13

Stem Cell Characterization

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1. Introduction

One of the main problems in stem cell studies is how researchers can identify and characterize a stem cell.

Identification and characterization of stem cells is a difficult and often evolving procedure. Stem cells not only must exhibit the appropriate markers, but also a healthy and robust stem cell population must also lack specific markers. In addition to the difficulty of this area of stem cell biology markers, profiles change based on the species, site of origin and maturity (totipotent vs. multipotent) of a given population. Furthermore, stem cell populations may consist of several specific phenotypes which are often indicators of the population's general health. Flow cytometry employs instrumentation that scans single cells flowing past excitation sources in a liquid medium. It is a widely used method for characterizing and separating individual cells.

This chapter tries to explain what stem cells are, as well as to summarize current knowledge on stem cell characterization and usage of stem cells markers.

2. Stem cells

All life forms initiate with a stem cell, which is defined as a cell that has the dual capacity to self-renew and to produce progenitors and different types of specialized cells in the organism. Scientists mostly work with two kinds of stem cells from animals and humans: embryonic stem cells and non-embryonic "somatic" or "adult" stem cells.

At the beginning of human life, one fertilized egg cell – the zygote – divides into two and two becomes four (Carlson, 1996). Within 5 to 7 days, some 40 cells are produced which build up the inner cell mass encircled by an outer cell layer subsequently forming the placenta. At this phase, each of these cells in the inner cell mass has the potential to give rise to all tissue types and organs – that is, these cells are pluripotent. Finally, the cells forming the inner cell mass will give rise to the some 1013 cells that constitute a human body, organized in 200 differentiated cell types (Sadler, 2002). Many somatic, tissue-specific or adult stem cells are produced during the foetal period. These stem cells have a more limited ability than the pluripotent embryonic stem cells (ESCs) and they are multipotent – that is, they have the potential to give rise only to a limited number of cell lineages. These adult

stem cells keep on in the related organs to varying degrees over the whole of a person's lifetime.

Stem cells are well-known from other cell types because of two important characteristics. First, they are unspecialized cells and the have ability to renew themselves through cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special abilities (Thrasher, 1966; Merok & Sherley, 2001). In some organs, such as the gut and bone marrow, stem cells divide regularly to repair and restore exhausted or damaged tissues. In other organs, however, such as the pancreas and the heart, stem cells only divide under special conditions.

3. Stem cells markers

In recent years researchers have revealed a broad range of stem cells that have unique capabilities to self-renew, grow indefinitely and differentiate or develop into multiple kinds of cells and tissues. Researchers now know that many different types of stem cells exist, but they are all found in very small populations in the human body, in some cases one stem cell in 100,000 cells in circulating blood. In addition, when scientists study these cells under a microscope, they are similar to other cells in the tissue where they are found. So, like the search for a needle in a haystack, how do scientists recognize these uncommon types of cells found in many different cells and tissues? The answer is rather simple, thanks to stem cell "markers."

What are stem cell markers? The surface of every cell in the body has specialized proteins called receptors that have the capability of selectively binding or adhering to other "signalling" molecules. There are many different types of receptors that differ in their composition and affinity for the signalling molecules. Generally, cells use these receptors and the molecules that bind to them as a way of communicating with other cells, and to perform their correct functions in the body. These cell surface receptors are commonly used as cell markers. Each cell type, for example a liver cell, has a certain combination of receptors on their surface that makes them distinguishable from other types of cells. Scientists have taken advantage of the biological exclusivity of stem cell receptors and the chemical properties of certain compounds to label or mark cells. Researchers owe much of the past success in finding and characterizing stem cells to the use of markers.

Stem cell markers are given shorthand names based on the molecules that attach to the stem cell surface receptors. For example, a cell that has the receptor stem cell antigen -1 on its surface is known as Sca-1. In many cases, a mixture of multiple markers is used to identify a particular stem cell type. So now, researchers often identify stem cells in shorthand by a combination of marker names reflecting the presence (+) or absence (-) of them. For example, a special type of haematopoietic stem cell from blood and bone marrow is described as ($CD34^{-/low}$, c-Kit^{+}, $Sca\text{-}1^{+}$) (Jackson et al., 2001).

