Firdos Alam Khan *Editor*

Stem Cell Production

Processes, Practices and Regulations

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ISBN 978-981-16-7588-1 ISBN 978-981-16-7589-8 (eBook)
https://doi.org/10.1007/978-981-16-7589-8

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

The use of stem cells as regenerative medicine is one of the most sought-after technologies for cell-based therapy required by many patients who need cell transplantations. To be able to successfully use these stem cells in humans, these stem cells need to be produced by Good Manufacturing Practice (GMP), FDA and WHO recommended procedures and guidelines. The production of clinical grade stem cells needs GMP compliant facilities, protocols, production systems and quality checking mechanisms. Over the past few years, there has been tremendous increase in the demand of clinical grade stem cells and differentiated cells for cell therapy, and considering this aspect, there are not many books available that deal with the entire process of production of stem cells as per GMP guidelines. There is no single book available that comprehensively describes the entire process of stem cell production from raw materials to finished products. In this book, we have included all the topics that are important for stem cell production and provide a unique source of information for anyone who wants to learn and understand the entire process of production of stem cell-based products. This book will help stem cell therapy researchers, stem cell therapy professionals, and biotechnology companies to learn the entire process of stem cell production.

There are 11 chapters in the book which covers topics such as (1) stem cell therapy: significance and applications of stem cell products in tissue engineering and regenerative medicine, (2) Good Laboratory Practice (GLP) facility and production of stem cells: design, constructions and automation for clinical grade production of stem cells (facility, regulatory requirements and approvals process), (3) Current Good Manufacturing Practice (cGMP) facility and production of stem cells: design, constructions, automation and scalability for clinical grade stem cell productions (facility, regulatory requirements process), (4) Current Good Manufacturing Practice (cGMP) inspection, approval and certification: ISO certification, FDA inspections and cGMP certifications for stem cell productions, (5) stem cell bioreactors: design, structure and operation of stem cell bioreactors, (6) stem cell culture and expansion: role of culture, types of cells (stem cell, immune cell, primary cell), growth conditions (adherent vs. suspension), media nutrients, growth factors and growth phase cycle, (7) upscaling of clinical grade stem cell production: upstream processing (USP) and downstream processing (DSP) operations of cell expansion, harvesting, detachment, separation, washing and concentration steps, and regulatory

requirements, (8) characterizations of clinical grade stem cells: microscopic, cellular, molecular and functional characterizations of stem cells and their products for regulatory requirements and approval process (FDA), (9) stem cell safety and sterility testing: testing for stem cell sterility (microbial, viral or other contaminations) and stem cell product safety and regulatory requirements and approval process (FDA), (10) stem cells packaging, storage and transportation: formulations, packaging, transportation and storage of stem cell products and (11) stem cell clinical trials and stem cell market: current clinical trials (autologous and allogeneic transplantation), global market for stem cell products, and market value, cell therapy business and challenges of current stem cells production and business. Each chapter provides detailed and updated information on each topic supported with beautiful illustrations.

Dammam, Saudi Arabia Firdos Alam Khan

Acknowledgements

I am grateful to the Almighty Allah for the blessings and guidance. I am thankful to Springer Publication for showing trust in me and providing me one more opportunity to edit this exciting book on stem cells. I appreciate the support of Dr. Bhavik Sawhney, Editor-Biomedicine, Springer Nature. I am thankful to all the members of the production team of Springer Nature for their support and cooperation.

I am grateful to all the authors and corresponding authors for their immense contributions and cooperation. I want to thank the entire management team of the Institute for Research & Medical Consultations (IRMC), Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia, for their support, especially to Professor Ebtesam Al-Suhaimi, Dean, IRMC, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia, for her constant encouragement.

I am grateful to all my teachers and mentors, especially Professor Nishikant Subhedar and the Late Professor Obaid Siddiqi FRS, for their immense contributions to shaping my research career. I am also thankful to all my friends, well-wishers and colleagues for their support and cooperation.

I am grateful to my entire family members, especially to my father Late Nayeemuddin Khan and mother Late Sarwari Begum, my brothers (Aftab Alam Khan, Javed Alam Khan, Intekhab Alam Khan, Sarfaraz Alam Khan), my sisters (Late Sayeeda Khanum, Faheemida Khanum, Kahkashan Khanum, Ayesha Khanum), my wife Samina Khan, and my sons (Zuhayr Ahmad Khan, Zaid Ahmad Khan, and Zahid Ahmad Khan) and my daughter (Azraa Khan), my father-in-law (Abdul Qayyum Siddiqi) and mother-in-law (Uzma Siddiqi) for their support.

Enjoy reading!

Contents

About the Editor

Firdos Alam Khan is a Professor and Chairman at the Department of Stem Cell Biology, Institute for Research and Medical Consultations (IRMC), Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia. He has obtained his Ph.D. in Zoology with a specialization in Neuroscience from Nagpur University, India. Over the past 24 years, Professor Khan is involved in teaching various courses such as Cell Biology, Pharmacology, Business of Biotechnology, Biomedicine, Cell & Tissue Engineering, and Bioethics & IPR to undergraduate and postgraduate students. He was previously associated with the Manipal Academy of Higher Education, Dubai Campus, United Arab Emirates. He was a Professor and Chairperson, School of Life Sciences and served as Chairman of Research & Development Program. He has published more than 75 research articles in several peer-reviewed journals.

1 Stem Cell Therapy: Significance and Applications of Stem Cell Products in Tissue Engineering and Regenerative Medicine

Kholoud Al Ghamdi

Abstract

This chapter concentrated mainly on the use of stem cells and their products in tissue engineering and regenerative medicine. It showed the promising therapeutic role of stem cells and their products for many chronic debilitating diseases. Combining the suitable stem cells/products with the suitable biomaterial to engineer the therapy of choice for certain diseases that has no cure so far with no or minimum side effects is the ultimate goal of TERM. Researchers all over the world are working in their laboratories and producing results on daily basis regarding the latest in this multidisciplinary field that requires collaboration of basic scientists, physicians, biomedical and software engineers and members of the community to come up with a solution for most/if not all the diseases affecting humanity.

Keywords

Stem cell therapy · Applications of stem cell products · Tissue engineering · Regenerative medicine

1.1 Tissue Engineering and Regenerative Medicine (TERM)

Tissue engineering and regenerative medicine (TERM) refers to the attempt to create functional human tissues from cells in a laboratory. Its aims to regenerate and/or repair tissues that had failed due to disease, genetic errors, congenital abnormalities,

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F. A. Khan (ed.), *Stem Cell Production*,
https://doi.org/10.1007/978-981-16-7589-8_1

and traumatic injuries. There are four important factors that are essential for the success of tissue engineering: (1) suitable cells from different cell sources, (2) suitable environment (scaffolds) to support these cells, (3) suitable biomolecules to help these cells to grow and differentiate, and (4) certain mechanical and physical forces to influence the development of the cells. The cells can be harvested directly from the target organs, developed from precursors or stem cells, or taken from cell lines grown in the laboratories. Supporting structures can be derived from donors' tissues or from natural or synthesized polymers. Biomolecules can be added directly or secreted from the cells residing on the scaffolds. These scaffolds may dissolve over time, or some may remain to provide support to the organ. Mechanical and physical stimuli of the engineered tissues regulate their function (gene expression and consequently protein formation) and their eventual differentiation and development.

In order for us to understand the mechanism upon which TERM functions, we need to understand the physiology of cell growth and development. Cells are the building blocks for tissues and tissues make organs. A group of cells make their own extracellular matrix (ECM) where many interactions and cell signalling occur. Through understanding how cells respond to and interact with these signals to form tissues and organs, researchers can manipulate or interfere with these processes to fix damaged cells or even create new ones.

While tissue engineering is basically formation of tissues through a successful combination of cells, scaffolds, and biomolecules, regenerative medicine is a broader term where tissue engineering is combined with other strategies such as gene therapy, cellular therapy, and immunomodulation to induce tissue regeneration (Lysaght and Crager 2009; Lindroos et al. 2011; Salgado et al. 2013; Porada et al. 2016).

TERM uses basically three strategies for it to reach its goal of establishing a three-dimensional (3D) cellular complex that resembles functioning tissues and is able to repair or regenerate damaged ones. These three strategies are: (1) cell systems such as stem cell transplantation, (2) cell-seeded biomaterials to be implanted in the body to repair or regenerate damaged tissues, (3) biomaterials to be implanted in the body to undergo the process of tissue integration.

1.2 Biomaterial Scaffolds as Essential Factors for the Success of TERM

Work is still in progress to optimize the best suitable biomaterial scaffolds to be used in tissue engineering from stem cells. However, this field is promising and there are many studies that proved the importance of these scaffolds in the success of stem cell therapy (Willerth and Sakiyama-Elbert 2008). They can enhance the differentiation and survival of the stem cells seeded in them. Scaffolds ideally should provide both cell support as well as the cues (chemical, mechanical, and biological) that are needed for these cells to respond to the environmental stimuli. There are many factors affecting the selection of the suitable scaffolds. First, the source of the biomaterial that the scaffold is manufactured from. Polymer scaffolds (in the form

of hydrogels, fibrous meshes, or porous sponges) are the most used and are divided into synthetic and natural (Wong and Mooney 1997). Natural polymers, including polysaccharides and proteins, promote good cell adhesion and growth and are biocompatible compared to synthetic ones. However, synthetic polymers are much more thermally stable and possess better mechanical properties. Lately, it has been shown that composite scaffolds made of a combination of two or more different phases of biomaterials overcome the above disadvantages of both synthetic and natural polymers (Sionkowska and Płanecka 2013). Second, the scaffolds should have the suitable mechanical properties (porous) that allows the cells embedded in it to exchange vital molecules easily. On the same time, scaffolds should be strong/stiff enough to bare handling in vitro and in vivo and to mimic the structure of the extracellular matrix (Hutmacher 2000). Third, the scaffold should be biocompatible providing the optimal environment for cell growth, differentiation, and migration with the least undesirable effects (Williams 2008). Fourth, the method of fabrication of these biomaterials also plays an important role in the success of cell therapy and should be selected carefully based on the desired purpose (Yarlagadda et al. 2005). They can support stem cells even after they are being transplanted for a long period. These methods of fabrication include different patterns and networks such as hydrogels, microcarriers, and three-dimensional (3D) bio-printable constructs. In conclusion, ideal biomaterial scaffolds both support embedded cells' growth, differentiation and infiltration and closely mimic the extracellular matrix, allowing the embedded cells to interact with their native environment (Wang et al. 2018; Skop et al. 2014; Lim et al. 2019).

1.3 Stem Cells and Their Products as Essential Factors for the Success of TERM

Three main classes of stem cells can be used for TERM as well as progenitor cells and adult tissue-derived cells. Stem cells include organ resident, lineage specific stem cells, culture-adapted pluripotent stem cells, and multipotent cells capable of mesodermal differentiation known as mesenchymal stem cells (MSCs). Stem cells are a major source for cells in TERM because of their indefinite proliferation and differentiation potentials (Mahla 2016).

Among these stem cells, MSCs derived from bone marrow, adipose tissue, blood, and amniotic fluid are the most widely used in TERM (Fontaine et al. 2016; Schäfer et al. 2016; Bertheuil et al. 2019). It has been reported that MSCs have been used in almost 100,000 patients and more than 1000 clinical trials (see ClinicalTrials.gov) for many diverse conditions such as autoimmune diseases, musculoskeletal defects, and myocardial infarctions.

Induced pluripotent stem cells (iPSCs) are adult somatic stem cells that underwent genetic reprogramming to embryonic stem cell-like state. This process was first successful by Japanese scientists (Takahashi and Yamanaka 2006). These embryonic stem cell-like cells have the capability to self-renew and differentiate and the privilege of being ethically acceptable. Initially iPSCs were genetically

reprogrammed from multipotent adult stem cells by retrovirally introducing four key transcription factors (Oct3/4, Sox2, Klf4, and c-Myc). Nowadays, more safe methods to introduce these factors to somatic cells have been developed and iPSCs are considered one of the major cell sources in TERM (Omole and Fakoya 2018; Malik and Rao 2013).

MSCs are of particular interest in TERM. In addition to their anti-inflammatory and immunomodulatory characteristics mentioned above, they exhibit many other features that make them superior to other stem cells when it comes to cell transplantation (Nasef et al. 2008; Williams et al. 2011; Bernardo and Fibbe 2013). First, they can differentiate multilinearly to many cells such as adipocytes, hepatocytes, osteoblasts, neuronal cells, chondrocytes, and myoblasts (Han et al. 2019). In addition to their ability to differentiate, they can be directly isolated from almost all adult and perinatal tissues as well as from solid organs (Kern et al. 2006; Pittenger et al. 1999; Marquez-Curtis et al. 2015; Ullah et al. 2015). A third important feature of MSCs is that they are less immunogenic compared to iPSCs and are rarely rejected by the host after transplantation since they do not express significant histocompatibility complexes and immune triggering molecules (Zhou et al. 2011). When transplanted, MSCs are also able to find and return to their original "niche/home" through their ability to respond to migratory stimuli from damaged areas (Karp and Leng Teo 2009; Andreas et al. 2014).

Historically, the first organ to be studied as a source of MSCs is the bone marrow and Arnold Caplan was the first researcher to grow MSCs from human tissues (Caplan 1991). Bone marrow-derived MSCs were cultured and differentiated to cells with osteogenic potentials (Haynesworth et al. 1992). Further studies demonstrated that the bone marrow was not the only source for MSCs, adipose tissue (Zuk et al. 2002; Rodriguez et al. 2005; Rodeheffer et al. 2008) and basically any vascularized tissue may have the potentail to be a provider for the multipotent MSCs (Romanov et al. 2003; Mansilla et al. 2006; Zheng et al. 2007; Crisan et al. 2008). Of the above sources for MSCs, adipose tissue is evidenced to be more favourable since it is easy accessible, abundant, and less painful to extract. Adipose-derived mesenchymal stem cells (ADSCs) can be expanded for long periods in cultures without losing their differentiation capacity indicating high proliferative ability (Gimble et al. 2007; Mazini et al. 2019). ADSCs have been used in many pre-clinical and clinical trials as potential source for repairing and replacing damaged neuronal and bone cells for example (Mizuno 2010; Ruetze and Richter 2014; Im et al. 2013).

Due to the popularity of MSCs as a mean of treatment of certain diseases and the robust number of studies regarding their efficacy, the International Society for Cell and Gene Therapy (ISCT), formerly known as the International Society for Cellular Therapy, ought to put a certain definition for these cells to be biologically identified as MSCs in the laboratory for research purposes (Dominici et al. 2006). The following are the minimal criteria for identifying MSCs: Being plastic adherent fibroblastic cells with the potential to differentiate to osteogenic, chondrogenic, and adipogenic cells. Expressing the cell surface markers CD73, CD90, and

CD105, and not expressing haematopoietic and endothelial antigens (CD14 or CD11b, CD19 or CD79α, CD34, CD45, HLA-DR).

Despite the popularity of MSCs and iPSCs in TERM, the exact mechanism for their cell-for-cell replacement is questionable. They recognized primarily as secretory and immunomodulatory agents (Sacchetti et al. 2007; Caplan 2017). It has been shown that their healing characteristics are mainly attributed to their paracrine action due to their ability to release extracellular vesicles (EVs) that play an important role in cell-to-cell communication and are directly involved in tissue regeneration (Barreca et al. 2020). Both MSCs and iPSCs exhibit the paracrine action through the release of EV. These vesicles act on neighbouring target cells by regulating their proliferation, viability, and survival. They also induce angiogenesis, downregulate proinflammatory cytokines, upregulate anti-inflammatory cytokines, and eventually reduce oxidative stress and further damage (Théry et al. 2018).

Extracellular vesicles are known to be released actively by almost all cell types in the body including stem cells, and they form an important mean for cell-to-cell communication via their secretory molecules along with cell adhesion molecules (CAMs) (Caby et al. 2005; Théry et al. 2006; Ratajczak et al. 2006; Turturici et al. 2014). They are classified based on their function and sizes to exosomes (<50 nm), membrane vesicles (50 nm–1 μm) and apoptotic bodies (>1 μm) (Colombo et al. 2014; György et al. 2011). EVs, based on their cell origin and internal and external stimuli, may contain proteins, nucleic acids, and bioactive lipids. They are coated by a lipid bilayer membrane that prevents them from being degraded by enzymes when released. However, many of these vesicles, when released, are broken down and some can reach their target cells and be engulfed through endocytosis, cell fusion or bound via ligand-receptor interaction after which they can exert their many actions on the target cells (Morel et al. 2004; Kupcova Skalnikova 2013; Candela et al. 2010).

1.4 Clinical Application of Stem Cells Therapy in TERM

1.4.1 Cardiovascular System

Stem cells and their products therapeutic potentials have been studied extensively in diseases of the cardiovascular system that are one of the main reasons for death worldwide. Shiba et al. (2012) showed that human ESCs-derived cardiomyocytes protected against arrhythmias and contracted synchronously with the guinea pig heart muscles. In addition, these ESCs-derived cardiomyocytes survived, matured, and enhanced the function of the infarcted heart in both mice (van Laake et al. 2007) and rats (Caspi et al. 2007; Laflamme et al. 2007).

iPSC is another source for cardiac cells. For example, both mouse (Ieda et al. 2010) and human (Fu et al. 2013; Wada et al. 2013) fibroblasts were reprogrammed into functioning induced cardiomyocytes-like cells (iCMs) in the presence of certain factors. Also, Menasché et al. (2008) injected autologous myoblasts along with coronary surgery in patients with ischemic cardiomyopathy. However, these cells

failed to improve the heart function and increased the number of early arrhythmias in the above patients. In another study from a homogenous sample conducted in one centre, Hagège et al. (2006) showed that skeletal myoblasts transplanted into a post myocardial infarction scar led to improvement of the heart function represented by increased left ventricular ejection fraction despite the arrhythmic risk that can be controlled through beta blockers.

Further, direct in vivo reprogramming of endogenous cardiac fibroblasts after injecting them with a reprogramming factor may convert them into iCMs internally. This shall decrease the tumorigenic risks of iPSCs and avoid cell transplantation in the future (Sadahiro et al. 2015).

Many studies lately showed that stem cells products released from extracellular vesicles (EVs) can overcome the pitfalls of cell-based therapy in cardiovascular diseases through inducing many cellular and molecular pathways (Pan et al. 2019; Arslan et al. 2013; Jiang et al. 2018). It has been shown that in some cases, MSCs products therapy is even of more effect compared to MSCs alone in reducing heart ischemia and enhancing cardiac repair (Lai et al. 2010; Shao et al. 2017). In addition, iPSC-EVs have demonstrated an important role in cardiovascular diseases protection (Feng et al. 2014). It has been shown in vitro that iPSCs products when infused to cardiomyocytes, can be protective against oxidative stress and can prevent cardiac cells apoptosis when transplanted in vivo (Wang et al. 2015). This is mainly attributed the iPSC-EVs miRNA content, precisely, CPCISX-9-derived EVs that have been shown to reduce fibrosis and promote angiogenesis in myocardial infarction (Xuan et al. 2019).

Scaffolds infused with stem cells and/or their products has been used in cardiovascular diseases lately. For example, iPSC-derived cardiomyocytes EVs were encapsulated in hydrogel patches. These engineered hydrogel patches slowly released their products after being implanted onto rats' hearts with myocardial infarction. Consequently, these rats showed improvement in their heart functions manifested both physiologically and anatomically (Liu et al. 2018). Furthermore, Wei et al. (2019) implanted heparinized electrospun polycaprolactone vascular grafts loaded with MSC-derived EVs into a segment of rat abdominal artery. These cell products loaded grafts were successful in enhancing the patency of the vessels in a rat model of hyperlipidemia by inhibiting thrombosis and calcifications.

1.4.2 Lungs

Repairing injured lung tissues by stem cells or their products has been an area of interest for many TERM scientists lately. It has been shown that MSCs derived from bone marrow, umbilical cord, and menstrual blood were effective in reducing fibrosis in many models of airway and lung injuries in rats and mice (Moodley et al. 2009; Kumamoto et al. 2009; Leblond et al. 2009; Xiang et al. 2017).

In addition to the direct effect of stem cell therapy on respiratory diseases, MSC-EVs have shown protective effects in various models of respiratory injuries. For example, MSC-EVs were successful in reducing hyperproliferating pathways in

the murine model of respiratory hypertension (Lee et al. 2012a, b, c). Also, Tang et al. (2017) showed that MSC-EVs, partially through their miRNA content, exerted an anti-inflammatory effect on a model of acute lung injury in mice. Potter et al. (2018) showed that although both MSCs and their products exert protective effects on the lungs, the mechanism of their molecular actions differs widely.

Extracellular vesicles from iPSC also showed important effects in lung diseases such as pulmonary fibrosis both in vitro and in vivo. iPSC-EVs containing antifibrotic hepatocyte growth factor increased the alveolar epithelial wound repair in vitro. It also attenuated fibrosis and collagen content in a model of acute lung injury in the rat after being administered intratracheally (Gazdhar et al. 2014).

1.4.3 Nerves

It is of urgent need to find cure to many chronic debilitating neurological disorders. TERM plays an important role in leading studies of such purpose (Lindvall and Kokaia 2006). Alzheimer's disease for example is a progressive neurological disorder characterized pathologically by deposition of amyloid beta plaques and neurofibrillary tangles made of tau proteins. Several studies have shown the effect of MSCs derived from bone marrow, umbilical cord, adipose tissue, or menstrual blood on improving both cognitive function and Alzheimer's disease pathology. These cells when injected intracerebrally in animal models of Alzheimer's disease, improved both functional (better spatial learning and memory) and neuropathological (fewer amyloid plaques and tau phosphorylation) aspects of the disease and this improvement is mostly attributed to their action on the microglial cells in the brain (Zhao et al. 2018; Lee and Landreth 2010; Lee et al. 2012a, b, c). Multiple sclerosis (MS) is another autoimmune neurological disorder characterized by relapses and remissions of its symptoms. Burt et al. (2009) reported that the use of autologous non-myeloablative haematopoietic stem cells in the relapsing remitting phase of MS was successful in reversing the neurological disability and sustained improvement. A third chronic disease of no cure so far is Parkinson's disease. It has been reported that after transplanting undifferentiated mouse ESCs into the striatum of rat model of Parkinson's disease, these cells differentiated into functioning dopaminergic neurons that restored the motor and behavioural functions in these rats (Bjorklund et al. 2002).

In addition to the cellular therapeutic effects of stem cells, their paracrine effects have been studied extensively lately in neurological diseases. Stem cell products are of particular importance in neurological disorders compared to stem cells themselves. MSC-EVs can cross the blood-brain barrier because of their lipid structure, hence they are able to reach to many targets in the brain. They are also able to last longer in the target tissues since they are less immunogenic and less detectable by the host immune system (Phinney and Pittenger 2017). Stroke is one of the most common neurological diseases that has low full recovery rates. In a rat model of stroke, Moon et al. (2019) compared rats treated with MSC-EVs to those treated with MSCs. MSC-EVs treated rats showed behavioural improvement. EVs were

concentrated in the target area and was less found in unwanted areas (lung and liver). EVs also contained the specific needed factors for angiogenesis and neurogenesis, concluding that MSC-EVs can be of superiority regarding safety and effectiveness when compared to cellular treatment for stroke.

Traumatic brain injury (TBI) is a common cause of morbidity and mortality worldwide, and there is an urgent need to develop treatments that prevent or decrease the progression of a secondary injury in TBI. It has been reported that MSC-EVs play an important role in reducing both apoptosis and cognitive impairment and promoting cortical vascular epithelial growth factor expression in rodents' models of TBI (Chuang et al. 2012; Kim et al. 2016). Furthermore, Patel et al. (2018) studied the effects of human adipose-derived stem cells (hASCs)-EVs on the prognosis of a rat model of TBI. EVs succeeded in upregulating anti-inflammatory molecules and downregulating proinflammatory molecules at the genomic level as well as in improving motor behaviours. Furthermore, Tsuji et al. (2019) group in Japan is currently preparing for their first human trial study involving the use of iPSC-derived neural precursor cells in the treatment of spinal cord injuries (SCIs). Also, the usefulness of MSC-EVs in peripheral nerve injury has been studied and a group of researchers found that these vesicles secreted from gingiva-derived MSCs promoted nerve growth and regeneration through partially repairing Schwann cells of injured mice sciatic nerves (Mao et al. 2019).

Genetically engineered scaffolds to treat neurological disorders especially SCIs gained attention lately. For example, a neurotrophin-3-containing chitosan-based scaffold, developed by Li et al. (2009), improved sensory and motor recovery in a rat model of SCI. This scaffold provided suitable environment to facilitate the activation of endogenous neural stem cells and decrease inflammation post injury (Duan et al. 2015). In another study of a rat model of SCI, Zeng et al. (2011) seeded 3D gelatin scaffolds with MSCs derived from bone marrow and transplanted them into a transected rat spinal cord. Few weeks later, the results showed that these scaffolds were biocompatible, and the seeded cells were able to reduce cavity formation, induce angiogenesis, and decrease inflammation. In another study, fibronectin secreted from MSC-derived neuron like cells engineered in gelatin sponge scaffolds succeeded at early stages in promoting neurite elongation of neural differentiating MSCs in vitro, concluding the potential future use of fibronectin in the treatment of SCIs (Zeng et al. 2016). In conclusion, the combination of scaffolds and stem cell products boasts their therapeutic effects in tackling debilitating neurological disorders.

1.4.4 Bone

Therapeutic combination of engineered biomaterials along with stem cell products to enhance bone growth and treat bone disorders has been experimented widely lately and gained much of attention. For example, a study by Xie et al. (2017) embedded bone marrow-derived MSC-EVs to decalcified bone matrix. These vesicles showed prior pro-angiogenic and pro-bone regeneration potentials in vitro. The modified EV

scaffolds were then evaluated in a bone formation model in mice, and they managed to enhance bone formation through promoting vascularization in the graft. Another study combined hADSC-EVs with poly lactic-co-glycolic acid scaffolds to produce a novel cell-free tissue engineered bone. The above engineered scaffolds enhanced bone restoration significantly in critical-sized calvarial defects in the mouse (Li et al. 2018). In addition, Kikuchi et al. (2018) showed that neural crest-like cells (NCLCs) derived from iPSCs were able to differentiate in vitro to dental MSCs that eventually gave rise to odontoblasts and dental pulp cells. These iPSC-NCLC-MSCs when transplanted into critical-sized calvarial defects in mice were able to differentiate into osteoblasts leading to bone regeneration without formation of any tumours. Ren et al. (2012) genetically modified hADSCs decreasing their expression of MHC I protein, hence decreasing their immunogenicity, and constructed tissue engineered bone that succeeded in repairing bone defects in pigs. This data shows promising results of the safe usage of genetically modified allogenic bone grafts as compared to autologous ones.

1.4.5 Cartilage

Repairing damaged cartilage is still not yet fully accomplished. However, engineered cartilage constructs containing suitable cells or cell products show promising therapeutic results. Park et al. (2017) reported the results of the first human clinical trial investigating the efficacy and safety of Cartistem (a composite of allogeneic human umbilical cord blood-derived MSCs and hyaluronic acid hydrogel) on osteoarthritic patients. Twelve weeks after the application of Cartistem to the lesion site, there were signs of cartilage repair seen arthroscopically. In addition, patient's pain scores on walking were decreased and no teratogenicity were observed 7 years after the trial. In addition, Li et al. (2017) seeded biodegradable scaffolds with bone marrow-derived MSCs forming engineered cartilage. This engineered cartilage was then wrapped with chondrocyte sheets and implanted into nude mice. It then showed typical cartilaginous features and succeeded in regenerating subcutaneous cartilage. Another study showed that after seeding ear-shaped biodegradable scaffold with both autologous microtia chondrocytes (25%) and bone marrow-derived MSCs (75%) and implanting the scaffold into a nude mouse, a human ear-shaped cartilage tissue with proper elasticity was constructed (Zhang et al. 2014a, b). Park et al. (2019) reported that in comparison to chondrogenic differentiated MSCs, undifferentiated MSCs were more effective in repairing critical-sized osteochondral defects in the rat femur, supporting the use of undifferentiated MSCs in cellular therapy for cartilage repair.

1.4.6 Tendon/Ligaments

Anterior cruciate ligaments tears and Achilles tendon defects are common sports injuries affecting many young age groups worldwide. Engineered tissues targeting

the repair of tendons and ligaments are under trials. A group of researchers succeeded in engineering functional ligaments analogues by assembling bone marrow-derived MSCs sheets on a knitted poly (L-lactide) scaffold (Ouyang et al. 2005) that can be used for the purpose of anterior cruciate ligaments repairs. In addition, Chen et al. (2009) reported that human ESCs-derived MSCs regenerated tendon tissues both in vitro and in vivo (patellar tendon regeneration model in rats) by secreting human foetal tendon-specific matrix and certain differentiation factors, indicating great potential for the use of ESCs in tendon injuries repairs. Another study reported enhanced tendon regeneration effects of tissue engineered construct composed of knitted silk-collagen sponge scaffold seeded with human ESCs-derived MSCs when exposed to appropriate mechanical stimulation, both in vitro and in vivo (Chen et al. 2010). In addition to the use of ESCs for tendon regeneration, Liu et al. (2017) reported that uniparental parthenogenetic stem cells can spontaneously differentiate to parthenogenetic MSCs that can be differentiated to functional tenocytes after their exposure to mechanical stretch. These tenocytes can be embedded on the surface of poly (lactic-co-glycolic) acid scaffolds and used for tissue engineered tendon regeneration.

1.4.7 Liver

Liver failure is a debilitating disease that decreases the patients' life expectancy if not treated by liver transplantation. Scientists are trying to find a cure for those who are not able to receive liver transplants possibly through tissue engineering. Chen et al. (2017) investigated the therapeutic effect of human menstrual blood-derived MSC-EVs on a mouse model of fulminant hepatic failure. It was reported that EVs (exosomes) expressed cytokines that markedly improved liver function and inhibited haptic cells apoptosis leading to overall hepatoprotective activity.

1.4.8 Urinary System

Diseases of the urinary system can affect a wide range of organs including kidneys, ureters, urinary bladder, and urethra. Cell therapy has been initiated in some urinary system organs disorders. For example, it has been reported that urine-derived stem cells, discovered by Bharadwaj et al. (2013), have proliferative and multipotent potentials and can be used as a cell source in urinary tract reconstruction (Zhang et al. 2014a). In addition, ADSCs have been used to improve voiding dysfunction in animal models of hyperactive or hypoactive urinary bladder of different aetiologies (Mousa et al. 2015). Also, Wu et al. (2019) reported that urine-derived stem cells exosomes played an important role in improving stress urinary incontinence in a rat model of the disease by enhancing both the activation of muscle stellate cells and the phosphorylation of extracellular-regulated protein kinases (ERK).

MSC-EVs showed a therapeutic effect subsiding acute and/or chronic kidney injury through various vesicles products that succeeded in reducing inflammation,

migration of macrophages, preventing cell death, promoting wound healing, and stimulating angiogenesis (Gatti et al. 2011; Bruno et al. 2017; Zou et al. 2016). Also, Lee et al. (2012a, b, c) reported that the intrarenal administration of iPSCs into a rat model of acute kidney injury, at a particular dose, attenuated tubular injury and improved renal function. Moreover, it has been recently shown the iPSC-EVs reduced cell death and inflammatory responses in an in vivo model of ischemia-reperfusion kidney injury. These vesicles also protected the mitochondria and reduced oxidative stress, suggesting that iPSCs can be a potential source of these protective vesicles in kidney disease (Collino et al. 2020).

1.4.9 Cornea

Corneal endothelium dysfunction is a leading cause of blindness worldwide and there is an urgent need to find a cure for this disease, especially with the scarce number of corneal donors and the high rejection rates. The major function of this single layered corneal endothelium is to prevent the corneal oedema. Recently, Yamashita et al. (2018) reported that human umbilical cord-derived MSCs were able to differentiate to corneal endothelial-like cells expressing major endothelial corneal markers. These corneal endothelial-like cells were able to maintain corneal thickness and transparency when transplanted into a rabbit model of keratopathy, suggesting the potential use of these cells in corneal endothelial disease. In addition, Wu et al. (2014) reconstructed highly proliferative tissue engineered lamellar cornea by embedding a combination of corneal epithelial cells and genetically modified ESCs into an acellular porcine corneal stroma and amniotic membrane. The transplantation of the engineered lamellar cornea into the rabbit accelerated wound healing and manifested epithelial barrier function, indicating that the above combination of cells could form a base for corneal tissue engineering.

1.4.10 Skin

Skin is exposed to many external environmental factors all the time. This makes it vulnerable to diseases. Stem cells and their products can be of therapeutic benefit in skin-related diseases. For example, Lataillade et al. (2007) combined surgical therapy with bone marrow-derived MSCs in a patient who was accidentally exposed to high dose of radiation. MSCs were infused at the site of necrotic ulcer/burn and successfully improved healing of the ulcer mainly through the secretion of anti-inflammatory mediators. In addition, it has been reported that bone marrow-derived MSCs, via their anti-inflammatory mediators' effects, reduced inflammation and promoted healing in a pig model of radiation-induced burn. They also promoted muscle growth and revascularization in severe cases where deeper muscular tissues were affected (Linard et al. 2018). Another important skin problem is UV light induced skin ageing (photoageing). Oh et al. (2018) studied the effect of iPSC-EVs on aged human dermal fibroblasts and showed that these vesicles (exosomes)

exerted a skin protective role against photoageing. Another important function of the skin is wound healing. However, this function is affected by the overall status of the body. For example, Shi et al. (2017) engineered a scaffold by seeding gingival MSCs onto a chitosan/silk hydrogel sponge and used it to cover a skin wound in a diabetic rat skin defect model. The above combination effectively speeded up skin wound healing (that was originally delayed as an effect of diabetes on these rats) via enhancing both angiogenesis and re-epithelialization. Since cutaneous wound healing can lead to scar formation, Liu et al. (2014) demonstrated a reducing post wound healing scar formation effect of bone marrow-derived MSCs and attributed it to the anti-inflammatory properties of these cells and their mediators. It was also found that the inflammatory regularity abilities are surprisingly in parallel with the apoptosis of the above cells. Moreover, the effect of a combination of fibroblasts and ADSCs embedded in a 3D tissue engineered skin system on epidermal morphogenesis has been studied. Results showed that having a mixture of cells in the engineered skin improved its epidermal morphogenesis manifested by the extensive proliferation of keratinocytes over the mixture compared to the lower proliferation seen in single mesenchymal cell types (Lu et al. 2012).

1.5 Future Perspective of Stem Cells and Their Products in TERM

This chapter concentrated mainly on the use of stem cells and their products in tissue engineering and regenerative medicine. It showed the promising therapeutic role of stem cells and their products for many chronic debilitating diseases. Combining the suitable stem cells/products with the suitable biomaterial to engineer the therapy of choice for certain diseases that has no cure so far with no or minimum side effects is the ultimate goal of TERM. Researchers all over the world are working in their laboratories and producing results on daily basis regarding the latest in this multidisciplinary field that requires collaboration of basic scientists, physicians, biomedical and software engineers and members of the community to come up with a solution for most/if not all the diseases affecting humanity.

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GLP Requirements of Stem Cells

2

Hassan Ahmed Khan

Abstract

Good Laboratory Practices (GLP) requirements of stem cells are implemented on the design, construction, equipment, quality control, and oversight approvals of stem cell laboratory. Planning to establish stem cell laboratory focuses on the budget, space, type of work to be done and availability of major equipment. Stem cell laboratory comprises of tissue culture laboratory, quarantine laboratory, quality control laboratory, and microscopy. These laboratories work independently with essential equipment and personnel with a lab management system maintained by information technology department. Obtaining appropriate clearance for the cultivation of hPSCs can be a lengthy and complicated procedure when establishing a new facility for the culture of the cells. There are different committees such as Stem Cell Research Oversight (SCRO), Institutional Review Board (IRB), Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC), which are the platforms that provide clearance for cultivation of hPSCs.

Keywords

Biosafety · Quarantine · Mycoplasma · Cryopreservation

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F. A. Khan (ed.), *Stem Cell Production*,
https://doi.org/10.1007/978-981-16-7589-8_2

2.1 Laboratory Design

2.1.1 Key Consideration in Planning and Design

2.1.1.1 Budget

The cost of a stem cell laboratory is determined by the type of research you want to do, the therapies or cells you want to use, and the equipment you want to purchase. One was constructed in Israel in 2005 for less than $1,000,000. It created stem cells for a clinical experiment, then 512 seriously ill cardiac patients from all over the world. One was recently built in Bangkok for $3.5 million and was quite contemporary and spacious, according to reports. A typical lab consists of 20–40 employees, with a senior researcher (the "principal investigator") in charge. The majority of people in a lab are doctorate or postdoctoral students seeking science professions. Many diverse projects are being investigated at the same time in labs. The majority of laboratories have yearly budgets ranging from $1 to $5 million (https://hbswk.hbs.edu/item/funding-unpredictability-around-stem-cell-research-inflicts-heavy-cost-on-scientific-progress). The California Institute for Regenerative Medicine was established in 2004 after voters approved a $3 billion bond package. The funding had significant direct and indirect economic benefits (through supply-chain effects): it is estimated that the program produced more than 56,000 jobs and added about $10 billion to the California state economy (Goldman et al. 2020).

2.1.1.2 Space

Space required for stem cell laboratory depends on the type of laboratory destined to establish. It depends upon whether it is newly established one or build out of shell space, rehab of existing laboratory.

2.1.1.3 Type and Scope of Work

Biosafety Requirement for Tissue Culture Labs

The majority of our facilities are Biosafety Level-2 (BSL-2). BSL-2 may be used to cultivate all cell lines devoid of infectious pathogens, including replication incompetent viral vectors for gene delivery, according to CDC standards. BSL-2 is also suggested for stem cell quality control laboratories.

2.1.1.4 Major Equipment

A cell processing lab just requires a few pieces of equipment. Maintenance and calibration of critical equipment should be done on a regular basis. When the laboratory only uses one device, backup equipment should be specified. Before being used, all equipment including backup equipment should undergo qualification and validation.

List of major equipment is given below:

Major equipment	• Biosafety Cabinet • Refrigerator • Water Bath • Balance • Centrifuge • −70 °C freezer • Plasma Extractor • Tubing Sealer • Tubing Stripper • −80 °C cryotransporter • Micropipette 100 and 1000 µl • Thermometer • Hemostats

Leemhuis et al. (2014)

2.2 Major Functional Areas

2.2.1 Tissue Culture Laboratory

The generation of primary cell lines from tissue biopsies or other materials is the role of the tissue culture laboratory. Before starting any studies, primary cell lines are screened for contamination, particularly mycoplasma infection. From adult cells of various sources, this group created human induced pluripotent stem cells (iPSCs). Characterization of iPSCs are done for further research and experimentation.

2.2.2 Quarantine Laboratory

New materials should be referred as "Quarantine" materials till they are free of contaminants such as bacteria, fungi, and particularly mycoplasma. This new material is kept in quarantine laboratory.

2.2.3 Quality Control Laboratory

The quality control (QC) laboratory is equipped with testing tools to guarantee that stem cells meet rigorous quality standards. All physical, chemical, or biological qualities must fall within a certain range, limit, or distribution in order to assure the product's quality and safety for its intended purpose. In addition, the assays that should be used to quantify these parameters, as well as the standards and tolerances that should be applied must be considered (Sullivan et al. 2018).

2.2.4 Microscopy Laboratory

The characterization of stem cells is done in the microscopy laboratory using a phase contrast microscope and a dissecting microscope.

2.3 Personnel

The personnel who have professional education in a laboratory with experience in clinical hematology and/or blood banking are the most suitable for the job. Although most processing activities for a new program may be handled by a single employee, a minimum of two qualified laboratory technicians is necessary, one of whom might be cross-trained from another unit. If necessary, the cell processing personnel can undertake QC testing on the product, although it may be more cost efficient to contract with the hospital's microbiology, flow cytometry and hematology laboratories, which perform these tests on a regular basis. In any case, QC personnel must be educated in the specific elements of evaluating hematopoietic progenitor cells (HPCs). We also propose employing or sharing part-time personnel who do not have processing duties to focus on Quality Assurance (QA) and Regulatory activities such as chart review, raw material inspection, product release for infusion, process improvement tasks, and internal regulatory compliance audits. One of the transplant physicians (Clinical physicians) frequently acts as the Medical Director of the cell processing lab, giving leadership and guidance to laboratory employees as well as serving as a consultant to less experienced transplant physicians. There should be staff in the unit to supervise and control everyday laboratory operations. In addition, a designated supervisor or manager should be in charge of staff scheduling and ensuring that standard operating protocols are properly followed (Leemhuis et al. 2014).

2.4 Freezer Rooms

Appropriate cryopreservation techniques for long-term storage are a prerequisite for the commercial and clinical use of stem cells. While efficient cryopreservation and storage techniques for hematopoietic and mesenchymal stem cells have been established, embryonic cells and iPS cells have shown to be more resistant (Hunt 2011).

Refrigerator or freezer is usually more efficient and less expensive than laboratory equipment. If you have enough space and more than three or four workers who use tissue culture, you should consider installing a cool room, which is more cost-effective and efficient. For stem cell laboratories, multiple temperature freezers are necessary, such as controlled rate freezers (−20 °C), low temperature freezers (−70 °C to −90 °C), and cryo-storage at −135 °C. Backup generators and warning systems are required for freezers.

2.5 Storage Areas

Chemicals and reagents used in a stem cell laboratory must be stored in designated storage rooms. The needs will be met by cabinets, shelves, and cabinets. Make sure that everything in your lab has a specific place. To assure that recently delivered materials are easily accessed by all lab members, designate distinct, clearly labeled storage areas for each reagent or group of reagents. Make a schedule for going through your chemicals and discarding expired and obsolete reagents from your storage area to decrease clutter.

In the laboratory, chemicals must be separated by hazard class and compatibility. All receiving chemical containers must have the proper manufacturer's labels, which must not be missing or damaged. Each chemical container should be labeled with the date it was received and the date it was opened (this is a requirement for peroxide forming chemicals). Long-term storage should not be done in work areas. It is definitely forbidden to store glass chemical containers on the laboratory work area floor. If space does not permit each chemical danger class to be stored in its own cabinet, each group should be separated by secondary containment (e.g., plastic trays), with special attention paid to providing stable, uncrowded, and closely monitored conditions. Always keep fuels away from oxidizers, at the very least. Hazardous chemicals (excluding cleansers) should not be stored under sinks. Use flammable storage lockers, corrosive storage lockers, shelves, or cabinets that have been permitted. Open shelves used to store hazardous chemicals must be securely fastened, painted, or coated with chemical-resistant materials.

2.6 Information Technology

Most laboratories will have one or more computers, which may or may not be networked. These computers meet the needs for cell line maintenance, primary culture, and experimentation, making retrieval and analysis simpler in the future. Plastics, reagents, and media are all kept in stock by computers.

2.7 Key Construction Considerations

2.7.1 Engineering Controls

Laboratories handle valuable and delicate items that can be harmed by temperature changes. In a stem cell laboratory, the importance of emergency and backup power supply systems cannot be emphasized. Every key safety system or element directly supporting the lab, such as equipment, ventilation, lighting, and control systems, must be online at all times to ensure a high degree of protection. The majority of systems and gadgets will be powered by UPS, backup generators, or both. When the main power supply goes down, emergency generators should come in automatically. Safety alarms must be installed in the facility to keep aware of the fire hazards.

Smoke detectors and sounders must be installed to meet any emergency situation. In addition, CO_2 and LN_2 delivery pipes are taken to the laboratory from "tank farm" or cylinders delivered to the laboratory and cryobank tanks.

2.7.2 HVAC

The quality of BSCs used in the construction of a tissue culture laboratory will have a significant impact on the cost of the project. The quantity and kind of BSCs, as well as any other heat-producing equipment like incubators and freezers, must be accommodated by air handlers. One of the most difficult aspects of the laboratory's design and operation is ensuring efficient and efficient air conditioning. In addition, while working in laboratories, where the heat cannot be regulated due to the installation of HVAC systems that are not capable of handling the heat produced by BSCs and incubators may be quite inconvenient. The direction of air flow (negative, positive, or neutral) is an important factor when constructing a cell culture laboratory and is crucial to its safe operations.

2.7.3 Electrical Capacity and Routing

Calculating the proper electrical capacity to ensure a safe working environment requires accurately forecasting the number of BSCs, freezers, and other important equipment, as well as determining how much power they need it and also how much heat they will generate. The thorough design includes the power needs for every equipment, as well as the location of power outlets, light switches, emergency power requirements, closeness of outlets to water faucets and coordination of outlet placement with the modular furniture designer. To accommodate all existing equipment as well as future growth, there must be enough and dedicated circuits. Backup generator circuits and alarm systems are required for incubators and freezers.

2.7.4 Interior Finishes

Vinyl flooring is both durable and attractive, making it an excellent choice for labs. Though foot traffic may not be considerable, rolling trolleys carrying heavy machinery, chemicals, and samples that require smooth and fast transit may be seen. The durability of vinyl flooring makes it more than a match for the excessive wear that occurs with heavy use. In comparison to harder floor materials, it is also more noise and pleasant underfoot. It's also simple to clean and maintain; depending on the design, any broken tiles or planks may be replaced with hardly any impact on the lab's performance. Gypsum ceiling is non porous ceiling recommended for laboratories. Paint and coatings should be washable and impermeable. Bench tops and furniture must be impermeable.

