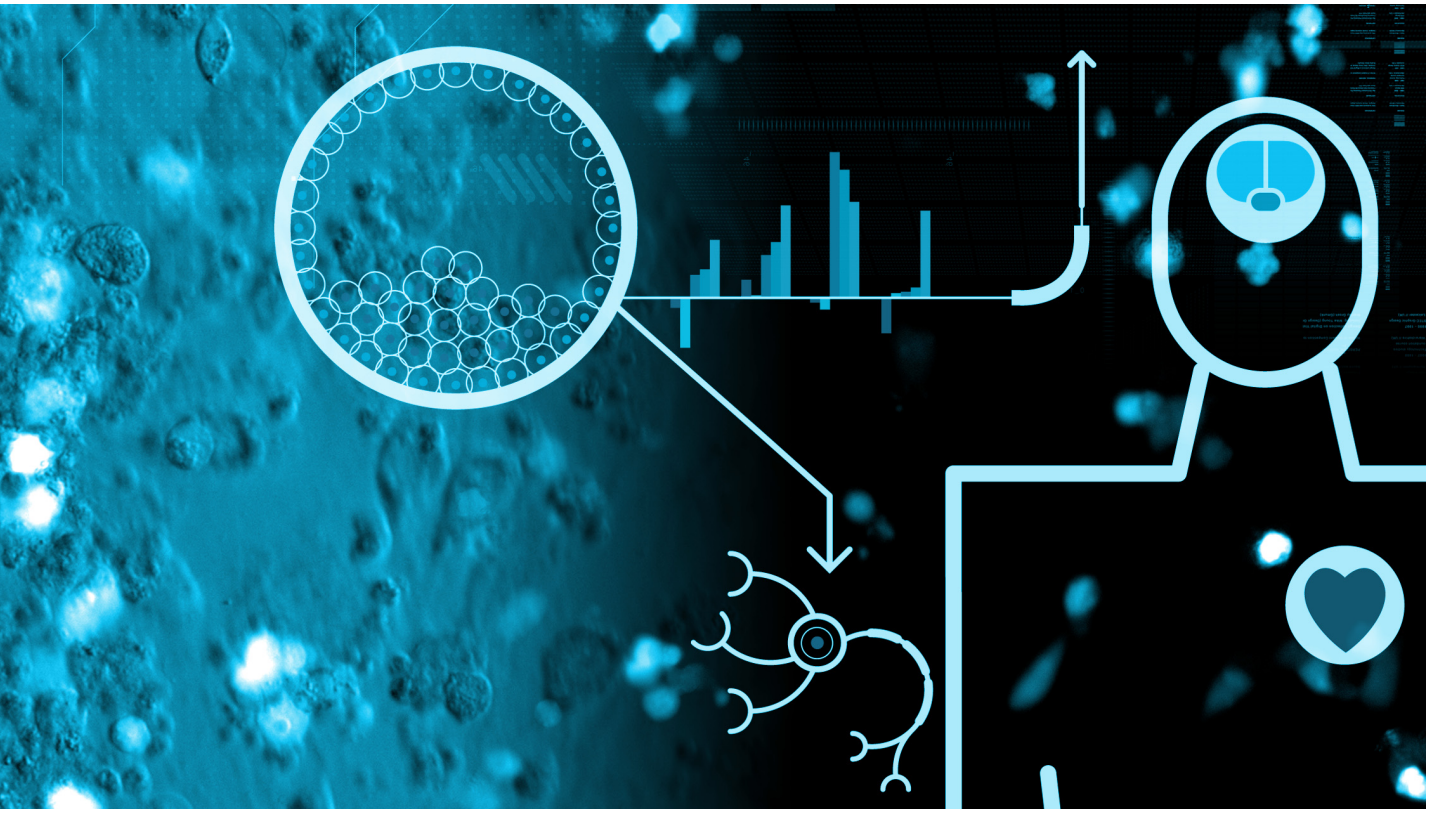


Stem Cell Basics for Life Science Researchers

BIO-RAD

Real-Time PCR Systems for Stem Cell Research

The CFX96™ and CFX384™ optical reaction modules convert a C1000™ thermal cycler into a powerful and precise real-time PCR detection system. Solid-state optical technology maximizes fluorescence detection of dyes in specific channels, providing sensitive detection, precise quantitation, and target discrimination for up to 5 gene targets for the CFX96 system and 4 targets for the CFX384 system.

This powerful platform together with CFX Manager™ software enables you to perform a variety of real-time PCR experiments, including monitoring pluripotency marker expression, multiplex experiments, and simultaneous detection of the presence of multiple stem cell differentiation markers.

Analysis

Electrophoresis and Western Blotting for Stem Cell Research

Bio-Rad offers several superior systems for separating and analyzing proteins isolated from your stem cells to achieve results faster. The Mini-PROTEAN® system includes everything necessary for identifying the critical markers used to verify your stem cells — a Mini-PROTEAN Tetra cell, Mini-PROTEAN® TGX™ precast gels for faster separations, Mini Trans-Blot® module, and PowerPac™ power supply. For more information on Mini-PROTEAN vertical electrophoresis systems, please visit **www.miniprotean.com**.

Analysis

Imaging Systems for Stem Cell Research

Bio-Rad Molecular Imager® Gel Doc™ XR+ and ChemiDoc™ XRS+ systems and the Criterion Stain Free™ system, together with Image Lab™ software, provide easy-to-use, automated image capture and analysis. Whether you are imaging western blots to view expression levels of a pluripotency gene or checking DNA constructs for the proper gene, these systems are the right choice. With these systems you can:

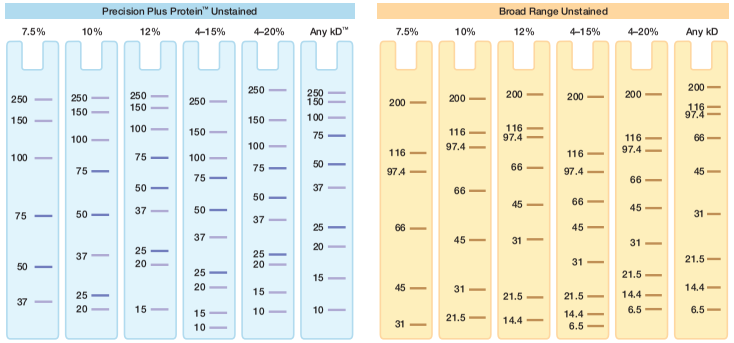
- Eliminate the guesswork of determining optimal camera and lighting settings for gel and blot imaging
- Make imaging and analysis repeatable and reproducible and maximize data quality
- Create custom analysis reports to make analysis automated, easy-to-use, and fast

Analysis

Automated Cell Counting for Stem Cell Research

The Bio-Rad TC10™ automated cell counter system provides accurate and precise quantitation of your mammalian stem or iPS cells in one simple step. To measure total cell count, load a TC10 counting slide with 10 µl of cell suspension. To assess cell viability, simply mix your sample with TC10 trypan blue prior to loading. Values are obtained in less than 30 seconds and can be printed on the optional TC10 label printer or can be downloaded to a USB key.

Preparation



Description	10 Gels/Box 10-Well, 30 µl	10 Gels/Box 15-Well, 15 µl	10 Gels/Box IPG Well/PREP*	2 Gels/Box 10-Well, 30 µl
Mini-PROTEAN TGX Precast Gels				
7.5% Resolving Gel	456-1023	456-1026	456-1021	456-1023S
10% Resolving Gel	456-1033	456-1036	456-1031	456-1033S
12% Resolving Gel	456-1043	456-1046	456-1041	456-1043S
4–15% Resolving Gel	456-1083	456-1086	456-1081	456-1083S
4–20% Resolving Gel	456-1093	456-1096	456-1091	456-1093S
Any kD™ Resolving Gel	456-9033	456-9036	456-9031	456-9033S

* 7 cm IPG strip/450 µl

Preparing Samples

Note: The TC10 cell counter demonstrates high reproducibility counting cells within the 5 x 10^4 to 1 x 10^7 cells/ml range and between 6–50 µm cell diameter.

Preparing Samples without TC10 Trypan Blue Dye

1. Pipet 10 µl of the cell suspension into the opening of either of the two chambers of the counting slide.

Preparing Samples with TC10 Trypan Blue Dye

1. In a micro test tube, combine 10 µl of the cell suspension with 10 µl of TC10 trypan blue solution. Gently pipet up and down 10 times to mix.
2. Quickly pipet 10 µl of the mixture into the opening of either of the two chambers of the counting slide.

Performing Cell Counts

1. Press Home and insert the slide completely into the slide slot until the instrument detects the slide and begins the count.

Important: Do not remove the slide or interrupt the instrument while it is performing the count.

2. For samples without trypan blue dye — On the Current Count screen, the TC10 counter provides the total cell count per ml.
3. For samples with trypan blue dye — On the Current Count screen, the TC10 counter provides the total cell count per ml, live cell count per ml, and percentage of live cells. The TC10 counter accounts for a 1:1 dilution with the dye and provides a dilution calculation based on live cells.
4. Once the instrument completes the cell count, remove the slide from the slide slot.

Additional Analysis Options

In the Current Count screen, use the up or down arrow keys to access the following screens: Dilution Calculator, View Image, and Print Count. Helpful hints for each screen are provided below. Press Enter to select the option.

Dilution Calculator

- In the dilution calculator, the current count is used as the starting cell concentration. The parameter that is ready to be modified is highlighted. Press the up or down arrow key to find the correct value, and press Enter to confirm the selection.
- To print the Dilution Calculator screen, use the down arrow key and select Yes.

View Image

- Use the up arrow key to zoom in on an image and examine cells in detail. To zoom out, use the down arrow key.the down arrow key.

