

Isolation and Enrichment of Stem Cells

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Abstract Stem cells have the potential to revolutionize tissue regeneration and engineering. Both general types of stem cells, those with pluripotent differentiation potential as well as those with multipotent differentiation potential, are of equal interest. They are important tools to further understanding of general cellular processes, to refine industrial applications for drug target discovery and predictive toxicology, and to gain more insights into their potential for tissue regeneration. This chapter provides an overview of existing sorting technologies and protocols, outlines the phenotypic characteristics of a number of different stem cells, and summarizes their potential clinical applications.

Keywords stem cells, differentiation, tissue regeneration, cancer, magnetic cell separation

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Abbreviations

ACL	Acute lymphoid leukemia
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
ASC	Adult tissue-specific stem cell
betaNGF	Beta Nerve growth factor
bFGF	Basic fibroblast growth factor
BMC	Bone marrow cell
BMP-4	Bone morphogenic protein
BTSC	Brain tumor stem cell
CABG	Coronary Artery Bypass Grafting
CC-IC	Human colon cancer-initiating cell
CR	Clinical remission
CSC	Coronary sinus catheter
CXCR4	Alpha chemokine receptor type 4
EFS	Event-free survival
EGF	Epidermal growth factor
EPC	Endothelial progenitor cell
EPCAM	Epithelial cell adhesion molecule
ESA	Epithelial-specific antigen
ESC	Totipotent embryonic stem cell
GBM	Glioblastoma stem cell
HCC	Hepatocellular carcinoma
HEF	Human embryonic fibroblast
hESC	Human embryonic stem cell
HGF	Hematopoietic growth factor
HLA	Human leukocyte antigen
HPSC	Human pluripotent stem cell
HSC	Pluripotent hematopoietic stem cell
iPS	Induced pluripotent stem cell
KIR	Killer inhibitory receptor
LIF	Leukemia inhibitory factor
LRP	Lineage-restricted progenitor cell
LTR-HSC	Longterm repopulatory hematopoietic stem cell
MAPC	Multipotent adult progenitor cell

MEF	Mouse embryonic fibroblast
mESC	Mouse embryonic stem cell
MMFD	Mismatched related family donor
MSC	Mesenchymal stem cell
MUD	Matched unrelated donor
NK	Natural killer
PSA-NCAM	Polysialic acid-neural cell adhesion molecule
RMS	Rostal migratory stream
SLAM	Slow as molasses
SP	“Side population” phenotype
SPC	Sphingosylphosphorylcholine
SSC	Spermatogonial stem cell
SSEA-1	Stage-specific antigen 1
TGF-beta1	Transforming growth factor beta 1
TH-EGFP	Tyrosine Hydroxylase- Enhanced green fluorescent protein

1 Introduction

Stem cells have the potential to revolutionize tissue regeneration and engineering. The hematopoietic stem cells were the first stem cells to be prospectively identified. Since then, an ever increasing number of new types of stem cells, including embryonic stem cell cells, tissue resident stem cells and cancer stem cells, have been identified and characterized. Currently, the derivation of induced pluripotent stem cells (iPS cells) from differentiated, post-mitotic cells that behave similar to ESC is further extending this exciting field. iPS cells may eventually combine the advantages of ESCs and autologous cell transplantation, allowing for a generation of patient specific derived stem cells for unrestricted tissue regeneration and without ethical issues.

Both general types of stem cells, those possessing pluripotent differentiation potential like ESC or iPS cells, as well as those with multipotent differentiation potential like tissue stem cells, are of equal interest. They can be useful in understanding general cellular processes in, e.g., embryogenesis, organogenesis, cancer or ageing, but also as a vehicle for the generation of transgenic mice for functional gene analysis and disease models. Further applications are in industrial research as cell based screenings for drug target discovery, drug discovery or predictive toxicology, and in clinical research as a potential source for tissue regeneration.

The reproducibility of culturing cells at a defined stage, as well as differentiating them to a certain endpoint, is a prerequisite for each of the listed applications. Therefore, a number of different protocols have been published for the isolation and enrichment of stem cells including selective culturing, immunopanning, flow cytometric sorting, or magnetic sorting.

In this chapter we give an overview of existing sorting technologies and protocols, outline the phenotypic characteristics of a number of different stem cells and summarize their potential for clinical applications.

2 Methods and Technologies

2.1 *Stem Cell Enrichment Using Flow Cytometry*

2.1.1 Flow Sorting

Flow cytometric cell sorting utilizes optical differences between target cells and nontarget cells. Light scattering and fluorescent properties are the optical parameters and are either intrinsic to the cell population (size and granularity for forward and sideward scatter) or generated by differential binding or incorporation of fluorescent dyes into cell populations.

Single cell suspensions in a flowing stream are embedded in a second fluid stream (sheath fluid) and are subject to a hydrodynamic focussing process that allows for passing an illumination and sensing unit within a defined distance. Cells in the center of the sample stream are illuminated, for example, by a laser beam, interact with the light and respond with light emission at different angles, intensities, and wavelengths. The optical signal of individual cells sequentially passing the sensor are compared with previously defined criteria for target and nontarget cells, and the fluid stream containing the cell suspension is split to direct different portions of the stream into different collection containers [1].

Different technologies are used to split the stream. Droplet sorters are the most widely used flow sorting technology. The fluid stream is broken into droplets – for example, by a vibrating nozzle. Some droplets contain cells, and droplets containing the desired target cells or unwanted nontarget cells can be identified through prior optical analysis and directed into collection containers.

Droplet frequencies of 2,000–100,000 per second can be achieved, limiting the sorting frequency to 50,000 (presort) cells per second.

Enclosed sorters are significantly slower ($<1000\text{ s}^{-1}$) than droplet sorters at significant lower costs and can be realized by different technology: catcher tube sorters move a collection tube into the liquid in air stream when a target cells has been detected; fluidic-switching sorters actuate valves in a branched fluid path, switching between different paths the cells can pass; and destructive sorters destroy nontarget cells, for example, by an intense laser beam.

The unique property of flow sorting is that a combination of multiple optical parameters can be used to identify the cell subset of choice.

2.1.2 Surface Staining

Stem cells differ from other cell populations by specific proteins expressed at the cell surface (cell surface markers). Monoclonal antibodies can selectively bind to cell surface proteins, and fluorescent dyes conjugated to the antibody thus tag the cell of interest. CD34 and CD133 cell surface molecules are frequently used to identify and sort human hematopoietic stem cells. The presence or absence of additional markers can be used to further define the target cell population (e.g., CD38-negative; see Sect. 4 for details).

2.1.3 Side Population Sorting

Stem cells are frequently described as being a “side population”, which is by definition a rare cell population distinguished from most other cells by specific characteristics. Stem cells differ from nonstem cells in their ability to transport Hoechst stains (Hoechst 33342) out of the cell. Hoechst 33342 is a DNA-binding fluorescent dye, excitable by ultraviolet light at 350 nm and emitting at 461 nm. A multidrug-like transporter in stem cells causes an increased efflux of Hoechst 33342 by an active biological process. Figure 1 shows a typical flow cytometric characterization of side population cells.

2.1.4 Aldefluor®

Stem and progenitor cells possess a different aldehyde dehydrogenase (ALDH) activity compared to nonstem cells. This enzyme converts a nonfluorescent substrate (an aminoacetaldehyde) into a fluorescent product (an aminoacetate) that is retained within living cells with an intact membrane. Cells with different ALDH enzyme activity can thus be differentially stained with the fluorescent product, and stem cells can be isolated based on their enzyme activity [2, 3]

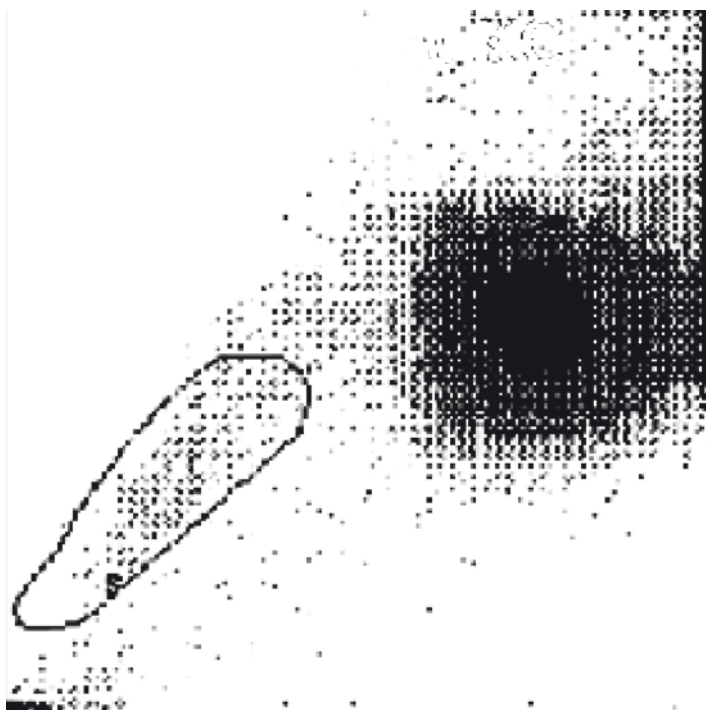


Fig. 1 Side population cells (*encircled*) are characterized by a low staining intensity for Hoechst dyes, and represent a low proportion of measured cells

2.2 *Magnetic Cell Sorting*

2.2.1 Introduction

Magnetic cell sorting has become a standard method for cell separation in many different fields. Numerous publications have demonstrated its use, from lab bench to the clinic; small to large scale; from abundant cells to rare cells with complex phenotypes; from human and mouse cells to many other species. Isolation of almost any cell type is possible from complex cell mixtures, such as peripheral blood, hematopoietic tissue (spleen, lymph nodes, thymus, bone marrow), nonhematopoietic tissue (e.g., solid tumors, epidermis, dermis, liver, thyroid gland, muscle, connective tissue) or cultured cells [4–11]. There are various magnetic cell separation systems currently available. They differ principally in two features: the composition and size of the magnetic particles used for cell labeling [5, 12] and the mode (i.e., “positive isolation”/enrichment or “negative isolation”/depletion) of magnetic separation.

2.2.2 Technology

The MACS® System is characterized by the use of nano-sized superparamagnetic particles (approx. 50 nm in diameter), unique separation columns, and MACS Separators providing the required strong magnetic field [5, 8, 10].

Magnetic cell separation using MACS Technology is performed in three steps as outlined in Fig. 2. The entire procedure can be performed in less than 30 min, and both cell fractions, magnetically labeled and untouched cells, are immediately ready for further use, such as flow cytometry, molecular analysis, cell culture, transfer into animals, or clinical cell therapy applications.

Dynabeads® represent an example of larger, e.g., cell-sized, magnetic beads, to be used in a tube-based system. They are super-paramagnetic and are made from a synthetic polymer [13, 14]. The starting sample is incubated with the beads, and the test tube is then placed in the field of a strong permanent magnet. Complexes of cells and beads are attracted to the wall of the tube, and the supernatant can thus be removed.

Both cell fractions can be used – bead-captured cells and untouched cells. Should captured cells be subjected to functional studies, the beads need be removed [15], e.g., by enzymatic cleavage or binding competition with affinity molecules (peptides, antibodies, biotin) disrupting the binding of antibodies to the target molecules.

2.2.3 Magnetic Separation Strategies

Magnetic cell separation is a very simple but flexible technique, with two basic strategies (“modes”): positive selection or negative selection (“depletion”). The optimal separation strategy depends on the abundance of target cells in the cell sample, their phenotype compared with other cells in the sample, the availability of

reagents, and a full consideration of how the target cells are to be used, including any restrictions with respect to purity, yield, and activation status.

Positive selection means that the desired target cells are magnetically labeled and isolated directly, representing the positive cell fraction (see Fig. 2). It is the most direct and specific way to isolate the target cells from a heterogenous cell suspension and requires a cell surface marker specific for the target cells. Positive selection is particularly well suited for the isolation of rare cells, such as hematopoietic stem cells, from complex cell mixtures, such as blood cells (for an example see Fig. 3).

Both fractions – labeled and unlabeled – can be recovered and used. Due to their composition of iron oxide and polysaccharide, MicroBeads are biodegradable and typically degrade and disappear rapidly when the cells are cultured. MicroBeads attached to receptors that are internalized and recycled to the cell surface may even be degraded much faster.

Depending on the cell type, on the target surface molecules used for magnetic labeling, and on the labeling moiety of the MicroBeads (mAb or ligand), the functional status of the cells can be influenced. This is inherent to labeling with Ab or ligands that recognize and crosslink cell surface receptors and thus may induce or suppress signal transduction. Labeling with antibody-conjugated MicroBeads has no additive effect compared to labeling with an unconjugated crosslinking Ab.

