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Laboratory Training Guidelines for Clinicians Undertaking Stem Cell Therapy

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Abstract

Stem cell-based regenerative therapies are an exciting and emerging field in medicine and dentistry. Since the 1960s, stem cell therapies have been successfully performed and approved in the form of bone marrow transplants and more recently, in skin and corneal grafting. However, the field of regenerative cell therapy has somewhat come to a halt due to safety, ethical and legal concerns associated with medical and scientific practices. In Australia, stem cell therapies, besides those mentioned above, are permitted provided that they are autologous in nature and that the procedure is performed by or under the supervision of a registered medical practitioner in a single treatment. However, the medical practitioner requires no formal stem cell training. We have identified this deficiency in optimal patient care and propose that education in the safe, legal and ethical delivery of stem cell treatments be part of a mandatory training course for medical/dental practitioners and the scientific team prior to the clinical use of stem cell therapies in Australia, and arguably, on a global scale. This involves the scientific and medical team having a sound understanding of the biology of stem cells, their appropriate applications and basic validation and laboratory techniques in order to provide effective and safe treatment for improved patient outcomes. As such, this article aims to propose a frame-work of scientific guidelines for dental and medical practitioners to undertake stem cell therapies.

Keywords: Adipose-derived stem cells; Mesenchymal stem cells; Regenerative medicine; Stem cells

Introduction

Stem cell therapy is becoming more frequent in many countries. Several issues regarding their use attracts both support and criticism from the various stakeholders, namely the public, treating clinicians, scientists, government agencies and commercial organisations. It is imperative that science establishes, at the minimum, validated methods that are reproducible to provide a framework for safety and efficacy. Once scientists have developed and published methods, it is again imperative that the treating clinician has a sufficient knowledge of the scientific terms, equipment and interdisciplinary approaches (Figure 1) used for stem cell treatments. Most clinicians have basic core university teaching in the fields of biology, chemistry and their interdisciplinary areas. However, specific awareness and understanding of stem cells is very limited for doctors, dentists and allied health practitioners.

The authors have collaborated in clinical stem cell treatments with an emphasis on peer reviewed evidence-based scientific methods and clinical outcomes to establish patient inclusion/exclusion criteria. RS is a developmental cell biologist and has completed a doctorate in embryonic stem cells methodologies. ERV is an oral and maxillofacial surgeon who completed training in embryonic stem cell lines and subsequently published the first clinical stem cell study demonstrating safety and efficacy of autologous mesenchymal stem cells in humans when injected into the head, face and oral cavity to treat neuropathic pain. NB is a counsellor with a special focus on psychological issues of stress, anxiety and depression and how it impacts patients undergoing stem cell treatment. Taken together, the authors have a combined 20+ years of experience in laboratory cell biology, stem cell culture techniques, and clinical and psychological assessments. In addition, the combined experience of the authors when they have presented lectures for university students, postgraduate medical and dental clinicians, and the public has led to many frequent questions on topics such as safety, quality assurance and laboratory methods. Government agencies, insurance and professional indemnity companies rely on evidence to make monetary decisions that largely permit, or do not permit, use of stem cells. The purpose of this article is to provide the scientific framework of knowledge for clinicians to fulfil these obligations in the therapeutic use of stem cells.

Types of Stem Cells

A stem cell, by definition, is a cell that is capable of self-renewal over multiple cell divisions, maintains a normal karyotype and retains the capacity to differentiate into a variety of cell types, depending on the stem cell's level of potency. Three main classes of stem cells exist: Embryonic, Adult and Induced Pluripotent stem cells, each of which serves important laboratory and/or clinical purposes.

Embryonic stem cells

Embryonic stem cells (ESCs) are those that are derived from the blastocyst-stage embryo. The blastocyst contains a population of cells known as the inner cell mass (ICM) that will eventually give rise to the >200 specialised cell types that make up the foetus and adult (Figure 2). These ICM cells can be isolated from the blastocyst and when cultured *in vitro* can be maintained as pluripotent ESCs. When the culture conditions are such that ESCs are permitted to differentiate, they will do so spontaneously and randomly into cells that constitute the three

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definitive germ layers (ectoderm, mesoderm and endoderm) of the embryo proper (Figure 2).

Pluripotency is a defining feature of ESCs: The gold standard for establishing pluripotency in non-human ESCs is tetraploid complementation. Here, diploid ESCs integrate into a tetraploid embryo to produce offspring that are exclusively produced from the ESC population, and which can contribute to germline transmission. Pluripotency can also be confirmed by production of viable chimeric offspring (offspring that are derived from two separately fertilized eggs) when ESCs are injected directly into the ICM of diploid blastocysts [1,2]. These methods are not ethical options to assess human ESC pluripotency, rather, teratoma formation of human ESCs (in immunodeficient mice) and spontaneous differentiation into cells that constitute the three germ layers is used, as well as human-mouse interspecies chimera formation [3].

An important characteristic of ESCs is that they can be directed to differentiate by providing relevant growth factors, cytokines and small molecules that are known to drive the differentiation of specific cell types. These properties of ESCs make them a desirable candidate population for studying the molecular mechanisms i.e. the science underlying cell fate.

ESCs were first successfully isolated and maintained in culture in 1981 from the mouse [4,5], and this was followed by the first successful isolation and maintenance of human ESCs in 1998 [6]. ESC research has since then leads to important advances in our understanding of normal and abnormal cell physiology as well as providing a platform for studying human drug design and screening. However, as ESCs are of embryonic origin, their use as cell-based therapies has been challenged by legal, ethical, biosafety and religious implications. Other sources of stem cells from patients themselves (known as autologous cell therapy), including adult and induced pluripotent stem cells are now at the forefront of modern stem cell-based regenerative therapies [7-9].

Adult stem cells: A focus on MSCs

Adult stem cells are those which are derived from developed organs and are named usually by their tissue of origin, for example, neural stem cells. These cells are found endogenously in specialised 'stem cell niches' within the foetal and adult body and serve to repair and rejuvenate damaged and/or aging tissue. Unlike ESCs, adult stem cells are multipotent which means that they can differentiate into multiple cell types but are conventionally restricted to the cell types within their lineage of developmental origin (Figure 2). These stem cells are currently at the forefront of autologous regenerative cell therapies, with mesenchymal stem cells (MSCs) currently being the most widely investigated stem cell type for such therapies.

