Stem Cells and the Basics of Immunology

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What You will Learn in This Chapter and Associated Exercises

Students will learn about different types of stem cells and their role in tissue engineering applications, including examples of cell type–specific differentiation protocols. A brief overview of immunology as applied to implantation of engineered tissue will be given. The concept of induced pluripotency and its implication for the field will be introduced. Students will then practice immunostaining their own samples using given choice of primary and secondary antibodies.

8.1 Concept of Stem Cell Potency

Stem cells can serve as precursors to many types of specialized cells and are capable of unlimited division without losing their stemness (Fig. 8.1). *Potency* specifies stem cell potential to differentiate into various cell types. Different degrees of stem cell potency exist. *Totipotent cells* are found in the very early morula stage of the embryo and are capable of forming all of the body cells as well as the placenta. *Pluripotent cells* originate from the inner cell mass of the blastocyst. They cannot form the placenta but are capable of forming all cells of the body. *Multipotent stem cells* can produce several types of cells functionally related to each other. *Oligopotent stem cells* can differentiate into only a few, usually phenotypically related, cell types. Finally, *unipotent cells* can serve as a continuous source of one cell type.

8.2 Embryonic Versus Adult Stem Cells

The previous paragraph described stem cells in terms of their potency. Another way to classify stem cells is by their developmental stage. There are two main categories: *embryonic* and *adult stem cells*. Embryonic cells, commonly abbreviated ESCs, are pluripotent cells that can be obtained from the inner mass of a blastocyst. They are capable of forming all cell types of the developing embryo except the placenta; so ESCs are *pluripotent* as per the definition given in the above section.

Adult stem cells, also sometimes called *resident stem cells*, reside within each type of tissue and can be either *multipotent* or *oligopotent*. They exist to replenish differentiated cells within each tissue type. Differentiated cells are very specialized units of each tissue, which constitute most of each tissue's cellular content. They are there to

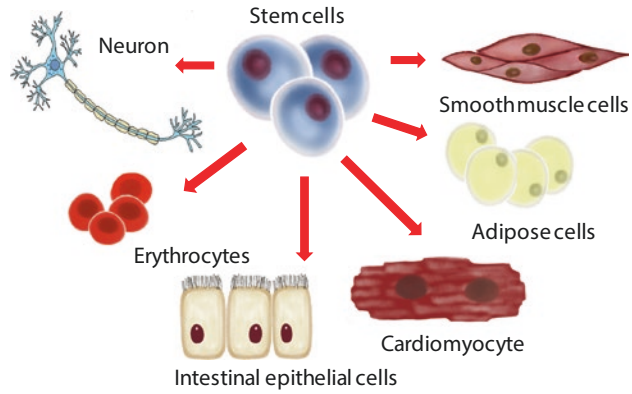

Fig. 8.1 A cartoon showing stem cells yielding different specialized cells

perform tissue-specific functions. Hepatocytes, for example, specialize in protein synthesis, neurons in conducting electrical impulses, smooth muscle cells in creating vessel wall tension, ventricular myocytes in heart contraction, and so on.

Fully differentiated cells are not capable of many divisions. Therefore, to replenish cells that are lost or damaged, all tissues contain a small amount of resident stem cells. Resident stem cells are responsible for maintaining a constant supply of newly differentiated cells in each tissue type. The rate of such cell renewal dramatically differs between the tissues—being very high, for example, for hematopoietic stem cells found in bone marrow, while being negligible for neurons or cardiac muscle. There are also *tissue-specific progenitor cells*. These cells give rise to specialized cells, but their capacity for self-renewal is limited.

8.3 Stem Cells in Tissue Engineering

The physiological rate of tissue renewal using progenitors or resident stem cells is not nearly sufficient to deal with large-scale tissue damage or anatomical defects. To create a sizable chunk of new tissue, the amount of newly differentiated cells needs to increase dramatically. For example, to repair an average human infarcted tissue, more than a billion new cardiac muscle cells are required.

Thus, to create an in vitro engineered tissue, one needs to first isolate stem cells and then amplify them. Among those initial sources can be embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), induced pluripotent stem cells (more below), or tissue-specific resident stem cells. Stemness of these cells ensures their ability to unlimited proliferation (Fig. 8.2). Once the desired amount of stem cells is achieved, they can be then differentiated into cells of choice for that specific tissue type. The last step is to seed these cells into scaffolds and culture them, so they can form a tissue (Fig. 8.3).