Researchers employ antibody molecules that selectively bind with the receptors on the surface of the cell as a way to identify stem cells. In former years a method was developed to attach to the antibody molecule another molecule (or tag) that has the ability to fluoresce or emit light energy when triggered by an energy source such as an ultraviolet light or laser

beam. Now, multiple fluorescent labels are available with emitted light that differ in colour and intensity.

Researchers exploit the combination of the chemical properties of fluorescence and unique receptor patterns on cell surfaces to identify specific numbers of stem cells. One approach for using markers is a technique known as fluorescence-activated cell sorting (FACS) (Bonner et al., 1972; Herzenberg, 2000; Julius, 1972). Researchers frequently use a FACS instrument to sort out the rare stem cells from the millions of other cells. By this method, a suspension of tagged cells (i.e. fluorescently-labelled antibodies are bound to the cell surface markers) is sent under pressure through a very fine nozzle. Upon exiting the nozzle cells pass through a light source, usually a laser, and then through an electric field. Operators apply a series of criteria. If the cell stream meets the criteria, they become negatively or positively charged. When cells are passing among an electric field, the charge difference permits the desired cells to be separated from other cells. The researchers now have a population of cells that have all of the same marker characteristics and with these cells they can conduct their research.

A second method uses stem cell markers and fluorescent antibodies to visually assess cells as they exist in tissues. Often researchers want to assess how stem cells appear in tissues and in doing so they use a microscope to evaluate them rather than the FACS instrument. In this case, a thin slice of tissue is prepared and the stem cell markers are tagged by the antibodies that have the fluorescent tag attached. The fluorescent tags are then activated either by special light energy or a chemical reaction. The stem cells will emit a fluorescent light that can easily be seen under the microscope.

4. Embryonic stem cells

An embryonic stem cell (ESC) is described by its origin. It is obtained from the blastocyst stage of the embryo. Embryonic stem cells are unique cell populations with the capability of both self-renewal and differentiation, and thus ESCs can give rise to any adult cell type. Pluripotent embryonic stem (ES) cells, like embryonal carcinoma cells, were first used as a tool to examine thoroughly early differentiation. However, the properties of ESCs identify them as being highly appropriate for making specific cell lineages in vitro. The ability of embryonic stem cells to almost limitless self-renewal and differentiation capacity has opened up the panorama of widespread applications in biomedical research and regenerative medicine.

ESCs are harvested from the inner cell mass of the pre-implantation blastocyst and have been derived from rodents (Martin, 1981; Evans & Kaufman, 1981; Doetschman et al., 1988; Graves & Moreadith, 1993), primates (Thomson et al., 1995) and humans (Thomson et al., 1998; Reubinoff et al., 2000).

4.1 Embryonic stem cell markers

Some approaches have been applied to characterize ESCs, but the most widely used approach is analysis of cell surface antigens by flow cytometry and evaluation of gene expression profile by RT-PCR or microarrays. Many cell surface antigens used to identify hESCs were first detected with antibodies prepared against pre-implantation mouse embryos and/or against mouse or human embryonal carcinoma cells (Pera et al., 2000).

Although the functions of those antigens in the continuance of undifferentiated human embryonal carcinoma cells are not necessarily clear, they may represent helpful markers for the recognition of pluripotent stem cells. These antigens include the globo-series glycolipid antigens, stage-specific embryonic antigen-3 (SSEA-3) and -4 (SSEA-4), keratan sulphate antigens TRA-1-60, TRA-1-81, GCTM2 and GCTM343, a set of various protein antigens comprising the two liver alkaline phosphatase antigens TRA-2-54 and TRA-2-49, Thy1, CD9, HLA class 1 antigens, Oct3/4, Nanog and the absence of hESC negative markers, such as SSEA-1 (Pera et al., 2000; Carpenter et al., 2003; Chambers et al., 2003; Draper et al., 2004; Heins et al., 2004; Nichols et al., 1998).

Some cell surface biomarkers are also listed in Table 1. The International Stem Cell Initiative (ISCI) established by the International Stem Cell Forum (http://www.stemcellforum.org.uk) carried out a comparative study of a large and different set of hESC lines derived from and maintained in different laboratories worldwide (Adewumi et al., 2007). Fifty-nine independent hESC lines derived from 17 laboratories in 11 countries were investigated for the expression of 17 cell surface antigens and 93 genes, which have been chosen as potential markers of undifferentiated stem cells or their differentiated derivatives (Adewumi et al., 2007). All of the independent hESC lines displayed a common expression profile for a specific set of marker antigens, despite the fact that they had different genetic backgrounds and were produced by different techniques in each laboratory. All examined cell lines expressed a comparable spectrum of cell surface marker antigens characteristic of hESCs, suggesting that there is a common set of markers that can be used to monitor, in general, the presence of pluripotent stem cells. SSEA-3 and SSEA-4 were expressed in all hESCs tests, indicating that these molecules are valuable operational markers of this cell type; however, a study revealed that they are not necessary for the pluripotency of hESCs (Brimble et al., 2007).