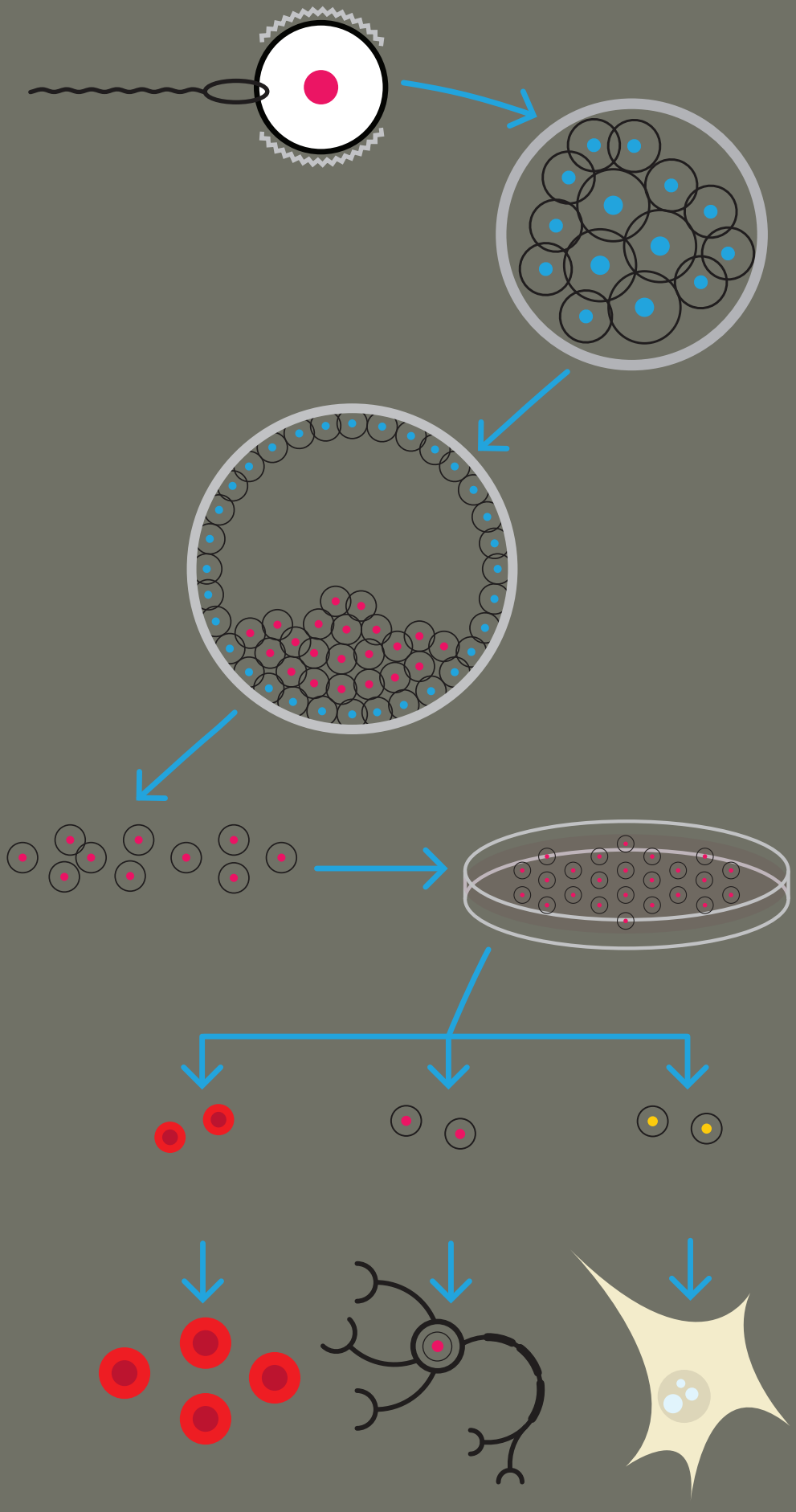
Compatible PCR Consumables for CFX96, CFX384 Systems and 1000-Series Thermal Cyclers

Tubes	
Low-profile 0.2 ml tube strips, clear	TLS-0801
Low-profile 0.2 ml tube strips, white	TLS-0851
48- and 96-Well Plates	
Multiplate™ low-profile 48-well unskirted PCR plates, clear	MLL-4801
Multiplate low-profile 48-well unskirted PCR plates, white	MLL-4851
Multiplate low-profile 96-well unskirted PCR plates, clear	MLL-9601
Multiplate low-profile 96-well unskirted PCR plates, white	MLL-9651
Hard-shell 96-well skirted PCR plates, white well	HSP-9655
Hard-shell 96-well skirted PCR plates, clear well	HSP-9601
384-Well Plates	
Hard-shell 384-well skirted PCR plates, clear well	HSP-3801
Hard-shell 384-well skirted PCR plates, white well	HSP-3805
Caps and Sealers	
Optical flat cap strips	TCS-0803
Microseal 'B' seals, optical	MSB-1001

Imaging System Selection Guide

	Application	Criterion Stain Free	Molecular Imager Gel Doc XR+	Molecular Imager ChemiDoc XRS+
Nucleic Acid Detection	Ethidium bromide stain	—	4	4
	SYBR® Green I stain	—	4	4
	SYBR® Safe stain	—	4	4
	Coumarin stain	—	4	4
Protein Detection, 1-D Gels	Stain-free gels	5	—	—
	Coomassie Blue stain	—	4	4
	Silver stain	—	4	4
	SYPRO Ruby protein gel stain	—	4	4
	Flamingo™ fluorescent gel stain	—	4	4
	Oriole™ fluorescent gel stain	—	5	5
Protein Detection, 2-D Gels	Coomassie Blue stain	—	3	3
	Silver stain	—	3	3
	SYPRO Ruby protein gel stain	—	3	3
	Flamingo fluorescent gel stain	—	3	3
	Pro-Q stain	—	3	3
	Cy2, Cy3, Cy5 label	—	—	—
Blot Detection	Coomassie Blue stain	—	4	4
	Silver stain	—	4	4
	SYPRO Ruby protein blot stain**	—	—	—
	Immun-Star™ chemiluminescence	—	—	4
	Chemifluorescence**	—	1	1
	Quantum dot**	—	1*	1*

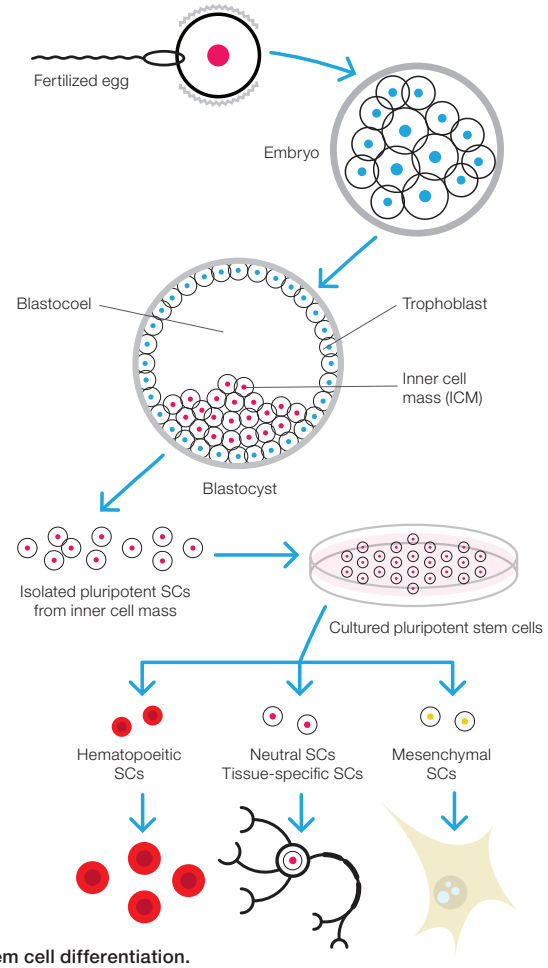
— Not recommended; 1–5, recommendation level (5 = highest).
* Custom filter required.
** Optimal with low-fluorescence PVDF membrane.



INTRODUCTION

Will stem cells, with their unlimited potential for growth, become the “fountain of youth” in the 21st century? Will physicians be able to grow new organs and tissues to replace those damaged by disease or age? Will diabetic patients finally find a cure? Will we eliminate Alzheimer’s disease? The answers to these questions are as yet unknown, but scientists are making remarkable advances in these areas of research using stem cells. In order to understand where we are today, we will review how we got here.

First, let's examine the important properties of stem cells that make them invaluable. During development, cells progressively lose the ability to differentiate. During the first stage of development the cells are totipotent and able to give rise to all cells of the organism. As development continues, the cells differentiate and become pluripotent (able to differentiate into all cells of the embryo except trophoblast). As the embryo develops, cells become multipotent (able to give rise to a particular cell type of a given lineage) or unipotent (terminally differentiated and only able to give rise to a single cell type or lineage). Pluripotent cells therefore can maintain prolonged undifferentiated proliferation (self-renewal) and have the potential to form one of any of the 220 different types of cells found in the human. How is this possible?



Stem cell differentiation.

Remember that upon fertilization, each cell in an organism carries identical genetic material or DNA. As an organism develops, this genetic material is altered by environmental factors, both internal and external, that decide their fate in the organism. The mass of cells formed four to five days after fertilization is known as a blastocyst. The blastocyst contains a mass of inner cells known as the embryoblast and a mass of outer cells known as the trophoblast. The trophoblast becomes the placenta and surrounds a hollow cavity called the blastocoel and an inner cell mass (ICM), a group of cells at one end of the blastocoel that develops into the embryo proper. Pluripotent embryonic stem (ES) cells are derived from the ICM.

Historical Perspective

Long before ES cells were cultivated in vitro, scientists were trying to understand regeneration. In the 1950s, Briggs and King developed a technique for replacing the nucleus of a frog's egg with the nucleus of another cell. In 1962, Gurdon used this technique to swap the nucleus of a fully differentiated small intestine cell into an egg to clone a frog. These experiments demonstrated that the changes seen in development are not due to permanent changes to the genetic code and that differentiation can occur in response to environmental factors. Subsequently, it was deduced that factors present in the oocyte must play a role in driving differentiation and development.

In the 1950s and 1960s, a tumor isolated from a mouse and known as a teratocarcinoma was studied in great detail. In 1964, Kleinsmith and Pierce demonstrated that these cells had unlimited self-renewal capabilities and multi-lineage differentiation, hence they were pluripotent. The establishment of stable cultures of these embryonic cancer (EC) cells by Kahan and Ephrussi (1970) was the precursor of ES cell culture. In the interim, EC cells were isolated from humans. Although these cells were useful for a variety of studies, most had limited developmental potential as therapeutics due to the genetic changes that occurred during tumor formation.

In the 1960s, McCulloch and Till conducted a series of experiments for measuring radiation sensitivity and used bone marrow cells for their transplantations. They observed spleen nodules containing dividing cells and subsequently traced their origin to a single stem cell (Becker et al. 1963, Siminovitch et al. 1963, Till et al. 1964). These early experiments form the basis for modern day adult and embryonic stem cell research.

Embryonic Stem Cells

Mouse ES (mES) cells were first cultivated successfully by Evans and Kaufmann (1981) and Martin (1981). The cells were derived from the ICM of mouse blastocysts and maintained in culture on a layer of mitotically inactivated mouse embryonic fibroblast cells known as "feeder cells" that provide optimal culture conditions. The addition of other molecules known as cytokines (LIF and BMP-2) was found to enhance continued cell growth (see Yu and Thomson (2008) review).