2.7.5 Tissue Culture Area

A sterile room with restricted traffic, separate from other laboratories, a preparation area, a washup area, space for incubators, storage facilities for liquids, glassware, specialist equipment, chemicals, cylinders, and a sink are all minimum requirements for a tissue culture facility.

2.7.6 Quality Control Laboratory

The equipment and standard operating protocols (SOPs) are necessary for executing a specified list of characterization assays that allows one to systematically examine the quality of cells in culture in the laboratory and make up the molecular biology/quality control laboratory. Cultures are examined for particular marker expression, cytogenetic structure (karyotype), and differentiation ability (Wesselschmidt and Schwartz 2011).

2.7.7 Quarantine Laboratory

It is worthwhile to set up a separate laboratory for quarantine and/or confinement. This lab must possess Class II microbiological safety cabinet, incubators, freezer, refrigerator, centrifuge, chemical and plastic supplies, and waste disposal. This laboratory must be isolated from the other laboratories by air lock system and must have negative pressure in the aseptic space. Imported cell lines or biopsies can be handled in the labs until they have been proven to be free of mycoplasma and restricted pathogens like HIV or hepatitis B.

2.7.8 Storage

The following items require storage, with sterile and non-sterile goods kept separate and clearly labeled. Sterile liquids, medium, serum, trypsin, glutamine, sterile and non-sterile glassware, sterile disposable plastics, screw caps, stoppers, sterile and non-sterile filters, gloves, disposal bags, and liquid nitrogen to replace the freezer are among the items stored.

2.8 Equipment

2.8.1 Tissue Culture Laboratory

Minimum set of requirements for tissue culture laboratory include biosafety cabinet, CO_2 incubators, pipettes, vacuum flask, water bath, and centrifuge.

2.8.2 Biosafety Cabinets

For potentially hazardous items (such as cell lines and virus-producing cultures), a biohazard cabinet of Class II or Class III should be utilized. However, in most laboratories, using a Class II microbiological safety cabinet is standard procedure.

2.8.3 CO_2 Incubators

The cost of a CO_2 incubator is higher, but the simplicity of use and greater control of CO_2 tension and temperature make it worthwhile. CO_2 incubators are used to incubate cell cultures. The size of a CO_2 incubator is determined by the number of people that will be utilizing it.

2.8.4 Pipettes

One of the most common jobs in the everyday handling of cultures is pipetting. Pipettors are used to transport chemicals in tiny quantities to containers. With these pipettors, reagents with a volume of 10–20 ml can be sampled in 5 µl–1 ml quantities from a universal container or in 4–200 µl volumes from a bottle.

2.8.5 Vacuum Flask

A peristaltic pump is used to remove used media or other chemicals from a culture flask, and the effluent can be collected in a vacuum flask with little risk of releasing aerosol into the atmosphere.

2.8.6 Water Bath

To maintain the liquid at the appropriate temperature, a water bath is used. The temperature of reagents put in a water bath is maintained by circulating hot water.

2.8.7 Low Speed Centrifuge

Centrifugation is used to raise the concentration of cells or to remove a reagent from a cell solution on a regular basis. For the most part, a simple bench-top centrifuge will serve.

2.8.8 Microscopy

2.8.8.1 Phase Contrast Microscope

It is critical to examine cultures on a frequent basis. A morphological change is the first indicator of a culture's degradation and the typical pattern of microbiological infections. If you plan to photograph living cultures, choose a microscope with phase contrast condenser, and objectives, as well as facilities for a CCD camera.

2.8.8.2 Dissecting Microscope

A dissecting microscope will be required to dissect tiny amounts of tissue. A dissecting microscope may be used to count monolayer colonies and is required for counting and selecting tiny colonies in agar.

2.8.9 Storage

While the therapeutic products of stem cells may not need to be stored for long periods of time, the master and working cell banks from which the products will be produced will almost certainly need to be stored for a long time. This will necessitate storage below the glass transition temperature, which is essential to stop molecular activities. This is usually accomplished by storing the material in a liquid or vapor state of liquid nitrogen. Mechanical refrigeration, which maintains temperatures below 135 °C, is now dependable, and stem cell tests show no major differences in liquid nitrogen up to 5 years (Hunt 2011).

2.8.10 Quality Control Laboratory

2.8.10.1 RT-PCR

Amplification and detection of particular DNA sequences are used in a number of accessory procedures in cell line validation, such as mycoplasma identification and DNA profiling. A heat cycler is used for PCR.

2.8.10.2 Flow Cytometer

This device can examine cell populations using a variety of metrics such as light scatter, absorbance, and fluorescence. The results of a multi-parametric study can be seen in two or three dimensions. These devices are commonly known as flow cytometers when utilized in an analytical mode, but the signals they create may also be employed in a fluorescence-activated cell sorter to separate individual cell populations.

2.8.10.3 Fluorescent Microscope

For mycoplasma identification and autoradiography, an upright microscope may be necessary in addition to a phase contrast microscope. Choose a high-quality research microscope with bright field optics up to 100× objective magnification, phase

contrast up to at least 40× and ideally 100×, and fluorescence optics with epi-illumination objective, which is very beneficial for observing routine mycoplasma preparations with Hoechst staining.

2.8.10.4 Confocal Microscope

When examined using confocal microscopy, cytological examinations of fluorescently labeled cells frequently benefit from enhanced resolution. This method permits the microscope to see an "optical section" through the material, displaying the image in a single focus plane and eliminating interference from neighboring cells that are not in the same focal plane. The data is saved digitally and may be processed in a variety of ways, including creating vertical sections across the sample, which is very important for analyzing three-dimensional cultures like filter wells or spheroids.

2.8.11 Quarantine Laboratory

2.8.11.1 Class II Biosafety Cabinet

The majority of cell culture operations are done in laminar flow hoods. To avoid contamination of cell cultures, laminar flow hoods or biological safety cabinets offer a clean working environment. Cell lines are examined at the quarantine laboratory under the Class II Biosafety Cabinet to determine the presence of any contamination, particularly mycoplasma.

2.8.11.2 CO_2 Incubator

The incubators provide the cells with a suitable environment in which to develop. For one class, a small- or medium-sized cell culture incubator is sufficient. Temperature control is required in the incubator. A CO_2 gas tank is linked to cell culture incubators. Inside the incubator, CO_2 gas is injected and dispersed by a fan or natural convection. The quarantine laboratory's CO_2 incubator is used to incubate the cell cultures to find out the presence/absence of contamination.

2.8.11.3 Phase Contrast Microscope

Growth phase of mycoplasma is observed under phase contrast microscope.

2.8.11.4 Water Bath

A cell culture facility needs a 37 °C water bath to warm up the medium and other chemicals needed for the cells. The warm water in the water bath provides an excellent habitat for bacteria and pollutants to thrive. As a result, the water bath must be cleaned on a regular basis and the water replenished with new, distilled water.

2.8.11.5 Low Speed Centrifuge

Clinical centrifuges are used to extract cells from the medium or other reagents and concentrate them. To avoid damaging the cells, a low-speed clinical centrifuge must

be utilized. Speeds of 80–100*g* (gravitational force) are sufficient for normal cell spinning. Higher speeds may cause cell damage.

2.8.11.6 Pipettes

Micropipettes are used to transmit tiny amounts of liquid ranging from 1 to 1000 μl. Micropipettes should be stored in separate clean boxes for cell culture activities alone. Micropipettes must be calibrated on a regular basis to guarantee accurate readings. Micropipette tips must be sanitized before use and are maintained in color-coded, sterile cartons.

2.8.11.7 Aspiration/Vacuum Flask

The hood is equipped with an aspirator pump that removes and pumps liquids straight into the disinfectant.

2.9 Quality Control

The QC should be consisting of equipment, staff, and SOPs for the production of reliable and reproducible experimental results.

2.9.1 Reliable Techniques

Identifying dependable procedures for the growth and characterization of cell lines is a crucial role of laboratory management while setting up a PSC lab. The essentials to creating a solid foundation include identifying well-established methods and employing well-characterized cell lines. New technologies and fast recent expansion in the stem cell field needed to be updated by the laboratory manager, particularly with regard to technologies that allow for the guided reprogramming of somatic cells to create induced pluripotent stem cells (iPSCs). There is a growing misconception that all that is necessary to establish a company is to produce some iPSC lines. Before starting an hPSC laboratory, we strongly recommend getting hands-on training in the art and science of human embryonic stem cell cultivation in an established laboratory, core, or training center. We also strongly recommend keeping a well-characterized hESC line (s) in the lab as the "gold standard" for any further PSC research.

2.9.2 Validated Reagents

The reagents which are used to grow and characterize hPSCs must be verified and proven to be safe, and clinical grades. It's necessary to test reagents specially, fetal bovine serum, knockout serum replacer, and mouse embryonic fibroblasts, are crucial to the PSC laboratory's effectiveness and efficiency.

2.9.3 Quality PSCs

The PSCs utilized in the lab must come from well-characterized stocks and exhibit the morphologic and genetic features of high-quality PSC lines, as detailed in numerous chapters of this book. In laboratories generating iPSC cultures, we emphasize the use of well-characterized hESC lines as the "gold standard." Having these authentic hPSCs in the lab will allow for reagent and procedure testing as well as confirmation of iPSC characteristics.

2.9.4 Checklist for Confirming Quality

Establishing a framework that permits cultures to be tested against established criteria on a regular basis can aid long-term success and offer trust and dependability in the experiments. hPSCs must be free of contamination, have a normal karyotype (number of chromosomes), express specified markers (tissue-specific markers), and be capable of developing into cells originating from all three germ lines.

2.10 Approvals

Obtaining appropriate clearance for the cultivation of hPSCs can be a lengthy and complicated procedure when establishing a new facility for the culture of the cells. The country, state, city, and institution where one is located all have a part in the sort of approval(s) necessary prior to starting culture. Beforehand, the first hPSC can be cultivated in the new laboratory, applications will need to be submitted to review boards and specialized committees, material transfer agreements will need to be acquired and completed, and employees will need to be educated. As a result, we recommend starting the approval process before or during the laboratory design phase.

2.10.1 Typical Institutional Review

2.10.1.1 Stem Cell Research Oversight Committee (SCRO)

The Stem Cell Research Oversight Committee's (SCRO) task is to guarantee that human stem cell research is well justified and that no unsuitable or unethical research is carried out. Its mission is to supervise ethical concerns surrounding the use of human stem cells in research facilities. The SCRO committee's review is different from other federal, state, and local regulations such as Institutional Animal Care and Use Committees (IACUC), Institutional Review Boards (IRB), and Institutional Biological Safety Committees (IBC).

It has been recommended that all stem cell projects in the following categories are required to obtain SCRO approvals:

- Stem cell research involving human embryonic stem cells, pluripotent cells, and their derivatives.
- Stem cell research involving human gametes and human embryos.
- In vitro human induced pluripotent stem cell research involving the generation of gametes, embryos, or other types of totipotent cells.

2.10.1.2 Institutional Review Board (IRB)

It has been found that IRB reviews and approves, require modifications in, or disapproves the derivation and use of human stem cells to assure appropriate procurement of gametes, blastocysts, fetal tissue, or somatic cells to generate new stem cell lines, including the procurement of blastocysts over clinical need from infertility clinics, blastocysts made through in vitro fertilization specifically for research purposes, and oocytes, sperm and somatic cells donated for the development of human embryonic stem cell lines through nuclear transfer.

To guarantee adequate permission from donors of sperm, oocytes, or somatic cells used to produce blastocysts for research, the IRB examines, approves, needs revisions in, or disapproves human stem cell operations. Human stem cell activities are reviewed, approved, required to be modified, or disapproved by the IRB to ensure donor privacy in compliance with HIPAA and CMIA rules for the use of personal health information for research purposes.

The IRB monitors the implementation of ongoing human subject procedures involving human stem cell research and demands periodic progress updates. The IRB evaluates and approves, needs changes to authorized human stem cell research, or rejects requested changes. The IRB has the authority to stop or terminate human stem cell research that has been approved.

2.10.1.3 Biosafety Committee Review (IBC)

The IBC is responsible for assessing operations using potentially hazardous biological agents, such as infectious agents, human and non-human primate materials (including established cell lines), CDC select agents, recombinant DNA, and human gene transfer research. The IBC ensures that research work using these substances is carried out safely for the researcher, laboratory worker, human research subjects, the general public, and the environment. In addition, the IBC verifies that the study is done safely, analyzes decontamination and containment levels, and confirms that rDNA research follows the NIH Guidelines for the use of Recombinant DNA.

2.10.1.4 Institutional Animal Committee (IACUC)

The IACUC oversees all animal use protocols, ensures compliance with federal legislation, inspects animal facilities and labs, and oversees training and education programs. The IACUC's main responsibility is to oversee the ethical and humane treatment of animals in research, testing, and education. Animals are used in human stem cell research, which requires IACUC clearance before the project can begin (https://research.uci.edu/compliance/hscro/other-uci-reviews-for-stem-cell-research.html).

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Current Good Manufacturing Practice (cGMP) Facility and Production of Stem Cell

3

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Abstract

In the last decade, the advancement of stem cell research as a new therapeutic approach in the field of tissue engineering, regenerative medicine, cell and gene therapy had urged researchers to produce clinical grade cell and tissue products. The process of translation from research to clinical application requires strict control of the product to ensure its quality, safety and efficacy. These clinical grade cell and tissue-based products should be manufactured in a current good manufacturing practice (cGMP) facility that is designed and accredited by the regulatory body for the production of cell and tissue. cGMP is the practice regulations or system that is internationally recognised and enforced by the regulatory agencies, that control the authorisation and licensing of the manufactured products such as pharmaceuticals, food and beverages, cosmetics, medical devices, cell and tissue-based products. The quality management system is the crucial part of cGMP to control the manufacturing process (including critical materials, sample collection, processing, culturing, storage quality control testing and release of the cell therapy products), facility management (including design, environmental control and monitoring, maintenance, equipment, personnel access, cleaning), validation, personnel training, competency and also records. The cGMP facilities for cell and tissue therapy should be designed at a higher standard and following the pharmaceutical manufacturer in order to produce a safe and high-quality product for human use. The facility must be designed in a proper size of cleanroom with different class or grade (Grade A, B, C and D) areas depending on air purity and particles. The environmental control,

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F. A. Khan (ed.), *Stem Cell Production*,
https://doi.org/10.1007/978-981-16-7589-8_3

monitoring and maintenance of the facility is critical to prevent contaminations and determines the operation efficiency. The standard operating procedures (SOP) for all related processes and procedures for the production of cell and tissue-based products should be developed and validated before the products can be manufactured and released for therapeutic use. In this chapter, we will discuss details on the cGMP quality system, facility management, manufacturing of the clinical grade cell and tissue-based products and ethical issues. All activities in the cGMP must be documented and audited regularly to maintain the consistency of the products and follow the standard guideline required by the regulator.

Keywords

Current good manufacturing practice · cGMP facility · Clinical grade production of stem cells

3.1 General Regulation for CGTP

Many different versions of GMP guidelines used worldwide, but most have adapted the Pharmaceutical Inspection Co-operation Scheme (PIC/S) guide to Good Manufacturing Practice (GMP). PIC/S currently has 53 Participating Authorities worldwide, including Europe, Africa, America, Asia and Australasia, and is open to any authorities having similar GMP inspection system. Brazil's National Health Surveillance Agency (ANVISA) has recently become the 54th Participating Authority to join the PIC Scheme in January 2021 (PIC/S 2021).

In recent years, the use of stem cells for general wellness, cosmetic and therapeutic application have become commonly used worldwide. Some countries have yet to enforce specific guidelines to regulate the manufacturing of CGTP. Most still consider CGTP as drugs and regulate them under GMP guidelines for pharmaceuticals (Bedford et al. 2018; Li et al. 2019; Tiwari and Desai 2018). Hence, some companies are using the regulatory loopholes and taking shortcuts in the manufacturing and marketing of stem cells for profit and maybe putting public health and safety at risk. Therefore, there is a pressing need to enforce guidelines specifically designed for CGTP manufacturing and product licensing to safeguard the public.

The U.S. Food and Drug Administration (US FDA) has published four guidance documents as a policy framework for regulating regenerative medicine products human cells, tissues, and cellular and tissue-based products (US FDA 2021). In Australia, the Therapeutic Goods Administration (*TGA*) regulates human cell and tissue-based therapeutic goods as biologicals (TGA 2021). In Malaysia, the National Pharmaceutical Regulatory Agency (NPRA) has drafted a CGTP guideline which will be enforced in the near future. All future manufacturing of CGTP will be regulated by this guideline (NPRA 2021).

3.2 What Is cGMP?

Current Good Manufacturing Practice, or commonly known as cGMP, is a set of internationally recognised principles and procedures used in the design, monitoring, maintaining and control of manufacturing facilities such as food and beverages (Institute of Food Science & Technology 2018), medical devices (Ramakrishna et al. 2015), dietary supplements (Mead 2012), cosmetics (Moore 2009), pharmaceutical (Nally 2007), cell and gene therapy products (CGTP) (Giancola et al. 2012). It is a compilation of different guidelines by international organisations and monitored by national regulatory authorities in different countries.

3.2.1 Primary Aspect in cGMP

The primary aim of cGMP is to consistently provide a high level of assurance of the quality, safety and efficacy of the manufactured products (Gouveia et al. 2015). To achieve these goals, the organisation should focus on several aspects of cGMP such as quality management, documentation, process control, validation, material control, personnel training, equipment, building and facility management (Seet et al. 2013). The cGMP guidelines are usually flexible, but the main focus is to help manufacturers implement effective manufacturing processes and to ensure that the end users receive safe, efficacious and good quality products. Different countries have their own regulatory authorities to ensure that manufacturers comply and operate according to the local GMP guidelines (Escano 2021).

3.3 Quality Management System in Good Manufacturing Practice

The Quality Management System is a system that documents processes, procedures and responsibilities for achieving quality objectives. It supports the manufacturing industries in the design and process development to enhance the quality of the products produced and to ensure that the final products meet the agreed specification and are fit for the intended use with acceptable safety, quality and efficacy. It also helps the manufacturer to meet regulatory requirements and for continuous improvement of the organisation (Choudhary 2016).

3.4 Quality System

The Quality Manual is usually one of the first document created when developing a Quality Management System. It is a top-level document comprising the organisational structure, procedures, processes and resources that control quality activities within the organisation. It should contain the Quality Policy, Objectives, Mission and Vision statement. It should include the scope of the QMS and the main

activities that are carried out by the manufacturer. Management and personnel responsibilities in the QMS should also be listed in the Quality Manual.

The Site Master File is another important document that describes the physical environments and references the procedures used in the management of the manufacturing facility. It is used to complement the Quality Manual, which covers the quality system used by the organisation. The contents of a Site Master file contain general information of the manufacturer, the manufacturing activities, quality management system, and mentions of procedures related to release of the final product, management of suppliers and contractors, quality risk management, product quality review, personnel, premises, equipment, documentation, production, quality control, distribution, complaint, product defects, recall and self-inspection.

3.5 Quality Assurance and Quality Control

Quality Assurance and Quality Control are two subsets of Quality Management, as shown in Fig. 3.1. Quality Assurance is an overall management plan to guarantee the integrity of the product. It is used to prevent errors and defects in the manufactured products. Generally, it comprises administrative activities and procedures that, if carried out correctly, can provide confidence that the product manufactured will fulfil the quality requirements. Quality assurance can be in the form of standard operating procedures (SOP) to manage the quality of raw materials, products, production process, maintenance process and inspection process (Seet et al. 2013; ASQ 2020; Wikipedia 2021). Other aspects of Quality Assurance also include implementation of non-conformance reporting (NCR), Corrective and Preventive Actions (CAPA), Management Review Meetings (MRM), vendor auditing, complaint, recall, process deviation management, risk management, change management, archiving system, maintenance of batch records, calibration records, personnel training and assessment, equipment and facility qualifications and logs, etc. (Kolkundkar et al. 2014).

Quality Control is the part of quality management that focuses on inspecting, testing and evaluating the manufactured product to assess its quality. It checks manufacturing records and the release procedure to ensure that the product undergoes the required testing before it is released for use. In general, it is a process of finding faults in the manufacturing process, and if faults are found, a

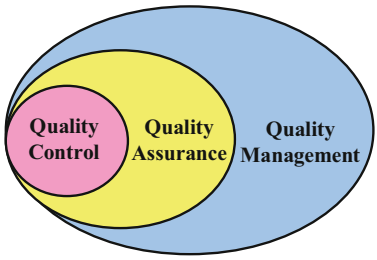

Fig. 3.1 Relationship between quality control, quality assurance and quality management

non-conformance report (NCR) should be generated, and corrective actions should be implemented (Seet et al. 2013; Escano 2021).

3.6 Document Preparation and Control

Documents are an essential part of the GMP quality management system. A good document control system will ensure that all documents used within the organisation have gone through proper revision, approval and the latest version. The document structure is usually broken down into a few levels. The top level is the organisation policy, quality manual or site master file, followed by standard operating procedures (SOP), work instructions, forms and records and finally, the CMS or any referencing material (Dawson 2014). For proper identification, each document should be given a document control number (DCN). This DCN should not be reused and must be unique for each document. For ease of tracking, a spreadsheet listing out all the documents with their corresponding DCN can be created. It can be used to track the location of the document, the current version, effective date, last reviewed date and any other information required by the document controller.

When preparing a document, the author can consider using text, flowchart, diagrams, illustrations, graphics, tables or any other means easy for the readers to understand. It is recommended to use simple, concise and unambiguous language. Do not write the procedures as a description but keep the sentences and paragraphs short, especially if the document is a manufacturing procedure meant to be used inside a cleanroom. Cleanroom operators need direct instructions to save time and reduce the error of interpretation. The author who is assigned to prepare the document should be well-versed in that topic. After completion of the document, a second person should review the document. If the document is a manufacturing procedure, the author and reviewer should test run the procedure, if necessary to ensure all details are included. When the author and reviewer are satisfied with the document, it should be sent to the Quality Department for final review and approval. The Quality Department and managers from other departments will decide whether training will need to be performed for the new procedure before the procedure is being made effective and issued for use (Djemal 1999).

Documents may need to be amended when changes are required in the procedure. All amended documents will need to be up-versioned, approved and made effective again before being issued. The outdated version needs to be retrieved and destroyed to prevent unintended use (Schneider et al. 2017). All major amendments made to the document should be tracked by a change log, highlighting, underlining or any other ways to identify the changes (AQM 2021). Those documents which does not require amendments will need to be reviewed at a set interval determined by the organisation. The revision period is generally set at once every 2 years (Dawson 2014). A summary of the document preparation and control work flow can be found in Fig. 3.2.

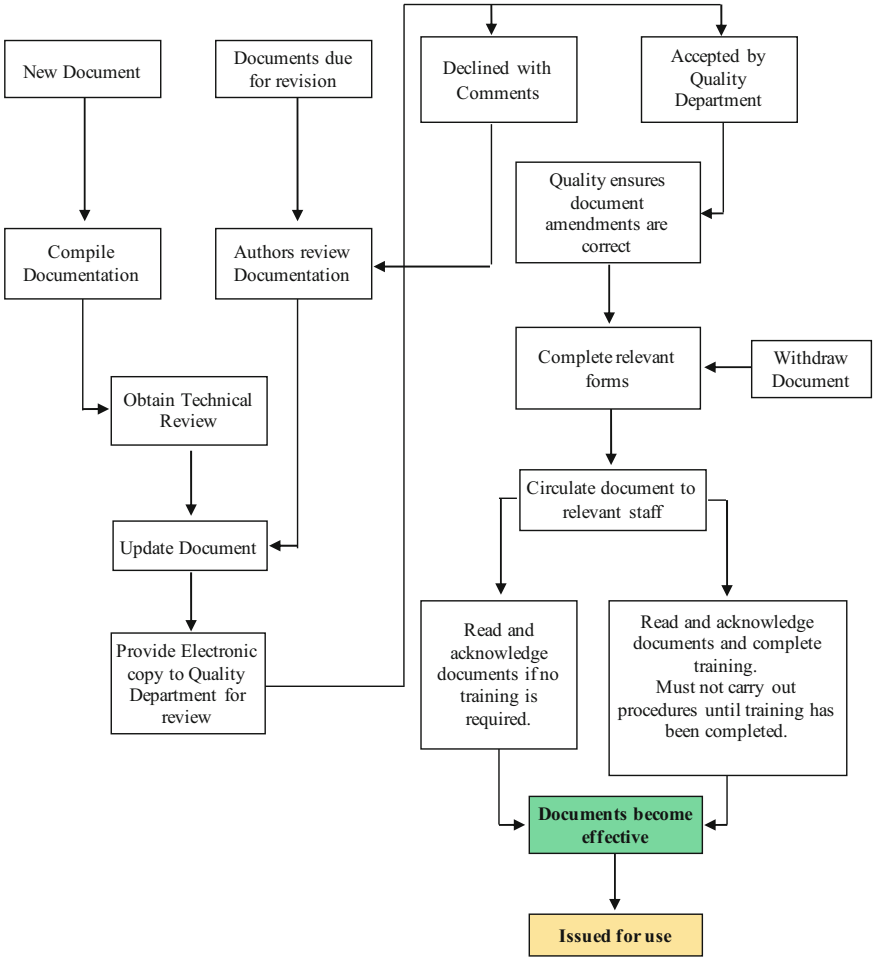

Fig. 3.2 Document preparation and control

Documents can be kept as either hardcopy or softcopy, depending on the need of the organisation. The softcopy or electronic documents can be shared in cloud storage for better access control. Any hardcopy documents except for forms need to be identified as a controlled copy. The document can be stamped as such, or they can be printed on specialised paper which can only be accessed by the document controller. All master documents also need to be backed up regularly for the purpose of audit traceability and to prevent accidental loss. The backup interval can be based on the activity of the organisation's document system. The more active the document system, the more frequent the backup needs to be performed.

3.7 Facility Design and Construction

One of the differences between the production of stem cells for research use and the production of stem cells for clinical use is the facility used to process or produce the stem cells. For research use, usually stem cells will be processed in a normal or research grade laboratory that does not follow the GMP guideline. Meanwhile, stem cell production will be done in the cleanroom with a certified cGMP facility for clinical use. The cGMP facility is designed to accommodate this manufacturing according to the national and international medicinal regulatory body guidelines. The initial purpose of the design of the cGMP facility must serve the manufacturing of stem cell and control the operation and maintenance of productivity. In the perspective of convenience, the manufacturing of the cGMP facility is designed in which it can accommodate different type of stem cell production later.

cGMP facility should be located, designed, constructed, utilised and maintained to ensure and confirm the safety of the product, reduce the error and allow the effectiveness of the cleaning and maintenance process. The main role of the cGMP facility is to lower the risk of contamination to the product and potentially adverse effect to the receipt. The cGMP facility is usually divided into three areas based on several features: air cleanliness classification and functionality, i.e. general area, unclassified area and classified area. The overall design of the cGMP facility is based on several factors, i.e. areas classification, gowning requirement, entry and exit plan and also product, material and waste flow (Table 3.1 and Fig. 3.3).

3.8 Facility Management

A stem cell-based cGMP facility should be well managed and planned to safeguard product safety, reduce the risk of contamination and manufacturing errors, and allow for effective cleaning and maintenance (Giancola et al. 2012). In order to provide a maximum safety and security to the facilities, the entrance and exit access to the facilities must be controlled. This also can prevent intrusion and also sabotage to the critical material and area in the cGMP facility. Access to all area of the cGMP facility is controlled, which will only be issued to authorised personnel. Full access staff can access all area in the facility. Contractors, cleaners and visitors have limited access to the specific area necessary only at the approval of the authorised manager or

Table 3.1 Typical area classification in the cGMP facility

Area	Air classification	Function
General	Unclassified	Management, general laboratory management supporting area
Unclassified	Unclassified	Supporting area for production, laboratory working area
Classified	Classified into different graded area	Production area, Storage area

Fig. 3.3 cGMP Facility in Centre for Tissue Engineering and Regenerative Medicine (CTERM), Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM). (**a**) Cleanroom grade B area is equipped with various equipment for cell manipulation; (**b**) Grade C corridor; (**c**) Grade D change room for change street cloth to scrub; (**d**) Waste disposal via waste hatch; (**e**) Production personnel working in cleanroom grade B

delegate. All doors are access restricted by mode of keylock and/or digital lock. The door access system (DAS) is controlled from the DAS workstation in a defined area by an authorised manager or delegate. Different levels of access, whether full, partial or visitor, can be programmed through the access control system and programmed by different responsible personnel according to their roles, training and abilities.

3.9 Environmental Control and Monitoring

The cGMP facility is also susceptible to various types of contamination that can occur during production. Thus, each facility must design a monitoring plan that considers environment over a specific time. The procedures for assessing the microbiological and particle cleanliness of air and surfaces are not specified in the EU GMP guideline, but it states that the air in the cleanroom is the main influence in contamination cases. cGMP facility can adopt the procedures following the ISO standard, ISO 14698:2003 for cleanroom as shown in Table 3.2, associated

Table 3.2 Cleanroom classification for cell therapy facility based on ISO 14644-1 and EU GMP, PIC/S

EU GMP, PIC/S Grade	**ISO 14644-1 classification numbers (N)**			
	At rest		**In operation**	
A	ISO 5		ISO 5	
B			ISO 7	
C	ISO 7		ISO 8	
D	ISO 8		Not defined	
EU GMP, PIC/S Grade	**Maximum allowed particles 1000 L (1 m^3) of air sample volumes**			
	At rest		**In process**	
	0.5 µm	**5.0 µm**	**0.5 µm**	**5.0 µm**
A	3520	20	3520	20
B		29	352,000	2900
C	352,000	2900	3520,000	29,000
D	3520,000	29,000	Not defined	Not defined

controlled environment and biocontamination control ISO 14644-2015 can be used to describe how to classify cleanrooms and related controlled environments and air cleanliness (ISO 2003, 2015). The cGMPs facility may also use EU GMP Annex 1 for GMP procedures where the areas were characterised using a scale of grade A, B, C and D based on the number of particles and microbiological contaminations, as shown in Table 3.2.

The environmental monitoring methods are based on viable and non-viable methods. The viable method involves active air particle sampling while non-viable methods can be included swab or contact plates and settle plates. It is designed to monitor operator aseptic technique and the cleanroom environment. Tryptic Soy Agar (TSA) and Sabouraud Dextrose Chloramphenicol (SDC) plates are used for the detection of bacteria and fungi, respectively. Different condition of operating cleanroom has different methods of requirement. The environmental monitoring programme for at rest differs from in-process environmental monitoring. At rest, the environmental monitoring must be performed after recommissioning, major HVAC maintenance, post-shutdown or at request. After the post-shutdown or maintenance cleaning is done on a specific schedule of maintenance/shutdown of the cleanroom, the at-rest environmental monitoring should be performed the next day after the cleaning day to allow the room to stabilise after a shutdown. However, the additional environmental monitoring programmes can also be carried out as necessary upon request, while in-process monitoring is performed during the production process, where the production personnel present and performing their normal work in the facility with all production equipment and services functioning.

To monitor the environment, the TSA and SDC plates are left open inside the Biological Safety Cabinet (BSC) for a maximum of 4 h to capture any falling microorganisms. At the end of the process, an air sampling procedure will be performed inside the BSC (Grade A environment) and outside the BSC (Grade B environment) along with a BSC work area surface contact plate. Operator aseptic

technique will be monitored by finger dab (five fingers) on each type of plates. The colony forming unit (CFU) for Grade A environment should be <1 CFU/m^3 for air sampling, <1 CFU/4 h for settle plate and <1 CFU/plate for surface contact plate. As for the Grade B environment, the air sampling should return ≤ 10 CFU/m^3. After sampling, the TSA plates will be incubated at 35 °C for 2 days for bacteria detection and SDC plates at 22 °C for 5 days for fungi detection (Martin et al. 2012). Non-viable air particle monitoring will also be performed inside and outside the BSC at the end of the process. The particle limits for Grade A environment for 0.5 and 5.0 μm particles are at 3520 and 20, and for Grade B environment at 352,000 and 2900, respectively (PIC/S 2018).

3.10 Equipment and Maintenance in cGMP Facility

To assure that the product is manufactured following the specifications and guidelines, all equipment should be suitable for its intended purposes, maintained appropriately, and technically applicable for use. Acquisition of any new equipment should be carried out to an approved specification. The basic critical equipment for stem cell production are the class II BSC, CO_2 Incubator, centrifuge and micropipettes, pharmaceutical refrigerator and freezer, water bath, inverted and light microscope, air sampler and particle counter. All these equipment must be qualified and performed such as Installation Qualification, Operational Qualification and Performance Qualification (IQOQPQ) (PIC/S 2021). The authorised manager must develop a maintenance schedule for all equipment. This schedule should specify requirements for routine maintenance, preventative maintenance, calibration, performance checks, and frequency of each type of maintenance and cleaning procedure. Schedules should be based upon the manufacturer's recommendations and the history of the item concerning reliability. Instruments should be re-calibrated based on time intervals rather than actual usage or operating hours. A documented history of all equipment maintenance should be kept. The authorised manager should ensure that persons performing the calibration are certified or approved. All calibrated equipment shall be tagged to indicate the currency of calibration where practicable. Non-conforming equipment shall be tagged as defective and, where portable, removed from the work area.

3.11 Calibration and Performance Check Procedures

Documented procedures should be prepared and used for the calibration and performance checks for all measuring instruments and measurement standards. The procedure should be based upon the manufacturer's instructions or published standard practices. Calibration procedures should include (a) Equipment to which the procedure is applicable, (b) Method to be used, (c) Frequency of calibration, (d) Calibration points, environmental requirements and special conditions,

(e) Acceptance limits, (f) Action taken if the results deviate from the acceptance limits.

3.12 Process, Personnel and Waste Flow

cGMP facility must identify the procedures for controlling the entry and removal of material and equipment into and out of the cGMP facility which includes movement from all classified and unclassified areas, including instructions for placing items into and out of the pass-through hatches. All personnel and material adhere to the aseptic technique of entry and exit of the facility. All waste should be removed from the cleanrooms and disposed of according to the predetermined procedure. All waste should be removed unidirectional via the exit route and waste material and incoming new consumables and patient material should not coincide.

3.13 Cleaning

Cleaning is the mechanical or physical elimination of visible and non-viable contamination caused by dirt, extraneous materials, or product residues. Cleaning is typically followed by a visual examination, followed by scientific testing to ensure that the cleaning is effective. Cleaning agents such as detergents and solvents are often required under specific pH, temperature, duration and solvent concentration criteria for effective cleaning. The cleaning reagents used should have broad-spectrum antimicrobial disinfectants and the rotation and combination of cleaning reagents should be practised avoiding microbe resistance and become ineffective. Table 3.3 shows an example of the combination and rotation of cleaning reagents for cleaning in the cleanroom. Cleaning of the working areas should be done at the end of the working shift. Some facilities may categorise production cleaning into minor and major. Minor cleaning is performed in the facility following a low-risk process such as aliquoting of reagents, media change and minimal cell manipulation, while major cleanings are to be performed following completion of production batches and high-risk manipulation. Maintenance cleaning can be done on a weekly, fortnightly and quarterly basis, according to a predetermined schedule. Cleaning should consist of equipment, work surfaces, floor, walls and ceilings.

3.14 Quality Control Requirement for Stem Cell Therapy Products

Cell therapy products contain living cells. These cells can be used to treat a person's diseased or injured cells by supplementing or replacing the original cells' function. In recent years, stem cell therapies have become widely available for clinical, general wellness and cosmetic application. Many stem cell manufacturing facilities have emerged to cater to the increasing demand for stem cell products. In the haste of

Table 3.3 Example of combination and rotation of cleaning reagents for cleaning in cleanroom

Monthly rotation	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Reagent X	√		√		√		√		√		√	
Reagent Y		√		√		√		√		√		√

meeting the market demands, we should not neglect the fact that quality control is an important and critical step in the manufacturing process to ensure product safety, consistency, effectiveness and good quality following GMP compliance. To comply with the GMP principles, we need to ensure that the entire process of cell preparations is controlled starting from collection and manipulation of raw materials, processing of products, quality controls, storage temperature, labelling, packaging and release (Fagioli and Ferrero 2015).

Stem cells play an essential role as a new therapeutic alternative in advanced therapies and regenerative medicine. This is due to their characteristics which are multipotent, easy to be harvested and expended, can be differentiated into multiple cell lineages, and their regenerative and immunomodulatory properties, and their ability to migrate to the area of injury (Guadix et al. 2019; Wuchter et al. 2015; Majumder and Olsen 2018). Stem cells can be categorised into three different types, namely embryonic stem cells (ESC), induced pluripotent stem cells (iPSC) and mesenchymal stem cells (MSCs). Since ESC raises ethical concerns, it is less widely used. Mesenchymal stem cells are plastic adherent and multipotent with the capability to differentiate into adipocytes, osteocytes and chondrocytes (Dominici et al. 2006). They also express specific surface antigens such as CD105, CD73 and CD90 and lack the expression of CD45, CD34, CD14 or CD11b, CD19 and HLA-DR and have the ability to express stem cell-specific genes such as *NANOG, OCT-4* and *SOX-2* (Dominici et al. 2006). With the prolonged expansion and multiple passaging, there is a possibility for the MSCs to lose their stemness properties (Saei Arezoumand et al. 2017). Therefore, it is pertinent to perform short studies using the same manufacturing protocol to ensure that the final product still exhibits the characteristics of MSCs. The maximum days in culture and maximum passage of the final product should be established during these studies to be used as part of the product release criteria. The shelf-life of the final product should also be evaluated to determine the optimal storage medium and temperature to plan for transportation and logistics (Seet et al. 2012; Ude et al. 2018).

3.15 Selection of Critical Material

3.15.1 Selection and Evaluation of Donor Suitability

The donor selection are dependent on the usage of the stem cells, either for autologous or allogenic use. In the autologous setting, donor serological testing is unnecessary because there is no risk of transmitting infectious diseases from the donor to the host (Wuchter et al. 2015). Nevertheless, it is highly recommended to perform serological screening to safeguard the operators and other samples being processed in the same cleanroom from cross contamination. In allogenic setting, it is mandatory to screen the donors cells as the infectious diseases can be transmitted to allogenic users. However, allogenic cells have certain advantages such as they can be produced in bulk, stored for later use and produced on-demand. Donor identification and screening start with a complete briefing of procedures to each potential donor

(Kolkundkar et al. 2014). Each donor will be evaluated for eligibility by their medical history, physical examinations, blood tests and infectious disease screening. Before donor samples are collected for the manufacturing processes, written informed consent should be obtained. The donors should be screened for Hepatitis B, Hepatitis C, HIV and Syphilis to safeguard the cleanroom operators, prevent sample cross-contamination and most importantly, the end user's safety. Donor sample collection will only be performed if the serology results are negative (Seet et al. 2014). The cells source plays an important role during this selection because donor's age can affect the quality of the stem cells (Kolkundkar et al. 2014). Therefore, the donor's age should be carefully considered to maintain the eligibility criteria within the specific range, especially for the allogenic donor.

3.15.2 Raw Materials Qualification

The materials used for the manufacturing process are known as ancillary materials (AM), which are the components, reagents or materials used that give an effect on the cell product but are not intended to be part of the final product. AMs include reagents for cell isolation, culture and cryopreservation media, and disposables such as plasticware. The term 'ancillary material' is not internationally recognised by regulators, and the nomenclature is varying among regions whereby the AMs are called 'raw materials' in Europe. Because the raw materials have direct contact with the cells destined for clinical use, it can affect the safety, potency and purity of the cell product. The raw materials qualification should focus on five parts which are: (1) identification, (2) selection and suitability for manufacturing, (3) characterisation, (4) vendor qualification and (5) quality assurance and control.

Raw materials used in GMP manufacturing processes, including reagents and consumables, play an important role in the safety and quality of the final product. All reagents used in the process should be acceptable grades, such as clinical grade, cell therapy grade or 'for further manufacturing grade' and must come with a Certificate of Analysis or equivalent. Unless there are no other alternatives, research grade reagents should be avoided. If a research grade reagent cannot be avoided, the organisation should perform a risk assessment and develop a mitigation plan to minimise the transfer of the said reagent to the end user. Furthermore, clearly defining the usage of the reagents in the clinical manufacturing process will help to clarify the risk level of the reagents further. For example, the culture medium used for the cell's expansion will be processed multiple times and further diluted may impose less risk compared to the cryopreservation medium used to store the cells before infusion.

Culture medium and reagents should also not contain any animal components. This is to reduce the risk of prion diseases transmission to human such as bovine spongiform encephalopathy or more commonly known as the mad cow disease. Moreover, the use of animal products such as foetal bovine serum (FBS) raises ethical concerns as the blood harvesting process is considered inhumane. Certain religious and cultural beliefs also prohibit the use of bovine and porcine-origin

products (Jochems et al. 2002; Easterbrook and Maddern 2008; Mellor and Gregory 2003).

Consumables used should also be of acceptable standards with appropriate certifications such as Certificate of Conformance, Sterility and Irradiation, etc. Consumables can be classified into three categories. The first category is consumables which have a direct contact with the product and may have a direct impact on the quality, safety and efficacy of the product. For example, cell culture vessel, serological pipettes, tubes, filters and micropipette tips. The second category is consumables that do not come into direct contact with the product but may affect the product's quality, safety and efficacy. Some examples of these products include gloves, masks, garment, cleaning reagents and surface wipers. The third category is consumables that do not affect the product's quality, safety and efficacy, such as cleanroom paper, sticky mats and other housekeeping items (Sivakumar 2015).

The first two categories are usually referred to as Critical Material. All Critical Materials, including medium and reagents, should be controlled when they arrived at the manufacturing facility. A stock management system should be established to determine if the material can be released for use after receiving proper documentation or passing required validations, quarantined for further testing or verification, or rejected due to unacceptable condition such as item expired, unacceptable delivery conditions, damaged, wrong item delivered and so on. To identify if the item delivered is acceptable, the organisation can develop a Critical Material Specification (CMS) sheet specifying the appearance, delivery/storage condition, acceptable criteria, validation tests and documentation required for each Critical Material (Seet et al. 2013; Khairul et al. 2012). Proper stock management will ensure that only approved materials are used in the manufacturing process to safeguard the product quality.

3.15.3 Master Cells Stock

Master cells stock or bank has been proposed as an alternative source of MSCs for tissue engineering and regenerative medicine applications due to the increasing demand for GMP compliance product. The MSCs from several healthy donors will be isolated, pooled and frozen in several hundred small aliquots or vials and will be repeatedly expanded into the final product if required. The extensive quality control testing will be performed on the several aliquots of the batches according to the release criteria which is similar to the quality control testing used in one-donor one batch approaches (Wuchter et al. 2015).

3.16 Production Protocol

Designing the product manufacturing process is important following the current GMP guidelines. The main steps of the production protocol are the cell isolation and expansion process; validation phases including all quality controls testing for the

characterisation, functionality, potency and safety of the MSCs; and the quality release to guarantee the safety of the final product for patient infusion (Torre et al. 2015). The manufacturing process design needs to be validated using the same protocol to ensure the final product meets the same specifications from batch-to-batch processes. The product that is consistently being manufactured needs to be verified, defined and approved according to quality standards. For autologous MSC-based products generate a single or one batch product for each process, while for the allogeneic process can generate multiple batches for each process. Manufacturing protocols should be validated together with the whole facility process, including cleaning procedure, environmental monitoring, transportation, disinfectants, media fill, etc. The staff must be competent to perform the activities and follow defined and authorised procedures for every stage of each manufacturing process.

3.17 Validation

GMP validation is part of the quality assurance programme. The organisation has to demonstrate that the products they produced are fit for their intended use by ensuring that all process, materials, activities, facility and equipment used in the manufacturing processes are able to reproduce the product with acceptable parameters.

3.18 Process Validation

All new manufacturing processes should be validated before a product is released to the market and should produce product with consistent quality (PIC/S 2021; GMP7.com 2019). Before starting an actual manufacturing process, all procedures should be simulated with nutrient broth, such as tryptone soy broth (TSB). This is used to evaluate the aseptic of the manufacturing process known as Media Fill Validation (MFV). Media fill validations need to return three consecutive passes before they can be considered a successful validation. After a successful MFV, an actual manufacturing process need to be also performed with three consecutive passes, to standardise the best culture condition and to demonstrate the safety and feasibility of the production protocol (Torre et al. 2015). This is sometimes referred to as Dry Runs as the products from these validations should not be used (PIC/S 2021). Cleanroom operators should also have their aseptic techniques validated from time to time. Only trained and competent personnel are qualified to perform the validation. Validation should be performed if there are significant changes in terms of facilities, equipment and processes that may affect the quality of the product.