	Mouse ES cells	Human ES cells
SSEA-1	+	-
SSEA-3	-	+
SSEA-4	-	+
TRA-1-60	-	+
TRA-1-81	-	+
GCTM-2	-	+
Alkaline phosphatase	+	+
Oct-4	+	+
GDF-3	+	?

Table 1. Marker expression and growth properties of mouse and primate pluripotent cells

5. Haematopoietic stem cells

Blood cells are responsible for continuous preservation and immune protection of every cell type of the body. This persistent and brutal work requires blood cells, along with skin cells, to have the greatest power of self-renewal of any adult tissue. The stem cells that form blood and immune cells are known as haematopoietic stem cells (HSCs). HSCs are among the best characterized adult stem cells and the only stem cells being regularly used in clinics.

A haematopoietic stem cell is a cell isolated from the blood or bone marrow that can renew itself, can differentiate a variety of specialized cells and can mobilize out of the bone marrow into circulating blood. Since HSCs look and behave in culture like ordinary white blood cells, it has been a challenge to identify them by morphology (size and shape). Even now, scientists must rely on cell surface proteins, which generally serve as markers of white blood cells.

5.1 Haematopoietic stem cell markers

HSCs have an identity problem. First, the ones with long-term replicating ability are rare. Second, there are multiple types of stem cells. Third, the stem cells look like many other blood or bone marrow cells. So how do researchers find the desired cell populations? The most common approach is through markers that emerge on the surface of cells.

A variety of markers has been found to help distinguish and separate HSCs. Early marker efforts focused on cell size, density and recognition by lectins (carbohydrate-binding proteins derived largely from plants) (Bauman et al., 1988), but more recent attempts have focused mostly on cell surface protein markers, as defined by monoclonal antibodies. For mouse HSCs, these markers contain panels of 8 to 14 different monoclonal antibodies that recognize cell surface proteins present on differentiated haematopoietic lineages, such as the red blood cell and macrophage lineages (thus, these markers are collectively referred to as Lin) (Spangrude et al., 1988; Uchid & Weissman, 1992) as well as the proteins Sca-1 (Spangrude et al., 1988; Uchid & Weissman, 1992), CD27 (Weissman et al., 2000), CD34 (Osawa et al., 1996), CD38 (Randall et al., 1996), CD43 (Moore et al., 1994), CD90.1 (Thy-1.1) (Spangrude et al., 1988; Uchid & Weissman, 1992), CD117 (c-Kit) (Ikuta & Weissman, 1992), AA4.1 (Jordan et al., 1996) and MHC class I (Bauman et al., 1988), and CD150 (Kiel et al., 2005).

Human HSCs have been described with respect to staining for CD34 (Civin et al., 1984), CD38 (Kiel et al., 2005), CD43 (Moore et al., 1994), CD45RO (Lansdorp et al., 1990), CD45RA (Lansdorp et al., 1990), CD59 (Hill et al., 1996), CD90 (Bauman et al., 1988), CD109 (Sutherland et al., 1996), CD117 (Gunji et al., 1993), CD133 (Miraglia et al., 1997; Yin et al., 1997), CD166 (Uchida et al., 1997), HLA DR (human) (Srour et al., 1992; Tsukamoto et al., 1995) and lacking expression of lineage (Lin) markers (Baum et al., 1992). It is important to note that lineage markers are cell surface antigens that can be used for immunophenotyping cells of a particular developmental lineage. Cells that do not express these marker antigens, or express them at very low levels, are said to be lineage marker negative [lin(-)].

While none of these markers recognize functional stem cell activity, combinations (typically with 3 to 5 different markers, see examples below) led to the purification of near-homogenous populations of HSCs. The ability to obtain pure preparations of HSCs, albeit in limited numbers, has greatly facilitated the functional and biochemical characterization of these important cells. However, now there has been limited impact of these discoveries on clinical practice, as highly purified HSCs have only rarely been used to treat patients. The irrefutable advantages of using purified cells (e.g., the absence of contaminating tumour cells in autologous transplantations) have been offset by practical difficulties and increased purification costs.