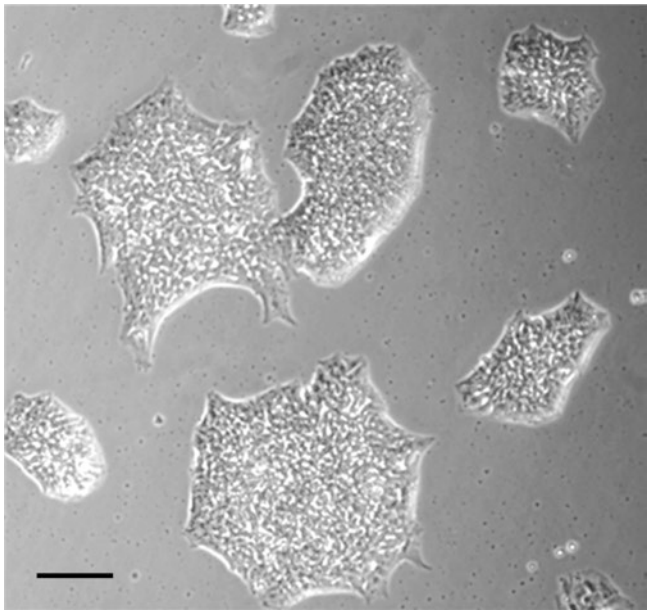

Fig. 8.2 Colonies of undifferentiated human embryonic stem cells that can be split and passaged multiple times without losing their stemness. Scale bar: 100 micron

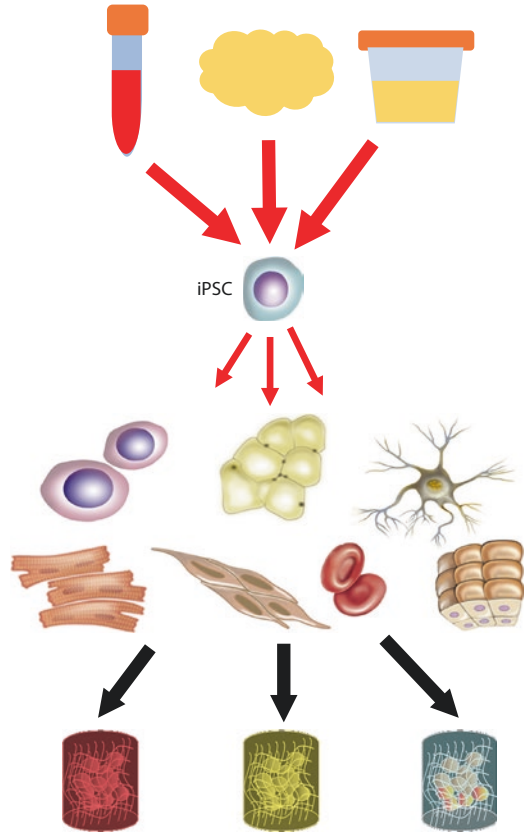

Fig. 8.3 Schematic representation of how patient's specific engineered tissues can be derived from stem cells found in blood, fat, or urine

It is important to note that in vivo, stem cells are located within spatially highly organized stem cell niches. This enables to keep their proliferation rates physiologically controlled. Such niches are yet to be created in ex vivo engineered tissues. Therefore, in cases when a piece of newly engineered tissue contains stem cells that are not fully differentiated, its implantation poses a high risk of teratoma formation. This is because, just like in case of cancer cells, stem cells lack mechanisms of self-limiting their proliferation.

8.4 Mesenchymal Stem Cells (MSCs)

MSCs are isolated from a fully developed organism; therefore, they belong to the category of adult stem cells. All organs contain MSCs and use them for local regeneration. One of the main ethical advantages of MSCs is that their isolation does not require the destruction of the embryo. MSCs are lineage-specific and less teratogenic than ESCs, but they also have limited longevity in culture and are capable of generating only a limited number of organ-specific cell types. Current tissue engineering protocols focus mainly on adipose tissue-derived MSCs (Ad-MSCs) due to their abundance, ease of access, and relative plasticity (Fig. 8.4).