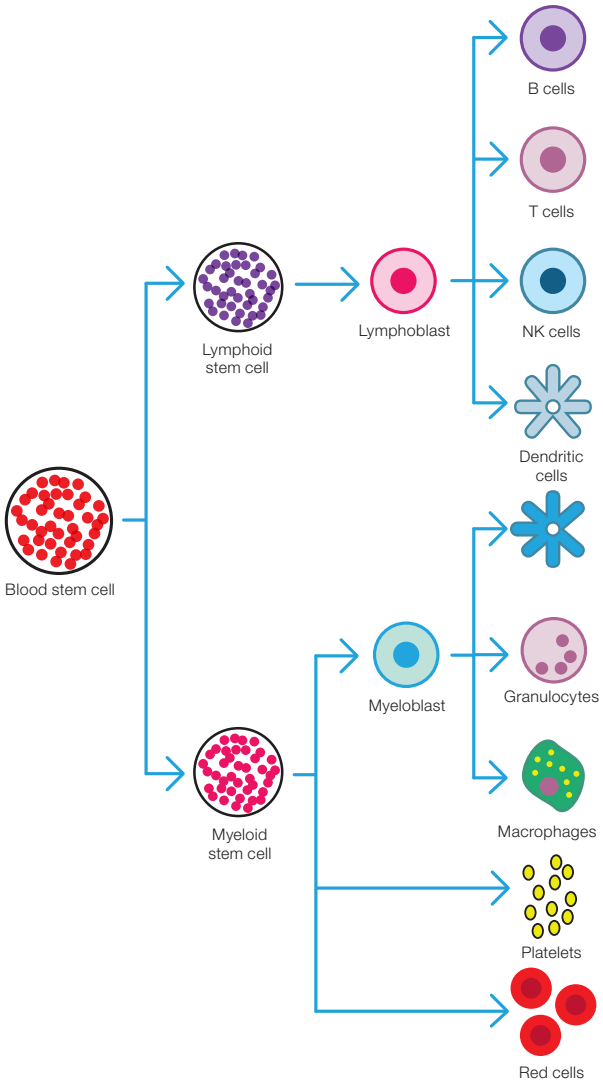
Human ES (hES) cells were successfully cultivated and maintained in an undifferentiated state by Thomson and Marshall (1998). Borrowing from research done in primates (Thomson et al. 1995, 1996) as well as work done in culturing human IVF embryos (Gardner et al. 1998), Thomson's team developed conditions optimal for growth and maintenance of hES cells. While the feeder cell layer used in culturing mES cells was similarly employed for the cells, it was discovered that biochemical pathways and growth factors involved in hES maintenance were quite different from the mES cells. Recent advances in growth conditions have now eliminated the need for feeder cells in many cases, replacing them with an artificial matrix and more defined growth media. Cells grown under these conditions are karotypically normal, i.e. the chromosomes appear normal when stained, and even after prolonged proliferation in the undifferentiated state can give rise to each of the three cell types found in the embryo.

WiCEll (www.wicell.org) was formed in 1999 as a repository for human stem cell lines. As the National and International Stem Cell Bank, they grow, characterize, and distribute hES cells and induced pluripotent stem (iPS) cells (see more below) to academic and industrial partners. The Stem Cell Unit at the National Institutes of Health also provides a database of federally approved stem cell lines against which any new hES or iPS cells can be compared. In Europe, the UK Stem Cell Bank (www.ukstemcellbank.org.uk) provides a repository of human stem cell lines. Its role is to provide stocks of these cells to researchers worldwide. The European Human Embryonic Stem Cell Registry (www.hescreg.eu), based in Berlin, provides the stem cell community with an in-depth overview on the current status of hES cell research in Europe. The Stem Cell Network – Asia-Pacific (SNAP) (www.asiapacificstemcells.org) was formed in 2007 with a mission of building a strong and dynamic stem cell research community in the Asia-Pacific region.

Adult or Somatic Stem Cells

Stem cells can also be isolated from the adult organism. These cells, known as somatic stem cells, are multi- or unipotent. They are found in different organs of the body, including the bone marrow, stomach, intestines, nose, and liver. Unlike embryonic stem cells, somatic stem cells differentiate into the cell type from which they originate. These cells are used by the body to maintain and repair the originating tissue. Scientists are still uncovering other areas of the body that contain these quiescent stem cells and are attempting to understand their role.

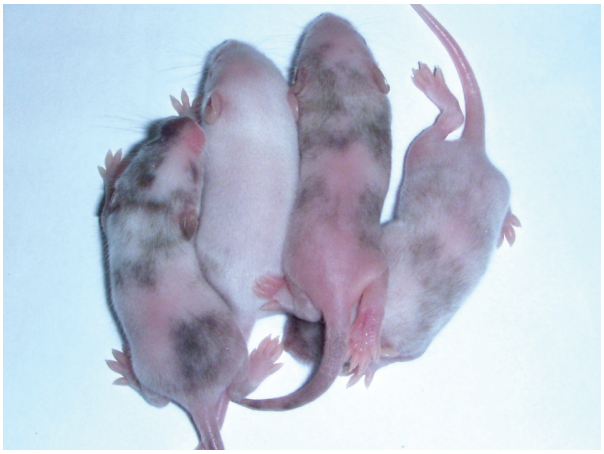
Adult stem cells have been used extensively for research as well as clinical applications. For instance, bone marrow contains stem cells that are constantly replenishing the blood system with specific cells required for survival. Bone marrow transplants are a common treatment in patients with severe leukemia and other blood-related diseases. Recent efforts are focused on stem cells in the brain for treatment of Alzheimer's and related dementia disease (Taupin 2009) and in the heart for treatment of cardiac problems (Barile et al. 2009).



Hematopoietic stem cell differentiation.

Induced Pluripotent Stem Cells

Remember again that all cells in the body, except for sex (germ) cells, contain approximately the same genetic material. In theory, if the "imprinting" that pushed a cell down a specific differentiation pathway could be erased, a cell could again become pluripotent. This imprinting can be caused by proteins such as transcription factors binding the DNA, the addition of molecules such as methyl groups to sites on the DNA, or the addition of methyl and acetyl groups or the molecule ubiquitin onto the histones. Removal of these changes can contribute to reverting the expression of genes in the cell so that it becomes pluripotent.



Chimeric mice produced by injection of MEF iPS cells into heterologous blastocysts.*

* Image courtesy of Dr Miguel Esteban, Stem Cell and Cancer Biology Group, Key Laboratory of Regenerative Biology, South China Institute for Stem Cell Biology and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510663, China.

In 2006, Takahashi and Yamanaka demonstrated that by introducing four genes, Oct4, Sox2, Kfl4, and cMyc, into a differentiated mouse cell, the cell could become pluripotent again. These "reprogrammed" cells were similar to mES cells, but they still contained some of the imprinting created through developmental differentiation. Additionally, these iPS cells did not support the development of viable chimeric mice upon injection into the blastocyst, the gold standard of the embryonic stem cell. However, by the next year Yamanaka's group overcame this hurdle and began producing chimeric mice from their iPS cells (Okita et al. 2007). These exciting discoveries propelled a number of studies that sought to understand the factors that control cellular reprogramming, including how to identify cells that share identical properties with ES cells, which factors control epigenetic changes ("the imprinting"), and which factors are absolutely necessary for reprogramming (Maherali et al. 2007, Okita et al. 2008, Mikkelsen et al. 2008). Human iPS cells were quick to follow (Wernig et al. 2007, Takahashi et al. 2007, Yu et al. 2007) as these details were revealed, and now human iPS cells have been created from such diverse sources as an 89-year-old patient with ALS (Lou Gehrig's disease) (Dimos et al. 2008) and a person suffering from Parkinson's disease (Soldner et al. 2009).

Originally iPS cells were generated using a viral construct to insert the genetic material into the chromosome. Although successful, the lack of control over the location of virally inserted material and the number of copies inserted makes viruses unsuitable for iPS cell production in the long run, so other methods were quickly developed. In 2009, Yu et al. eliminated the need for continuous presence of vectors and transgene sequences that were previously required for reprogramming by using nonintegrating episomal vectors to generate iPS cells. It has also been shown that direct DNA delivery of the genes or the proteins that are produced by these genes can induce a pluripotent state (Zhou et al. 2009). Studies involving generation of iPS cells using microRNAs and small molecules are underway (Huangfu et al. 2008, Judson et al. 2009). Other efforts (Nakagawa et al. 2008, Wernig et al. 2008) have shown that not all the genes described initially are required to induce pluripotency; in fact, at least one paper described that Oct4 alone was enough to reprogram neutral stem cells (Kim et al. 2009). These discoveries are moving the use of iPS cells closer to clinical application.

A Look Towards the Future

The future of the use of stem cells in healthcare depends upon understanding the underlying mechanisms that propel a cell down the differentiation pathway. It has become more evident that diseases such as cancer or birth defects are due to errors that occur in these pathways. Starting with diseased cells and working back to the native state, investigators will get a clearer picture of the mechanisms involved.

Additionally, stem cells and especially iPS cells offer the possibility of a renewable source of replacement cells for one's own body. Many articles are being published in the area of heart disease where these cells could replace damaged or diseased cardiac cells. Much of the recent work is being performed in rat and mouse models. Stem cells taken from bone marrow have been shown to differentiate into cardiomyocytes to become coronary arteries, arterioles, and capillaries.

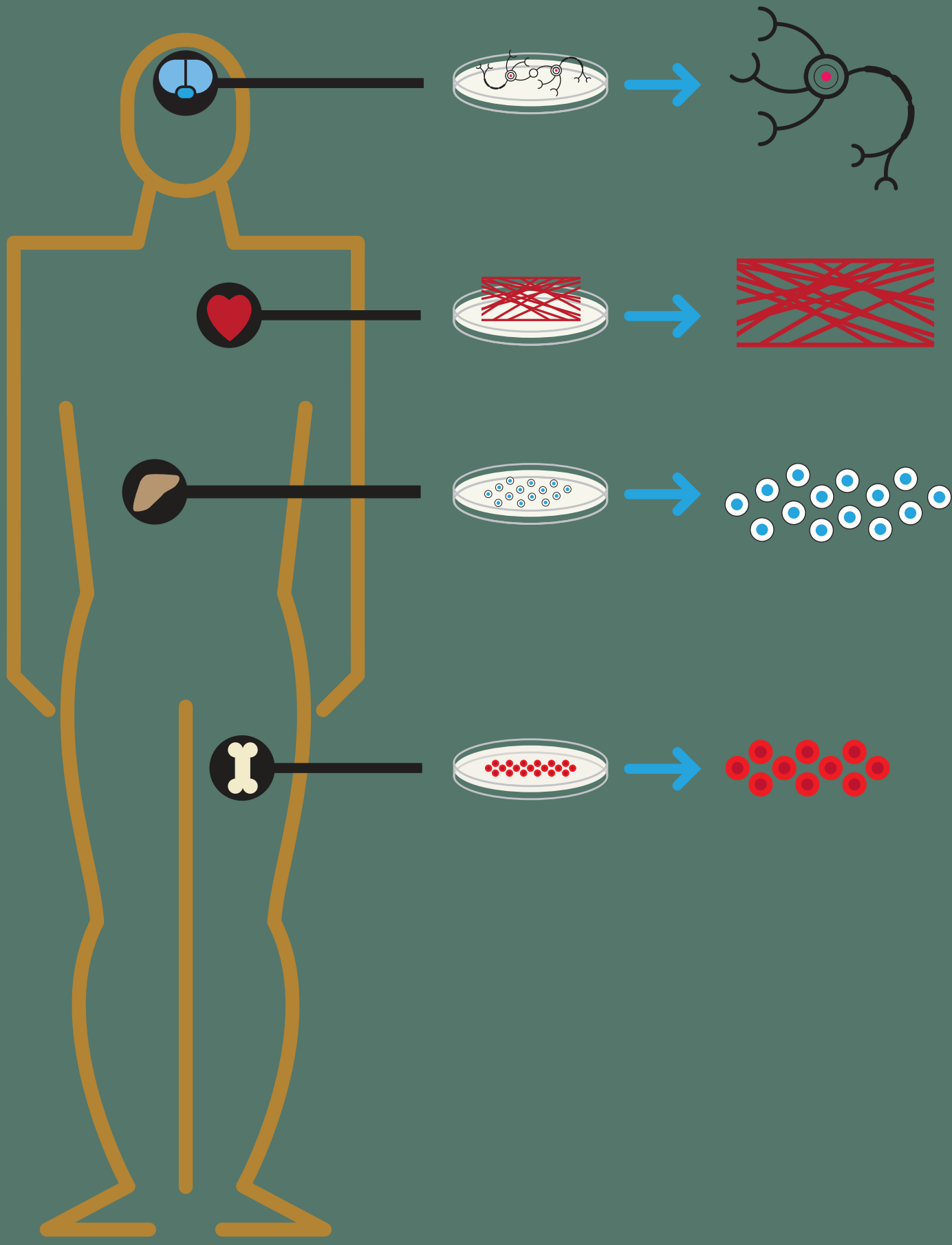
Stem cells may also provide a treatment for many brain diseases. The most likely candidate is Parkinson's disease in which the cells can be transplanted directly into the specific location in the brain. Other diseases that might be impacted include ALS, multiple sclerosis, Huntington's disease, Alzheimer's disease, as well as strokes and spinal cord injuries.