MSCs are found throughout the body (including but not limited to bone marrow, adipose tissue, dental tissue, salivary glands and peripheral blood) [10-13], making them a relatively easy-to-obtain source of stem cells. MSCs have been shown to differentiate into a range of mesoderm-derived tissues including bone, muscle and adipose tissue, as well as transdifferentiate into ectoderm- and endoderm-derived cell types [11,14,15]. Importantly, MSCs secrete immunomodulatory factors such as cytokines and growth factors [16,17] that have been shown to aid in the immune response. Evidence also suggests that these factors may modulate the body's own stem cells to differentiate and repair damaged tissue [18,19]. Importantly, MSCs express cluster of differentiation (CD) molecules CD73, CD90 and CD105, but lack haematopoietic markers CD45 and CD39 [16], and express low levels of Major Histocompatibility Complex (MHC) I and MHC II. These properties prevent MSCs from inducing an immune response making

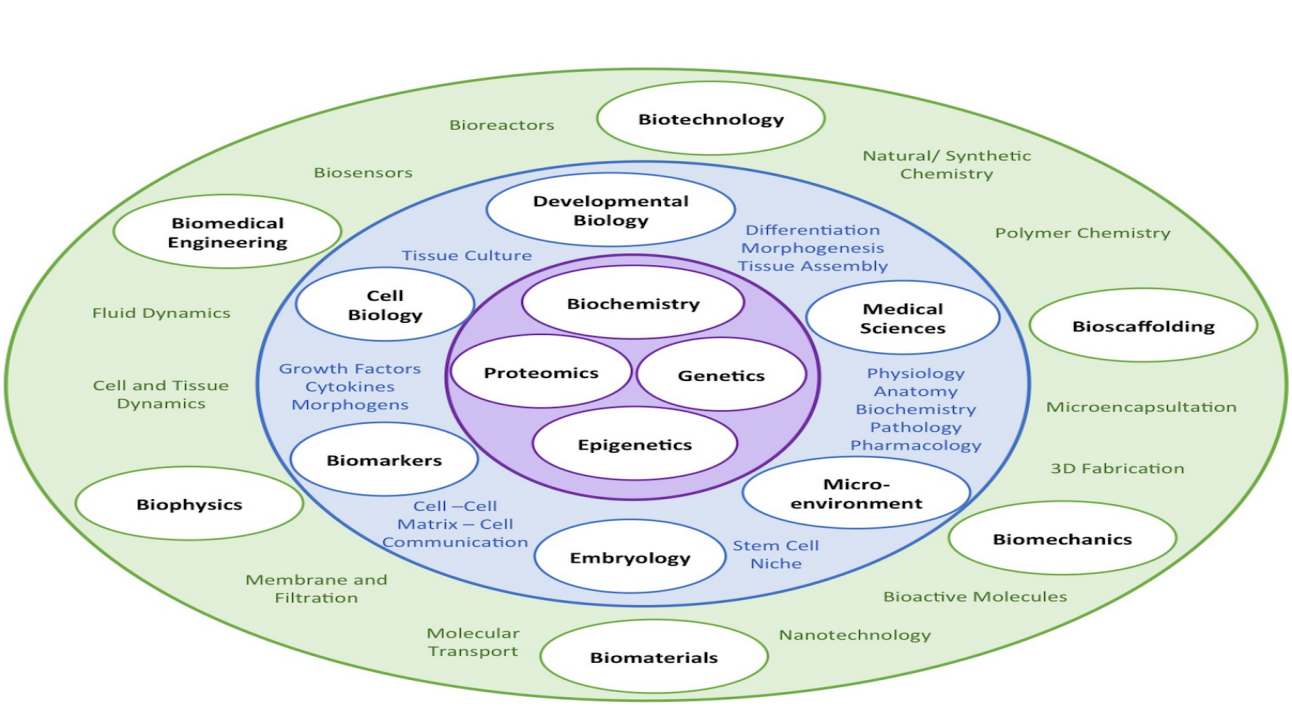


Figure 1: Interdisciplinary approach to stem cell and regenerative therapy.

them an attractive source of stem cells for autologous and allogeneic (cells from a foreign donor) cell therapy.

Fat tissue contains a rich supply of MSCs, known as adipose-derived stem cells, commonly obtained from patients through lipoaspiration. The stromal vascular fraction (SVF) within the lipoaspoaspirate contains a heterogeneous population of cells including red blood cells, endoethelial cells, leukocytes and MSCs. The SVF can be obtained through two primary methods: enzymatic isolation [20] or mechanical isolation [21]. Enzymatic isolation incorporates the use of collagenase to break down the lipoaspirate, producing two distinct phases: An adipose phase on the top and an aqueous phase (containing the cellular portion of interest) on the bottom (Figure 3). Centrifugation followed by filtration and lysing of red blood cells yields a population of pure SVF cells.

However, in order to be used for human therapy, GMP-grade human collagenase must be used, and this is an expensive component. The mechanical isolation process has thus been developed as an alternative method to extract SVF. This process relies on the emulsification of the lipoaspirate in order to mechanically agitate the tissue and release adipose-derived MSCs from adipose stores. This method not only eliminates the need for enzymatic digestion (thus reducing the time and cost associated with obtaining SVF rich in stem cells), but it can be done in a closed system (using a series of sterilized separators and syringe tubing) straight from the patient, eliminating the possibility of cross-contamination as well as the need for cells to be exposed to an artificial culture environment.