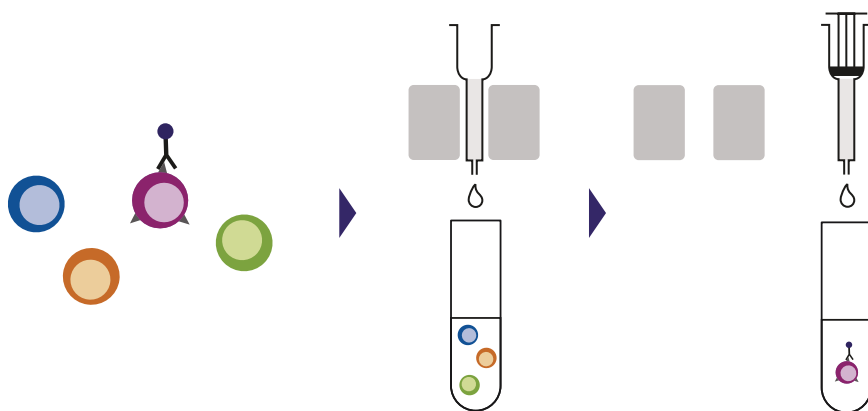


Fig. 2 Principle of high-gradient magnetic cell sorting. The procedure comprises three steps. Magnetic labeling (*left*): The cell preparation and labeling methods are similar to those used in flow cytometry. Individual cells of a cell suspension are immunomagnetically labeled using MACS MicroBeads, which typically are covalently conjugated to a monoclonal antibody (mAb) or to a ligand specific for a certain cell type. Magnetic separation (*middle*): The cell suspension is passed through the separation column that contains a ferromagnetic matrix and is placed in a MACS Separator. The separator contains a strong permanent magnet creating a high-gradient magnetic field in the magnetizable column matrix. Labeled target cells are retained in the column via magnetic force, whereas unlabeled cells flow through. By simply rinsing the column with buffer, the entire untouched cell fraction can be eluted. Elution of the labeled cell fraction (*right*): After removing the column from the magnetic field of the MACS Separator, the retained labeled cells can easily be eluted with buffer

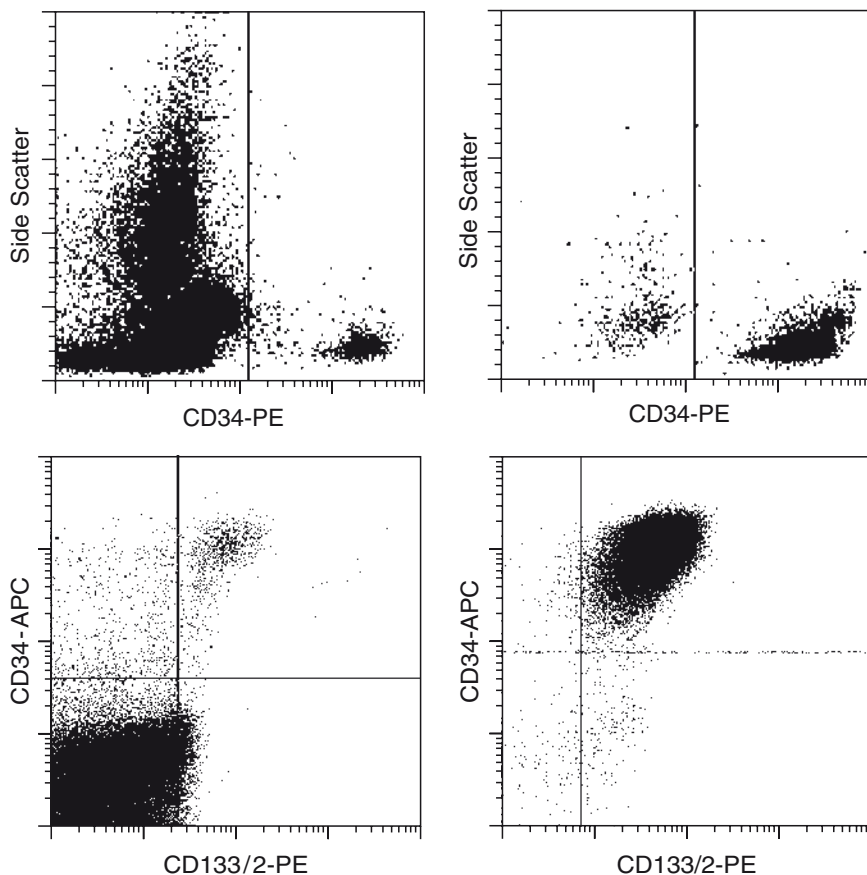


Fig. 3 FACS diagrams showing isolation of stem cells by positive selection with CD34 and CD133 directly conjugated antibodies and the CliniMACS Plus Instrument. CD34 cells were enriched from mobilized leukapheresis product (*upper row*), CD133 cells from bone marrow aspirate (*lower row*). Diagrams show the cellular composition before (*left column*) and after (*right column*) enrichment. Mononuclear cells from peripheral blood (PBMC), cord blood, bone marrow, fetal liver or leukapheresis harvest are obtained by density gradient centrifugation using Ficoll Paque®. For CliniMACS separation, hematopoietic stem and progenitor cells are directly magnetically labeled using MACS MicroBeads specific for CD34 and CD133, respectively. After enrichment, 99.2 or 96.7% pure stem cell fractions are obtained starting from frequencies of 0.92 and 3.1%

In summary, positive selection should be considered for (1) excellent purity, especially for enrichment of rare cells, (2) excellent recovery, and (3) fast procedures.

Depletion or negative isolation, on the other hand, means that the unwanted cells are magnetically labeled to eliminate them from the cell mixture, whereas the nonmagnetic, untouched fraction contains the cells of interest (Fig. 4). Potential effects on the functional status of cells can thus be minimized. A single depletion procedure can remove up to 99.99% of the magnetically labeled cells, leaving a highly pure fraction of unlabeled cells.

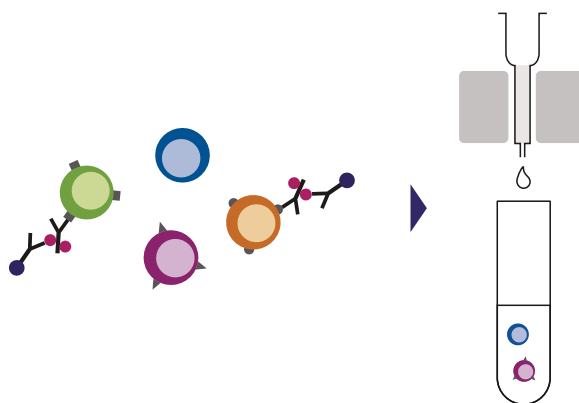


Fig. 4 Depletion strategy. The unwanted cells are labeled with immunomagnetic MicroBeads or a cocktail thereof and applied to the column. Labeled cells are eliminated on the column, and the untouched fraction with the cells of interest is collected in the flow-through

In particular, this strategy may be advantageous if functional studies have to be performed with the target cells, such as T cell activation studies or gene expression profiling. If the desired target cells are heterogeneous or do not have a well-defined phenotype, removing well-characterized cells by depletion is an efficient way to isolate the target cell population. Commonly used examples of depletion approaches include the depletion of cancer cells from autologous stem cell grafts and the depletion of T cells and B cells from allogeneic stem cell grafts.

In summary, a depletion strategy should be considered (1) for the removal of unwanted cells, (2) if no specific antibody is available for target cells, (3) if binding of antibody to target cells is not desired, and (4) for the subsequent isolation of a cell subset by means of positive selection (see below).

Multiparameter magnetic cell sorting is the strategy for isolating target cells that cannot be defined by a single cell surface marker, but by multiple cell surface antigens. Using only magnetic separation, sequential isolation of even complex targets cells can be achieved, combining both depletion and positive selection steps. There are several different routes for multiparameter magnetic sorting.

Commonly, a first step is “debulking” of the start population by using a panel of reagents directed against multiple cell surface antigens to deplete for several markers simultaneously.

Second, depletion may be followed by positive selection. The nonretained cells from the first separation are again magnetically labeled and enriched on a second column. In order to obtain highest purity, different stringencies may be used for the two separations. The depletion step can be performed on a steel-wool column with the highest retention rates for labeled cells and the enrichment step is performed on an iron-sphere matrix column with the lowest unspecific retention rates for unlabeled cells. This reduces the probability that labeled cells will be carried over from the first separation step into the second.

A third option is sequential positive selection. This can be accomplished by using colloidal superparamagnetic particles, which can be rapidly released from the cell (MultiSort MicroBeads) using an enzyme. Since the specificity of the enzyme is unique to the magnetic particles, cell surface molecules are not modified. MultiSort MicroBeads are typically used for a first positive selection. After this first step, release of the MultiSort MicroBeads takes less than 10 min. The cells are then ready for further labeling and another separation cycle.

The concept of positive selection followed by depletion is very attractive for the depletion of contaminating tumor cells or alloreactive T cells from purified CD34⁺ hematopoietic progenitor cells for therapeutic autologous or allogeneic stem cell grafting. This concept requires either the combination of MultiSort MicroBeads and MicroBeads or the use of MicroBeads followed by larger magnetic beads.

2.2.4 Magnetic Labeling Strategies and Reagents

Direct labeling is the fastest way of magnetic labeling. Only one labeling step is required if a monoclonal antibody specific for a certain cell surface antigen can be directly coupled to the MicroBeads (Fig. 5, left).

Direct labeling minimizes the number of washing steps and thereby prevents cell loss. For many human, mouse, rat, and nonhuman primate cell surface markers, antibody-conjugated MicroBeads are available as one-step reagents.

Indirect labeling (Fig. 5, right) is performed if no direct MicroBeads are available, if a panel of antibodies directed against multiple cell surface antigens is used, or if two-step magnetic labeling is significantly more efficient compared to one-step labeling, for example, with weakly expressed antigens or antibodies of low affinity.

Cells are labeled with a primary antibody that is unconjugated, biotinylated, or fluorochrome-conjugated. In a second step, three different indirect magnetic labeling methods can be used:

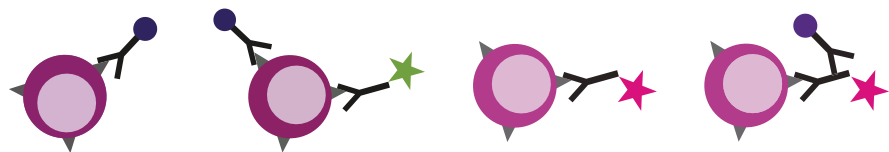


Fig. 5 Principles of magnetic labeling with superparamagnetic MACS MicroBeads. Direct labeling (*left*): One-step magnetic labeling, where a cell surface-antigen specific mAb is directly conjugated to the MicroBeads. Indirect labeling (anti Ig) (*right*): Two-step magnetic labeling with a primary cell surface-antigen specific Ab and anti immunoglobulin Ab-conjugated MicroBeads. Like any staining reagent, each magnetic bead reagent must be titrated for optimal cell separation, using different concentrations of MicroBeads for one otherwise standardized separation and determining the concentration with the best performance with respect to purity and yield of the cells of interest

1. MicroBeads conjugated with antiimmunoglobulin antibody to detect unlabeled primary antibody
2. MicroBeads conjugated with streptavidin or antibiotin antibody to detect biotinylated primary antibody
3. MicroBeads conjugated with anti fluorochrome antibody (e.g., antiFITC) to detect fluorochrome-labeled primary antibody

A cocktail of antibodies can also be used for isolating or depleting a number of cell types concurrently. This amplifies the magnetic labeling and thus, indirect labeling may be the method of choice if dimly expressed markers are targeted for magnetic separation.

2.2.5 Superparamagnetic MicroBeads

MACS MicroBeads are superparamagnetic particles made of an iron oxide core and a dextran coating. They are nano-sized, ranging between 20 and 150 nm in diameter (see Fig. 6), and form colloidal solutions, i.e., they remain dispersed [5, 8]. Superparamagnetism means that in a magnetic field the iron oxide cores magnetize strongly like ferromagnetic material, but when removed from the magnetic field the particles do not retain any residual magnetism. The dextran coating of the MicroBeads permits chemical conjugation of biomolecules. Numerous highly specific mAb, fluorochromes, oligonucleotides and various other moieties have all been covalently linked to MicroBeads, thereby transferring additional biochemical and physical properties to them [5, 6].

The nano-sized iron-dextran particles confer several unique features on MACS Technology. MACS MicroBeads are biodegradable and do not alter cell function. Effects on the functional status of cells by magnetic labeling with MicroBeads are primarily dependent on the target cell surface antigen and on the degree of cross-linking by mAb or ligands conjugated to the MicroBeads, but not on the MicroBeads themselves. Cells labeled with MicroBeads have been used for numerous functional in vitro assays, experimental transfers into animals, and therapeutic transplantations in humans.

2.2.6 Column Technology and Separators

MACS MicroBeads are extremely small, and the amount of magnetizable material bound to cells is very low. Specific devices are required to generate a high-gradient magnetic field powerful enough to retain the labeled cells. MACS Technology uses high gradient magnetic cell separation units consisting of a strong permanent magnet of 0.4–1 Tesla and a separation column with a matrix of iron spheres.

