# Chapter 2

# Stem Cell Banks: Preserving Cell Lines, Maintaining Genetic Integrity, and Advancing Research

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## Abstract

The ability to cryopreserve and successfully recover cell lines has been critical to the conservation of all cell lines, especially the preservation of pristine early-stage cultures and the preparation of well-characterized cell banks. Indeed, the systematic storage and establishment of cryopreserved banks of cells for the stem cell research community is fundamental to the promotion of standardisation in stem cell research and their use in clinical applications. In spite of the significant potential for the use of stem cells in research and therapy, they are challenging to maintain and have been shown to be unstable after prolonged culture that often results in permanent alterations in their genetic make-up, which ultimately alters the phenotype of the culture. This chapter will review the principles of cell bank production, techniques for the scale-up of human pluripotent stem cells, quality control, and characterisation methods for banked cell lines.

**Key words:** stem cell banking, master cell bank, working cell bank, stem cell characterisation, pluripotent stem cell characterisation, pluripotent stem cell quality control methods

## 1. Introduction

The establishment of large cryopreserved stocks or "banks" of microorganisms and cell cultures has been key to the reliability of industrial processes and to the manufacture of products based on the use of these cultures. Working in a similar way, cell culture collections, such as the UK Stem Cell Bank (UKSCB), that supply human stem cell cultures to researchers around the globe have promoted standardisation of methods that ensure that these cell lines remain available for decades. When setting out to establish a "Stem Cell Bank," it is important to identify its core function so that subsequent development can be directed appropriately. The cost associated with establishing and operating a cell bank is influenced by

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the amount and type of quality control measures employed by the bank. Depending on the goal of the cell bank, these control measures may encompass mandatory quality standards including good manufacturing practices for therapeutic use in humans (1–5).

There is still much progress to be made in the optimisation of methodology in the areas of culture, preservation and characterisation of human stem cell lines. It is fundamental that those responsible for supplying stem cell lines to the stem cell community for research and clinical purposes work closely with leading stem cell researchers in order to remain in the forefront of these evolving methods. Projects funded by an international consortium of research funding bodies called the International Stem Cell Forum (www.stem-cell-forum.net/ISCF) have already promoted such developments (6, 7). Other organisations such as the International Society for Stem Cell Research (www.isscr.org/) have initiated high level guidance (*Guidelines for the Conduct of Human Embryonic Stem Cell Research, 2006, Guidelines for the Clinical Translation of Stem Cells, 2008*) as, has the US Academy of Sciences (*Guidelines for Human Stem Cell Research, 2005*). Key developments required to improve the standardisation of research and suitability of human pluripotent stem cells (hPSCs) for clinical applications will include development of robust methods for feeder-free culture (see Chapter 9) and passage of stem cells as single-cell suspensions (see Chapter 10) that do not result in genetic instability of the stem cell lines.

Characterisation of stem cell lines will no doubt be further enhanced through the application of proteomics, genomics and transcriptomics. Proteomic analysis has already been used to screen for phenotypic consistency in the analysis of human embryonic stem cell (hESC) cultures (8, 9), and has also been proposed for the quality control of cell lines (10). A major issue yet to be addressed is the development of robust and standardized in vitro and in vivo methods for evaluation of pluripotency in hPSC lines that can be used routinely in stem cell research laboratories.

This chapter summarises the various activities and issues involved in the preparation of master- and working-cell banks and quality control testing of lot-batches of human pluripotent stem cell (hPSC) lines that ensure the preservation of genetic integrity and increase experimental reproducibility.

## 2. The Principle of Master- and Working-Cell Banks

It is a commonly held belief that the banking of stem cell lines is simply a matter of growing up an extremely large batch of cells, aliquoting these cells into cryogenic vials, and freezing down one large lot that can then be used for experimental work and sent to collaborators. For specific projects, this may provide a short-term

solution. However, in a research lab, with ongoing research programmes, it is important to be able to recover early passage stocks periodically in order to avoid the potentially detrimental effects on research data of experimenting with cells that have been in culture for prolonged periods of time. Irreversible genetic changes have been observed in hPSC cultures upon continuous culture over an extended period of time. Furthermore, resource centres that supply researchers with stem cell lines, as well as those supplying cells for clinical use, are expected to supply cells of consistent quality for many months and years with regards to characteristics and passage level.