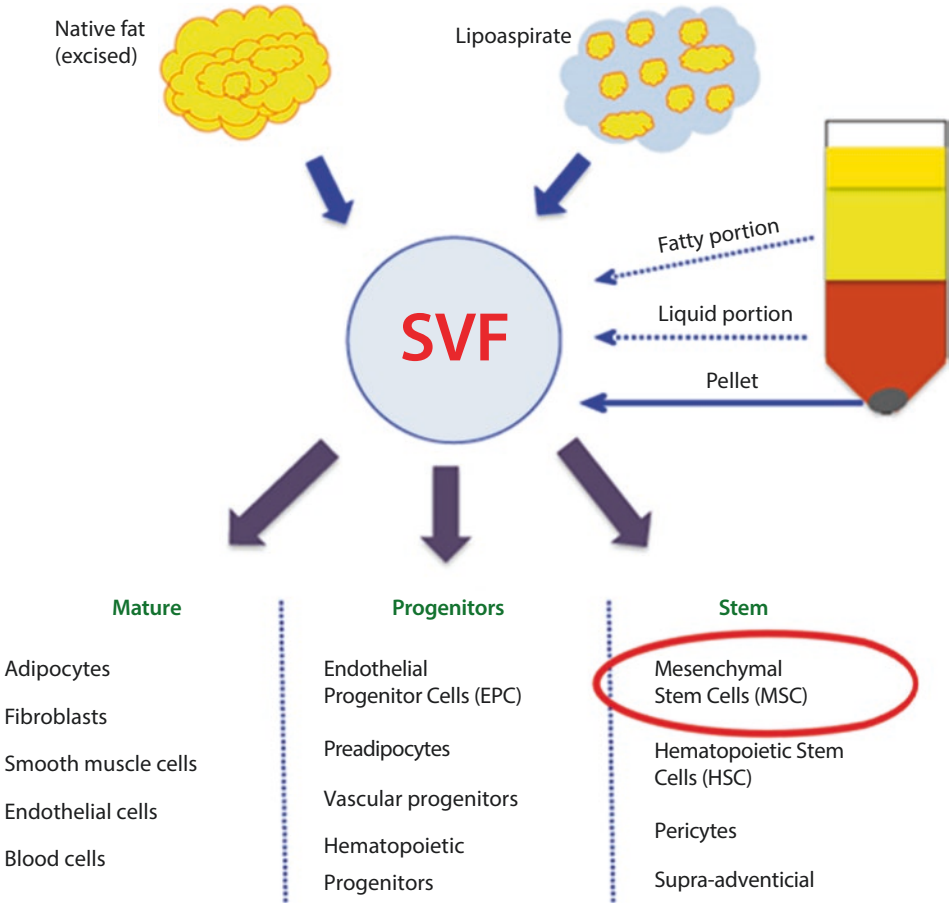

Fig. 8.4 Types of cells that can be derived from Ad-MSC

8.5 Basic Protocols for Maintenance and Growing Stem Cell Colonies

Undifferentiated stem cell colonies are usually grown using mouse embryonic fibroblast-conditioned medium (MEF-CM). More recently, several artificial media that do not use animal-derived products have also been developed. Stem cell colonies are often cultured on *layers of feeder cells*. The latter are fibroblast-like cells that provide attachment and other factors needed for stem cell maintenance. There are also protocols that enable culturing stem cells in *feeder-free conditions*. For example, primary adipose tissue-derived stem cells (Ad-MSCs) can be cultured under feeder-free conditions, using tissue culture-treated plates and daily supplementation with growth medium and human serum. Isolated, sparsely plated cells are grown to form individual colonies. Afterward, they are re-plated every 3–5 days to maintain pluripotency.

8.6 How to Differentiate Stem Cells into Tissue-Specific Phenotypes

As noted above, transplanting undifferentiated pluripotent stem cells can be dangerous because these cells are teratogenic. It was also shown that placing stem cells in the environment of an organ does not necessarily "educate" them to become specialized cells of that tissue/organ. Instead, these cells form a cluster, which does not engraft with the rest of the tissue. In order to avoid such problems, pluripotent cells need to be pre-differentiated before transplantation.