As scientists learn more about these cells the list of treatable diseases will expand. The future may well hold the promise of the fountain of youth.

Interesting web sites include:
http://library.thinkquest.org/24355/data/details/1952.html

http://blogs.nature.com/reports/theniche/2009/03/what_a_week_for_ips_human_cell_1.html

www.texasheart.org/Research/StemCellCenter/index.cfm



ISOLATION AND MAINTENANCE OF STEM CELLS

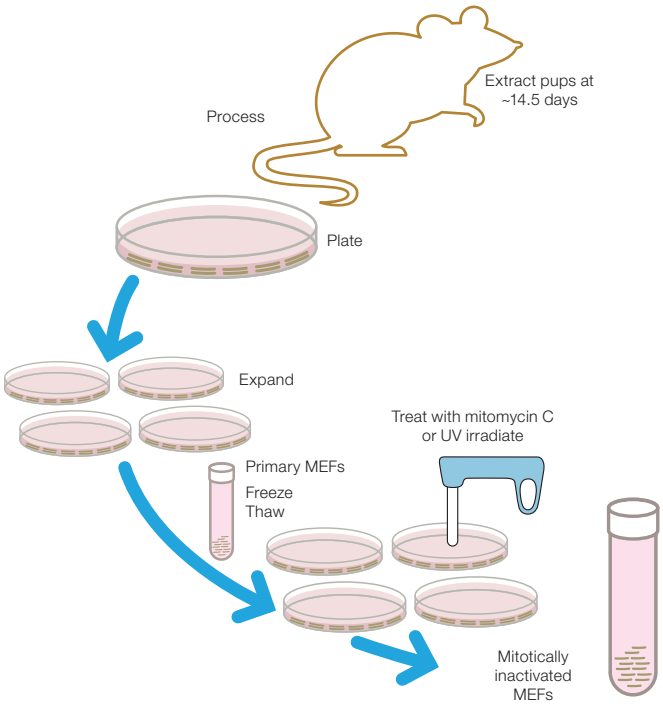
Stem cells are found in both embryonic and adult tissue. Many companies now offer stem cells along with media containing the growth factors necessary for maintenance. In general, standard tissue culture techniques are followed.

Until recently, embryonic cells were always grown on a layer of mouse embryonic fibroblast (MEF) cells. These cells can be purchased from ATCC or isolated by retrieving embryos from a pregnant animal. To isolate from an animal, the head, liver, intestines, heart, and all viscera are removed and the remaining tissue is minced and sheared. The resulting suspension is divided among T175 flasks and grown at 37°C with 5% CO_2 for 3–4 days. Before they can be used as feeder cells, the MEF cells must be mitotically inactivated using either UV light or treatment with the chemical mitomycin-C. This prevents the MEF cells from overwhelming the slow-growing stem cells in the culture. The MEF cells can be frozen before or after inactivation and then used as the stock for either generating more feeder cells or for supporting ES cells. Standard plating density for MEF cells to support ES cells is 1.2 x 10^5 cells/cm^2 or about 1.2x10^6 cells in a 35 mm dish.

Methods to culture ES cells without feeder layers have been developed recently. This method requires coating tissue culture plates with BD Matrigel (BD Biosciences), a gelatinous protein mixture isolated from mouse tumor cells. Many companies sell media that is formulated for use in maintaining stem cells in their original state.

The following link provides tips for maintaining embryonic stem cells in BD Matrigel medium.

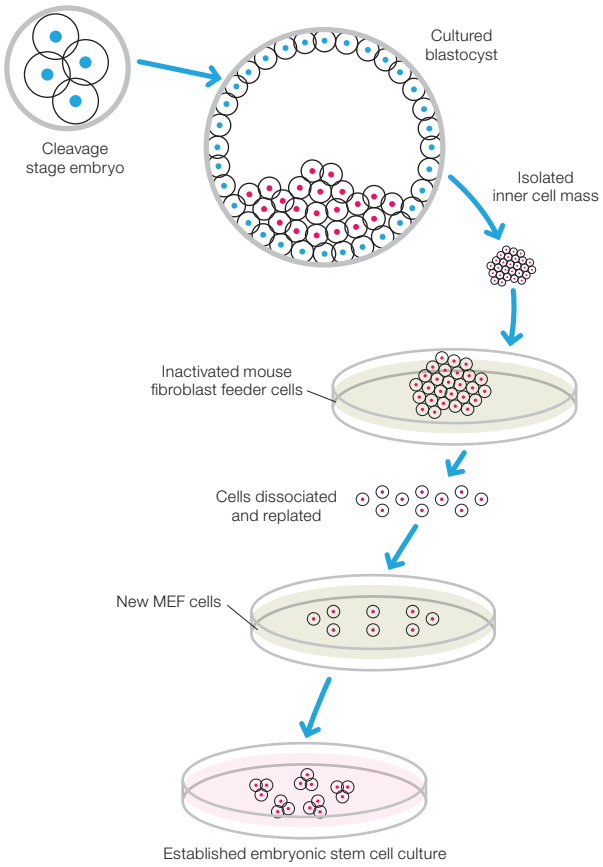
www.jove.com/index/browse.stp?tag=Mouse%20embryonic%20fibroblast%20(MEF)



Preparation of mitotically inactivated MEF cells for use as feeder cells.

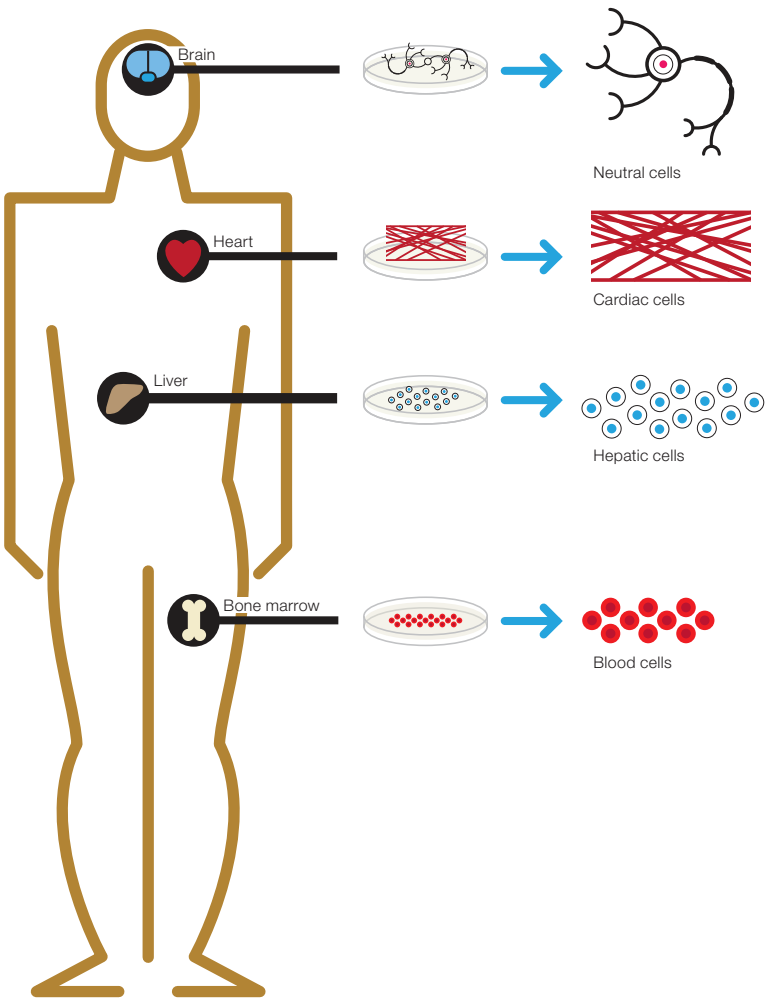
Embryonic Stem Cells

Human embryonic stem cells are available through commercial vendors.



Cultivation of embryonic stem cells.

Embryonic stem cells from other model organisms such as mouse and rat are also available through several different vendors (see end of chapter for more information). Alternatively, these can be established by culturing zona-free blastocysts or morulae embryos in a feeder layer for 3–4 days. The ICM that propagates is harvested and the tissue dissociated before being replated and expanded. Once expanded to a high enough density, the cells are verified by screening for the proper surface markers, a normal karyotype, and the ability to form a teratoma in an immune-compromised mouse.



Sources of somatic stem cells in the human body.

Somatic Stem Cells

Somatic stem cells are found in most major organs and tissues; however, the number of cells that can be isolated varies greatly depending upon the origin. For example, cardiac stem cells are quite rare, while hematopoietic stem cells occur in high enough numbers that they are routinely isolated and used in medical procedures.

Isolation of somatic stem cells is done either by an enrichment process or through a depletion process. Enrichment is commonly used for isolating cells originating from the hematopoietic system. The isolated cells are used for treating patients with a number of diseases including multiple myeloma and leukemia. In this case, apheresis is a common technique used to collect the appropriate cells from circulating blood cells. In a research environment or when cells are isolated from bone marrow, density gradient centrifugation or bead-based isolation methods may be used. Beads are coated with antibodies to surface markers found on the cells of interest. The cells expressing these markers bind to the beads allowing the other cells to be washed away. The bound cells are eluted from the beads and cultured under proper conditions.

In a depletion process, cells from the tissue of interest are plated in media that will allow growth of only the stem cell. This process is typically used for isolation of adherent cell types such as cardiomyocytes. In this case, cardiac cells are prepared from tissue biopsies and grown in media that select only for stem cells. The cells resulting from the culture procedure are verified for stemness by all the methods cited previously.

Induced Pluripotent Cells

Methods for creating iPS cells are continuously evolving (see Introduction for details). At the time of writing, the presence of a single transcription factor, Oct4, was enough to induce pluripotency in a certain cell type (Kim et al. 2009). The introduction of this factor can be done through a virus (Kim et al. 2009), electroporation, or the transcription factor itself. Protocols for doing these are identical to those used in standard molecular biology experiments.