In the biotechnology industry, where microorganisms and cell cultures have been used for manufacturing purposes for many decades, the establishment of a well-characterises, cryopreserved, seed stock, the master cell bank, as the source for all future work, is considered fundamental good practice. Individual vials of the master cell bank are then used to generate large "working" cell banks from which individual vials are used to initiate cultures for each production run or period of experimentation. This master/working bank system has been key to assuring long-term provision of high-quality cells for both research and industry and should be considered best practice for any stem cell culture laboratory (11, 12). The physical process of cryopreservation is not always well understood by stem cell workers and this can lead to failure to recover cells and even loss of cell lines. Furthermore, where preservation is suboptimal certain abnormal cells may be selected or induced giving rise to an altered cell culture. Reviews of preservation techniques and protocols used for pluripotent stem cell lines have been published respectively by (13, 14).

### 2.1. Feeder-Cell Banks

In order to maintain and expand undifferentiated hPSCs in culture these cells are usually co-cultured on inactivated fibroblasts of human or mouse origin known as feeder cells (see Chapter 8). It is not generally useful to extend the master- and working-stock principle to primary mouse embryonic fibroblasts (MEFs) because they have limited lifespan in culture and increased passaging affects their performance as feeder cells. MEFs are usually inactivated between passages 3 and 5 as they become senescent, depending on the mouse strain from which they were harvested, around passages 5–7. However, the preparation of large, pooled stocks of feeder cells at a consistent passage can improve the reliability of these cells in supporting the undifferentiated growth of PSC cultures. It is also important to obtain these primary cell cultures from animal colonies maintained under stringent animal husbandry standards and screened for a panel of infectious agents.

Human diploid fibroblasts, on the other hand, can be carried in culture much longer and passaged many more times than their murine counterparts, whilst retaining their capacity to support undifferentiated growth of hPSC lines. They therefore lend

themselves to the production of master- and working-cell banks while providing a xenogenic-free alternative to MEFs.

Following preparation, feeder-cell banks should be subjected to functional assessment and to microbial quality control (QC) tests prior to their use as feeder-cells in PSC cultures. This will help prevent the contamination of the PSC line with bacteria, fungi, mycoplasma carried by the feeder-cells.

### 2.2. Scale-Up Techniques

Currently, the preferred method of hPSC culture requires co-culture with feeder cells and manual passaging methods that do not lend themselves readily to culture scale-up and production of large banks of cells. At the UKSCB, undifferentiated hPSC banks are generally prepared using a 6-well plate format where the hPSCs are in co-culture with feeder cells and subcultured manually. In order to produce a bank of high quality hPSCs, cells from the same cell line are harvested and pooled from several plates prior to cryopreservation in an attempt to create a homogeneous bank of cells. Enhancing the "homogeneity" of cryopreserved cells promotes vial-to-vial consistency of these seed stocks, thus minimising variation that can arise in a single-well of any given plate.

Reduction of colonies to small clumps or single-cell suspensions using enzymes will facilitate the scale-up of cultures and the preparation of large banks of cells (15–18). While enzymatic passaging is preferable to manual passaging, especially when scaling-up cultures, chromosomal abnormalities (19–23) have been associated more commonly with cultures that are passaged using enzymatic methods. Until systems that sustain genetically stable cultures are developed and well-qualified, this may well remain the most important barrier to scale-up.

Scale-up of hPSCs is further complicated by the use of feeder-cell co-culture systems, which are still the most common method of hPSC culture. Effective scale-up of undifferentiated cells will only be achieved following the optimisation of culture conditions that do not use feeder cells. It is a challenge, however, to develop feeder-free systems that are capable of fully replacing the complex and as yet unknown mixture of components provided by feeder-cells. Currently, commonly-used systems require the coating of culture surfaces with extracellular matrices derived from animal sources, such as Matrigel™ or recombinant, xeno-free cell adhesion protein preparations of laminin (24) or vitronectin (25). However, these feeder-free systems will require significant development and validation for routine scale-up of hPSC cultures.

Despite these constraints, a number of scale-up methods for hPSC culture have been described (26–32). The ability to produce robust, reproducible protocols for scale-up is a fundamental requirement for the production of these cells, in both their undifferentiated and differentiated states, for use in cellular therapies.

