiation studies are conducted on high-quality, undifferentiated starting cell populations.

3 Stem Cell Identification

CD34 Positive marker is expressed in all human bone marrow (BM) progenitor cells.

HSCs can be isolated using flow cytometry based on surface marker expression. There are several criteria that have to be taken into consideration when the isolation of HSCs has to be performed. For most purposes, multipotent hematopoietic progenitors should be CD34+/CD38-/CD45RA-/CD71- in the expression of surface markers. CD133+, CD90+ (Thy-1), ALDH+, and Sca-1+ are also expressed in progenitor stem cells and are considered positive or expressing markers. The absence of expression of CD2-, CD3-, CD19-CD41-, CD16-, CD14-, and CD15- is also a hallmark of hematopoietic progenitor stem cells and are considered negative markers thus should be non-expressing for bone marrow progenitors and included in the list of mature blood lineage (Lin-) markers.

4 Stem Cell Expansions

4.1 Expansion of Human CD34+ Hematopoietic Progenitor Cells

- Cell culture media is prepared by combining the Basal Medium and the Supplement Mix of the PromoCell HPC Expansion Medium DXF (Sigma C-28021) according to the manufacturer's instructions. An appropriate volume of PromoCell Cytokine Mix E (Sigma C-39890/C-39891) is carefully added to generate the completely supplemented Expansion Medium. The complete and supplemented media should be pre-equilibrated at 37 °C and 5% CO_2 for 30 min by placing the media in the 37 °C incubator.
- To the pre-equilibrated medium, the freshly isolated HPCs plated at a density of 5000 cells/ml. When using cryopreserved HPCs, thawing of the cells is necessary for 2 min in a 37 °C water bath. Post-thawing, the HPCs are transferred into the

pre-equilibrated medium at a density of 5000 cells per ml. It is recommended that at least 9 ml of medium is added per vial of cryopreserved cells.

- The cells are incubated for 96 h at 37 °C and 5% CO_2. If necessary, partial medium change can be performed. Essentially, cells are removed from the incubator and pipetted up and down several times to prepare a single cell suspension. The entire content of the tissue culture plate is then transferred into a 50 ml tube. The cells were next centrifuged for 10 min at 240 x g. Then, carefully aspirate the upper two-thirds of the medium. Gently resuspend the cells in the remaining third of the medium and replenish to the original volume with fresh cytokine-supplemented HPC Expansion Medium DXF.
- Cells are to incubate for another 6–8 days to allow sufficient expansion. About two-thirds of the medium is replaced as described above every 72.
- Cells are next collected from the tissue culture plate containing the expanded HPCs. It is better to pipette up and down the culture media several times in order to release loosely attached cells and to achieve a single-cell suspension. The HPCs cells are finally harvested by centrifugation at 240 x g for 10 min following which the supernatant is discarded.
- The cells are finally resuspended in Promo Cell HPCs Expansion Medium DXF (Sigma C-28021) and the cell number is determined using a hemocytometer and trypan blue staining. The HPCs are then ready for a new experiment.

5 Stem Cell Differentiation

HSCs have a few unique properties, the combination of which defines them as such. The core properties are the ability to choose between self-renewal or differentiation which is essentially embarking on becoming a mature hematopoietic cell. HSCs migrate in a regulated fashion and the population is significantly controlled by apoptosis (programmed cell death). The balance between these activities determines the number of stem cells that are present in the body. Hematopoietic stem cells can be differentiated in vitro by using relevant cytokines that would promote the differentiation into a specific lineage. Colony-stimulating cytokines and other cellular components signal via their cognate receptors to regulate hematopoiesis. These cytokines are termed hematopoietic cytokines.

