# Identifying the Stem Cell

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## INTRODUCTION

Stem cell research is an exciting new field in all areas of biology and medicine, including dermatology. First, we explain the most important terms used in stem cell biology, followed by a short summary of the techniques most commonly used to enrich and identify adult stem cells from the skin (epidermal stem cells) and from the bone marrow (hematopoietic stem cells (HSCs) and skeletal stem cells, also known as mesenchymal stem cells (MSCs)).

## BASIC TERMINOLOGY IN STEM CELL BIOLOGY

### Stem cells

Stem cells are undifferentiated cells without any tissue-specific function that have the potential to differentiate into certain tissues determined by their genetic and epigenetic program. The most important feature of stem cells is their ability to self-renew by asymmetric cell division, which recreates the stem cell itself and another cell with more restricted progeny and with limited proliferation and/or differentiation potential.

### Potency

Potency can be easily understood through embryonal development. During embryonal life, the differentiation potential of stem cells becomes more and more restricted. *Totipotent cells* of the early embryo can build a complete organism as well as extraembryonal tissues such as the placenta. *Pluripotent stem cells* exist in the inner cell mass of the embryo and are capable of creating all tissues in the body. *Multipotent and unipotent* stem cells exist in the embryo as well as in postnatal adult tissues. Multipotent stem cells can be found in the hematopoietic and in the stromal compartment of the bone marrow; the former produce blood lineages, whereas the latter are responsible for creating connective tissue cell types, such as adipocytes and osteoblasts. Finally, unipotent stem cells can differentiate along only one lineage. Those found in epithelial tissues replenish the epithelial cells of the skin, gut, or airways.

### Plasticity and transdifferentiation

During embryonal development, tissues develop from three mesodermal layers: endoderm, mesoderm, or ectoderm. An old dogma of stem cell biology is that stem cells isolated from a particular tissue can only renew and differentiate into cells of the same mesodermal lineage. This view has been challenged by several studies demonstrating that tissue-specific stem cells have the *plasticity* to cross lineage restrictions and become cell types of other lineages; this process is called *transdifferentiation* (Mezey *et al*., 2000).

### Induced pluripotent stem cells

Terminally differentiated cells can be induced into pluripotency by a genetic manipulation that introduces a specific combination of transcription factors into the cell. These induced pluripotent stem cells (iPSCs) can become any tissue-specific adult stem cell (Takahashi and Yamanaka, 2006). Details on iPSCs and their exciting potential are presented by Dinella *et al*. (2014).

## STEM CELL IDENTIFICATION ASSAYS: ADVANTAGES

- FACS-based separation: fast, relatively easy, reproducible.
- Side population analysis: fast, easy, and reflects functionality.
- Label-retaining assays: allow *in situ* detection of stem cells.
- Functional assays: measure "real" stemness.

## LIMITATIONS

- FACS-based separation: multiple antibodies can affect viability and function of sorted cells.
- Side population analysis: reproducibility depends on cellular functionality (viability, etc.).
- Label-retaining assays: only the H2B-GFP technique allows isolation of living cells.
- Functional assays: complicated, serve only as validation for other separation techniques.

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### Origin of stem cells

Depending on the tissue source, stem cells arise from a variety of places; accordingly, they are referred to as embryonic stem cells, fetal stem cells, amniotic stem cells, cord-blood stem cells, adult stem cells, and so on (Figure 1).

### Distribution of adult stem cells: the stem cell niche

Stem cells are found in a special microenvironment called the stem cell niche. This is a well-defined area in tissues where stem cells reside and receive a unique combination of extracellular signals directing them to maintain their "stemness," that is, to preserve their stem cell characteristics.

A characteristic niche of epidermal stem cells is the so-called bulge region of the hair follicle, but they can also be found in the interfollicular epidermis. Hematopoietic stem cells are located either in close proximity to osteoblasts in the endosteal niche or around sinusoidal vessels in the vascular niche within the bone marrow (Morrison and Scadden, 2014). Mesenchymal stem cells reside within the basement membrane of capillaries and postcapillary venules and form a subpopulation of contractile perivascular cells called pericytes (Sacchetti *et al.*, 2007) (Figure 2).