3.18.1 Media Fill Validation

MFV test consists of a simulation of the manufacturing process using a microbial growth medium instead of the cells and reagents to validate the production process. This simulation is to identify any weakness in manufacture protocol that can cause microbiological contamination to the final cell product and also to qualify the operators. The selected medium should support a wide-ranging of microorganisms with a low number, i.e. 10–100 CFU/unit or less and pass a 'growth promotion test' before starting the process simulation. Any microbial growth must be detected within 3 days for bacteria and up to 5 days for yeasts and moulds. The guideline recommends the use of Tryptic Soy Broth (TSB) for aerobic bacteria, yeasts and moulds. The used TSB medium should be clear enough to observe turbidity and filterability if the cell manufacturing process requires a filtration step (Serra et al. 2014).

The materials used for validation must be the same as those used for the manufacturing process, sterile, pyrogen-free, latex-free (if necessary) and cell culture tested. Even materials used for environmental monitoring such as contact and settle plates should be the same utilised in the manufacturing process. MFV carried out in the GMP facility, where the production activities will be used for cell manipulation.

All steps in the manipulation processes (such as preparation of culture medium, medium changes and trypsinisations, seeding of cells into a biomaterial/scaffold, processing of raw material, i.e. bone marrow, cartilage tissue, etc. and/or final product) and including the movement of materials inside and outside the biohazard hood should be performed, but incubation times can be shortened in which the product is not opened or manipulated. The media fill samples will be incubated at 22.5 ± 2.5 °C for a minimum of 7 days followed by incubation at 32.5 ± 2.5 °C for a total minimum incubation time of 14 days. The validation result should clarify either the microbiological test has been correctly performed or either it can find any possible weakness in the process or personnel activities. After the appropriate investigations and eventual corrective actions (if needed), media fill validation should be repeated. If the acceptance criteria are all satisfied, the ability to proceed with the routine activities of production can be restored and a manufacturing process can start.

3.18.2 Material Validation/Use Test

Some of the materials such as reagents, culture medium and growth supplements used during the manufacturing process needs to be validated prior to use to reduce risk of non-functionality creating additional cost and loss of time and efficacy with relevant specification (Kolkundkar et al. 2014). This validation process is referred to as the use test and should be performed on each batch of the item received. The source, origin and suitability of the starting material are critical to manufacture a consistent MSCs therapy product. This testing should be done before the manufacturing process starts. The functionality of culture media, reagents and

growth supplement will be tested in the standard cell culture setting either in the research laboratory or GMP facility. The cells proliferation and cells growth recorded in the use testing validation form for inspection and to determine if there are any problems within the material batch before it can be released for manufacturing. Other material such as labels and packaging material should also be validated.

3.18.3 Dry Run

The dry run validation is a simulation of the manufacturing process using the actual sample and materials to validate the production protocol. This validation is important to improve and strengthen the protocol. Dry run validation should be done for three consecutive passes before the manufacturing can start. During this validation, production personnel also will be trained to familiarise themselves with the production protocol. The different production protocol needs dry run validation separately. Dry runs will identify any ambiguous instructions and mistakes within the production protocol. Once all validation documentation has been verified, the documents will be approved, effective and signed by the responsible parties involved. No further changes to the documentation are made without repeating the approval process on any change.

3.19 Equipment and Facility Validation

Equipment plays an important role in stem cell manufacturing facilities. They are required to produce consistent and good quality products. Hence, before a piece of equipment is put to use, it needs to undergo Installation Qualification (IQ), Operational Qualification (OQ) and Performance Qualification (PQ) to ensure suitability for its intended use. Aside from equipment, the facility itself will also need to be validated. Among the validation which needs to be performed include, but not limited to, Heating, Ventilation and Air Conditioning (HVAC) system validation, cleaning validation, cleanroom validation, operator capacity, and any other validations deem necessary by the organisation (US FDA 2011).

3.20 Quality Control Testing and Release of the MSCs

During manufacturing, the organisation should establish in-process quality control checkpoints which are deem suitable for their processes such as sterility results of aliquots (if any medium or reagents need to be aliquoted), culture medium which are prepared by the addition of other components, blood culture results (if the manufacturing process requires the use of blood products) and monitoring for cell culture contamination at intervals set by the organisation. Critical stop points such as cell culture contamination should be built into the system to protect the quality of the

final product. If the cell culture was found to be contaminated, the manufacturing process should be terminated immediately, and the contaminated product is discarded according to the organisation's waste management procedure. However, if culture medium aliquots or blood culture return a positive sterility result, a second test sample should be sent for confirmation and the cell culture should be monitored closely for signs of contamination since contaminated culture medium will most definitely lead to cell culture contamination (Seet et al. 2014). When in doubt, terminate that batch of cell culture.

Aside from in-process quality control, the organisation need to establish release criteria for their final products. These can include, but not limited to, cell count, cell viability, number of maximum allowable passages, and total days in culture and cell characterisation. Micro-contamination, mycoplasma, endotoxin and Gram stain are also standard testing required to release the MSCs (Galvez et al. 2014). It is highly recommended for the micro-contamination, mycoplasma and endotoxin testing to be performed by a third-party testing laboratory. Contamination by external factors can cause toxic reactions; therefore, it is crucial to ensure the absence of any pyrogens in MSC-based products before being used to human. The MSC-based final product must be sterile by evaluating the sterility of hMSC-based products by directly inoculating the sample into the growth media. This test requires an incubation step for 14 days, exceeding the short shelf-life (48–72 h) of the cells (Guadix et al. 2019). Since the samples are usually sent a few days prior to product release, the risk of contamination cannot be neglected during the days until product release. Since stem cell products have a short shelf-life and need to be administered as soon as possible after release, a rapid sterility test such as Gram staining can be performed in-house as part of the release criteria (US FDA 2020).

The characterisation of MSCs is the most critical criteria according to the requirements by the health authorities. The evaluation which includes cell's identity, viability, purity, potency, proliferative capacity, genomic stability, tumorigenicity and efficacy are the minimum criteria for the MSCs characterisation (Martín et al. 2014; Ra et al. 2011; Guadix et al. 2019). MSCs can be characterised at different points during expansion process such as at the isolation stage, reference sample, intermediate product, final product, retention sample, master cell bank (MCB) and working cell bank. According to The International Society of Cellular Therapy (ISCT), three minimum criteria of MSC are: (1) MSCs must be plastic adherent (appearing under the microscope as fibroblast cells); (2) MSCs must express CD73, CD90, CD105, Oct-4, Rex-1, Sox-2, and there must be an absence of expression of CD45, CD34, CD14 or CD11b, CD79 alpha or CD19, and human leukocyte antigen (HLA)-DR surface molecules; (3) MSCs must have high capability to differentiate to adipocytes, chondroblasts and osteoblasts under standard in vitro culture conditions (Dominici et al. 2006). The cell differentiation is evaluated by specific staining which are Von Kossa or Alizarin Red staining for osteogenic differentiation through calcium deposition, Oil Red O staining for adipocyte differentiation through the presence of lipid droplets, and Alcian Blue staining for chondrogenic differentiation through the presence of cellular aggregates in the culture.

3.21 Personnel Training and Competency

All personnel working in the organisation who are directly involved in the manufacturing process or whose activities affect the quality of the product will need to undergo relevant training and assessment to achieve competency in performing their designated duties. A job description specifying duties and responsibilities should be issued to new personnel when working with the organisation (Frost 2019). Relevant department managers should identify training requirements for the personnel and a training plan developed for them. All new personnel should complete the training assigned to them before performing any of the related tasks unsupervised. Before completing the training and achieving competency, supervision by competent personnel is mandatory.

Training can be conducted by having the trainee read and understand specific documents, attend lectures and observe the trainer perform certain processes or operate certain equipment. At the completion of the training, the trainee will be required to undergo competency assessment in a verbal interview, written assessment or successfully performing and completing specific tasks. After completing the initial training, all personnel will also need to undergo ongoing training in response to workplace changes or attend periodical refresher training (Sullivan et al. 2019; Lynch 2020). Additional training can be provided if the personnel has not passed the initial training and requires retraining or when new procedures are introduced.

3.22 Regulatory Requirement Process

GMP is the set of procedures operated through the organisation's quality management system (QMS) to confirm the quality, safety and efficacy (QSE) for medicinal product manufacturing, including cell-based therapeutic products (Medcalf et al. 2014). Stem cell products, which are classified under Advanced Therapy Products (ATPs) or Cellular & Gene Therapy Products (CGTPs), respectively in EU and US, are required to be manufactured in cGMP accredited facility and the whole production process need to be controlled and quality assured through the cGMP quality control and management system. The cell manufacturing process needs to ensure that the process of donor tissue procurement, laboratory culturing, cryo-storage, quality control, and delivery of cell products to consumers by cold chain and compliant to good delivery practice (GDP) has been designed to suit existing and higher demand of future (Teng et al. 2014; Lee et al. 2017). The code of rules under GMP is defined by territorial authorities and the guidelines encompass a minimum set of practices to ensure compliance. cGMP is the acronym used by the United States of America (US) regulatory authority, the Food and Drug Administration (FDA), to refer to 'current Good Manufacturing Practice(s)' while European Medicines Agency (EMA) which coordinates compliance to these standards and harmonises GMP activities at European Union (EU) level refer it as GMP only. The addition of the word 'Current' is used to implicate that the practices and guidelines

will be improved from time to time and the manufacturers are expected to enhance their QMS according to the latest guidelines, regulations and practices.

cGMP sets the minimal standards of both the manufacturing process and the resources utilised. The resources include physical facilities, human resource, raw materials and also the documentation used. It is pertinent to understand that the cGMP evolved based on previous standards and regulation, supplemented with issues learned from real-life incidents. Thus, the cGMP only defines a minimal standard that need to be followed by the pharmaceutical or cellular therapeutics manufacturers and it is important to improve the QMS based on the knowledge, practice and experience from similar product manufacturing. cGMP system for cell-based therapeutics is widely developed based upon practice for pharmaceuticals. The certification of compliance to cGMP by authorities will provide faith to clinical practitioners and the end user that the product is manufactured under the minimal standard for usage. The manufacturing and marketing of CGTPs are controlled by the relevant national authorities based on the *International Conference on Harmonisation of Pharmaceuticals for Human Use* and also based on harmonisation of scientific and technical aspects by various other international organisations including the International Council for Harmonisation (ICH), International Pharmaceutical Regulators Programme (IPRP), the Pharmaceutical Inspection Co-operation Scheme (PIC/S), Asia-Pacific Economic Cooperation (APEC) and International Coalition of Medicines Regulatory Authorities (ICMRA), which standardises the potential interpretations and applications of the corresponding national recommendations. The harmonisation at the global level enables the development and registration of safe, effective and high-quality CGTPs in a resource-efficient manner. This process also avoids repeated animal testing, clinical trial and post-marketing surveillance, which reduce the cost and time taken for commercialisation of new CGTPs, without compromising safety and effectiveness. In line with that, national or local authorities may inspect the facilities in another country to confirm that the foreign cGMP facility confirms to the standards sets by it before the marketing authorisation for the imported product is given.

A cGMP facility is a facility that is built and operated in compliance with current *cGMP* for manufacturing of drugs or cellular products (Giancola et al. 2012). It includes the production area, gowning and de-gowning area, quality testing area and various storage areas such as raw materials, materials released for production usage, finished product, and support laboratory areas (Dietz et al. 2007). The design of the cGMP facility should incorporate the concept of quality by design, whereby the facility design itself should be able to support the production of CGTPs in accordance with the requirements of cGMP guidelines. The design of cGMP facility for CGTPs production must be able to manufacture clean, safe and effective products while avoiding the risk of contamination and errors during production. In addition, the design also shall ensure biosafety, eliminate contact between the personnel and any pathogenic or harmful agents, and prevent the release of harmful materials into the environment. Among the important features that ensure the compliance to cGMP quality standards are maintenance of differential pressure between different room classification, separation of air handling systems for different production units,

restricted and tightly controlled access to various area within the facility, and unidirectional flow of personnel, raw materials, products and waste materials (Abou-El-Enein et al. 2013). Another important feature in cGMP facility or any cleanroom facility is the design that avoids the generation of any unwanted particles and eases the cleaning process, such as seamless floor, minimal ledge, easy to clean flooring and wall, and moveable equipment.

In ensuring various environmental parameters such as temperature, humidity, pressure, airflow pattern and content of airborne particle of the cleanrooms and the GMP facility is well monitored, controlled and maintained, both the environmental monitoring system (EMS) and building monitoring system (BMS) are required. These systems continuously monitor the environmental parameters that is critical in maintaining the cleanliness of the facility, complying with the user requirement for CGTPs manufacturing and storage, and protecting the personnel, the product and the environment from contamination and hazardous material exposure. These systems assist the facility manager to monitor the performance of various equipment, heating systems, air-conditioning systems, ventilation and personnel access controls (Fig. 3.4).

The national regulatory body also set the minimal tests that should be conducted and the quality of the product that should be achieved before the product is authorised for marketing as therapeutics, in this case as CGTP. Among the information and characterisation needed by the regulatory body are product type, indication, route of administration, cell origin (autologous versus allogeneic), biological

Fig. 3.4 cGMP Facility Management and Monitoring in CTERM, UKM. (**a–c**) Building and equipment monitoring system; (**d**) CCTV security camera; (**e**) Equipment for environmental monitoring (air sampler and particle counter); (**f**) Equipment maintenance and service by authorised personnel; (**g**) Facility personnel cleaning

characterisation of the cell, purity, potency, dosage, mechanism of action, manufacturing process, homing to target area, viability and longevity, biodistribution, tumorigenicity risk, side effects, data on GMP compliance manufacturing, and safety and efficacy data if the market authorisation is being requested (Mendicino et al. 2019). In addition, the pre-clinical study data is required for products applying to conduct a clinical trial. The main pre-clinical study data are accreditation record of the GLP compliance animal facility, evidence of rigorous and independent peer review and regulatory oversight of the pre-clinical study data, small and big animal safety data, comprehensive animal toxicology data, proof of desired effect on animal, side effects, biodistribution, and physiologic integration or differentiation, if any. The animal model should be chosen in a way that allows the prediction of response to the same CGTPs by a human. Biocompatibility and safety data for any supplementary substances that are administrated together or as a part of the cell-based medicinal product, such as cellular components, biomolecules, biomaterials and/or chemical substances also need to be provided, if any (Salmikangas et al. 2015).

The clinical trial also should be proven to be conducted according to Good Clinical Practice (GCP). Each batch of CGTPs or ATPs should pass a very strict and specific test control depending on the characteristics of the cell therapy product, in accordance with the national guidelines such as *European Pharmacopoeia* (EurPh) or *The United States Pharmacopoeia* (USP). Tissues and cells used as source material for cell therapy products should comply with the requirements set by the regulatory bodies too. An important factor in sourcing tissue or cell is screening for various infectious diseases and eliminating the risk of infectious or genetic diseases transmission. Besides that, aseptic manufacturing process validation and qualifying starting material is of utmost importance for the preparation of microorganism-free CGTPs since terminal sterilisation or removal/inactivation of microbial contaminants from the finished product is not possible. (World Health Organization 2002). Sterility testing is required to be conducted before every batch release, as much as possible. Sterility testing is not possible to be performed right before the product release due to the short shelf-life of the CGTPs and long incubation period for sterility test (14 days). In this case, the sterility testing should be performed earlier during cell culture to obtain at least a 3-day interim result before the product release and possibly in the last manipulation step before the product release such as rapid gram stain (Ridgway et al. 2015).

Another challenge in the manufacturing and marketing process of stem cell products is the need to ensure that products produced in GMP laboratories are delivered to consumers without experiencing any deterioration in product quality and efficacy. This criterion requires ultra-cold chain delivery. It is usually stored at below −130 °C and the delivery process can be done at −80 °C over a period with minimal quality deterioration. Since the delivery and storage conditions of cell products such as temperature and sterile conditions are very important in ensuring its safety and efficiency, the whole process of manufacturing, packaging, labelling, shipping and storage is part of the authority approval requirements. Therefore, the cold chain delivery process must be stable, comply with the rules, have continuous quality

control and be able to meet marketing needs such as delivery frequency, delivery period and delivery location. Approval from the authorities needs to be regained if the delivery process or operator needs to be changed. The need to ensure that the cold chain delivery process is stable and approved by the authorities also makes the production cost of stem cell products higher and more complicated than traditional medicines (Teng et al. 2014).

Another challenge faced in the marketing of stem cell products is the high cost of stem cell therapy, but these costs are not borne by insurance companies and are not paid for by the government health system. This is because most cell therapies, although approved by the authorities and guaranteed to be effective, are new treatment methods and are considered alternative treatments to existing treatments and are not recognised by health insurance agencies and government health systems as recognised treatment practices. In addition, cell therapy products sometimes require additional equipment to process stem cell products before they are injected into patients. These constraints lower demand and result in increased production costs. Most cell therapy products that have been approved at this time are for autologous use. The manufacture of stem cell products for own use, in a personalised medical concept, also causes the cost of cell therapy to be very high compared to conventional treatments or allogenic cell therapy (Lee et al. 2017).

The regulatory framework is also a challenge to the industry to bring cell therapy from research laboratories to market. The existing framework is based on the old practice whereby all products for therapeutic use have to undergo multi-phase randomised controlled clinical trials. This clinical trial method requires a large number of patients, including control patients, as well as incurs high costs because treatment cannot be charged to patients. Most stem cell therapies are studied to treat diseases that are difficult to treat and are rare. This causes difficulties in finding suitable patients in large numbers for clinical trials (Lee et al. 2017). In addition, each country has a different framework and different definitions (for cell types and processes) for obtaining approval for stem cell treatment. These different frameworks have led industry players to take opportunities in countries with loose regulations or weak enforcement to offer therapeutic cell treatments not only to local patients but also overseas patients. This method has become widespread under the auspices of medical tourism and, to some extent, has reduced the need to obtain approval from the authorities before this stem cell therapy is offered to patients. In addition, one of the definitions that has always been an issue is that cells are minimally manipulated. The definition of minimally manipulated and processed cells above the minimum remains unclear as this field is still new, and diverse cell procurement and processing methods are still being developed. This results in confusion in classifying a stem cell product in applying for approval for treatment with the stem cell product (Rosemann et al. 2019; Gopalan et al. 2020; Nagai 2019).

Despite legal constraints to market unapproved products, a handful of societies demanded unapproved cell therapies be used by critically ill patients and had no other choice provided they bear any risks that may arise. Consequently, the United States has allowed treatment in the study stage by critically ill patients since May 30, 2018. But most countries still do not support or take similar measures (Nagai

2019). Regulatory barriers have also led to industry demand for a simpler and less costly regulatory framework. Countries such as Japan, the United States and the European Union have begun to adopt simpler frameworks whereby the number of patients, time periods and costs involved in clinical trials can be dramatically reduced. But this simpler framework is devoted to regenerative medicine treatments and innovative medicine, including cell therapy products, which treat rare diseases, no standard treatment for the disease or such cell therapy products show excellent benefits over existing treatments, as well has been proven safe for human use in previous clinical studies. These conditional marketing approval products are allowed to be used in treating patients with patients paying for such treatment, and the effects of the treatment need to be closely monitored. A post-marketing survey should be conducted and the results of this survey should be reported to the authorities to obtain full approval (Nagai 2019).

3.23 Ethics, Legal and Social Issues in Stem Cell Research

Although stem cell therapy is not new considering the established bone marrow transplantation protocol for haematopoietic pathologies, the trial for using haematopoietic stem cell transplant for different indications is still ongoing. It is important to note that the focus of the discussion here is on the use of stem cells for novel therapies. Interest in novel stem therapies stems from the breakthrough in stem cell isolation, characterisation, expansion and differentiation techniques and science. The mounting publications of stem cells research and reviews in the literature have far fetching effects on the public. Stem cells therapy was touted as the next medical and biotechnology revolution. The social media have picked on the prospect of stem cell therapy and reported them with overzealous promise. Often the scientific reports are even sensationalised in public media. This had triggered both the interest and controversies from patients and public in general and provided them hope or myths. Plant stem cells made its way in beauty products added to the confusion.

In the early days of stem cells research, much of the controversy was in the source of the stem cells and how they were derived. The stem cell source in the central of the debate was the embryo. Research in embryonic stem cells was seen as ‘playing god’, and destroying and devaluing life. Much of the debate has quietened down after a consensus was achieved with major religious bodies on the use of embryonic stem cells for therapeutic and not reproductive use, and stricter regulation on the process of obtaining embryos from informed consented donors implemented. Various guidelines on stem cell research has been established and are being regularly updated as the science evolves. The definition and distinction of the various stem cell types have further allayed some of the initial confusion. In a way, stem cell research in the scientific community is highly regulated by academic institutions and government bodies due to the complexity and the red tape involved in obtaining these cells and the high cost associated with such research.

However, the adult stem cells such as mesenchymal stromal cells (previously known as mesenchymal stem cells) are relatively easier to work with. The ease of

extracting or isolating crude adult stem cell populations from the human body has tempted many clinicians and aestheticians to offer stem cells as therapeutics or 'supplements' to their patients. Many saw this as an extension of their current procedures and services, and stem cell was not a medical product and that infusing stem cells will not differ much from the established standard of blood transfusion especially if they are derived autologously. The typical example here is the use of crude cell population derived from fat during liposuction also known as stromal vascular fraction. If the need for furthering cell culture and propagation, a simple Class II tissue culture facility and aseptic practice was deemed sufficient for such purpose. Hence, aesthetic clinics and beauty centres became one of the early proponents of stem cell therapy.

With the advent of induced pluripotent stem cells and allogenic mesenchymal stromal cell therapy, the race was on again to set up large-scale cell production facilities. Good Manufacturing Practice (GMP) was seen as a hurdle to just meet regulatory demands. Many ventures capitalist rode on this trend following the examples of international corporations such as Mesoblast Ltd., Athersys and Pluristem Therapeutics. Private cord blood banking, blood processing or fractionation companies and immune cell therapeutics companies became involved in such stem cell therapy as an extension to their existing production lines. Against this background, many researchers, clinicians or businessmen with an entrepreneurial bent joined venture with venture capitalist to set up their own GMP compliant facilities for cell production. The first stem cell trial was initiated by Geron Corporation using embryonic stem cells back in the 2010. In the millennium, advertisements offering stem cell therapy were already widespread in the private settings as regulation on such novel therapeutical product was still developing. As such, the results of such therapies were not published apart from the few anecdotal clinical testimonials published on commercial websites. Nevertheless, there were a handful of publication reporting the adverse effect of stem cell therapy when the patients ended up seeking treatment in public or tertiary hospitals.

3.24 Unapproved Stem Cell Therapy

Due to the different regulatory landscape for cell therapy in different countries, there may be a definition gap to what is accepted as unapproved therapies (Turner 2015a, b, c). It is also important to recognise that while stem cells are derived from the body and may not be seen as a product or drug and hence, the perception that its use is associated with an existing medical procedure under the discretion of the treating physician. However, the bulk of unapproved therapies involves manipulation such as culturing and propagation in the laboratory. Thus, it ought to be regarded as a medicinal product or drug. The chemistry, manufacturing procedure and controls (CMC) should be defined and controlled to ensure the product is safe, effective and consistent. In general, the definition I would like to put forth is that unapproved therapies are 'therapies were prescribed without undergoing proper clinical trial or still in clinical trial and/or that the manufacturing process and the

product has yet to undergo proper validation'. Most of the critics on unapproved therapies are on the ethics of direct to customer marketing, unfounded therapeutic claims and misleading scientific information. Two scientific societies that strongly oppose unapproved and unregulated stem cell therapies are the International Society of Cell Therapy (ISCT) and International Society for Stem Cell Research (ISSCR).

Industry player's/private physician's perspectives on unapproved stem cell therapy

- Product is safe (GMP compliant)
- Similar to blood transfusion/bone marrow transplant
- Customer's right to treatment
- Meeting an unmet medical need
- Customer's willingness & consent obtained
- To slowly grow the field until more evidence is eventually obtained
- To charter the route for using cells as supplement

Regulator's/academic researcher's perspectives on unapproved stem cell therapy

- Not to put people in unnecessary risks (protecting citizens) esp. the vulnerable or ignorant groups
- International Regulatory Guidelines & sentiments
- Risks outweighs benefits
- No solid (objective) evidence besides testimonials (subjective)
- No pre-clinical trial/clinical trial data
- No standard formulation/protocol
- No long-term studies
- Lost time for proven therapy
- Selling hope
- Lack of rigorous and independent expert reviews
- Threatens and undermines the progress of basic research and clinical trials

3.25 Risk, Limitation and Adverse Effects Associated with Stem Cell Therapy

While autologous stem cell transplantation logically has fewer issues to deal with compared to those derived from allogenic source, there are still concerns as to the possible contaminant and genetic alterations introduced during in vitro manipulation of the cells. The science behind the mechanism of the different type of stem cells, its efficacious dose, and its long-term effect in the body have not been fully investigated or understood. Moreover, scientists are still uncovering the full potential of stem cells by varying the protocol of stem cell preparation before implantation. Safety of implanting cells differentiated from embryonic stem cells or induced pluripotent stem cells must be ensured by rigorous genetic screening to rule out risk of tumour formation. Even with stem cells such as mesenchymal stromal cells which are

considered relatively low risk due to the assumption of their short-term persistence in the body, reports have shown the evidence of long-term engraftment of at least a fraction of these cells in the body which warrants longer term follow-ups. Other possible adverse effects associated with stem cell therapy published by companies offering mesenchymal stromal cell infusion in their website includes fatigue, headache and nausea.

A few of these adverse effects' reports were published in international journal cautioning the potential risks of stem cell therapy, but more importantly they served as lessons to be learnt for developing safer and efficacious stem cell therapy. A paper published in PloS Medicine in 2009 reported the case of a boy who had developed a very slow growing form of cancer called glioneural neoplasm in the brain and spinal cord from the multiple injections of neural stem cells 4 years ago to treat his neurological condition called ataxia telangiectasia (AT). The neural stem cells were derived from the brains of aborted foetuses and the tumour was shown to be derived from non-host origin (Amariglio et al. 2009).

In an article published online in Scientific American in 2012, it was reported that a woman underwent a face-lift procedure 3 months ago where mesenchymal stromal cells derived from her abdominal fat along with a dermal filler were injected in the area around her eyes developed bone fragments in the tissues around the area of the eye. This was believed to be due to the interaction between the mesenchymal stromal cells and a mineral component of the dermal filler, calcium hydroxyapatite, which plastic surgeons have safely used for more than 20 years to reduce the appearance of wrinkles (Jabr 2012). In a letter to the editor published on June 22, 2016, at The New England Journal of Medicine, it was reported the case of a man who underwent intrathecal infusions for the treatment of residual deficits from an ischemic stroke, what was described as consisting of mesenchymal, embryonic and foetal neural stem cells. He eventually developed progressive lower back pain, paraplegia and urinary incontinence. Magnetic resonance imaging (MRI) revealed a lesion of the thoracic spinal cord and thecal sac. Neuropathological analysis revealed a densely cellular, highly proliferative, primitive neoplasm with glial differentiation (Berkowitz et al. 2016).

In March 2017, in a brief report published by The New England Journal of Medicine, three women had macular degeneration disease but still fairly functional vision prior to the procedure and were blinded by the next day after autologous fat stem cell injection into the eye. One woman became totally blind, and the others were declared legally blind (Kuriyan et al. 2017). In June the same year, a letter to the editor in the Journal of Neuro-Ophthalmology reported about a man who was injected with stem cells into the left frontotemporal region of the scalp with the aim of enhancing hair growth had immediately developed shortness of breath and severe pain at the injection site. He then experienced acute loss of vision in the left eye and paralysis of the right hand. MRI revealed an acute left frontal cerebral infarct and left fundus showed changes consistent with a central retinal artery occlusion (Burke et al. 2017). Although the number of severe adverse effects reported was seemingly low considering the number of patients treated in the private setting so far, underreporting cannot be ruled out.

3.26 Conclusion and the Way Forward

As of any surgical procedure and drugs there are potential risks and contraindications in stem cell therapy. Often patients are informed and have consented to these procedures and claimed their rights to try such unapproved stem cell therapies. Hence, as the society become more informed of the risk and benefits, they can make better informed decision. This underpins the importance of scientific communication to the lay public in a more effective manner. Ultimately, a cooperation among the public, industry, government and academia will be required to progress this novel technology in a safe, sustainable and efficacious manner in a market-driven economy that can advance and benefit humankind in a significant manner (Matthews and Iltis 2015).

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Global Regulatory Frameworks and Quality Standards for Stem Cells Therapy and Regenerative Medicines

4

Sudhir Sawarkar and Asawari Bapat

Abstract

Regenerative medicine consists of Cell, Gene and Tissue (CGT) therapies, novel therapeutic technologies that can treat and sometimes cure various illnesses and ailments. Many stem cell facilities and regenerative clinics operating in the United States are immeasurable globally, primarily throughout Latin America, Europe, Asia, China, Israel and the Middle East. In recent times, cell and gene therapies have shown tremendous clinical effectiveness and have been authorized for commercial use by regulators. They are mainly being used to treat blood malignancies, metabolic disorders and immune system disorders. Cell, gene and tissue therapies are globally regulated by authorities that govern patients' safety, potency, and efficacy in each region. Regulators of medicines, devices and therapies across the globe require producers of the therapy products to meet several criteria and comply with regulations before commercializing a cell-based treatment. Occasionally, a few unscrupulous clinics and bad players advertise untested treatments as "cures"—for a fee—and have preyed on individuals eager to find a remedy for their specific ailment after traditional medicine fails to offer one. Unchecked, few bad players lead the patients towards a dangerous trend of stem cell tourism, where the cost to patients is high, and so the danger involved with these experimental therapies and the profit margin for the clinic are substantial. This chapter summarizes the regulatory environment and industry compliance for regenerative therapies globally for all the stakeholders involved in this process. Authorities globally are establishing guidelines and restrictions on

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F. A. Khan (ed.), *Stem Cell Production*,
https://doi.org/10.1007/978-981-16-7589-8_4

regenerative therapies on a risk-based basis. These include regulatory oversight of the investigational product's preclinical and clinical evaluation, compliance with applicable premarket or post-market approval requirements, and demonstration that the product is manufactured per current good manufacturing practices (cGMP) to ensure the product's safety, purity, and potency before reaching a patient. These scientifically rigorous regulatory products and procedures must substantiate any claims made regarding the safety, potency and efficacy of stem cell therapy. Apart from ICH and country-specific pharmacopoeial standard-setting organizations, many more international organizations are currently establishing standards in their respective fields of competence and then implementing them to worldwide standards or guidelines. This chapter will serve as a quick reference guide to international regulatory and quality requirements related to cell, gene and tissue therapies and regenerative medicine.

Keywords

Stem cells · Regulatory framework · Compliance · Cell therapies · Gene therapies · Regenerative medicine

4.1 Introduction

Regenerative medicine has been a current hot topic in medicine and biotechnology. However, this field has many challenges, mainly due to new, disruptive, and complex technologies. The ability and dexterity to use these new therapeutic approaches and technology remain a constant upstream and downstream challenge. This is an arduous task; due to a lack of oversight, many regulators are unaware of these techniques. Evolution is glacial. The regulators are providing stricter guidelines to ensure the safety, potency and effectiveness of therapies, delaying the applications and licencing of such technologies if any mishaps may occur. In 1954, Dr. Joseph Murray performed the first human organ transplant, transferring a kidney between identical twins (Barker and Markmann 2013). The procedure was successful, resulting from almost 50 years of research and experience in transplantation and grafting. This started a new era of treating ailments. Organ transplant technology has been in use for an extended period and has saved millions of human lives. Organ transplant has many disadvantages, including difficulties finding donors of the right age, organs of the appropriate age, and organ rejection (Sampogna et al. 2015). Regenerative medicine has also addressed the similar problem of transplanting organs from chronically sick donors. These organs have been associated with tissue degeneration, making them ineffective (Slingerland et al. 2013).

Additionally, organ transplant is symptomatic. It stems from the necessity to treat the disease. To treat a disease, it is critical to repair or replace the tissue or cells. That is to say, the organs have to be rebuilt. During the 1960s and 1970s in the nineteenth

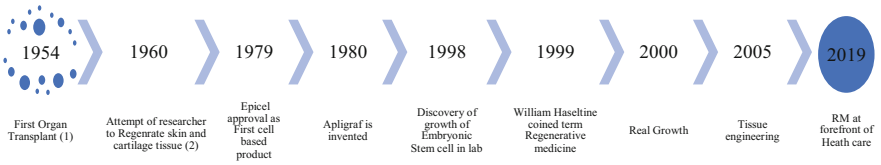


Fig. 4.1 Chronology of regenerative medicine's history

century, tremendous amounts of research were undertaken on tissue and cell regeneration methods.

Consequently, a new area of medicine known as tissue engineering (TE) and regenerative medicine has emerged (RE). Tissue engineering is a field that combines engineering and biology in order to develop biological tissues that heal, improve tissue or organ function (Langer and Vacanti 1993). TE is a subfield of regenerative medicine (RM), which, according to Dr. Heather Greenwood et al., is a rapidly growing interdisciplinary field of research and clinical applications centred on the repair, replacement, regeneration of cells, tissues, or organs in order to restore impaired function caused due to congenital disabilities, diseases, trauma and ageing. Regenerative medicine has now advanced much further than traditional organ transplants and replacement therapies. Regenerative treatments include tissue engineering, cell therapy, gene therapy, stem cell transplantation, and cell and tissue reprogramming (Greenwood et al. 2006) (Fig. 4.1).

Currently, stem cell research has established itself as a critical component of biomedical technology. In the area of biomedicine, stem cells have tremendous promise. This includes options such as disease modelling, developmental biology, medication discovery, tissue engineering and testing for hazardous toxicity. Stem cells are capable of regenerating damaged cells, tissues and organs. On one side are the many advantages of stem cell technology, and on the other side, they raise several ethical, legal and social concerns (Cuende et al. 2020). The fundamental challenge in stem cell research is related to human embryonic stem cell (hESC) lines. Both society and government are apprehensive about the human embryo and human cells and tissues (Lo and Parham 2009).

Additionally, the novel stem cell processing and manufacturing introduces the possibility of a cell developing tumorigenic properties as stem cells have an unlimited proliferative capacity. Contamination and genetic changes are possible because of in vitro modification and immunological incompatibility (Zakrzewski et al. 2019). These potential risks may have a detrimental impact on a patient's health and society at large. The technology of stem cells is still considered to be in its infancy. Therefore, major pharmaceutical firms have so far been hesitant to invest in this field in recent years. Most big pharmaceutical companies are now acquiring or investing in smaller companies, resulting in potentially marketable stem cell or regenerative medicine therapies for the future. This trend has been rising, especially after the Pandemic, which has been still ongoing.

The use of human embryonic stem cells (hESCs) is limited for several reasons. Notably, it originates from human embryos and is eliminated at the blastocyst stage.

This is highly correlated with ethical concerns. Since it is not autologous, there is a risk of host immunological rejection (Taylor et al. 2011). Recently, the area of induced pluripotent stem cells has seen a rise in attention (iPSCs). iPSCs are adult stem cells that can be programmed and differentiated via the introduction of a set of genes. Therefore, a pluripotent cell line specific to the patient can be generated. Takahashi and Yamanaka generated pluripotent stem cells from mouse embryonic and adult fibroblast cultures (Takahashi and Yamanaka 2006). Following this, the number of clinical research using iPSCs has increased dramatically, with some progressing to clinical trials.

One of the critical aspects of governance on social media that came into force is Google who implemented a ban on the digital advertisements of new, unproven medical treatments. On Friday, September 6, 2019, Google stated that it would "prohibit advertising for untested or experimental medical methods such as most stem cell therapy, cellular (non-stem) therapy, and gene therapy". According to the firm, the therapies have "dangerous health consequences" and therefore have "no place" on its platform (Google 2019). According to the current estimates, the worldwide regenerative medicine market is worth $5444 million in 2016 and is expected to grow to $39,325 million by 2023, recording a compound annual growth rate (CAGR) of 32.2% from 2017 to 2023 (Kunsel and Chandra 2018). Nonetheless, regenerative medicine and stem cell therapy present the potential to cure a broad range of serious human diseases. There are, however, global disparities in stem cell research, regulations, and practice compliance.

Consequently, developing internationally uniform standards to govern research is almost impossible. There is an ongoing effort to establish guiding concepts for all laws and standards to prevent the early commercialization of untested stem cell treatments and to promote new information based on sound scientific reasoning while considering all ethical considerations. This chapter discusses the regulatory and industry requirements for regenerative medicine and stem cell research to collect, manufacture, process, test, store and ship these products. For safeguarding human health and controlling product quality and safety, national, regional or worldwide regulatory frameworks need to be strengthened. This chapter closely looks at current regulatory scenarios globally, which offer a ready reckoner for professionals associated with these novel technologies. Global regulatory organizations develop industry standards for the stem cell research and therapy sectors. For example, International Standardization Organizations (ISO), European Pharmacopoeia, ICH, BSI, AABB (American Association of Blood Banking), Standards Coordinating Body (SCB) and other organizations develop standards for stem cell-related medical equipment, laboratory processes, clinical practices, and storage and transit practices.

4.2 International Regulatory Framework for Stem Cell and Regenerative Medicine

Regulators worldwide are attempting to create legislation that meets the requirements of their respective countries. Restriction and prohibition of specific activities in regenerative medicine regulatory frameworks are driven mainly by country-specific value systems and religious views. Numerous aspects, such as restrictions regarding human embryonic stem cells for research and their alterations, are universally accepted. Internationally regulatory requirements decide the future of regenerative medicine and stem cell therapy (Qiu et al. 2020). In a region where there are tighter restrictions, the development has almost paused, and incidences of patients travelling to get the treatment from the other region where these treatments are available have increased. Regions like the USA, China, EU, Israel, Japan and Canada are the hotspots for regenerative medicines. This chapter summarizes the regulatory and quality landscapes for stem cell and regenerative treatments on a worldwide scale. Let us look at the regional legislation that now regulates the field of regenerative medicine.

Deepak Srivastava has said that Google's new policy prohibiting advertising for speculative treatments is a much needed and welcome move to prevent the promotion of unethical medical goods such as untested stem cell therapies (ISSCR 2019). Public health, scientific research confidence, and the discovery of genuine novel treatments are threatened by the premature marketing and commercialization of untested stem cell products. "This decision is a pleasant surprise for all of us", the University of California, Davis stem cell scientist Paul Knoepfler told the press. Because the advertisements attracted many of the patients adversely impacted by the therapies, he adds, this is a significant issue. Andrew Ittleman, the lawyer, told "The Post" that the regulation would hurt genuine treatment claims. "It puts Google in the position of becoming a quasi-regulatory", Ittleman adds. As a result, genuine businesses will suffer collateral harm (Yeager 2019).

There are no regulations which are directly relevant to research utilizing "human embryonic stem cells" at the United Nations or European level or any other union, on both these levels. But there are opinions and laws or regulatory efforts regulating the use of human cloning methods. Hence, these perspectives and findings are helpful to govern research on human embryonic stem cells. Additionally, regulations linked to Horizon 2020, the EU's Eighth Framework Programme for Research and Innovation are essential at the European Union and UN/UNESCO levels.

The International Bioethics Committee (IBC) of UNESCO said on April 6, 2001, that the regulation of human embryonic stem cell research was an ethical problem and that each community had the right, but also responsibility, to debate it in its way. Considering this, the IBC encouraged governments to promote open and informed public debates at the national level. The committee recommended that the state control embryo research in countries that ensure ethical compliance. To use "surplus" embryos for stem cell research, the committee advises that donors provide their free and informed consent and that ethical bodies authorize the study. The IBC has also called for a comprehensive analysis of the advantages and risks of various stem

cell production methods. The study highlighted the need of utilizing nuclear transfer in conjunction with medical research (Smith and Revel 2001).

Following new scientific discoveries of significance, such as the induced pluripotent stem cells, the IBC called for risk-averse legislation based on the precautionary principle at both the national and international levels in its report from October 2, 2015 (International Bioethics Committee 2015). According to the study, to be "uncontroversial", embryonic stem cell research must gain wide cultural and ethical acceptance. Induced pluripotent stem cells, rather than embryonic stem cells, are one step in this direction (International Bioethics Committee 2015).

4.2.1 Europe

Europe is a varied continent. Although the European Union has a common regulatory framework, individual nations may develop their own. This is also true with regenerative medicine rules—the position of a nation on human stem cell research and therapy. We can classify the nations in the following classes of cell research and regenerative medicine. (1) Very Permissive, (2) Permissive with Restrictions, (3) By default restricted, (4) Very restricted and (5) Unregulated or unlegislated. Human embryonic stem cells (hESC) are permitted in different parts of Europe. Only a few nations permit the production of human embryos only for scientific purposes. A large number of nations only permit the extraction of human embryonic cells from excess IVF (in vitro fertilization) embryos. Only a few European nations prohibit the use of hESC. Some European countries allow the import of hESC cell lines under stringent restrictions and regulations. Europe undoubtedly is one of the top regions with much work happening on human stem cells and regenerative medicines. The number of clinical trials, reports on discoveries, directions, tissue research, transplants, advanced treatments, biobanks, and data protection shows the advancement of regenerative medicine development in Europe (Campo-Ruiz and Slørdahl 2013). It was announced in 2011 that all fundamental biological processes for the creation of plants and animals are not patentable under the European Patent Convention. The European Parliament also backed the non-patentability of biological processes such as animal and plant breeding in 2012. Due to the non-patentability of innovation, this resolution affected investment. In 2012, Europe's regulatory framework for clinical trials, medical devices, and pharmaceuticals underwent significant revisions.

Europe has centrally Human Tissues and Cell Directive (2004/23/EC), Technical Directive, Biopatent Directive (98/44/EC) from 2006, and Legislation on Medicinal Products for Human and Veterinary Use. Many European nations adopted their regulatory frameworks in response to the non-uniform application of these regulatory frameworks. Article 6 of the Biopatent Directive is ambiguous on whether some innovations are presently patentable. When there is no legal definition of either "embryo" or "industrial or commercial objectives", Article 6 prohibits their usage. This has allowed for many interpretations. In 2011, the EU Parliament decided that all hESC procedures are non-patentable, making financing for such research

difficult. Horizon 2020 (European Union 2014) was the largest EU research and innovation initiative from 2014 to 2020. Horizon Europe, the 9th Framework Programme, succeeded as Horizon 2020 (H2020) in January 2021. The European Commission proposed the 9th Framework Programme or Horizon Europe in June 2018 with a €100 billion budget as part of the EU long-term budget (Multiannual Financial Framework) for the years 2021–2027. In March/April 2019, the European Parliament and Council achieved a political and preliminary agreement on Horizon Europe.

The significant players influencing stem cell policy, financing and their views as of early 2013 may be summarized as follows: DG SANCO (Health and Consumers) and DG R and I (Research and Innovation) of the European Commission. Whereas, DG SANCO seems to be focused on safety and is unlikely to propose new stem cell legislation. DG Research and Innovation argues for ongoing financing of stem cell research under Horizon 2020. The JURI (Legal Affairs) and ITRE (Industry, Energy and Research) are the committees of the European Parliament. While JURI voted against financing non-patentable research, ITRE decided to continue funding it under the Framework Programme (Campo-Ruiz and Slørdahl 2013). European research projects using hESCs are in danger of losing EU funding if public opinion stays split. This may jeopardize ongoing international research collaborations and clinical trials, and funding choices for years to come.

Legislative and governance frameworks for stem cell research (specially hESC) in prominent European nations (Campo-Ruiz and Slørdahl 2013).

1. Austria (Unregulated), No particular law regarding hESC research, although there is a 2004 Reproductive Medicine Act. hESC research is illegal. Medically aided reproduction is the only use for embryos and gametes in stable heterosexual couples. hESC procurement and therapeutic and reproductive cloning are illegal. Adult stem cells get more funding than hESCs. Surplus embryos are advocated by the Austrian Bioethics Commission (majority position with dissenting opinion). The Austrian Bioethics Commission and Federal Chancellery advise the Federal Chancellor on all social, natural scientific and legal problems resulting from scientific advances in human health and biology.
2. Belgium (Regulated and permissive), There are three critical regulations with this respect. (1) 2003 Law In vitro embryo research, (2) 2007 Law on medically assisted reproduction and surplus embryos and gametes, and (3) 2008 Law on the procurement and use of human body material for human medical applications or scientific research purposes. The regulatory body which controls stem cell research is the Advisory Committee on Bioethics, Federal Service of Health, Food Chain Safety and Environment, Federal Agency for Medicines and Healthcare Products. hESC research is permitted but not patented or commercialized. Embryos may be produced for study or from excess IVF embryos.
3. SWEDEN (Permissive with restriction), IVF embryos or aborted foetuses may be used in hESC research. Cloning is illegal. Associated Regulations are

(1) 2002 Law on biobanks in medical care, (2) 2003 Law on ethical reviews of research on humans, and (3) 2006 Law on genetic integrity.
Associated Regulators are the National Council on Medical Ethics, the National Board of Health and Welfare and Swedish Gene Technology Advisory Board.