Scale-up systems commonly used for non-stem cell cultures include roller bottles that are continuously rotated and can be

supplied with ridged surfaces to increase the culture surface area (available from numerous suppliers). Stacked, static-flask systems are also available (e.g. Cell Cube™ [NUNC]) and there is a range of well-established scale-up systems for the culture of cell lines that grow as cell suspensions. Such systems are not readily amenable to the standard methods of culture and passage of hPSCs as enzymatic recovery of cells without altering their karyotype and potency needs to be developed for such systems. Most animal cell culture, scale-up systems are focused, maintaining growing cultures of homogenous cell suspensions and use an internal impellor device within the suspension of cells (e.g. spinner flasks), growth of cells in porous membrane compartments (e.g. "miniPerm"™ vessels, dialysis tubing systems, hollow fibre systems), or the culture of cells in agitated flexible culture bags (e.g. "Wave" bioreactors [GE Healthcare]). Despite the difficulty in growing human stem cell lines as single-cell suspensions, adherent cells, such as hPSCs, could be grown on "microcarriers", which could enable their growth in bioreactor formats normally used for suspension cell cultures.

A novel scale-up approach is adopted in low sheer-stress systems, sometimes called "microgravity" culture systems. Here, a gas-permeable membrane replaces the usual direct air-medium/gas–liquid interface and the cells are rotated in suspension to prevent gravitational settling (33). hPSCs grown in such systems form embryoid body-like structures. Other systems that enable metabolic activity to be measured without turbulence in the growth medium allow the cells to be grown in "orbital" or "free-fall" modes, or a combination of these approaches, as the culture develops (34). Examples of such systems that are commercially available include the Rotating Wall Vessel™ (Cellon) and Nova Pod™ (Novathera, now Medcell).

Automated systems for cell culture are now beginning to be applied to the culture of stem cell lines (35). Currently available systems focused on the automation of the cell culture process are primarily designed for enzyme-mediated passage of cultures (e.g. "CompacT", "SelecT" and "Cello" [Technology Automation Partnership]); however, some are designed to passage cultures, such as hPSCs, that grow as colonies (e.g. "Cellhost" and the BioLevitator™ [Hamilton]).

## 3. Quality Control and Characterisation

There are a number of central issues for the key quality control of all cell cultures since they are important for reliable research data and the quality and safety of products derived from such cultures.

Of these, the most critical characteristics are:

- Viability
- Identity (the cells are what they are purported to be)

- Purity (freedom from microbiological contamination)
- Stability on growth or passage in vitro

### 3.1. Viability

The ability of a cell culture to recover from the cryopreserved state is often determined using a dye-exclusion test such as trypan blue-exclusion. Whilst this is a useful indicator of the viability of cells, any one parameter will only give a narrow dataset on the overall status of a cell culture (36). If time and resources allow, it may be helpful to add additional parameters of viability, such as early markers of apoptosis (for example annexin IV expression). It is critical for the validity of any research programme that the culture recovered from a cell bank is representative of the original stock. In the case of hPSCs, this is indicated by demonstration of the typical phenotypic markers of the stem cell type, and importantly, the functional potential for pluripotency (see characterisation below).

### 3.2. Identity and Authenticity of Cell Lines

There is a long-established tradition of passing cell lines from one laboratory to another. Unfortunately, due to variation in local culture procedures and reagents, genetic alteration may occur following extended passaging, resulting in significant and permanent changes in the characteristics of the cell line. Furthermore, accidental cross-contamination, or mislabeling of cultures, can lead to the generation and publication of invalid data. Such events may go unrecognized for many years and the consequences may be far-reaching and cumulative, thus, resolution of such problems may be very time-consuming. In the history of cell culture, many examples of cell line cross-contamination have been identified (37, 38). Unfortunately, new generations of researchers do not appear to have learnt the lessons from these early publications since the problem of cross-contaminated cell lines, in particular, is still a significant issue (39–42). For human stem cell lines, and hPSCs in particular, the morphology and surface marker phenotype of cultures is very similar. Thus, identification and discrimination of different hPSC lines based on morphology and phenotype alone is almost impossible.