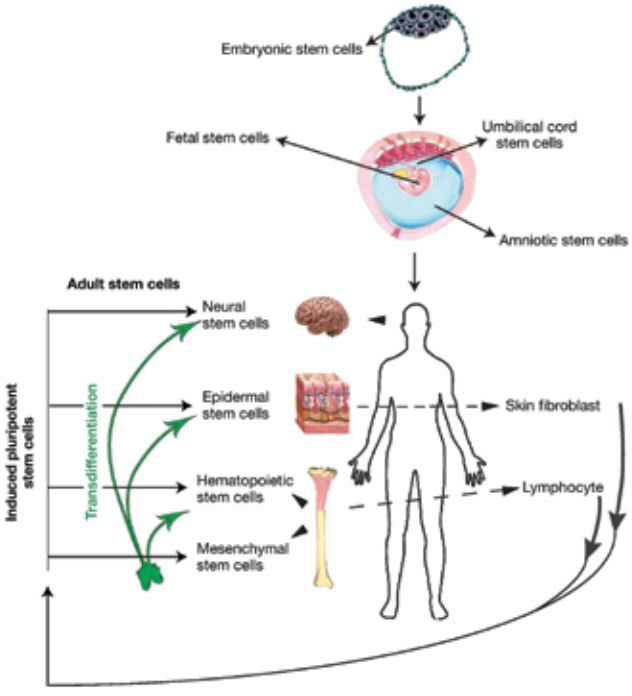


**Figure 1. Types of stem cells from early embryonic development through adulthood and ways of reprogramming an adult cell.** Embryonic stem cells are derived from the inner cell mass of the blastocyst by culturing. Fetal, amniotic, and umbilical stem cells come from a developing fetus, the amniotic fluid, or the cord blood, respectively. The different types of adult stem cells are found in their corresponding organs. Adult stem cells might be able to differentiate into mature cells of other tissues, a process called transdifferentiation. Mesenchymal stem cells, for instance, may become hematopoietic, epidermal, or neural stem cells. Mature tissue cells such as fibroblasts or lymphocytes can be reprogrammed as well by making them induced pluripotent stem cells first and guiding their differentiation toward other tissue cell types afterward.

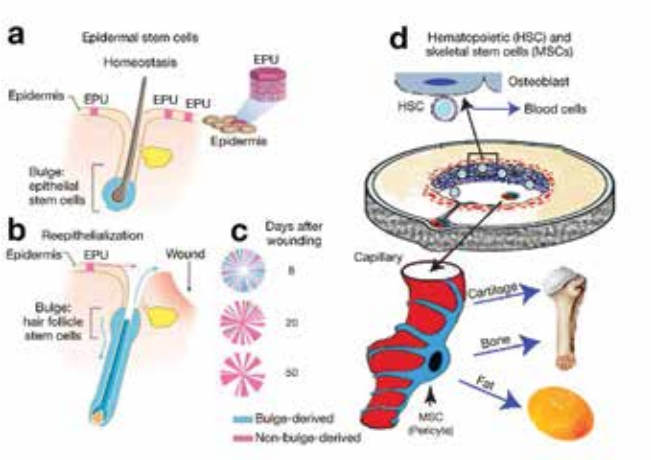


**Figure 2. Localization of stem cells within the epidermis and the bone marrow.** (**a**) During normal conditions, epidermal renewal is dependent on cell proliferation within epidermal proliferative units (EPUs), which are clonal populations of cells roughly arranged in hexagonally shaped columns that produce a single outer squame (cornified keratinocyte). Epithelial stem cells in the hair follicle bulge do not contribute to epidermal renewal. (**b**) Following full-thickness wounding, bulge cells contribute cells to the epidermis for immediate wound closure (light blue upward arrow). Bulge cells also are required for hair follicle cycling (light blue downward arrow). (**c**) Over time, bulge-derived cells diminish, whereas non-bulge-derived cells appear to predominate in the reepithelialized wound. (**d**) Hematopoietic stem cells reside in close proximity to sinusoid vessels and the endosteum (this relationship is highlighted), whereas skeletal stem cells form a subpopulation of pericytes around capillaries. Figure 2a–c reprinted from Cotsarelis (2006).