4. BULGARIA (Permissive with restrictions), Under the Health Act, excess IVF embryos may be utilized for research purposes with the donor's informed permission (s). Human reproductive cloning is illegal. Regulations controlling stem cell research are (1) 1987 Regulation for the artificial reproduction, (2) 2000 Law on drugs and pharmacies in human medicine, (3) 2003 Law on the transplantation of organs, tissues and cells, (4) 2003 Law on blood, blood donation and blood transfusion, (5) 2004 Health Act and (6) 2007 Law on assisted reproduction. Associated regulating bodies are Bulgarian Central Ethics Commission (CEC), Ministry of Health, Commission on Ethics in Drug Trials, Ministry of Health, Specialised Committee for Approval of Conducting Clinical Trials, Ministry of Health Bulgarian Drug Agency, Commission on Research Ethics, Ministry of Education and Sciences and Executive Agency for Transplantation, Ministry of Health.
5. CYPRUS (Permissive with restrictions), If the embryo is adequately protected, hESC research utilizing IVF embryos is permitted. A ban on cloning has been there. Governing Regulations are (1) 2001-2 Law on medicinal products for human use, (2) 2001 Law 31 Oviedo Convention research on pharmaceuticals, medical devices, medical radiation and imaging, surgical procedures, medical records, and biological samples, epidemiological and psychological investigations, and (3) 2005 Operational guidelines for pharmaceuticals, medical devices, and biological samples, while the governing body is National Bioethics Committee.
6. HUNGARY (Permissive with restrictions), However, the excess IVF embryo must be utilized within 14 days after fertilization and with informed permission. Reproductive cloning is illegal. Regulation related to stem cell research are (1) 1978 Criminal Act modified in 1998 articles on violation of regulations of research on embryos and gametes, (2) 1992 Act on the protection of the foetus life, (3) 1997 Healthcare Act Chapter IX (Articles 165–187), (4) 1998 Ministerial order on special procedures of human reproduction, the treatment of embryos, gametes and their storage, (5) 2002 Law on biomedical research on human subjects, (6) 2008 Act on the protection of human genetic data and the regulation of human genetic studies, research and biobanks. While Stem Cell Committee, Health Science Council, Health Science Council, Human Reproduction Commission (HRB), Scientific and Research Ethics Commission (TUKEB) and Health Science Council regulate the medical research.
7. ICELAND (Permissive with restriction), The 2003 Act addresses IVF embryos and their usage in research, but not hESC research. Reproductive cloning (by SCNT) is forbidden. Biobanks are not governed by legislation. Regulations governing stem cells are (1) 1996 Act on artificial fertilization, human gametes and embryos for stem cell research human, and (2) 2008 Biobanks Regulation

on scientific research in the biomedical field, body controlling stem cells are National Bioethics Committee, Ministry of Welfare.

8. IRELAND (Permissive with restriction), In vitro fertilized embryos may be used for hESC research up to 14 days after fertilization. A ban on cloning has been in this law; an "embryo" is defined as a live group of cells arising from fertilization. The body regulating stem cell research is the Commission on Assisted Human Reproduction, Department of Health.
9. ITALY (Permissive with restriction), Informed permission is required for hESC research utilizing IVF embryos up to 8 days old. Allowed hESC imports illegal cloning. Regulation controlling such activities 2004 Law on medically assisted reproduction. The Governing body is National Bioethics Committee.
10. LITHUANIA (Very Restrictive), After December 2001, hESCs can no longer be made or obtained. A ban on cloning using imported cell lines before May 1, 2007, is permitted for hESC research only if necessary for new medical and scientific information. Associated Regulation 1996 Law on donation and transplantation of human tissues, cells, and organs, 2000–2007 Labiomedical research ethics search, Associated Regulating Body in this country, Lithuanian Bioethics Committee.
11. LUXEMBOURG (Permissive with restriction), Human hESC research utilizing IVF embryos or aborted foetuses is permitted. Cloning for reproduction is illegal. The production of human embryos for scientific purposes is illegal. There is no associated regulation. The Associated Regulatory Body in this country is the National Ethics Commission (CNE), National Committee of Research Ethics (CNER) Ministry of Health.
12. THE NETHERLANDS (Permissive with restriction), However, the excess IVF embryo must be utilized within 14 days after fertilization and with informed permission. Human cloning is illegal. Associated Regulations in the country are the 1999–2006 Act on medical research involving human subjects, the 2002 Embryo Act, 2006 Regulation on stem cell transplantation and associated regulations. Associated authority to control stem cell research and regenerative medicine is the Health Council of the Netherlands, Central Committee on Research Involving Human Subjects (CCMO).
13. NORWAY (Permissive with restriction), hESC research using IVF embryos up to 14 days old is allowed. It is unlawful to create embryos for research. Cloning for reproduction is prohibited. Therapeutic cloning is prohibited, but embryo sex selection is allowed. Associated Regulations are (1) 1996 Act on organ donation, (2) 2003 Act on biobank for therapeutic purposes, (3) 2003 Biotechnology Act, (4) 2005 Regulations on the withdrawal, testing, processing, storage, and distribution of human blood and blood components, and on the handling of health data in blood donor registers, (5) 2008 Health Research Act, (6) 2008 Regulations on quality and safety requirements for the handling of human cells and tissues. Associated Regulators in this country are Norwegian Biotechnology Advisory, National Committee for Medical and Health Research Ethics, Ministry of Health and Care Services.

14. POLAND (Permissive with restriction), Research on IVF embryos is allowed if it is part of IVF therapy or aims to diagnose genetic disorders of human embryos, advance treatment for infertility, or improve knowledge of causes of congenital infertility. IVF embryo research is permitted if it is part of IVF therapy or seeks to identify genetic abnormalities of embryos, infertility treatment utility, or increase understanding of causes of congenital illnesses and miscarriages. Informed permission is required. Human embryos can only be implanted after 10 years. Isolating embryos for longer than 14 days or cloning are prohibited. If appropriate, stem cell lines may be created via SCNT diseases and miscarriages. Gamete donors must provide informed consent. There are no Associated Regulations for embryonic research legislation. Associated Regulators or advisory body is Commission of Bioethics at Wroclaw Medical University.
15. PORTUGAL (Unlegislated), There is no legislation on stem cell research. There are associated regulations in this country. There is a 2006 Law on medically assisted procreation.
    National Council for Medically Assisted Reproduction (CNPMA), National Council for Ethics in Life Sciences (CNECV) regulates human-assisted reproduction and ethics.
16. ROMANIA (Very Restrictive), Human embryonic stem cell research is banned unless it is explicitly intended to improve the embryo's therapeutic and medical condition. Prohibited is the creation of embryonic stem cell lines allowing for the study import of embryonic cells. Associated Regulations in this county are (1) 1998 Law on the removal and transplantation of human tissue and organs, (2) 2004 Law on good conduct in scientific research, technological development, and innovation, (3) 2011 Law related to the ratification of the convention on human rights and biomedicine and the additional protocol on the prohibition of human cloning. Associated Regulators in this country are the Bioethics Commission, Ministry of Health, National Ethics Council for Scientific Research, Technological Development and Innovation, Ministry of Education and Research.
17. SLOVAKIA (Very Restrictive), The research on human embryonic stem cells is limited to clinical findings (non-interventional trials). Other applications and the import and export of human embryo tissues, stem cells, and lines are banned. Associated Regulations is (1) 1994 Law on health, care and associated Regulators is National Ethics Committee, Ministry of Health.
18. SLOVENIA (Unlegislated), No legislation on stem cell research, and associated Regulations are (1) 2000 Law on biomedically assisted fertilization, (2) 2007 Act on quality and safety of human tissues and cells, for the purposes for medical treatment. The Associated Regulator in this country is National Medical Ethics Committee.
19. SPAIN (Permissive with restriction), If the research is done within 14 days after fertilization, hESC research is permitted. The creation or implantation of human embryos in animals and animal embryos in humans are all prohibited. It is illegal to choose the sexes of embryos unless in extreme cases. There is no difference in

(stem) cell treatment associated regulations, which are (1) 2006 Law on techniques for human-assisted reproduction, (2) 2006 Law on quality and safety norms for donation, obtention, evaluation, processing and distribution of human cells and tissues, (3) 2006 Law on imports and exports of biological samples, (4) 2006 Law on human-assisted reproductive technology, (5) 2007 Law on biomedical research, (6) 2010 Law regulating the Guarantees Commission for the donation and use of human cells and tissues and the registry of research projects. Associated Regulators in this country are the National Commission on Assisted Human Reproduction, Ministry of Health, Social Services and Equality, National Bioethics Committee, Ministry of Health, Social Services and Equality, Guarantees Commission for the Donation and Use of Human Cells and Tissues, Ministry of Economy and Competitiveness.

20. SWITZERLAND (Unlegislated), there is no law on hESC research. As a result of outdated legislation, embryonic research is de facto banned. Human embryos are legally defined as "conceived children" and therefore cannot be used in research. The Polish Parliament considers hESC research "unlawful". Human embryos cannot be utilized for non-therapeutic research. Associated Regulations is 2003 Federal Act on research involving embryonic stem cells.
    The Associated Regulator in this country is National Advisory Commission on Biomedical Ethics.
21. TURKEY (Permissive with restriction), In vitro hESC research is only permitted. Embryo creation for the study is illegal. Associated Regulations are (1) 1979 Law on organ transplantation, (2) 2005 Regulation on cord blood banks, (3) 2006 Circular on stem cell and guidelines for non-embryonic clinical therapeutic stem cell research, Ministry of Health, (4) 2008 Directive on the coordination of the centres for embryonic stem cell research, 2011 Regulation on clinical research and trials, AND (5) 2012 Regulation on the transplantation of organ and tissues. The Associated Regulator in this country is the Ministry of Health.
22. UNITED KINGDOM (Permissive with restriction), In 2020, UK got separated from the EU, and the UK has established its separate regulatory system. The hESC research has formal permission. However, there is no restriction on IVF, embryonic stem cell research, or embryonic stem cells. Human genetics is not governed by legislation. Cloning is illegal. Associated Regulations are the (1) 1990 Human Fertilisation and Embryology Act, amended in 2009, (2) 2001 Human Reproductive Cloning Act, (3) 2004 Human Tissue Act. Associated Authorities are Human Fertilization and Embryology Authority (HFEA), Human Tissue Authority (HTA), Medicines and Healthcare products Regulatory Agency (MHRA), Health Research Authority (HRA), Emerging Science and Bioethics Advisory Committee (ESBAC), Department of Health.

Regulatory and political procedures differ from one country to the next throughout Europe and across the world. The REMEDIE (SATSU The University of York 2008) study project examined the many ways in which tissue sourcing, specifically the procurement of oocytes for research, is subject to regulatory supervision and the

extent to which this control is successful. It also found some significant variations in the political cultures that shape the sector as one moves from the United States to Europe and China. National sovereignty plays a crucial role in this matter, deflecting efforts towards harmonization regarding controversial topics such as regenerative medicine. Consensus on controversial topics such as those relating to regenerative medicine is fragile and frequently challenged on moral and political grounds. Furthermore, in this fast-moving area of science and technology, consensuses are prone to collapsing quickly when innovation disrupts previously established moral limits. In addition, other factors such as historical constraints, the existence of pressure groups, the dominant political ideology, individual leadership of policymakers, and their ability to develop pragmatic regulations that "do the job" while avoiding controversy are all factors that influence ethical consensus on regenerative medicine.

4.3 India

Recent years have seen a rise in physicians' reports administering unlicenced stem cell and regenerative medicine to patients (Bhutia 2017). India now lacks specific legislation governing stem cell and regenerative medicine, and as a result, there is a possibility that some of the physicians abuse this position. The National Stem Cell Guidelines, which govern stem cell research and treatment, are the sole standards that offer guidelines on stem cell and regenerative medicine (National Guidelines for Stem Cell Research – 2017 2017). National Stem Cell Guidelines for India were established in 2007 by the Indian Council of Medical Research (ICMR) and Biotechnology (DBT). The Stem Cell Guidelines from 2013 and 2017 have been revised. The National Stem Cell Guidelines for 2013/2017 are more stringent than those for 2007. These rules were initially titled "Therapy" in 2007 but were renamed in 2013 and 2017 (Tiwari and Desai 2018).

Consequently, no therapy suggestions can be made until the efficacy of the treatment has been proven. Except for haematopoietic stem cell reconstitution for approved reasons, all stem cell use in patients is presently experimental (ICMR-DBT National Guidelines for Stem Cell Research 2013 and 2017). In a similar vein, minimally modified stem cells derived from bone marrow/peripheral blood may be used in certain approved medical circumstances. CDSCO approval is required to provide stem cells to individuals who have undergone substantial or more than modest alteration. It is difficult to define what constitutes minimum manipulation and what constitutes substantial or more than minimal alteration. Some chances of stem cell treatment can be misused by some entrepreneurs (Tiwari and Raman 2014). Although clinical trials are required before patients use adipose-derived Stromal Vascular Fraction (SVF), it is being marketed as a minimally manipulated stem cell product in India.

The other critical point is that violations of these regulations are not punishable by law. Numerous physicians have debated that these are mere suggestions (Bhutia 2017). Furthermore, neither the ICMR nor the DBT is empowered to compel doctors

to follow the recommendations. The ICMR's role as a government-appointed health advisory body is limited to planning, coordinating, and promoting biomedical research. Likewise, the DBT promotes biotechnology throughout the country but has little enforcement power over clinics. The ICMR and DBT are well aware of the guidelines' flaws, most notably their incapacity to regulate unproven stem cell treatments (Tiwari and Raman 2014). To address the issue mentioned above, they highlighted the need of including many authorities and acts in the suggestions, especially those related to controlling misleading advertising, which may assist in avoiding claimed stem cell therapies. In the stem cell business, direct-to-consumer advertising is a significant problem. Exaggerated claims about the benefits of stem cell therapy have attracted many patients to stem cell clinics worldwide. This may be one of the reasons why stem cell standards place a greater emphasis on preventing misleading advertising (Best Media Info 2017). Such practices of unproven claims must be nipped in the bud.

India's regulatory framework:

1. **National Stem Cell Guidelines for Research, 2017**
   These are prepared by the Indian Council of Medical Research (ICMR) and the Department of Biotechnology (DBT); neither of these organizations has authority over physicians; those who violate the recommendations cannot be penalized since the standards lack legal backing.
2. **Regulations on deceptive advertising**
   (a) Regulations 2002 of the Indian Medical Council (Professional Conduct, Etiquette and Ethics), The Indian Medical Council (MCI) and state medical councils.
   Clinicians are banned from promoting medical goods and services; violators may lose their licence to practise for a certain period, as determined by the Medical Council of India/state medical councils.
   (b) The 1954 Drugs and Magical Remedies (Prohibited Advertisements) Act.
   The Directorate General of Health Services bans deceptive advertising about medicines and magical cures; those found guilty face up to 12 months in jail, a fine, or both.
   (c) The 1940 Drugs and Cosmetics Act, Schedule J.
   According to the Central Drugs Standard Control Organization (CDSCO), medication cannot make promises about preventing or curing illnesses classified in Schedule J, such as cancer, genetic disorders, or Parkinson's disease.
   (d) Self-regulation: The Advertising Standards Council of India (ASCI) maintains merely a code of conduct since it is not a government agency with the legal authority to penalize offenders; nevertheless, relevant regulating agencies may respond to its ASCI complaints (Best Media Info 2017).
3. **Clinical Practice Regulations.**
   (a) Under the Medical Council of India Act, 1956, the Medical Council of India/state medical councils regulate clinical practices via licences to practise. Regulations of the Indian Medical Council on Professional Conduct,

Etiquette, and Ethics, 2002. The MCI/state medical boards control doctors' ethical behaviour.

(b) Section 304-A of the Indian Penal Code; Under the Civil Law of Torts, law enforcement authorities such as the police and courts are responsible for medical malpractice and negligence.

(c) Under the Consumer Protection Act of 1986, consumer forums defend customers' (patients') rights against substandard goods and services (medical services); they may be utilized exclusively against clinicians who provide compensated services, i.e. private medical practitioners.

(d) Proposed Amendments to the 1945 Drugs and Cosmetics Regulations CDSCO shall classify as a medicine any stem cell or stem cell-based product produced with significant, or more than minimum, modification; this class excludes weakly modified stem cells their homologous usage.

4.4 China's Rules

In China, it is unclear whether stem cell-based treatments are classified medicines or medical technology, resulting in conflicting stem cell-based intervention legislation and regulations (Rosemann and Sleeboom-Faulkner 2016). China's National Health and Family Planning Commission (NHFPC) was the Ministry of Health [MOH], which later became the National Health Commission [NHC] and the National Health Commission (NHC) are governed by two primary regulatory agencies: the Guidelines for Quality Control and Preclinical Studies of Stem Cell Preparations (Trial) (Lv et al. 2020) and Administrative Measures for Stem Cell Clinical Research (AMSCCR) (Lv et al. 2020). However, no legal opinion or court judgement in China unequivocally defines it as a drug. As a result, whether stem cell-based therapies are drugs or medical technology has a significant effect on the regulation of advertising and promotion in this space, as they may be marketed and promoted as medications or medical services.

4.5 Africa

Africa is lagging behind other continents in terms of clinical stem cell research. At present, Africa accounts for just 2.5% of mesenchymal stem cell clinical trials, compared to more than 80% in Asia, Europe, and North America (Chen 2016). In Africa, there are very few clinical trials using pluripotent stem cells. Nonetheless, efforts are being undertaken to expand the number of clinical trials investigating the potential of stem cells to treat cardiac diseases (Pepper and Slabbert 2015). However, these cells are primarily used in vitro as experimental models rather than for therapeutic reasons.

South Africa has established legislation to regulate stem cell research. However, since this legislation is rarely strictly enforced, medical professionals involved in

stem cell tourism often abuse it to further their interests (Pepper and Slabbert 2015; Meissner-Roloff and Pepper 2013). The National Health Act and the Medicines Control Act both provide laws regulating the use and research of stem cells in South Africa. The National Health Act covers explicitly the use of embryonic stem cells, induced pluripotent stem cells, and adult stem cells derived from mature somatic tissues, such as bone marrow. Adult stem cells are expressly regulated under Chapter 8 of the National Health Act. South Africa's National Health Act, 61 of 2003, allows for stem cell research and therapeutic use only with the donor's consent (Oosthuizen 2013).

As mentioned in the recommendations of a workshop on the ethical issues of sophisticated regenerative therapies for infertility (Ilkilic and Ertin 2010), Egypt has widespread approval for the generation of stem cells through cloning. On the other hand, Tunisia has an opposed legal position on stem cell research, banning any experimental activity on embryos since an embryo is regarded to possess the potential to grow into a fully-fledged autonomous life. This is stated in Tunisia's 1997 Opinion No. 1 by the Medical Ethics Committee, which also bans cloning for medical or reproductive purposes 1,1 of the law, saying that embryos may be used exclusively for reproductive purposes. The bulk African and Arab countries do not have explicit stem cell research legislation. According to the United Countries Education Report (UNESCO Cairo office 2011), stem cell research is more culturally and spiritually motivated in these countries. In general, it seems as if stem cell research regulation in Africa is controlled by vague laws that do not precisely describe how research should or should not be conducted. This hurts the progress of stem cell research on the continent and promotes unethical actions in the field.

4.6 United States of America

The use of stem cell products and other regenerative therapies can substantially enhance catastrophic injuries and serious diseases. FDA has authorized some therapies, but the vast majority of treatments currently have not been approved; despite the strict regulation, many of these unapproved therapies have resulted in life-threatening infections, chronic pain or even death in patients (Bauer et al. 2018). Over 700 clinics in the United States offer unapproved stem cell and regenerative medicine treatments (SCRIs) for conditions such as Alzheimer's disease, muscular dystrophy, autism, spinal cord injuries, and, most recently, COVID-19 (Turner 2018). These businesses often produce and market products derived from a patient's cells (autologous) or donated (allogenic) amniotic fluid, placental tissue, or umbilical cord blood, among other birth-related cells and tissues. The FDA had given manufacturers and marketers of SCRIs until May 31, 2021, to comply with the agency's regulations governing human cell and tissue products, including submitting such items for FDA review when necessary (The Pew Charitable Trusts 2019). The discretionary period granted by FDA is now over, and all the stakeholders are required to comply with the regulations applicable to the product.

The role of the FDA in regenerative medicine has now become quite prominent. The FDA, however, does not have the authority to regulate medical practice. This is because regenerative medicine products (cells and tissues) are intrinsically linked to care (The Pew Charitable Trusts 2019). The FDA's traditional product classification system is currently being overhauled to provide a framework for products with human cells and tissues. Along with product purity and potency, other FDA-regulated goods must demonstrate safety and effectiveness. These products have specific evaluations and approval processes applicable to each category and require clarity for their lifecycle for all stakeholders, including the product manufacturers. The regulations have evolved in tandem with medical progress and public health concerns (The Pew Charitable Trusts 2019).

Human cells, tissues, and products derived from cells and tissues (HCT/P) are used for therapeutic uses. The FDA began using its regulatory authority on a case-by-case basis many decades ago, after the deaths of two women from rabies and Creutzfeldt–Jakob disease following corneal transplants. The FDA first tried to categorize human tissue products as biologics, medical devices, pharmaceuticals or physical commodities subject to premarket approval by the FDA (PHSA). The donation, processing and marketing of HCT/P began in 1993. Additionally, new criteria were developed for donor screening, record keeping, inspections and recalls. If a product complies with specific criteria stated in CFR Title 21, Section 361 of the PHSA rules, it is exempt from premarket clearance or approval, whereas those that do not comply are controlled in the same way as other medicines or devices under Section 351 of the PHSA regulations.

CFR Title 21, sections 1270 and 127,141, formalized this provision.

(a) The Federal Food, Drug, and Cosmetic Act governs medications and medical devices that are "intended to diagnose, cure, mitigate, treat or prevent disease" or "intended to change the structure or function of the body (Federal Food, Drug, and Cosmetic Act of 1938, 21 U.S.C. § 321(g)(1); See Id. § 321(h)(2)-(3) n.d.)". With few exceptions, medications and medical devices must get FDA premarket clearance before being sold in the United States (Federal Food, Drug, and Cosmetic Act of 1938, 21 U.S.C. § 355(a); Id. § 360e(a) n.d.). This requires manufacturers to demonstrate the product's safety and effectiveness via suitable and well-controlled clinical studies (Federal Food, Drug, and Cosmetic Act of 1938, 21 U.S.C. § 355(d); Id. § 360e(d), n.d.).
(b) The Public Health Service Act (PHSA) Section 351 governs interstate commerce in biological goods such as vaccinations, therapeutic proteins and similar items designed to prevent, treat or cure illness or condition. Biological products must adhere to the same safety and efficacy criteria as pharmaceuticals and medical devices and are authorized by the FDA after submitting a biological licence application.
(c) Section 361 of the Public Health Service Act authorizes the FDA to create and enforce regulations "to prevent the importation, transfer or spread of infectious diseases". Numerous products derived from human cells and tissues are controlled under Section 361 and not under Section 351.

(d) The twenty-first Century Cures Act: Among numerous provisions aimed at fostering innovation and lowering barriers to the development and approval of new medical products, this legislation directed the FDA to establish an expedited process for evaluating regenerative medicine advanced therapies, dubbed the RMAT designation, and to consider the use of alternative forms of evidence in such evaluations (FDA's Framework for Regulating Regenerative Medicine Will Improve Oversight n.d.).

The Part 1271 regulations have been interpreted in the field over time as establishing three tiers, or categories, of cell and tissue products; two are virtually unregulated by the FDA, while the third is subject to the exact licencing requirements as drugs, medical devices or biological products. Manufacturers developing regenerative products may seek FDA guidance on applicable regulatory tier to place their product by submitting a formal designation request to the Tissue Reference Group (composed of representatives from the FDA's Center for Biologics Evaluation and Research and Center for Devices and Radiological Health); or to the agency's Office of Combination Products (SOPP 8004: Tissue Reference Group, 2020). For many years after the proposed and finalization of the Part 1271 rules, the responses to requests for designation from these two organizations served as the primary source of guidance for the FDA's interpretation and implementation of the HCT/P regulations. In 2013, the FDA started limiting the scope of products exempt from regulation and increasing the number of enforcement letters sent to companies it suspected claimed such exemptions illegally (*Food and Drug Administration Letters to Companies* n.d.). It published a series of draft guidance papers in 2014 to clarify its regulations by defining guiding the stakeholders on how the FDA defines these goods. Taken together, the guidance documents demonstrated the agency's intention to seek premarket clearance for a broader range of HCT/Ps, including some that had been on the market for many years. In response to additional comments and feedback from the industry, the FDA convened a public hearing in September 2016 to discuss these studies and the agency's more comprehensive framework for regulating HCT/Ps.

FDA's efforts were bolstered further by the passage of the twenty-first Century Cures Act in December 2016. Along with $30 million in NIH money for regenerative medicine research, the legislation directed FDA to establish a new programme called the RMAT designation to expedite the development, review, and approval of these products, as well as crucial FDA Authorization Legislation.

4.6.1 Regenerative Therapy Guidelines

The FDA published four guideline papers in November 2017 that supplement current legislation and provide the regulatory framework for regenerative medicine. The FDA's guidance documents do not establish legally binding duties. Rather than that, they provide the agency's current thoughts and suggestions on a topic and how the legislation will be implemented. Stakeholders are strongly encouraged but not

required to consult and adhere to regulations. Its foundation for regenerative medicine is detailed in white papers and guidelines published by the FDA.

1. The United States Food and Drug Administration's "Same Surgical Procedure Exception Under 21 CFR 1271.15(b): Questions and Answers Regarding the Scope of the Exception: Guidance for Industry" (2017) describes the Food and Drug Administration's exemption from oversight for "same surgical procedures", as defined in the Code of Federal Regulations, Title 21, Part 1271. It encompasses the methods through which goods may be treated while still adhering to the exception.
2. The United States Food and Drug Administration's "Regulatory Considerations for Human Cells, Tissues, and Cellular and Tissue-Based Products: Minimal Manipulation and Homologous Use: Guidance for Industry and FDA Staff" (2017) defines critical regulatory terms, including minimal manipulation and homologous use. This section discusses how to apply these criteria to a human cell or tissue product and outlines the FDA's compliance and enforcement policies for human cell and tissue products.
3. U.S. Food and Drug Administration, "Evaluation of Devices Used With Regenerative Medicine Advanced Therapies: Guidance for Industry" (2019), describes how FDA will approach the evaluation of devices used in the recovery, isolation or delivery of regenerative medicine advanced therapies (RMATs).
4. The United States Food and Drug Administration's "Expedited Programs for Regenerative Medicine Medicines for Serious Conditions: Industry Guidance" (2019), describes the expedited development programme for qualifying regenerative therapies, referred to as RMAT designation. The United States Food and Drug Administration's "Evaluation of Devices Used with Advanced Regenerative Medicine Therapies: Industry Guidance" (2019) describes how the FDA will approach the evaluation of devices used in the recovery, isolation, or delivery of advanced regenerative medicine therapies (RMATs).
5. FDA has provided strategic steps to move forward towards compliance and regulations for all the stakeholders in regenerative medicine and healthcare innovations towards compliance and towards obtaining approvals for products. CBER announced, "2021–2025 Strategic plans" under the leadership of Dr. Darkmarks regenerative medicine, cell therapy, gene therapies and FDA approval processes. According to the experience of stakeholders, they have found the FDA, CBER and OTAT helpful in providing information and guiding the stakeholders right from the initial proof of concept. The strategy is to contact and connect with regulators at the earliest.

The FDA highlights the difference between goods that need complete medication approval and those that do not while also simplifying the review process and easing certain regulatory burdens on product inventors (Marks and Gottlieb 2018). The agency's declared overarching aim is to balance accelerating the development of new treatments and to ensure that they are adequately safe and effective. The agency states that developing the regulatory framework guidelines considered preventing

communicable disease transmission between donors and recipients, the processing controls required to prevent contamination, preserving tissue integrity and function, and ensuring clinical safety and efficacy (Final Guidance Document: Regulatory Considerations for Human Cells, Tissues, and Cellular and Tissue-Based Products: Minimal Manipulation and Homologous Use 2020).

4.6.2 FDA Ends Their Discretionary Period and Is Focusing on Unscrupulous Providers

The Food and Drug Administration has recognized the need for tighter regulation in the area of regenerative medicine and issued revised one in November 2017. It has been said that due to increase in regenerative medicine demand, there is also entry of some unscrupulous elements who made deceptive claims to patients about unproven and, in some cases, dangerous procedures. This demonstrates the critical importance of establishing a clear regulatory framework for developers and holding those who violate these rules (USFDA Statement 2017).

Additionally, November 2017 marked the start of a 3-year transition phase. The FDA will focus its enforcement efforts on products already on the market (Regulatory Considerations for Human Cells, Tissues, and Cellular and Tissue-Based Products: Minimal Manipulation and Homologous Use 2020). While the FDA will not impose their compliance on treatments, it deems to be at low risk, but on those it deems to be at high risk.

4.6.3 Risk-Based Regulatory Tiers for Regenerative Therapies

Regenerative treatments will continue to be regulated by the FDA on a risk-based basis. Products will continue to be classified into three broad regulatory categories, with treatments presenting the least risk to patients put in the lowest tier and those posing the most risk placed in the highest tier and subject to the entire premarket approval procedure.

The framework's most significant modifications concern how the agency established the tier borders. These differences have important implications for developers of regenerative treatments since the premarket approval procedure may take years and require a considerable financial investment.

4.6.4 Lowest-Tier Products

HCT/Ps in the lowest regulatory tier includes instances in which cells and tissues are transplanted as part of therapies in first- or second-degree relative, or for reproductive use or are collected and transplanted, reinfused inside the same patient (autologous) during the same surgical operation, referred to as the "same surgical procedure exemption". These goods are excluded from the FDA regulations regulating facility

registration, product listing, donor eligibility and tissue handling—collectively referred to as Part 1271 regulations. Generally, these rules are intended to prevent disease transmission and pollution (Same Surgical Procedure Exception under 21 CFR 1271.15(b): Questions and Answers Regarding the Scope of the Exception, 2017).

The same surgical technique exemption is conditional on three factors: The exception is solely applicable to autologous use—that is, the HCT/Ps must originate from the patients or a first- or second-degree relative, or for reproductive use. The operation or treatment must be seen as a single surgical procedure. The HCT/P must stay in its unaltered state (Guidance Document: Same Surgical Procedure Exception under 21 CFR 1271.15(b): Questions and Answers Regarding the Scope of the Exception, 2017).

While FDA permits specific handling and processing of the HCT/P, these operations are limited to size, shape, cleansing and rinsing. Beyond this, the product is deemed to have changed from its initial state and is stated as more than minimal manipulation.

4.6.5 Middle-Tier Products

To be considered for the intermediate tier, a product must fulfil four criteria: (21 CFR PART 1271—Human Cells, Tissues and Cellular and tissue-Based products Subpart A-General provisions Sec. 1271.10, 2020) Can be manipulated in a minimally invasive manner (see "Definitions: Minimal invasiveness" below). Be designed for "homologous use"—that is, for a purpose comparable to that of the donor in the receiver. Except in restricted, particular situations, not be mixed with other drugs. Not have a systemic impact or rely on the metabolic activity of live donor cells to operate correctly unless the product is intended for personal use, a donation to a first- or second-degree relative, or reproductive use. While middle-tier treatments are excluded from the premarket approval procedures applicable to highest-tier goods, they must comply with the Part 1271 standards for infection and contamination control. The FDA's interpretation of the words "minimum manipulation" and "homologous usage" contributes significantly to the ambiguity and subsequent debate over whether a treatment belongs in the middle or top tier. These ideas are not obvious, and the implications of failing to qualify for the intermediate tier—and therefore falling into the most rigorous regulatory tier—are significant for product developers and, by extension, for the patients who may get such therapies. A more rigorous evaluation process assists in ensuring that goods are safe and effective. This is critical since these products may offer serious hazards to patients. However, a substantial regulatory burden can also slow the pace of innovation and potentially keep effective therapies from reaching the market.

4.6.6 Minimal Manipulation

The Food and Drug Administration's (FDA) criterion for assessing whether a human cell and tissue product (HCT/P) has been minimally altered varies according to whether the HCT/P is deemed as structural or nonstructural cells or tissue (Regulatory Considerations for Human Cells, Tissues, and Cellular and Tissue-Based Products: Minimal Manipulation and Homologous Use, 2020). According to the agency, structural tissues offer physical support or act as a barrier, cover, cushion or conduit inside the donor. Bone, skin and adipose tissue (fat) are all examples. A structural tissue must not undergo processing that alters critical properties such as strength, flexibility, cushioning, or covering to be minimally modified. Processing may involve testing, sterilization, preservation, cutting and grinding. Grinding or sculpting bone into screws, for example, is considered minimum manipulation since it does not impair the capacity of the bone to support the body. However, processing adipose tissue to harvest stem cells does not satisfy the criteria of minimum modification since it removes the components of the tissue that enable it to operate structurally as fat. By contrast, nonstructural cells and tissues mainly perform metabolic or biochemical functions in the body. Reproductive cells, blood stem cells and bone marrow are all examples. For these products, any procedure that modifies a critical biologic feature that affects the product's function in the donor (for example, altering a tissue's metabolic activity or the capacity of cells to proliferate) may be considered more than minimum modification. Thus, processing blood to increase the concentration of stem cells satisfies the criteria of minimum modification since it does not impair the stem cells' capacity to repopulate bone marrow after administration to the recipient. However, reprogramming those identical cells to develop into a certain kind of cell goes beyond the basic steps of modification (66 FR 5447 - Human Cells, Tissues, and Cellular and Tissue-Based Products; Establishment Registration and Listing Page 5447–5469, 2001).

4.6.7 Structural Versus Non-structural Cells, Tissues

4.6.7.1 Homologous Use

In addition to being minimally processed, a product in the middle tier must also be intended for homologous use, which is defined as the repair, reconstruction, replacement or supplementation of cells or tissues with an HCT/P that performs the same primary function in the recipient as it does in the donor.

Under this definition, adipose tissue could be used as part of breast reconstruction procedures but could not be used as part of a treatment for a degenerative or inflammatory disorder, similarly, amnion.

(a) Structural tissue: Bone, Skin, Amniotic membrane, Umbilical cord, Adipose tissue, Cartilage, Tendons, Ligaments.

(b) Non-structural tissue: Reproductive cells, Blood stem cells or blood progenitor cells, Lymph nodes, Thymus, Peripheral nerves, Parathyroid and thyroid glands, Bone marrow, Pancreatic tissue.

The membrane covering an embryo could be used as a cover or selective barrier for the passage of nutrients but could not deduce scarring or inflammation as part of wound repair. By defining a cell or tissue's basic functionaries strictly, FDA further narrows the scope of HCT/Ps that fall into the middle tier, thereby classifying a broader range of products in the top tier.

4.6.8 Highest-Tier Products

The highest-tier products require full premarket approval. Regenerative treatments that do not satisfy the low or medium tier criteria are subject to the same premarketing procedures as any other medication or device, including the need to undertake clinical trials to show safety, potency, and effectiveness. As part of its stated aim of promoting novel regenerative treatments, the FDA has made efforts to decrease regulatory obstacles and streamline the route to market without compromising safety and effectiveness requirements.

Among those measures was creating the RMAT designation, a fast-track regulatory route allowed by the twenty-first Century Cures Act. FDA may simplify the road to market for qualified goods by performing a rolling assessment of application components and offering extensive assistance to stakeholders throughout the process. As part of its framework, the agency published two draft guidance papers relating to this designation in February 2019.

Along with the RMAT-related guidance papers, FDA has committed to implementing new regulatory ideas that would streamline the preapproval process, such as collaborative development models that will enable numerous small-scale investigators or manufacturers to collaborate (Marks and Gottlieb 2018). Additionally, the FDA is implementing an informal meeting programme called INTERACT (Initial Targeted Engagement for Regulatory Advice on CBER Products). CBER is the FDA's Center for Biologics Evaluation and Research, which will enable potential sponsors to communicate their concerns and questions early in the development process (INTERACT Meetings (Initial Targeted Engagement for Regulatory Advice on CBER Products) 2020).

4.6.9 Opinion of Stakeholders About FDA Framework

According to stakeholders, the FDA's framework clarifies regenerative medicine and will significantly affect the sector's trajectory. Additionally, most stakeholders said the framework represented an efficient, risk-based approach to industry regulation, but this was not universal. Nonetheless, the majority viewed the agency's focus on regenerative therapies as a net positive, arguing that tighter regulation at this stage

would lend legitimacy to the field and provide regulatory certainty, which are critical for developers seeking investment as well as payers who will ultimately decide whether to cover these new treatments.

Stakeholders expressed particular appreciation for the agency's explanation of how key concepts such as "minimum modification", "homologous usage", and "such HCT/Ps" would be defined for specific kinds of goods. The majority said that this transparency was critical for ensuring that goods are created and appropriately regulated. The final guidance will significantly impact companies that rely on adipose- or amnion-derived stem cells, which have previously been the focus of FDA litigation. The agency's declaration that such products are medicines should be regulated as pharmaceutical medicines. For years, many of these companies worked on the assumption that their goods would not be subject to premarket assessment and clearance. The majority believed that the framework is well-grounded in scientific and public health principles, and was created transparently with significant involvement from stakeholders.

4.7 Japan

Japan established stem cell and regenerative medicine as an essential field to study. In May 2013, the Japanese legislature passed the Pharmaceuticals and Medical Devices Act and stated its intention to make necessary reforms to promote the area of regenerative medicine. The government moved quickly on this agenda, passing the Act on the Safety of Regenerative Medicine (RM Act) and amending the Pharmaceuticals and Medical Devices Act (PMD Act) in November 2013, which became effective in November 2014 (Reform of 2013). The "drug" route is the initial channel for regenerative medical products. Market approval for drugs and medical devices was governed in Japan by the Pharmaceuticals and Medical Devices Act, which established the criteria for market approval issued by the Pharmaceuticals and Medical Devices Agency (PMDA) (i.e. safety and efficacy) and detailed rules requiring applicant compliance at both the pre- and post-marketing stages. As part of the reform, the category of "Regenerative Medical Product" was established.

Additionally, Japan has an expedited process for approving regenerative medical goods. This expedited review process enables market approval based on a limited number of patients and surrogate results. Accelerated approval is just the beginning of the product development process. To get expedited approval, it is essential to show an appropriate R&D design for the whole product cycle, including post-market research and trial plans, well-defined effectiveness indicators, and, ultimately, an acceptable safety and efficacy threshold to justify prescription to patients. The RM Act categorizes regenerative medicine into three risk categories (Class I to III), with each Class requiring hospitals and clinics providing regenerative medicine to adhere to its own set of procedural standards.

4.8 Korea, South

South Korea is another APAC (Asia-Pacific) nation committed to regenerative medicine deployment. South Korea, like Japan, has approved regenerative treatments conditionally, including exempting them from Phase I trials if positive results are published in peer-reviewed publications. Additionally, the South Korean Ministry of Food and Drug Safety accepts post-marketing data on the effectiveness of treatments for difficult-to-treat or life-threatening illnesses.

4.8.1 South Korea Released New Law on Advanced Regenerative Medicine and Advanced Biopharmaceuticals

On 28 August 2020, the Advanced Regenerative Medicine and Advanced Biopharmaceuticals Safety and Support Act (acronym "Advanced Regeneration Bio Act") and its associated Enforcement Decree went into effect.

The Enforcement Decree for the Advanced Regenerative Medicine and Advanced Biopharmaceuticals Safety and Support Act was recently issued to define the extraordinary power granted by the legislation to implement the "Advanced Regenerative Bio Act", with the following principal contents:

1. Definitions of sophisticated regenerative medicine and human cells, including the scope of their use (Scope of advanced regenerative medicine). The four types of treatment include cell therapy, gene therapy, tissue engineering, and fusion therapy (Scope of human cells). Cells and tissues from humans or animals, as well as those subjected to cell modification, cell processing, and cell manufacturing (Risks of clinical studies), provides risk assessment recommendations to medical institutions for use in establishing research programmes.
2. Establishment of a Fundamental Plan (Basic plan) outlines the first quinquennial Basic Plan for Advanced Regenerative Medicine and Advanced Biopharmaceuticals, as well as the procedures for establishing its annual implementation plan coordinating with related government departments, and operating a policy review committee for cross-governmental public-private governance cooperation.
3. Advanced regenerative medicine clinical trials (Institutions for regenerative medicine) specifies the paperwork, submission procedures, and required documents for research plans, as well as the application processes and methodologies for research plan assessments (Reviews by the Review Committee), prescribes a screening process for research ideas submitted by regenerative medicine institutions (Constitution of an expert subcommittee inside the review committee). Four expert subcommittees will be established, one for each of the four treatment categories, to guarantee a more professional assessment procedure. The subcommittees will evaluate advanced regenerative medicine research programmes for efficacy, safety, and ethical validity. Establish a review

committee secretariat for oversight of the review committee's activities, including registration of research proposals, organization of review committee meetings, and distribution of review results.

(Cell processing facilities) establish criteria for the facilities, equipment, and human resources required to be approved as cell processing facilities, as well as rules regulating cell processing, such as the need to record and report throughout the cell processing process.

(Safety management institution) the creation and operation of an information system for the systematic collection and management of clinical trial data generated by regenerative medicine institutions, as well as the design of patient care plans (test subjects), safety management, and long-term follow-up surveys.

4. Strengthening of the management system to suit the unique characteristics of novel biopharmaceuticals.

Requirements for advanced biopharmaceutical manufacturing and importing facilities sets the required requirements for establishments, storage, laboratories, and record rooms for approval and reporting.

Standards for the approval of human cell management companies specify the standards for facilities, equipment, human resources, and the quality management system components.

A long-term follow-up study of advanced biopharmaceuticals developed procedures for selecting medicines that need long-term monitoring, reporting adverse events, and documenting dose data.

4.9 Australia

Certain health practitioners provide stem cell treatments as a therapeutic or cosmetic technique to their patients. The TGA (Therapeutic Goods Administration) is the regulatory body and is concerned that a small number of practitioners may be offering unproven, illegal, and potentially hazardous treatments. The TGA has increased its oversight of this industry by imposing penalties for non-compliance. This protects consumer health and safety, as well as the reputations of legitimate clinics and practitioners. Furthermore, it fosters therapeutic innovation.

- In Australia, autologous human cell and tissue (HCT) treatments, such as stem cell therapies, are regulated to distinguish legitimate practitioners providing evidence-based clinical treatments from unscrupulous business practices offering expensive, unapproved, and unproven therapies and to protect patients from potentially harmful treatments.
- Permit continued access to cell therapies with sufficient clinical evidence showing their safety and efficacy. Enhance the prescriber–patient relationship by determining the best appropriate treatment for a patient's condition.

4.9.1 Access to the Provision of Cell Treatment to Patients

According to the rules, cell products may be supplied only if one of the following regulatory criteria is fulfilled.

TGA Products approvals include the following: The TGA approves a product and is placed on the Australian Register of Therapeutic Goods (ARTG). Suitably competent practitioners may use this product for appropriately selected patients by the authorized clinical indications.

- Manufacturing products for use in hospital treatment: Medical and dental practitioners are permitted to handle and deliver some kinds of stem cell therapies in a hospital setting that meets the requirements for prohibited goods. These goods are not subject to TGA regulation.
- Provisioning outside of hospitals: To qualify for the TGA exemptions, the product must be produced with very little processing (minimal modification). These goods must nevertheless comply with specific TGA standards. The majority of stem cell products do not satisfy this standard.
- Clinical research: Products may be provided in registered clinical studies, which need prior informed permission from patients for the experimental or exploratory therapy. Patients do not have to pay for these therapies, and the production facility must adhere to good manufacturing practices (GMP) standards and be licenced by the TGA.
- Compassionate provisioning: The Special Access Scheme and Authorized Prescriber routes are accessible to severely sick individuals or patients who do not have other therapeutic options.

Regulatory supervision of practitioners of these therapies is appropriate with the safety concerns associated with these treatments. For instance, stem cell therapies administered in hospitals under the supervision of a licenced medical and dental practitioner are exempt from TGA monitoring due to hospitals' pre-existing governance requirements. By contrast, private clinics operating outside of hospitals are controlled by the TGA based on the intricacy of their manufacture or processing of cell treatments and the level of danger to patients.

Practitioner requirements for stem cell therapies under the TGA rules.

4.9.2 Reporting Adverse Events

The TGA requires medical and dental practitioners who provide stem cell therapies to record any adverse events during or after treatment. This will contribute to developing a safety profile for such therapies, which is necessary for clinical innovation. The TGA requires reporting adverse events for therapeutic products (e.g. prescription medications and medical devices) and plays a critical role in ensuring their safe use in Australia.

4.9.3 Important Risks to Consider

As a result of stem cell therapies, the following adverse effects have been documented. The TGA should be notified of any adverse events that occur during or after the administration of a stem cell therapy, including but not limited to the following:

- Tumours.
- Infection caused by aberrant bone development.
- Allergic response.
- Unfavourable immunological events.

4.9.4 Advertising

All HCT products, including stem cell therapies, must adhere to patient-centred advertising standards. Advertisements for autologous HCT products, including stem cell therapies, are prohibited.

4.9.5 Other Regulatory Oversight

The TGA is in charge of therapeutic products regulation, which includes stem cells. The TGA, on the other hand, has no jurisdiction over the clinical practice. Additional criteria for stem cell therapies may exist with the following regulatory bodies:

- Regulation of Australian Health Practitioners (AHPRA).
- Medical and dental boards or councils at the state, territorial, and national level.
- Administration and management of public hospitals by the state and territory.
- Licencing private hospitals by state and territory.
- Commission de la concurrence et de la consommation de l'Australie (ACCC).