When the columns are placed between the poles of the magnet of a MACS Separator, high magnetic gradients up to some 10^4 T/m are generated in the vicinity of the ferromagnetic matrix. The magnetic force is then sufficient to retain the target cells labeled with a very small number of MicroBeads. Once the column is removed

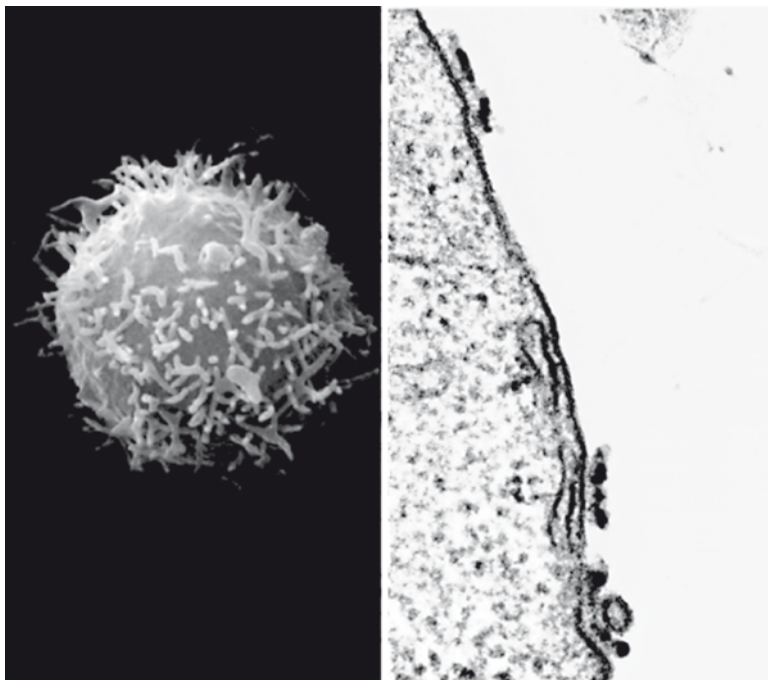


Fig. 6 Scanning (*left*) and transmission (*right*) electron micrograph of a CD8⁺ T cell. The cell was isolated with MACS Technology using CD8 Ab-conjugated superparamagnetic MicroBeads (EM courtesy of Prof. Groscurth, Zürich, Switzerland). Some superparamagnetic MicroBeads attached to the membrane are visible on the micrograph image. They are about 50 nm in diameter, form colloidal solutions, and are biodegradable. Their small size enables high kinetics of the MicroBead-cell reaction and minimizes unspecific binding. Thus, cell enrichment of more than a 10,000-fold is possible from frequencies below 10^{-8}

from the magnet, the column matrix rapidly demagnetizes, and retained cells can be easily and completely eluted simply by rinsing the column with buffer.

MACS Columns for research use are available in various sizes (Fig. 7) for fast (5–30 min) processing of different amounts of cells. Up to 2×10^{10} cells, containing up to 10^9 target cells can be routinely handled. This is in striking contrast to fluorescence-activated cell sorting (FACS, see Sect. 2.1.1), where cells are sorted one after the other, limiting the sorting speed to about 50,000 cells per second, that is, 10^8 cells in 33 min, or a leukapheresis pack with 10^{10} cells in 56 h.

With the autoMACS and autoMACS Pro Separators, column-based magnetic cell separation can also be automated in order to standardize frequent cell separations.

2.2.7 Clinical-scale Cell Separation

Magnetic cell separation technologies have provided novel tools to use specified cell populations for treatment of patients. Desired effects such as reconstitution of



Fig. 7 Hardware and instruments. A variety of different MACS Separators and Columns is available, each individually designed for specific applications. The *OctoMACS* Separator (*left*), for example, is a device for separations of up to 10^8 labeled cells and up to 2×10^9 total cells in combination with LS columns. The *autoMACS Pro Separator* (*right*) is an automated benchtop magnetic cell sorter for high cell numbers or multiple samples. It is capable of sorting up to 10 million cells per second from samples of up to 4×10^9 cells

the immune system can be utilized while sparing unwanted effects of nontarget cells such as immune reactions vs patient tissue [16]. Two devices for isolation of stem cells by magnetic cell separation technologies are available. They differ in the size of magnetic particles used (see Sect. 2.2.2).

CliniMACS® Plus Instrument

The CliniMACS Plus Instrument is an automated cell separation device based on MACS Technology. It enables the operator to perform large-scale magnetic cell separation in a closed and sterile system (Fig. 8).

The use of clinical-grade isolation or depletion of cells has grown dramatically over the past few years, and is now a standard technique established in many cellular therapy centers. The CliniMACS Plus Instrument is a flexible system for separating cells labeled with clinical-grade MicroBeads. Cells are processed and labeled in a closed bag system using standard clean-room techniques. The processed cells are then attached to a tubing set and processed using the preset programs of the CliniMACS Plus Instrument. Target cells are recovered in a transfer pack or cell culture bag ready for downstream processing, again using a closed system.

Stem cells isolated with the CliniMACS Plus Instrument are used for stem cell grafts (“graft engineering”) to reconstitute the immune system in the context of tumor therapies (chemotherapy, whole body irradiation) and for regeneration of patient tissues (regenerative medicine, tissue engineering; see Chap. 5 for details). Graft engineering procedures can be performed by both positive isolation (CD34 or CD133 enrichment) and negative isolation (CD3/CD19 depletion); see Sect. 2.2.3.



Fig. 8 CliniMACS® Plus Instrument. The CliniMACS System is an automated cell separation system for clinical-scale magnetic enrichment of target cells or depletion of unwanted cells in a closed and sterile system. For separation, a single-use tubing set, including a separation column, is attached to the CliniMACS Plus Instrument. Then the cell preparation bag containing the labeled cells is connected to the tubing set. After starting the separation program, the system automatically applies the cell sample to the separation column, performs a series of washing steps, and finally elutes the purified target cells. The CliniMACS® System components (Reagents, Tubing Sets, Instruments and PBS/EDTA Buffer) are manufactured and controlled under an ISO 13485 certified quality system. In Europe, the CliniMACS System components are available as CE-marked medical devices. In the USA, the CliniMACS System components including the CliniMACS Reagents are available for use only under an approved Investigational New Drug (IND) application or Investigational Device Exemption (IDE). CliniMACS® MicroBeads are for research use only and not for use in humans

Isolex® 300i

Baxter Healthcare Corporation has adapted Dynabead-based stem cell isolation to an automated process in a clinical scale. The Isolex 300i Magnetic Cell Selection System allows for separation of CD34-positive cells. Magnetic beads are removed from the isolated stem cells using a competing peptide [15, 17, 18].

2.2.8 Evaluation of Separation Performance

Different technologies for isolation and enrichment of stem cells are available, and thus, cell separation performance parameters are useful to compare those methods.

The most evident performance parameter for isolation of stem cells is the purity of target cells, i.e., the frequency of stem cells within a given processed target cell population:

$$\text{purity} = \frac{\# \text{ stem cells}}{\# \text{ all cells}} 100\%.$$

Nevertheless, purity of stem cells alone is not a sufficient performance parameter, as one always needs a specific number of stem cells for either basic research or clinical applications. Thus recovery of almost all of the stem cells contained in the initial cell product is desirable:

$$\text{yield} = \frac{\# \text{ stem cells_in_processed_sample}}{\# \text{ stem cells_in_unprocessed_sample}} 100\%.$$

It is obvious that 100% purity of target cells with 100% yield during processing would be optimal, at best combined with a low processing time. In practice, and for a given technology, optimizing one parameter can only be done at the expense of another, moving a coordinate within the area of a triangle (see Fig. 9).

Purity and yield characterize the processed cell product. Both may significantly depend on the input product, e.g., abundance of target cells before processing. Additional parameters have been defined that characterize a relative separation performance:

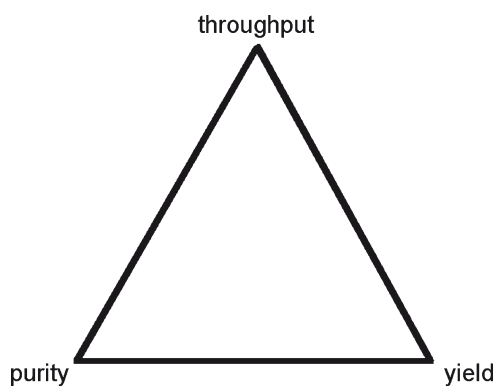


Fig. 9 The separation triangle. Each point within the triangle represents a possible parameter set in three dimensions (purity, yield, throughput). For a given process performance, parameters can only be optimized by compromises in other parameters, i.e., 100% purity and 100% yield of target cells cannot be combined with maximum throughput

$$\text{Enrichment rate } f_E = \frac{\%pos_{pos} / \%neg_{pos}}{\%pos_{ori} / \%neg_{ori}}$$

$$\text{Depletion rate } f_D = \frac{\%pos_{ori} / \%neg_{ori}}{\%pos_{neg} / \%neg_{neg}}$$

In these equations, %pos means the frequency of cells “positive” for a specific marker, e.g., CD34, and %neg means the frequency of cells “negative” for the same marker (100%–%pos).

Stem and progenitor cells are usually very rare in cell samples being used for isolation. Thus, high enrichment rates are required to obtain optimal purity. For a given technology the final purity will depend on the input frequency of target cells (see Fig. 10). Using MACS Technology, enrichment rates of up to 5,000 can be achieved.

Typical depletion rates are 5–200, and for a positive isolation strategy they assess how many labeled target cells are lost into the flow-through fraction. For a negative isolation strategy the depletion rates measure how effective labeled non-target cells are removed from the sample.

Both enrichment rate and depletion rate use frequencies of cell populations for calculation and do not take into account possible bulk cell loss during processing. Graft engineering procedures thus typically use different parameters for evaluation of separation performance, based on absolute cell numbers. The probability P defines the fraction of nontarget cells (e.g., CD34-negative cells) that are still contained in the final cell product:

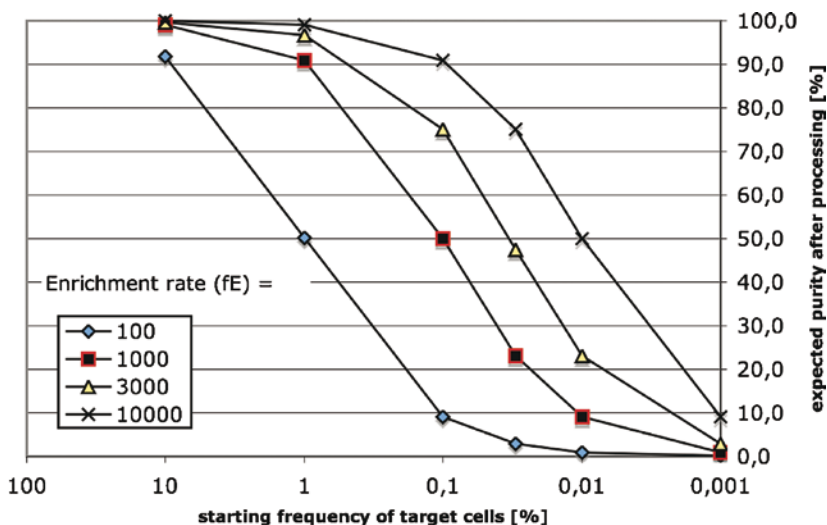


Fig. 10 Dependence of purity on enrichment rate and starting frequency. The final purity of a cell separation procedure depends on the frequency of target in the unprocessed sample and on the enrichment rate of the respective separation technology used. With MACS Technology, enrichment rates of up to 5,000 can be achieved. In conclusion, high purity is only achievable with a high enrichment rate and moderate starting cell frequency

$$P = \frac{\# \text{neg}_{\text{pos}}}{\# \text{neg}_{\text{ori}}},$$

where “#neg” is the number of negative (i.e., nontarget) cells. A typical probability of a CliniMACS separation procedure using CD34 as a target molecule to carry over nontarget cells to the final cell product is below 0.4×10^{-4} , i.e., > 99.96% of CD34-negative cells are removed.

P is usually very small for high performance cell separation systems. Therefore, the logarithmic scale is used:

$$-\log P = -\log 10 \frac{\# \text{neg}_{\text{pos}}}{\# \text{neg}_{\text{ori}}}.$$

CliniMACS CD34 procedures typically achieve a >3.5 log depletion of CD34-negative cells.

When stem cell isolation is used clinically for graft engineering of hematopoietic stem cell grafts for allogeneic transplantation, the removal of T cells is of utmost importance for patient safety. T cells in the graft may cause life-threatening immune reactions versus patient tissue (graft vs host disease, GVHD). Therefore, graft engineering performance is frequently characterized by the efficiency of T cell depletion rather depletion of all CD34-negative nontarget cells.

When a stem cell isolation system, such as the CliniMACS Plus Instrument, is characterized with regard to nontarget cell carry-over (e.g., $-\log P$ of 3.5), the stem cell purity of the final product mainly depends on the starting frequency, and Fig. 11 may be used to predict stem cell purity for samples with different stem cell content.

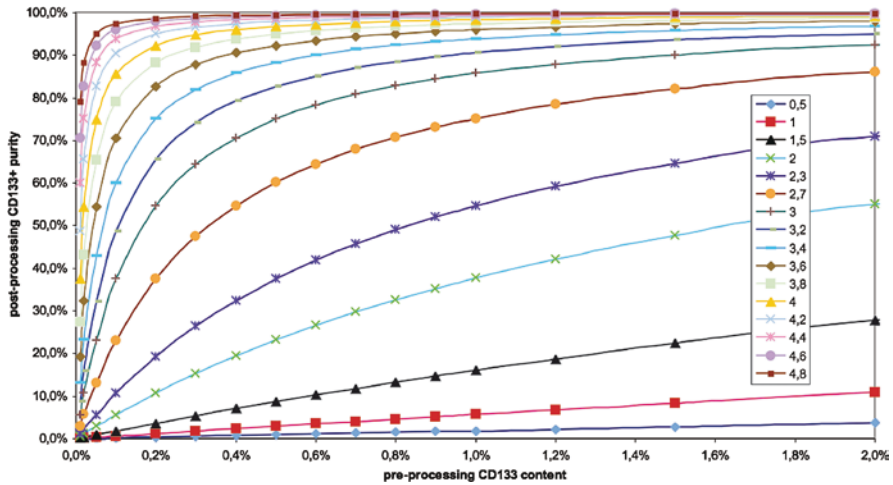


Fig. 11 Dependence of purity on depletion efficiency of nontarget cells and preprocessing stem cell content. The final purity of a cell separation procedure depends on the frequency of target cells in the *pre-processing stem cell* sample and on the depletion efficiency of nontarget cells of the respective separation technology used. With MACS Technology, depletion efficiencies of up to 4.5 orders of magnitude can be achieved

3 Isolation and Enrichment of Embryonic Stem Cells

3.1 Introduction

Embryonic stem cells (ESC) are not continuously present in an organism but can be derived during a very limited period of time from the inner cell mass of blastocysts. The indefinite in vitro self renewal of mouse ESCs (mESC) and moreover their pluripotency, that is, the capacity to differentiate into every cell type in the body, was described for the first time more than 25 years ago [19, 20]. Later on, ESCs were derived from a number of different species and finally also from human preimplantation embryos [21]. Due to their unique properties, ESCs have been used in a variety of different fields: (1) in basic research to understand general cellular processes in, for example, embryogenesis, organogenesis, cancer, or ageing, and as a vehicle for the generation of transgenic mice for functional gene analysis and disease models; (2) in industrial research as cell-based screenings for drug target discovery, drug discovery, or predictive toxicology; (3) in clinical research as a potential source for tissue regeneration. The recently described derivation of induced pluripotent stem cells (iPS cells) from differentiated, postmitotic cells that behave similar to ESCs have sparked the whole field even more [22]. With iPS cells combining the advantages of ESCs and autologous cell transplantation, the generation of patient specific derived stem cells for unrestricted tissue regeneration and without ethical issues can be envisaged.