## IDENTIFICATION AND ISOLATION OF STEM CELLS

### Cell surface markers

The maintenance of stem cell properties requires the simultaneous expression of several molecules, some of which might only be found in self-renewing cells. The combination of these specific markers indicates the character of tissue stem cells and helps differentiate between a more restricted progenitor and a terminally differentiated cell. Therefore, determining the presence or absence and quantitatively assessing these markers is very useful and is an integral part of several stem cell isolation techniques. For example, the human hair follicle–derived epidermal stem cells are *negative* for CD45 and c-kit, *positive* for CD34 and CD200, and have a *low expression* of CD71 while producing α-6-integrin at a *high level* (Terunuma *et al.*, 2008). Similar to FACS, magnetic bead–based assays can be used to enrich stem cells based on their surface marker expression. Cell surface markers can be used to identify stem cell markers *in situ* using immunostaining.

### Side population

Side population (SP) analysis distinguishes stem and progenitor cells from other somatic cells based on their ability to remove foreign molecules from the cells. In a mixture of cells, all cells take up DNA-binding fluorescent dyes (such

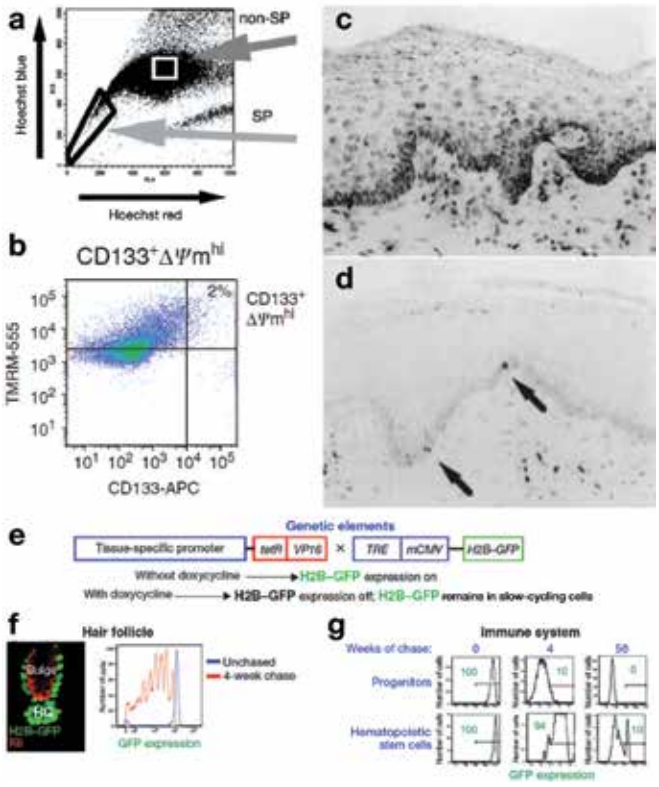


**Figure 3. Various stem cell enrichment techniques.** (**a**) Side population (SP) cells of the epidermis are shown on an FACS plot. SP cells are found in the lower left corner of the plot representing a cell population that stains negative with two different DNA-binding Hoechst dyes. (**b**) Measuring mitochondrial membrane potential (MMP) can also aid enrichment of stem cells. High TMRM-555 intensity corresponds to high MMP. CD133-expressing epidermal cells show high MMP. (**c**,**d**) *In situ* evaluation of a label-retaining assay using BrdU. Proliferating cells that pick up BrdU are shown in the epidermis during the pulse and later in the chase period. Initially all proliferating cells are labeled, but with time only cells with stem cell characteristics will retain the BrdU tag. (**d**) Measuring mitochondrial membrane potential (MMP) can also aid enrichment of stem cells. High TMRM-555 intensity corresponds to high MMP. CD133-expressing epidermal cells show high MMP. (**e**) Schematic representation of the genetic strategy to mark slow-cycling cells with GFP-labeled histone H2B. Transgenic mice harboring a tissue-specific promoter driving the tetracycline-repressor (tetR)–VP16 transgene are crossed to transgenic mice expressing a tightly regulated tetracycline-responsive regulatory element (TRE)–mCMV–H2B–GFP element. Without doxycycline, H2B–GFP is uniformly present in the nuclei of all cells within the tissue of interest. With doxycycline, H2B–GFP expression is inhibited, resulting in a twofold dilution of existing fluorescence with each division. By chasing for different time periods in the presence of