Hematopoietic cells are under the tight control of a group of hematopoietic cytokines. Each cytokine exerts multiple actions mediated by receptors whose cytoplasmic domains contain specialized regions initiating the various responses that include survival, proliferation, differentiation commitment, maturation, and functional activation. It has been postulated that cytokines, signaling via their cognate receptors, may play an instructive role in lineage specification in hematopoiesis. Individual cytokines can be lineage-specific or can regulate cells in multiple lineages. The simultaneous action of several cytokines is often required for proliferative responses. Example of such cell types includes progenitors of megakaryocyte and their progenitor stem cells. These cytokines also control basal and emergency hematopoietic cell proliferation. The three major cytokines, erythropoietin,

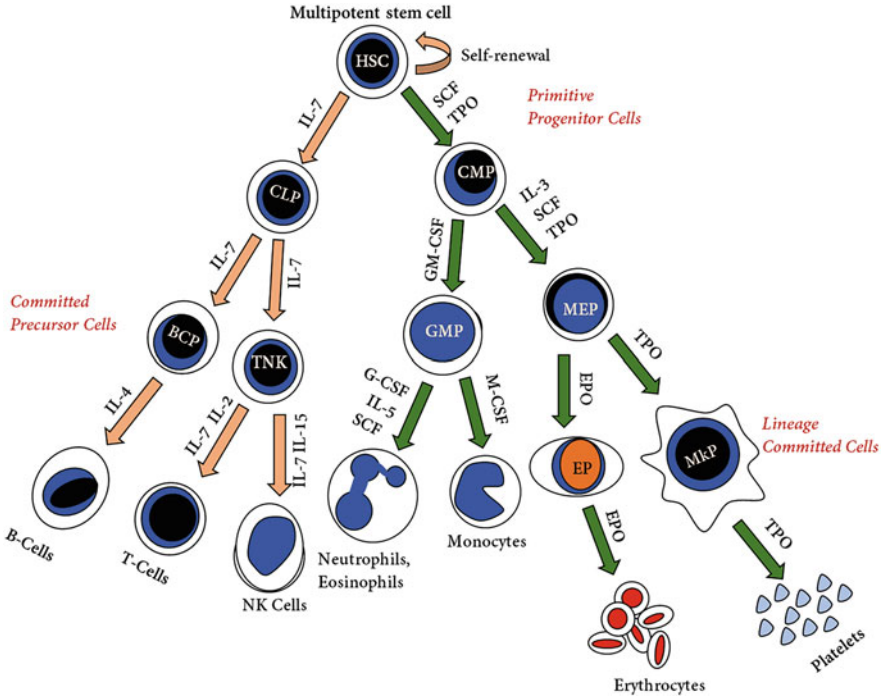

Fig. 3 A schematic representation of the lineage adopted by hematopoietic stem cells. Essentially, they are capable of both self-renewal and/or differentiation when the signal is received. The CLP (common lymphoid progenitor) and CMP (common myeloid progenitor) are considered to be primitive progenitor cells whereas BCP (B cell precursor), TNK (T and natural killer cell precursor), GMP (granulocyte-macrophage progenitor), MEP (megakaryocyte erythroid progenitor), and BCP (B cell progenitor) are partially differentiated progenitor cells. Different cytokines promote differentiation and are controlled by a cascade of the signaling pathway

granulocyte colony-stimulating factor, and granulocyte-macrophage colonystimulating factor, are now been used in routine clinical practice to stimulate cell production, and thus are routinely employed in patient management.

Hematopoietic cytokines are a large family of extracellular ligands that stimulate hematopoietic cells to differentiate into eight principal types of blood cells. Hematopoiesis requires the synergistic action of several cytokines that act both as positive and negative regulators functioning within a complex network of actions. Some cytokines have very narrow lineage while many others have rather broad and overlapping specificity ranges. Cytokines whose predominant action appears to be the stimulation or regulation of hematopoietic cells include GM-CSF, G-CSF, M-CSF, interleukins, EPO, and TPO. There are several other cytokines that exert profound effects on the formation and maturation of hematopoietic cells, which include stem cell factor (SCF), flt-3/flk-2 ligand (FL), and leukemia inhibitory factor (LIF). Other cytokines or ligands such as jagged-1, transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α) also play significant roles in modulating hematopoiesis (Fig. 3).