Protocols

Detailed protocols for isolating MEF cells can be found in Robertson (1987) and Hogan et al. (1994).

Protocols for stem cell isolation and culture can be found at the following links:

www.seas.harvard.edu/biomat/resources/protocols/hES_MEF_EB.pdf

Niimi et al. 2005, supplementary material. www.biotechniques.com/multimedia/archive/00042/NiimiSuppl_page_nos3_42422a.pdf

Companies

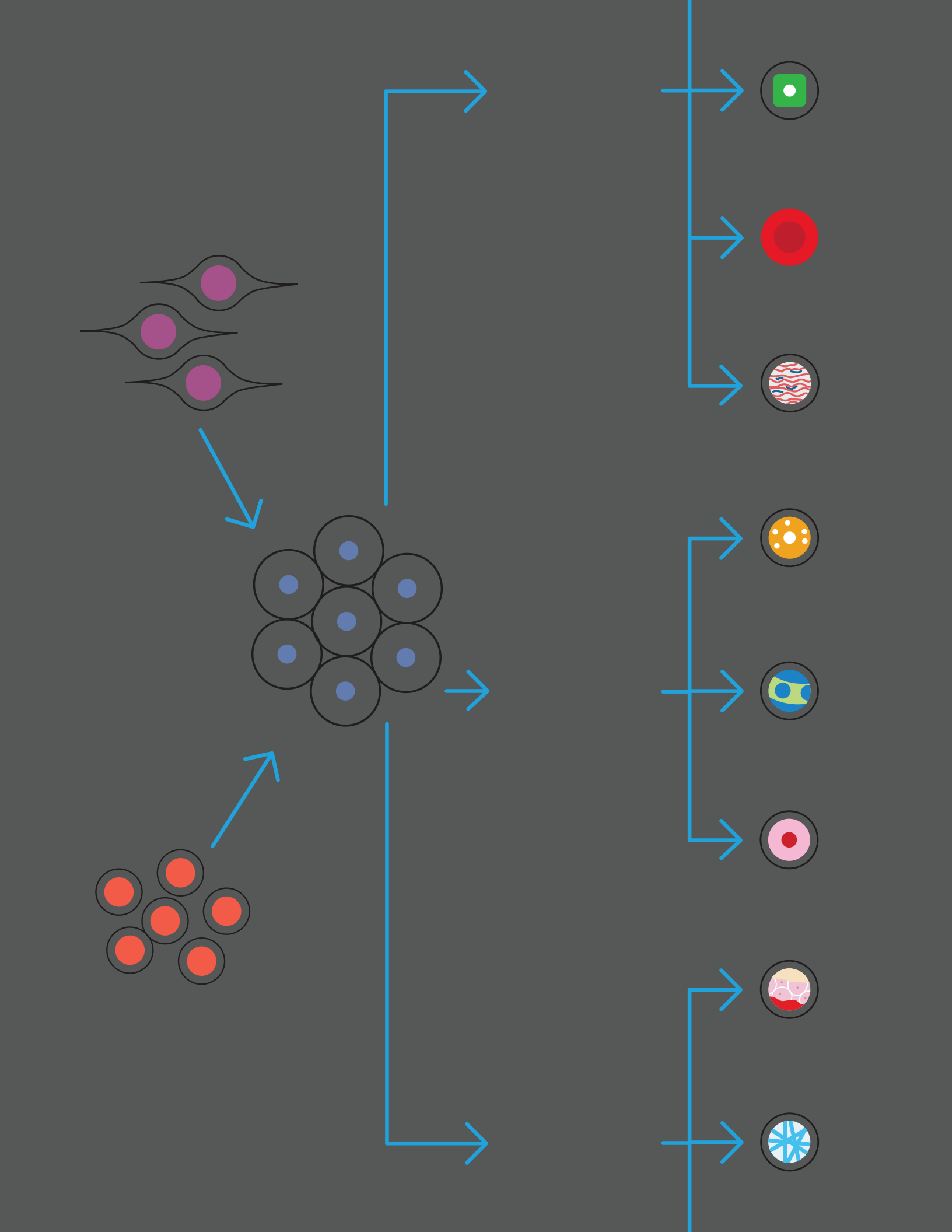
Some resources for obtaining stem cells are:

WiCell Research Institute — www.wicell.org

Celprogen, Inc — www.celprogen.com

Zen-Bio — www.zen-bio.com

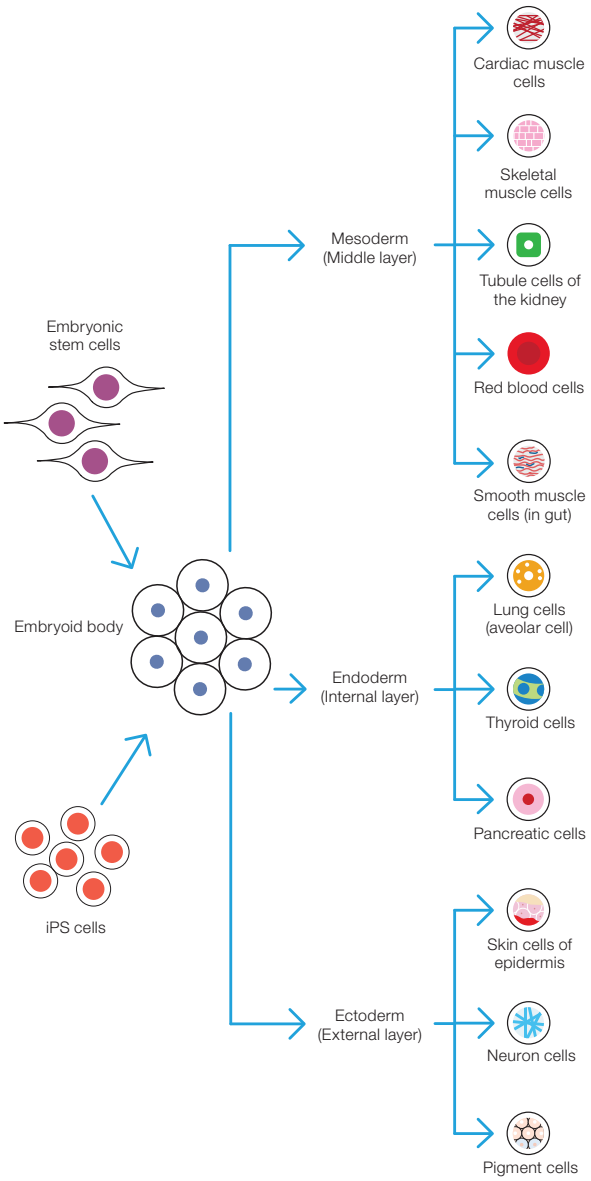
StemCell Technologies — www.stemcell.com



DIFFERENTIATION

One of the greatest hurdles early human embryonic stem cell researchers faced was determining how to grow the cells in an undifferentiated state for long periods of time. Early techniques did not always transfer from one lab to another successfully, resulting in a variety of very different culture conditions that were nontransferable within the community. Due to the wide variety of starting conditions, the original techniques for differentiating stem cells into one of a wide range of specialized daughter cells and their success rates were even more varied than the assorted conditions for general stem cell growth.

In recent years, great efforts have been made to standardize stem cell culture techniques and, while there is no single way of culturing stem cells or differentiating them into specialized cells, methods are becoming more generalized and widely applicable. For instance, the use of a synthetic serum replacement medium combined with bovine fetal growth factor (bFGF) instead of fetal bovine serum has decreased spontaneous rates of differentiation in a stem cell culture and eliminated many of the lot-to-lot variation problems. With more consistent culture conditions in place, methods for differentiation are becoming more standardized as well. A generalized overview of the current methods for differentiating hES cells into several important cell types is given below.



Differentiation of ES and iPS cells.

The Embryoid Body

The first stage of human embryonic stem cell differentiation is the induction of individual stem cell colonies to form an embryoid body (EB). All downstream differentiated cells are derived from this initial structure. EBs are created by enzymatically detaching undifferentiated stem cell colonies from the dish and transferring them into bFGF-free media (Itskovitz-Eldor et al. 2000). The colonies are kept in suspension and form spherical EBs in about four days. Each EB contains all three germ layers, the ectoderm, mesoderm, and endoderm, just as a developing embryo.

Cells of the Ectoderm

The central nervous system, hair, and the epidermis are all derived from the ectoderm. The default differentiation pathway for mammalian stem cells is to form neurons. Therefore, the cells of the nervous system have been the most studied of all of the cell types that can be derived from hES cells. There are several protocols for producing neural progenitor cells from undifferentiated cultures. One of these protocols (Zhang et al. 2001) is discussed here.

In order to induce EBs to form neurons, the culture medium is replaced with neural basal media containing bFGF, heparin, and N2 supplement. N2 supplement consists of transferrin, insulin, progesterone, putrescine, and selenite. Two days later, attachment of the differentiating EBs is induced by plating them onto dishes coated with laminin or polyornithine. After an additional 10–11 days in culture, the EBs differentiate into primitive neuroepithelial cells. The identity of the cells can be confirmed by staining for PAX6 (paired box gene 6, a transcription factor), SOX2 (sex determining region Y-box 2, another transcription factor), and N-cadherin (a calcium-dependent cell adhesion molecule specific to neural tissue).

From here it is possible to differentiate the neuroepithelial cells into specific cell types of the central nervous system, including motor neurons (Li et al. 2005), dopaminergic neurons (Yan et al. 2005), and oligodendrocytes (Nistor et al. 2005).

Cells of the Mesoderm

Cells of the mesoderm form most of the body's internal supporting structures, including the blood, muscles, bone, and heart. Because heart disease is a major public health concern, much stem cell research has been devoted to studying cardiomyocytes. Only about 10% of the cells of an EB can form these cells, therefore, the process of producing a pure cardiomyocyte culture involves many rounds of cell isolation. Cardiomyocytes develop spontaneously from 10 day old EBs that have been plated onto gelatin-coated plates (Kehat et al. 2001). Fortunately, cardiomyocytes are easy to identify because of their

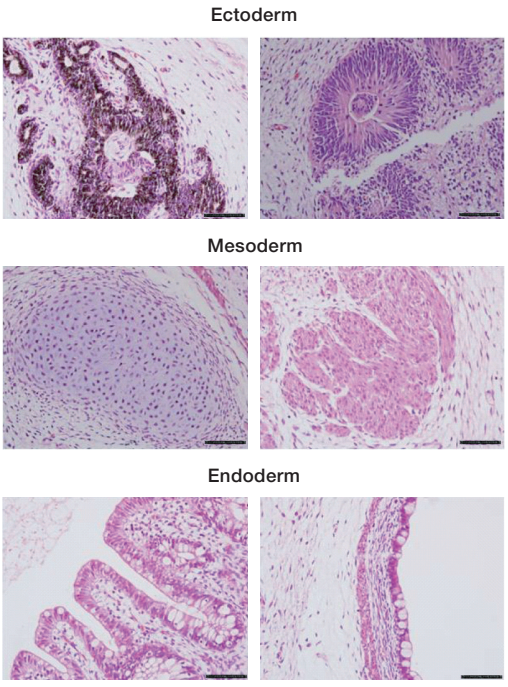
hallmark rhythmic contractions that can be observed using a phase microscope. Cardiomyocytes can be separated from the rest of the differentiating culture using flow cytometry and antibodies against cardiac markers such as cardiac myosin heavy chain, alpha-actinin, desmin, and cardiac troponin I (Xu et al. 2002).