There are many published protocols that detail the sequence of steps for exposure of pluripotent cells to specific active molecules in the growth media that direct the conversion of stem cells into cardiomyocytes, hepatocytes, or neurons, to mention a few. Many factors, including the density of culturing, passaging, and re-seeding times, can affect the differentiation process. Below we give examples of three detailed differentiation protocols.

I. *To induce the development of cardiomyocytes.* The cell line of undifferentiated human ESC (hESC) is expanded under standard feeder-free conditions using mouse embryonic fibroblast-conditioned medium (MEF-CM) supplemented with human bFGF (4 μg/L). When undifferentiated hESC colonies occupy approximately two-thirds of the surface area, they are dispersed using 0.2 mL/cm^2 Versene and re-plated to form a dense monolayer. After a confluent monolayer is formed, hESCs are switched to the RPMI-B27 medium and serially pulsed with 100 μg/L activin A on day 1 and bone morphogenetic protein-4 (BMP4, 10 μg/L) from days 1 to 5. Thereafter, the differentiating cultures are grown in the RPMI-B27 medium supplemented with insulin and vitamin A. Spontaneous beating activity commenced on days 10–20. *More details in ref* [1].

II. *To induce the development of hESC into definitive endoderm (DE) and subsequently hepatocytes.* Stem cells are cultured in serum-free conditions with the RPMI medium containing activin A (100 ng/mL), Wnt3a (50 ng/mL), 2 mM L-glutamine, and 1% penicillin/streptomycin for 24 h. Then the medium is changed to the RPMI medium with activin A (100 ng/mL), sodium butyrate (0.5 mM), and B27 supplement for 6 days. DE cells are then passaged on collagen type I-coated 12-well plates in IMDM supplemented with 20% fetal bovine serum, FGF4 (20 ng/mL), hepatocyte growth factor (HGF) (20 ng/mL), BMP2 (10 ng/mL), BMP4 (10 ng/mL), 0.3 mM L-thioglycerol, 0.5% dimethyl sulfoxide, 100 nM dexamethasone, and 0.126 U/mL human insulin for 2 weeks. *More details in ref* [2].

III. *Neuronal differentiation* can be induced via two methods: embryonic body (EB) formation or growth of adherent neurons. To make EBs, stem cells are trypsinized, counted, and grown in suspension (5×10^6 cells in 10 mL) in a 100 mm Petri dish. This culture is then placed on a shaker at 50 rpm in a 37 °C incubator. After 2 days, 20 μl of 10 mM all-trans retinoic acid is added and incubated with shaking for an additional 3 days. At day 5 formed EBs contain pre-committed neurons, which can be grown out by plating them onto poly-L-ornithine and fibronectin coated-dishes and grown in a neurobasal medium with B27, bFGF, and EGF. *Details in ref* [3].

Alternatively, to obtain adherent differentiated neurons, 10,000 ESCs are plated to an 8-well chamber slide coated with 0.1% gelatin. These cells are then cultured in Neurobasal/F12/DMEM supplemented with B27, glutamate, and N2 supplement. *Details in ref* [4].

8.7 Basic Immunology of Graft Rejection

Let's say we were able to isolate stem cells, amplify them to a desired quantity, differentiate them into a chosen cell phenotype, and finally make a piece of functional tissue (more details as to how this last step is achieved can be found in ▶ Chap. 9). When this engineered piece of tissue will be implanted into an experimental animal or a human patient, it is important to consider how the immune system of the recipient will react to it. Below we will briefly describe the basics of immunology as it applies to grafting [5]. There are three phases of acquired immune rejection. Hyperacute rejection occurs within minutes of transplantation. In this case, rejection is induced by pre-existing host antibodies that bind to the graft's antigens. This binding activates the complement system and a sequence of events ensues, including an influx of peripheral blood mononuclear cells, the formation of platelet thrombi, small vessel thrombosis, and finally, damage and/or destruction of the graft. Acute rejection occurs within 1–2 weeks after transplantation and is characterized by capillary rupture and severe graft infiltration by monocytes/macrophages, lymphocytes, and dendritic cells. This reaction is mediated by cytotoxic T lymphocytes. Chronic rejection occurs months or years after transplantation and is associated with deposits of immunoglobulin and C3 complement molecules on the basement membrane of graft cells. When using stem cell-derived grafts, all three phases of rejection should be considered, but acute rejection is the most important. Therefore, let's consider it in more detail.