5.1 Cancer Stem Cells

Cancer is characterized as a monoclonal disease originating from a single apparently transformed cell. It is accepted that a single cell undergoes transformation and then escapes immune surveillance and continues to proliferate to form a colony of cancerous cells. Despite great advances in our understanding of tumor initiation and progression, the identity of the "cell of origin" of cancer remains elusive. It is now established that these cells have self-renewal properties analogous to stem cells. The cancer stem cells (CSC) constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and maintain the tumor. These cancer stem cells have the capacity to both divide and expand the cancer stem cell pool and to differentiate into the heterogeneous non-tumorigenic cancer cell types that in most cases appear to constitute the bulk of the cancer cells within the tumor (Clarke et al. 2006). Solid tumors are an enormous cancer burden and a major therapeutic challenge. The cancer stem cells (CSC) provide important cues to cellular mechanisms that may explain the probable reasons for the chemotherapy refractoriness and dormant behavior often observed for many of these neoplastic growths. There is increasing evidence that diverse solid tumors are hierarchically organized and sustained by a distinct subpopulation of CSCs (Visvader and Lindeman 2008).

An alternative theory for the origin of CSCs suggests that they arise from normal somatic cells that acquire stem-like characteristics and malignant behavior through genetic and/or heterotypic alterations. For example, cancer cells gain stem-like characteristics through epithelial-mesenchymal transition (EMT). If cancer stem cells are relatively refractory to therapies that have been developed to eradicate the rapidly dividing cells within the tumor that constitute the majority of the non-stem cell component of tumors, then they are unlikely to be curative and relapses would be expected. If correct, the cancer stem cell hypothesis would require that we rethink the way we diagnose and treat tumors, as our objective would have to turn from eliminating the bulk of rapidly dividing but terminally differentiated components of the tumor and refocused on the minority stem cell population that fuels tumor growth (Clarke et al. 2006). Several cell surface markers such as CD44, CD24, and CD133 are often used to identify and enrich CSCs. A network comprising microRNAs along with Wnt/β-catenin, Notch, and Hedgehog signaling pathways regulates the CSC properties. Since CSCs are resistant to different types of chemotherapies and radiation treatments, it is hypothesized that CSCs play a crucial role in cancer metastasis. CSCs are believed to be an important target for novel anticancer drug discovery (Yu et al. 2012). Initially, CSCs were believed to represent a small fraction of the total cell population in a solid tumor; however, it has been claimed that as many as 25% of cancer cells may have the properties of CSCs. An alternative theory for the origin of CSCs suggests that they arise from normal somatic cells which acquire stem-like characteristics and malignant behavior through genetic and/or heterotypic alterations. For example, cancer cells gain stem-like characteristics through epithelial-mesenchymal transition (EMT). The induction of EMT in immortalized human mammary epithelial cells (HMLEs) resulted in the acquisition of

mesenchymal traits and expression of stem-cell markers, which are similar to those stem cell-like cells isolated from HMLE (Yu et al. 2012).

It has been reported that freshly isolated patient-derived $CD133^+$ CSC were highly tumorigenic. When as few as 500 cells were implanted in athymic mice, orthotopic tumor formation was observed. In contrast, as many as 10^6 CD133-tumor cells did not result in any tumor formation. Most importantly, it was shown that CSC does not represent a homogeneous population of tumor-initiating cells. Instead, it was defined that a subpopulation of migrating CSC that were characterized by expression of the CXCR4 receptor and are critically involved in tumor metastasis. Indeed, the elimination of this subpopulation of CSC virtually abrogated the metastatic activity of CSC. It is thus confirmed that cancer stem cells arise from normal stem cells possibly by a series of mutations. Some evidence also indicates that CSC may also arise from mutations of the progenitor cells. Cancer stem cells are capable of undergoing symmetric and asymmetric divisions, which consequently leads to the clonal expansion of the tumor cells. This leads to the production of a population of the progeny of differentiated cells that constitute a major portion of the tumor cell mass. CD133 is universally expressed in many cell types that include cells of normal tissue as well as hematopoietic stem cells. As demonstrated for hematopoietic and endothelial progenitors, CD133 is also expressed on early progenitors but usually is no longer detectable upon differentiation (Singh et al. 2004). In tumor cells, CD133 has been used for the identification of a subpopulation of highly tumorigenic cells as demonstrated for neural cancers and, more recently, also for cancers of the colon (Singh et al. 2004). The expression of CD133 is no longer expressed on undifferentiated tumor cells once they undergo differentiation. Thus, the hypothesis is that a subpopulation of CD133+ cells that bear self-renewal capacity as they can be clonally expanded are exclusively tumorigenic and is able to differentiate into CD133 positive tumor cell clone.