doxycycline, only slow-cycling stem cells or long-lasting, nondividing, terminally differentiated cells of the tissue will remain labeled. (**f**) Histology (left) and FACS (right) analysis of H2B–GFP label retention in the hair follicle when *Keratin 5* (K5) is the tissue-specific promoter. Left: At the start of new hair growth, green nuclei, reflective of label-retaining cells, can be detected within the stem cells of the outer layer of the hair follicle bulge (the inner layer is marked by keratin 6; K6), as well as in the hair germ (HG) at the base of the bulge. (Right) by analyzing GFP expression during a 4-week chase, populations of cells exhibiting a twofold reduction in GFP fluorescence, reflective of cell division, can be detected by FACS. (**g**) FACS analysis of H2B–GFP label retention in the immune system. Hematopoietic progenitors lose H2B–GFP retention after 56 weeks of chase, whereas 10% of stem cells retain label at this time point (green numbers and the lines represent the percentage of cells expressing GFP). Panels **a**,**c** and **d** reprinted with permission from Terunuma *et al*. (2003); **b** reprinted with permission from Charruyer *et al*. (2012); **e**–**g** reprinted with permission from Fuchs and Horsley (2011).

as Hoechst dyes), but only stem cells (expressing special ATP-binding cassette–containing pumps, a type of membrane-bound active transporter) will be able to remove the dyes and will remain unstained or weakly stained when analyzed by flow cytometry. In several tissues dye-excluding SP cells represent a stem cell–enriched fraction. This method is remarkably sensitive and able to pick up rare events in a heterogeneous cell population. A pitfall is that the detection of dye-excluding stem cells requires active cellular metabolism and is largely dependent on cell viability, which is adversely affected by the presence of DNA intercalating dyes. Therefore, the isolated SP cells are not always suitable for further functional stem cell analysis (Terunuma *et al*., 2003) (Figure 3a).

### Mitochondrial potential

More recently, another FACS-based assay measuring mitochondrial membrane potential was shown to enrich cells with stem cell characteristics. By adding a potentiometric dye to a mixture of cells followed by FACS analysis, stem cells can be separated by their higher mitochondrial membrane potential compared to more differentiated cells (Schieke *et al*., 2008; Charruyer *et al*., 2012) (Figure 3b).

### Label-retaining assays

Label-retaining characteristics have been often used to enrich stem cells by a pulse–chase method. In the "pulse phase," radiolabeled nucleotide or BrdU is administered to animals or cell cultures. This will label all proliferating cells in the S phase of the cell cycle when DNA replication takes place. Labeling substances are then taken away for a prolonged period of time before the tissues or cultures are examined. Fast-cycling cells are constantly dividing; thus, their label