Induced pluripotent stem cells

The discovery of induced pluripotent stem cells (iPSCs) has been an important breakthrough in the field of stem cell and regenerative medicine. Once a cell is said to be terminally differentiated, it means that the cell has reached its differentiation capacity. However, in 2006, this phenomenon was challenged by a team lead by Shinya Yamanaka, who questioned whether a terminally differentiated cell could be reprogrammed to a pluripotent embryonic stem cell-like state. The team used retrovirus technology to deliver 4 genes known to be important in mouse ESCs maintenance (Oct4, Sox2, c-Myc and Klf4, collectively known as Yamanaka Factors) to mature mouse fibroblasts [22]. This resulted in reversion of the fibroblasts to cells which displayed ESC marker expression, pluripotency, and growth and morphology properties like ESCs. These cells were termed induced pluripotent stem cells (iPSCs). In 2007, iPSCs were developed using the same methods in human cells [22,23]. It was this important discovery that has changed the face of personalised regenerative medicine, and which won Yamanaka and colleague Gurdon the 2012 Nobel Prize for Physiology or Medicine. Scientists are now working on viral-free reprogramming methodologies to deliver these factors in a safer, more efficient way for potential use as a clinical therapy. Besides their potential clinical application, iPSCs have been a very useful tool for studying the development and progression of (rare) genetic diseases as well as a useful platform for human drug design and screening.

Another breakthrough technology in the field of personalised regenerative medicine is that of the CRISPR-Cas9 system for genome

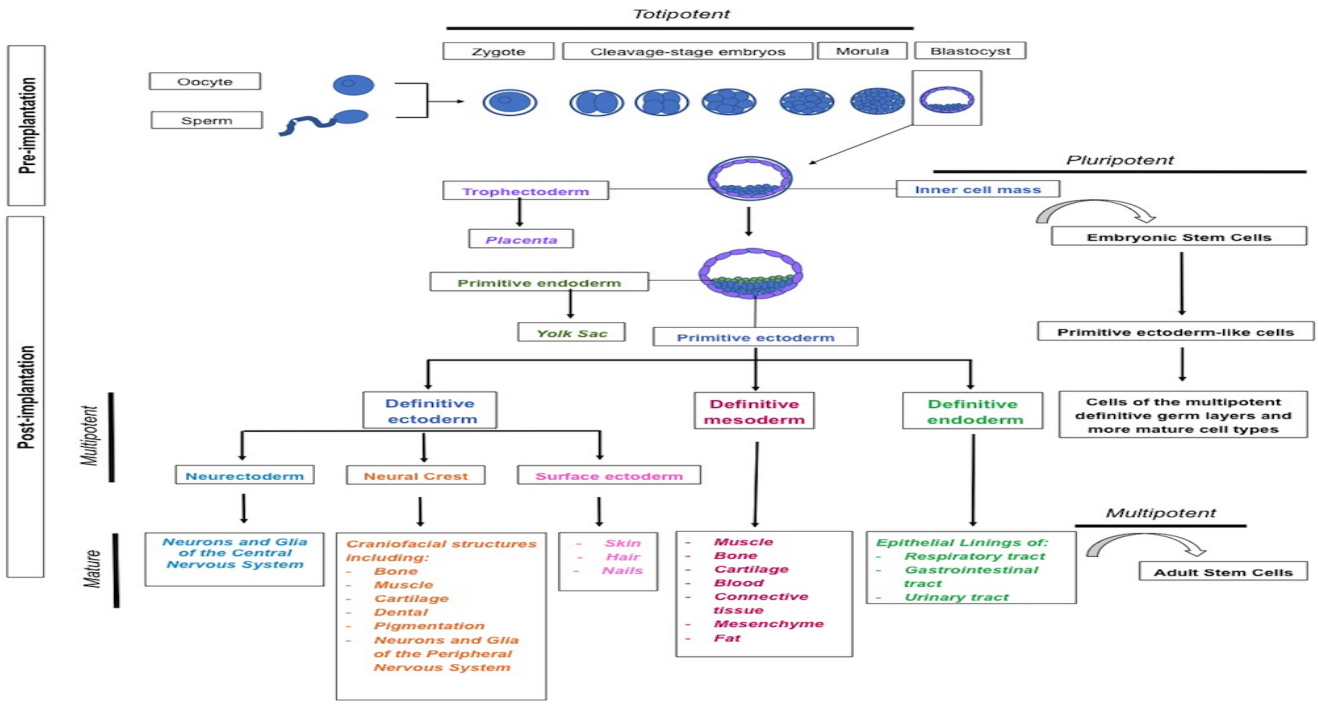


Figure 2: Mammalian embryonic development and derivation of embryonic and adult stem cells. Mammalian development begins with fertilisation of the oocyte by the sperm to produce a single totipotent embryo known as the zygote. The zygote subsequently undergoes a series of cell divisions, eventually forming the blastocyst. The blastocyst is composed of two cell populations: An outer multipotent trophectoderm layer that goes on to form the placenta, and an inner pluripotent inner cell mass population that further differentiates into either the primitive endoderm (which forms extraembryonic structures such as the yolk sac) or the pluripotent primitive ectoderm. Following implantation, the primitive ectoderm undergoes gastrulation to form the three multipotent definitive germ layers: Endoderm, mesoderm and ectoderm, which subsequently differentiate into progenitors and mature cells that comprise the adult and foetal body. Pluripotent embryonic stem cells can be derived from the inner cell mass population of the blastocyst, whilst multipotent adult stem cells can be derived from developing or mature organs of the foetal and adult body.

editing [24]. Briefly, a small RNA strand guides the DNA cutting enzyme Cas9 to a specific site of interest on the DNA. Here, Cas9 produces a double stranded break that disrupts gene function at the specific location at which the DNA was cut at. The DNA then undergoes repair in one of two ways: By

i) Re-joining the cut ends (which now have a deleted portion due to the enzymatic break in the sequence) or

ii) If a new DNA sequence is delivered, the cell will use this sequence to join the strands back together.

Together with iPSC technology, CRISPR-Cas9 technology has implications for personalised regenerative medicine, as scientists now have the ability to

i) Ethically obtain pluripotent cells from a patient and

ii) Genetically modify the cells to remove problematic, or add clinically-useful, genes before introduction back into the patient. This area of research is still very much in its experimental stages, with pre-clinical and clinical studies underway [25,26]. When the combined technologies have been developed with validated safety and efficacy it is expected this will lead to a quantum shift in human medical treatments and veterinary applications including species protection.