HSC assays, when combined with the ability to purify HSCs, have provided increasingly detailed insight into the cells and the early steps involved in the differentiation process.

Several marker combinations have been developed that describe murine HSCs, including [CD117^{high}, CD90.1^{low}, Lin$^{neg/low}$, Sca-1^{pos}] (Morrison & Weissman, 1994), [CD90.1^{low}, Lin^{neg}, Sca-1^{pos}Rhodamine123^{low}] (Kim et al., 1998), [CD34$^{neg/low}$, CD117^{pos}, Sca-1^{pos}, Lin^{neg}] (Osawa et al., 1996), [CD150 ^{pos}, CD48^{neg}, CD244^{neg}] (Kiel et al., 2005) and side-population; cells using Hoechst-dye (Goodell et al., 1996). Each of these combinations allows purification of HSCs to near-homogeneity. Similar strategies have been widened to purify human HSCs, employing 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 mostly experimental and clinical use normally employs enrichment for one marker, usually CD34. CD34 enrichment yields a population of cells enriched for HSC and blood progenitor cells, but still contains many other cell types. However, limited trials in which highly FACS-purified CD34^{pos} CD90^{pos} HSCs were used as a source of reconstituting cells have demonstrated that rapid reconstitution of the blood system can reliably be obtained using only HSCs (Negrin et al., 2000; Vose et al., 2001).

None of the HSC markers currently used are directly linked to crucial HSC function, and consequently, even within species, markers can differ depending on genetic alleles (Spangrude & Brooks, 1992), mouse strains (Spangrude & Brooks, 1993), developmental stages (Morrison et al., 1995) and cell activation stages (Randall & Weissman, 1997; Sato et al., 1999). In spite of this, there is an obvious connection with HSC markers between divergent species such as humans and mice. However, unless the current efforts at defining the complete HSC gene expression patterns will yield usable markers that are linked to essential functions for maintaining the stemness of the cells (Ramalho et al., 2002; Ivanova et al., 2002), functional analysis will remain necessary to identify HSCs clearly (Domen et al., 1999).

Mouse	Human
CD34low/-	CD 34+
SCA-1+	CD59+
Thy1+/low	Thy1+
CD38+	CD38low/-
C-kit+	C-kit -/low

Table 2. Proposed cell surface markers of undifferentiated haematopoietic stem cells

5.2 Side population

When in adult mouse haematopoietic tissue, unpurified bone marrow cells are labelled with the membrane-permeate DNA binding dye Hoechst 33342, a very small fraction of cells extrudes this dye via a membrane pump (Goodell et al., 1996, 1997, 2001). Analysis of these cells on a flow cytometer equipped with an ultraviolet (UV) laser source allows finding of these cells; when Hoechst-labelled cells are analysed simultaneously through blue and red emission filters, the SP forms a dim tail extending from the normal G1 cell populations. These cells can reconstitute the bone marrow of lethally irradiated mice at an ED_{50} (Effective Dose 50) of fewer than 100 cells, indicating that they are highly enriched for totipotent stem cells. The SP cell subpopulation is also enriched for cells expressing the murine stem cell markers Sca-1 and c-kit, further suggesting that they contain very early haematopoietic progenitors (Goodell et al., 1996).

The SP fraction expresses an ABC transporter, Bcrp-1(ABCG2), on the cell surface and this transporter contributes to efflux of the Hoechst dye from the cells, leading to low levels of staining (Zhou et al., 2001). Interestingly, the bone marrow and peripheral immune system in ABCG2 transporter knockouts animals, is normal, suggesting that the capability to efflux Hoechst 33342 is characteristic of stem cells, but not essential for function (Uchida et al., 2002). Similar SP subpopulations have been observed in primates and humans (Kim et al., 2002; Allen et al., 2002). The SP phenotype, therefore, has become a significant marker for stem cell activity in the identification of these cells and in their physical isolation by fluorescence-activated cell sorting.

6. Mesenchymal stem cells

Stem cells from adult tissues are an interesting source for cell therapy, gene therapy and tissue engineering. These cells normally have limited lineage potential in comparison to embryonic stem cells and this can be advantageous from the viewpoint of controlling cell growth and differentiation in certain therapeutic applications (Barrilleaux et al., 2006; Barry & Murphy, 2004; Haynesworth et al., 1998).