CSCs were believed to represent a small fraction of the total cell population in a solid tumor; however, it has been claimed that as many as 25% of cancer cells may have the properties of CSCs. There are several different explanations regarding the origin of CSCs. As discussed earlier, one of the strongest theories is that genetic mutation or environmental alterations is the primary cause that induces the transformation of CSCs from normal stem/progenitor cells and then develops the ability to generate tumors. Some CSCs exhibit similarities to normal stem/progenitor cells in cellular property, phenotype, function, and even cell surface markers. It is becoming evident that a cancer treatment that fails to eliminate cancer stem cells may allow the regrowth of the tumor. In cases of disease relapse post-eradication of the tumor tissue by chemotherapy indicates that cancer stem cells remain viable after the destruction of the tumor tissue. Recent studies have indicated that CSCs have the intrinsic characteristics to become resistant to cancer therapies. Cancer research attempts to identify mechanisms that drive drug resistance. Similar to normal stem cells, the CSCs confer the abilities of self-renewal and can serve as a core reservoir of cells, thus making the disease of cancer incurable. The overexpression of stem cell-specific transcription factors may contribute to the pathological self-renewal characteristics of cancer stem cells while the surface molecules mediate interactions between cells

and their microenvironment. Several other studies implicate that there are several other stemness-related markers and proliferative pathways that may promote cancer development and maintenance. Therapeutic strategies that specifically target cancer stem cells are urgent and should eradicate tumors more effectively than current treatments and reduce the risk of relapse and metastasis.

5.1.1 Cancer Stem Cell Isolation

The existence of cancer stem cells (CSC) has been documented for the past two decades. However, the studies related to CSCs pave a number of issues due to rare cell populations and difficulties in their isolation which for the most part due to their similarities to common stem cell markers. CSCs are frequently found in the core of solid tumors and their microenvironment plays an important role in tumor maintenance, renewal, division, and development. Thus, in vivo tracking is an important technique for functional studies of cancer stem cells. CSCs are commonly resistant to chemotherapy and can introduce dormancy in tumor cells for extended periods of time. Therefore, proper detection, isolation, and characterization of the undifferentiated stem cells residing within solid tumors are the potential markers for the development of cancer-targeted therapies in recent years. Remarkable similarities and identities between normal and cancer stem cells make it a challenge to delineate specific methods or markers to distinguish them individually and identify markers that can be therapeutically challenged. Tumors generally originate from the transformation/alterations of normal stem cells; hence some markers continue to be common to both types of stem cells. In a similar vein, the signaling pathways may regulate self-renewal in stem cells and cancer cells, and thus cancer cells may include "cancer stem cells" – a rare type of cells with indefinite potential for self-renewal that induces tumorigenesis. Due to ethical reasons, it has been difficult to isolate cancer stem cells from human patients. During primary resection of the neoplastic tumor, the isolation must be organized with the laboratory team which is often difficult in hospital settings. Most of our knowledge regarding the characteristics and salient properties of CSC has been gathered from mouse xenograft studies.

Mouse Xenografts CSC Isolation (Adopted: Dobbin and Landen 2013)

CSCs constitute a small subset of the cancer cells in a heterogeneous tumor. As such, it is necessary to dissociate a tumor sample into a single-cell suspension to be able to isolate CSCs from the rest of cancer. Although some studies have taken advantage of marker-positive and -negative populations identified within cell lines, it is generally believed that for a population to be considered a CSC, increased tumorigenicity from a patient-derived tumor and the capacity to reproduce the original tumor are required. This adds additional challenges, as dissociation of a solid tumor into a single-cell population is traumatic to cells and time-consuming. In this method, it will be described how to mechanically dissociate a tumor tissue sample that comes either from a human patient or a mouse xenograft.