Stem Cell Maintenance and Differentiation

Stem cells are characterised by their ability to self-renew over extended periods of time in culture but are poised for differentiation when conditions are favourable. As such, the culture conditions in which the cells are grown in are vital for producing the appropriate cell population, be it stem cell maintenance or differentiation. General maintenance requires cells to be anchored onto the tissue culture plate allowing cells to attach and proliferate over time (Figure 4). As to prevent over-confluency and cell toxicity, cells must be sub-cultured to remove waste product from the medium, preventing a drop in pH, and to replenish nutrient supply to maintain optimal proliferation and growth (Figure 4). When ~70% confluence is reached, i.e., 70% of the surface of the plate contains viable cells, cells are dislodged (usually by enzymatic cleavage of surface proteins) and re-seeded on a newly prepared plate for continuous maintenance or for differentiation experiments.

As like any living cell, stem cells require basic salts, vitamins, amino acids, energy sources, growth factors and other proteins to remain viable. Dulbecco's Modified Eagle's Medium (DMEM) is a standard osmotically-balanced formulation of the minimal requirements for mammalian (including MSC and ESC) cell culture. When supplemented with 10-20% serum (often obtained from foetal bovine serum (FBS)) or serum replacer (SR), it contains all the necessary micro and macro-molecules and growth factors required to keep cells alive (known as basal medium). To this, a cocktail of growth factors, cytokines, small biologically-active and/or pharmacological molecules can be added to promote maintenance or directed differentiation of stem cells. For example, human ESCs maintain pluripotency and self-renewal when cultured in basal medium in the presence of bFGF and Nodal/Activin [27,28], however they will differentiate in the presence of BMP4. Similarly, MSCs can be maintained as multipotent MSCs (for up to 50 passages) in the presence of bFGF [29,30].

Unlike ESCs, MSCs do reach senescence when cultured *in vitro* for extended periods of time (due in part to decreased telomere length [31] as well as an increased probability of spontaneous malignant transformation) [32]. Thus, minimal culturing is recommended if MSCs are to be used for cellular therapies. Another limitation to culturing MSCs is the use of animal sources for the serum component. Animal serum is thus far the most widely used source of important growth factors to keep MSCs alive and viable *in vitro*. However, its use raises several concerns for *in vivo* human cellular therapies as follows:

(i) Serum is poorly defined and varies from batch to batch;

(ii) There is a risk of pathogenic contamination;

(iii) Xenogenic proteins (proteins from another species) can induce an immune response in the host;

(iv) Serum is expensive and there is limited availability.

Ideally, autologous human serum would be the most appropriate source of growth factors to maintain human MSC viability.

Autologous and allogeneic human serum has been shown to support the growth of MSCs, as has serum obtained from umbilical cord blood and placenta. However, large volumes are required to produce numbers of cells that are clinically relevant. Use of allogeneic sources also runs the risk of introducing pathogens into the host that may not be detected in standard blood screening. Human platelet lysate has also been shown to contain factors important for maintenance of MSCs, however some studies have shown that whilst cells remain

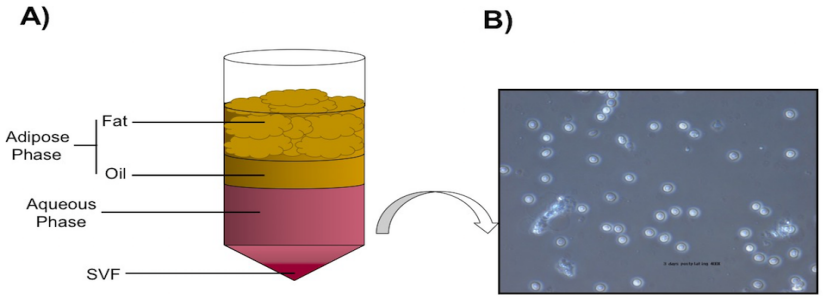


Figure 3: Lipoaspirate containing an adipose phase and aqueous phase following enzymatic and mechanical isolation of SVF. (A) Following manual separation of the stromal vascular fraction (SVF) from lipoaspirate samples, two distinct phases are formed in the sample. Briefly, the lipoaspirate sample is emulsified by passing it through syringes between small diameter separators to break-up the fat and release the adipose-derived mesenchymal stem cells (MSCs). Following centrifugation, an adipose phase and an aqueous phase (containing the MSCs in the SVF) is produced. The SVF is used during the stem cell therapy procedure, as well as for the cell validity laboratory testing. (B) SVF from a patient was plated in phosphate buffered solution (PBS) for 3 days and imaged using light microscopy under a 40X objective (with 10X eyepiece=400X total magnification).

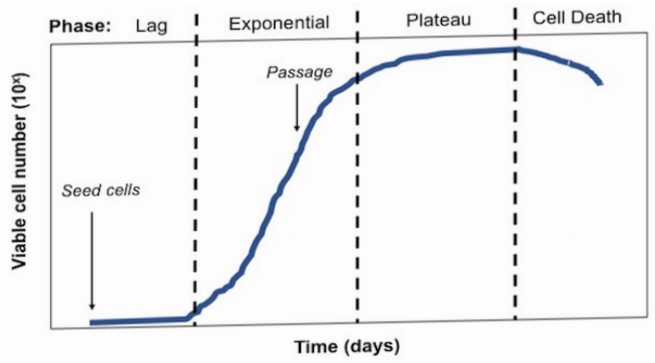


Figure 4: Typical growth curve for mammalian cell growth. When mammalian cells are cultured under conditions that promote proliferation, initially a lag phase in proliferation occurs as the cells adapt to the *in vitro* environment. Following the lag phase, cells begin to actively proliferate and the population number increases exponentially. This phase is considered the phase when cells are most viable, and therefore cell validity data should be collected during this phase. For continuous culture, cells should be re-plated (passaged) when they reach 70% confluence to maintain cell viability. If the cells are not passaged, they will continue to proliferate, such that they begin to cover the tissue culture plate, eventually resulting in 100% confluence. As competition for adhesive substrate and nutrient supply increases, proliferation begins to plateau, before decreasing: If the medium is not changed regularly, nutrients supplied in the growth medium can no longer support normal healthy growth and proliferation of cells. At the same time, metabolic by-products produced by the cells result in changes in the environment (e.g. decreases in pH), resulting in an unstable cellular environment, and thus cell death.

self-renewing and proliferative, they lose their multipotentiality and immunosuppressive capacity. Several FDA-approved, commercially-available serum-free media are also available, however, these can be expensive and their constituents are often undisclosed.