In 1961, bone marrow was shown to have haematopoietic progenitor cells (Till & McCulloch, 1961). In the early 1970s, many investigators confirmed that bone marrow also had cells with fibroblastic morphology that could differentiate into bone, cartilage, fat and muscle (Prockop, 1997). These cells have been variously designated as marrow stromal cells or mesenchymal stem cells, and abbreviated as ''MSCs.'' It has been demonstrated that individual cells from the bone marrow stromal population possessed multilineage potential (Pittenger et al., 1999). Since the recognition of MSCs in bone marrow, cells with the same multilineage potential have been isolated from other tissues, including trabecular bone (Noth et al., 2002; Sottile et al., 2002) , adipose tissue (Lee et al., 2004; Zuk et al., 2001) and umbilical cord (Secco, 2008). The presence of MSCs in adipose tissue has generated special interest because harvesting fat tissue is generally less invasive to the donor than harvesting bone marrow and larger quantities may be available. Adipose-derived MSCs are also called adipose-derived stem cells (ADSCs) and adipose-derived adult stromal or stem cells (ADAS cells). But in addition to ADSCs, umbilical cord is also another interesting source for MSCs and has recently gained some attention.

6.1 Mesenchymal stem cell markers

Cell surface proteins may characterize particular cell types or lineages. In some cases, the role of a specific surface protein and its role in the biology of the cell type is known. However, often the function of the protein has not been determined, but the protein has been shown to be related to a certain type of cell and can serve as a marker. Exclusive diagnostic surface markers for human MSCs have not been identified, however, several surface markers have been found to be commonly associated with hMSCs, including STRO-1, CD105 (endoglin), CD166 (activated leukocyte cell adhesion molecule, ALCAM) (Barry & Murphy, 2004; Gronthos et al., 2001) and more recently CD271 (low affinity nerve growth factor receptor, LNGFR) (Buhring et al., 2007; Quirici et al., 2002). Surface marker antigens can be used to distinguish the cells in a specific preparation and monitor their differentiation. Surface markers that are exclusively positive for a different cell type, for example, the haematopoietic surface markers CD45 and CD34, can be used to search for

contamination of MSC preparations with other cell types. Surface markers have also been used for positive and negative immunoselection of MSC cell populations (Buhring et al., 2007; Simmons & Torok-Storb, 1991).

The expressed genes that appear on the hMSC surface include receptors for growth factors, matrix molecules and other cells, and point out how the hMSC will interact with its environment. The flow cytometry analysis also indicates the homogeneity of the hMSC population or whether it is a mixture of different cell types. A wide-ranging, yet incomplete, list of the surface molecules on hMSCs is provided in Table 3.

Surface antigens	
Positive	CD13, CD29, CD44, CD49b(Integrin alpha 2,5), CD54(ICAM1), cd71(Transferrin Rec), CD73(SH-3), CD105(Endoglin.SH-2), CD106(VCAM), CD166(ALCAM)
Negative	CD3, CD4, CD6, CD9, CD10, CD11a, b, CD14, CD15, CD34, CD45, D18 (Integrin beta 2), CD31 (PECAM), CD49d (Intergrin alpha 4), CD50 (ICAM3), CD62E (E-Selectin), CD117(c-kit), CD133

Table 3. Mesenchymal stem cells markers

7. Neural stem cells

Neurogenesis is defined as the procedure of generating new neurons from neural stem cells (NSCs),which consists of the proliferation and fate determination of NSCs, migration and survival of young neurons, and maturation and integration of recently matured neurons (Ming & Song, 2005).

NSCs are defined as undifferentiated cells that developmentally originate from the neuroectodermal layer during early embryogenesis. After neural tube closing, these undifferentiated precursor cells and their immediate progeny compose the neuroepithelial layer that surrounds the lateral, third and fourth ventricles in the midbrain and forebrain, and the central canal in the spinal cord. They are the main source of cells that later form all major structures of the brain and spinal cord (Maric & Barker, 2004).

NSCs have recently attracted a great deal of attention because of their inherent ability to generate all major classes of cells of the nervous system. NSCs have therefore been supposed as a useful resource for potentially repairing and restoring the physiological functions to damaged, diseased or aging neural tissues (Gang, 2000; Anderson, 2001; Temple, 2001; Vaccarino et al., 2001; Vescovi et al., 2001; Weissman et al., 2001).