An alternative method for deriving cardiomyocytes is to transfect a stem cell culture with a viral vector containing a drug-resistance gene driven by the alpha-myosin heavy chain promoter. Subsequent selection for drug resistance enables the selection of cells that are differentiating only into cardiomyocytes (Zhao and Lever 2007).

The differentiation of stem cells into various blood cells has long been of important clinical interest for cancers of the blood, such as leukemia. Much work has been done with hES cells to develop techniques for differentiating them into most of the cells of the hematopoietic system. However, these complex techniques are beyond the scope of this review. More information can be found in Keller et al. (1993) and Kaufman et al. (2001).

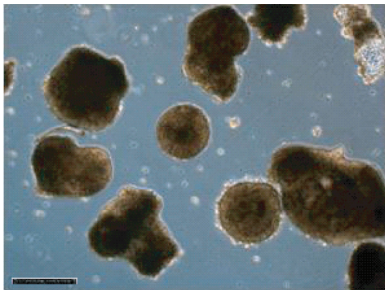
Cells of the Endoderm

The endoderm forms many of the internal organs, including the pancreas and the liver. The high rates of diabetes and liver disease have made the production of insulin-secreting cells and hepatocytes key goals in the field of stem cell research.



Teratomas composed of tissues derived from the three germ layers produced after injection of the same cell line into immunodeppressed mice.*

* Images courtesy of Dr Miguel Esteban.



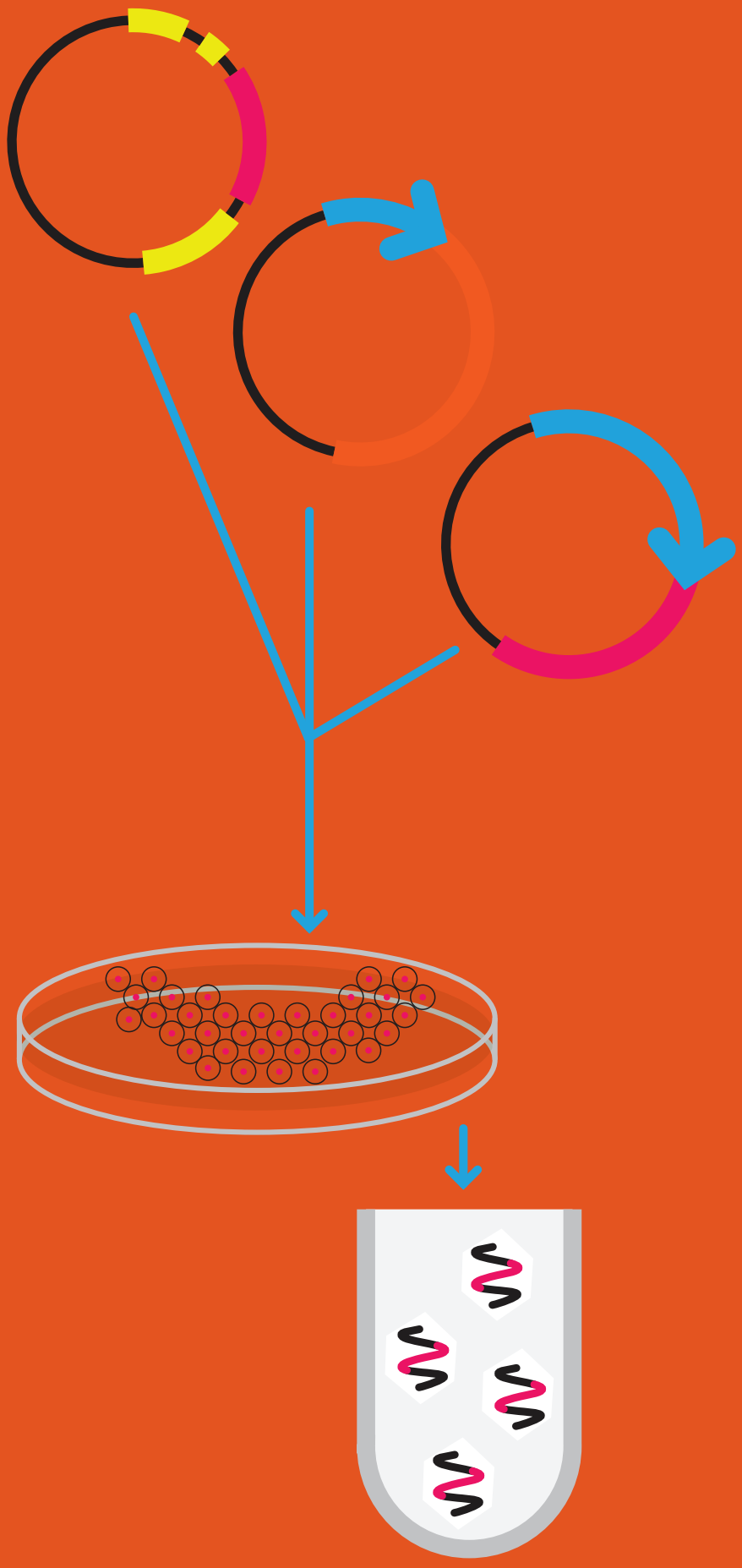
Embryonic bodies after growing in suspension for eight days. The dissociated cells are from the same iPSC cell line.*

* Image courtesy of Dr Miguel Esteban.

Diabetes is caused by the destruction of the insulin secreting beta-cells of the islet of Langerhans in the pancreas. Currently, it is possible to replace these lost cells with those of donors. However, there is very little of this material available for transplantation and the transplantations are not usually a permanent cure, often requiring additional transfusions of beta-cells for the patient to remain asymptomatic. It is now possible to make human embryonic stem cells into all pancreatic cell lineages (Guo and Hebrok 2009). However, the beta-like cells produced in the complex differentiation process are not efficient insulin producers and are not as completely responsive to cell signaling as native beta cells (Furth and Atala 2009). Rapid progress in this line of research combined with the efforts of the iPS cell community make a stem cell–derived treatment for diabetes a real possibility in the near future.

In contrast, progress toward developing liver cells (hepatocytes) for transplantation has not been as successful. Stem cells can be differentiated into hepatocyte-like cells using several methods (Hay et al. 2008, Basma et al. 2009, and others). Even though all of these differentiated cells express many of the canonical hepatocyte markers, it is still unclear which of these differentiation techniques and resultant cell types, if any, will be suitable for transplantation in the future (Flohr et al. 2009).

The ability of stem cells to differentiate into nearly any cell in the body gives them the potential to be the source of therapies for many currently incurable illnesses. As research moves forward, standardized techniques for stem cell culture and differentiation will be developed. These new techniques will lay the foundation for future stem cell therapies.



TRANSFECTION

Transfection is a method used to deliver molecules into cells. In embryonic and somatic stem cells, this method is used to introduce a new protein via a plasmid construct or through homologous recombination, or to knock down the expression levels of a protein via the RNAi pathway. In order to reprogram somatic cells so that they become pluripotent, a small number of genes (1–4), or their protein products, must be transfected into the cell. The method used for transfection varies depending on the molecule being delivered and the application. The most widely used methods are viral-mediated delivery, lipid-mediated delivery, and electroporation. Biolistics is also a technique that may be useful for transfer of materials into stem cells.

In general, stem cells are considered difficult to transfect. Unlike immortalized cells, they have not adapted to the environment of the tissue culture plate. No single transfection method will work for all stem cells, and even within a lab, the method of choice may not be reproducible.

Viral-Mediated Delivery

This method was the first method used and is by far the most efficient way to introduce new genes into a somatic cell to reprogram it to become a pluripotent cell (Takahashi and Yamanaka 2006, Okita et al. 2007, Takahashi et al. 2007, Wernig et al. 2007). This section will focus on the methodology used to produce the iPS cells; these are commonly used techniques that can be used to introduce genetic material into many cell types.

Retroviruses such as lentivirus have been the most commonly used for reprogramming; however, the lack of control over their integration site into the chromosome has led to the development of other vectors. A sendai virus, which carries out replication in the cytoplasm, has also been used to reprogram cells to the iPS state (Fusaki et al. 2009). Additionally, scientists at the Whitehead Institute (Carey et al. 2009) engineered a polycistronic virus that reduces the integration events while still producing multiple proteins.

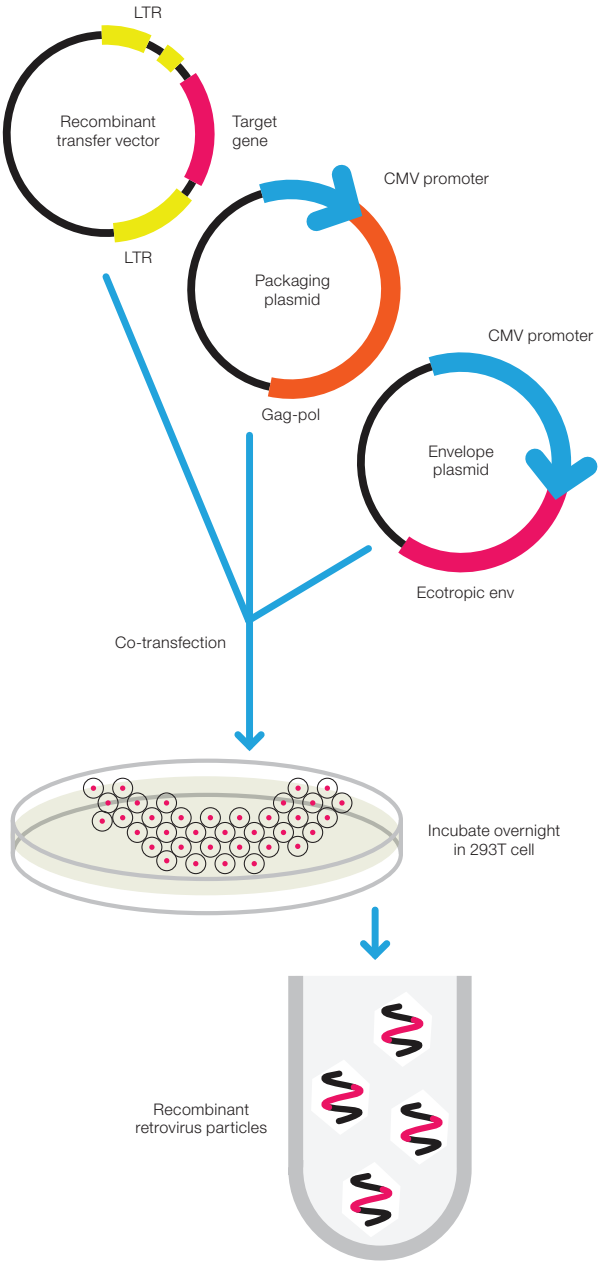
Although viral-mediated gene delivery is highly effective, the production of the virus itself is time consuming. Lentiviral production is accomplished using three separate constructs — a transfer vector, a packaging plasmid, and an "envelope" plasmid. The virus is produced by first cloning the DNA of interest into the transfer vector. The appropriate clone is selected and transfected with the packaging vector and "envelope" plasmid into 293T cells overnight to propagate the virus. Supernatant from the cells containing the amplified virus is then titered to determine the concentration. Virus prepared in this manner can generally be stored for several weeks at 4°C.