Transplantation of a graft made of cells from a genetically unrelated organism (called allogeneic cells) results in a robust immune response and, consequently, graft rejection. T-lymphocytes recognize foreign antigens in the form of peptides, which are presented in association with self-major histocompatibility complex (MHC) molecules. There are two primary classes of MHC molecules. MHC class I antigens are found on the surface of every nucleated cell; they display fragments of proteins synthesized within the cell to cytotoxic T lymphocytes (CTL) or $CD8^+$ T cells. Cell surface expression of MHC I antigens is the predominant reason for immune detection and rejection of allogeneic grafts. In comparison, MHC class II antigens are expressed only on a few specialized cell types, including macrophages, dendritic cells, and B lymphocytes. These cells are called professional antigen-presenting cells. The function of MHC II molecules is to display peptides of exogenous proteins to T-helper cells or $CD4^+$ T cells. Direct allorecognition arises when both types of host T-lymphocytes ($CD8^+$ and $CD4^+$) are stimulated by donor antigen-presenting cells. This stimulation occurs through a direct interaction between host T-lymphocyte receptors and MHC I and II antigens expressed on the surface of donor cells (◘ Fig. 8.5). Indirect allorecognition occurs when donor MHC peptides are processed and then presented by host MHC II molecules. Host antigen-presenting cells internalize and break down donor MHC molecules, display those peptides onto their MHC II molecules, and present them to host $CD4^+$ T cells. This process triggers an immune response against grafts expressing

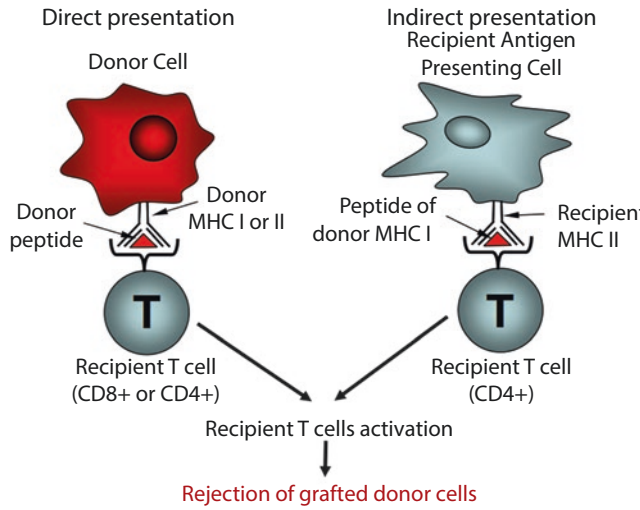

Fig. 8.5 Schematic representation of direct and indirect recognition of donor antigens to the recipient T cells

donor MHC, both I and II. All-in-all, the presence of MHC molecules is the defining factor that largely determines the degree of acute graft rejection.

8.8 Induced Pluripotent Stem Cells

Until very recently, our ability to amplify and differentiate human stem cells was focused on stem cells obtained from discarded human embryos, freshly excised pieces of tissue from surgical procedures, or from recently deceased donors. In all these three cases, engineered tissues made from these cells will be rejected by the recipient's immune system as per the previous paragraph. The only solution to this problem was to (a) find the best match as far as immunocompatibility and (b) subject graft recipient to a lifelong regimen of immunosuppression drugs. Fortunately, sustained research efforts in the cell biology field led to a giant breakthrough. In 2006, a team of Japanese researchers led by Shinya Yamanaka published a study [6] in which they successfully reprogrammed intact mature cells from connective tissue into stem cells by introducing only a few genes. Synthesis and action of four regulatory proteins (OCT3/4, Sox2, c-Myc, and Kfl4) were sufficient to govern such de-differentiation. These regulatory proteins have since been called Yamanaka factors. Next came a possibility of what is called "direct reprogramming," a procedure that enables one to convert specialized cells of one type into cells of another tissue without going back to stem state. This approach requires genetic or chemical modifications and is called re-differentiation. It allows to save time in generating patient-specific cells and is currently one of the most actively developing branches of cell biology [7, 8].