The broad application of ESCs, but also the fact that they are kept in culture for a prolonged time, has led to a number of different protocols for their derivation, isolation, and enrichment at a pluripotent stage or after differentiation into a certain cell type.

The techniques involved for the isolation and enrichment are generally the same as described above, including selective culturing, immunopanning, flow cytometric sorting, or magnetic sorting.

3.2 Selective Culturing of Embryonic Stem Cells

For historical reasons, the most eminent protocol for enrichment of pluripotent mouse and human ESCs is based on selective culturing. A detailed description for the derivation of ESCs can also be found elsewhere in this book (see Itskovitz-Eldor). In brief, mouse ESCs are derived from embryonic day 3.5 blastocysts by letting them attach and expand on mitotically inactivated murine embryonic fibroblast layer in a medium containing leukemia inhibitory factor (LIF) [23]. Expanded blastocysts are repeatedly trypsinized and single clones derived. As the original protocol was quite inefficient, with a success rate of up to 30% and strong dependency on the mouse strain, many improvements have been introduced. Such improvements include use of specifically conditioned medium [24], genetically modified blastocysts [25], microdissection of the blastocyst [26], treatment with pharmacological drugs [27], and use of serum replacement (SR) [28].

Human ESCs have been derived using similar protocols as originally described for mouse ESCs. However, at least the first hESC lines had a higher tendency for spontaneous differentiation and a lower proliferation rate which made the handling much more difficult – up to the point that individual colonies need to be selected by a micropipette according to their undifferentiated morphology and then mechanically dissociated into clumps in order to proliferate them at an undifferentiated stage [21].

Despite almost 10 years of research, currently available hESCs show heterogeneous phenotypes, and the consistency of culture is still a challenge for many labs. No generally applicable culture protocol has evolved [29]. Different laboratories culture hESCs either feeder-free (“matrix culture”) [30], with mouse embryonic fibroblasts (MEF) [21] or with different kinds of human fibroblasts (HEF) as feeder cells [30]. Also, the propagation of hESCs is either done by mechanical (“cut and paste”) or enzymatical dissociation of cell colonies using serum-containing or serum-free/xeno-free media. The main difficulties still arise from the observation that singularized hESCs tend to differentiate spontaneously if culturing conditions are not tightly controlled.

To address these problems, a study (ISCI II) has been started which is coordinated by the International Stem Cell Forum (<http://www.stemcellforum.org/>) and follow the ISCI I ring study which originally aimed to characterize 59 human embryonic stem cell lines [31]. The ISCI II study is carried out in four reference laboratories and seeks to clarify if certain media are able to support pluripotent growth of hESC for 40 passages (1 year) while maintaining a stable karyotype.

3.3 Isolation and Enrichment of ESCs Based on Surface Markers

As one way to standardize culturing of ESCs and to synchronize undifferentiated but also differentiated ESCs, populations can be envisaged by using cell sorting techniques which are based on the expression of stage-specific surface markers.

With regard to sorting of pluripotent embryonic stem cells, different monoclonal antibodies reacting with surface markers of undifferentiated (pluripotent) ESCs have been described. These markers differ partly between mouse and human ESCs. For mouse ESCs, these are mainly E-cadherin (CD324) and SSEA-1 (CD15).

For human ESCs, CD90, GCTM2, GCTM343, SSEA-3, SSEA4, CD9, TRA-1-60, TRA-1-81 and HLA A/B/C have been suggested [31]. The enrichment of pluripotent ESCs has been used for different purposes. For example, a synchronization of mESC cultures by sorting with SSEA-1 (CD15) MicroBeads has been described by Cui et al. [32].

In another report, immunomagnetic sorting has been used to separate pluripotent mESC from mouse embryonic feeder cells with a primary SSEA-1 antibody. In a slightly different approach, Annexin V MicroBeads have been used to remove apoptotic cells from mESC during normal cultivation or differentiation [33].

Similarly, SSEA-3 has been used for flow separation of undifferentiated human ESC [34]. Based on SSEA-3 expression, the authors propose a cellular differentiation

hierarchy for maintenance cultures of hESC. While SSEA-3⁺ cells represent pluripotent stem cells, normal SSEA-3⁻ cells have exited this compartment, but retained multilineage differentiation potential. However, adapted SSEA-3⁺ and SSEA-3⁻ cells cosegregate within the stem cell territory, implying that adaptation reflects an alteration in the balance between self-renewal and differentiation.

SSEA-3 and SSEA4 have not been classified as ultimate markers of pluripotency, due to their slow kinetics upon differentiation. Search for those markers is still ongoing and several groups claim to have identified such fast downregulated markers [35].

Besides the selection of pluripotent stem cells to ease and standardize the propagation of undifferentiated ESC, the capability of undifferentiated hESCs to form teratomas is a risk factor worth considering when applying hESC-derivatives to cellular therapy. Again, cell sorting techniques might help to enrich target cell types and to deplete unwanted cell types or undifferentiated hESCs. Lastly, the removal of residual pluripotent mESC from differentiated cells can also be used to purify ESC-derived cell populations. Hedlund et al., for example, have reported the selection of murine dopaminergic neurons by sorting of TH-EGFP positive and SSEA-1 negative cells before transplantation [36].

3.4 Sorting of Cell Types Derived from Embryonic Stem Cells

A number of protocols have been reported for the targeted differentiation of ESCs to progenitor or postmitotic cell types. By exposure of pluripotent ESCs to growth factors, such as basic fibroblast growth factor (bFGF), transforming growth factor beta1 (TGF-beta1), activin-A, bone morphogenic protein 4 (BMP-4), hepatocyte growth factor (HGF), epidermal growth factor (EGF), beta nerve growth factor (betaNGF), or retinoic acid, almost every somatic mouse and human cell type has been generated [37]. This includes neurons, glia, skin, muscle, bone, and many others [37, 38].

However, the characterization of the derived cell types is often limited to surface marker description which is obviously not an unambiguous proof for a given cell type. Also, most protocols do not direct the differentiation exclusively to one cell type, but to multiple routes of differentiation and a mixture of different stages of differentiation. This again makes it desirable to enrich specific cell types of interest or to deplete unwanted cell types. A great number of surface differentiation markers – essentially all those which are also used for the characterization or isolation of somatic cells – have been described for mouse or human ESC-derived progenitors or differentiated cell types, amongst others: A2B5, PSA-NCAM, CD56 (NCAM), O1, O4, CD309 (VEGFR-2/KDR/Flk-1), Sca-1, CD117 (c-kit), CD34, CD133 (Prominin), CXCR4, CD324 (E-cadherin). Enrichment of mESC-derived hematopoietic/endothelial (hemangio) precursor cells has been achieved by indirect immunomagnetic sorting [39] and in another report, direct labeling with Sca-1

MicroBead-conjugated antibodies has been used for mESC-derived vascular progenitors [40]. Recently, it was also shown that CD56-positive neural cells derived from hESCs can be sorted magnetically with good survival rates [41].

Notably, epitopes like CD324 (E-cadherin) might also be used as markers for particular differentiation stages. Considering this, a general feature of surface marker-based cell sorting becomes apparent. It does not essentially have to be a marker exclusively expressed on a certain cell type at a certain differentiation stage. A unique expression in relation to the other cell types present in a given organ or cell culture can be sufficient for cell sorting.

The success of efficient enrichment of undifferentiated cells depends – besides other factors – on the turnover rate of these markers, especially when differentiation of ESCs starts, on the number of marker protein per cell, and on the specificity and avidity of the monoclonal antibodies.

As already mentioned above, by using a negative sorting strategy, early markers of differentiation can also be used to enrich untouched undifferentiated cells by depletion protocols [36].

3.5 Sorting Based on Genetically Modified Embryonic Stem Cells

Despite the obvious advantages of marker-based cell sorting, so far, magnetic cell separation has just started to be used for the enrichment or depletion of pluripotent ESCs and ESC-derivatives. Cell separation by flow cytometry is already used more routinely, especially with the help of genetically modified mESCs which express EGFP under control of a given cell-type specific promoter [42, 43]. Interestingly, the approach of using genetically modified ESCs to enrich differentiated derivatives can also be used for magnetic cell sorting (Fig. 12). For example, David et al. [44] reported the labeling of stably transfected ES cells expressing a human CD4 molecule lacking its intracellular domain (DeltaCD4) under control of the phosphoglycerate kinase promoter for magnetic cell sorting. The membrane-bound protein allowed for immunomagnetic sorting with purities greater than 97%. The viability of selected cells was demonstrated by reaggregation and de novo formation of embryoid bodies developing all three germ layers.

It was concluded that expression of DeltaCD4 in differentiated ES cells can be used for a rapid high-yield purification of a desired cell type for tissue engineering and transplantation studies.

Combined selections of GFP-expressing and surface marker-positive cells have recently been described for the enrichment of mESC-derived cardiomyocyte precursors. Here, GFP-expression was controlled by promoters of mesodermal- or cardiomyocyte-specific transcription factors and coselection performed with antibodies against the surface markers CD309 (VEGFR-2/KDR/Flk-1) or CD117 (c-kit) [45–47].

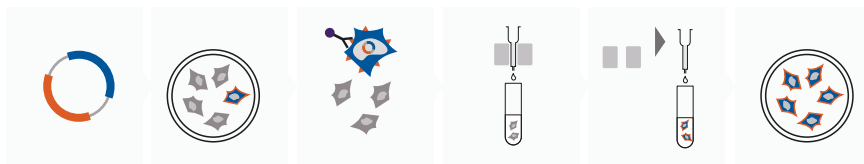


Fig. 12 Immunomagnetic enrichment of ESCs or derivatives thereof using genetically modified embryonic stem cells. ES cells are stably transfected with a vector carrying a certain cell type-specific promoter, which drives the expression of a vector-coded surface resident protein. This surface marker can then be used for immunomagnetic labeling and separation by MACS Technology

3.6 Concluding Remarks

In the past, isolation and enrichment of ESCs and their derivatives was mainly achieved by selective culturing. With the advent of genetically tagged ESCs, and supported by an increasing availability of antibodies reacting with cell surface markers expressed only on specific cell types or at certain differentiation stages, flow and magnetic cell sorting has become more popular. The surface marker-based sorting of cells offers great potential to optimize further the routine culturing but also the differentiation protocols of ESCs. Both the starting population as well as intermediate and postmitotically differentiated ESCs can be enriched to high purity. Especially magnetic cell sorting with its advantage of swift processing of high cell numbers, also in a closed setting, will help translate ESC research to clinical applications. The recently generated induced pluripotent stem cells (iPS cells), which essentially behave like ESCs and are thought to pave the way for autologous tissue regeneration approaches, will greatly profit from the knowhow currently generated with ESCs.

4 Adult Stem Cells

4.1 *Stem Cells from the Hematopoietic System with Hematopoietic Differentiation Potential*

For many applications that are under development for future clinical applications, mice are used as model organisms, facilitating the translation from basic in vitro research to the in vivo environment. Cell populations include cells with hematopoietic and nonhematopoietic differentiation potential, as well as pluripotent stem cells and differentiated progenitors.

Blood contains a complex mixture of cells, such as erythrocytes, the oxygen-transporting cells, the white blood cells comprising the cells of immune response,

such as lymphocytes (T cells, B cells, dendritic cells, etc.) and macrophages, as well as the platelets that trigger blood clotting in case of tissue damage. Hematopoietic stem cells (HSCs) generate all these cells and can thus be considered as being multipotent and capable of regenerating the complex hematopoietic system. HSCs give rise to more specialized progenitor cells with more limited differentiation potential, which are the progenitors of red blood cells, platelets, and the two main categories of white blood cells, the lymphoid and the myeloid progenitors.

4.1.1 Phenotype and Isolation of Mouse Hematopoietic Stem Cells

Several marker combinations have been identified that describe murine HSCs, including negative or low expression of lineage commitment markers such as CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4, and Ter-119, and high expression of markers such as stem cell factor receptor CD117 (c-kit/SCFR) and Sca-1 [48, 49]. This cell population is then called KSL. Additional markers have been defined to be not or only weakly expressed on the KSL population, such as CD90.1 and CD34.