5.1.2 Materials

- Tumor sample preferred at least 1 cm of viable tissue
- Serum-free RMPI cell culture media (or preferred media)
- 10 cm Petri dish
- Scalpel handle and #22 blade
- 50 ml conical tube
- Tissue forceps
- 70 μm sterile mesh filter
- 10 ml, 5 ml, and 1 ml pipette tips
- 16-gauge needles and 3–5 ml syringes
- Hemocytometer, trypan blue

5.1.3 Protocol

- Start processing the tumor without delay to maximize viability. Ideally, post-tumor resection, the tumor should immediately be subjected to processing which should begin within 30 min of removal. If this can be achieved, then the tumor tissue can be implanted in mice or culture plates in another 30–60 min. Sometimes, this is not possible due to the unavailability of the personnel and lateness in pathologic review. Prior collaboration should be initiated between the surgeon and the pathologist to prevent any compromise in patient care and early retrieval of the excised tissues. On the other hand, mouse tumors can generally be obtained more quickly, as they are resected immediately after sacrifice, can be identified grossly, and processing can begin immediately.
- Patient or mouse-derived tissue specimens should be placed in a 10-cm dish with 1 ml cold media. The dish must be kept on ice or an ice pack during dissection.
- The solid mass has to be next disintegrated/minced using forceps to hold the specimen firmly and use the back of a number 22 scalpel blade to scrape the specimen downward, and away, such that cells are loosened from the tumor mass into the 10-cm dish. During this process, strands of connective tissue can be isolated, and should ideally be removed from the cell collection dish.
- The process of scraping should be continued till the specimen is exhausted and can no longer be held with the forceps and a large "slurry" population has been collected in the 10-cm dish.
- The cell slurry is further disintegrated first with a 10 ml serological pipette, then to a 5 ml pipette, then a 1 ml pipette, until the slurry passes easily in and out easily.
- A 16-gauge needle on a 3- or 5-ml syringe is then used for complete mechanical dissociation.
- At this point, the cells should be ready for downstream experiments such as injection into mice, or for tissue culture. If a single-cell suspension is required for performing flow cytometric analysis, the next few steps are necessary.
- The cell suspension has to be next filtered using a 70-μm filter placed on top of a 50 ml conical tube. The cell suspension should be poured slowly onto the filter and allow the cell suspension to accumulate in the collection tube. Clogging can happen quickly if the suspension has largely unbroken tissue mass; thus, the prior

steps are extremely important. In such eventuality, the suspension should be passed through 200 μm and 100 μm filters, and then only through 70 μm filters.
- After collection of the entire cell suspension, 50 μl of the cells are taken out and used for counting and assessment of the viability. The remaining cell sample is centrifuged at 3000 rpm (1500 × g) for 10 min at 4 °C.
- While samples are centrifuging, the viability and cell number in the suspension are determined by using a hemacytometer and trypan blue staining.
- Media must be aspirated off followed by resuspension of the cellular pellet in cell culture media or PBS as required for downstream applications. The appropriate volume to be used is dependent on the applications to be utilized at the later time point.

5.1.4 Alternate Protocol Mouse Xenografts CSC Isolation: Chemical Dissociation (Adopted: Dobbin and Landen 2013)

The decision to use a mechanical-based dissociation or a chemical-based dissociation is primarily a personal preference and is also guided by the density of the tumor. Avoiding chemical dissociation is always a better preference, as this can be corrosive, and has been reported to reduce viability when adding this method. Some tumor types are more amenable to mechanical dissociation with high viability while others tend to be more appropriate for chemical digestion. This varies not only with tumor type, but the site of collection and patient heterogeneity. Therefore, the decision to add chemical dissociation is often a case-by-case basis, depending on the success of mechanical dissociation. If the tumor is especially dense, starting with chemical dissociation may provide maximal yield and viability. Alternatively, a combination can be used, whereby cells are first mechanically dissociated using the above protocol, followed by chemical digestion of firm residual tumor that was not released by this method. The principles of chemical dissociation are using an enzyme to digest the physical bonds between the tumor cells and the extracellular matrix.