Stem cells that were reprogrammed from a patient's own fully differentiated cells are called *induced pluripotent stem cells* or *iPSC*. Today, multiple types of differentiated cells have been reprogrammed into iPSC, including fibroblasts, white blood cells, adipocytes, epithelial kidney cells, and many others.

Discovery of iPSC means that it is now possible to derive a person's own pluripotent stem cells from small samples of their blood, skin, fat, or even urine. iPSC obtained from these samples can be then amplified in vitro and re-differentiated into

desired specific types of cells. In layman terms, it is now possible to derive a person's own nerve or heart cells from urine sample. This has been a truly exciting development from the perspectives of both science and medicine as it turns humanity's dream of creating entirely new organs from a person's own cells into a reality. It has also opened the doors to what is now called personalized drug testing. The latter uses engineered tissue made from an individual's own cells to test the effects of the drugs and their combinations.

As of today, protocols to create engineered tissues from iPSC are very time-consuming and labor-intensive. They are also very expensive. However, every year, thanks to efforts of thousands of researchers from across the world, these protocols are becoming more and more affordable, reproducible, and scalable. This progress enables the hope of using iPSC-derived tissues for clinical treatments in a not-so-distant future.

8.9 Immunohistochemistry/Immunostaining

Epitope recognition by specific antibodies developed by the body can be used as a method to identify certain proteins within or on the surface of the cells. It involves immunization of an animal with an epitope of interest (which can be a full protein or its fragment). Immunoglobulins that develop against the epitope are called *primary* antibodies. *Secondary* antibodies can then be used to recognize *primary* antibodies based on antibody type and the animal in which the primary antibody was developed in. The secondary antibody can have a fluorescent label or an enzyme attached to it, enabling colorimetric or fluorescence-based detection. Figure 8.6 shows the main players involved in a typical immunostaining protocol. To obtain good quality immunostaining, it is important to optimize fixation methods, timing and concentration of blocking agents, and concentration and duration of exposure to both primary and secondary antibodies. It is also recommended to have both positive (i.e., a sample in which epitope of interest is present in high amounts) and negative controls. For the latter, the same exact sequence of steps is applied, but the primary antibodies are omitted. This enables ruling out the non-specific staining by the secondary antibodies.

8.10 Choice of Antibodies

Primary antibodies have to be raised in a host species that is different from the species of the antigen in order to avoid cross-reactivity with endogenous immunoglobulins. For example, let's say we are trying to stain mouse cells for the presence of connexin 43. The choice should be a primary antibody that is raised in a species other than mice. For example, the primary antibody raised in goat (or rabbit or horse) would be a good choice and the secondary antibody should be then anti-goat IgG.

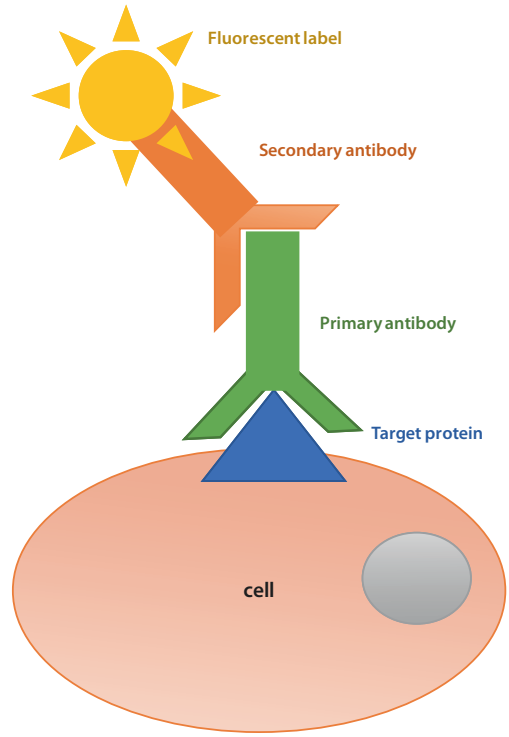

Fig. 8.6 A cartoon showing a basic sequence of epitope labeling used in immunostaining protocols

Session I

Demonstration

The instructor shows steps involved in the isolation of adipose mesenchymal stem cells from adipose tissue and immunostaining for stem cell markers. Briefly, a fresh visceral or subcutaneous animal fat is washed with PBS to remove most of blood cells. Fat tissue is minced using a scalpel into slurry and transferred into a 50 mL tube with 1–2 mg/mL collagenase I solution in PBS. After 30 min incubation using vortexing, serum-containing media are added to stop the digestion, following 800 xg for 10 min. Top fatty layers are discarded, while the remaining clear solution is filtered through a 100-micron filter and centrifuged for 5 min at 200 g. Pellet is resuspended in erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 5 min, followed by another round of centrifugation and cell count. Cells are seeded at ~2000 cells/cm^2 and cultured using available standard cell culture media supplemented with antibiotics.