Another strategy for defining hematopoietic stem and progenitor cells is the use of SLAM markers. A specific set of these markers, the “slam code,” is supposed to characterize hematopoietic stem cells and more committed progenitors for their potential [50]. SLAM cell surface markers delineate differentiation steps in early hematopoiesis. Originating with multipotent hematopoietic stem cells (HSCs), differentiation steps include multipotent progenitor cells (MPPs) and lineage-restricted progenitor cells (LRPs). Each is characterized by a different complement of SLAM markers: HSCs are CD150⁺ CD48⁻ CD244⁻; MPPs are CD150⁻ CD48⁻ CD244⁺; LRs are CD150⁻ CD48⁺ CD244⁺. It should be noted that CD48 is a ligand for CD244, and thus CD150⁺ CD48⁻ is sufficient to distinguish HSCs from MPPs and LRs.

Other ways to define these cells apart from by surface marker expression is the use of fluorescent mitochondrial and DNA-binding dyes, such as rhodamine-123 and Hoechst 33342. Primitive hematopoietic cells are able to transport the dye outward, resulting in a Hoechst^{low} phenotype.

Because of its characteristic flow cytometric profile, the Hoechst^{low} stem cell population has been designated as the “side population” (SP) phenotype [2]. SP cells are lineage-negative, which means that negative preselection approaches can be used to deplete mature cells from the sample, thus reducing the flow cytometric sorting time required to isolate SP cells. The SP phenotype has been attributed to high expression of membrane transporters. Although several multidrug transporter molecules are expressed in primitive cells, one transporter molecule, ABCG2 (or BCRP1), has been shown to be necessary and sufficient to mediate the Hoechst dye efflux ability of SP cells. Since ABCG2 expression is highest in primitive cells and gets downregulated during differentiation, this molecule might also be a potentially useful marker to identify and isolate primitive HSCs.

Other approaches have been made to define and isolate better the population of long-term repopulating HSCs (LTR-HSCs) – the most primitive HSCs in mouse

bone marrow. Chen and colleagues isolated a population of LTR-HSCs based on the expression of Sca-1 and CD105 in combination with Rhodamine 123 staining [51–53].

In addition to the hematopoietic potential described for stem cell populations KSL and SP, these populations also show a certain nonhematopoietic differentiation potential, although this issue is still controversial. Highly purified HSCs from mouse bone marrow have been reported to contribute to hematopoietic regeneration and also to hepatic regeneration with functional differentiation producing serum transaminases and bilirubin, as well as certain amino acids, such as phenylalanine [54]. Furthermore, these cells have been used to regenerate cardiac [55] and muscle [56] tissue and have been shown to contribute to neovascularization [57] as well as regeneration of the neural system [58]. However, the mechanism of their contribution has not yet been fully elucidated.

4.1.2 Phenotype and Isolation of Human Hematopoietic Stem Cells

Human CD34 was the first differentiation marker recognized on hematopoietic stem and progenitor cells from hematopoietic sources, such as fetal liver, cord blood, peripheral blood, and bone marrow. It is therefore the classical marker used to obtain enriched populations of human hematopoietic stem and progenitor cells (HSCs/HPCs) for research and clinical use. CD34 is expressed on approximately 1–3% of the nucleated cells in normal human bone marrow (BM) and on 0.1–0.5% of the nucleated cells in human peripheral blood. The majority of human cells capable of producing multilineage hematopoietic engraftment in myeloablated recipients express CD34. The engraftment potential of enriched populations of human CD34⁺ cells has also been demonstrated clinically in numerous autologous and allogeneic transplantation trials (see Chap. 5). The CD34⁺ subset also includes hematopoietic stem cells and more committed progenitor cells, such as lymphocyte progenitor cells, but is not expressed on the majority of terminally differentiated cells. Cytokine treatment and/or cytotoxic therapy increase the level of CD34⁺ cells in the blood to more than 1%. CD34⁺ cell mobilization regimens have become well-established methods to collect by leukapheresis sufficient amounts of HSCs for clinical transplantation (see Chap. 5 and references therein). Human CD34⁺ cells can be isolated by FACS or by immunomagnetic methods using monoclonal antibodies against CD34 coupled to superparamagnetic MicroBeads.

For the immunomagnetic depletion of mature cells from stem cell-enriched fractions, cells expressing lineage commitment markers can be depleted in a single negative selection step by using combinations of lineage-specific antibodies, such as CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a (Glycophorin A). Furthermore, CD34-enriched but CD38-depleted populations have been used to enrich for early hematopoietic progenitor cells [59, 60].

The usefulness of CD34 as a hematopoietic stem and progenitor cell marker for human cells is well established. There is evidence, however, of the existence of a very primitive population of CD34⁺ cells with HSC and lymphopoietic potential in human cord blood and adult hematopoietic sources. Thus far, the phenotype of

primitive CD34⁺ HSCs has been characterized by the concurrent absence of CD38, and the positive expression of CD133 [61]. CD133 has been described as a marker of more primitive hematopoietic stem and progenitor cells. It was originally found on HSCs and HPCs deriving from human fetal liver, bone marrow, and peripheral blood [62]. Phenotypical analysis of CD133-expressing cells (CD133⁺ cells) revealed a high expression on primitive hematopoietic and myeloid progenitor cells [63].

Functional studies showed that CD133 is lightly or not at all expressed on late progenitors, such as pre-B cells, CFU-E (colony forming units-erythrocytes), CFU-G (colony forming unit-granulocytes). Long-term culture-initiating cells (LTC-ICs), the most primitive human hematopoietic cells that can be assayed in vitro, are highly enriched among CD133⁺ cells [64, 65]. Thus, CD133⁺ cells in the hematopoietic system appear to be ancestral to CD34⁺ cells, especially as the latter can be generated in vitro from CD133⁺ CD34⁻ cells [66]. Furthermore, CD133⁺ cells from cord blood display a higher proliferative activity [66, 67] and a more primitive gene expression profile [68] than CD34⁺ cells.

Thus far, the phenotype of CD34-negative HSCs has been characterized by the concurrent absence of CD38, lack of lineage-specific cell surface antigens, as well as by expression of CD133 [69]. In contrast, CD133⁻ CD34⁺ cells were shown to mostly consist of B cell progenitors, late erythroid progenitors [61], and other more committed hematopoietic progenitors [64]. CD34, although well established, might therefore not be the best choice as a marker for the isolation of primitive human hematopoietic stem cells, due to its variable expression on late hematopoietic progenitors (see Fig. 13).

Enumeration of hematopoietic stem cells by phenotyping, although useful, does not always predict the abundance, viability, and hematopoietic potential of the cells that support hematopoiesis after transplantation, in particular after cryopreservation, expansion in culture or other ex vivo manipulations. Analysis of the functional properties of HSCs can be done by diverse in vivo and in vitro assays, e.g., repopulation assays in mouse, by which the transplanted cell (population) is tested for its ability to regenerate the complete hematopoietic system. In vitro assays are commonly used to investigate the differentiation potential of HSCs and their progenitors in the myeloid lineage, e.g., by the HSC-CFU assay.

In addition to the hematopoietic potential of stem cells isolated from hematopoietic sources, such as bone marrow, cord or peripheral blood, a nonhematopoietic differentiation potential has been described for this population. Therefore, these cells are also of great interest for tissue engineering and regenerative research applications.

4.2 Stem Cells from the Hematopoietic System with Nonhematopoietic Differentiation Potential

Ongoing investigations have led to the proposal that HSCs, as well as other stem cells from the hematopoietic system (bone marrow, peripheral blood, cord blood), have the capacity to differentiate into a wide range of nonhematopoietic tissues.

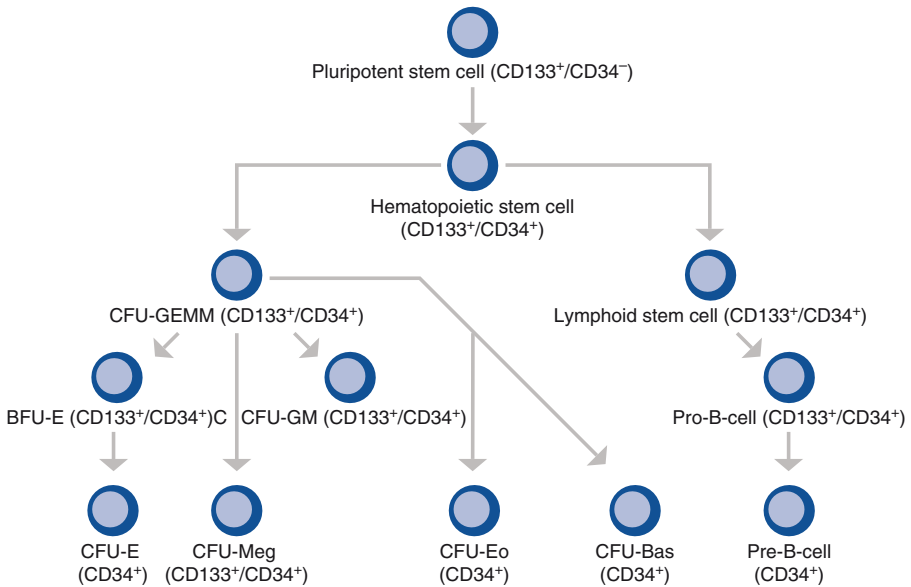


Fig. 13 In contrast to CD34, no expression of surface marker CD133 can be found on late progenitors, such as pre-B cells, colony forming unit erythrocytes (CFU-E), and colony forming unit granulocytes (CFU-G). CD133 and CD34 are coexpressed on early hematopoietic progenitors with multipotent differentiation potential, such as colonies consisting of granulocytes, erythrocytes, macrophages and megacaryocytes (CFU-GEMM), granulocytes and macrophages (CFU-GM), as well as the early burst forming unit erythrocytes (BFU-E)

One example is the hemangioblast, the common progenitor of HSCs and endothelial progenitor cells (EPCs), which can differentiate not only into blood cells but also into endothelial cells [70, 71].

4.3 Vascular Tissue

Vascularization of tissues is a major challenge of tissue engineering. In the last decade, a number of experimental data and clinical observations have suggested that bone marrow represents a reservoir of immature cells that permanently reconstitute the hematopoietic system and also participate in regeneration and repair of many peripheral tissues. These stem or progenitor cells are activated and mobilized to the blood stream by environmental stimuli for physiological and pathological tissue regeneration. Asahara first described the isolation of human progenitor cells from peripheral blood, their ability to differentiate to endothelial cells *in vitro* and to form new blood vessels and thus contribute to vascular repair. This cell population was termed endothelial progenitor cells (EPCs) [72] and has been defined by the expression of the markers CD34 and CD309 (VEGFR-2/KDR/Flk-1), as well

as in combination with CD133 to distinguish between early and (matured) EPCs in human [73]. In mouse, the phenotype for EPCs is described as Lin⁻ Sca-1⁺ c-kit⁺ CD309 (VEGFR-2/KDR/Flk-1)⁺ [74].

Considering the importance of blood vessel development for organogenesis, vasculogenesis by EPCs may be an essential cascade for tissue and organ regeneration following pathological damage in various critical diseases [75].

Regeneration of vascular tissue is also an important topic in therapeutic research, especially for the potential treatment of atherosclerosis and the revascularization of ischemic tissues, for example, in the heart or peripheral vascular disease. Due to the role EPCs play in postnatal neoangiogenesis and neovascularization, they have come into focus for tissue engineering applications and for the potential treatment of ischemic or injured tissue [74, 76], as well as after myocardial infarction [77, 78].

In mice, it has been shown in serial studies that EPCs can be mobilized from bone marrow in response to endogenous and exogenous stimuli and can therefore be isolated from populations of Sca-1⁺ cells from mouse blood and can “home” and incorporate into foci of neovascularization [57]. Bone marrow-derived Sca-1⁺ CD309 (VEGFR-2/KDR/Flk-1)⁺ progenitor cells isolated from mouse peripheral blood showed the potential to differentiate into endothelial and epithelial cells in vivo after induced lung injury [79]. EPCs from mouse bone marrow have been enriched by their expression of CD117 (c-kit, SCFR) and play a key role in therapeutic angiogenesis. After transplantation of CD117⁺ cells into the ischemic hindlimbs of mice, the cells survived and were incorporated in microvessels within 14 days in contrast to the CD117⁻ cells [80].

In humans, CD133⁺ cells isolated from bone marrow [81], cord blood [76, 82], mobilized [71, 83] and unmobilized peripheral blood [84] are capable of giving rise to endothelial cells in vitro.

Vascular progenitor cells isolated from umbilical cord blood by CD34 expression showed in vitro differentiation potential to endothelial cells and smooth muscle cells. Implantation studies in nude mice showed that both cell types contribute to the formation of human microvasculature in vivo [85]. Isolated from cord blood, CD133⁺ cells incorporated into capillary networks, augmented neovascularization, and improved ischemic limb salvage after transplantation into nude mice suffering from ischemic hind limb [76].

CD133⁺ cells have been used in studies that show significantly improved vascular network restoration in an ischemic hind limb rat model [86]. Biodegradable scaffolds are also being employed for the three-dimensional tissue engineering of microvessels, also using CD133⁺ cells [84].