5.1.5 Materials

- Enzymatic digestion solution (0.25% Trypsin/EDTA). The ideal digestion solution will depend on cell type to maximize viability. Commonly used digestion solutions include hyaluronidase at 0.05 mg/ml and collagenase at 0.5 mg/ml.
- Tumor fragments
- RPMI-1640 medium with 10% fetal bovine serum
- 1X PBS
- Trypan blue
- 70 μm sterile mesh filter
- 10 cm Petri dish
- No. 22 scalpel blade and blade handle
- 50 ml conical tube
- Tissue forceps
- 10 ml, 5 ml, and 1 ml serological pipette

5.1.6 Protocol

- After extraction of the tumor from the mouse or the patient, place the tumor in 10 ml of 1X PBS in a 50 ml conical tube and store the sample on ice until ready to begin dissociation
- Add 10 ml, 0.25% trypsin/EDTA with or without hyaluronidase and collagenase to the 50 ml conical tube. If the sample is small, can use 5 ml PBS and 5 ml chemical digestion solution in a 10 ml conical tube.
- Begin to dissociate the sample using the No. 22 scalpel blade and cut up the tumor sample in a chopping motion taking care not to crush the sample.
- Place the sample in the conical tube and incubate at 37 °C for 20 min.
- Further dissociate the tumor sample by pipetting up and down the solution using a 10 ml serologic pipette until it passes freely, then a 5 ml and 1 ml pipette.
- Neutralize the trypsin-cell solution with 20 ml, RPMI-1640 medium with 10% FBS (or 10 ml in a 15 ml conical tube).
- Using the 5-ml pipette, pass the cell suspension through a 70 μm sterile mesh filter placed over a new 50 ml conical tube to generate a single-cell suspension
- Once the entire cell suspension has been collected, collect 50 μl to use for counting and assessment of the viability, and centrifuge the remaining sample at 3000 rpm (1500 × g) for 10 min at 4 °C.
- While samples are centrifuging, determine the number and viability of cells in the suspension using a hemocytometer and trypan blue exclusion.
- Aspirate off media and resuspend the cellular pellet in cell culture media or PBS as desired for downstream applications, in the appropriate volume needed for these applications, based on cell density calculated by trypan blue exclusion.

5.1.7 Cancer Stem Cell Markers

Cancer stem cells have been appreciated as a small subpopulation of cells within tumors with capabilities of self-renewal, differentiation, and tumorigenicity when transplanted into an animal host. CSCs similar to adult and embryonic stem cells may express markers that are not expressed in normal somatic cells and are thus marked to contribute toward a "stemness" phenotype. Thus, stemness-related CSC surface markers that are highly expressed in stem cells are also expressed in human cancers include TRA-1-60, SSEA-1, EpCam, ALDH1A1, Lgr5, CD13, CD19, CD20, CD24, CD26, CD27, CD34, CD38, CD44, CD45, CD47, CD49f, CD66c, CD90, CD166, TNFRSF16, CD105, CD133, CD117/c-kit, CD138, CD151, and CD166. Among these, CD44 and CD133 are the most widely used markers in CSC research and are therapeutic targets in cancers. Of many cell surface markers, CD44, CD24, and CD133 are often used to identify and enrich CSCs within the niche of the tumor microenvironment. Several stemness-related markers have recently been identified and in fact, they are neither transcription factors nor cell surface proteins and include ALDH, Bmi-1, Nestin, Musashi-1, TIM-3, and CXCR. Furthermore, a regulatory network consisting of microRNAs and Wnt/β-catenin, Notch, and Hedgehog signaling pathways have also been documented to control the CSC properties. Emerging scientific evidence (Yu et al. 2012) has strengthened the clinical relevance of CSCs.

5.1.8 Stem Cell Therapy and Remaining Challenges

Although there are some successes documented so far, there are several major challenges that must be addressed before stem cells can be used as cell therapies to treat a wider range of diseases.