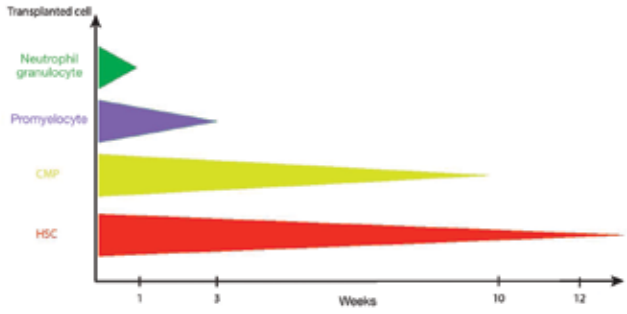


**Figure 4. Long-term repopulation (LTR) ability of hematopoietic stem cells (HSCs).** LTR ability serves to validate various stem cell isolation methods. To determine that the transplanted cells are indeed stem cells, we must choose an adequate follow-up period after transplantation. Assume that we are looking in the blood for neutrophil granulocytes, one of the end products of HSCs. Neutrophils differentiate from HSCs through various stages, such as common myeloid progenitors (CMPs) and promyelocytes. An observation period of less than 12 weeks can yield misleading findings. After 3 weeks, for instance, detected neutrophils can originate from HSCs but they might as well be the product of more differentiated cells such as CMPs or promyelocytes. If we transplanted a CMP instead of an HSC we might falsely identify a stem cell marker that belongs to a more differentiated cell type.

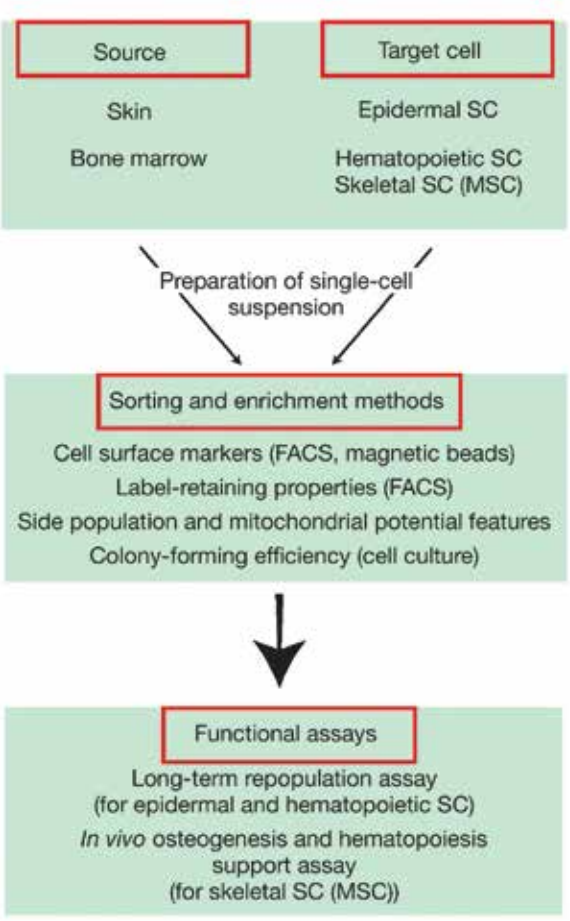


**Figure 5. A summary of stem cell isolation and validation techniques.**

is diluted with each division. Consequently, the amount of original label steadily decreases to the point of becoming undetectable. Slow-cycling cells divide less frequently, retain more label, and are therefore identified as label-retaining cells (LRCs) (Figure 3c,d). These labeling techniques allow *in situ* histological detection of stem cell–enriched cell populations, but are not suitable for isolating living LRCs from tissues. A relatively new, elegant method that solves this issue utilizes the expression of histone-linked green fluorescent protein (Fuchs and Horsley, 2011) (Figure 3).

### Colony-forming efficiency and holoclone identification

The colony-forming efficiency assay is an *in vitro* method that also helps enrich stem cells. It is performed by first creating a single cell suspension from the target tissue, followed by a simple dilution step and subsequent inoculation of culture dishes with a low number of cells (typically 10–100 cells/cm$^2$). The colonies that form are enriched in stem cells and the number of colonies is believed to correlate with the initial number of stem cells in the tissue.