Protocols for viral production can be found at the following sites:

http://biology.kenyon.edu/slonc/gene-web/Lentiviral/Lentivi2.html

www.broadinstitute.org/genome_bio/trc/protocols/trcLentiVirusProd.pdf

http://web.mit.edu/jacks-lab/protocols/lentiviralproduction.htm



Preparation of retrovirus.

Lipid-Mediated Delivery

This method is generally performed using commercially available lipid reagents. The lipid is mixed with the molecule being transfected and added directly to the plated cells. The lipid-encapsulated molecule is thought to move into the cell via endocytosis. Each lipid reagent must be optimized for the type of cell that is used. Efficiencies and toxicity of the lipids vary depending upon the reagent and the cell type. The disadvantage of this method is the introduction of an exogenous substance (the lipid) into the cell, which may alter biochemical pathways affecting pluripotency of the cell.

Electroporation

This is a physical method in which an electrical shock is applied to the outer membrane to temporarily disrupt the lipid bilayer, which allows molecules to enter the cell. Since this method introduces only the molecule of interest, it is the method of choice for many investigators working in the area of gene therapy. The technique should be optimized depending on the cell type used. For example, the optimal conditions identified for electroporating hES cells, which resulted in 10–30% of cells showing strong, transient GFP expression, were a voltage of 250 V, a capacitance of 200 µF, a resistance of 1000 Ω, and an exponential decay waveform. On the other hand mES cells required voltage of 220 V, a capacitance of 950 µF and a resistance of 1000 Ω (Zsigmond 2009).

Bio-Rad has an extensive array of optimized electroprotocols for delivery of materials into a variety of cell types and cell lines. More protocols for electroporation can be found at www.bio-rad.com/transfection/.

Bio-Rad's Gene Pulser MXcell™ electroporation system and electroporation buffer for transfection.

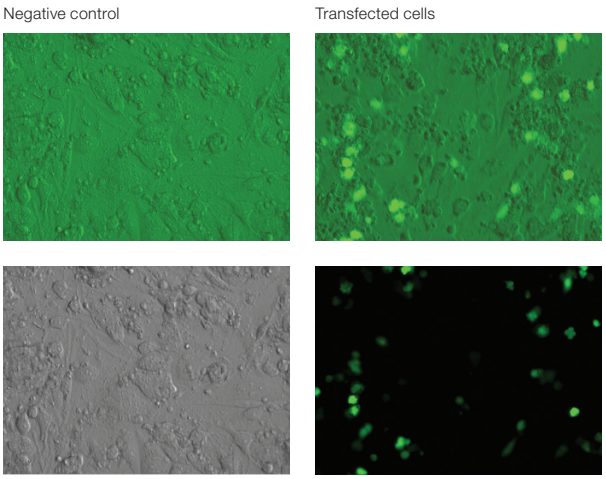
Biolistics Particle Delivery

This method uses a high pressure burst of helium to "shoot" the molecules of interest into the cells. The molecules of interest are first coated onto micron-sized beads. The method entails three steps: coating microparticles with DNA, drying them onto a macrocarrier disk, and propelling them into the target cells. The macrocarrier disk is accelerated with high-pressure helium into a stopping screen, which frees the microprojectiles to bombard the cells. Cells penetrated by the microparticles are likely to become transfected.

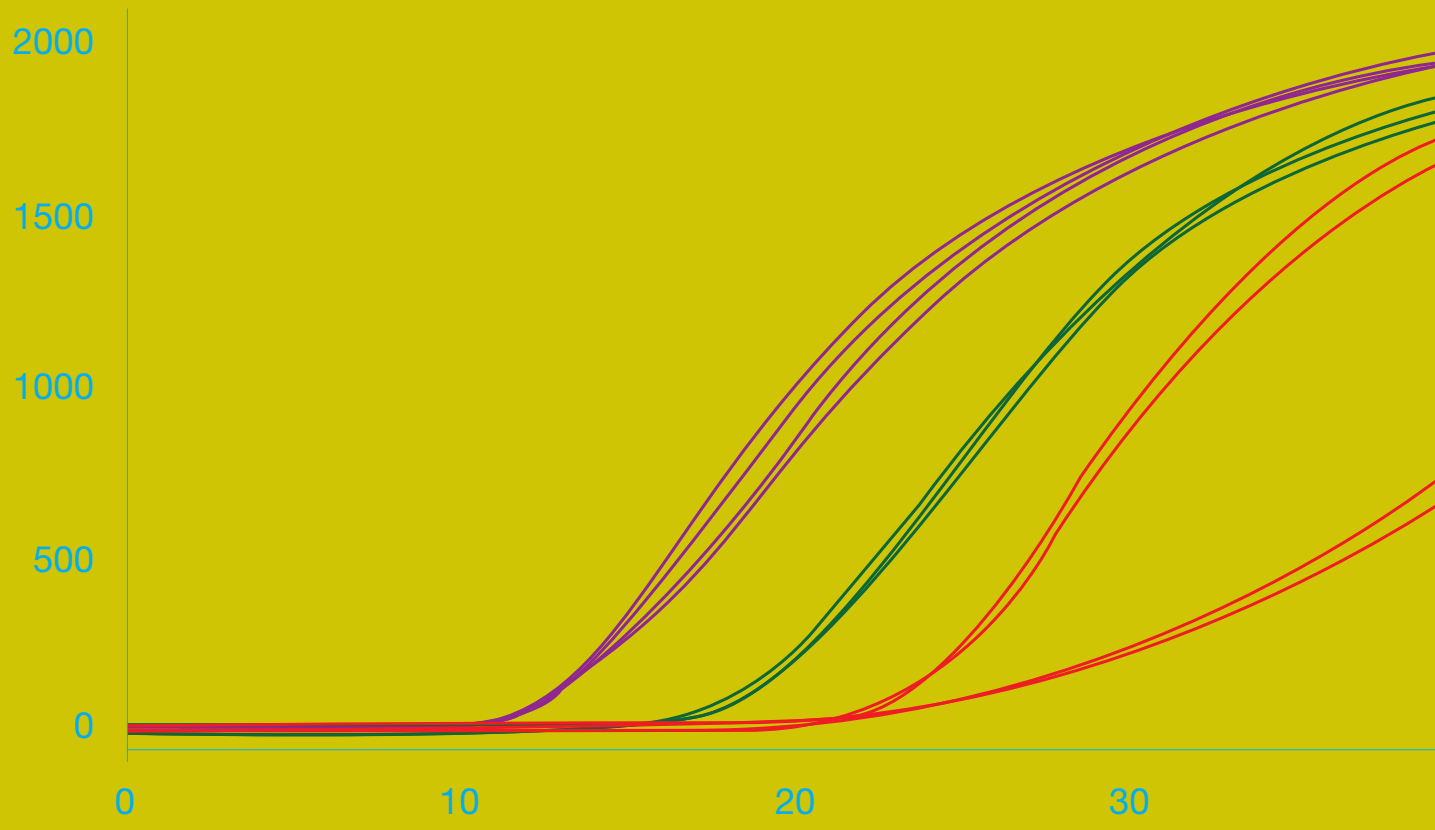
Like the other techniques, a certain amount of optimization is required for this method. This includes optimizing the density of cells plated, the amount of microparticles used in each blast, the pressure used to deliver the particles, and the amount of vacuum applied.

A major advantage of the biolistic approach is the ability of the particles to be carried through many layers of cells. As ES cells differentiate into embryoid bodies, this technique could be employed.

More information on this technique can be found at www.bio-rad.com/biolistics/. Specific protocols for transfecting neuronal cells can be found at www.natureprotocols.com/2009/07/09/transfection_of_rat_or_mouse_n.php.



Mouse ES cells grown in the presence of STO feeders (ES+) were electroporated using the Gene Pulser Xcell™ electroporation system with an exponential waveform at 240 V and 75 µF.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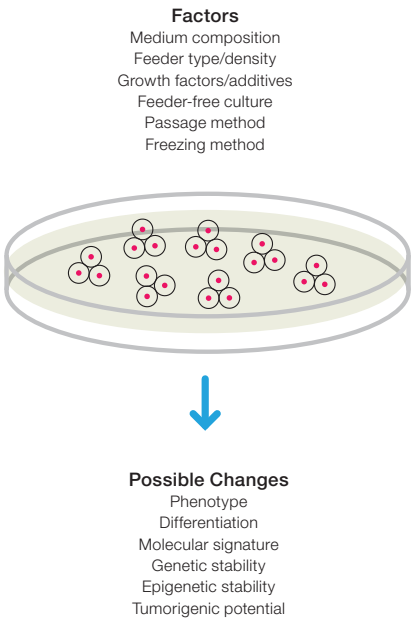
ANALYSIS

Analysis of stem cells falls into two main categories: first, monitoring the genomic integrity of the cells and second, tracking the expression of proteins associated with pluripotency. Genomic analysis of stem cells is necessary to ensure that stem cells maintained in culture for long periods of time have not become unstable through chromosomal loss or duplication or changes in their epigenetic profiles. Proteomic analysis ensures that the cells are expressing the factors necessary to maintain pluripotency. Finally, analysis of the differentiated state through both genomic and proteomic means will ensure identification and propagation of the proper cell type.



Factors affecting stem cells in culture and their effects.

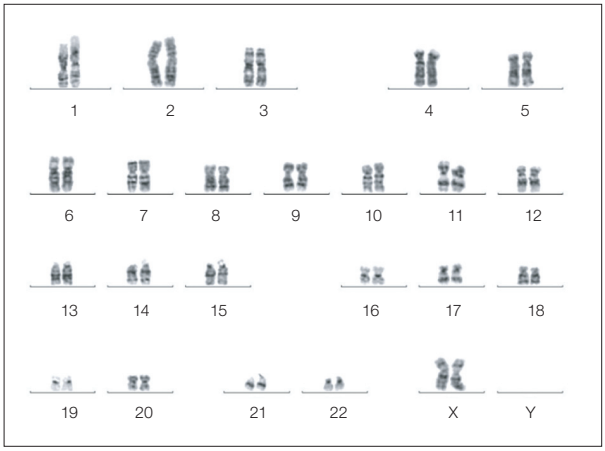
Three main genomic techniques are used for this type of analysis: karyotyping, SNP analysis, and epigenetic profiling. Healthy stem cells also express a known set of cell surface and cytoplasmic proteins associated with pluripotency. Flow cytometry, immunocytochemistry, RT-PCR/RT-qPCR, and western blot analysis are the techniques most commonly used for confirming expression of these pluripotency-related proteins.

It should also be noted that analyzing the genetic, epigenetic, and protein expression profiles is crucial during stem cell differentiation and the creation of iPS cells. A stem cell that has been differentiated into, for example, a motor neuron, will have a fundamentally different profile than its progenitor. Similarly, somatic cells being induced into pluripotency must be screened for expression of stem cell markers.