4.10 Canada

The regulatory system in Canada is logical, proactive, and transparent. Canada is well-positioned to advance scientific research and technological innovation in regenerative medicine. Federal and provincial incentives are spurring private investment in cell manufacturing facilities.

Health Canada is an ICH member and adheres to the ICH standards. Additionally, Health Canada is a member of the International Pharmaceutical Regulators' Forum's Cell Therapy and Gene Therapy Working Groups. This includes talks on Advanced Therapy Medicinal Product Clusters with the FDA, the European Medicines Agency, and officials from Asia-Pacific. Acts, Regulations, Guidelines, and Policies

constitute Canada's regulatory framework. Each of these four organizations has a role in the regulatory oversight of cell and gene therapy products in Canada.

Health Canada is legally authorized to control and supervise the manufacture and marketing of medicines under the Food and Drugs Act. Regulations include legally binding interpretations of the Food and Drug Administration's Food and Drug Regulations, the Medical Device Administration's Medical Device Regulations, and the Cells, Tissues, and Organs Act's Regulations. They provide specific instructions on requirements for each product and category. Defend the regulations and show how to adhere to them. They are quicker and easier to implement since they are not legally binding and allow for freedom of interpretation. Policies may be used to broaden or narrow the scope of regulatory provisions. Generally, misconceptions are addressed or simplified.

Cell therapy is not regulated in Canada and is not expressly covered by the Food and Drug Act. In Canada, the Medical Devices Regulations (MDR) may apply to a marketed or promoted device or combination product used to process CGTPs, and provincial medical practice regulations govern the distribution of CGTP to patients. CTOR is also a term that refers to a subset of cell treatments directed towards allogeneic cells, tissues, and organs that meet specific HHS requirements. Schedule D of the F&DR classifies goods as medicines if they have been significantly modified, are intended for non-homologous use, or have a systemic or metabolic effect (i.e. Biologics Drugs). This is consistent with the methods used by other nations. Division 1A (Licencing for Establishments) and Division 2 (Good Manufacturing Practices) include rules regulating biologics, biologic products, clinical trial applications, and clinical trial processes (New Drugs). A medical device or a pharmaceutical product may be a combination of medical equipment and pharmaceutical products. They come under MDR if they are devices; otherwise, they fall under F&DR. CTOR is a word that refers to minimally modified allogeneic cells, tissues, and organs that are not part of a combination product, have no systemic or metabolic effects and have shown safety and efficacy via historical use or clinical studies. Regardless, the regulations currently lack examples showing the requirements for minimal little manipulation or homologous use.

Cell therapy products are regulated by Health Canada's Biologics and Genetic Therapies Directorate (BGTD). The BGTD is a Department of Health and Human Services' Health Products and Foods Branch (HPFB). The BGTD is responsible for supervising all biological drug products in Canada, analogous to the FDA's Center for Biologics Evaluation and Research in the United States, before referring some recombinant products to the Center for Drug Evaluation and Research. Two centres within BGTD are responsible for biologics oversight and evaluation: the Center for Evaluation of Radiopharmaceuticals and Biotherapeutics oversees gene therapy products and somatic cell therapies. In contrast, the Center for Biologics Evaluation oversees cells and tissue for transplantation, including traditional and novel stem cell treatments and recommendations. The Medical Devices Bureau, a part of the Therapeutic Products Directorate, is responsible for medical devices, including cell scaffolds.

The Government of Canada unveiled a new pricing strategy for medicines and medical equipment in May 2018 after a series of consultations. The strategy covers biosimilars and medical devices. It found, among other things, that since Canada currently provides 8 years of exclusivity for all commercially available medicines, the previously proposed Orphan Drug Framework is superfluous for further protection.

CTOR (Cells, Tissues, and Organs) products are allogeneic cell therapy products that fulfil six requirements (little manipulation, allogeneic, homologous, acts locally). Moreover, CTOR allows for a less rigorous regulatory approach to a single medicinal drug that has been proven to be safer or has a well-established safety profile and therapeutic use (e.g. bone marrow transplantation). The CTOR guidelines were designed to minimize health hazards associated with recipients of Cells, Tissues, and Organs (CTOs), namely transmissible illnesses. These recommendations are based on current practices in Canada and include Canadian Standards Association-established national standards. Health Canada is responsible for product safety and not for the process of transplantation.

As a consequence of the 1980s, Canadian Blood Services transfused contaminated blood products, which resulted in up to 2000 patients with HIV and about 30,000 with hepatitis C virus (HCV); now, there is a significant focus on preventing infectious disease transmission. The Kroner Report included suggestions for improving the safety and monitoring of CTOR-transplanted goods.

CTO product's quality, safety, and effectiveness are highly dependent on raw material or source material screening and control, and regulatory focus is on disease prevention. Donor screening regulations include medical and social history, physical examination, diagnostic tests, and autopsy (if applicable). Additionally, laboratory testing for transmissible illnesses is required. In an ideal environment, specimens for infectious disease testing would be obtained as soon as possible after donation. Health Canada's CTO guideline paper details the recommended period for specimen collection. CTOR requires the presence of anti-HIV-1, anti-HIV-2, HbsAg, anti-HBc IgG, anti-HBc IgM, anti-HCV, anti-HTLV-Ia, and anti-HTLV-Iia infectious disease indicators. Additional testing includes IgG to evaluate the source material's quality may also be conducted. While autologous cells, tissues, and organs are excluded from CTOR, this does not imply that they are uncontrolled. Health Canada is in charge of regulating autologous treatments, regardless of whether they are slightly modified.

4.11 United Arab Emirates

The UAE biotechnology and pharmaceutical sectors are heavily regulated—mainly at the federal level and, to a lesser extent, at the Emirate level. Abu Dhabi and Dubai have the most advanced biotechnology and pharmaceutical laws and regulations among the seven emirates, while the other emirates generally follow their cues in terms of policy and legislation.

As part of the globalized economy, the United Arab Emirates (UAE) has established itself as an established destination for healthcare. As a result, much of its new law reflects the influence—and direction—of global markets in the pharmaceutical and medical sectors on jurisdictional developments. The healthcare industry has seen rapid development in recent years, assisting the UAE in becoming a centre for medical tourism. Additionally, the nation's policy seeks to lead and assist in healthcare initiatives by establishing sustainable public-private partnership models in this sector. The therapies for Regenerative Medicine are practised as Halal and Sharia-compliant under the local Sharia jurisdiction.

In 2019, the Abu Dhabi Department of Health released the DOH Standard on Stem Cell Therapies and Products and Regenerative Medicine. This standard establishes the Department of Health's criteria for the safe, effective, and high-quality provision of stem cells, stem cell-based products, related cellular therapy, somatic therapy and tissue engineering products, and regenerative medicine products for human use in the Emirate of Abu Dhabi (DOH 2019). The United Arab Emirates allows adipose stem cells, minimally manipulated and cultured cells for therapeutic use (DOH 2019).

Circular 304/2015 of the Ministry of Health and Prevention on regulating cord blood and stem cell storage facilities was issued. Decision No. (6) of the Council of Ministers for the Year 2020 endorsing the regulations governing Cord Blood and Stem Cell Storage Facilities, this section establishes licencing requirements for health institutions that intend to undertake activities using cord blood, stem cells, and other perinatal cells derived from bone marrow, peripheral blood, perinatal sources, and cord blood. Additionally, these regulations provide a set of requirements for compliance with the regulations. The emirate is developing standards for the quality and safety of human stem cells and tissues for patient wellness. The aim is to establish a framework for assessing the performance of licenced institutions to ensure the delivery of safe and high-quality services while protecting donors, recipients, and the general public. This decision includes the norms and criteria utilized throughout the collection, processing, and testing.

Except for haematopoietic stem cells and adipose stem cells, these restrictions do not apply to foetal tissue, embryonic stem cells, blood or blood products, reproductive cells, eggs, or solid or human organs. These regulations apply to national public and private health care institutions that engage in any of the activities specified under the scope of application. These facilities—cord blood, cord tissue, perinatal tissues, and adult stem cell banks—are covered in the scope or publication of this judgement (Council of Ministers' Decision No. (6) of the Year 2020 on Endorsement of the Regulations of Cord Blood and Stem Cells Storage Centers 2021; DOH STANDARD ON STEM CELL THERAPIES, PRODUCTS & REGENERATIVE MEDICINE April 2019 2019).

4.12 Kingdom of Saudi Arabia

Numerous Arabic nations undertake stem cell research, as demonstrated by the hundreds of scientific publications in this area. Saudi Arabia is a pioneer in stem cell research, having begun research on stem cells more than two decades ago (Al Douri et al. 1996), with many institutions, including King Saud University and King Faisal Specialized Hospital and Research Center. Additionally, many following research institutions, such as the King Abdullah International Medical Research Center, a specialized stem cell research department, a stem cell registry with over 10,000 donors, and the Cord Blood Bank, contribute significantly to this field (Fakhoury et al. 2015). Saudi Arabia was the first country in the region to establish ethical guidelines regulating stem cell usage and research. The Research Ethics Law, which was adopted in 2010, and its implementing regulation, which was implemented in 2012, include all ethical principles regulating stem cell research (Alahmad et al. 2020). KSA also follows Sharia law and implements the requirement of Halal for product processing and manufacturing; Jordan allowed suit in 2014, enacting stem cell-specific law (Alahmad 2017). Apart from these national rules, some research institutes, such as King Faisal Specialized Hospital and Research Center and King Abdullah International Medical Research Center, have their institutional requirements.

4.13 Israel

Israel is a global leader in regenerative medicine research and development. According to the ARM's annual report, over 900 firms worldwide are researching regenerative treatments, with 24 companies engaged in the cell, gene, and tissue therapy in Israel as of 2018. This places Israel as the world's most active nation in this field per capita and fourth in absolute numbers in Europe.

Israel was recognized as a global leader in stem cell research by Science Magazine in 2002. Israeli researchers have maintained their position as leaders in this area. Israel excels in both embryonic and adult stem cell research. Blood, bone, liver, pancreas, heart, and nerve cells are all subject to study.

Israel was one of the first nations to enact laws prohibiting genetic tampering for human reproductive cloning (1998). Additionally, Israeli legislation prohibits germline gene therapy for "creating a human". The Knesset (Israel's parliament) extended the existing 5-year ban to March 2009 in March 2004. Because the legislation makes no mention of therapeutic cloning, it is de facto allowed. At the moment, no therapeutic cloning is being conducted. The Israeli parliament's approach is based on recognizing that research in this area is still in its infancy. As a result, it is essential to evaluate the moral, legal, social, and scientific implications of genetic interventions. Their effect on human dignity on Israel law is inspired by Jewish beliefs that are orthodox yet highly liberal in terms of medicine. In Judaism, the primary goal is to heal and save lives. This tradition motivates doing stem cell research for medicinal purposes (Revel 2002).

Israel is also a participant in several international stem cell fora. Additionally, the Israeli Cell Therapy consortium is a founding member of an expanding set of networks dedicated to stem cell research, cell therapy, synchrotron science, and water treatment technologies (Israel's Innovation Authority 2019).

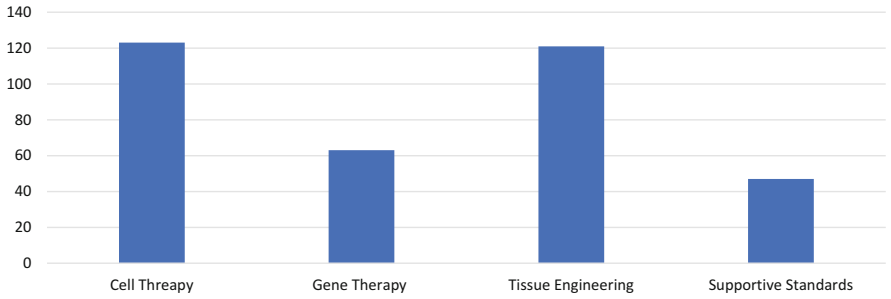
4.14 Quality Standards for Regenerative Medicine

Regenerative medicine may both benefit and harm people. Before a product can be sold, its potency, safety, and efficacy must be shown. This field requires strict quality control (QC). ISO provides a set of standards and requirements in manufacturing the product, and each component is checked against set parameters. They are assuring that the products are correctly produced in each lot, in each batch and follow processes for GMPs. Local governments establish industrial norms based on these standards and regulations. Authorities also check certifications such as ISO and GMPs. ICH standards are often compared to US and EU GMPs. The following section provides the detailed requirement of quality standards as per US and EU GMPs and ICH, which offer an overall benchmark in implementing details for processes in Regenerative Medicine.

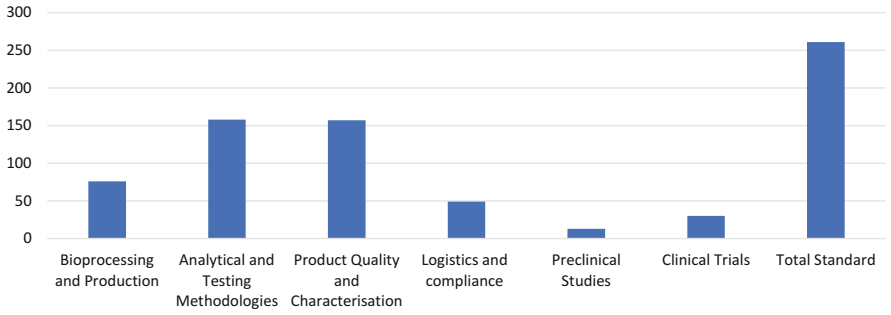
Nexight Group LLC and SCB collaborated on a study titled The Regenerative Medicine Standard Landscape in 2018. The project was funded by the United States Food and Drug Administration (FDA). This paper summarizes the regenerative medicine standardization landscape (Nexight Group LLC & SCB 2020). It summarizes current and in-development standards, serving as a resource for companies looking to enhance their operations by identifying accessible standards.

The following are details about Standards related Regenerative medicine.

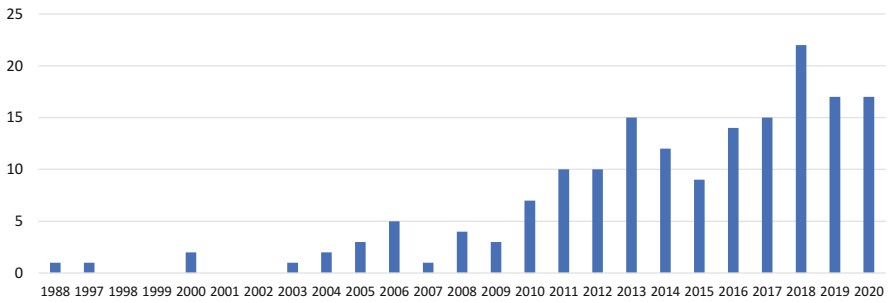
1. **Existing Standards And Standards Under Development.**
   (a) Ancillary materials in cell therapy manufacturing.
   (b) Tissue-engineered medicinal products (TEMPs).
   (c) The best methods for producing, evaluating, and administering gene therapy products.
   (d) Methods for cell counting and measurement.
   (e) Quality assurance and risk evaluation of cell therapy products.
   (f) Thermoelectric initiator materials characterization and testing.
   (g) DNA sequencing and molecular diagnostics method of obtaining DNA.
   (h) Genomic DNA, reference panels, gene therapy reagents, and virology standards.
   (i) Equipment requirements for universal cell culture.
   (j) TEMPs for infectious agent evaluation.
   (k) Structural scaffolding for tissue engineering and pore testing (Graphs 4.1, 4.2, and 4.3).



Graph 4.1 Existing standard by regenerative medicine sector



Graph 4.2 Existing standards number by functional area



Graph 4.3 Standards published yearly

2. **Standard-setting organizations globally for regenerative medicine and related fields.**
   (a) International Organization for Standardization (ISO).
   (b) American Standard of Testing and Materials (ASTM) International.
   (c) American Association of Blood Banking (AABB).
   (d) European Directorate for the Quality of Medicines and Healthcare (EDQM).
   (e) International Society for Advancement of Cytometry (ISAC).

(f) International Society for Biological and Environmental Repositories (ISBER).
(g) American Type Culture Collection (ATCC).
(h) American Society of Histocompatibility & Immunogenetics (ASHI).
(i) British Standards Institution (BSI).
(j) Association of German Engineers (VDI).
(k) American Association of Tissue Banks (AATB).
(l) Clinical & Laboratory Standards Institute (CLSI).
(m) EuroFlow Consortium.
(n) European Federation for Immunogenetics (EFI).
(o) Foundation for the Accreditation of Cellular Therapy (FACT).
(p) International Federation for Adipose Therapeutics and Science (IFATS).
(q) Human Cell Differentiation Molecules.
(r) International Conference on Harmonization of Technical Requirements.
(s) Human Use (ICH) International Council for Commonality in Blood Banking Automation (ICCBBA).
(t) Standards Coordinating Body for Gene, Cell, and Regenerative Medicines and Cell-Based Drug Discovery (SCB).
(u) International Society for Stem Cell Research (ISSCR).
(v) The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH).
(w) International Society for Cellular Therapy (ISCT).

3. **Significant contributions of various International Standard bodies (Table 4.1).**
4. **Essential Standards for Regenerative Processing area:**
   Particle pollution in the air is kept to acceptable levels in cleanrooms and other regulated environments. Air pollution reduction benefits the following industries like Airborne Health, Microelectronics, Medical gadgets and foods (Table 4.2).
5. **Quality Standards in Supply chains of Regenerative Medicine** (Cryoport 2019).
   Although no one, including the FDA, knows what the new regulatory requirements will be, today's rules, standards, and guidance papers offer a helpful guide.

Table 4.1 Significant contributions of various International Standard bodies

Agency name	Contribution
European Medicines Agency	• The guideline for human cell-based medicinal products is human cell-based medicinal products (EMEA/CHMP/410869/2006).
	• Reflection paper on stem cell-based medicinal products (EMA/CAT/571134/2009).
	• Reflection paper on in vitro cultured chondrocyte containing products for cartilage repair of the knee (EMA/CAT/CPWP/568181/2009).
	• Guideline on Xenogeneic cell-based medicinal products (EMEA/CHMP/CPWP/83508/2009).
	• Reflection paper on clinical aspects related to tissue-engineered products (EMA/CAT/573420/2009).
	• Guideline on safety and efficacy follow-up and risk management of advanced therapy medicinal products (EMEA/149995/2008).
	• Guidance on the use of bovine serum in the manufacture of human biological medicinal products (CPMP/BWP/1793/02).
	• Minimizing the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (EMA/410/01).
	• CHMP/CAT position statement on Creutzfeldt–Jakob disease and advanced therapy medicinal products (CHMP/CAT/BWP/353632/2010).
	• Position Paper on Re-establishment of working seeds and working cell banks using TSE compliant materials (EMEA/22314/02).
	• Note for guidance on plasma-derived medicinal products (CPMP/BWP/269/95).
	• Quality: Excipients—Guideline on excipients in the dossier for application for marketing authorization of a medicinal product (EMEA/CHMP/QWP/396951/2006).
The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH)	• ICH Q2 (R1) Validation of analytical procedures: text and methodology (CPMP/ICH/381/95).
	• ICH Q5A (R1) Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin (CPMP/ICH/295/95).

(continued)

Table 4.1 (continued)

Agency name	Contribution
	• ICH Q5C Stability testing of biotechnological/biological products (CPMP/ICH/138/95).
	• ICH Q5D Derivation and characterization of cell substrates used for the production of biotechnological/biological products (CPMP/ICH/294/95).
	• ICH Q5E Comparability of biotechnological/biological products (CPMP/ICH/5721/03).
	• ICH Q7 Good manufacturing practice for active pharmaceutical ingredients (CPMP/ICH/4106/00).
	• ICH Q8 (R2) Pharmaceutical development (CHMP/ICH/167068/04).
	• ICH Q9 Quality risk management (EMA/CHMP/ICH/24235/2006).
	• ICH Q10 Pharmaceutical quality system (EMA/CHMP/ICH/214732/2007).
	• ICH S6 (R1) Preclinical safety evaluation of biotechnology-derived pharmaceuticals (CHMP/ICH/731268/1998).
	• Guideline on clinical trials in small populations (CHMP/EWP/83561/2005).
	• Points to consider on applications with
	– 1. Meta-analyses
	– 2. One pivotal study (CPMP/EWP/2330/99)
	• ICH E1 The extent of population exposure to assess clinical safety (CPMP/ICH/375/95).
	• ICH E3 Structure and content of clinical study reports (CPMP/ICH/137/95).
	• ICH E4 Dose-response information to support drug registration (CPMP/ICH/378/95).
	• ICH E6 (R1) Good clinical practice (CPMP/ICH/135/95).
	• ICH E7 Geriatrics (CPMP/ICH/379/95).
	• ICH E8 General considerations for clinical trials (CPMP/ICH/291/95).
	• ICH E11 Clinical investigation of medicinal products in the paediatric population (CPMP/ICH/2711/99).
European Pharmacopoeia in setting standards	• Ph. Eur. Monograph on human haematopoietic stem cells (Cellular stirpes haematopoietic Humanae) Version 7.2.
	• Ph. Eur. monograph on Method of analysis (2.7.23.) Numeration of CD34/CD45+ cells in haematopoietic products. Version 7.2.

(continued)

Table 4.1 (continued)

Agency name	Contribution
	• Ph. Eur. monograph on Method of analysis (2.7.28.) Colony-forming cell assay for human haematopoietic progenitor cells. Version 7.2.
	• Ph. Eur. Monograph on Nucleated Cell Count and Viability (2.7.29.).
	• Ph. Eur. Monograph on Nucleic Acid Amplification Techniques (2.6.21.).
	• Ph. Eur. Monograph on Flow Cytometry (2.7.24.).
	• Ph. Eur: (2.6.27) Microbiological control of cellular products.
	• Ph. Eur: (2.6.1.) Sterility.
	• Ph. Eur: (5.1.6) Alternative methods for control of microbiological quality.
	• Ph. Eur. Monograph Mycoplasmas (2.6.7.).
	• Ph. Eur. Monograph on Bacterial endotoxins (2.6.14.).
	• General chapter 5.2.12 raw materials for the production of cell-based and gene therapy medicinal products.
British Standards Institute (BSI) in setting standards	**BSI's Significant Standards and Publications**
	EN ISO 22442-1:2015 BS EN ISO 22442-1: 2015.
	• Medical devices are made from animal tissues and derivatives of animal tissues. Risk management in action.
	• EN ISO 22442-2:2015 BS EN ISO 22442-2: 2015.
	• Medical devices are made from animal tissues and derivatives of animal tissues. Controls over the source, gathering, and management of information.
	• BS EN ISO 22442-3:2007 BS EN ISO 22442-3:2007.
	• Medical devices are made from animal tissues and derivatives of animal tissues. Validation of viral and transmissible spongiform encephalopathy (TSE) agent removal and inactivation.
	• Regenerative medicine PAS 84:2012.
	• PAS 83:2012 in the European Union and the United States of America, developing human cells for therapeutic purposes Guide.
	• PAS 83: The British Standards Institute (BSI) published PAS 83 Guidance for cell-based treatments in 2006, spanning basic research

(continued)

Table 4.1 (continued)

Agency name	Contribution
	through clinical application. This book details the process and juxtaposes it with current laws and regulations to illustrate the area and highlight significant shortcomings.

Table 4.2 Essential standards for regenerative processing area

Standard reference/ identification	Description of standards
BS EN ISO 14644-1	Cleanrooms and associated controlled environments. Part 1. Classification of air cleanliness by particle concentration.
BS EN ISO 14644-2	Cleanrooms and associated controlled environments. Specifications for monitoring and periodic testing to prove continued compliance with ISO 14644-1.
BS EN ISO 14698-1: 2003	Cleanrooms and associated controlled environments. Biocontamination control. General principles and methods.
BS EN ISO 14698-2: 2003	Cleanrooms and associated controlled environments. Biocontamination control. Evaluation and interpretation of biocontamination data PD 6609:2007 Environmental cleanliness in enclosed spaces. Guide to in situ high-efficiency filter leak testing.
BSEN 14175-1:2003	Fume Cupboards.
BS EN ISO 14698-2: 2003	Cleanrooms and associated controlled environments. Biocontamination control. Evaluation and interpretation of biocontamination data PD 6609:2007 Environmental cleanliness in enclosed spaces. Guide to in situ high-efficiency filter leak testing.
BS ISO 48:2007	Rubber, vulcanized or thermoplastic. Determination of hardness (hardness between 10 IRHD and 100 IRHD).
BS EN 14175-2:2003	Fume cupboards. Safety and performance requirements.

Standards Reference	Description
ISO 9001:2015, EMA 2013/C 343/10, ICH Q10, and Annex 5	A comprehensive quality systems approach to the distribution of regenerative therapies, supply chain systems, equipment, and logistics. Its risk-based approach to quality is consistent with QbD components relating to risk management through equipment performance and in-field logic.
Quality Systems Design ICH Q8–10	A framework provides a quality management system based on QbD (Quality by design) and Process Analytical Technology (PAT). This method is well-suited for risk management in complex logistical situations and in-field risk management.
QbD, Risk Management:	Providing commercial regenerative medicine products requires real-time information technology. It will be critical for novel therapies to be launched successfully if

(continued)

Standards Reference	Description
	informatics can gather, evaluate, predict, and provide automated alerts and pre-defined escalation processes based on the nature of the data collected. Procedures based on QbD provide the foundation for this approach.
Document Control: ISO 9001:2015, EMA 2013/C 343/10, 21 CFR Part 11, ICH Q10, and those for whom Annexe 5 lays the groundwork:	A mechanism for maintaining records. Controls over the collection and handling of data business practices that are consistent criteria for establishing the accuracy, authenticity, and inalterability of electronic records, Electronic Equipment and Software Calibration/Validation Additionally, ICH Q7 mandates calibration validation of the equipment and software used to gather temperature, location, and equipment traceability information is critical for Chain of ComplianceTM and Quality by Design procedures.
Equipment Validation and Traceability 21 CFR 210, 211, EMA 2013/C 343/10, and ICH Q7. Cleaning and maintenance of equipment must adhere to the requirements of CFR 211.67.	Every aspect of regenerative medicine must be traceable, from the Chain of custody to the Chain of condition, the Chain of identification, and the Chain of Compliance TM. 13 Assembling a Chain of Compliance TM competence in support of the distribution of regenerative medicine requires consideration of 21 CFR 210, 211, EMA 2013/C 343/10, and ICH Q7. C
Facilities and Personnel 21 CFR 210, 21 CFR 211, and EMA 2013/C 343/10	All applicable Good Manufacturing Practice (GMP) standards for clinical and commercial product distribution. 21 CFR 210 and 211 provide critical requirements for equipment maintenance and traceability in support of chain of compliance standards.
Distribution and Logistics	These regulations support absolute temperature and logistics management requirements, including equipment performance and temperature control.
EMA 2013/C 343/10, who Annex 5, ISTA 3A, 7D, and 7E	These should be supplemented by a risk-based approach to minimize in-field risk associated with third-party handling and should involve active temperature, equipment, and location monitoring at a minimum.
Contamination and Packaging EMA 2013/C 343/10>, 21 CFR 211.67, and 21 CFR 1271.190	These standards address a growing worry regarding cross-contamination in cell and gene therapies. Consensus dictates that all equipment used to distribute blood and cell components and the finished product must have been exclusively utilized to carry human clinical and commercial products; this is essential for reducing the possibility of non-human commodity contamination.

4.15 Conclusion

The area of regenerative medicine provides a vast potential to grow. It is evident from many publications, white papers, and clinical studies around regenerative medicine that this discipline is rapidly progressing and proliferating patient's lives. The scientific community is confident that the risks associated can be mitigated by setting up appropriate regulations and standards for collecting, developing, producing, manufacturing, labelling, testing, infusing, and administering these new biological medicines. Despite a few untoward incidents caused by using non-proven regenerative medicines, the possibilities of doing good are more substantial.

As could be anticipated, the rate of development of such new technology is far faster than the establishment of regulatory frameworks.

While robust RM legislation/frameworks are in place in the EU, the United States, China, Japan, South Korea, Australia, and India, there is a critical need to assess the situation continuously. This would ensure modification of legislation in line with new challenges. This may be accomplished via frequent dialogues of stakeholders at the international level and through private-public partnerships. Most authorities adopt a risk-based approach, with exemptions and stringent compliance requirements for specific categories in RMs. According to the current findings, global restrictions are being implemented over new technologies and products. The rapid development of these new technologies is closely monitored by regulators, non-profit organizations, and patients, all of whom have a vested interest in this rapidly developing technology.

The field of regenerative medicine has a promising future. Although change occurs at an accelerated rate, the pandemic has taught all the stakeholders to come together for an extensive sharing of experiences, lessons learned, and gather incidents across the area to bridge the gaps and focus on regenerative medicines as part of "Novel" therapies.

The horizon for this kind of treatment is expanding, and now scientists are discussing Biological Therapy. Biological therapy is a kind of treatment that involves the use of components of the body's immune system to combat illness. Additionally, biological therapy is utilized to protect the body against specific adverse effects associated with particular therapies. Biological treatment often entails the administration of chemicals known as biological response modifiers (BRMs). Typically, the body generates trace quantities of these chemicals in response to infection and illness. Scientists can manufacture BRMs in massive quantities using current laboratory methods to treat cancer and other illnesses such as rheumatoid arthritis and Crohn's disease. Additionally, biological treatment may target particular molecules on cancer cells to kill them, or it may target proteins that promote cancer cell development. Biological treatments may be administered orally, intravenously, or by injection, depending on the agent.

Looking at future advancement, there is a hard-pressed need for all nations and regions to unite to study, research, analyse, and explore these technologies and perform risk assessment and impact of these new-age therapy together for the good of humankind.

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Stem Cell Bioreactors: Design, Structure, and Operation of Stem Cell Bioreactors

5

Firdos Alam Khan

Abstract

A bioreactor is a manufactured device or system that helps to produce biologically active substances such as yeast, bacteria, or mammalian cells under controlled environment. Bioreactor is basically a vessel constructed where chemical process is carried out in the presence of cells or organisms to produce large quantity of cells or their byproducts. The need for bioreactors arises when you need to produce large quantity of biological products, which is however not possible in the research laboratories. These bioreactors come with various sizes ranging from 2 L to 100s of L. These bioreactors are used to produce biopharmaceuticals, vaccines, or monoclonal antibodies, cell production and expansion, enzyme production, tissue engineering, algae production, protein synthesis, and also stem cell production. In this chapter, we will discuss significance, application, design, types, and operation of bioreactor especially focusing to mammalian or stem cell culture.

Keywords

Stem cell bioreactors · Design of bioreactors · Structure of bioreactors · Operation of stem cell bioreactors

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F. A. Khan (ed.), *Stem Cell Production*,
https://doi.org/10.1007/978-981-16-7589-8_5

5.1 Introduction

A bioreactor is a manufactured device or system that helps to produce biologically active substances such as yeast, bacteria, or mammalian cells under controlled environment (Stephenson and Grayson 2018). Bioreactor is basically a vessel constructed where chemical process is carried out in the presence of cells or organisms to produce large quantity of cells or their byproducts (Stephenson and Grayson 2018). For large-scale production of biological active substances such as cells or tissues or organisms, the bioreactors are designed and constructed. These bioreactors come with various sizes ranging from 2 L to 100s of L. These bioreactors are used to produce biopharmaceuticals, vaccines, or antibodies and also used in converting raw materials into useful byproducts such as in the bioconversion of corn into ethanol.

5.2 Bioreactors and Its Application

Bioreactors are generally used to produce mammalian cells, stimulate cell differentiation and tissue formation under controlled environment supplied by nutrients. These bioreactors have been extensively applied to culture and expansion of mammalian cells, chimeric cells, induced pluripotent stem cells, and human mesenchymal stem cells, respectively. Furthermore, another advantage of bioreactors is that they allow delivery of biological and chemical ingredients on control manner to regulate the production of different types of cells with high purity and better functionality. The well-defined controlled environment in the bioreactors has many advantages such as it improves standardization and reproducibility of the products and also helps to produce in large quantities. It also produces clinically relevant cells with high purity and superior functionality. Over the past years as the field of regenerative medicine has grown up globally, so as the use of bioreactors also increased immensely. The different types and sizes of bioreactors have been designed and produced to meet the demands received from various biopharmaceutical and biotechnology companies to produce clinical grade stem cells for the cell therapy.

5.3 Types of Bioreactors

Bioreactors can be designed and produced based on the two major factors, one is that which types of cells need to be produced for example, bacterial cells, yeast and mammalian cells and second one how much volume (liters) of the cells need to be produced. Based on these factors, bioreactors are classified into six types such as fluidized bed bioreactors, and packed bed bioreactors, continuous stirred tank bioreactors, bubble column bioreactors, airlift bioreactors, and photo-bioreactors.

5.3.1 Continuous Stirred Tank Bioreactors

The bioreactor is also known as back-mix reactor or mixed flow reactor as this continuous-flow stirred tank reactor is used in chemical and environmental engineering. This bioreactor mainly used to assess the key unit operation variables to reach a specified output. This bioreactor can be used to in the forms of liquids and gases chemical compositions.

5.3.2 Bubble Column Bioreactors

The bubble column reactor is used wherein mixture of gases can be distributed in the liquid form by using a suitable distributor which travels upwards direction produced bubbles.

5.3.3 Airlift Bioreactors

Airlift bioreactors are used where the injection of a gas is made in the culture medium which can cause the broth to circulate between the riser and an interconnected down comer compartment of the bioreactor.

5.3.4 Fluidized Bed Bioreactors

In a fluidized bed bioreactor, mixture of culture medium is moved in upward direction through a packed bed of immobilized cells suspends them inducing a fluid-like behavior.

5.3.5 Packed Bed Bioreactors

Packed bed bioreactors are used in cell and tissue engineering applications. These bioreactors support the growth and expansion of different types of cell lines for long period of time under various culture conditions.

5.3.6 Photo-Bioreactors

In photo-bioreactor, light source is used to cultivate phototrophic microorganisms. These phototrophic microorganisms apply photosynthesis process to generate biomass by converting light energy into biomass using light and CO_2.

5.4 Design of Bioreactors

Bioreactor is basically a steel vessel which is covered by thermal jacket to prevent the loss of heat. The center of reactor is fitted with aerator panel to mix the cell culture which is equipped with motor where speed of rotation can be increased or decreased (Fig. 5.1).

These inlets allow the entry of air, media, and nutrients inside reactors which are monitored by the computerized system. In case outlet, which releases effluent outside. The temperature, pH, dissolved oxygen, and pressure of the reactor is monitored through sensors which are submerged in the culture media.

5.5 Function and Operation of Bioreactors

5.5.1 Culture Condition

The bioreactor is generally used to culture cells which are growing in the media and nutrients need to be regularly and steadily mixed all the time to be able to produce uniform and pure products. In case, the nutrients are not properly mixed in the bioreactor, the growing cells will not get sufficient amount of nutrients which may

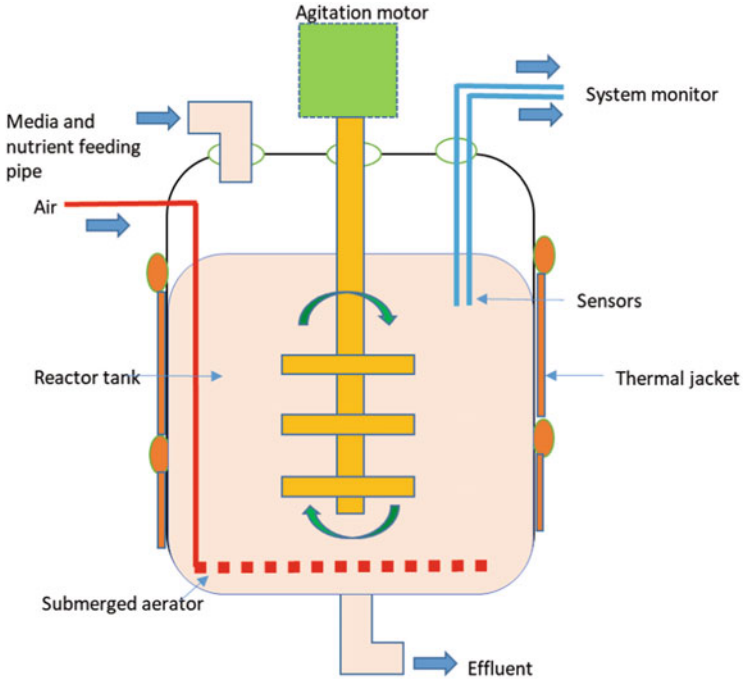

Fig. 5.1 Diagrammatic representation of the bioreactor for cell culture

Table 5.1 Rate of mixing in the bioreactor

Organism types	Rate of mixing
Bacteria, yeast, fungi	500–1500/min
Mammalian or plant cells	30–300/min

Table 5.2 Temperature range in different organisms

Type of organisms	Temperature range
Bacteria, yeast, fungi	+20 °C to +60 °C
Mammalian or plant cells	+25 °C to +37 °C

result in not getting the desirable products, and there will be deviation from one lot to another lot which is not acceptable as per the GMP guidelines. In some case, if the pH of the mixture is too high, which will have negative impact on both quality and quantity of the cell production and is not acceptable as per the GMP guidelines. Such deviations in the bioreactor not only reduce the efficiency of the bioprocess, but can also promote genetic modifications which may result in the financial loss to the company as such product is not approved for the consumption. In addition, temperature in the bioreactor is also very critical factor for the product development as any minute variation in the temperature may negatively impact the cell growth and culture. Hence, it's very important to regulate and maintain the bioreactor temperature uniformly and consistently. Another important issue is the stirring of the mixture in the bioreactor. It's very important to continuously mix the air and nutrients with growing cells, so that all growing cells get uniformly same amount of air and nutrients. The speed of stirrer is critical, as very slow and very high speed may not good for the cells and may cause damage to growing cells. It has been found that depending on the cell line, cell cultures may have a much more intense response to overly vigorous stirring which may lead to cell death. The mixing rate for different organisms is shown in Table 5.1.

5.5.2 Temperature Monitoring During Culture

Temperature is critical factor for microorganisms and cell growth inside bioreactor and optimum temperature has been defined for the cell culture growth. The presence of enzyme in the nutrients and media is important for cell growth and action of enzyme may be changed with the change in the temperature. It's very important to maintain the reactor temperature uniformly and consistently during the cell culture growth and production. It has been found that mammalian cells are normally grown comfortably in the narrow range of 37 °C and any variation from 3 to 4 °C may cause damaging impact on the cell production and there is great possibility that growing cells might die due to high temperature. Platinum resistance sensors are used to check and monitor the bioreactor temperature. The typical temperature range in different organisms is shown in Table 5.2.

To avoid an overheating of the vessel, bioreactor is now active cooling system which quickly helps to reduce temperature and brings back to required temperature.

But none the less, the temperature must be constant throughout the cell production stage or cell cultivation. In some unique condition, where the products such as penicillin or recombinant proteins, changing in the temperature at the end of the growth phase may be useful to activate certain important genes for product formation. In addition, the temperature of the vessel is rarely dropped at the end of the bioprocess which allows finished product to remain stable for future use.

5.5.3 pH Monitoring During Culture

Like temperature, the measuring and controlling of pH of the growing cells inside bioreactor is critical for the bioprocesses, as any change in the pH may significantly alter growth conditions and may produce negative impact on cell growth and cell cultivation. It has been noted that culture media generally possess buffer substances that may cause the change in pH of the mixture, and any increase in the acidic pH may damage the cells and cells will eventually die. A typical pH range for different organisms is depicted in Table 5.3.

In the bioreactor, pH sensor is fitted to measure pH during the bioprocess and this pH sensor is also known as a combination electrode for pH. To properly control the pH of the mixture, the bioreactor has option for acid and an alkaline which is connected to the culture vessel via tubes and pumps.

5.5.4 Addition of Nutrients During Culture

Nutrients are the most constituent for the cell cultivation, and based on the different cell types, array of nutrients have been used and developed. The basic composition of a nutrient medium typically consists of water, glucose, carbon, nitrogen, and phosphorus, salts and trace elements. In certain specific conditions, there is also need for vitamins, essential amino acids to be added in the culture media. It's very important to maintain the quality of nutrients used in the cell cultivation and any variation in the quality of nutrients will have impact on the quality of cells product.

5.5.5 Providing Air During Culture

During the bioprocess of cell cultivation, it's very important to provide the growing cells with required amount of sterile oxygen into the culture medium. It has been recommended that constant stirring is required during the whole process of cell culture for successful and uniform gas distribution into cell culture media. While gas

Table 5.3 pH range for different organisms

Type of organisms	pH range
Bacteria, yeast, fungi	4.5–7.0
Mammalian cells	6.7–7.4

release into the mixture, which may lead to production of bubbles in the mixture which indicate that gas is mixing in the cell culture media. The bubble formation is important as it shows that microorganisms and cell cultures absorb the oxygen that has been dissolved in the nutrient mixture. The demand for oxygen may vary from cells to cells and level of oxygen can be monitored regularly.

Unlike to microorganisms, cell cultures are not only needed oxygen in the mixture but also get influenced by pure nitrogen and pure oxygen present in the mixture. The precise composition of the various gases in the mixture generally depends on type of the cell culture. The gases conditions in the vessel can be monitored through computerized control system. It has been suggested that during the starting time of the bioprocess, the amount of oxygen required is minimum but its requirement increased when cell cultivation progresses. In addition to ensuring a constant supply of the desired oxygen, the bioreactor should be also delivered the right amount of gas at the right time. It has been suggested that the gases amount will be varied during the bioprocess and it's directly related to amount of cells which are grown in the vessel. During the mixing of different gases, the air bubbles will form which will uniformly be distributed in the vessel which is very critical for obtaining the optimum cell products. The efficiency of cell production is also depended on the mixing of oxygen during culture, which means, if oxygen is not properly distributed in the vessel, it will produce a negative impact on the quantity and quality of the cell production.

5.5.6 Monitoring Pressure During Culture

The vessel pressure is critical factor for cell cultivation. It has been found that higher the pressure in the vessel will cause the more oxygen to dissolve in the mixture. The cell culture vessels which are made of glass can only take pressure up to 0.5 bar, which is not sufficient for the cell cultivation and if you increase the pressure, there is great possibility that glass bioreactor will get burst. Unlike glass culture vessels, stainless steel bioreactors are designed for higher pressures. Hence, it's highly recommended that all bioreactors should be made of steel material. Even, in the steel made bioreactor, outlet should be provided so that access gas can be released from the bioreactor. The standard steel vessel can take pressure up to 2 bar.

5.6 Application of Bioreactors in Stem Cells Production

The bioreactors have been extensively used in the production of clinical grade stem cells for the regenerative medicine, tissue reconstruction, and cell transplantation application. As large number of stem cells or differentiated cells are required for the regenerative medicine, it can only be produced by using bioreactors. The cells can be produced for both allografts and allogeneic transplantation. As per WHO, FDA, all the clinical grade stem cells or differentiated cells must be produced as per GMP compliance and guidelines. GMP compliance manufacturing facility needs a huge

investment, and biotechnology companies and hospitals need to create separate GMP facilities. The advantage of GMP facility allows you to produce millions of cells in the bioreactor which are needed for the cell transplantation. The bioreactors are capable of supporting industrial-scale, ultra-high-density cell suspension cultures with controlled microenvironments, standardization, and uniformity of culture conditions in order to generate homogenous populations of stem or lineage-specific cells. There are different types of bioreactors such as Rocking bed (wave motion) (Shekaran et al. 2016) which has size (1–500 L), has advantages—versatile single-use bags, and has limitations—limited scale-up potential for hMSCs. Stirred tank (Surrao et al. 2016; Gasperini et al. 2014; Lawson et al. 2017; Markert and Joeris 2017) has size (100 mL–1000 L), can produce large volumes with limitation of cell death due to force and may be useful for hMSCs, hASCs, hiPSCs, and murine ovary cell production. In the case of rotating wall vessel (Varley et al. 2017), which has size (100 mL–10 L) with advantages of low turbulence and can simulate microgravity but has produced less than 10 L of cells with hMSCs. In case of perfusion bioreactor (Nguyen et al. 2016a, b; Ball et al. 2016) has size (100 mL–5 L) and has advantage of limited turbulence and can be automated with limitations that may affect cells due to large force and useful for hMSCs production. In case of isolation/expansion automated systems (Mock et al. 2016; Priesner et al. 2016) have size (100 mL), and it has many advantages such as versatile single-use bags, automated cell isolation, manipulation, and expansion, GMP-compliant. But it can only produce 100 mL of human lymphocytes.

In addition to non-anchoring or attached cells, bioreactor can also be used to culture adherence or anchored cells. There are different types of cells which can only be grown in adhesion form, not in the suspension form. It's challenging to grow the anchored cells in the bioreactors and it's now possible to grow anchored cells in the bioreactors by using hollow fibers in perfusion systems, encapsulation, or microspheres. The attachment of anchored cells with this methods increase surface area of suspension bioreactor. In addition, packed bed bioreactors can also be used to grow mesenchymal stem cells. Moreover, there are few studies which have shown that adherent cells such as bone marrow derived mesenchymal stem cells can be successfully cultured on protein coated microspheres (Stephenson and Grayson 2018). It has been reported that cells which are grown on these microspheres retain their morphological and functional properties. By using this approach, it's possible to produce the cells in large volume (1000–2000 L) of cells by using Mobius (EMD Millipore) stirred tank bioreactor. This bioreactor is commercially available in sizes ranging from 50 to 2000 L. There is an issue with this method, as high speed is required for such bioreactor which may cause the stem cells differentiation into different cell types and this problem can be tackled by encapsulating them into microspheres. This type of encapsulation method is commercially expensive method (Stephenson and Grayson 2018).