Tissue engineering may offer patients new options if replacement or repair of an organ is needed. However, most tissues will require a microvascular network to supply oxygen and nutrients. One strategy for creating a microvascular network would be promotion of vasculogenesis in situ by seeding vascular progenitor cells within a three-dimensional biodegradable construct. Isolated CD34⁺ CD133⁺ endothelial progenitor cells (EPC) from human umbilical cord blood were expanded ex vivo as EPC-derived endothelial cells (EC). EPC-derived EC formed capillary-like structures and microvessels when seeded on scaffolds in combination with

human smooth muscle cells, indicating that EPCs may be well suited for creating microvascular networks within tissue-engineered constructs [82].

The work of Suuronen and colleagues demonstrates a novel approach for the expansion and delivery of blood CD133⁺ cells resulting in improved implantation and vasculogenic capacity. Adult human CD133⁺ progenitor cells from peripheral blood were expanded and delivered within an injectable collagen-based matrix into the ischemic hindlimb of athymic rats. Controls received injections of phosphate-buffered saline, matrix, or CD133-negative cells alone. Immunohistochemistry of hindlimb muscle 2 weeks after treatment revealed that the number of CD133-positive cells retained within the target site was more than twice as great when delivered by matrix than when delivered alone ($P < 0.01$). The transplanted CD133⁺ cells incorporated into vascular structures, and the matrix itself was also vascularized. Rats that received matrix and CD133-positive cells demonstrated greater intramuscular arteriole and capillary density than other treatment groups ($P < 0.05$ and $P < 0.01$, respectively). Compared with other experimental approaches, treatment of ischemic muscle tissue with generated CD133-positive progenitor cells delivered in an injectable collagen-based matrix significantly improved the restoration of a vascular network [84].

CD133-positive vascular progenitor cells (hVPCs) from the human fetal aorta were able to differentiate into mixed populations of mature endothelial cells, smooth muscle cells, and pericytes after stimulation of progenitor cells. When embedded in a three-dimensional collagen gel, hVPCs reorganized into cohesive cellular cords that resembled mature vascular structures. Transplantation of such cells into the ischemic limb muscle of immunodeficient mice indicated the therapeutic efficacy of a small number of transplanted hVPCs that markedly improved neovascularisation and inhibited the loss of endogenous endothelial cells and myocytes, thus ameliorating the clinical outcome from ischemia [87].

4.4 *Multipotent Mesenchymal Stem Cells (MSCs)*

A brief review of the history of MSC research is given in the article by Geraerts and Verfaillie (see p. #). For tissue engineering applications, it is crucial to start with a defined cell population to develop standardized protocols and obtain reliable results. Therefore, a broad range of approaches for the isolation of defined stem cell populations from different tissues have been developed (Table 1).

Several cell surface antigens have been used for the isolation of MSCs, such as antifibroblast antigen [88], CD117 [89], CD105 [90, 91], Stro-1 and CD146 [92], CD133 [93], CD271 [61] and MSCA-1 (W8B2) [61]. Clone W8B2 recognizes the mesenchymal stem cell antigen 1 (MSCA-1), a so far unknown antigen. MSCA-1 was shown to be restricted to mesenchymal stem cells (MSCs) in the CD271^{bright} population in bone marrow. These CD271^{bright}CD45^{dim} MSCs have a much higher clonogenic capacity compared with the CD271⁺CD45⁺ fraction in bone marrow [61]. MSCA-1 is therefore suited to identify MSCs with a high proliferative potential. Remarkably, CD105⁺ cells, isolated from bone marrow, also showed the capacity to form bone in vivo without prior cultivation or differentiation [91].

Table 1 Strategy of isolation for human and mouse MSCs

<i>Strategy for the isolation of fresh MSCs</i>	<i>Cell source</i>	<i>Reference</i>
<i>Human primary cells</i>		
Positive selection of CD271 (LNGFR/p75NTR)	Bone marrow	[61, 99]
Positive selection of CD117 ⁺ cells	Bone marrow	[89]
	Amniotic fluid/amniocentesis cultures	[97]
Positive selection of CD133 ⁺ cells	Peripheral blood, bone marrow, cord blood	[93, 100]
Depletion of CD45 ⁺ CD31 ⁺ cells	Lipoaspirate/stromal vascular cells (SVF)	[101, 102]
Positive selection of CD34 ⁺ cells	Lipoaspirate, stromal vascular fraction	[103]
Isolation of CD34 ⁺ CD31 ⁻ cells	Lipoaspirate/stromal vascular fraction	[104]
Positive selection of Stro-1 ⁺ cells	Bone marrow	[92, 105–110]
Positive selection of Stro-1 ⁺ cells	Bone marrow, fetal liver, fetal brain	[109]
<i>STRO-1</i>		
Positive selection of Stro-1 + CD146 + cells	Bone marrow and dental pulp	[92]
Stro-1/CD106 (VCAM)+	Bone marrow	[110]
Positive selection of CD63 (HOP-26) + cells	Bone marrow	[108, 111]
Positive selection of CD49a (α 1-integrin subunit) + cells	Bone marrow	[100, 108, 112]
Positive selection of CD166 (SB-10) + cells		[108]
SSEA-4	Bone marrow	[113]
Positive selection of GD2 (neural ganglioside) + cells	Bone marrow	[114]
Depletion of GlyA + CD45 + cells	Bone marrow	[99, 116]
Depletion of GlyA + CD45 + cells	Maternal blood	[117]
<i>Mouse primary cells</i>		
Lineage depletion	Bone marrow	[56, 118]
Depletion of CD45 + cells	Bone marrow	[119]
<i>Cultured MSCs</i>		
	<i>Cell source</i>	
Positive selection of CD117 + cells	Amniotic fluid/amniocentesis cultures	[97]
Positive selection of Sca-1 + cells	Bone marrow	[100]
Positive selection of CD49a (α 1-integrin subunit) + cells	Bone marrow	[100]
Positive selection of CD271 (LNGFR/p75NTR)	Adipose tissue	[120]
Depletion of CD11b + cells	Bone marrow-derived MSCs after culture	[95]
Depletion of CD45 + CD34 + cells	Bone marrow-derived MSCs	[94]

Mouse MSCs are often heterogeneous populations that are contaminated by lymphohematopoietic (CD34⁺, CD45⁺) cells [94], hematopoietic stem cells and macrophages [95], until late passages. Contaminating cells have been depleted

from MSC cultures by their expression of CD11b [95] or by their expression of CD34 and CD45 [94] as well as by depletion using a combination of Anti-Ter119 and CD45 MicroBeads [96]. Multipotent plastic-adherent fetal stem cells have been positively selected from amniocentesis cultures by their expression of CD117 [97] and showed broad differentiation potential. MSCs expanded from mouse bone marrow culture are also described to be positive for Sca-1, CD117 (c-kit), and CD105 – among other markers [98].

4.5 Multipotent Adult Progenitor Cells (MAPCs)

Unique cells in human and rodent postnatal marrow are the extremely rare (1 in 10^7 to 1 in 10^8 marrow cells) multipotent adult progenitor cells (MAPCs). MAPCs were selected by depletion from adult bone marrow of hematopoietic cells expressing CD45 (human and mouse) and glycophorin-A (human) or Ter-119 (mouse), followed by long-term culture on fibronectin with EGF, PDGF and low-serum condition. The emerging cell population did not undergo proliferative senescence, due to telomerase expression and maintenance of long telomeres that showed no shortening over 80 doublings. For more details on MAPCs see the article by Geraerts and Verfaillie in this book (see p. ###).

4.6 Tissue Resident Stem Cells and Cancer Stem Cells

Several varieties of tissue resident stem cells and progenitor cells have been identified and also partly isolated in vivo and in vitro. All are characterized by their dual ability to both self-renew and to reconstitute and differentiate into a given number of different somatic or postmitotic cell lineages, depending on their potency. Included are stem cells for oocyte, intestine, breast, kidney, skin, pancreas, hair, lung, ovary, teeth, or stomach formation. In the following, only the most prominent tissue stem cells – neural, cardiac, spermatogonia, and liver (hepatic) stem cells – are described in more detail.

In general, when considering the isolation of tissue resident (stem) cells, an appropriate processing of the tissue prior to cell sorting is crucial. The dissociation might influence the relative composition of cell types or even lead to complete loss of certain cell types, e.g., of large or fragile cells by shear stress or vulnerable cells by high concentrations of proteases. Further, rare cell types might be lost because of incomplete dissociation, making a careful perfusion of the respective organ mandatory. Strong aggregation and adhesion of certain cell types to each other might lead to false interpretation of markers as well. Finally, dead cells and cell debris can influence the purity and recovery of sorted cell types. But not only cells can be harmed by tissue dissociation. Already the protease sensitivity of certain epitopes

will influence the sorting outcome when approaches based on surface markers are used. Loss of antigen epitopes can either decrease the yield of target cells, or the outcome might change when using a separation strategy combining several markers. It is therefore important to choose the appropriate protease for each experiment according to the antigen epitope used for isolation. We have carefully analyzed the influence of different concentrations of papain and trypsin on cell viability, recovery and epitope integrity. Papain is often viewed as a mild protease, while trypsin treatment is regarded as harsh and causing detrimental effects on epitopes. We could show that this perception does not apply to a number of antigen epitopes and that even the opposite can be the case [121].

To address the problems concerned with enzymatic dissociation of brain tissue, for example, an enzyme mix has been developed for whole mouse brain tissue or of specific regions, such as the subventricular zone (NTDK, Miltenyi Biotec). In addition, to facilitate and standardize mechanical tissue dissociation, the process can be performed with semiautomated mechanical dissociation systems, such as the gentleMACS[®] Dissociator (Miltenyi Biotec). Thereby, fluctuations in the yield of viable cells caused by different mincing of the tissue can be avoided.

4.6.1 Neural Stem Cells

The existence of neural stem cells in the rodent brain is widely accepted, as it has been shown that there are restricted regions in the postnatal and adult brain where developmental processes such as neuronal generation and migration continue [122, 123]. Prominent neuronal migration is evident in the cerebellum, hippocampus and rostral migratory stream (RMS) [124]. For humans, a comparable migratory system was not found until 2007 when Curtis et al. presented data demonstrating the presence of a human RMS, which is organized around a lateral ventricular extension reaching the olfactory bulb, and illustrating the respective neuroblasts [125].

CD133, which has emerged as an important surface marker for many stem cell types, was originally described as “Prominin” in murine embryonic neural stem cells (later termed Prominin-1) [126, 127] and as an antigen on human fetal and adult hematopoietic stem and progenitor cells [128]. CD133 antibodies were used to isolate human neural stem cells from fetal brain but not from later developmental stages [129–131]. Likewise, Prominin antibody stained murine neural stem cells in very early (E11.5 and E12) embryos [126, 132]. Lee et al. [133] reported the isolation of a CD133-positive cell population with neural stem cell properties from postnatal murine cerebellum. Whether or not it persists in the adult cerebellum remains elusive. Finally, Pfenninger et al. showed that CD133 is present on neural stem cells in the embryonic brain, on an intermediate radial glial/ependymal cell type in the early postnatal stage, and on ependymal cells in the adult brain [134].

Markers for neural and glial (astrocytes and oligodendrocytes) precursor cells like PSA-NCAM and A2B5 have also been identified and used in many studies [135, 136].

4.6.2 Cardiac Stem Cells

With respect to mouse cardiac stem cells, the report by Hierlihy and colleagues in 2002 [137] was the first identifying a stem cell-like population in adult hearts. Their findings were based on the specific ability of stem cells to efflux Hoechst dye, as shown for many different types of stem cells, also known as side population (SP) [2]. In 2003, Beltrami et al. thoroughly described a population of rat cardiac stem cells (CD117 (c-kit)⁺ cells) found in clusters and residing among cardiomyocytes in adult hearts [138]. In vitro, cardiac c-kit⁺ cells were able to undergo self-renewal and differentiation into cardiac cell lineages, i.e., cardiomyocytes, endothelial, and smooth muscle cells. These c-kit⁺ cells, when implanted in mouse hearts following myocardial infarct, retained the capacity for differentiation into cardiomyocytes in vivo. Oh et al. employed a different stem cell marker, Sca-1, to identify yet another population of resident cardiac progenitor cells in adult hearts [139, 140]. Similarly, these Sca1⁺ cells were found to be capable of differentiation into cardiomyocytes in vitro and in vivo. Then, Pfister et al. demonstrated that, among mouse cardiac SP cells, cardiomyogenic differentiation is restricted to cells negative for CD31 expression and positive for Sca-1 expression (CD31⁻/Sca-1⁺ SP cells) [141]. Besides the described stem cell types, a fourth population of cardiac stem cells is characterized by its expression of the transcription factor Isl1 in rat, mouse, and human myocardium [142]. Isl1-expressing cells are also present in the adult mammalian heart, but they are limited to the right atrium, are found in smaller numbers than in embryonic hearts, and have an unknown physiological role [143]. The most important part of the heart with respect to obstructive heart failure, however, is the left ventricle.

In addition to the Isl1-expressing human cardiac stem cells, Bearzi et al. described their isolation and expansion from human myocardial samples obtained by a minimally invasive biopsy procedure. Following their findings, human cardiac stem cells are positive for the stem cell antigen c-kit, but negative for the hematopoietic and endothelial antigens CD45, CD34, CD31, and KDR. CD45 and KDR are typically expressed in a subset of bone marrow c-kit⁺ cells that have the ability to migrate to the heart after injury; CD31 (PECAM-1) on mature endothelial cells, platelets, and on some white blood cells, such as monocytes, NK cells, granulocytes, B cells, and T cell subsets [144]. Smith et al. use a simple explant outgrowth and cardiosphere expansion method [145]. While the description of mouse cardiac stem cells is quite accepted, these first findings on human cardiac stem cells have to be fully confirmed.