As mechanical isolation in a closed-loop system has been shown to be effective, safe [21] and meets the requirements of the TGA in regards to minimal culturing of the cells, this is the recommended method for human MSC isolation and preparation for autologous cell therapy.

Storage, Freezing and Thawing of MSCs

Live cells are commonly stored in a humidified incubator at 5% CO_2 concentration at 37°C, as to attempt to mimic the *in vivo* mammalian environment. If cells are not required immediately for experimentation, they can be cryopreserved in liquid nitrogen (-196°C) for many years to maintain viability. The most common freezing medium contains 10% cryoprotectant agent such as dimethyl sulfoxide (DMSO) in 90% FBS or growth medium (90% DMEM/10% FBS). Ideally, cells are frozen at a slow rate of 1°C/minute as to minimize cryo-injury in the form of crystal formation and osmotic damage to cells. Cells are then stored in liquid nitrogen until they are required, at which time they are thawed. The freeze-thaw process is a critical procedure that, if not done correctly, can result in apoptosis due to plasma membrane, mitochondrial and lysosomal damage [33]. There is also evidence to suggest that the freeze-thaw process may induce alterations in the cells' epigenetic landscape [34]. However, there is still much controversy over whether the freeze-thaw process reduces the immunomodulatory response, phenotype or metabolic activity of MSCs [35-38]. If autologous and allogeneic cell therapies are to become off-the-shelf therapies, more research is required to delineate the long-term optimal and therapeutic-grade cryopreservation conditions for MSC storage, freezing and thawing.

For short term storage, studies have shown that MSCs suspended in plasmalyte A (a solution which is physiologically similar to human plasma) remain 82-95% viable for up to 2 h when stored between 4°C and 22°C. This is particularly useful for situations where cells need to be transported for therapeutic use, such as to rural areas or between hospital or operating rooms.

Establishing a Cell Culture and Clinical Research-based Lab

A laboratory by definition is a facility where researches in the form of experimentation and measurement procedures take place. The requirements for a lab depend on the type of research being conducted. For the purposes of this article, we will focus on the minimal requirements to validate patient data for a research- and clinical-based wet lab.

Laboratory Safety

First and foremost, safety for the patient, clinician and scientist is of the utmost importance when working in a lab. Safe work practices should be in place for personnel working in the laboratory in order to reduce the risk of potential hazards (Table 1). As a minimum requirement, personal protective equipment (PPE) is required to be worn by scientific and clinical personnel in the form of gloves, gowns and non-porous, closed-in foot-ware. Additional PPE such as protective eye-ware may be required when handling certain substances such as liquid nitrogen. Appropriate training, in the form of standard operating procedures (SOPs), spill and waste protocols and equipment handling should be undertaken by all staff operating any equipment, chemicals and/or biological tissue.

Psychological management of personnel in a laboratory environment

Guidelines to achieve validation of laboratory results are dependent on the personnel. Due to the demand placed on personnel it is important for the laboratory manager or clinician to identify certain psychological changes in their staff if it occurs. Personnel working in a laboratory may have to demonstrate high levels of performance due to the nature of peer scientists and the request for urgent laboratory results by the treating clinician. This results in stress and potential mistakes.

Hazard Type	Potential Harm	Resolution
Physical	Manual handling Electrical Thermal Machinery Noise Radiation Lasers Sharps and needles	• Training • Personal Protective Equipment (PPE) • Lab design and machine location • Standard Operating Procedures (SOPs)
Chemical	Toxins Mutagens Carcinogens Flammables Corrosives Aerosols	• Training • PPE • SOPs in place • Material Safety Data Sheet (MSDS) accessibility • Appropriate chemical storage • Labelling • Appropriate ventilation: fume hood exhaust system set up if using hazardous chemicals
Biological	Microorganisms Contamination Animal and biological tissue Body fluids	• Training • PPE • SOPs in place • Meet the requirements of physical containment (PC) lab • Biosafety cabinet for tissue culture

Table 1: Classes of potential hazards associated with wet laboratory work.

The key psychological issues to be identified are stress, anxiety and depression. Stress can affect all aspects of the laboratory scientist such as their emotions, behaviour, thinking ability and physical health. Stress response varies from person to person and can affect the whole body. Chronic stress can cause depression with symptoms of a change in appetite, fatigue, emotional well-being, feeling of worthlessness, sleep problems, loss of motivation, feeling sad and withdrawing. Anxiety can result in increased heart rate, rapid breathing, and perspiration, panic attacks, tightening of the chest, tremor and shakes. If these symptoms are observed then the guideline is to seek advice from a professional counsellor or psychologist.

Minimal requirements for validating cell types

The key to effective and safe stem cell therapy is ensuring that the patient is delivered relevant and viable cells of interest. From the recommended liposuction procedure, the lipoaspirate product contains a heterogeneous mix of adipose, haematopoietic and mesenchymal cells of different subpopulations and maturities. Following manual processing, it is important to validate whether the SVF sample obtained contains (1) viable cells, (2) cells of interest (i.e. MSCs) and (3) cells that have maintained their potency and ability to differentiate. We therefore recommend mandatory validity tests for cell viability, marker expression, expansion and differentiation potential as a minimum requirement for stem cell clinical therapies. The following recommendations have been based off the International Society for Cellular Therapy's minimal criteria to define human MSCs [39].

Cell viability and count

Cell viability is critical for effective cell-based therapies, however currently the optimal cell number for effective therapy is unknown. The cheapest method to assess cell viability and number is using the Trypan Blue exclusion test on a haemocytometer (Figure 5). This method is based on the principle that living cells that maintain an intact cell membrane are unable to take up trypan blue and therefore, under a light microscope, remain transparent. In contrast, cells which take up trypan blue due to a damaged cell membrane appear blue under the light microscope: A percentage of clear: blue cells can be calculated to give an indication of the % of viable cells in culture. More advanced fluorescence-based methods of counting live verses dead cells and total cell number are available, such as Millipore's Muse®.