However, with the accelerated interest in and growth of the NSC field, there has been growing uncertainty around the understanding of what cell phenotype actually makes up a neural stem cell. NSCs in their undifferentiated shape are characterized by a unique bipolar morphology that can help identify them from the heterogeneity associated with early culture. Derivation from human foetal material gives rise to an apparently mixed population of NSCs, exhibiting both classic bipolar NSC morphology and other cell morphologies.

7.1 Neural stem cell markers

The major research limitation is that the cellular preparations used as a source of NSCs are themselves naturally heterogeneous and consisting of both NSCs and self-renewing, but

more lineage restricted, progenitors; accordingly making the retrospective studies of NSC biology skewed to an unknown degree. Adding to this is the increasing evidence that implies clear functional differences between neural stem and progenitor cells (Galli et al., 2003; Cai & Rao, 2002). Consequently, there is a critical need to use strategies to identify and isolate pure populations of NSCs and other type cells with the aim of resolving their shared or unique biological properties with respect to cell-fate determination and lineage progression.

NSCs are immunoreactive for a range of neural precursor/radial glia markers such as Nestin, Vimentin, RC2, 3CB2, Sox-2 and brain lipid-binding protein (BLBP). However, subtle differences exist between mouse and human NSCs. For example, hNS cells display moderate levels of glial fibrillary acidic protein (GFAP) expression unlike mouse NSCs (Conti et al., 2005), reflecting the differences between the species in vivo (Malatesta et al., 2000; Rakic, 2003).

So, as mentioned above, the cells which are gathered from neural tissue are heterogeneous and identifying cells is required. Therefore, some markers that are used in studies are listed below:

Neural stem cells	GFAP, Nestin, Prominin, SOX-2
Proliferating cells	Ki-67,BrdU, PCNA
Immature neurons	beta Tubulin,DCX,PSA-NCAM
Radial glia	GLAST, RC2
Mature neurons	NeuN, MAP-2, NF, BLBP
Oligodendrocyte precursors	NG2
Oligdendrocytes	O4, MBP, RIP

Table 4. Neural stem cells markers

8. Spermatogonial stem cells

Germ cells are specific cells that transfer the genetic information of an individual to the next generation. Making functional germ cells is vital for continuation of the germ line of the species. Spermatogenesis, the process of male germ cell production, takes place in the seminiferous tubules of the postnatal testis and is an extremely productive system in the body. In the mammalian testis, more than 20 million sperms per gram of tissue are created daily (Amann, 1986). The high productivity relies on spermatogonial stem cells (SSCs). Similar to other kinds of stem cells in adult tissues, SSCs are self-renewing and produce daughter cells that assign to differentiate throughout the life of the male (Meistrich & van Beek, 1993). In addition, in mammals, SSCs are unique among stem cells in the adult body, because they are the only cells that undergo self-renewal and transmit genes to subsequent generations. Furthermore, SSCs provide an excellent model to study stem cell biology due to the availability of a functional assay that clearly identifies the stem cell (Weissman et al., 2001).

Spermatogonial stem cells derive from primordial germ cells (PGCs), which in turn originate from epiblast cells (embryonal ectoderm) (Lawson KA et al., 1992). Soon after the development of the PGCs, they migrate from the base of the allantois, along the hindgut, finally reaching the genital ridges. The PGCs increase in number during migration, when

these cells have reached the genital ridges; their number increases to about 10,000 per gonad (Tam and Snow, 1981). PGCs are single cells that under certain culture conditions can make colonies of cells which morphologically are similar to undifferentiated embryonic stem cells (ESCs) (Resnick JL et al., 1992).When they have arrived in the genital ridges, the PGCs are surrounded by the differentiating Sertoli cells, so seminiferous cords are formed.

The germ cells present within the seminiferous cords are different morphologically from PGCs and are called gonocytes (Clermont and Perey, 1957; Sapsford CS et al., 1962; Huckins & Clermont, 1968) or various subsequent types of pro-spermatogonia (Hilscher B et al;1974). Shortly after birth, the gonocytes restart proliferation to give rise to adult types of spermatogonia (Sapsford CS et al., 1962; Huckins & Clermont, 1968; Vergouwen RPFA et al., 1991; Novi & Saba, 1968; Bellye AR et al., 1977). This happening indicates the start of spermatogenesis.