4.6.3 Spermatogonial Stem Cells

Germ cells are defined by their innate potential to transmit genetic information to the next generation through fertilization. Males produce numerous sperm for long periods to maximize chances of fertilization. Key to the continuous production of large numbers of sperm are germline stem cells and their immediate daughter cells, functioning as transit amplifying cells [146]. Several possible options for preservation

and reestablishment of the reproductive potential have been described. Apart from fertility preservation, SSC studies are useful for other applications as well, such as gene targeting [22, 147], transgenerational gene therapy, and cell-based organ regeneration therapy [148, 149]. A marker for mouse spermatogonial stem cells has recently been described by Seandel et al. [150]. The authors show that highly proliferative adult spermatogonial progenitor cells (SPCs) can be efficiently obtained by cultivation on mitotically inactivated testicular feeders containing CD34⁺ stromal cells. SPCs exhibit testicular repopulating activity *in vivo* and maintain the ability in long-term culture to give rise to multipotent adult spermatogonia-derived stem cells (MASCs). Furthermore, both SPCs and MASCs express GPR125, an orphan adhesion-type G-protein-coupled receptor.

4.6.4 Hepatic Stem Cells (HpSC)

Widespread use of liver transplantation in the treatment of hepatic diseases is restricted by the limited availability of donated organs [151]. Stem cells are a promising source for liver repopulation after cell transplantation. However, it is still not clear whether or not the adult mammalian liver contains hepatic stem cells [152].

According to Schmelzer et al., human hepatic stem cells (hHpSCs), which are pluripotent precursors of hepatoblasts and thence of hepatocytic and biliary epithelia, are located in ductal plates in fetal livers and in Canals of Hering in adult livers [153, 154] and can be isolated by positive immunoselection for the epithelial cell adhesion molecule CD326 (EpCAM⁺). The hHpSCs are approximately 9 mm in diameter, and express cytokeratins 8, 18, and 19, CD133/1, telomerase, CD44H, claudin 3, and albumin (weakly). They are negative for α -fetoprotein (AFP), intercellular adhesion molecule 1(ICAM-1), and for markers of adult liver cells (cytochrome P450s), hematopoietic (progenitor) cells (CD45, CD34, CD14, CD38, CD90 (Thy1), CD235a (Glycophorin A), and mesenchymal cells (vascular endothelial growth factor receptor and desmin) [153, 154].

As for rodent HpSCs, Yovchev et al. studied progenitor/oval cell surface markers in the liver of rats subjected to 2-acetylaminofluorene treatment, followed by partial hepatectomy (2-AAF/PH). Further, they compared hepatic cells isolated by two surface markers, epithelial cell adhesion molecule (EpCAM) and thymus cell antigen 1 (Thy-1). They found that CD326 (EpCAM)⁺ and CD90 (Thy-1)⁺ cells represent two different populations of cells in the oval cell niche. EpCAM⁺ cells express the classical oval cell markers (α -fetoprotein, cytokeratin-19, OV-1 antigen, α 6 integrin, and connexin 43), as well as cell surface markers identified previously by the same researchers (CD44, CD24, CD326 (EpCAM), aquaporin 5, claudin-4, secretin receptor, claudin-7, v-ros sarcoma virus oncogene homolog 1, cadherin 22, mucin-1, and CD133). Oval cells do not express previously reported hematopoietic stem cell markers Thy-1, c-kit, CD34, or CD56, the neuroepithelial marker neural cell adhesion molecule 1 (NCAM-1). It was shown that Thy-1⁺ cells are mesenchymal cells with characteristics of myofibroblasts/activated stellate cells. Transplantation

experiments reveal that EpCAM⁺ cells are true progenitors capable of repopulating injured rat liver [155, 156].

4.6.5 Cancer Stem Cells (CSCs)

Although the concept that cancers arise from “stem cells” or “germ cells” was first proposed about 150 years ago, it is only recently that advances in stem cell biology have given new impetus to the cancer stem cell hypothesis. What has become clear in the past 10 years is that tumor cells are functionally heterogeneous. They are organized in a hierarchy of heterogeneous cell populations with different biological properties. Specifically, only a minority of tumor cells has the capacity to regenerate the tumor and sustain its growth when injected into an immune-compromised mouse model [157].

In the last 5 years, investigation of solid-tumor stem cells has gained momentum. Using similar approaches and principles as for ALM of serial dilution and serial transplantation, solid-tumor stem cells have been prospectively identified in several tissues, such as blood, brain, colon, liver, lung, pancreas, prostate, skin, and breast cancers. The experimental strategy most often combines sorting of tumor cell subpopulations, identified on the basis of the different expression of surface markers, with functional transplantation into appropriate animal models.

Blood or Hematopoietic CSCs

John Dick and colleagues isolated and identified CD34⁺ CD38⁻ leukemic stem cells (LSCs) from human AML by FACS and demonstrated that these cells initiated leukemia in NOD-SCID mice compared with the CD34⁺ CD38⁺ and CD34⁻ fractions [158]. An engrafted leukemia could be serially transplanted into secondary recipients, providing functional evidence for self-renewal. Xenotransplantation, followed by serial transplantation, is now regarded as an essential criterion in defining cancer stem cells. The ability to recapture tumor pathophysiology is an important defining functional criterion of cancer stem cells prospectively isolated [157].

Breast CSCs

A minor, phenotypically distinct tumor cell population has been isolated that is able to form mammary tumors in NOD-SCID mice, whereas cells with alternative phenotypes are nontumorigenic even when implanted at significantly higher cell numbers, thereby demonstrating enrichment of tumor-initiating cells in selected fractions. The tumorigenic cells can be serially passaged, demonstrating self-renewal capacity, and are able to generate tumor heterogeneity, producing differentiated, nontumorigenic progeny. Thus, like AML, breast cancer growth appears to be driven by a rare population of

tumor-initiating cells [157]. Breast cancer stem cells have been reported to be ESA⁺ CD44⁺ CD24^{-/low} Lineage⁻ [159–161]. ESA stands for “epithelial specific antigen,” also known as EpCAM (epithelial cell adhesion molecule) or CD326. Usage of those markers allows for a more than 50-fold enrichment to form tumors (0.6% of cancer cells).

Brain CSCs

Singh et al. [161, 162] reported the identification and purification of cancer stem cells from human brain tumors of different phenotypes that possess a marked capacity for proliferation, self-renewal, and differentiation. The increased self-renewal capacity of the brain tumor stem cell (BTSC) was highest among the most aggressive clinical samples of medulloblastoma compared with low-grade gliomas. The BTSC was exclusively isolated with the cell fraction expressing the neural stem cell surface marker CD133. The CD133⁺ fraction among highly aggressive glioblastomas (GBMs) ranged from 19 to 29%, and among medulloblastomas ranged from 6 to 21%, and correlated closely with an in vitro primary sphere formation assay (which was used to quantify stem cell frequency).

Lung CSCs

Lung cancer stem cells were first identified by Kim et al. [163] who describe a niche in the bronchioalveolar duct junction of adult mouse lung that harbors stem cells from which adenocarcinomas are likely to arise. More importantly, these double-positive cells appear enriched in FACS-sorted Sca-1⁺/CD34⁺ cell populations and show enhanced capacity for both self-renewal and differentiation. Subsequently it was shown that the human lung cancer-derived A549 cell line also harbors CSCs with a side population (SP) phenotype revealing several stem cell properties [164]. Very recently, Eramo et al. [165] found that the tumorigenic cells in small-cell and nonsmall-cell lung cancer are a rare population of undifferentiated cells expressing CD133.

Liver CSCs

Although liver CSCs have been identified in hepatocellular carcinoma (HCC) cell lines, no data have shown the presence of these cells in human settings until very recently. Now, Yang et al. [166] have delineated CSCs serially from HCC cell lines, human liver cancer specimens, and blood samples, using CD90 as a potential marker. The number of CD90⁺ cells increased with the tumorigenicity of HCC cell lines. CD45⁻ CD90⁺ cells were detected in all the tumor specimens, but not in the normal, cirrhotic, and parallel nontumorous livers. In addition, CD45⁻ CD90⁺ cells were detectable in 90% of blood samples from liver cancer patients, but none in normal subjects or patients with cirrhosis.

Prostate CSCs

According to Richardson et al. [167], prostatic stem cells are $\alpha 2\beta 1^+/\text{CD133}^+$. Collins et al. [168] have shown that cancer stem cells in prostate have been identified with a $\text{CD44}^+/\text{integrin } \alpha 2\beta 1^{\text{high}}/\text{CD133}^+$ phenotype. Approximately 0.1% of cells in any tumor expressed this phenotype, and there was no correlation between the number of $\text{CD44}^+/\alpha 2\beta 1^{\text{high}}/\text{CD133}^+$ cells and tumor grade. In addition, Miki et al. have shown that expression of CXCR4 was also detected in CD133^+ cancer cells.

Colon CSCs

O'Brien et al. [169] and Ricci-Vitiani et al. [170] showed that the tumorigenic population in colon cancer is restricted to CD133^+ cells, which are able to reproduce the original tumor in permissive recipients. Ricci-Vitiani et al. state that the vast majority of the samples analyzed showed the presence of rare cells ($2.5 \pm 1.4\%$) clearly positive for CD133 while CD133 expression in normal colon tissues was extremely rare (barely detectable upon extensive analysis of histological sections using CD133/1 and CD133/2 antibodies). O'Brien et al. state that purification experiments established that all colon cancer-initiating cells (CC-ICs) were CD133 -positive; the CD133 -negative cells comprising the majority of the tumor were unable to initiate tumor growth.

Melanoma CSCs

According to Fang et al. [171] and Kamstrup et al. [172], a small subpopulation of CD20^+ melanoma cells harbors multipotent stem cells. Most interestingly, CD20 has been identified by gene expression profiling as one of the top 22 genes that define aggressive melanomas. In metastatic melanomas, they have identified individual CD20^+ tumor cells. Monoclonal antibodies against CD20 have become a standard treatment for nonHodgkin's lymphoma. CD20 seems to be a potential target for melanoma as well, although a correlation between differentiation ability and tumorigenicity is still under investigation by comparing CD20^+ with CD20^- fractions.

Pancreas CSCs

Li et al. [173] identified a highly tumorigenic subpopulation of pancreatic cancer cells expressing the cell surface markers CD44 , CD24 , and epithelial-specific antigen (ESA; EpCAM; CD326). Pancreatic cancer cells with the $\text{CD44}^+ \text{CD24}^+ \text{ESA}^+$ phenotype (0.2–0.8% of pancreatic cancer cells) had a 100-fold increased tumorigenic potential compared with nontumorigenic cancer cells, with 50% of animals injected with as few as 100 $\text{CD44}^+ \text{CD24}^+ \text{ESA}^+$ cells forming tumors that

were histologically indistinguishable from the human tumors from which they originated.

As a conclusion, the field of tissue stem cells (apart from hematopoietic stem cells) is still at the very beginning. This is partly due to the fact that potential markers for these tissue stem cells have not or have only recently been defined and are still intensively debated. Also, standardized processes for appropriate dissociation of tissues are currently hardly available, resulting in poor comparability of sorting results. It can be estimated that sorting of tissue resident (stem) cells will play a dramatically increasing role in the future, because this will offer the option for a detailed analysis and understanding of malignant and disease-causing cells, as well as of cell types urgently needed for tissue regeneration and engineering approaches.

5 Clinical Applications of Stem Cells

5.1 *Allogeneic Hematopoietic Stem Cell Transplantation*

To date, the major application of enriched and purified stem cells is the transplantation of hematopoietic stem cells derived from bone marrow or peripheral blood. For a number of patients suffering from malignant and nonmalignant diseases, allogeneic stem cell transplantation, i.e., when the stem cells originate from a healthy individual, is the only curative treatment option [174].