Flow Cytometry

Of equal critical importance is the type of cell being used in the treatment. For this measurement, cell characterisation in the form of phenotype, proliferative and differentiation capacity as well as marker expression is used. The gold standard for single cell marker expression analysis is flow cytometry. Flow cytometry is a laser-based technique which utilizes a cell's physical (size and granularity) and biochemical (cell surface expression) properties to identify cell type at the level of the single cell in real time. In a given sample, >10,000 cells per second can be assessed using this technique.

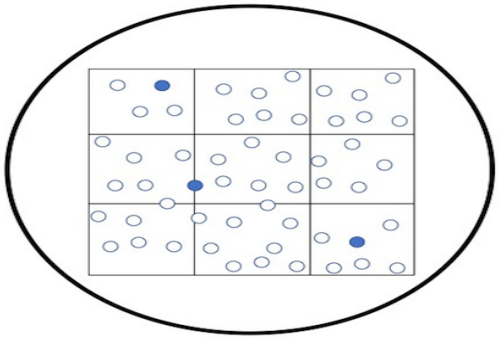
A flow cytometer is composed of three main parts: A fluidics, optical and electronic system. The fluidics system is responsible for the flow of single cells past a light source. As the cells flow past the light source, the light is scattered and detected by sensors that measure the forward scatter (indicative of cell size) and side scatter (indicative of cell granularity) (Figure 6). Based off this information, cell populations can be identified. Further characterisation of specific cell populations is done through fluorescence detection to determine whether a cell expresses a particular antigen marker. Cells are incubated with fluorophore-conjugated antibodies against the antigen of interest (for example, the MSC protein markers CD73, CD90 and CD105). Laser-excitation of the fluorophores causes a corresponding wavelength to be emitted and this is detected by sensors specific to each wavelength. As a result, cells can be separated based on their size, granularity and protein expression (Figure 6).

Fluorescence Microscopy

Another technique used to visualise protein expression is fluorescence microscopy which relies on similar principles of laser excitation-emission described above. Whilst flow cytometry is a quantitative measure used for identifying the percentage of cells that express a particular protein, fluorescence microscopy is used to qualitatively visualise the spatial location of the protein of interest. Basic fluorescence microscopes use either LED, Argon or Mercury lamps and excitation and emission lasers and filters, such that one fluorophore can be imaged at a time. A composite figure can be obtained by merging the individual channels together to produce a 2D image. More advanced microscopy in the form of confocal microscopy can be used to increase the optical resolution and contrast of images, to obtain finer detail of, for example, specific organelles. Confocal microscopy can also be used to create a 3D image of the cell through Z-stack imaging.

Biomarker Analysis

Biological markers are an emerging field of early intervention and preventive medical treatment. The major chronic disease states of cancer, arthritis, autoimmune, cardiovascular, respiratory and neurological deterioration commences with cellular changes. The nature of current sophisticated technology can now identify these changes before significant tissue and organ damage occurs with its clinical signs and symptoms. Two methods that have translated from the science laboratory to clinical diagnosis include multiplex assay systems and mass spectrometry. *In vitro* and *ex vivo* laboratory testing has provided the means to demonstrate these remarkable technologies



Cell count = *average cell number * dilution factor * chamber volume*

Cell viability = *(number clear cells / total cell number) * 100*

Figure 5: Manual cell counting using haemocytometer. Following a 1:5 dilution of trypan blue: single cell suspension, the sample is loaded into a haemocytometer and a cell count is performed under the light microscope. Typically, the average total number of cells is obtained by averaging how many cells lie in one third of the haemocytometer grid (i.e. the average of three squares). Cells that appear blue under the microscope are recorded as dead cells, whilst transparent cells are healthy cells. Total cell count, % viable cells and % dead cells can be calculated.