As per the published data, it has been found that maximum 3 L of mesenchymal stem cells and adipose-derived stem cells can be produced in the bioreactors (Stephenson and Grayson 2018). In another report, it has been found that more than 50 L of stem cells can be produced in the bioreactor with some modifications.

Another advantage of this method is that stem cells don't lose their stem cell-like characteristics as these cells retained pluripotency and showing the stem phenotypic markers (CD44 and CD90), when compared with cells cultured under traditional conditions. But, this method has some disadvantage as this method requires certain growth factors and animal serum which is clinically not accepted as per the FDA guidelines.

Another strategy for better production of clinical grade stem cells is to produce them in the self-assembled aggregate forms. It has been suggested that cellular aggregates showed better and improved survivability and tissue forming capabilities (Stephenson and Grayson 2018). The impact of bioreactors on the mesenchymal stem cell aggregation and cell size has been examined by using commercially available WAVE Bioreactors™, and this bioreactor provides better stirring capabilities.

5.6.1 Induced Pluripotent Stem Cell Expansion

Over past few years, different types of bioreactors have been designed and constructed for producing embryonic and induced pluripotent stem cells such as in rotating flasks bioreactors, rotating wall bioreactors, stirred tank bioreactors, and WAVE Bioreactors™ (Wang et al. 2014; Kropp et al. 2017). It has been found that cell method with aggregation approach is considered to be the best method to generate natural pluripotent stem cells with natural characteristics. While producing pluripotent stem cells, care should be taken to avoid differentiation of pluripotent stem cells into different terminally differentiated cells. The differentiation of pluripotent stem cells can be avoided by providing them with specialized media and growth factors with proper monitoring and supervision. The stem cell aggregate size can be maintained to avoid differentiation. It has been found that stem cell aggregate sizes are regulated by chemically using Rho-kinase inhibitors and mechanically by using physical disruption techniques. Furthermore, the dissolved oxygen concentrations and dilution rate in the cell mixture also effect the stem cell (Abecasis et al. 2017).

5.7 Future Prospects in the Advances of Bioreactors

One of the limitations in the currently used cell culture is 2-dimensional form of cell production, which is not closely related to natural process of stem cells development. In fact all the body cells and tissues are grown in 3-dimensional form and cells grown in 3-dimensional form are physiologically and functionally better than 2-dimensional form. Hence, recently efforts are made to design bioreactors in such way that stem cells can be grown 3-dimensionally. Three-dimensional bioreactors support the production of different cells with applications. The 3-dimensional approach can be used to produce large number of cells and tissues. One of the challenges of the 2-dimensional approach, is that due to large size, it's difficult to

provide the nutrients and growth factors to all parts of the cells, whereas in case of 3-dimensional approach cells receive nutrients and growth factors and required amount of oxygen as all the sides are exposed to them. Efforts are also being made to design bioreactors to enhance the functionality of the cells by incorporating them with biomimetic physiological stimuli and sensors in the cell construct. These sensors help to monitor the health, growth, and viability of the cells on time-scale. The use of computer and artificial intelligence based approach also helps engineers to design automatic bioreactors with better efficiency and productivity of functional cells and tissues (Stoppel et al. 2016; Luciani et al. 2016; Dikina et al. 2017; Guo et al. 2016; Mellor et al. 2017). The application of computational modeling found to be effective in improving the predictability of the clinical grade cell product (Shakeel et al. 2013; Guyot et al. 2016; Nguyen et al. 2016a, b).

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Stem Cell Production: Processes, Practices, and Regulation

6

Stem Cell Culture and Expansion: Role of Culture, Types of Cells, Growth Conditions, Media Nutrients, Growth Factors, Growth Phase Cycle

Daniela Lisini, Simona Frigerio, Sara Nava, and Simona Pogliani

Abstract

The latest scientific advances in the field of cell and molecular biology allowed the development of a new category of therapies, based on the cells (gene therapy, cell therapy, and tissue engineering), collectively known as Advanced Therapies Medicinal Products (ATMPs). ATMPs can be defined as products which consists of cells that have been subject to substantial manipulation or that are not intended to be used for the same essential function(s) in the recipient and the donor. ATMPs are characterized by complex manufacturing bioprocesses, under Good Manufacturing Process (GMP) rules, and offer new therapeutic opportunities for different diseases, including those of genetic origin, tumors, and neurological diseases, that currently have limited or no effective conventional therapeutic options. To date, Cell Therapy Medicinal Products are the most developed and used as drugs for the cure of different pathologies, so we will focus our discussion on the description of this type of medicinal products.

Keywords

Advanced Therapy Medicinal Products · Good Manufacturing Practice · Cell culture · Immunotherapy · Regenerative medicine

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F. A. Khan (ed.), *Stem Cell Production*,
https://doi.org/10.1007/978-981-16-7589-8_6

6.1 Introduction

The latest scientific advances in the field of cell and molecular biology allowed the development of a new category of therapies, based on the cells (gene therapy, cell therapy, and tissue engineering). This innovative field of the medicine permits the use of new strategies for the cure of different diseases.

Over the past decade, the technological revolution in the field of biomedical research has led to the development of new clinical practices and a variety of highly sophisticated and rapidly changing drugs, collectively known as Advanced Therapies Medicinal Products (ATMPs). ATMPs are drugs of high novelty, complexity, and technical specificity, which differ from conventional drugs since they consist of cells (Regulation EC No 1394/2007 of the European Parliament).

ATMPs can be defined as products which "consists of cells that have been subject to substantial manipulation or that are not intended to be used for the same essential function(s) in the recipient and the donor" (cited from Regulation EC No 1394/2007 of the European Parliament). Then a first distinction between cell products is the degree of manipulation: for example cells that have undergone a separation process, sterilization or lyophilization are not considered an ATMP, while those administered to the patient after substantial manipulations, including isolation and expansion processes, fall within the definition of ATMPs. The other definition criterion is the use of the non-homologous cells: cells that are used with a function different from the original function are considered ATMPs, even in cases where the patient is the donor and recipient at the same time.

ATMPs are characterized by complex manufacturing bioprocesses and offer new therapeutic opportunities for different diseases, including those of genetic origin, tumors and neurological diseases, that currently have limited or no effective conventional therapeutic options (Directive 2001/83/EC of the European Parliament; Commission Directive 2009/120/EC; Council Directive 90/385/EEC; Directive 2004/23/EC of the European Parliament; Regulation (EU) 2017/1569).

Sub-classifications of advanced therapies are different between regions: whereas in US legislation the ATMPs are classified in only two categories (gene vs. cellular therapy), in EU, ATMP can be classified in four categories:

1. **Gene Therapy Medicinal Products** (GTMPs): in this context DNA is directly used pharmacological substance, as recombinant genes are inserted into cells. GTMP is defined as "an active substance which contains or consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding, or deleting a genetic sequence; its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence" (cited from Regulation EC No 1394/2007 of the European Parliament).
2. **Somatic Cell Therapy Medicinal Products** (CTMPs): It is defined as "biological medicinal product which contains or consists of cells or tissues that have been subject to substantial manipulation so that biological characteristics, physiological functions or structural properties relevant for the intended clinical

use have been altered, or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor" (cited from Regulation EC No 1394/2007 of the European Parliament). Cell selection, ex vivo expansion of cells, generation of clones with both anti-neoplastic and anti-infectious activity can be considered for example substantial manipulations. Cells most often used in the development of CTMPs are adult stem cells, unspecialized cells that can be selected from various tissues of the body and have high differentiation capacity. These cells are autologous when obtained from a donor and reinfused in the same person, or allogeneic, when donor and recipient are two different people.

3. **Tissue Engineering Products** (TEPs): It is defined as "a product that contain or consist of cells or tissue engineered by different methods, including viral or bacterial plasmid vectors" (cited from Regulation EC No 1394/2007 of the European Parliament). They can be used to repair, regenerate, or replace tissues. The first products developed include artificial skin, bone, and cartilage.
4. **Combined Advanced Therapy Medicinal Products**: It is defined as "a product that contain one or more medical devices as an integral part of the cell or tissue based medicinal product" (cited from Regulation EC No 1394/2007 of the European Parliament).

ATMPs are manufactured through very complex processes, in aseptic conditions in facilities, named cell factories. The entire production process must follow the Good Manufacturing Practices (GMP) rules in Europe, or US Good Manufacturing Practices (GMP; Eudralex) guidelines and require preliminary tests conducted under Good Laboratory Practices (GLP) rules and clinical trials conducted in Good Clinical Practice (GCP) before marketing authorization.

Uniqueness is a specific characteristic of an ATMP, as every single preparation derives from one sample and represents in almost all cases a unique product that is considered a transplant rather than a batch of a pharmacological industrial production, and for this reason may not be replaced with another batch or another preparation. Despite this characteristic ATMPs are characterized by unique pharmacologic, metabolic, and immunologic features and can be used for the treatment of potentially many different diseases, thus ATMPs must be produced outstanding the stringent rules for chemical drugs.

The development of an ATMP must be carried out through a series of steps aimed to demonstrate the quality, safety, and efficacy of the final product. Typically, the development process starts at the end of both in vitro and in vivo studies on disease models, to demonstrate product safety and to provide initial indications to plan a clinical trial. In parallel with non-clinical studies, a strong manufacturing process development comprehensive of scale-up evaluation and validation steps must be carried on. The Investigational Medicinal Product (IMP) would first be tested for safety (Phase I trials) and then for dose finding and the initial evaluation of efficacy (Phase II trials); during clinical testings the shelf lives of IMPs must be defined. For confirmation of efficacy, Phase III (pivotal) trials must be performed to obtain the final Marketing Authorization (MA); the last step (Phase IV trial) occurs post-approval, to confirm safety and efficacy data (Fig. 6.1).

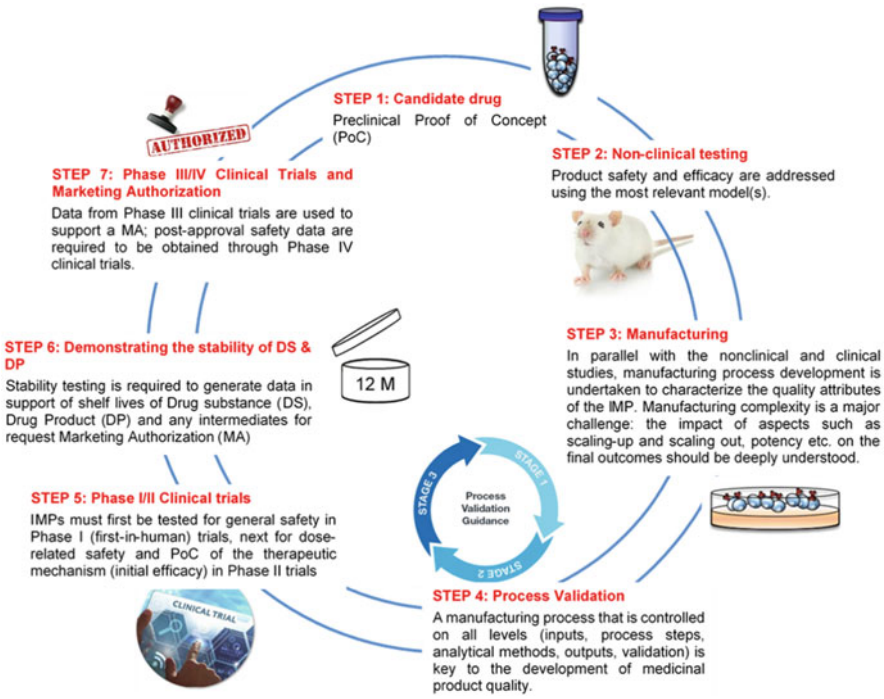


Fig. 6.1 Development of an Advances Therapy Medicinal Product (ATMP). The development of an ATMP involves different steps aimed to show the product's quality, safety, and efficacy. Generally, the development of a CTMP starts at the end of preclinical proof-of-concept (PoC) studies in both in vitro and in vivo models of disease

The production of GMP-compliant cells for use as drug in the cure of many diseases is a complex, long and often expensive process: to efficiently manufacture ATMPs, not only the targeted cell quantity and quality but also the production costs must be taken into account.

To date CTMPs are the most developed and used as drugs for the cure of different pathologies, so we will focus our discussion on the description of this type of medicinal products.

6.2 Types of Cell Used as Cell Therapy Medicinal Products: An Overview

Almost half of the medicinal products currently in development are CTMPs used for immunotherapy treatments, followed by CTMPs prepared for tissue repair. To date several different pathologies can be treated with these drugs, but oncologic diseases remain the principal target, while cardiovascular diseases are also widely treated with CTMPs; other disease areas are inflammation, musculoskeletal system diseases, neurology, diabetes, ophthalmology, dermatology, and other therapeutic areas.

CTMPs can be grouped by their mechanism of action, such as immunological activities (anticancer and immunoregulatory cells) or regenerative cells. As CTMPs development is a dynamic and fast-growing sector, the following breakdown may not be exhaustive of the future landscape. Figure 6.2 summarizes the cell types used as CTMPs.

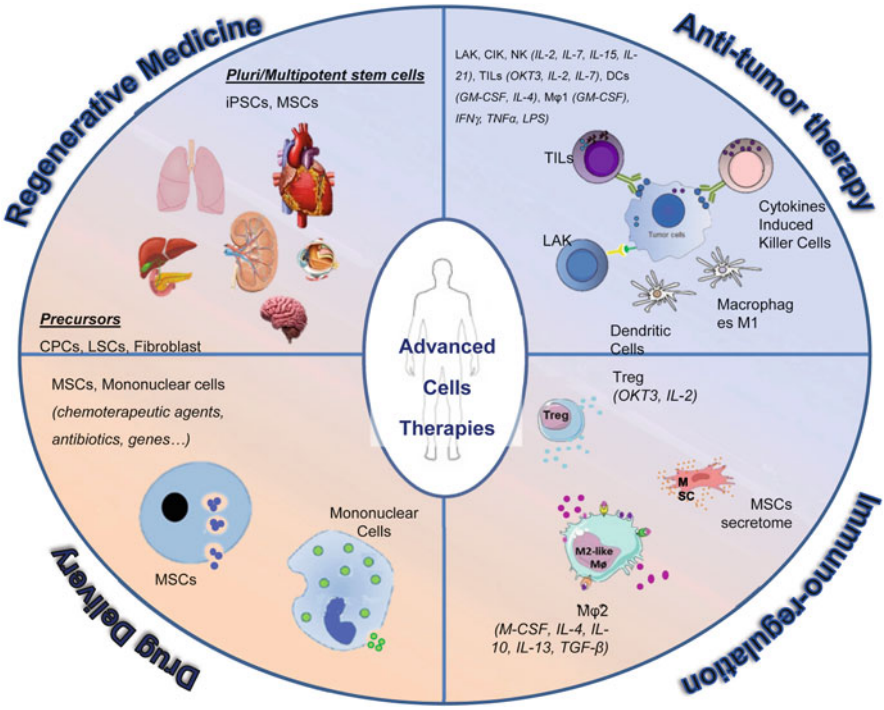


Fig. 6.2 Excursus of cell types used as Cell Therapy Medicinal Products (CTMPs). Pluri- or multipotent stem cells (MSCs and iPSCs) and precursor cells (CPCs, LSCs, and fibroblasts) are the principal candidates for *regenerative medicine*. These cells are utilized in clinical trials for the correction of several damaged organs such as cardiac tissue, lung, liver, pancreas, nervous system, and kidney. *Antitumor activity* can be exerted by a broad range of immunological cells, educated in vitro by specific pro-inflammatory cytokines (IL-2, IL-7, IL-15, IL-21, IFNγ, TNFα, LPS). Immune cells can also be made antigen specific. An appropriate education of some type of immunological cells by immunoregulatory cytokines (IL-4, IL-10, IL-13, TGF-β) can convert them from pro-inflammatory/antitumor mediators to anti-inflammatory/*immune-regulator* (i.e., type 1 vs type 2 macrophages or effector T cells vs T regulatory cells). Cells can also be used as drug in *drug-delivery* approaches. MSCs and mononuclear cells have been demonstrated to be able to uptake and release different types of molecules without affecting their chemical activities and with the advantage of specific homing to damaged tissues. Among cell types, MSCs are the most eclectic cells, because they can be used for regenerative medicine, immunomodulation drug delivery and therefore antitumor therapy. *iPSCs* induced pluripotent stem cells, *MSCs* mesenchymal stromal cells, *LAK* lymphokine-activated killers, *CIK* cytokine-induced killer, *NK* natural killer, *TILs* tumor infiltrating lymphocytes, *DCs* dendritic cells, *Mϕ* macrophages, *IL* interleukin, *CPCs* cardiac precursors cells, *LSCs* limbal stem cells, *GM-CSF* granulocytes macrophage-colony stimulating factor, *IFN* interferon), *TNF* tumor necrosis factor, *LPS* lipopolysaccharide, *Treg* T regulatory, *TGF* transforming growth factor

6.2.1 Immune Cells

The growing development of adoptive immunotherapy in the last years provided the possibility to use a new therapeutic approach for the treatment of different diseases. Immune cells are used mainly for the treatment of malignant hematological diseases as well as solid tumors, but are also promising as regulatory cells in autoimmune diseases.

Historically ex vivo expanded allogeneic cytotoxic T lymphocytes were the first immune cells used for donor lymphocyte infusions in the setting of allogeneic Hematopoietic Stem Cell Transplantation (HSCT) to improve immune reconstitution, decrease infection risk, and increase antitumor immune surveillance. Lymphokine-Activated Killer (LAK) cells were the first ex vivo expanded CTMPs utilized starting from 1980 for adoptive immunotherapy. Few years after starting the use of LAK cells, an innovative clinical trial aimed to cure patients affected by metastatic renal cell carcinoma with ex vivo purified and expanded autologous Natural Killer (NK) cells plus continuous infusions of interleukin (IL)-2 has been started (Hercend et al. 1990). In the next years other trials have deepened the use of autologous NK cell infusions together with IL-2, after a high-dose chemotherapy treatment (Miller et al. 1997; Burns et al. 2003). Over the past decades, immunotherapy with other cell types have been developed as Cytokine-Induced Killer (CIK) cells, Peripheral Blood Mononuclear Cells (PBMCs) and specific T and NK subsets.

The first CTMP developed for the treatment of non-viral cancers was represented from Tumor Infiltrating Lymphocytes (TILs) to cure melanoma-affected patients. TILs are effector T cells with the ability to penetrate tumor masses and to kill cancer cells. In 1988, some authors described the use of autologous TILs, isolated from cancer biopsies and expanded with IL-2, for the cure of melanoma-affected patients, but without success; indeed the treatment resulted in a limited regression, both in terms of rate and duration. Today TILs are also used, but as experimental therapy limited at few cases. With the exception of melanoma and cholangiocarcinoma, TILs have not been successfully applied, their use being limited by the small number of cells obtained for the treatment and the lack of significant innate antitumor immunity increase (Dafni et al. 2019).

Macrophages (MΦs) are also widely used as CTMPs for the treatment of oncologic diseases. MΦs are cells characterized by high plasticity, with many different functions, as tissue development and homeostasis, clearance of cellular debris, pathogens depletion, and immune response modulation (Varol et al. 2015). Postnatal development of macrophages occurs through the Macrophage-Colony Stimulating Factor (M-CSF) or Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)-dependent differentiation of circulating monocytes (Anderson et al. 2021). MΦs arise from myeloid progenitors in the Bone Marrow (BM); when at adult stage, MΦs can encompass a broad spectrum of phenotypic aspects, depending on the cytokine milieu and the surrounding tissue niche (Lavin and Merad 2013). On the basis of the future function, MΦs can be classified in two categories, M1 and M2, where M1 are the classically activated MΦs, while M2 are the alternatively activated MΦs (Mosser et al. 2008).

Starting from the year 1987 and up to 2010, were employed for the treatment of patients with different pathologies almost only autologous MΦs. Fidler and colleague are the first users of MΦs to treat C57Bl6 mice bearing a B16 subcutaneous melanoma (Lee et al. 2016). Later the employ of MΦs in the field of advanced therapy has been developed on three different fronts: (1) ex vivo generated MΦs, which uses the innate characteristics of these cells; (2) MΦs as delivery system for small molecules, plasmid DNA, or other therapeutic compounds; and (3) engineered MΦs, to obtain a large number of cells ex vivo or to implement the therapeutic benefits.

Finally, in the field of anticancer therapy, Dendritic Cells (DCs) are "professional" Antigen-Presenting Cells (APCs) able to process/present antigens for priming CD4+ and CD8+ T-cell subsets and to generate cytokines that support the immune response; in this sense DCs operate as a bridge between innate and adaptive immunity. DCs used as drug for patient treatment were differentiated in a few steps from monocytes isolated from leukapheresis, used as starting material; monocytes are then loaded with an antigen and matured using an appropriate, cytokine-based maturation cocktail. The subsequent administration to the patient can occur via the intradermal, intravenous, intranodal, or subcutaneous route. The ex vivo DC preparation provides the important advantage that antigen loading and activation occur under controlled conditions.

The production of the DCs often requires adjuvants, such as imiquimod, IL-2, or KLH, to improve the potency and the timing of the antitumor response. DC-based therapies can be limited by the high production costs and the complexity of the preparation process, potentially linked to an insufficient DCs cell number for patient treatment and unsatisfactory product quality. However, DCs is the first CTMP to receive Food and Drug Administration approval (Provenge, approved for prostate cancer). Based on data from the clinical trials, it seems that DCs-based therapies are well tolerated with very low toxicity (Olsen et al. 2021).

Natural regulatory T cells (Treg) are a subset of CD4+ T cells of thymus origin; since their discovery, immunological applications of Treg in many clinical contexts have been explored. To date the best specific markers of Treg are the IL-2 receptor α chain (CD25) (Sakaguchi et al. 1995) and the forkhead box P3 (Dazzi et al. 2007). Treg cells play a crucial role in the maintenance of peripheral tolerance, in the modulation of susceptibility to autoimmune disease and tumor immunity (Sakaguchi et al. 1995; Shimizu et al. 1999). Moreover, Treg are crucial in the induction of transplantation tolerance and in the setting of the microbiological response. New strategies to improve Treg function can broadly ameliorate the treatment of many disorders. Continued improvements in Treg manufacturing strategies are currently underway (Mukhatayev et al. 2021). Treg may be divided into two subgroups: a cytokine-independent and antigen-independent naturally occurring population that derives directly from the thymus, and the so-called adaptive Treg cells that derive from CD4+CD25– T-cell precursors in the periphery and are recruited by the cognate antigen and immunoregulatory cytokines (Waldmann et al. 2006). Treg can be isolated in vivo and expanded in vitro or directly induced in vitro.

6.2.2 Cells for Regenerative Medicine

Regenerative medicine is a relatively new and highly interdisciplinary branch of medicine concerning the repair and the regeneration of cells, tissues, and organs to repair physiological functions compromised by causes such as congenital defects, diseases, trauma, or aging.

The knowledge in developmental biology, stem cell biology, and biological engineering allowed an improvement in the field of regenerative medicine: in recent years, the scientific community has placed particular attention to the research and development of a new category of CTMPs for regenerative use in the field of cardiac diseases, bone and cartilage regeneration, dermal repair, and neurological diseases. The capacity of CTMPs to restore tissue functions has been broadly studied in the last few years (Goula et al. 2020).

Mesenchymal Stromal Cells (MSCs) have displayed a great capacity to repair tissues and to restore their function; it seems that MSCs have many exploitable potentials, in particular for pathologies in which a definitive therapy has not yet been identified such as neurological diseases as Parkinson and Alzheimer, heart disease, osteoarthritis, AIDS, diabetes, and amyotrophic lateral sclerosis.

MSCs are characterized by their ability in self-renewal and differentiation into tissue-specific specialized cells. According to International Society for Cellular Therapies (ISCT), minimal criteria for defining a cell as multi-potent MSCs are: (1) plastic adhesion capacity in standard culture conditions; (2) high expression of the markers CD29, CD44, CD73, CD90, CD105, CD166, and very low expression of CD14, CD34, CD45, and CD31 markers; (3) differentiation capacity in vitro into adipocyte, osteoblast, and chondroblast lineages (Czapla et al. 2019).

MSCs therapeutic effects are supposed to be mediated by anti-apoptosis, pro-angiogenesis, and pro-neurogenesis mechanisms, but also anti-inflammatory activities. In the field of regenerative medicine, fibroblasts were also often used as CTMPs; fibroblasts have been traditionally described as adherent cells sharing several features with MSCs. There are no specific markers to distinguish fibroblasts from MSCs: membrane glycoproteins like CD29, CD44, CD73, CD90, CD105, and CD166 are all present at high levels both by MSCs and fibroblasts. Dermal fibroblast also seems to have capacity to differentiate towards the adipogenic, chondrogenic, and osteogenic lineages.

Producers of extracellular matrix, cytokines, growth factors, and proteases are not only attended in the steady-state physiology or tissue homeostasis. The high number of fibroblasts that can be extracted from the skin and their ex vivo expansion, their versatility, multilineage differentiation potential, and immunosuppressive properties make them an attractive alternative to MSCs for some regenerative therapies, as treatment of burns, facial scars, diabetic foot ulcers, and gingival repair (Costa-Almeida et al. 2018; Ichim et al. 2018).

Recently other two cell types have been developed for using as innovative CTMPs: Limbal Stem Cells (LSCs) for the treatment of patients affected by functional/anatomical loss of corneal epithelial stem cells and Cardiac Progenitor Cells (CPCs), in case of tissue loss after the damage. LSCs are located at the cornea-

conjunctival zone named limbus zone. They are responsible for differentiation and proliferation of corneal epithelial cells and have the function to cover and protect the cornea. Injury and deficiency of LSCs leads to the lack of corneal epithelialization with consequent photophobia, lacrimation, edema, and aniridia. Limbal cells deficiency is usually manifested by vascularization and chronic inflammation of cornea. In the most serious cases, the ocular surface compromise leads to severe clinical pictures such as "dry eye." The identity of LSCs has not yet been fully characterized because no specific markers has been identified. Many of adjacent non-stem cells share common physical characteristics and surface molecules of LSCs and for this reason it is not possible to adequately isolate them. Therefore, most studies were focused on characterization of the stem cell microenvironment that influences differentiation capacity and the features of the LSCs. Despite the problems related to the characterization, the LSCs administration, both of autologous or allogeneic origin, is now recommended in case of corneal injury (Joe and Yeung 2014; Barut Selver et al. 2017).

CPCs are a heterogeneous group of cells located in cardiac tissue with the ability to differentiate into cardiomyocytes, endothelial and smooth cells through different mechanisms. Characterization of CPCs occur through the evaluation of the surface glycoproteins expression Sca-1, NKX2-5 and GATA4 and the markers OCT4, SOX2 and Nanog, typical markers of pluripotency/stem cell (Yadav and Mishra 2019). CPCs are widely used as alternative therapeutic approach for the treatment of cardiovascular diseases, as acute myocardial injury. It seems that CPCs have a limited ability to survive, proliferate, and integrate in the damaged heart, therefore the development of techniques that improve the survival of transplanted CPCs is critical to maximize the regenerative potential. The paracrine ability of CPCs to release factors into the surrounding environment makes them suitable candidates for regenerative medicine (Amini et al. 2017).

More recently, Pluripotent Stem Cells (PSCs) were studied with the aim to develop stem cells-based therapy. PSCs are characterized by their high proliferation and self-renewing capacity (potentially infinite) in vitro as well as by the ability to differentiate in cells of the three layers, ectoderm, mesoderm, and endoderm.

Until 2007, PSCs could only be isolated from human embryos (Hasegawa et al. 2010). The obtained PSCs, known as Embryonic Stem Cells (ESCs), have considerably improved the knowledge in this research area and the perspectives for clinical application. However, the use of ESCs is still limited, due to ethical problems, as well as the immunological rejection of the differentiated ESCs upon allogeneic HSCT (Takahashi et al. 2007; Takahashi and Yamanaka 2006; Moradi et al. 2019).

ESCs can be obtained also from embryonic and adult fibroblasts, as described by Takahashi and Yamanaka in 2006, reprogramming the cells by the overexpression of different ESCs-enriched transcription factors (Oct4, Sox2, Klf4, and c-Myc). The obtained cells, named induced PSCs (iPSCs), are derived in this case from adult somatic cells genetically modified to an ESC-like status; these cells show a high self-renewal capacity (stemness) and the ability to differentiate in the three embryonic germ layers (pluripotency). Shortly after this important finding, many studies displayed the capacity to generate iPSCs from different organisms and tissue

types, including humans (Takahashi and Yamanaka 2006; Yamanaka 2012; Lowry et al. 2008; Yu et al. 2007).

Different methodological strategies have been carried out to induce pluripotency in somatic cells, mainly using integrative approaches (allogeneic DNA sequences encoding reprogramming factors are placed in the genome of the starting cells), and non-integrative approaches (without steady genetic modifications) (Yamanaka 2012). In particular, non-integrative methods allow to improve the safety of the medicinal product, minimizing the possibility to acquire secondary mutations. On the contrary, integrative methods despite showing the highest efficiency are not recommended due to the lack of safety (e.g., insertional mutagenesis and presence of viral components) (Hou et al. 2013; Moradi et al. 2019).

6.2.3 Cell Types for Different Approaches

Beyond the use of CTMPs for immunotherapy treatments and regenerative medicine, some types of cells, mainly MSCs, can be used in different approaches. Several studies have shown how chemotherapy and total body irradiation, which patients undergo during the transplant conditioning regimen, damage the microenvironment of the BM and compromise the function of stromal cells in support of hematopoiesis. These clinical observations led to the hypothesis that cell therapy with MSCs grown in vitro could provide valid support in patients given a HSCT, linked to the ability of MSCs to reconstitute the stroma and to facilitate the reconstitution of the repertoire of hematopoietic progenitors. Results obtained initially in animal models and later confirmed in humans have demonstrated the validity of the hypothesis. In the oncohematological field, Lazarus and colleague conducted the first clinical trial injecting cells from BM into patients with hematological cancers. These observations paved the way for the implementation of experimental clinical protocols in which the HSCT was supported by the co-infusion of MSCs from the recipient, in the case of autologous transplantation, or of donor origin in the case of allogeneic transplantation. Recent clinical studies have shown that this strategy is able to accelerate hematopoietic reconstitution, favoring both platelet engraftment and that of neutrophil granulocytes, and to prevent the onset of acute Graft-versus-Host Disease (GvHD) or improve its course through the immunosuppressive action of MSCs (Lazarus et al. 2005). Additionally, for their anti-inflammatory ad immunomodulatory properties, in the last year, MSCs have found application in anti-COVID cell therapy (Gentile et al. 2020; Musial and Gorska-Ponikowska 2021).

An innovative therapy strategy is based on the capacity of some cell to deliver drugs, offering the advantage that drugs, loaded into the cells or linked to the cell surface itself, can be transported specifically to the site of damage thanks to cells-homing ability. It has been shown that different cell types when exposed in vitro to high doses of chemotherapeutic drugs are able to load and then release them into the culture medium. Bonomi et al. (2015) demonstrated for the first time that CD14+ monocytes are able to internalize the chemotherapeutic drug Paclitaxel (PTX) and release it in the media at concentrations that inhibit the growth of glioblastoma (GB) tumor cells in vitro.

Moreover, it has been shown that neural stem cells of the olfactory bulb, PBMCs, and MSCs are also able to load PTX. MSCs seem, to date, to be the best candidates for chemotherapy drugs delivery to the inflammation site as MSCs are easily available and expandable in large quantities in vitro, and have adequate dimensions for the internalization of drugs and an inherent homing capability when injected in vivo (Bonomi et al. 2015; Schiariti et al. 2017).

6.3 Cell Sources

Cells used as CTMPs can be isolated from different sources, mainly from hematological samples and tissue specimens. Most of the immune cells as T, NK, CIK, DCs, and MΦ cells were generally obtained from circulating PBMCs or leukapheresis products, whereas T and NK cells can be isolated also from BM or Cord Blood (CB). Other cell types such as TILs, Fibroblasts, LSCs, CPCs, or iPSCs require human bioptic specimens as starting material. MΦ can also be isolated from cavity lavages (alveolar, peritoneal). Skin represents the main source of fibroblasts and, then, of iPSCs.

MSCs can be isolated and then expanded starting from different tissues, for example BM, CB, Adipose Tissue (AT) (surgical fragments and lipoaspirate samples), skeletal tissue, peripheral blood, pancreas, liver, lung, dental pulp, skin, and placenta. Currently BM is the most common source used for obtaining MSCs but considering that the collection of BM samples is a highly invasive procedure and that the number of cells at the end of the expansion step is low, AT is currently the most widely used MSCs source (Bunpetch et al. 2017).

The control of the starting material is critical during the manufacturing of MSCs. At least the following test must be performed: (1) Plasma test for syphilis; (2) Detection of anti-Hepatitis B status; (3) Detection of anti-Hepatitis B status; (4) Detection of anti-Hepatitis C status; (5) Detection of anti-HIV 1/2 status; 6. RT-PCR to detect HIV-1/HCV/HBV nucleic acids (alternative to 1–5 assays).

6.4 Cell Culture and Expansion

It is one of the main problem in the CTMPs development to obtain a large amount of cells, enough for the treatment of a single patient. Most of the immune cells and product prepared for advanced therapies (as T cells, TILs, NK, and others) cannot be obtained through direct harvesting from donors but required a large-scale in vitro expansion protocol (Jin et al. 2012). Moreover, some preparation, e.g., DCs from apheresis products, result in a dramatic collapse after differentiation steps, thus forcing the use of very high numbers of starting cells. For these reasons in order to obtain a large amount of cells enough for clinical application, it is mandatory to set a scalable manufacturing protocol, that allow the control and the monitoring of the entire process (Bunpetch et al. 2017; Vymetalova et al. 2020).

To ensure and maintain the quality requested by regulatory authorities and consistency across batches and cell factories must be identified the critical parameters throughout the production process: these usually include process variables like (1) cell isolation and purification methods; (2) media composition; and (3) expansion, activation, and differentiation methods. Production protocols generated in the last years allowed excellent results, also through the passage of the in vitro expansion steps from flasks to bag or culture vessels and mainly to bioreactors (See below).

6.4.1 Cell Isolation and Purification Methods

The isolation and purification methods, as well as the conditions of expansion in culture, can have a considerable influence in cell bioactivity, therefore an optimal standardization of these processes becomes important for the production of cells as CTMPs for clinical use. The isolation of cell types from hematological sources (i.e., PBMCs) can be done by centrifugation method based on cell size, using or not density gradient reagents. As Ficoll Hypaque, this method allows at the same time to eliminate granulocytes, platelets, and red blood cell from starting material. There are now available several closed and automated systems to isolate specific cell types, as PBMCs, and to remove contaminant cells by density gradient method, such as the Sepax II (GE Healthcare, Chicago, IL, USA) and the Elutra (Terumo BCT, Lakewood, CO, USA) techniques. However, these devices cannot isolate different cell subsets with the same size (for example, healthy T cells vs. leukemia cells and CD4 T cells vs CD8 T cells). Cells isolation from bioptic specimens is usually based on simple plastic adherence technique: Bone Marrow Stromal Cells (BMSCs), Adipose Tissue derived MSCs, Stromal Cells from Cord Blood, LSCs, CPCs, Fibroblast, and iPSCs are usually isolated by exploiting their adhesion capacity to the support.

Purification steps are often required after isolation to discriminate specific cell subsets and to improve the production process, especially for non-adherent cells such as immune system cells (e.g., CD8 T cells, CD4 T cells, CD56 NK cells, CD14 monocytes). Purification process can be a critical step in order to favor the expansion of the cell type of interest, thus obtaining the most pure product possible. Many techniques, that exploit different technologies, including physical separation via centrifugation, immune-magnetic, immune-fluorescent, and acoustic-based selection were increasingly used for purification.

Antibodies can be used to purify cells from hematological sources, as DCs, T cells, or BMSC/CB-MSCs, based on the expression of specific membrane glycoprotein. These antibodies are usually conjugated to fluorochromes and both magnetic- and agarose-coated beads. Fluorescence-Activated Cell Sorting (FACS) is a new strategy employed for cell subsets isolation. FACS has limitations: sorting a rare population or a large cells dose may be very time-consuming; moreover, the technique is not suitable for separating cells which subsequently need to be expanded; indeed, being the system an open circuit, exposes the product to the

risk of microbiological contamination. To improve the safety level of the system were developed new instruments, as the WOLF Cell Sorter (Nanocollect Biomedical, CA, USA) and MACsQuant Tyto (Miltenyi Biotec, Bergisch Gladbach, Germany), that required disposable cartridges; the disadvantages of these tools included the costs and the processing time, too high.

Magnetic Activated Cell Sorting (MACS) is currently the most used method in many CTMP production processes: cells are marked using specific magnetic beads for the desired cell subset and sorted by a magnetic column. The subpopulation of interest can be either negatively or positively selected. In the case of negative selection, the magnetic beads bind all undesired cells that remain trapped in the magnetic field, whereas the desired cells flow out of the column. In the case of positive selection, the magnetic beads directly bind the desired cell subset, which is trapped and collected; in this case magnetic beads remain trapped on the target cell subset and this can invalidate the desired application. Dynabeads, also frequently used for cell purification, are uniform polystyrene spherical beads characterized by a diameter of 1–5 μm, whereas the MACS beads have a diameter of approximately 50 nm. Dynabeads can be tied to an antibody that identifies a specific protein on the membrane of the target cell. Dynabeads can also indirectly bond the cell: the complex Streptavidin-Dynabead can link a biotinylated primary antibody, or a secondary antibody on the Dynabead linked to the primary antibody. The complex Streptavidin-Dynabead-primary antibody permits a higher yield of the bound with cells (de Wynter et al. 1999; Jain et al. 2013).

Many commercial kits now available for cells purification are helpful. Magnetic separators, for example the ClinExVivo and Clinimacs Plus (Miltenyi, Bergisch Gladbach, Germany), are now also available to isolate a specific cell subset and to detach the beads after purification. A very recently developed tool, the CliniMACS Prodigy® (Miltenyi), permits the isolation of the cell subset, the large-scale expansion, and all the washing passages, in a closed and completely automated system. Below we reported some specific examples of isolation and purification methods.

6.4.2 Isolation of MSCs

As described above, MSCs can be isolated from a broad range of tissues. Traditionally, isolation and expansion of MSCs is carried out by adhesion on plastic in adequate complete culture medium. This procedure is simple, but often does not permit to separate desired cells from other subpopulation as endothelial cell, pericytes, and hematopoietic cells. It's therefore crucial to define an isolation process that allows to eliminate "contaminant cells" as soon as possible, ensuring a high quality of the medicinal product. Thus other isolation methods have been exploited. A variety of protocols starting from hematological sources provide the isolation of cells by density gradient using Ficoll, Percoll or dextran as well as advanced cells sorting methods as flow cytometry or MACS technology. Flow cytometry and MACS technologies are based on the expression of membrane glycoproteins as

CD105, CD73, CD90 and the lack of CD45, CD34, and HLA-DR markers (Chu et al. 2020).

MSCs can be isolated also from AT specimens or AT lipoaspirates or other solid tissues. In this case tissues are generally disrupted by enzymatic digestion or mechanical dissociation before being plated. MSCs sprouted from tissues in few days and after tissue removal MSCs grown up to 80% confluence. Then MSCs are expanded as described below (Lisini et al. 2019, 2020; Czapla et al. 2019).

The primary plating density of MSCs can be a critical condition for optimal expansion of cells from BM and CB; most authors use high primary seeding densities of 1–2 $\times$ 10^5 cells/cm^2 (Bieback et al. 2008). In the case of other tissues such as AT samples, a maximum of 150 μL/cm^3 is plated. This isolation method can be also applied to other adherent cells such as fibroblasts and CPCs (Aydoğdu et al. 2021; Andriolo et al. 2018, 2021).

6.4.3 Limbal Stem Cells Isolation

LSCs are obtained from limbal biopsy tissue surgically removed from limbus zone. Two isolation methods have been described: biopsy samples can be mechanically/enzymatically dissociated and single cells suspension grown for 2–3 weeks, until cells spread to form a confluent monolayer; alternatively biopsy samples can be placed directly on a substrate represented by Human Amniotic Membrane (HAM), a non-immunogen biomembrane that for many years has been applied to facilitate wound healing. Deepithelialized HAM seems to better support limbal epithelium growth. HAM can be obtained from cesarean section from healthy donors. HAM preparation was performed under aseptic condition, separating HAM from chorion tissue and decidua and cutting the tissue in small pieces. Then HAM is mounted on a nitrocellulose membrane with epithelial side facing up and stored at −80 °C until use (Tsai et al. 2000; Joe and Yeung 2014; Barut Selver et al. 2017; Zhang et al. 2016).

6.4.4 Treg Isolation

High cells purification rate is crucial for certain cell types. For example the association of CD4, CD25, and the CD127 biomarkers can be used to selectively enrich human Treg cells for potentially in vivo therapy. To date, the GMP-compliant purification of autologous Treg occurs mainly using immunomagnetic beads, offering this method a high grade of safety; on the contrary, this methodological approach fails in the selection of a cell subset represented at low levels and in the multiparametric isolation. In these cases, it is better to use fluorescence activated cell sorting (FACS), although the system works in an open circuit, exposing the product to the risk of microbiological contaminations. The disadvantage of isolating Treg by using immunomagnetic beads is that the obtained Treg cell subset may include activated effector T cells; these cells, if expanded together with Treg during the cell culture, can give some problems in an advanced cell therapy approach, as it can exponentially grow and induce graft damage. The addition of a CD8 depletion

step can be useful to by-pass this problem. Moreover, the addition of the immunosuppressant rapamycin during cell culture can help to avoid the proliferation of effector T cells, resulted in a final product of a pure population of Treg with increased suppressive function (Namdar et al. 2010; Fraser et al. 2018).

6.4.5 MΦs Isolation

For the production of ex vivo MΦs, that can be modified for therapeutic purposes, are usually used two different methods: starting from whole blood or from BM samples it is possible to differentiate the monocytes into MΦs by adding M-CSF in the culture medium; alternatively, MΦs can be directly isolated from alveolar or peritoneal lavages. During the culture steps, MΦs can be further exposed to immune stimulators, for example LPS and/or cytokines, inducing different polarizations that mimic in vivo phenotypes (Mosser et al. 2008).

The classical MΦ collection methods make possible to obtain only small numbers of MΦ subtypes. Other methodological approaches, including polyacrylamide beads and proteose peptone, generate MΦs with different phenotypes. Regardless of the isolation method used, it is possible to obtain only a limited number of monocytes and MΦs. During culture steps, monocytes are unable to divide and are characterized by a very low proliferation capacity; moreover, monocytes have a short life, as they differentiate into MΦs (Italiani and Boraschi 2014).

6.4.6 iPSCs Isolation

Many different studies showed the capacity to obtain iPSCs from various organisms and tissues, including humans (Takahashi et al. 2007; Takahashi and Yamanaka 2006; Lowry et al. 2008; Yu et al. 2007). iPSCs can be obtained by reprogramming of any somatic cell (Sohn et al. 2012); however as the starting material is a critical feature for the ATMPs development, most of the ongoing clinical trials are based on skin fibroblasts and peripheral blood CD34+ cells derived iPSCs that guarantee easy cell isolation and high quality for derived iPSCs. By now, guidelines that give indication regarding the best somatic cell type are lacking. It seems that hematopoietic progenitor cells, as CD34+ cells, have a higher reprogramming efficiency than mature hematopoietic cells. The use of CD34+ cells resulted in the development of a highly reproducible iPSC production process (Sharma et al. 2019; Eminli et al. 2009).

The most wide source of CD34+ used to generate iPSC is peripheral blood, easy to obtain from all patients, despite the relatively low yield of CD34+ cells; another source of CD34+ can be cord blood, but unfortunately this source is less available. Moreover, peripheral blood is to date more suitable than cord blood, due to the GMP-compliant protocols now available for the CD34+ expansion and the iPSC reprogramming (Sharma et al. 2019).

The main characteristics of iPSCs is the self-renewal capacity, which makes them infinitely expandable; hence, even if the methodological approach employed to generate iPSCs is characterized by low efficiency, it is possible to isolate and expand a small number of very high-quality iPSC, obtaining optimal iPSCs lines for the use in cell therapy context. When iPSCs are generated from patients with a genetic defect, there is the possibility to correct the defect before cell administration, using special genome-editing technologies, for example a clustered short palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9) system (Steyer et al. 2018).

6.5 Culture Media

The complete culture medium for the isolation and expansion of GMP-compliant CTMPs contains a combination of the following: basal medium, supplements (i.e., serum or platelet lysate), cytokines, monoclonal antibodies (mAbs), and/or other small molecules. The components of the complete culture medium affect the morphology, the expansion time and the final number of cells, purity, viability, phenotype, function of final cell product, due to the specifics of the manufacturing process (Fekete et al. 2012).