To ensure the correct identity and authenticity of a cell line, two primary factors must be addressed: firstly, cell line provenance (i.e., a traceable and documented origin, starting from the laboratory in which they were derived), and secondly, direct characterisation of the cells that can confirm cell line authenticity. A range of specialised tests may be employed to determine cell line identity including, HLA typing (43), isoenzyme analysis and karyology (44), but the level of specificity achieved using these methods is not very high (31). Genetic profiling of cell lines using multiplex PCR DNA Short Tandem Repeat (STR) profiling is the preferred method as it allows identification of specific cell lines with a high

degree of specificity. This method has been utilised for a range of human tumor cell lines (45, 46) and is recommended as part of best practice in the quality control of human stem cell lines (7). Numerous companies now provide inexpensive DNA profiling services for human cells, making this technology available to all stem cell laboratories. It is important to note that, whilst these methods are human-specific, and therefore there is no cross-reactivity with the DNA derived from mouse feeder cells, it is possible that, when hPSCs are cultured with human feeder cells, the cell line DNA profile could be contaminated with the human feeder cell DNA. Thus, the specificity of the authentication method needs to be taken into account or evaluated. It is also important to remember that, whilst a DNA profile provides a highly specific DNA "bar code" that discriminates between different cell lines, this technique is only valuable for the formal authentication of a cell line when it can be compared with other material from the original donor/s and/or profiles from other qualified sources of the same cell line. Sharing genotypic data of this type between stem cell banks is therefore to be encouraged (4); however, national laws on release of individual, specific, genetic data may need to be considered before open publication of such information.

### 3.3. Microbial Contamination

Bacteria and fungi are common environmental contaminants that can infect and destroy cell cultures. A standard sterility-testing method using bacteriological broth cultures, such as outlined by Stacey (44), can be used on a regular basis to give assurance that general environmental contamination is not occurring. However, such methods do not have the capability to identify all possible bacterial and fungal contaminants that may arise; therefore it is best practice not to culture cells with antibiotics and to adhere to this principle most stringently when preparing cultures for banking.

The most common organisms known to cause unrecognized contamination are *Mycoplasma* and *Acholeplasma spp*. These organisms require special isolation media and growth conditions as described in Stacey (44). Details of standard methodologies for the above referenced testing methods can be obtained from the US and European Pharmacopoeia (47–50).

Any cell line can also harbour viral contamination arising from the original tissue or biological cell culture reagents. As a precaution, all cell cultures should be treated as potentially infectious, with appropriate containment and disposal according to local and national safety rules. Numerous viruses have been reported to establish persistent infection in cell lines (31). Viral contamination is clearly significant in that it may not only represent a hazard to laboratory workers, but also, where cells are persistently infected, it is likely to alter cell biology of the host culture (e.g., altered or deregulated biochemical pathways, transformation) and may cross-infect other cell lines in the laboratory.

It is not feasible to test cell banks for all potential viral contaminants but, where there is a very low risk of such contamination based on a risk assessment (51, 52), standard good laboratory practices, including containment of cell cultures (sealed culture vessels, use of biological safety cabinets, etc.), should provide adequate protection from contamination of cell cultures (11). Best practice guidance from the International Stem Cell Banking Initiative indicates that centres distributing stem cell lines to researchers, should aim to test for the most prevalent harmful human blood-borne viral pathogens (7).

It is suggested that all cell cultures suspected of harbouring microbial contamination be discarded in order to avoid health risks to laboratory workers and to assure the quality of the research. However, if the culture is irreplaceable, there may be a case for its maintenance in the laboratory under appropriate containment and isolation procedures prescribed to the infectious nature of the contaminating organism.

### 3.4. Characterisation and Stability Testing

Characterisation of each bank of cells will depend on the cell type and key phenotypic and genotypic markers for the particular stem cell line. *The master cell bank receives detailed characterisation with fundamental quality controls performed on working banks.* An international consensus, testing regime for master stocks of human stem cell lines has been established by the International Stem Cell Banking Initiative (7) and is shown in Table 1. Additional characterisation of the working cell bank and/or cultures passaged to anticipated maximum passage levels may be required to confirm the characteristics of the line (for example, assessing the chromosomal integrity of the line).