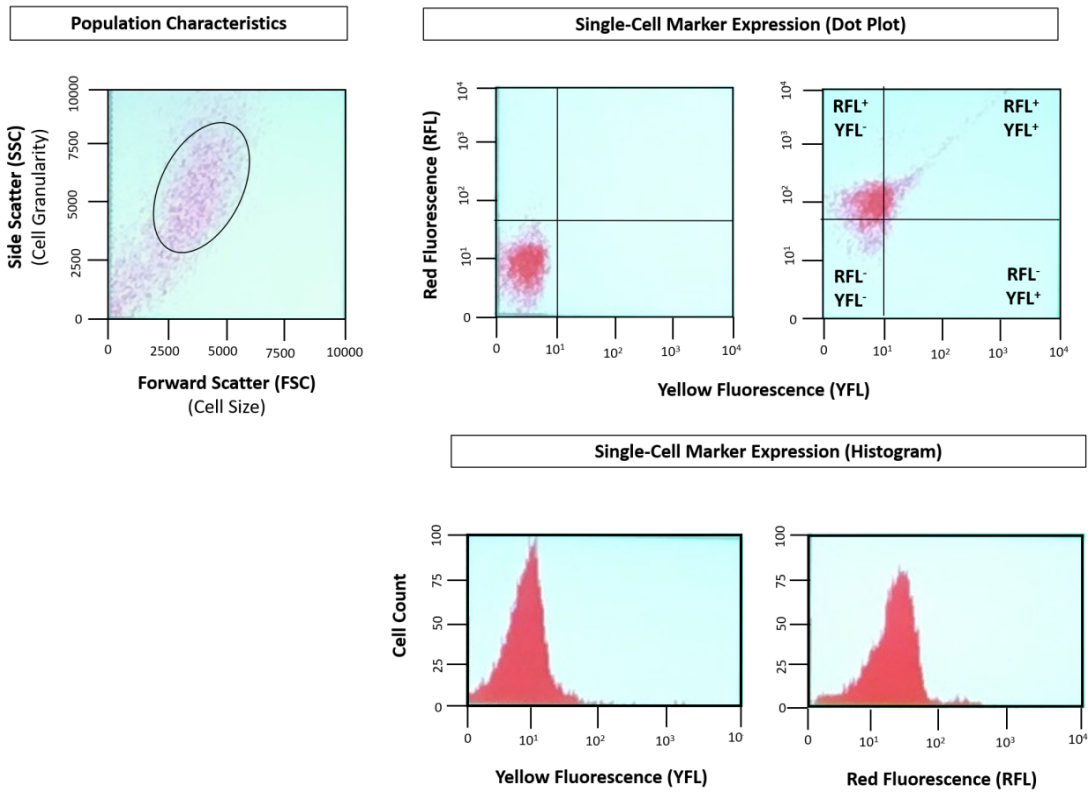


Figure 6: Typical flow cytometry plots for assessing single cells and cell population characteristics. Forward and side scatter are used to gate a population of interest based on size and granularity respectively. The gated population can then be assessed by co-expression or single expression of markers of interest using a fluorescence/antibody-based system. The total number of cells and their expression profile can then be quantified.

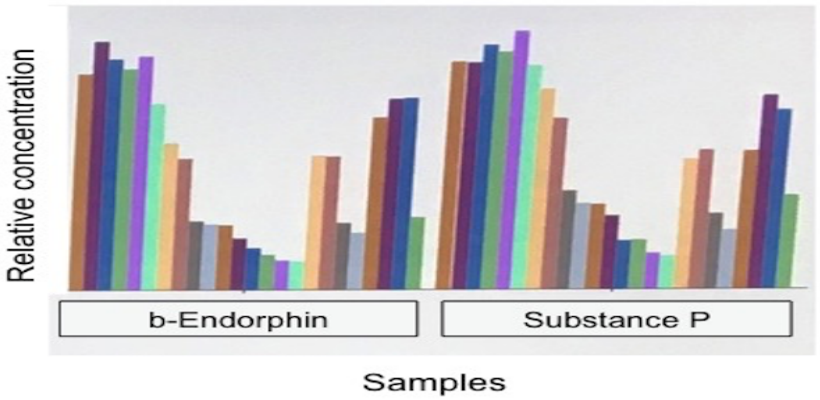


Figure 7: Example of multiplex assay data obtained from a patient's blood and saliva samples. Blood and saliva samples were taken from a male patient and assessed for the expression of β-endorphin and Substance P alongside a series of standards.

for the clinic. Biological samples required for biomarkers include blood, saliva, urine, sweat and breath analysis (Figure 7).

Multiplex assays

These assays use highly selective antibodies to the cell or molecule of interest. A fluorescent reporter binds to the antibody. The reporter has spectra where they can be detected by emission cut-off photosensors after narrow band laser excitation (or light emitting diode) as described above in fluorescence microscopy. Multiplex assays are suited for rapid screening of known biomarkers. An increasing number of known biomarkers for stem cell expansion and differentiation show this technology to be clinically useful (Table 2).

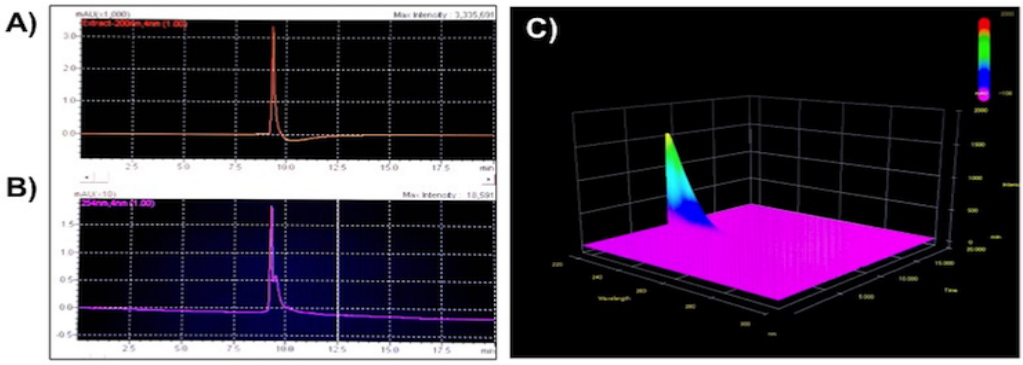


Figure 8: Chromatogram of HPLC gradient of a single compound termed palmitoylethanolamide (PEA). PEA has protective neurological effects and well-studied for is anti-inflammatory and antineuropathic pain actions. The chromatogram shows that it is a pure compound for clinical use and not contaminated with other chemicals. (A) UV absorbance at 200 nm and (B) at 254 nm. (C) These 2D traces can be obtained by a UV detector, or derived from a 3D photodiode array detector that scans from UV to visible wavelengths for more comprehensive analysis.

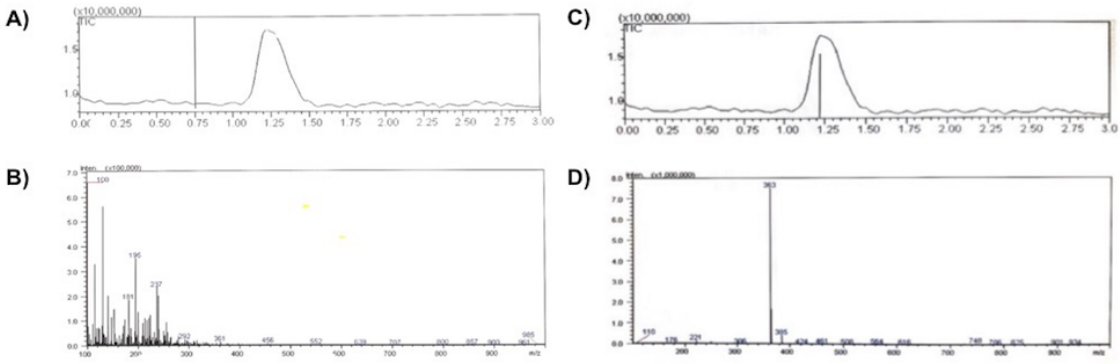


Figure 9: Mass spectrometric analysis of thyrotropin releasing hormone (TRH). TRH is a small molecular weight (MW 362.38 Daltons) tripeptide with an amino acid sequence pGlu- His-Pro-NH2 with the compound having multiple biological effects as an antidepressant, antisuicidal and anti-ageing peptide. (A) Infusion of pure TRH shows a peak at 1.00-1.25 min. and a scan line at 0.75 min to identify the baseline background spectrum of the solvent (0.1% acetic acid in MilliQ water). (B) Mass spectrogram of the background solvent, scan range 100- 1000. (C) Scan of the peak conducted at 1.25 min. (D) Mass spectrogram of the peak at 1.25 min showing absolute confirmation of peak identity and purity of TRH with a single mass /charge ratio (m/z) of 363 Daltons representing the MW of 362.38 Daltons+1 H+ (one proton from acetic acid).