Most traditionally used media for research-grade isolation and expansion of different type of cells are Serum-Containing Media (SCM), where the basal medium is usually supplemented with Fetal Bovine Serum (FBS) to ensure the presence of critical factors. The presence of FBS is essential to obtain cells of high quantity and quality. However, the use of serum to prepare cells for therapeutic purposes can be a serious problem as it can introduce the possibility of (a) safety risks to the patient, related to the use of animal-derived component, (b) great process variability due to lot-to-lot differences. Media containing small amounts of serum should be enriched with other factors essential for the target cell type. For the preparation of cell used as CTMPs it is strongly recommended by the regulatory authorities to use Serum-Free Media (SFM) because the use of serum poses huge risks for CTMPs production, as components of animal origin can cause risks for adventitious infections such as prions and viruses. Moreover, in a GMP-compliant approach is highly discouraged the use of antibiotics, especially at late culture passages, due to potential allergies of the recipient and to avoid resistance. In all the cases in which the use of antibiotics is essential, it is absolutely crucial to demonstrate the absence of residues in the final product (Gottipamula et al. 2013).

SFM is often supplemented with proteins and/or peptides tailored to the specified application and does not require additional serum; often serum is replaced products of human derivation. Human platelet lysate (hPL) is an example of a human-derived product. hPL is produced in most cases from expired platelets obtained from volunteer donors in certified transfusional centers; currently some companies prepare controlled, ready-to-use and GMP-compliant hPL (Cook Reagent, Indianapolis, IN, USA).

Protein Free Media (PFM), such as Chemically Defined Media (CDM), are media without peptides/proteins as well as serum/platelet lysate, that can be supplemented with short low molecular weight components such as those of acid enzymatic hydrolysis. CDM can be supplemented with proteins or peptides, but these are

generally well known in terms of chemical structure and biochemical activity; usually proteins/peptides are recombinant. Media without components from non-human animals are classified as "xeno-free" media, while those without components from any animal, including humans, are classified as "without components of animal origin" media.

Due to the requirement for known composition of all components, CDM is almost always xeno-free or animal-derived component free.

Historically, SCM media such as RPMI 1640/FBS or SFM media such as AIM-V (Life Technologies, Carlsbad, CA, USA) or X-VIVO (Lonza Biosciences, Basel, Switzerland) have been used for the culture of immune cells. FBS, on the other hand, is often used in translational research for its capacity to promote superior growth kinetics, but as stated above, not for the preparation of CTMPs. AIM-V and X-VIVO media also contain human serum albumin (HAS) and thus these are inherently chemically undefined. Although RPMI, AIM-V, and X-VIVO are generally intended for immune cells culture, however, they may no longer be appropriate for expansion of T or DC cells.

For this reason, specialized and fully characterized media have been developed for cell manufacturing protocols. CTS OpTmizer (Life Technologies) is a complete, xeno-free, serum-free culture medium developed specifically for T-cell expansion, while serum-free media for DCs, CellGro DC Media (CellGenix, Freiburg, Germany) and DC Generation Medium (Promocell, Heidelberg, Germany) have also been developed and used in clinical trials. Also for NK cells complete media were developed (Stem Cell Grow Medium, SCGM, CellGenix and NK MACS Expansion Medium, Miltenyi).

The most common basal media for research-grade adherent cells (MSCs, fibroblasts, LSCs, CPCs) isolation and expansion are alpha-MEM or DMEM mainly completed with FBS. GMP-compliant protocols involve in the most cases the use of hPL that allows, like serum, the maintenance of the morphology, proliferation, and differentiation capacity of adherent cells. Xeno/serum free, GMP-compliant media capable of stimulating efficient isolation and expansion of adherent cells without altering their therapeutic functions, have also been developed. MesenCult™-ACF Plus Medium (STEMCELL Technologies, Vancouver, Canada), CTS StemPro MSC SFM and StemPro MSC SFM XenoFree (ThermoFisher Scientific, Waltham, MA, USA), MSC NutriStem® XF (Biological Industries, Israel), MesenCult™ MSC (Stemcell Technologies), Prime-XV MSC Expansion (Irvine Scientific, CA, USA), and StemMACS™ MSC (Miltenyi Biotec) are example of media developed and marketed for this finality (Mebarki et al. 2021; Chu et al. 2020; Bunpetch et al. 2017).

For the maintenance and expansion of pluripotent stem cells, such as iPSCs, the quality and consistency of the culture medium are critical.

Originally developed by Chen et al. (2011), Essential 8TM (E8) Medium is a fully defined, xeno-free and feeder-free medium formulated for the growth and expansion of human pluripotent stem cells. It has been shown to support iPSC growth for >50 passages (cGMP-compliant E8 medium, GibcoTM, ThermoFisher, Waltham, MA) ensuring high proliferation rates (doubling time of approximately

20 h), the typical iPSC morphology in cultures, and pluripotency in multiple pluripotent stem cell lines. Essential 8TM Medium has been extensively tested and unlike most feeder-free media, Essential 8TM Medium does not require the presence of BSA (bovine serum albumin) or HSA (human serum albumin) which contributes to batch-to-batch variability. Consequently, it is suitable for GMP iPSC manufacture. It is important to note that the different basal media, as well as different supplements, may have different effects on cell expansion and/or differentiation, therefore the choice of the complete medium is critical and must be carefully evaluated.

6.6 Cell Expansion, Activation, and Differentiation

In vitro culture steps aimed to cell expansion, activation, or differentiation have also a considerable influence for the production of CTMPs able to satisfy all the therapeutical needs.

6.6.1 Immune Cells

The growth and expansion of several immune cells types (e.g., NK, TIL, T cells, Treg) is completely dependent on physical contact with a feeding layer/Antigen-Presenting Cells (APCs) usually consisting of adherent, growth arrested, but viable and bioactive cells used as a substratum to support the selective expansion of cell subsets, particularly those consisting of a very low number of cells after the isolation procedure.

The cells of the feeder layer, irradiated or treated so that they do not proliferate, support the growth of target cells by releasing growth factors into culture media. In addition, they also play important roles in processes such as detoxification of the culture medium or synthesis of extracellular matrix proteins needed to control cell growth in culture and as a substrate for cell attachment.

To support selective expansion of target cells in culture, feeder layers are metabolically active and able to synthesize needed ligands or cytokines. The repertoire of growth factors of a given feeder layer differs from one to another. However, there are common critical factors (Llames et al. 2015). The feeder cell population must be optimized in the context of immune cells preparation for patient treatment.

PBMCs, DCs, B cells, and monocytes/macrophages are the most used feeder layer/APCs. PBMCs are also frequently used as feeder layer cells, mainly pooled from different healthy donors; unfortunately, the use of different donors are difficult to obtain and relatively expensive. Furthermore donor-derived allogeneic PBMCs represent a potential source of variability due to the use of different donors. Ideally, a more consistent and non-variable power source or even growing conditions without feeders could be the optimal solution.

B cells are also used as feeder layer/APCs, with the advantages over DCs of their comparative abundance and facility of expansion (Putnam et al. 2013). CD40L-

expressing fibroblasts were used as feeder layers for B cells whose proliferation requires the costimulation signal determined by CD40/CD40L interaction (Alzhrani et al. 2017). It is mandatory that starting material used for preparation of CTMPs is approved and checked in compliance with GMP, and this is considered a limitation for this approach, mainly when many different donors are used. B cell banking has been suggested to address this challenge; the limitation is that the donor/recipient of human leukocyte antigen (HLA) may not be fully covered by this bank.

Recently has been developed a novel feeder cell culture consisting of artificial Antigen-Presenting Cells (aAPCs). These are genetically modified cells lines that, in a more controlled way than natural APCs, constitutively express antigens that drive the activation and expansion of specific cell types. aAPCs are particularly effective in promoting the expansion of NK cells. An example of aAPCs is represented by K562 cell line genetically modified to express CD86, CD137L, and membrane-bound IL-15. In general, the use of feeder layers/aAPCs involves challenges that include: (a) costs for the production of GMP certified feeders layers/ aAPCs, (b) potential non-total removal of the feeder layer at the end of the cell culture, (c) the potential donor-to-donor variability and (d) with the use of autologous feeder cells from critically ill patients, the limiting factor could be the number of activating cells present in the starting material.

Different technologies were also developed in feeder layers/APCs-free culture conditions, including beads-based activation (both magnetic and non-magnetic) and antibody-based activation.

In commercial immunotherapy production of cell therapies, microbead-based activation reagents are the most common activation reagent. They appear to produce consistent activation leading to streamlined manufacturing workflows.

For example, the CD3/CD28 dynabeads (ThermoFisher) employ magnetic beads bound to anti-CD3 and anti-CD28 antibodies for activation. These beads produce robust expansion but the removal of the magnetic beads prior to patient treatment remains difficult. To reduce the complexity of manufacturing, several non-magnetic cell activation reagents have been developed. MACS GMP TransAct CD3/CD28 beads (Miltenyi Biotec), a colloidal polymer nanomatrix covalently attached to humanized recombinant agonists of human CD3 and CD28, or the removable MACS GMP ExpAct Treg beads (Miltenyi Biotec) with a cell-to-pearl ratio of 1: 4, specific for the activation of Treg are an example. Miltenyi Biotec's TransAct beads can be simply removed through centrifugation.

Immunocult T-Cell Activators (Stemcell Technologies) is an example of antibody-based activator. It is a tetrameric antibody complex based on crosslinking of CD3, CD28, and CD2 cell surface ligands via a central linker domain. Immunocult T-cell Activator can be simply removed through centrifugation (Iyer et al. 2018). In addition to the activation methods, the expansion capacity of immune cells is strongly influenced by the choice of the cytokines used during the expansion phases; on the contrary specific stimuli linked to cellular expansion can influence cellular characteristics with potentially decisive implications for clinical efficacy.

The cytokine most used for its high ability to induce the growth of T lymphocytes is IL-2. Over the years, cytokines such as IL-7, IL-15, and IL-21, members of the

common γ-chain family, have been used for the expansion of T cells ex vivo. The interest in the study of these cytokines is also linked to their ability to generate long-lived memory T cells. However, other cytokines have been studied for their ability to expand T cells ex vivo. T-cell culture combined with IL-12, IL-7, or IL-21 allows for the generation of minimally differentiated CD8 T cells and results in a central memory and effector differentiation when combined with IL-15. IL-12 however has limited impact on T-cell differentiation when combined with IL-2 and is not directly involved in T-cell memory formation or survival. IL-18 cytokine, studied to evaluate ex vivo T-cell expansion, showed no benefit with regard to the preservation of minimally differentiated T cells. Overall, it can be concluded that IL-2 represents the gold standard cytokine for ex vivo T-cell expansion and IL-7, IL-15, and IL-21 are three key cytokines for influencing cell differentiation (Márton et al. 2020).

TILs can be also isolated from tumor biopsy and expanded, in vitro, mainly using IL-2; however, cultures are successful only from a few donors (Mellman et al. 2011; Tran et al. 2008). To avoid this problem studies based on T-Cell Receptors (TCR) or Chimeric Antigen Receptors (CAR) have therefore been developed.

In these studies, normal T cells from oncological patients are isolated by apheresis and transduced to generate TCR or CAR cells as reported in the review articles (Barrett et al. 2015; Mellman et al. 2011). For transduction and proliferation of the manufactured T cells, Interleukin-2 receptor (IL-2R), a surface protein expressed on T cells, can be upregulated in vitro. Exogenous CD3 and IL-2 antibodies, but also PBMCs as feeder cells, represent the complete environment for T-cell activation and proliferation. This combination is reported as a standard "Rapid Expansion Protocol" (REP). Studies are currently underway to improve the REP method. IL-7, IL-15, anti CD-28 antibodies added to the culture medium, pre-stimulation with peptide mix that focuses on the pp65 protein, scaffolds that mimic the physiological signal of activation of T cells, DNA-based platforms for presenting anti-CD3 and anti-CD28 antibodies or bispecific antibodies, are recent alternative to determine an increase in cellular proliferation in the same culture vessel (Sadeghi et al. 2011; Zhang et al. 2020; Keskar et al. 2020; Guo et al. 2020).

In the context of immunomodulatory therapies, Treg is a cell subset that can be used as "polyclonal" or "alloantigen-specific." To selectively expand alloantigen-specific Treg cells in humans in a short time, Putnam et al. developed a manufacturing process under GMP conditions using allogeneic CD40L-activated B cells followed by polyclonal restimulation to multiply the yield (Putnam et al. 2013).

Coated anti-CD3/CD28 antibodies supplemented with high amounts of IL-2 (200–1000 IU/mL) can also be used for stimulation of polyclonal Treg. Activated Tregs must then be expanded ex vivo for several days (Sharma et al. 2019). Treg can be also expanded with commercially available product, such as TexMACS GMP Medium (Miltenyi Biotec) supplemented with IL-2, pooled human AB serum and removable MACS GMP ExpAct Treg beads (Miltenyi Biotec). For the ex vivo expansion of alloantigen-specific Tregs, donor APCs or aAPCs (K562 cell-based artificial APCs) pulsed with given antigen in presence of high-dose IL-2 are used. Cell expansion is continued for several days.

A protocol to generate CIK cells was first described by Schmidt-Wolf and colleague (Schmidt-Wolf et al. 1991). CIK cells were generated in 21 days from PBMCs cultured in presence of interferon (IFN), recombinant IL-2, a monoclonal antibody (mAb) against CD3 and IL-1. The manufacturing protocol promoted a specific induction of T cells with phenotype similar to NK cells (T-NK cells) as effector cells.

Ex vivo NK cell expansion using a K562 leukemia feeder cell line modified to express membrane-bound IL-15 has reported promising results as reported by several groups.

This method was subsequently improved by Denman and colleagues through the use of a K562 cell line expressing membrane-bound IL-21 and 4-1BBL. The optimization of the method results in rapid cell expansion without inducing senescence or loss of cytotoxic activity (Denman et al. 2012).

Ex vivo generation of mature DCs (mDC) for therapeutic vaccination does not imply a large-scale expansion but require differentiation steps; mDCs preparation takes place in shorter and less variable times than those necessary to obtain T lymphocytes; mDC are obtained generally starting from selecting the monocytes CD14+ subpopulation by an immunomagnetic process from leukapheresis. Fully mDC are obtained in 7–10 days of culture: differentiation of CD14+ cells in immature DC (iDC) is obtained in 5–7 days providing GM-CSF and IL4; iDCs are activated and loaded with disease-specific antigens generally represented by pulsation with antigenic peptides, DNA/RNA transfection or viral transduction. The transfection process involves lower costs but requires an open system that makes viral transduction a preferred choice. Incubation of DCs with autologous tumor lysates was generally adopted to reduce production costs. It has also been shown that whole cell lysates are superior to synthetic peptides or proteins in the clinical setting. Finally DCs, in the presence of pro-inflammatory stimuli (using a cytokine cocktail such as TNFα, IL-6, and IL-1β, with or without the addition of prostaglandin), are induced to maturation to generate a population of immunogenic mDC (Nava et al. 2015; Boudousquié et al. 2020). mDC can also be obtained in 2–3 days cell culture as demonstrated by several groups (Fast-DC) (Nava et al. 2020). Fast-DC production has advantages in the clinical setting as it reduces the time between the time of patient recruitment and the start of treatment. Rapid protocol of DCs production would also reduce costs, variability between preparations, and the risk of contamination.

MΦs are a heterogeneous population of innate myeloid cells exhibiting remarkable plasticity; depending on the types of stimuli that MΦs are exposed, these cells, with distinct physiological and pathological roles, will be able to polarize to M1 (pro-inflammatory) or M2 (anti-inflammatory) MΦs. Exposure to factors such as GM-CSF, IFNγ, TNFα, LPS, or other molecular models associated with pathogens (Jaguin et al. 2013) promotes the polarization of M1 MΦs. M1 MΦs, through the secretion of the CXCL9 and CXCL10 chemokines (van Dalen et al. 2018), increase the recruitment of Th1 cells in the inflammation site and promote a pro-inflammatory Th1 response through the secretion of cytokines such as TNFα, IL1β, and IL-12. Furthermore, to enhance T-cell responses, M1 MΦs upregulate genes involved in

antigen processing and presentation, as well as co-stimulatory molecules (Martinez et al. 2014). These functions have the potential to participate in antitumor immunity (Italiani and Boraschi 2014).

Exposure to M-CSF, IL-4, IL-10, IL-13, transforming growth factor (TGF) -β, glucocorticoids, or immune complexes induces the polarization of M2 MΦs (Italiani and Boraschi 2014). M2 MΦ generally promote Th2 responses and play a critical role in normal immune function and tissue homeostasis. However, some subsets of M2 MΦ also play a critical role in promoting tumor progression (Atri et al. 2018).

6.6.2 Cells for Regenerative Medicine

Most of the CTMPs prepared for tissue repair, as MSCs, fibroblasts, CPCs, and LPCs, grow as monolayer in adhesion to the support, represented by plastic in the case in which the expansion takes place in plates/flasks/multi-later systems, or synthetic fibers when some types of bioreactors are used.

It's described that, after isolation, the adherent cells expansion requires low, or very low, plating density to maximize the proliferation rate and to maintain cell functions. After the first culture phase, cells are usually cultured at concentration of 1000–4000 cells/cm^2 to obtain a high number of final product. Plated cells must be checked twice weekly, medium changed every 4–5 days and cells harvested when reached 80% confluence (after about 10 days), using an enzymatic solution (i.e., TrypLE Express, Thermo Fisher Scientific, USA) and then reseeded for large-scale expansion (Zheng et al. 2013; Fernández Muñoz et al. 2021; Milinkovic et al. 2015). With the use of bioreactors, thanks to the synthetic fibers support there is no need to harvest cells between passages: this is a great advantage in the field of a GMP-compliant production process, decreasing the risks of microbiological contaminations.

The number of sequential passages (detachment) that the final product has undergone (or the expansion time in the case of an expansion in bioreactors not requiring detachment) must be considered as critical parameter during the setup of an expansion process, because may affect the quality of cultured adherent cells causing senescence, slowing of proliferation rate and consequent loss of multipotentiality. For this reason to limit the number of population doublings to less than 20 seems to be appropriate (Sensebé et al. 2013). Pluripotent stem cells with their ability to propagate indefinitely and differentiate in other cellular types such as neurons, cardiac, pancreatic and liver cells, represent a single cellular source to be used in the field of regenerative medicine. To define a culture protocol for iPCs under GMP conditions is the main objective for the culture of these pluripotent cells (Rivera et al. 2020).

Cell culture matrix should be used to support iPSC growth. Some researchers explored the use of synthetic peptide-based surface-coating Synthemax II-SC (Corning, New York, NY, USA), certifiable under cGMP, or other extracellular matrices (ECM) manufactured under cGMP guidelines (Rivera et al. 2020). Between the different matrixes tested, Vitronectin (VTN) was shown to efficiently support

human pluripotent stem cell self-renewal. VNT is a xeno-free recombinant human protein used as ECM substrate. Compared to native vitronectin, the vitronectin variant, VTN-N, lacks the terminal SMB-N functional domain and supports human pluripotent attachment and survival better than wild-type vitronectin.

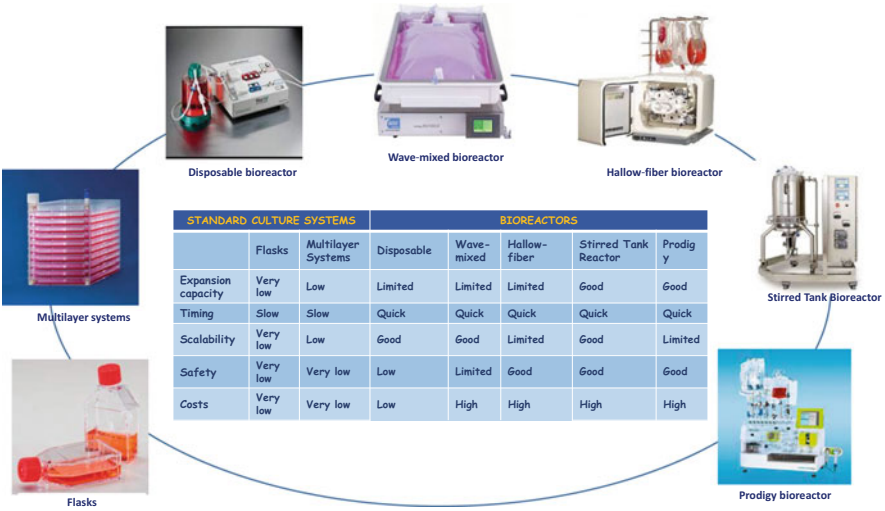
A culture system that supports self-renewal and high cell proliferation rates in compliance with GMP regulations, represents an optimal system for in vitro iPCs maintenance. The CTSTM-VTN-N matrix allows iPCs pluripotent stem cells, even for long culture times, to grow homogeneously and without inducing spontaneous differentiation. Cell survival is the priority of iPCs during selection and expansion. Genetic and epigenetic iPCs stability is influenced by the culture conditions and above all by the cellular enzymatic dissociation during the expansion processes. Therefore enzyme-free dissociation methods have been developed to successfully derive and expand iPCs. Dissociation of iPCs must be done quickly to avoid cell mortality and to keep cell cultures out of the incubator for long periods of time. In human embryonic stem cells, Rho-associated protein kinase (ROCK) inhibitor increases the survival of dissociated cells by preventing apoptosis (Mandai et al. 2017). Therefore, ROCK inhibitor (ROCKi) addition to the medium after cell dissociation is recommended.

6.7 Closed Culture Systems

The optimization of a culture protocol that meets GMP rules can be a very complex process.

One of the most important aspects to take into consideration is the high number of cells required for their application in cell therapy areas; the different cell types used as CTMPs are present within adult human tissues in very low concentrations, therefore, in order to obtain a sufficient number of cells to guarantee therapy, an adequate ex vivo cell expansion is required.

Traditionally, at preclinical levels, cell isolation and culture takes place in flasks or plates, but for large-scale production of CTMPs this approach can present some difficulties. In fact it requires the management of a large number of flasks to be kept in incubator for long periods, which involves high costs in terms of personnel and a large number of "open circuit" manipulations by the operator, with a greater risk of microbial contamination of the final product. Furthermore, as mentioned above, to obtain a high number of cells in flasks would require culture until late passages, exposing cells, such as MSCs, to the risk of senescence or accumulation of genetic and epigenetic alterations with the potential risk of cell transformation, making them unsuitable for clinical use. For this reason, many studies in the field of cell therapy have focused on more appealing culture methods. For the expansion of CTMPs new technology platforms, summarized in Fig. 6.3, have been developed. Gas Permeable Bags (GPB), such as VueLife (Saint Gobain, Blois, France), Charter Medical (Winston-Salem, NC, USA), and OriGen (Austin, TX, USA) are offered by different companies. GPBs, designed to ensure high gas transfer rates to cells maintaining low



STANDARD CULTURE SYSTEMS			BIOREACTORS				
	Flasks	Multilayer Systems	Disposable	Wave-mixed	Hallow-fiber	Stirred Tank Reactor	Prodigy
Expansion capacity	Very low	Low	Limited	Limited	Limited	Good	Good
Timing	Slow	Slow	Quick	Quick	Quick	Quick	Quick
Scalability	Very low	Low	Good	Good	Limited	Good	Limited
Safety	Very low	Very low	Low	Limited	Good	Good	Good
Costs	Very low	Very low	Low	High	High	High	High

Fig. 6.3 The figure shows the different technologies used to expand CTMPs. Production processes development begins using flasks or multilayer systems at preclinical level (standard culture systems), then the scale-up of the processes occur mainly using bioreactors, to obtain adequate cells number for patients treatment. The blue table indicates the best system concerning the expansion capacity, the timing of expansion, the scalability, the safety of the final product, and the costs

water permeability, appear to be optimal for T cells and DCs culture (Zuliani et al. 2011; Jin et al. 2012).

Also G-Rex® bottle systems (Wilson Wolf, New Brighton, MN, USA) were employed specifically to expand non-adherent immune cells such as T and NK cells, or PBMCs. G-Rex bioreactors are tissue culture vessels which, in 8–10 days of culture, allow cell expansion starting from a low initial seeding density. Cell expansion is permitted by the presence of a gas permeable membrane at the basis and efficient gas exchanges at the liquid-cell interface. Corning Cell Stacks (Corning, NY, USA) or Nunc Cellfactory (Nunc, part of Thermo Fisher Scientific Inc., Waltham, MA, USA) are multilayer system frequently used for cells growing in adhesion on a plastic support, as MSCs.

GPB and G-rex systems, by mimicking flask culture, represent a simple and cost-effective way of cell culture that can be used to translate a process from a preclinical to an early clinical setting. However, their lack of automation, in-process monitoring capacity, and finally the high costs to meet GMP regulations, limit their commercial use on a large scale (Tarte et al. 2010).

Fully closed and automated bioreactors can be used for simpler and safer processes.

Bioreactors represent a promising solution. Their use allows, in a short time, an increase in cell growth with a reduction in culture volumes. Compared to static flasks cultures, bioreactors ensure efficient gas exchange, correct supply of culture

medium, pH correction and/or real-time monitoring of culture conditions. In addition, the use of automatic bioreactors (1) reduces the risk of microbial contamination by increasing the safety of the final product; (2) allows to obtain a very high number of cells in a short time; (3) reduces variability between batches and operators; (4) improves the traceability of cell culture data continuously by monitoring and automatic control of all parameters (Heathman et al. 2015; Das et al. 2019).

Different types of bioreactors have been developed to date. Wave-mixed bioreactors have become the most common platform for commercial scale-up of T cells for application in immunotherapies. They use oscillating wave motion to allow, with low lateral movement, efficient mixing and oxygen transfer within the reactor (Singh 1999). A large number of wave-mixed bioreactors were developed including the Smart Rocker (Thermo Fisher), Allegro (Pall, Port Washington, NY, USA), Biostat RM (Sartorius, Aubagne, France), and Xuri Cell Expansion System (GE Healthcare).

Mixed wave bioreactors are closed automated instruments that promote high cell expansion by reducing the amount of manual work and ensuring continuous monitoring of parameters such as pH and Dissolved Oxygen (DO) and the perfusion of the culture medium. Limitation of these systems is that they require a first cell expansion before the culture reaches the required volume to be transferred into the wave-mixed. Quantum Cell Expansion System from Terumo BCT is another example of closed automated system based on a hollow-fiber bioreactor which can culture either adherent or suspension cells. The system can monitor pH, DO, control temperature and gases; each disposable cartridge consists of over 11,500 hollow fibers generating an area of 2.1 m^2, making them an attractive choice for use in cell therapy manufacturing. However like the previous system, Quantum requires MSCs isolation and first expansion up to 20×10^6 outside the isolator, thus increasing the potential risk of contamination (Martin-Manso and Hanley 2015).

Other bioreactors such as Octane Biotech's Cocoon™ Bioreactor and CliniMACS Prodigy (Miltenyi Biotec) are examples of culture systems aimed to fully isolate and expand adherent or suspension-grown cells. In these bioreactors, seeding, expansion, perfusion, digesting/harvesting, concentration, washing and formulation of final product are completely closed and automated within a single chamber. CliniMACs Prodigy, for example, selects immune cells by magnetic separation, allows their expansion and final product formulation. However, even if these systems are advantageous because they are easy to use and because they reduce the risk of contamination, the use of a single system that carries out the entire process may not be as convenient as having individual steps of a process separated (Iyer et al. 2018). For culture expansion of immunotherapies there are also several alternative technologies that can potentially be used. Stirred Tank Reactors (STRs) are commonly used for the production of monoclonal antibodies. The production scale ranges from hundreds of milliliters (e.g., rotating bottles) to thousands of liters.

The STR System could be useful for T-cell culture in an allogeneic setting, where scale-up will be more important (vs. scale out with patient-specific therapies). For this reason, GE Healthcare has developed the Xcellerex XDR line ranging in volume from 10 to 2000 L in single-use format and PBS Biotech (Camarillo, CA, USA) has

developed a new series of bioreactors that use a vertical wheel to enable homogeneous mixing with less shear force compared to standard STRs. PBS systems can be an attractive choice for adherent cells grown on microcarriers or in aggregates. Due to their design, efficient mixing, adaptability to patient-specific or allogeneic workflows, compatibility with DO and pH probes, their use could be explored in the immunotherapy field.

6.8 Cryopreservation

Most cell product cannot be administrated immediately after the preparation, due to the timeline of the clinical trial or the waiting for the results of the quality controls on final batch or other different causes, and must be cryopreserved. Many CTMPs often are stored in multiple aliquots (which may vary in size) before use, for periods which may range from weeks to years in order to make CTMPs more flexible. Potentially the storage can significantly affect the viability, functionality, and potency of cells prepared for cell therapy: for these reasons suitable cryopreservation protocols for long-term storage are an indispensable prerequisite for clinical and commercial application.

Cryopreservation of cells at extremely low temperatures drastically reduces metabolic activity and thus preserves cell health. For cell cryopreservation temperatures of −196 °C for liquid nitrogen and −156 °C for vapor nitrogen are recommended. However, vapor storage is preferred in GMP-compliant approaches, as it avoids microbial contamination. During cryopreservation, intracellular ice formation must be minimized to prevent cell rupture (Aijaz et al. 2018). For this purpose, different cryopreservation solutions and different storage temperatures as well as maximum stability times of the starting (if requested) and final product have been studied to support the good quality of CTMPs so that, after thawing, cells can maintain the same characteristics of viability and functionality that had before cryopreservation.

Freezing must take place in a controlled manner through the use of controlled rate freeze. Mainly controlled rate freezing devices assure the temperature decrease of 1–2 °C/min. The gradual temperature decrease prevents crystal formation in the cells ensuring a high cell viability after thawing. Generally, a specific freezing curve is created for each type of CTMPs. In addition, speed-controlled rate freezer, with integrated computer, provide data traceability for every run. Traceability can be critical to determine for example, root cause(s) in case of deviation/out of specifications occur. Alternatively to speed-controlled rate freezing procedure, CTMPs can "classically" be cryopreserved, first at the temperature of −80 °C for 24 h in isopropyl alcohol containers (designed with the aim to induce a cooling rate of −1 °C/ min) and then transferred to liquid or vapor nitrogen containers. This easy cryopreservation method guarantees good results in terms of viability, functionality, and potency of cells (Harris 2016). In compliance with cGMP regulations, continuous temperature monitoring and storage system alarms are required.

CTMPs can be cryopreserved in different solutions, as human Plasma AB or saline solution supplemented with human serum albumin, containing 10% dimethyl sulfoxide (DMSO; WAK-Chemie Medical GmbH, Steinbach, Germany). DMSO is the most common cryoprotectant (Hunt 2011). DMSO is mainly used at a concentration of 10%, but 5% and 2% concentration sometimes gave satisfactory results. DMSO minimizes ice formation. However, it is toxic to cells. For this reason it is necessary to take measures to minimize both the contact time and the osmotic shock for cells, mainly during the freezing/thawing phases. Residual DMSO in final product must be avoided as possible; any residues must be in compliance with the maximum dose recommended by the pharmacopeia.

Sucrose, glycerol, trehalose, methylcellulose, and sericin are cryopreservatives alternative to DMSO. They have been tested, but resulted less efficient than DMSO as they reduce cell viability (Shivakumar et al. 2015). PRIME-XV FreezIS DMSO free (Irvine Scientific) and STEM-CELLBANKERTM are alternative DMSO-free cryoprotectants used (Aijaz et al. 2018; Hunt 2011). In addition to DMSO, the cryopreservation liquid must also contain additives, generally proteins, that allow cells protection during cryopreservation. The best additive is represented by FBS; however, according to GMP, xeno-free reagents or human albumin are recommended as alternative to reagents with animal components.

In some cases, commercial pre-formulated preservation solutions, as Cryostor™ CS10 (BioLife Solutions, Inc., Bothell, WA) were used. Cells are generally cryopreserved in cryovials or cryobags of variable volumes. Cryovials or cryobags must be suitable for low temperature storage and transportation and must prevent the entry of unwanted materials when used. The choice of cells concentration depends on the cell type, on type of treatment and is based on preclinical studies results. To prevent cross-sample contamination and microbial contamination during storage and subsequent manipulation, bags and cryovials should be equipped with external thread cap (Chu et al. 2020).

The stability time, that is the maximum storage time within a CTMP maintains the optimal characteristics of vitality, functionality and potency, must be validated. Based on the stability results, it is possible to program therapeutic treatments in the context of a clinical trial (Harris 2016) and allows to plan therapeutic treatments for potential use in future.

Transport of CTMPs for clinical application is generally performed by specialized personnel. During transportation, some external factors can influence the quality of CTMPs, so critical parameters are essential to be considered both for frozen and unfrozen samples. Watertightness, sterility, temperature stabilization, shock resistance, gas stability, shielding and monitoring of ultraviolet radiation are requirements to be considered during transport as demonstrated by Aoyama. For this reason, containers used for transportation must be periodically controlled and internal data loggers are generally used in order to check temperature. Any transport anomaly must be investigated to guarantee the quality of the product: in accordance with GMP regulations, the traceability of each stage of transport is essential (Aoyama 2017; Hunt 2011).

After cryopreservation and prolonged storage recovery of maximal numbers of viable and functional cells is critical for CTMPs used in clinical trial. A reproducible methodology to thaw samples is necessary to avoid loss of viability, functionality and minimizing osmotic shock. The most common and reproducible methodology is generally a rapid thawing of the sample at 37 °C followed by dilution and rapid washing of the DMSO with cold media as 10% DMSO can be toxic to cells especially when samples reach room temperature (Harris 2016).

Cryopreservation is a major potential obstacle to maintaining intact the quality of particular sensitive cells, such as Treg or iPSCs. Treg and iPSCs typically suffer from very low post-thaw recoveries. Inadequate cryopreservation methods cause poor recovery during thawing, resulting in a low population survival rate. The poor cryopreservation efficiency of iPSCs also leads to the formation of abnormal cells, while it has been shown that suboptimal freezing and thawing protocols for Tregs, through the loss of CD62L, limit their ability to control the onset of GVHD (Florek et al. 2015).

6.9 Conclusions

In the last years advanced therapies, mainly based on the administration of CTMPs, have been increasingly used for the treatment of a large variety of diseases. Most of CTMPs are administrated to patients enrolled in clinical trials, while only few products received marketing license. The reason may be, at least in part, linked to the approval process, that requires skills, experience, time, and investment; the development of a CTMPs requires multiple strategies that allow the transition from research-based culture protocols to a completely defined large-scale production process of a medicinal product used as a drug. These strategies include (but not be limited to) the choice of the most suitable cell type, the optimal GMP-compliant medium to isolate and expand CTMPs, the definition of all the production process steps aimed to maximize therapeutic effects, and the choice of the quality control strategy to improve the safety of CTMPs and to minimize the risks for the patients.

Despite the difficulties, the development of CTMPs is needed and will be increasingly crucial in the next future, to improve the possibility of cure, particularly for those diseases for which there are currently no other effective drugs

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7 Upscaling of Clinical Grade Stem Cell Production: Upstream Processing (USP) and Downstream Processing (DSP) Operations of Cell Expansion, Harvesting, Detachment, Separation, Washing and Concentration Steps, and the Regulatory Requirements

Achim Cchitvsanzwhoh Satheka

Abstract

This chapter has discussed very important issues in stem cell production for instance, the background of stem cell production, and upscaling of clinical grade stem cell production. The chapter has further highlighted on critical areas of clinical grade stem cell production such as the upstream processing (USP) and downstream processing (DSP) operations of cell expansion, harvesting, detachment, separation, washing and concentration steps, and the regulatory requirements. Then the chapter has provided a recap that has summarized the expanded chapter sections. Finally, this chapter provides a call to action on upscaling of clinical grade stem cell production.

Key words

Upscaling of clinical grade stem cell production · Upstream processing · Downstream processing of cell expansion · Harvesting · Detachment · Separation · Regulatory requirements

7.1 Introduction

Basically, life emanates from a collection of atoms that leads to a molecule which its collection gives organelles. It is the collection of organelles that rise to give what we call a cell; this is a building block of life which every living organism is made up

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F. A. Khan (ed.), *Stem Cell Production*,
https://doi.org/10.1007/978-981-16-7589-8_7

of. Simply, a cell is a basic unit of life. For instance, in humans, an average human being is made up of around thirty trillion cells. Cells are much smaller or in other ways, they are microscopic. For instance, it would require 50 human cells to fill the dot on the letter "i". Cell production in human being is an ongoing process, for example, since the time you read the word **"abstract"** above, over one million cells have been produced in your body. This is the reason why you can get tired while just being seated, because your body is continuously at work, producing or replenishing cells. If this process is not controlled within the body, it then therefore leads to tumors that will advance into what is called cancer. With this regeneration activities happening continuously in our bodies, in exactly 7 years from today, each one of us will be made up of a different set of cells which are not there right now.

More interestingly, there are different types of cells in human body; for instance, on cells that make up human beings or any other multicellular organisms, most cells are specialized through differentiation to perform specific functions within the organisms (Hay 2013). Cell specialization helps organized groups of cells to perform tasks that in return sustain life. For example, in human beings, red blood cells are specialized to carry oxygen (O_2) throughout the human body; carrying of O_2 is a specialized function of red blood cells. However, red blood cells cannot divide like other types of cells (Solomon 2015).

Then there is this other type of cells which has a very unique ability to become or develop into specialized cells by dividing over and over again to produce new cells, and also by changing into other cell types in the body (Hay 2013; Marieb 2015). This unique group of cells is what is known as the stem cells. Basically, there are three types of stem cells namely:

1. Embryonic stem cells popularly known as ES cells
2. Adult stem cells otherwise known as AS cells
3. Induced pluripotent stem cells which are acronymized as iPS cells

In our century, the twenty-first century, the stem cells are uniquely useful as they are being used to replenish cells in the body as well as tissues that have either been destroyed or lost through diseases (Center for Disease Control [CDC] 2018). Recently, under the Coronavirus Disease of 2019 (Covid-19), stem cells have been useful in the development process of Covid-19 vaccines. For instance, stem cells have been used to test some Covid-19 vaccines in their early stages by inserting messenger Ribonucleic Acid (mRNA) into the stem cells to test or verify if the mRNA has no toxicity and the safety of the mRNA on humans. CDC (2021) indicated that the mRNA is placed into stem cells to ensure correct production of the spike protein from the severe acute respiratory syndrome coronavirus version 2 (SARS-CoV-2).

Now looking at how important the stem cells are becoming, this chapter therefore is to highlight a thorough background of stem cell production by looking at details from atoms to a fully developed organism (human being) which needs the products of stem cell production (Fig. 7.1). Basically, the chapter focuses on the upscaling of clinical grade stem cell production. It will critically look into:

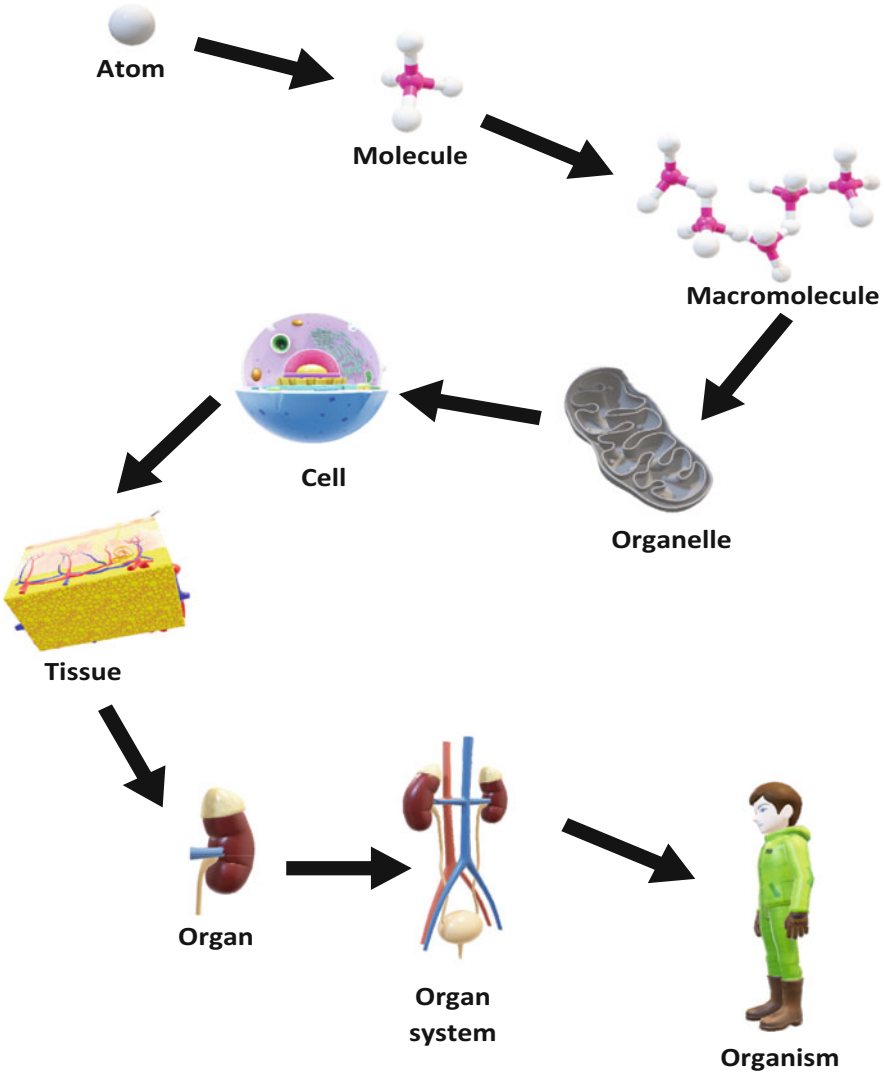

Fig. 7.1 The figure indicates the journey that leads to a fully fledged human being from an anatomical genesis. The stages of organization of organisms from atomic level to organismic level

1. Upstream Processing (USP) Operations of Cell Expansion
2. Downstream Processing (DSP) Operations of Cell Expansion
3. Harvesting
4. Detachment
5. Separation
6. Washing and Concentration Steps
7. Regulatory Requirements

The chapter discusses the background of stem cell production, then upscaling of clinical grade stem cell production. Then it highlights on chapter sections such as Upstream Processing (USP) and Downstream Processing (DSP) operations of cell expansion, harvesting, detachment, separation, washing and concentration steps, and the regulatory requirements. Then the chapter provides a recap that summarizes the expanded chapter sections. Finally, this chapter provides a call to action on upscaling of clinical grade stem cell production.

7.2 Background

This section creates a basic understanding of the connection between life and stem cell production technique. The section is divided into two subsections, namely:

1. Atomic to organismic level
2. Stem cell production
3. Upscaling of clinical grade stem cell production

7.2.1 Atomic to Organismic Level

Human body is a sum of its parts which can be studied at a variety of levels of organization from the simplest level to the most complex level as indicated in Fig. 7.1. For instance, the most complex and well-organized human body structure starts with a simple collection of atoms. Two or more atoms make a molecule. A collection of molecules gives rise to biologically significant large molecules basically found in the cell, for instance, macromolecules. Two or more macromolecules rise up to organelles which aggregate together to carry out specific functions in the cell (Hay 2013). Collection of organelles now rise to a basic unit of life, cell. Cell is a basic unit of life as it can be an organism or a living matter on its own; for instance; we have single celled organisms which are also called unicellular organisms such as archaebacteria, eubacteria, and yeast. On the other hand, there are other organisms that are made up of two or more cells and are called multicellular organisms. A good example of multicellular organism is a human being who is made up of approximately between 31 and 37 trillion cells which is a rough estimation of totaling all cell numbers of all organs in human body (Solomon 2015).

Now, a collection of cells which are fundamentally smallest living things make up a tissue. There are four basic types of tissues for instance, muscle tissues, nervous tissues, epithelial tissues, and connective tissues (Marieb 2015). A collection of tissues give rise to an organ which otherwise is known as a collection of cells with specific functions to play in the body, for example the heart, the lungs, and the liver are all organs.

A collection of organs functioning together gives an organ system. Organ systems are also referred in other texts as body systems. They are made up of organs that work collectively to perform specific functions for the whole body for example

circulatory system which is made up the heart, the blood and the blood vessels which functionally performs transportation of materials throughout the body. The circulatory system is well known in transportation of oxygen throughout the body hence the name circulatory system as it circulates oxygen, hormones, fuel, immune cells, nutrients, waste products, and other materials to the rest of the body. There are a lot of organ systems, namely the digestive system, the respiratory system, circulatory system, nervous system, endocrine system, excretory system, muscular system, skeletal system, integumentary system, reproductive system, and immune system (Marieb 2015; Solomon 2015).

A collection of organ systems or body systems functions together makes up an organism for instance, a human being. For example, the cardiovascular system (circulatory system), excretory system (urinary system), and the lymphatic system together helps the human being to keep water balance in the body. Note that the journey to a fully-fledged organism (as illustrated in Fig. 7.1) originates from an atomic level where elements such as hydrogen, oxygen, calcium, carbon, and phosphorus make up a single atom. A group of atoms makes a molecule which its collection results into macromolecules which their collection gives an organelle; a collection of organelles gives a cell.