#### 3.4.1. Phenotypic Characterisation

Early work on characterisation of hPSCs by flow cytometry and immunocytochemistry, using fluorescent antibodies often raised against embryonal carcinoma cells, established that hPSCs expressed certain phenotypic markers including OCT-4, Nanog, SSEA-3, SSEA-4, TRA-1-81, TRA 160 and alkaline phosphatase, and unlike mouse embryonic stem cells, were shown to be negative for SSEA-1 (53–55). A recent international study to characterise a large number of hPSC lines from numerous laboratories around the world established the consistent expression of such markers in hPSC cultures and provided a consensus for the characterisation of hPSCs including profiles of both surface markers and RNA expression (3). A selection of such antibody markers should be used to characterise banks of human stem cell lines. It is important to remember that these markers identify an undifferentiated state in the culture; if differentiation occurs, the presence of certain antigens is altered, with decreased expression of OCT-4 (which is associated with the ability of a hPSC cell to self-renew) and increased expression of SSEA-1. Those who have

**Table 1**
**Outline of a typical testing regime for master cell bank of a human embryonic stem cell line**

| Test specification | Examples of test methods |
|---|---|
| Identity matches parent cell line | Short tandem repeat (STR) DNA profile<br>Human leukocyte antigen (HLA) genotype |
| Bacteria/fungi | Inoculation of microbiological culture media to detect growth of bacteria and fungi |
| Mycoplasma | Direct culture in broth and agar and indirect test using indicator culture/DNA stain |
| Karyotype | Giemsa-band analysis<br>Fluorescent in situ hybridization |
| Recovery | Post-thaw, trypan blue dye-exclusion<br>Viable colonies recovered (qualified efficiency of recovery of each bank/lot should be given) |
| Pluripotency | Formation of teratomas in immune-deficient (SCID) mice<br>Formation of embryoid bodies, in vitro "directed" differentiation |
| Growth characteristics | Doubling time<br>Growth rate |
| Cell antigen expression | Flow cytometry or immunocytochemistry for a range of hPSC markers |
| Cell gene expression | Gene-expression profiling using DNA microarray or Q-PCR analysis |
| Genetic stability | Single nucleotide polymorphisms. Comparative genome hybridization by DNA microarray methodology (see also karyotype) |

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worked with hPSC cultures will recognize that hPSC cultures often contain a population of differentiated cells that will vary to some degree from one time-point in culture to another and that it is not feasible or realistic to set absolute, stringent criteria for the expression of such markers.

*3.4.2. Pluripotency*

Key to the scientific and therapeutic potentials of human stem cell lines is their capacity to generate cells representative of all three human germ layers (endoderm, ectoderm and mesoderm), and thus, their potential to generate all the tissues of the human body – pluripotency. This is clearly a critical characteristic for cell banks to address; any human "stem cell line" claimed to be pluripotent must demonstrate this characteristic using accepted techniques. The current "gold-standard" technique is the formation of teratomas from hPSCs injected into immune-compromised

(typically SCID) mice (see Chapter 17). The teratomas should reveal cells and tissue-like structures representative of all three germ layers. Other options to determine the pluripotency of hPSCs include the preparation of embryoid bodies showing evidence of markers from all three germ layers, and in vitro, directed differentiation into cell populations associated with each germ layer. It is important to remember that all of the described experimental procedures provide evidence for pluripotency but do not categorically confirm the ability of stem cells to generate all the cells and tissues of the human body. Even for the standard teratoma method, it is known that mouse strain and age and the method of preparation of the inoculated cells can influence the outcome of the assay. The lack of a robust, qualified laboratory assay for pluripotency is a significant challenge to researchers and stem cell banks alike. Establishing robust standardized assays that can be used for characterising cell banks remains a fundamental goal in regenerative medicine.

#### *3.4.3. Genetic Characterisation*

Another key requirement for hPSCs is a diploid karyotype (46:XY for male and 46:XX for female). In vitro culture, however, appears to promote the generation of clones of genetically abnormal cells that can take over a culture and rapidly replace the original diploid cells. Banks of cells should therefore routinely be assessed for karyotype. The guidance published by the International Stem Cell Banking Initiative gives recommended approaches for such karyological studies of hPSC lines (7). A number of other karyotyping methods are currently being used in clinical and research work but remain to be qualified for routine quality control of banks of human stem cell lines.

#### *3.4.4. Stability Testing via Extended Culture*

A desirable adjunct to the cell banking process is to passage cells beyond their anticipated limit of use in order to further evaluate the stability of the line. For hPSCs, this may include how well they maintain the undifferentiated phenotype, the consistency of the undifferentiated growth when compared with cells from the master bank and the confirmation of pluripotency. Ideally, this will involve establishing "extended cell banks" at intervals of around ten passages and comparing the characteristics of these banks to determine what, if any, drift has occurred in the culture over time. However, this would involve considerable time and effort, which would have to be balanced against the benefits. It might be better for banks to recommend that recipients of their lines should periodically check their cultures for chromosomal integrity.

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