Validation type	Validation technique
Viability Are the cells healthy and alive? Is the phenotype expected?	Trypan Blue exclusion assay Flow cytometry Cell counting assay (e.g. Merck Muse®) Microscopy
Cell type What type of cells am I working with? Do the cells express expected (marker) genes and proteins?	Flow cytometry Microscopy Gene expression assays such as qPCR
Expansion Do the cells undergo population doublings in vitro? Are the cell numbers expected given the initial seeding number?	Trypan Blue exclusion Flow cytometry Cell counting assay (e.g. Merck Muse®) Microscopy
Differentiation Are the cells capable of turning into bone, cartilage and fat [36]?	Flow cytometry Microscopy
Cytokine profiling Do the cells secrete factors the modulate the immune response?	Enzyme-Linked Immunosorbant Assay (ELISA) Multiplex assays (e.g. Merck Milliplex®)

Table 2: Minimal requirements for validating stem cell populations for use in therapy.

High pressure liquid chromatography and mass spectrometry (HPLC-MS)

High pressure liquid chromatography (HPLC) has been a fundamental method for separation chemistry for decades. The analyte of interest is injected onto a column with beads containing high carbon bonding molecules that allows concentration of the chemical forming a distinct peak that can be measured for its retention time against a reference standard and the peak area for its concentration (Figure 8). The technique also separates multiple chemical compounds of interest in biological fluids such as drugs, peptides and biomolecules. A high-pressure solvent typically containing a gradient of water with increasing methanol causes elution of the compound(s). Mass spectrometry (MS) is a method for identification of the molecular weight of compounds (Figure 9). When HPLC is combined with MS it offers a powerful tool for the concentration, separation and identification of drug and peptide pharmacodynamics and pharmacokinetics. Single and triple quadrupole HPLC-MS is particularly suited to screening studies of stem cell epigenetics.

Bioscaffolds and 3D Printing

Degradable biological scaffolds provide structural support for stem cells to grow into a functional tissue. The scaffold can have intrinsic mechanical tensile properties that guide stem cell behaviour and differentiation. Moreover, scaffolds can have peptides and growth factors incorporated into the structure for additional stem cell guidance [40,41]. Materials to construct the scaffold include alginate, a seawood derivative often used in dental impressions, and polyacrylic acid that is used in surgical sutures. Alginates can be mixed at room temperature for direct stem cell placement and its growth factors. Resorption of alginate can be days to weeks. An advantage of the polyacrylic and polylactic acid materials is their ability to be spun with selective mechanosensitive properties.

3D printers are becoming sophisticated in design and degree of accuracy. Very accurate representation can be achieved by CAD/CAM software allowing for anatomical restoration in medical and dental augmentation of bone defects to be achieved. 3D printers will augment the design and fabrication of bioscaffolds in a reproducible manner for medical devices, but also allow for personalized devices when coupled with CAD/CAM systems. Most 3D printers melt and extrude the filament materials at 180-290°C that is currently too high for stem cell growth factor integration in its manufacture. One further material of interest is polycaprolactone, an inexpensive material with the advantage of its low melting point at 60°C and excellent rheological and viscoelastic properties. Numerous structures obtained by this material are slow degrading implantable devices, nanoparticle carriers and porous scaffolds. Further development of polycaprolactone derivatives with lower melting points that do not damage cells or protein-like structures would overcome this limitation.

Conclusion

Stem cells have gained much attention over recent years for their unique potential to assist in the regeneration of aging, diseased and dysfunctional tissue. In order for stem cell therapies to be administered as approved treatments, scientists and medical practitioners must have a basic scientific understanding of what stem cells are and how they communicate with each other and their environment in order to provide patients with the safest and most effective treatment possible. Underlying most, if not all medical interventions is the laboratory testing that occurs behind the scenes to validate and ensure that a therapy gives a high level of confidence for the clinician and patient. Stem cell therapy should not be exempt from these expectations. Government and professional health organisations acknowledge there is raised public awareness for optimal patient care and prevention of treatment risks and complications. It is well recognised that preventive mechanisms for comprehensive care includes ongoing mandatory professional education and training when undertaking new technologies. Balancing public demands for quick access to stem cell therapy can be achieved with knowledge, training and continued research.

Countries are continuing to introduce new regulations for patient safety and proof of efficacy (For an overview of international regulations see) [42]. In Australia, for example, new regulations allow autologous stem cell therapy, provided that the cells are minimally manipulated, and the procedure is performed in a single treatment by or under the supervision of a registered medical or dental practitioner. As previously mentioned the purpose of this article is to provide a set of guidelines in order that clinicians from medical, dental, veterinary and allied health disciplines are familiar with laboratory equipment and techniques that are fundamental for patient safety and treatment efficacy. An advantage with establishing this framework of standard laboratory techniques, combined with clinical data measurements, is the development of an expanding database with laboratory and treatment variables (using de-identified information) that would be extremely valuable for research and improved outcomes on a global perspective.

To the authors' knowledge there are currently no national or international standards or regulations regarding the training for clinicians in stem cell therapy. This is a concern, given that stem cells are a unique cell type that, if used safely and the data is reported appropriately, have the potential to provide effective treatment for a number of conditions. Based on the information guidelines presented in this paper, the authors propose that stem cell training is a prerequisite for clinical treatments and institution of a research database of laboratory and treatment variables for improved outcomes.

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