The following is an overview of each of these techniques and why they are important for stem cell research. The WiCell Research Institute and the National Stem Cell Bank provide detailed stem cell protocols for each technique on their website: www.wicell.org/index php?option=com_content&task=category&sectionid=7&id=246&Itemid=248.

Karyotyping

Karyotyping refers to examining chromosomes for any abnormalities. When a stem cell population is exposed to stressful circumstances, such as enzymatic passaging and feeder-free growth, this can lead to karyotypic abnormalities (Inzunza et al. 2004). Thus it is crucial to monitor a culture for the accumulation of genetic abnormalities. It is recommended that a stem cell line be karyotyped every 10–15 passages to ensure that no chromosomal duplication, deletions, or translocations have occurred. Traditional karyotyping uses a dye (for example, giemsa or quinacrine) to stain the chromosomes of a metaphase cell in distinct banding patterns, which are then used to match the chromosomes and identify any abnormalities. Recently, spectral karyotyping (SKY) has become popular. SKY is a hybridization-based combinatorial technique that uses fluorescently-labeled DNA probes specific for each chromosome. This method can detect translocations within and between chromosomes much more accurately than traditional karyotyping (Liehr et al. 2004).



Normal karotype of the human iPSC clone.*

* Image courtesy of Dr Miguel Esteban.

SNP Analysis

Single nucleotide polymorphisms, or SNPs, are the most common type of genetic variation (Brookes 1999). A SNP is a single base pair mutation within a region of DNA. SNPs have been evolutionarily conserved and for this reason can be used to identify members of a family or subclass within a species. SNPs can accumulate in a stem cell population over time and can lead to a dominant phenotype that has a survival or growth advantage, but has lost pluripotency or gained tumorigenicity. SNP genotyping with high-density oligonucleotide arrays (Gunderson et al. 2005) can specifically identify a stem cell line's origin and monitor its genomic integrity. SNPs can be identified using PCR, microarrays, or DNA sequencing.

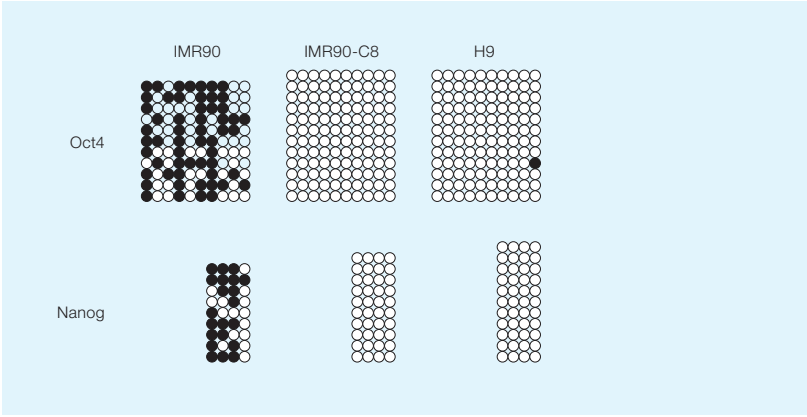
Epigenetic Profiling

It is becoming clear that a stem cell's pluripotency is dictated to a large extent by its epigenetic profile (Bibikova et al. 2006). DNA methylation and histone modification regulate the accessibility of DNA to the transcriptional machinery and thus regulate gene expression (Jaenisch and Bird 2003).

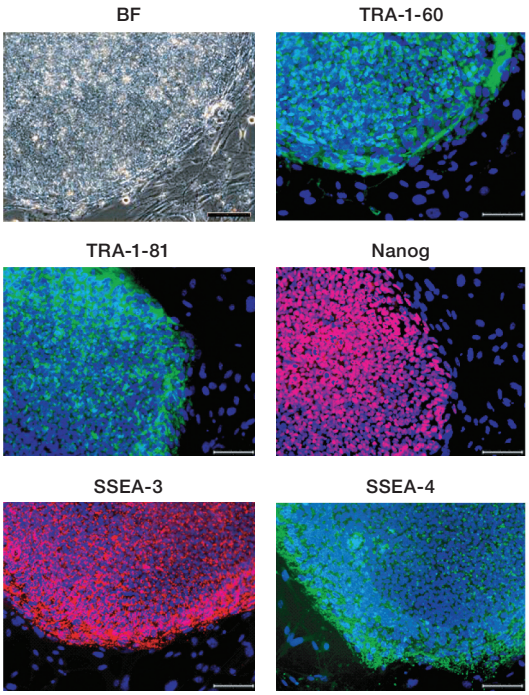
Analysis of DNA methylation patterns is performed by treating sheared DNA with bisulfite under controlled conditions such that all cytosines are converted to uracils while leaving methyl cytosines unchanged. After the conversion, the DNA can be analyzed for global methylation patterns using either a chip array or DNA sequencing. Chromatin immunoprecipitation (ChIP) analyzes patterns of histone modifications by cross-linking the DNA to histones, using formaldehyde. The cross-linked chromatin is then sheared and purified using antibodies against a specific protein or histone modification. Analysis of the cross-linked regions can be performed using qPCR, microarrays, or DNA sequencing. New stem cell lines can be initially characterized by examining the epigenetic state of a few key genes such as H19, Xist, Oct4, Notch 1, Dlk1/MEG3, and PWS/AS (Loring et al. 2007).

Flow Cytometry and Immunocytochemistry

Flow cytometry with cell sorting is a method for sorting a heterogeneous population of cells based on their light scattering characteristics (Watson 2004). Fluorescently tagged monoclonal antibodies are often used as a method of distinguishing a specific cell population and sorting it from another. Stem cells express a number of cell surface proteins that are considered to be markers of pluripotency and many antibodies are available for their detection (Adewumi et al. 2007). Glycolipid antigens SSEA-3 and SSEA-4 and the keratin sulfate antigens TRA-1-60 and TRA-1-81 are some of the most commonly used antigens for identifying and sorting live stem cell populations. Fixed stem cells can also be sorted using these markers as well as others, such as the transcription factors Oct-3/4 and NANOG. As stem cells differentiate, the loss of these markers and the expression of new markers can be used to track the progress and overall condition of a stem cell population. For instance, the protein nestin is a marker for neural progenitor cells, BNP (brain natriuretic peptide) is a marker for cardiomyocytes, and CD31, CD34, and CD45 are markers for hematopoietic cell types (see Deb et al. 2008, and references therein). While flow cytometry and sorting allow for the separation of a stem cell culture into distinct subpopulations, immunocytochemistry uses these antibodies to label fixed cells without disturbing the cell culture as a whole. This enables the visualization of an individual cell within the larger context of a stem cell colony and enables the user to evaluate the subcellular localization of the molecular markers (Myers 2006).



DNA methylation profile of the Oct4 and Nanog proximal promoters in the indicated cell types.*



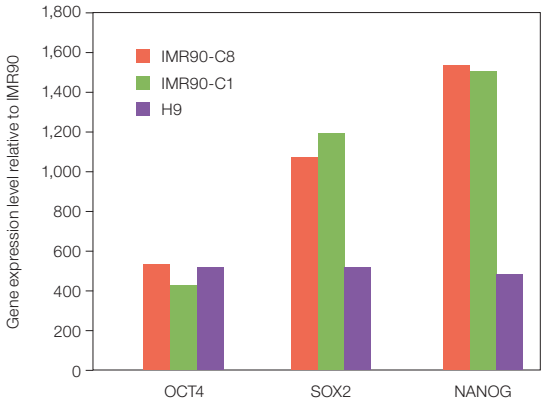
iPSC clone generated from the human fibroblast cell line IMR90 using medium containing vitamin C (Vc). Phase contrast photographs and immunofluorescence microscopy for the human ESC surface markers TRA1-60, TRA-1-81, SSEA-3, SSEA-4, and the transcription factor Nanog. Nuclei are shown in blue.*

* Images courtesy of Dr Miguel Esteban.

RT-PCR/RT-qPCR

RT-qPCR provides a rapid, sensitive, and quantitative method for monitoring the gene expression profile of a cell population. (For a comprehensive review of real-time PCR see Real-Time PCR Applications Guide, Bio-Rad catalog #170-9799). RT-PCR/qPCR first requires isolation of the stem cell RNA. The RNA is then converted into cDNA using the enzyme reverse transcriptase. The cDNA for a gene of interest is amplified by the addition of gene-specific DNA primers and Taq polymerase, enabling the detection and quantitation of the levels of gene expression in a given cell population. Stem cells express a number of unique genes that are used as markers for pluripotency. These genes are mainly transcription factors that are involved in creating and maintaining the undifferentiated state. Loss of expression of these genes correlates with a loss of pluripotency. Some of the best known transcription factors expressed in stem cells are Oct-3/4, NANOG, FOX2D, SOX2, and UTF-1 (Bhattacharya et al. 2004). Other important genes expressed in stem cells are LIN28, a regulator of translation and microRNA production (Büssing et al 2008) and hTERT human telomerase reverse transcriptase (Ju and Rudolph 2006).

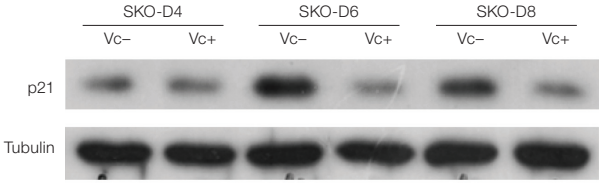
RT-qPCR is also a valuable tool for evaluating the state of differentiated cells. For example, GFAP (glial fibrillary acidic protein) and MAP-2 (microtubule associated protein 2) are markers for neural progenitor cells (Gage 2000). RT-qPCR is such a widely used technique for analysis of stem cells as well as differentiated cells that primers have been designed and validated for nearly every gene of interest. The qPrimer Depot (http://primerdepot.nci.nih.gov/) and the Quantitative PCR Primer Database (http://web.ncifcrf.gov/rtp/gel/primerdb/) are excellent resources for finding validated primers for nearly every human gene.



Real-time qPCR analysis of endogenous OCT4, SOX2, and NANOG and markers for the three germ layers in EBs from two periosteum cell lines.*

Western Blotting

Western blotting is an analytical technique for measuring the amount of a specific protein in a complex sample. The proteins are separated using 1-D gel electrophoresis and then transferred to a membrane (nitrocellulose or PVDF). Antibodies specific to the protein of interest are then used to probe the membrane. Detection is through a two-step process which utilizes a reporter system consisting of an enzyme linked to an antibody that interacts with a colorimetric substrate to produce a band on the membrane. For stem cell scientists studying the mechanism of differentiation, western blotting is especially useful in determining the success of transfection experiments. When a gene is either introduced into the cell or knocked down using RNAi, detecting and quantitating its protein level through a western blot procedure coupled with measuring the transcript level by RT-qPCR is a common way to assess transfection efficiency. More importantly, western blots can be used to study the effects of transfection on downstream protein expression during differentiation or culture maintenance (Rubinson et al. 2006).



Western blot for p21 shows reduced senescence in MEFs transduced with SKO factors and treated with vitamin C (Vc) compared to control.*

* Images courtesy of Dr Miguel Esteban.

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