8.1 Spermatogonial stem cell markers

Since the establishment of the transplantation technique, several new markers and characteristics of spermatogonial stem cells have been identified that can be used to isolate a population from the testis that is enriched for spermatogonial stem cells - Tables 5 and 6.

Markers for positive selection of spermatogonial stem cells	CD9 (Kanatsu-Shinohara M et al., 2004), integrin alpha 6 (Shinohara T et al., 1999), integrin beta 1 (Shinohara T et al., 1999), THY-1, CD24 (Kubota H et al., 2003)
Markers for negative selection of spermatogonial stem cells	c-kit, MHC1, Ly6A(Sca-1), CD34 (Kubota H et al., 2003)

Table 5. Overview of markers that have been successfully used to isolate spermatogonial stem cell populations from the testis by either positive or negative selection

8.2 Testicular side population

So far, four groups have separated a side population of testicular cells; meanwhile, different results were drawn as to whether these were spermatogonial stem cells. The first group reported the existence of a testicular side population. Amazingly, they did not find this population to be capable of colonizing a recipient testis after transplantation and concluded that it did not contain spermatogonial stem cells (Kubota et al., 2003).

Then, two other groups found the testicular side population to be enriched for spermatogonial stem cells (Falciatori et al., 2004; Lassalle et al., 2004). A fourth group then explained that testicular side population cells contain Leydig cell progenitors (Lo et al., 2004; de Rooij.,2004) and later failed to find spermatogonial stem cells in this population (Lo et al., 2005).

The controversial results can probably be explained by the strictness of the FACS gating and the different procedures used to separate the side population. It may be possible to isolate a very pure population of spermatogonial stem cells from the testis using the side population technique, alone or in combination with membrane markers (van Bragt et al., 2005),

however, for this to be possible more research needs to be performed to determine the optimal procedures and combinations of markers.

A(s) and	GFRalpha-1(Von Schonfeldt et al., 2004, Hofmann et al, 2005) FC, MACS, IHC, ISH, WM
A(s), A (pr) and A(al)	PLZF (Buaas et al., 2004) (Costaya et al., 2004) FC, ISH, IHC, WM, Mu; OCT4 (Pesce et al., 1998) FC, ISH, IHC, WM TG; NGN3 (Yoshida et al., 2004) ISH, TG, WM, ISH; NOTCH1 (Von Schonfeldt et al., 2004) RT–PCR, IHC, SOX3 (Raverot et al., 2005) KO, IHC; c-RET (Meng et al., 2000) IHC, MACS
A spermatogonia	RBM (Jarvis et al., 2005) RT–PCR, IHC
Spermatogonia	EP-CAM (Anderson et al., 1999) FC, IHC, MACS
Premeiotic germ cells	STRA8 (Oulad Abdelghani et al., 1996) RT–PCR, ISH, IHC, WM EE2 (Koshimizu et al., 1995) WB , IHC
Cells on basal membrane and interstitium	CD9 (Kanatsu-Shinohara et al., 2004) FC, IHC, MACS
Spermatogonia, spermatocytes and round spermatids	GCNA1 (Enders & May., 1994) FC, WB, IHC
Premeiotic spermatogonia and postmeiotic spermatid	TAF4B (Falender et al., 2005) FC, KO, IHC

A(s), A-single; A (pr), pair of spermatogonia; A (al), A-aligned spermatogonia; FC, flow cytometry (including FACS); Mu, mutant mouse; TG, transgenic mouse; KO, Knockout mouse; IHC, immunohistochemistry; WM, whole mount immunostaining; WB, Western blot; ISH, in situ hybridization; RT–PCR, reverse transcriptase– PCR; MACS, magnetic-activated cell sorting

Table 6. Overview of markers used to identify spermatogonial stem cells

9. Epidermal stem cells

The skin is the body's strong outer cover that maintains the inside of the body being moist and protects the body from outside assaults by physical, environmental and biological factors. Skin and its associated hair follicles and glandular structures, sebaceous and sweat glands, are made by a stratified epithelium where the position of the cell within the tissue relates to its state of differentiation. The terminally differentiated stratum corneum, hairs and oil-filled sebocytes have a limited lifespan and are constantly shed from the body throughout the adult life. This continual shedding requires that the epithelium is replenished and restored by a stem cell population during normal maintenance of the skin and also in response to injury (Fuchs & Horsley, 2008; Watt et al., 2006). By definition, adult stem cells (ASCs) have the ability to both self-renew and make differentiated progeny (Lajitha, 1979). In healthy skin, epidermal stem cells divide uncommonly, but upon skin injury, stem cells quickly divide to repair the lesion.