Homework

Students are tasked to search the literature for most suitable antibodies and protocols to stain stem cells from the demo session. A list of available primary and secondary antibodies is given to students to narrow down their choices.

Session II

Team Exercises

Students stain adipose explant stem cell cultures with stemness markers of their choice, followed by imaging using a fluorescent microscope.

Homework

Students are tasked with making a PowerPoint presentation that includes images of stained cell cultures with and without primary antibodies.

Sample Protocol

Immunostaining

1. Fix samples by incubating in ice-cold 1:1 methanol-acetone solution for 10 min. Rinse twice with PBS.
2. Block specimen for 60 min in blocking solution (PBS, 5% normal serum, 0.3% Triton™ X-100). Use serum from the same species as the secondary antibody. Alternatively, use 1% albumin.
3. Aspirate blocking solution and apply diluted primary antibody. Incubate overnight at 4 °C.
4. Rinse three times in PBS for 5 min each. Incubate specimen in fluorochrome-conjugated secondary antibody diluted in PBS, 1% BSA, 0.3% Triton™ X-100 for 1–2 h at room temperature in dark. Rinse in PBS.
5. Cover samples with a small amount of antifade reagent or Mowiol. Place a coverslip on the top and seal with clear nail polish.
6. Examine samples using appropriate excitation/emission settings. Store slides at 4 °C protected from light.

Take-Home Message/Lessons Learned

After reading this chapter and performing the requested assignments and exercises, students should be able to:

- Define degrees of cell stemness and terms to describe them
- Understand three phases of immune rejection and the key players involved in rejection of implanted engineered tissue
- Choose appropriate primary and secondary antibody for a specific sample
- Name four Yamanaka factors
- Understand the complexity of differentiation protocols and their cell-type specificity

Self-Check Questions

Q.8.1. Choose the correct sequence of terms describing the decreasing capacity of stem cells to give rise to different phenotypes.

A. Totipotent, pluripotent, multipotent, oligopotent, unipotent
B. Pluripotent, totipotent, multipotent, oligopotent, unipotent
C. Multipotent, totipotent, pluripotent, unipotent, oligopotent
D. Pluripotent, totipotent, multipotent, oligopotent, unipotent

Q.8.2. All the following statements about MHC class I are correct, EXCEPT
- A. MHC class I antigens are found on the surface of every nucleated cell.
- B. MHC class I display fragments of proteins synthesized within the cell to cytotoxic T lymphocytes (CTL) or $CD8^+$ T cells.
- C. The function of MHC I molecules is to display peptides of exogenous proteins to T-helper cells or $CD4^+$ T cells.
- D. Cell surface expression of MHC I antigens is the predominant reason for the rejection of allogeneic grafts.

Q.8.3. Discovery of induced pluripotent stem cells (iPSCs) is considered a scientific breakthrough mainly because it
- A. Suggested that stem cells can give rise to different types of specialized cells
- B. Overthrew long-standing dogma that differentiation process cannot be reversed
- C. Demonstrated that stem cell differentiation can occur in both animal and human subjects
- D. Was the first time that stems cells' existence was experimentally shown

Q.8.4. When stem cell colonies start to merge, it is critical to disperse and re-seed stem cells at lower densities because
- A. Stem cells will start to differentiate
- B. pH of the media will become more alkaline
- C. The amount of cells will exponentially increase
- D. Contamination will be imminent

Q.8.5. Choose the correct statement.
- A. There are five phases of acquired immune rejection.
- B. Immunoglobulins that develop against the epitope of interest are called primary antibodies.
- C. Acute rejection is mediated by cytotoxic T macrophages.
- D. Transplantation of undifferentiated pluripotent stem cells is safe and can never lead to teratomas.

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