For many years, the clinical outcome of an allogeneic transplantation has been determined to a major degree by the matching between donor and recipient of the genes encoding histocompatibility antigens – the human leukocyte antigen (HLA) system in human beings. However, the “ideal” donor, an HLA-matched sibling, can only be found for about 30% of the patients. With the help of worldwide registries of unrelated donors, the probability of identifying a matched unrelated donor (MUD) depends on the diversity of HLA antigens within a population and on the race, and ranges from about 75% for Caucasians to less than 50% for ethnic minorities [174]. A MUD donor search is time-consuming, and may take more than 3 months in most cases or even longer, so that not every patient might benefit from a potentially life-saving allogeneic transplantation. For those patients who do not have a matched related or unrelated donor or who are at high risk for disease progression during the donor search, an alternative approach is the use of mismatched related family donors (MMFD). Most of these donors share only one HLA haplotype with the patient and are referred to as haploidentical donors. In fact, virtually every patient has a potentially suited haploidentical donor among parents or children. However, in the past haploidentical transplantations have been hampered by clinical complications linked to the high degree of donor–recipient HLA disparity, such as graft failure, prolonged and profound immunodeficiency, and severe acute or chronic graft vs host disease (GVHD). Acute GVHD is a major cause of death after allogeneic transplantation (Fig. 14). Additionally, chronic graft vs host disease is

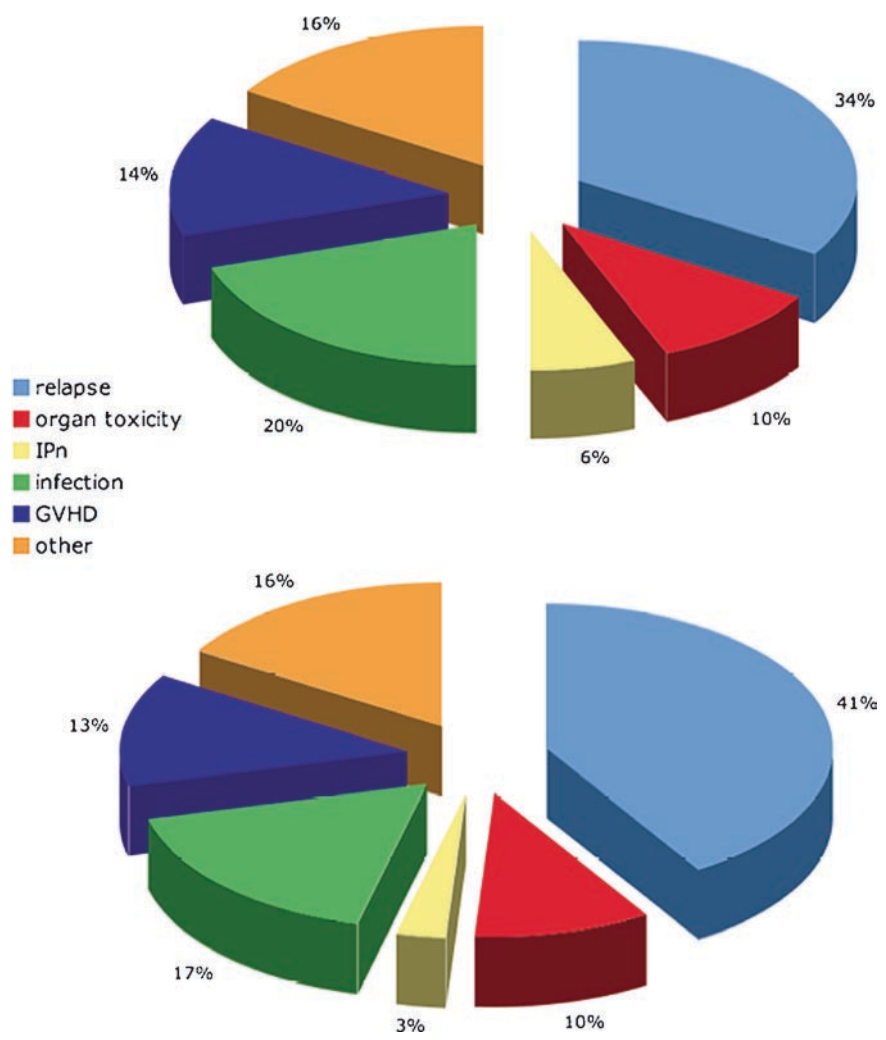


Fig. 14 a,b Causes of death after allogeneic stem cell transplantation (2001–2006). Graft vs host disease (GVHD) is a major complication after allogeneic stem cell transplantation. A total of 14% and 13% of patients die of GVHD after unrelated and HLA-identical sibling donor transplantation, respectively. **a** Unrelated donor transplantation. **b** HLA-identical sibling donor transplantation (Source: CIBMTR Newsletter, Dec 2007)

the major predictor of long-term outcome and overall survival after allogeneic stem cell transplantation [175]. Since the severity of GVHD, among other factors, depends on the degree of histocompatibility between donor and recipient and mediated by alloreactive donor T cells, a thorough T cell depletion is mandatory in haplo-identical transplantation, where only one HLA haplotype is shared between patient and mismatched family donor.

In recent years both experimental concepts and technical developments have eventually paved the way for the clinical application of haploidentical stem cell transplantation.

In 2001, Martelli and Reisner [176] published their pioneering work about the “megadose” concept, demonstrating that transplantation of high doses of purified stem cells can overcome the HLA barrier between donor and recipient and can lead to tolerance.

At the same time, the finding that growth factors can release high amounts of stem cells from bone marrow into the peripheral blood, and the development of effective in vitro T cell depletion systems have made haploidentical transplantation technically feasible. In vitro CD34⁺ selection is one of the most potent technologies for an effective T cell depletion, resulting in a ten- to hundred-thousand-fold depletion of T cells from the graft [177]. The use of mobilized peripheral stem cells in addition to bone marrow, or as a sole source of stem cells in combination with effective CD34⁺ selection strategies, allowed for the transplantation of megadoses of highly purified stem cells. This resulted in high engraftment rates and complete prevention of GVHD without the need for a post-transplant immunosuppressive treatment [174].

The CliniMACS Plus Instrument is an automated cell selection system based on MACS® Technology (see Chap. Methods and Technologies). It enables the operator to perform clinical-scale magnetic cell selection of target cells or the depletion of unwanted cells in a functionally closed and sterile system. A number of publications and reports substantiate the reliable and excellent performance (Table 2) of CD34⁺ cell separation with the CliniMACS Plus Instrument, which provides a high recovery and purity of target cells as well as efficient T cell depletion rates.

The Laboratory of Hematology at the University of Perugia is one of the most experienced transplant centers for haploidentical transplantation. Recently, Aversa and coworkers gave an update of the clinical outcome after haploidentical transplantation for adult patients suffering from acute leukemia [181]. The event-free survival (EFS) after haploidentical transplantation is closely related to disease and disease status at transplant. In the Perugia experience, 62 patients with acute lymphoid leukemia (ALL) transplanted in clinical remission (CR) have about 25% probability of surviving event-free. Results are better for the 83 patients with acute

Table 2 CliniMACS CD34 separation results from PBSCs of allogeneic donors [177–180]

	<i>n</i> > 500	<i>n</i> = 293	<i>n</i> = 136	<i>n</i> = 30	<i>n</i> = 335	<i>n</i> = 73
Purity CD34 (%)	95–99	97.5	90	96	93	92
Recovery CD34 (%)	70	77	81	64	81	71
CD3 log depletion	>5	4.6	4.6	5	4.8	5.1
CD19 log depletion	nd	nd	3.2	3.2	3.3	3.7

nd = not determined

myeloid leukemia (AML) transplanted in CR: the 3-year EFS ranges between 40%, for those who were transplanted in second or later CR, to 50% for patients transplanted in first CR. With no chronic GVHD, all these long-term survivors enjoy an excellent quality of life. It has been shown by the Perugia group [182, 183] that natural killer (NK) cell alloreactivity can exert an antileukemic effect in the absence of killer inhibitory receptors (KIR). Donor vs recipient NK-cell alloreactivity impacts favorably upon survival in AML patients: Thirty patients in any CR who received a transplant from an NK cell alloreactive donor enjoy 67% EFS vs 18% in 31 patients transplanted from nonNK alloreactive donors [181].

5.2 Autologous Hematopoietic Stem Cell Transplantation in Autoimmune Diseases

Recently, the transplantation of highly enriched stem cells has emerged as a novel treatment option for some therapy-refractive autoimmune diseases. In a recently published article [184], Vonk et al. report on promising data of the outcome after high dose immunosuppressive therapy and autologous CD34⁺-selected cell transplantation in severe systemic sclerosis (SSc), also referred to as scleroderma. This disease is a generalized autoimmune disease causing morbidity and reduced life expectancy, particularly in patients with rapidly progressive diffuse cutaneous SSc. Since no proven treatment exists, autologous hematopoietic stem cell transplantation (the stem cells are derived from the patient) is considered as a new therapeutic strategy in patients with poor prognosis. CD34⁺ cell enrichment is performed in order to remove any autoreactive T and B cells. Vonk and colleagues [184] report that after a median follow-up of 5.3 (1–7.5) years, 21 out of 26 patients (81%) demonstrated a clinically beneficial response. Event-free survival, defined as survival without mortality, relapse or progression of disease, was 64.3% at 5 years and 57.1% at 7 years. Alexander and coworkers [185] report on the long-term outcome (median follow-up period: 60 months) of seven patients suffering from refractory systemic lupus erythematosus (SLE) who had been treated by transplantation of CD34⁺-enriched stem cells after a profound immunoablation. The presented data show that the long-term therapy-free clinical remissions observed in SLE patients after complete immunoablation and ASCT are accompanied by a loss of immunological memory and a fundamental reset of the immune system.

5.3 Stem Cells for Tissue Regeneration in Cardiac Diseases

Current pharmacological, interventional, or surgical approaches fail to regenerate nonviable myocardium. As a consequence, restoration of functional myocardium following cardiac infarction remains an ambitious challenge for clinicians. The heart has the ability to elicit a regenerative response designed to restore cardiac function

through replacement of damaged cells. There is rising evidence that this is accomplished by the activation of resident cardiac stem cells [144, 186] or through the recruitment of a stem cell population from other tissues such as bone marrow [187, 188].

Hence, stem cell therapy is a promising new strategy for myocardial repair. Since the availability of autologous cardiac stem cells in large numbers is poor and requires *ex vivo* expansion, highly proliferative, totipotent embryonic stem cells (ESCs) seemed to be a promising alternative. However, there are persisting ethical and legal issues as well as concerns about the tumorigenic and infectious potential of allogeneic ESCs. Furthermore, the early deaths of ESCs as a result of ischemia currently impede their use in clinical studies [189].

The discovery of adult tissue-specific stem cells (ASCs), which have the ability to transdifferentiate into other tissues, led to extensive use of these cells for hematological, cancer and myocardial infarct-related administration [190].

In skeletal muscle the so-called satellite cells function as progenitor cells and are responsible for normal muscle growth and regeneration [191]. Transplantation of skeletal muscle into mouse myocardium alone [192, 193] and in combination with CABG into human heart [194–197] proved to be feasible, safe, and efficient in restoring functional myocardium. However, although improved symptoms and LVEF were achieved [198, 199], the lack of gap junction formation of the graft may lead to failure in electromechanical coupling and, thus, to a higher arrhythmic risk. There are several strategies aiming at an enhancement of electromechanical coupling, e.g., by modifying the expression of connexin-43 [200]. Further investigation may provide long-term improvement.

Bone marrow (BM), among several other organs, possesses multipotent adult stem cells with high plasticity, as demonstrated convincingly in the mouse model of infarcted myocardium [201]. Only recently it has been demonstrated in a meta-analysis including 18 randomized controlled trials and cohort studies that BMC transplantation seems safe and is associated with modest improvements in physiological and anatomical parameters in patients with both acute myocardial infarction and chronic ischemic heart disease, above and beyond conventional therapy [202].

Mesenchymal stem cells (MSCs) are bone marrow–populating cells (stromal cells), which possess an extensive proliferative potential and the ability to differentiate into various cell types, including osteocytes, adipocytes, chondrocytes, myocytes, cardiomyocytes and neurons [203] (see above). Apart from bone marrow, MSCs are located in other tissues, such as adipose tissue, peripheral blood, cord blood, liver, and fetal tissues. Their multilineage potential and their ability to elude detection by the host's immune system, as well as their relative ease of expansion in culture, make MSCs a promising source of stem cells for transplantation [204]. However, it was recently shown in mice that the developmental fate of bone marrow-derived MSCs is not restricted by the surrounding tissue after myocardial infarction, but by induced calcification and/or ossification [205].

Probably the simplest approach to use stem cells for myocardial cell therapy is to harvest mononuclear cells either from bone marrow or mobilized peripheral blood [206–210]. However, the functionality of the different cells has not yet been

clearly identified. When delivered intracoronarily, improvement in cardiac function was sparse [211–213]. Furthermore, concerns exist regarding limited efficiency due to the small numbers of progenitor cells in nonenriched peripheral blood and bone marrow that are delivered intramyocardially. The risk of foreign tissue differentiation following local stroma cell injections was also found with mononuclear cells [205]. Thus, neither the preferred source and type of stem cell nor the optimal method of delivery of stem cells to the target area have been defined so far. Positively selected human CD34⁺/CD133⁺ cells from mobilized peripheral blood for intravenous injection in nude rats resulted not only in a substantial increase in left ventricular ejection fraction but also in a fivefold increase in the number of capillaries compared with the control [214].

Hematopoietic stem cells (HSCs) expressing CD133, a marker for more ‘primitive’ multipotent stem cells, are considered to be particularly important in the context of myocardial repair [186, 201, 215–217]. The cell surface antigen CD133 is expressed on primitive HSCs and endothelial progenitor cells (EPCs), which collaborate to promote vascularization of ischemic tissues [218]. CD133⁺ cells can integrate into sites of neovascularization and differentiate into mature endothelial cells. For enriched CD34⁺ cells, homing into the border zone of infarcted myocardium has been demonstrated [219].

With respect to functionality, a direct side-by-side comparison of human CD133⁺ bone marrow cells and human skeletal myoblasts in a myocardial ischemia model in immuno-incompetent rats demonstrated similar functional improvement in both groups, although only the myoblasts reached robust engraftment [220].

Since intracoronary administration of mononuclear cells did not yield the expected functional benefit [213], and the heart is easily accessible during CABG procedures, several groups started to inject mononuclear [221], purified selected cord blood-derived [222, 223], bone marrow-derived, or blood-derived CD133⁺ stem cells [78, 224–226] intramyocardially during surgical intervention.

In 2003, Stamm et al. published first clinical results of a phase I study on patients suffering from chronic ischemic heart disease, treated with CD133⁺ stem cells in conjunction with Coronary Artery Bypass Grafting (CABG) confirming safety and feasibility [77]. Functional benefit could also be demonstrated in a subsequent controlled randomized trial [227]. The therapeutic potential of CD133⁺ cells was further confirmed by the positive response of 10 patients with end-stage chronic ischemic cardiomyopathy, only treated with cell injection [228].

In summary, results from current trials support conducting large randomized trials to evaluate the impact of cell therapy on patient-related outcomes.

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