There has been important progress in the recognizing of epidermal stem cells (ESCs) since the 1970s, when the idea of interfollicular epidermis was firstly suggested; later, much work was focused on the specific region of the hair follicle outer root sheath, mainly the bulge

region. Hair follicle stem cells are multipotent, capable of giving rise to all cell types of the hair, the epidermis and the sebaceous gland (Morris et al., 2004).

9.1 Epidermal stem cells markers

Recognizing the ESCs is major progress in the field of skin biology which lets scientists examine their biochemical properties, lineage and their relation to other cells. There is evidence of ESCs in the bulge region of the hair follicles (Myung et al., 2009a, 2009b; Zhang et al, 2009), as well as in the interfollicular epidermis (Abbas & Mahalingam, 2009; Ambler & Maatta, 2009). When the epidermis undergoes severe damage, it may fully regenerate from the ESCs of the bulge (Watt, 2006). The ESCs present in the bulge and interfollicular epidermis are potentially interconvertible, but under normal conditions they only differentiate a more confined progeny.

ESCs can be identified in vivo by label retention or in vitro by clonogenicity, but neither of these methods allows easy isolation of stem cells for analysis. Therefore, there is a strong need for specific ESCs markers to be identified.

Identifying stem cells by their cell cycling properties has limited potential. Therefore, several research groups have undertaken wide attempts to characterize a set of stem cell specific markers. Much of this research has focused on the bulge region, as this is the most clearly defined stem cell niche in the skin (Fuchs & Horsley, 2008; Watt et al., 2006).

Many efforts have been made in recent years to recognize ESCs. The potential candidate hair follicle stem cells markers include integrin beta 1, keratin 15, keratin 19, CD71, transcription factor p63 and CD34 (Ma et al., 2004). Keratinocyte shows the characteristics of keratin intermediate filaments. In the epidermis, keratins 5 and 14 are expressed in the basal layer, while keratins 1 and 10 are found in the suprabasal layer. The hair follicle stem cells expressed the above keratins and keratins 6, 16 and 17 (Al-Refu et al., 2009; Hoang et al., 2009), and desmosomal proteins, including desmoglein, may serve as negative markers of ESCs (Wan et al., 2003).

In 2001, p63 was identified as a marker for ESCs; p63 is a transcription factor belonging to a family that contains an additional two structurally-related proteins, p53and p73 (Pellegrini et al., 2001). Although p53 fulfils an important role in tumour suppression, p63 and p73 participate in morphogenetic processes (Klein et al., 2010). Their expression is evidenced in ESCs.

CD34 is also a specific marker for bulge keratinocytes. The mouse bulge marker CD34, often used for isolating murine bulge cells, is expressed below the bulge region in human hair follicles (Ohyama et al., 2006). As mentioned before, CD34 is also a specific marker for haematopoietic stem and progenitor cells, however, much more work is needed to clarify specific markers for ESCs.

10. Conclusion

Flow cytometry is able to rapidly check thousands of cells stained with antibodies conjugated to fluorescent dyes. Each cell is individually assessed for a mixture of features such as size and biochemical and/or antigenic composition. High accuracy and sensitivity,

combined with the large numbers of cells that can be examined, allows resolution of even very minor subpopulations from complex mixtures with high levels of statistical validity.

As mentioned earlier, the main problem with stem cell research is that a specific marker for each stem cell is not available for researchers and markers usually are common between some cell populations. Therefore, it is clear that we should wait to hear more from future studies to resolve this issue and introduce new and specific markers for each individual stem cell.

11. References

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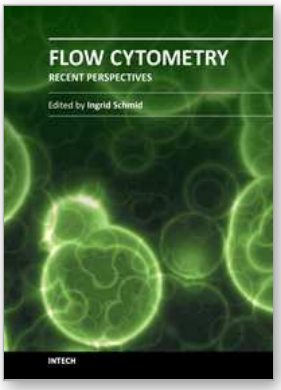

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"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

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