First and foremost is the identification of an abundant source of stem cells. Identifying, isolating, and growing the right kind of stem cell with proper stimulation and differentiation, particularly in the case of rare adult stem cells, is an extremely painstaking and difficult process. Pluripotent stem cells, such as embryonic stem cells, can be grown indefinitely in the lab and have the advantage of having the potential to become any cell in the body, but these processes are again very complex and have been tightly regulated. Induced pluripotent stem cells, while promising, are also limited by these conditions. In both cases, considerable work remains to be done to ensure that these cells can be isolated and used safely and routinely within a clinical environment.

Second, as with organ transplants, a close match between the donor tissue and the recipient is crucial; the more precisely the tissue matches the recipient, the lower the risk of rejection. This would ensure that the recipient could avoid the lifelong use of immunosuppressants. The discovery of iPS cells has opened the door to developing patient-specific pluripotent stem cell lines that could later be developed into a needed cell type without the problems of rejection and immunosuppression that occur from transplants from unrelated donors.

Third, a system for delivering the cells to the right part of the body must be developed. Once in the right location, the new cells must then be encouraged to integrate and function together with the body's other cells. The immune reaction post-transplantation is another aspect that requires careful vigilance. Thus, a comprehensive management plan has to be developed to successfully include stem cell therapy as a routine clinical treatment.

Although many signs of progress have been made in delineating the molecular basis of cancer, the progress in cancer detection and treatment, mortality is still high and there is no definitive cure despite great improvements made in various therapies. Stem cell-based regenerative therapies have raised hopes for novel therapeutic approaches. Stem cells are cells that can perpetuate themselves through their ability of self-renewal and to generate mature cells of a particular tissue through differentiation.

6 Conclusions

Stem cells are the foundation for every organ and tissue in the human body. There are many different types of stem cells originating from different tissues within the body and are generated at different stages of growth and development. The embryonic stem cells that exist only at the earliest stages of development are important as various types of tissue-specific stem cells that appear during embryonic development and continue to remain in human tissues throughout life. Stem cells occur in many different somatic tissues and are important participants in cellular physiology. Cell

populations derived from stem cells are organized in an ordered fashion, with the stem cell placed right at the apex of the developmental pathway. Stem cells have three distinctive properties: self-renewal, the ability to develop into multiple lineages, and the capacity to proliferate extensively. The combination of these three properties makes stem cells unique and different from matured differentiated somatic cells.

The specialized class of somatic stem cells or adult stem cells supports and serves as an internal repair system that generates replacements for cells that are lost through normal wear and tear injury, or disease throughout the life of an organism. Populations of adult stem cells have been identified in many organs and tissues and are generally associated with specific anatomical locations. These stem cells may remain quiescent (nondividing) for long periods until activated by a normal need for more cells to maintain and repair tissues.

Every day, new scientific evidence indicates that stem cells are present in many more tissues and organs than once thought of. These stem cells are capable of developing into more kinds of cells than previously perceived. Efforts are being invested to harness stem cells and to take advantage of self-renewal capabilities, with the goal of devising new and more effective treatments for several diseases. The future of stem cell biology is still unclear but it is definite that there are vital research questions to be answered and that those answers will hold great promise for the future of mankind.

To effectively administer stem cell therapies to diseases, stem cell researchers manipulated stem cells so that the cells have the necessary characteristics for successful differentiation, transplantation, and engraftment. A stupendous degree of research is in progress to develop procedures to utilize stem cell populations for the betterment of human health and include procedures like the induction and development of blood vessels (vascularization) for the regeneration and repair of solid tissues. The attribute of self-renewal is especially notable because its subversion is highly relevant to oncogenesis and malignancy. Increased self-renewal capacity, along with the inherent growth potential of the stem cells, might also be the primary cause of the development of aberrant cell growth which can be alternatively considered a neoplastic phenotype. Stem cell therapies although having tremendous promise is still in their infancy. It still assures hope for the future.

7 Cross-References

- ▶ Common Reagents and Medium for Mammalian Cell Culture
- ▶ Culture of Neuron and Glia Cells
- ▶ Isolation and Primary Culture of Various Mammalian Cells
- ▶ Mammalian Cells, Tissues and Organ Culture: Applications

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