When epidermal stem cells are studied, colonies formed by keratinocytes can be further subcultured and colonies in this secondary culture reevaluated. Cells with high replicative capacity and a low level of terminal differentiation will form large colonies with smooth edges called holoclones. They represent a further enriched population of epidermal stem cells (Barrandon and Green, 1987).

### Functional stem cell assays

The ultimate test for stemness is the ability of sorted/enriched cells to maintain tissue homeostasis, that is, to produce tissue-specific differentiated cells indefinitely. Methods that assess *in vivo* functionality of transplanted epidermal and hematopoietic stem cells are called *long-term repopulation assays*. The more differentiated a progenitor cell is, the longer it can live when transplanted. If a hematopoietic stem cell is transplanted into an irradiated host (there is no functional host bone marrow left), it can repopulate the bone marrow and will last the lifetime of the recipient. However, if a developed white cell (or red cell) is transplanted, it will remain only as long as that cell type lives. Thus, if a labeled cell (such as green fluorescent protein labeled) is transplanted into an irradiated animal and the blood is sampled, the presence of the fluorescence at different time points will indicate the lifespan of the transplanted cell. If the fluorescence is still present after 12 weeks, the transplanted cell must have been a hematopoietic stem cell because no other progenitors could live this long (Figure 4). Often, these assays are designed as competitive repopulation assays, meaning that long-term repopulation is assessed against a competitor cell type so that quantitative results can be achieved (Ghadially, 2012). A similar technique is available to measure the long-term repopulation ability of epidermal stem cells (Schneider *et al.*, 2003). The test for MSC stemness is somewhat different. Here MSCs are transplanted *in vivo* and their ability to form bone and support hematopoiesis is evaluated after 12 weeks. The amount of created bone and hematopoietic islands correlates with the number of MSCs implanted (Robey *et al.*, 2014).

## SUMMARY

Stem cell research is becoming increasingly complex. Identifying stem cells can be challenging and often requires the simultaneous use of various isolation techniques. Being aware of the basic terms and selection techniques used in stem cell biology can help to better understand stem cell–related research and may facilitate the formulation of new, collaborative ideas in dermatology. A summary of stem cell enrichment and identification techniques is presented in Figure 5.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

## CME ACCREDITATION

This activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education through the joint sponsorship of the Duke University School of Medicine and Society for Investigative Dermatology. The Duke University School of Medicine is accredited by the ACCME to provide

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## SUPPLEMENTARY MATERIAL

A PowerPoint slide presentation appropriate for journal club or other teaching exercises is available at http://dx.doi.org/10.1038/jid.2014.393.

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## QUESTIONS

This article has been approved for 1 hour of Category 1 CME credit. To take the quiz, with or without CME credit, follow the link under the "CME ACCREDITATION" heading.

**1. The following is a unique feature of stem cells:**

A. Fastest dividing cell type in tissues.
B. Greatest proliferative capacity.
C. Pluripotent nature.
D. Able to terminally differentiate.

**2. Which of the following is true?**

A. Side population analysis requires active transport of Hoechst dyes inside the cell.
B. Label-retaining assays can be used to isolate living human stem cells.
C. Long-term repopulation assays can only be used to study murine cells.
D. Use of the H2GFP technique allows isolation of living stem cell–enriched cell fractions.

**3. What is the best assay to validate a new stem cell isolation technique?**

A. Side population analysis.
B. Label-retaining assay.
C. Colony-forming unit assay.
D. *In vivo* repopulation assay.

**4. Which of the following is true?**

A. Induced pluripotent stem cells are reprogrammed embryonic cells.
B. Epidermal stem cells are mostly found in the hair bulb.
C. Skeletal stem cell–derived cells support hematopoiesis.
D. Plasticity refers to the ability of terminally differentiated cells to become stem cells.

**5. Which of the following markers do human epidermal stem cells stain for?**

A. CD45 and keratin 10.
B. CD71 and CD271.
C. CD31 and CD146.
D. CD34 and CD200.