Cell is a fundamental unit of life and a human being as earlier on stated is a lump sum of different cells. There are different types of cells in human body which function distinctively too. For instance, in human body alone, there are cell types such as the bone cells, muscle cells, fat cells, endothelia cells, skin cells, nerve cells, blood cells, stem cells, and others.

It is very significant to discuss these details of how the body of an organism or a human being comes into being before discussing one component of the body which does an amazing working throughout the entire life of an organism (humans) and that's the cell. It is even necessary before this chapter discusses how to upscale clinical grade production of cells, specifically stem cells to firstly understand how these cells are made and what function do they perform in the body.

Figure 7.1 has illustrated the journey of an atom to the level where collection of these atoms start to be called cells up through the organism level.

7.2.2 Stem Cell Production

As stated, the body has various types of cells which conduct various functions as well, and one of the cell types is the stem cell. Stem cell is just a cell which has a unique ability of developing into a specialized cell type in human body (Fang and Eglen 2017; Nguyen et al. 2016). All cell types in the body have specific function which they play in the body for life sustenance, for example, the red blood cells are specialized in carrying and distributing oxygen to all parts of the body. However, the stem cells are unspecialized or in other ways, they are undifferentiated cells and have that special ability of becoming any type of the specialized cells as required by the body at a particular time (MayoClinic 2021). Stem cells emanate from embryos and adult body tissues. Stem cells exist in humans throughout their entire life. Adult stem

cells are sometimes referred to as somatic cells or tissue-specific stem cells. As it has been highlighted earlier on that human body is in constant production of new cells that replenish the destroyed or diseased cells, stem cells regenerate damaged tissues and repair wounds in humans that comes after an injury or an illness.

Stem cells can be artificially grown in a laboratory setting whereby samples of stem cells are extracted from either an adult human or an embryo and then placed in a controlled culture (Uysal et al. 2018). The stem cells that are placed in controlled culture have the ability to divide and reproduce similar unspecialized cells. These stem cells that are in controlled culture and are able to divide and reproduce are referred to as stem cell line. The stem cell line can be stimulated to become specialized cells through what is called direct differentiation (Cossar 2011).

7.2.2.1 Categories of Stem Cells

Basically, stem cells are grouped based on their potential to undergo differentiation in order for them to become a different type of cells other than their current status. There are basically five categories of stem cells, namely unipotent stem cells, oligopotent stem cells, multipotent stem cells, pluripotent stem cells, and totipotent stem cells (MayoClinic 2021). Table 7.1 shows the categorization of stem cells in details.

7.2.2.2 Main Sources of Stem Cells

There are categorically two major sources of stem cells from adult body tissues and embryos. In addition to these two major sources, there is a third source of stem cell that has surfaced of late that emanates from scientific genetic reprogramming techniques. The three sources of stem cells are as follows:

1. Embryonic stem cells popularly known as ES cells
2. Adult stem cells otherwise known as AS cells
3. Induced pluripotent stem cells which are acronymized as iPS cells

Table 7.1 Categorization of stem cells based on their differentiation potential

Category	Literal meaning	Site of extraction	Differentiation potential
Unipotent	One-potential	Adult muscle stem cells	Can only be differentiated into one type of cells—their own type
Oligopotent	Few-potential	Adult lymphoid/myeloid stem cells	Can be differentiated into few cell kinds
Multipotent	Many-potential	Adult hematopoietic stem cells	Can be turned into closely related cells
Pluripotent	Several-potential	Early embryo cells	Can be turned into any type of cell
Totipotent	Entirely-potential	Few cells that emanate when zygote starts to divide	Can differentiate into all possible cell types

Embryonic stem cells (ES Cells) are taken from an earliest stage of pregnancy, thus, 3–5 days after male sperms fertilize the female egg and forms a blastocyst. At 4–5 days a blastocyst has 150–200 cells and is basically having two parts: the outer layer which later becomes part of the placenta and the inner cell mass layer which later becomes a fully-fledged human being. It is this inner cell mass where the ES cells are found and are referred to as totipotent cells as they have the capacity to develop into any kind of all possible cell types. ES cells can develop into blood cells, skin cells, tongue cells, or any other cell in the body.

Adult stem cells (AS cells) are also referred to as somatic stem cells (SS cells) or tissue-specific stem cells (TSS cells) and are found through the human's entire life cycle as they are needed by the body whenever it is deemed fit. Somatic cells exist in a non-specific form though they are more specialized as compared to ES cells, however, when these AS cells needed, they can now be turned into specialized cells such as skin cells or muscle cells (Kropp et al. 2017). As the body is continuously renewing its tissues, AS cells are there to provide maintenance and repair to the worn-out body tissues. These AS cells can be found from the brain cells, the bone marrow, the liver, the skin, the skeletal muscles, the blood vessels, and even the blood itself (Nogueira et al. 2021).

Induced pluripotent stem cells (iPS cells) are artificially created in laboratory setting using skin cells and other tissue-specific cells; they carry the same ability and potential as the embryonic stem cells.

Lastly, a new group of stem cells called the Mesenchymal Stem Cells (MSCs) is known to come from the connective tissues or stroma which is found around body organs such as the ligaments and cartilages (Jossen et al. 2018). MSCs have the capability of being stimulated to develop into body tissues such as the cartilages, fat cells, and the bones.

7.3 Upscaling of Clinical Grade Stem Cell Production

It is undisputed that stem cells are very vital throughout human life cycle. Stem cells are very significant and needed not only in day to day renewal and repair of tissues in humans but also in life sustenance from embryo stage of life to adulthood till death. With wide scientific research that have been conducted world over, stem cells have shown to have a great potential of solving a wide range of human's health problems such as:

1. Regenerating damaged tissues caused by injuries or accidents or even illnesses
2. Creation or growth of specific tissues or organs such as kidneys for transplant purposes
3. Cardiovascular disease treatment such as repairing the broken blood vessels in human body that comes due to high blood pressure
4. Brain disease treatment: Parkinson's and Alzheimer's disease could be treated by just generating ES cells which can repair the brain cells that are affected to cause these two health conditions

5. Cell deficiency therapy such as development of healthy new heart cells to donate to patients with heart problems that require transplanting
6. Blood disease treatments such as the use of adult hematopoietic stem cells to treat medical conditions such as leukemia and anemia

There are a lot more other significant roles which stem cells are currently being applied to in order to solve numerous problems that human race is facing in the twenty-first century. It is against this background that stem cell production needs to be fast tracked for mass production away from the laboratory setting production so as to help in solving the health problems that humankind is currently facing. Therefore, upscaling of clinical grade stem cell production is inevitable. Stem cells are being used as biological drugs in regeneration and inflammatory modulation in clinical settings. For instance, to replace disease-induced loss of pancreatic β-cells, hepatocytes or cardiomyocytes cells would require approximately between $1–10 \times 10^9$ efficient cells per individual patient while in terms of transfusion medicine, it would require approximately 2.5×10^{12} red blood cells per patient to sort out anemic problems via in vitro blood production (Kropp et al. 2017). Such huge numbers of required stem cell production per individual patient cannot be met using ordinary and conventional approaches, moreover, almost equivalent number of the stated required cell numbers per patient are readily needed to be available before the actual treatment (Cherian et al. 2020).

Scaling up clinical grade stem cells translate to manufacturing of therapeutic cells on a larger and expanded scale that is normally achieved through the use of technologies such as bioreactors. Cherian et al. (2020) indicated that cell-based therapies such as the use of stem cells require scale-up manufacturing methodologies such as propagation using microcarriers in stirred-tank bioreactors as opposed to the use of conventional planar tissue culture techniques. Development and optimization of reduced process-related risks such as the relative short batch-like production and cell transformation procedures would help in both industry and clinical grade production of stem cells (Kropp et al. 2017; Cherian et al. 2020). Table 7.2 below summarizes the universal techniques that are being used in upscaling stem cell production.

The different types of bioreactors stated in Table 7.2 have the ability of scaling up clinical grade stem cells with densities between 10^6 and 10^7 cells/mL. In addition to these bioreactor techniques' capabilities of expanding stem cell production for mass production, they can also produce the desired stem cells without unnecessary agglomeration of aggregates and hydrodynamic-induced damages of the cells (Cherian et al. 2020). Basically, the stem cell scaling up methods fall into two categories, namely; firstly, the conventional two-dimensional (2D) dependent approach that relies on cell immobilization on microcarriers and the matrix-free cell-only aggregation, and secondly the three-dimension (3D) culture system which is also popularly known as the suspension culture which use the matrix-free cell-only aggregates approach (Zweigerdt 2009).

Table 7.2 Universal techniques that are being used in upscaling stem cell production

Large-scale production technique	Approach used	Source of stem cells	Technical aspect	Operation mode
Stirred-tank bioreactor (STBR)	Two-dimension (2D) system culture	Mesenchymal Stem Cells (MSCs)	Cylindrical vessels with impeller that gives constant movement	Repeated batch (semi continuous)
Rotating wall bioreactor	Three-dimension (3D) system culture	Human Embryonic Stem Cells (hESCs)	Uses embryoid bodies and aggregates (cylindrical culture vessel is used)	Perfusion
Spinner flask	Two-dimension (2D) system culture	Human Embryonic Stem Cells (hESCs) and Human Induced Pluripotent Stem Cells (hiPSCs)	Uses aggregates	Repeated batch
Wave bioreactors	Three-dimension (3D) system culture	Human Pluripotent Stem Cells (hPSCs)	Uses microcarriers or self-assembling aggregates	Repeated batch
Shaking Erlenmeyer flasks	Three-dimension (3D) system culture	Human Pluripotent Stem Cells (hPSCs)	Uses microcarriers or self-assembling aggregates	Repeated batch
Vertical wheel	Three-dimension (3D) system culture	All stem cell sources	Uses microcarriers	Batch (nutrients are provided at initiation only and the volume is kept constant)

7.4 Upstream Processing (USP) Operations of Cell Expansion

Upstream in cell expansion refers to the fermentation process that includes the dispersion, media preparation as well as cell culturing. Upstream processing (USP) is the very first step that emanates from early stem cell isolation and cultivation through cell banking up to culture development of the cells to the final harvesting stage where the needed quantity of cells are obtained. USP process is an early bioprocessing for mass production of cells and uses equipment such as bioreactors and cell therapy aseptic isolators. USP of cell expansion is very significant in upscaling clinical grade production of stem cells meant for therapeutic purposes. Cell expansion in USP operation is affected by the nutrients and minor elemental impurities, the procedures taken in cell expansion that demand media ingredients onto the overall process hence affecting the final product yield such as longer time

and high amounts of product-related impurities (Wasalathanthri et al. 2020). Therefore, it is very important for scientists, biopharma researchers and manufacturers to pay more attention on the best practices that can improve USP operation procedures in order to maintain both the quality and consistency of the cell culture's raw materials for minimization of variability that happen during USP operations of the cell expansion.

There are well-established procedures that allow USP operations in biopharmaceutical companies to manufacture vial to large-scale bioreactor production without compromising the quality and viability of the stem cells being produced. The steps for cell expansion vary from company to company or among biopharmaceutical industries; however, the cell expansion basic steps remain the growth of the target cells from vial to an initiation bioreactor stage to ensure that the cells are close together for expansion phase. At expansion phase, a close look and cell culture conditions' observation is critical as to ensure healthy and robust growth of the cells as per designed process (Kropp et al. 2017). Closer observation on cell culture's conditions should mainly focus on controlling the conditions over each bioreactor in terms of optimal temperatures as well as the availability of oxygen (O_2), carbon dioxide (CO_2), and other key gases. In addition to temperature and availability of key gases, stability, purity, and composition of the culture media such as the carbohydrates (e.g., glucose), supplemental vitamins and amino acids must be critically observed (Jang et al. 2017; Kropp et al. 2017).

In the event that this initial stage of using the bioreactor has not been well conducted, the whole USP operations will fail and resulting in death of the cells. In such circumstances, then the requirement is to discard every raw material used, and immediately stop the whole process as it cannot yield enough cells to enable continuation to another level or phase of cell expansion (Cherian et al. 2020). At this point then, it is very important to restart the whole USP operations of cell expansion with the initial cell inoculum.

7.4.1 Upstream Processing Areas, Systems, and Equipment

The requirement for an USP success is that USP processing areas must at all cost be very sterile. The USP dispensing room's materials for production must be well weighed and measured in traceable, sanitary and controlled arena before initiation of the USP operation (Carmelo et al. 2015). Basically, the biomanufacturing USP operation raw materials get into the manufacturing facility or the dispensing room in well-packaged containers that are fit for current good manufacturing practice (cGMP). Current good manufacturing practices help in checking USP operations' proper design, monitoring, and control of the whole cell expansion or manufacturing process as well as the facilities being used (Czapla et al. 2019). For instance, the suitable cGMP process arena should ensure clean materials such as the glass or plastics which have no cardboard or any fiber shedding materials to avoid contamination. If the packaged production raw materials are delivered to the facility in very bulky containers, it is good for the manufacturers to just take the needed amount to

the dispensing area for weighing and measuring. It is good to make the weighing and measuring of the needed USP operation raw materials closer to the dispensing room to minimize long movements with the material which can lead to contamination somehow (Upstream Processing 2021; Abbasalizadeh et al. 2017).

Both the cell raw material and non-cell raw material are to be mitigated from cross-contamination risks during the dispensing process through segregation to avoid exposing these raw materials to adventitious viruses which can compromise the USP operation. In addition, both animal-derived raw materials and non-animal-derived raw materials should be prepared in separate dispensing booths to ensure high levels of precautions in reducing contamination risks during preparation of USP operations for cell expansion.

Dispensing process varies from company to company or manufacturer to manufacturer as some companies or manufacturers use electronic weighing and measuring gadgets such as scales that provide both electronic recording and verification of data, while other companies or manufacturers use manual equipment to weigh and measure the required raw materials for an USP operation. When a biomanufacturer is using a manual system to weigh and measure the USP operation raw materials, there are two individuals to conduct the weighing and measuring; one individual acts as the operator of the weighing and measuring process, another individual verifies the weighing and then records the data on a paper (Fernandes-Platzgummer et al. 2016). Verification is needed so as to make sure that the required amount of the correct raw material has been dispensed. To avoid cross-contamination, it is advisable to have one raw material in the dispensing booths at a particular time. The raw materials in dispensing rooms are needed in different amounts depending on the amounts of yields required at the end, for instance, it can range from just a 1 mL of supplements to over 500 kg of salts.

The biomanufacturing personnel or technicians are trained to be able to use the sophisticated equipment in the facility. For instance, in the dispensing rooms there are equipment such as the airflow booths that are used for weighing or measuring bulky raw materials in correct amounts; drum inverters post hoist as well as the vacuum tube lifters that are used for handling larger solid raw materials (Lin et al. 2017). Upon completing the dispensation process, the equipment that have been used must be kept clean and safe by the technicians or the operators; for instance, spatulas pipette tips, scoops, weighing boats as well as the graduated cylinders should be discarded or otherwise cleaned.

7.4.2 Clean in Place and Steam in Place Systems

As highlighted in Sect. 7.4.1, all equipment that are used in USP operation for cell expansion must be kept clean or discarded soon after completion of the dispensing process. The two common systems to be used are:

1. Clean in Place (CIP)
2. Steam in Place (SIP)

CIP is an automatic cleaning process of vessels, in-line devices, piping and all processing equipment with minimal manual setup, and shutdown and this is done without an operator's intervention during the cleaning process. On the other hand, SIP is a timed sterilization process for both upstream and downstream processes that biopharmaceuticals used in cleaning utensils or apparatus using steam. The cleaning process of equipment used in USP operation in cell expansion is very necessary because most bioreactors are multi-use and need not to have debris or remaining media or chemicals from the previous batch. Therefore, whenever an USP operation is completed, all equipment used are cleaned using various means such as CIP and SIP or even manually so that during the next use for another production, they should be clean without growth of microorganisms such as bacteria due to media that can cling to the walls of the equipment (Galipeau et al. 2021; Stephenson and Grayson 2018).

7.4.3 Cell Isolation and Cell Culture

Isolation of cells (Fig. 7.2) depends on the cell mechanisms that allow formulation of novel biological drugs which in the process help in fighting against specific diseases or disorders in humankind. In some instances, cell isolation protocols require bulky numbers of cells that as well need to be disturbed physically to enable further isolation and proliferation for further analysis. Firstly, the matrix is disrupted through treatment of the tissue sample with proteolytic enzymes and also *chelatin* agents. Proteolytic enzyme digests the protein in the extracellular matrix while the *chelatin* binds the calcium (Ca^{2+}) for the matrix to break cell-cell adhesion. This graduates to gentle agitation in order to obtain single living cells. However, the yielded products at this stage still have different types of cells that further need to be singled out or separated. Therefore, suspension is conducted to isolate the different cell types. Centrifugation is performed so that the cells that have the capacity to adhere to the plastic or glass should be separated (Zhu and Murthy 2013).

An advanced methodology applied to cell separation uses an antibody combined to a fluorescent dye. An electronic fluorescence-activated cell sorter machine has a capability of sorting out a pool of cells for instance 1000 unlabeled cells can be sorted once per second (one thousandth per second); this is achieved by passing the cells through a laser beam which allows the fluorescence of each single cell to be rapidly measured. This is not the only method being employed for cell isolation, there are several other methods which are used in cell isolation process such as the use of the laser capture microdissection which is simply called LCM. LCM isolation method has a capability of producing pure cells from a pool of heterogenous cells or cytological preparations. LCM method is very outstanding in the sense that it can extract DNA, RNA, and protein biomolecules which are useful in downstream techniques such as the polymerase chain reaction (PCR), proteomic analysis, and microarray analysis (Lim and Shin 2013).

7.4.4 Cell Proliferation and Expression

Proliferation of cells means the increase in number of cells due to a direct normal cell growth and cell division. When vaccines are being formulated or any other biological drugs, specific cells are always targeted and then isolated to their advantages to boost the health of the targeted patients. For example, the Coronavirus Disease of 2019 (Covid-19) messenger ribonucleic acid (mRNA) vaccine targets the muscle cells hence being administered on the upper arm muscle so that when the mRNA gets inside the muscle cells, then these cells get instruction to make spike protein similar to the spike protein on the surface of coronavirus which causes Covid-19 (CDC 2021).

Now, in cell proliferation, when the target cell has been isolated as in Fig. 7.2, it is then firstly grown artificially in the laboratory setting. Thereafter this laboratory level, it then graduates to biopharmaceuticals level for clinical grade upscaling in order to create the needed biological drugs. Cell proliferation is regulated by

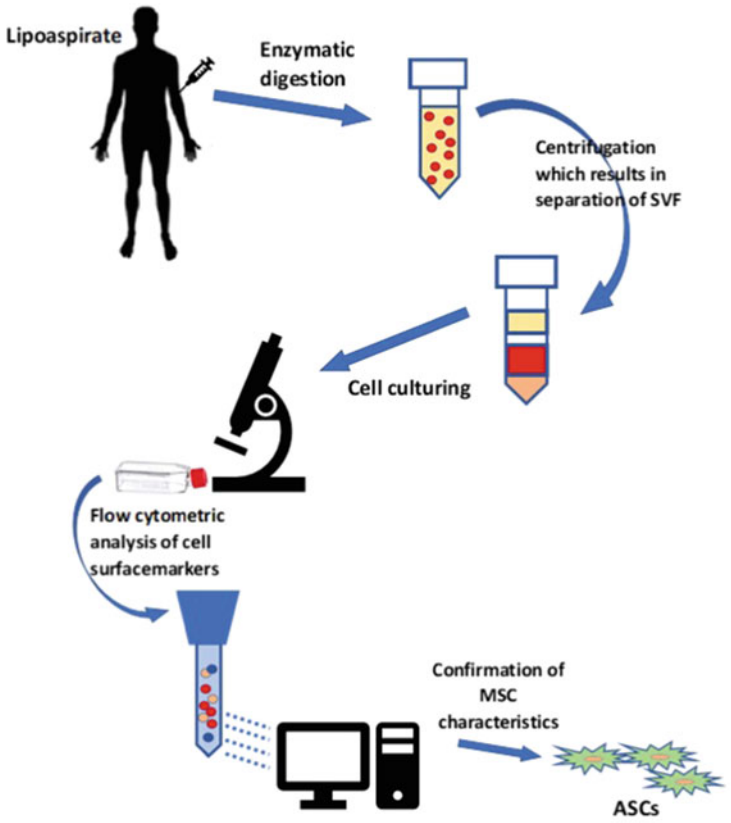

Fig 7.2 The MS cell culturing process

conditions such as pH, temperature, O_2 and nutrient levels internally. In biopharmaceutical industries, standardized method that ensures proper reproducibility of cell proliferation employs bioreactor approaches in order to perfectly achieve USP operations in cell expansions (Zhu and Murthy 2013). Bioreactors are known to have been very significant nowadays in the expansion of red blood cells (RBC), chimeric antigen receptor (CAR) T cells, mesenchymal stem cells (MSCs) as well as the induced pluripotent stem cells (iPSCs). This is possible because the bioreactors provide the excellently controlled microenvironment that triggers easy growth of cells (Rafiq and Masri 2017) (see Fig. 7.2).

7.4.5 Cell Banking

The gathering, processing and storage of specific stem cells, for instance, cell lines in order to provide life-saving biopharmaceutical products is what is referred to as cell banking. The process of cell banking gives a reproducibility platform for cells to be manufactured at clinical grade with high-quality, efficient and safe biological therapeutics which possess proper characterization. Cell banking ensures total safety, risk free and non-invasive cell products. One of the best techniques in cell banking is the use of cryopreservation method which employs very low temperatures to store cell lines for over 25 years. Of late the cryopreservation method has been further improved and has even attained the ability to remove ice crystals, nucleation and minimizes the rate of water transport in the stored cells which were previously posing risks of injuring the stored cells. The improved version of cryopreservation is called the cryopreservation agent (CPA) and has the ability to preserve the fine structure of the cells for indescribable period of time while maintaining quality and viability of the cells (Zhu and Murthy 2013).

7.5 Downstream Processing (DSP) Operations of Cell Expansion

Gottschalk (2011) defined Downstream Processing (DSP) as the series of operations that are needed in order to obtain biological materials such as tissue culture fluid or cells and derive from such biological materials a pure and homogenous protein product. DSP is simply the unit operations that come after cell growth and expansion including completion of other product formation. DSP is a multi-step procedure that incrementally increases the target's purity through exploitation of both the chemical and physical properties that make it very different from contaminants (Gottschalk 2011). The DSP operations of cell expansion generally comprise of the following:

1. Harvesting
2. Clarification steps for removing bulk contaminants (e.g., particulates, carbohydrates, oils, etc.)
3. Capturing and polishing steps

4. Washing
5. Cell concentration
6. Formulation
7. Final fill or finish stage
8. Preservation

The DSP operations for stem cell therapies are the follow-up steps that take place after cellular expansion stage and these steps as indicated above will vary depending on the therapy and the biomanufacturing company or the technologies being used. Generally, a swing-bucket rotor centrifugation phase otherwise known as the centrifugation phase is included in DSP operations in order to remove the large aggregates, unwanted proteins, cell debris, volume reduction for concentrate as well as formulation of cellular suspension that gives a patient-friendly ability or preservation-friendly buffer capabilities (Mettler-Toledo 2021) (see Fig. 7.3).

DSP operations for cell expansion might as well include formulation phases that signify the transition from drug substance to the drug product. There are some considerations that need to be taken into account for DSP operations for cell expansion at biopharmaceuticals and biomanufacturing companies such as management of biohazards wastes, management of multiple waste streams as well as management of resources that are used for both DSP operations and the facility. Piloting at laboratory level for DSP operations for cell expansion is one significant step which is performed before manufacturing scale that involves the process analytical technology (PAT); this ensures the optimization, trouble-shooting, and scale-up of DSP operation for cell expansion.

As highlighted above as well as in Fig. 7.3, the whole process of downstream processing vary from product to product, method used as well as the biomanufacturing company which is performing the DSP. However, there are some steps which cannot be missed in most of the DSP operations; firstly the harvesting and filtration phase of DSP operations which enables separation of products from large debris at the same time being able to optimize retention of both the yield and quality of the product (See Fig. 7.3).

The second step of significance to be noted during DSP operations is primary capturing phase which enables the maintenance of desired product while retention of impurities being minimized. This step benefits not only the main product but also the by-products, which are retuned with minimized impurities. At primary capture phase, there are no bulk debris as the harvesting step ensures removal of such big debris.

The third worthy noting phase in DSP operations is buffer exchange and up-concentration which involves two steps, namely ultrafiltration (UF) and diafiltration (DF). On UF step, there is a concentration of diluted product stream as well as the separation of molecules in solution according to membrane pore size and/or molecular weight cutoff. On the other hand, the DF step involves exchanging of products from the existing buffer to a novel buffer after a final formulation buffer and sometimes even for following process. Combination of UF and DF results into buffer exchange.

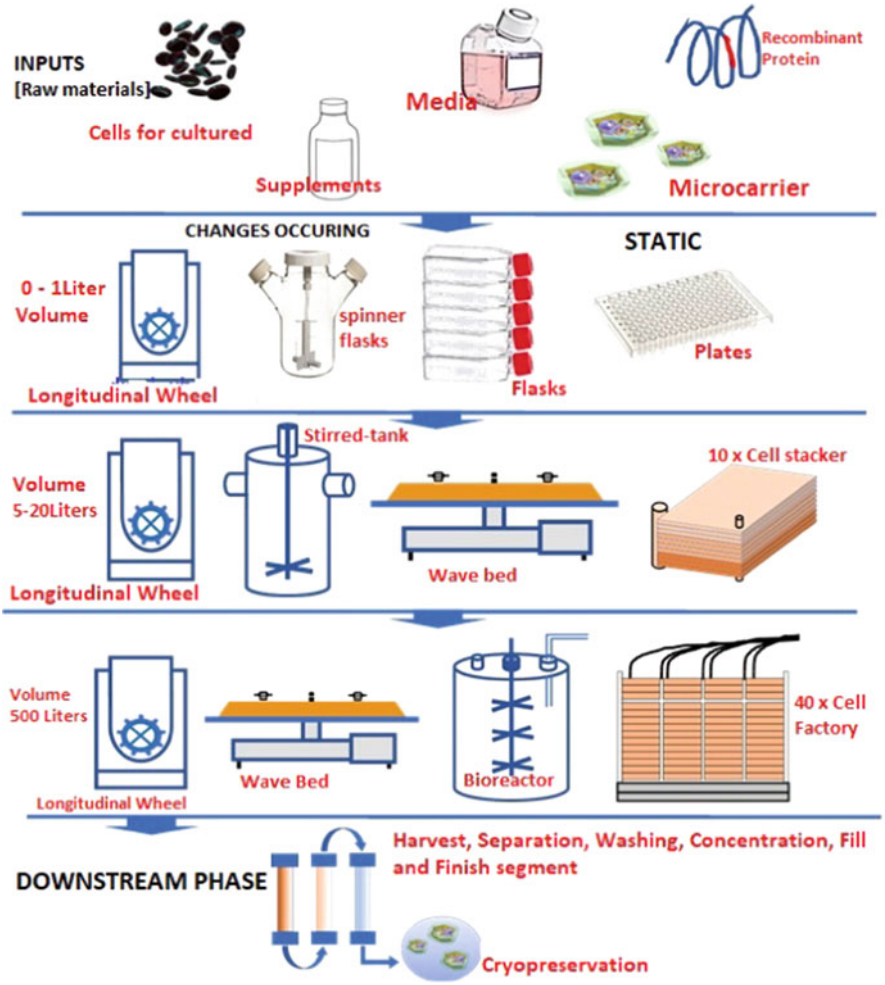

Fig. 7.3 Stem cell expansion at clinical grade

The fourth step which is significant too, is the purification which involves clearance of impurities or contaminants. This significant step removes residual impurities in a systematic manner where each purification stage contributes to the residual removal while retaining as much products as possible. Purification step in DSP operations for cell expansion helps in product purity achievement while potential yield loss is highly minimized in the process. The fifth step of DSP operations in cell expansion is the bioconjugation. This is a molecule-dependent step that involves a novel class or generation of biologic molecules that are specifically designed to increase efficacy through combination of two or more distinct types of therapeutical molecules. For example, anti-drug conjugates (ADC) are the

bioconjugates that are synthesized by an antibody that has been biochemically altered and is covalently connected to an active pharmaceutical ingredient (API).

Lastly, formulation step is a significant step in DSP operations for cell expansion whereby a transition is done on a drug substance (DS) to a formulated drug product (DP) and this is where the DP molecule (cell) is made ready for clinical administration. At this stage, the environment, solvent, or the physical stage is made friendly for the cell or the therapeutical product in preparation for a clinical administration. This is an important step in DSP operations in cell expansion as it stabilizes both DS and DP as well as formation of a platform that characterizes the final concentration, polymerizations, encapsulation, adjuvant synthesis, adsorptions, and some other specific events.

7.6 Harvest

In cell expansion, the term harvest basically means the act of removing the cells from the vessel which was used for culturing for instance, after the cell expansion. The devices that are used for culture where harvesting can be conducted from include but not limited to:

1. Stirred-tank bioreactors
2. Gas-permeable membrane reactors
3. Hollow-fiber reactors
4. Packed-bed reactors
5. Shaking Erlenmeyer flasks

Harvesting process requires optimization of each step of harvesting hence known to be a process-specific act. For instance, when harvesting human mesenchymal stem cells (hMSCs) from culture vessels like stirred-tank bioreactor on microcarriers, there is a need to combine the enzymatic dissociation with the mechanical force via impeller agitation in order to detach cells in situ from the microcarrier.

Primary recovery is the first step done in cell harvesting where separation of cells from the media that carries the target active pharmaceutical ingredients (API) is performed; this leads to recovery of protein products from the culture. Fermentation process or cell culture ends when the harvesting process is initiated. During cell culture, it is important to closely monitor the process so as to make sure of the progress of the culture to the end. The monitoring can be achieved through taking of samples at an interval to monitor in order to determine the progress. Once the cell culture is over, then harvesting is initiated. Quality and quantity of the product that has been accumulated in the culture vessel, for instance, the bioreactor predetermine cell culture or fermentation process. Basically, the maximum high-quality product is obtained when numbers of cells being collected in the vessel decreases or in other ways when the cells start to die in bulk. Consequently, lysed cells start producing enzymes that could digest the product hence posing risk to the whole downstream process operation of cell expansion.

Fig. 7.4 Cell centrifugation at 120 revolutions per second for 14 min to separate debris from the cells. This centrifugation is being conducted in a molecular biology laboratory. (Photo credit: Achim Cchitvsanzwhoh Satheka)

When cell culture process ceases, harvesting process is initiated with the first step normally being the centrifugation which involves spinning of the culture from the culture vessel for instance the fermenter or the bioreactor. Allowing settlement of the suspended contents of the culture vessel leads to the slow sinking of the cells to the bottom; these cells are attracted to the gravitational force due to their large size. Spinning of the culture vessel triggers the activity of gravity hence big debris or cells easily spun out of the mix and consequently discarded (see Fig. 7.4).

The next step in the process of harvesting cells is the filtration step which is very significant as it ensures removal of large debris. As the step's name suggests, filtration uses a filter which performs size exclusion or mechanical straining activities where larger particles are trapped within the filter membrane. Filtration process in harvesting happens in two phases; the first filtration phase is called the depth filtration. At depth filtration phase, the larger particles that have been prevented from passing through the filter membrane also known as the retentate are trapped, whereas the small particles pass through and make their way to the filtrate. API products make it through as filtrate together with other small particles such as proteins. However, there are other particles that are smaller and have the capability of passing through the filter but are trapped because of inertia impaction. When initiating harvesting process, the suspension shows a cloudy appearance, however, after centrifugation step and the depth filtration the translucence of the suspension becomes high and clear hence these steps are also referred to as clarification steps.

The end result of the depth filtration step is the separation of the debris and the cells from the suspension. The second filtration phase in harvesting process is called

the sterile grade membrane filtration which removes the smaller particles as well as potential microbial contaminants. Sterile grade membrane filtration has a pore size of $\leq$0.22 micrometer or microns (μm) and with this size, the sterile grade membrane filtration has the capability of removing any bacteria. After the sterile grade membrane filtration which is also the last step in cell harvesting process, the suspension or the mixture or the product now attains a translucent appearance making it ready for purification (Plouffe et al. 2007).

7.7 Detachment

Generally, cells adhere or stick to surfaces due to attraction forces that are found between molecules and the substrate, hence great need to eliminate such interaction of cells and other molecules of interest. Elimination of adhesion of cells with other molecules or surfaces can be achieved through physical, chemical, optical, or electrical means; this is called detachment in cell expansion processes (Murthy and Radisic 2013) (see Fig. 7.5).

Murthy and Radisic (2013) indicated that cells can adhere to extracellular matrix through adhesion receptors found in the membrane of the cells to the proteins of the extracellular matrix. An increased impeller agitation during cell harvesting process helps in increasing the rate of cell detachment. This method of cell detachment during cell harvesting process minimizes cell-damaging shear forces risks, hence provide a >95% harvesting efficiency. In the event of in situ detachment protocols need to be scalable larger for instance, in a situation of an industrial scale bioreactors should be well and excellently established on theoretical mixing technique. When in situ detachment procedure is well undertaken, it provides an extra step outside the culture vessel, hence minimizing contamination risks as well as saving time between steps in cell harvesting process (Rafiq and Masri 2017).

7.8 Separation

Separation simply means the act of moving or diving or isolating cells into their constituent or distinct elements. For instance, isolation of pure stem cells from a heterogenous suspension in a clinical grade cell expansion process is one fundamental stage which is commonly referred to as separation stage (Zhu and Murthy 2013). There are a number of technologies and approaches that have been devised to facilitate the cell separation step in stem cell production. Some of the technologies used in separation of cells at clinical grade stem cell production include:

1. Physical parameters-based technologies that utilizes aspects such as size or density of cells or the substrates of interest.
2. Affinity-based technologies that utilizes magnetic couplings, electrical or chemical aspects of the cell or substrate of interest.

Fig. 7.5 Addition of reagents using a pipette in order to relieve cell adhesion through chemical means in a laboratory setting. (Photo credit: Achim Cchitvsanzwhoh Satheka)

On physical parameters-based approaches, stem cell separation is done using the density gradient centrifugation (DGC) method which allows processing large quantities and easy scaling up; an example being either *ficoll-paque* or *percoll*. Another physical parameters-based approach is the density gradient centrifugation-negative selection which provides facile and high purity; a good example is the *RosetteSep*. Pre-plating method is another method utilized in stem cell separation techniques for instance human embryonic stem cells (hESCs) as well as in the human adipose-derived stem cells (hADSCs).

Conditioned expansion media is another method which is employed in stem cell separation technologies and is able to process large quantities, scaling up easily and facile; for instance, in hMSCs from the bone marrow. Dielectrophoresis (DEP) method is used in conventional stem cell separation technologies to sort out cells based on viability as well as sorting out cell without dilution of cells in fluid for example live adipose-derived stem cells (Yang et al. 2000). Another method is the field flow fractionation (FFF) which provides label free on stem cells in separation process and this is used for instance in hMSCs from fetal membrane. Another separation method from the stem cell separation technologies is the flow cytometry (FACS) which is very powerful, and has very high resolution, sensitivity as well as precision; a good example of FACS separation method is used in hESC-derived

neural cell populations (hESCDNCP) and also the human hematopoietic stem progenitor cells (Mettler-Toledo 2021).

Lastly, the conventional stem cell separation technologies' magnetic activated cell sorting (MACS) method is a simpler method as compared to FACS separation method; however, MACS method targets stem cells that contain magnetic pieces. MACS has a capability of processing the target cells in parallel and achieves separation faster. MACS achieves cell purity of up to 75%.

Of late there have been novel methods for stem cell separation technologies. The first emerging method of separating cells at clinical grade is by using the aqueous two-phase system that uses temperature responsive polymer. This method of cell separation is very simple, scalable as well as having a capability of the system being reused for instance, the antibodies. Examples of aqueous two-phase system cells used are CD34 + KG1.

SELEX is another emerging novel stem cell separation technology which has an ability to select stem cells without any molecular information whatsoever, reduces in vitro testing costs as well as being able to capture both protein and non-protein targets. Examples of cells in SELEX separation method are the mesenchymal stems cells from humans.

Lastly on emerging stem cell separation technologies, microfluidics method is a novel method in separation of cells which is automatic; thus, with less operator handling, it has the ability of label free on cells. Furthermore, microfluidics method of cell separation has the ability to run several processes on one chip, miniaturization as well as mass processing. Examples in fluidics method include negative DEP fluidics, cell affinity chromatography-based microfluidics as well as the droplet microfluidics (Pierzchalski et al. 2013).

7.9 Washing and Concentration Steps

Swing-bucket rotor centrifugation has been employed for cell washing and concentration in stem cell production. To perform washing and concentration procedure using swing-bucket rotor centrifugation, a biological safety cabinet is required for the subsequent resuspension activities. For instance, resuspension step demands discarding spent media and then resuspending it in a recommended buffer (Li et al. 2021). This process is normally repeated for several times so as to eliminate any unnecessary particles and/or excessive reagents. The swing-bucket rotor centrifugation has limitations in as far as product sterility maintenance is concerned, achievement of the highly concentrated product as well as low cell quantity yielding.

Another alternative cell washing, and concentration approach involves continuous counterflow centrifugation system and also filtration system. This alternative cell washing and concentration approach is suitable to large-scale stem cell production for both counterflow centrifugation and filtration systems which are automated. Counterflow centrifugation system keeps the cells in suspension while washing them, clearing debris from supernatant and the buffer exchanging. Counterflow

centrifugation system of cell washing and concentrate have capabilities of obtaining >80% cell recoveries and also >90% cell viabilities (Rafiq and Masri 2017).

Part of good manufacturing practice (GMP) in clinical grade stem cell product is through following the washing and concentration steps. In biomanufacturing industry, washing and concentration cannot be done manually as the opening and closing of the culture vessels can compromise the quality and viability of the final product. Opportunistic contaminants might get their way into the culture vessels through continuous opening and closing of the culture vessel when passing through each and every step of washing and concentration. So automated upstream and downstream devices have been developed to accommodate all the washing and concentration steps in a single enclosed device where a final product is obtained at the end after completing the entire washing and concentration procedure. These single devices that have been developed to address cell culture and wash-and-concentration gap follow the GMP approach, hence such devices reduce risks that complicate validation process (Bacon et al. 2020).

Tangential flow filtration (TFF) is an alternative cell washing and concentration technique which can be turned to be automatic, upscaled, and require small capital investments. Basically, steps followed in TFF cell washing and concentration process include enzymatically dissociation of anchorage-dependent cells into a single-cell suspension; then followed by the separation of cells from the microcarriers through size exclusion process.

In cell washing and concentration, automation procedures are being implemented using two procedures, namely;

1. Modular procedure or technique where several devices with each device made to perform its major function and is considered part and parcel of the processing chain.
2. Integrated procedure or technique where several processes are integrated into a single device that performs all the cell washing and concentration steps.

Both procedures pay attention to the buffer exchange and volume reduction protocols. In a laboratory setting, cell washing and concentration steps involve benchtop centrifugation (See Fig. 7.4) which is followed by the repeated manual pipetting (See Fig. 7.5) in order to get the cell suspension. It is possible to apply the manual process to either biopharmaceutical companies or any biomanufacturing industry where the cell washing and concentration steps can manually be carried out onto a biosafety cabinet in a GMP cell manufacturing facility.

7.10 Regulatory Requirements

Upscaling clinical grade stem cell production is regulated almost in all countries around the world except for other few countries where scientific or biopharmaceutical industry or biomanufacturing industry is never seriously monitored. There are regulations that manufacturers all around the globe are aware concerning handling of

clinical grade stem cell production. Stem cell production gives hope to the field of regenerative medicine as it holds the future of organ transplant, injured or diseased cell replacement, treatment of multiple sclerosis, cardiovascular diseases, and Parkinson's disease (Jose et al. 2020). This therefore calls for serious checks for biopharmaceuticals and biomanufacturing industries that are in upscaling clinical graded production of stem cells. Both developed and developing countries must approve the safety, efficacy as well as the licensing requirements of both manufacturing and selling of stem cell products or therapies. Most western countries such as the United States of America (US) and the European Union (EU) have written rules and regulations for monitoring and reviewing the manufacturing procedures companies use in stem cell production to ensure both potency and purity of the stem cell products are maintained at all times (George 2011; Jose et al. 2020; Lee 2011).

In both the US and the EU, regulatory bodies are set to examine and assess the production processes which the biopharmaceutical companies are following to produce stem cells on large scale. Besides regulatory agencies, stem cell therapies are subjected to passing certain criteria and undergo particular examinations. Regulatory agencies in both the US and EU focus much on the following areas in order for them to approve stem cell therapies or stem cell products and by-products:

1. Product stability
2. Batch consistency
3. Product safety
4. Product efficacy through preclinical and clinical studies up to market authorization

However, in developing countries such as African and Asian countries some countries are not very strict in pushing the biopharmaceutical industries to stick to the set criteria and regulations for stem cell therapies process and production. For example, George (2011) observed that in India and some Asian countries, there is lack of excellently defined framework for stem cell-based products (SCBP). Lack of frameworks for SCBP has not spared both African countries and Latin American countries.

Regulatory framework in EU basically focuses on three main areas; firstly, the definition of cell therapy as clinical products and their framework demands inclusion of SCBP's specific requirements. Secondly, the EU regulatory framework emphasizes on cell therapies as mandatory for such cell therapy products which in the end there is a need to describe the special requirements for approval of cell therapy trials. Lastly, the EU regulatory requirement looks at the establishment of standard quality, tests, processing, harvesting, preservations, storage, and the distribution of hSCs.

In the US, the regulatory framework firstly demands avoidance of the use of contaminated cells for example cells that are contaminated with an acquired immune deficiency syndrome (AIDS) or hepatitis. Secondly, the US framework requires prevention of improper handling or processing that might end up in contaminating

the cells. Lastly, the US framework encourages clinical safety of all the tissues or cells that might be processed, used for functions other than normal function.

There is a need for the whole world to have one harmonized stem cell therapies' regulatory framework. Some of the proposed regulatory framework for clinical grade stem cell production include:

1. Clinical use of stem cells should not be permitted until efficacy and safety procedures are openly established.
2. Clinical use of stem cells should not be permitted until origin, safety, and the composition of the stem cell product are adequately labeled and defined.
3. Clinical use of stem cells should not be permitted until conditions for storage and use are provided in proper details.

Acknowledgement All the illustration diagrams were done by Daniel Moyenda Manduwa except for Figs. 7.4 and 7.5 which are pictures provided by the author.

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8 Characterization of Clinical-Grade Stem Cells: Microscopic, Cellular, Molecular, and Functional Characterization of Stem Cells and Their Products According to Regulatory Requirements for FDA Approval

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Abstract

Stem cells have unique properties that make them an exceptional source for regenerative medicine. Various types of stem cells and their products are used for therapeutic purposes, and they differ in their morphology, molecular markers, and functional characteristics. Nevertheless, to comply with regulatory requirements, strict criteria must be fulfilled. Here, we provide a comprehensive overview of the characterization criteria for human mesenchymal stem cells (hMSCs), human neural stem cells (hNSCs), human pluripotent stem cells (hPSCs), and human hematopoietic stem cells (hHSCs) that constitute the starting material for cell therapy products.

Keywords

Clinical-grade stem cells · Characterization · Pluripotent stem cells · Mesenchymal stem cells · Neural stem cells · Hematopoietic stem cells · Microscopic · Cellular · Molecular · Functional characterization

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F. A. Khan (ed.), *Stem Cell Production*,
https://doi.org/10.1007/978-981-16-7589-8_8

8.1 FDA Regulations and Recommendations for Cell Therapy Product Characterization

Cell therapy products have unique characteristics due to the nature of living cells. Cells are able to express various molecules on their surfaces and secrete various factors into the medium that can be crucial for cell therapy or lead to adverse effects. These molecules and factors can change over time and through changes in culture (Gharibi and Hughes 2012; Wagner et al. 2010). The proper characterization of cells is indispensable to prevent unintended or even harmful effects on patients. Stem cells have the potential to differentiate into desired cell types, although there is a risk of the formation of spontaneously differentiated cells. These cells can cause adverse effects after administration to the patient. Furthermore, stem cells might lead to the development of tumors when not sufficiently differentiated. Differentiated cells might also develop undesired autonomous functions, such as cells with the characteristics of cardiomyocytes forming a focus that generates electrical activity uncoordinated with the rest of the heart (Considerations for the design of early-phase clinical trials of cellular and gene therapy products; Guidance for Industry 2015). Another feature is the migration of differentiated cells after administration to the patient. Undesired effects can include cell migration to various organs and tissues that are not target locations. Assays described in this chapter are examples of methods that can be used for in-process and release testing to prevent these unintended effects. However, potency assays and other relevant assays should be based on the particular use of the final product and on the mechanism(s) of action (Guidance for Industry Potency Tests for Cellular and Gene Therapy Products 2011).

8.2 Characterization Strategies for Cell Therapy Products

8.2.1 Identity

Identity may be established either through the physical or chemical characteristics of the product, inspection by macroscopic or microscopic methods, specific cultural tests, or in vitro or in vivo immunological tests (CFR Sec. 610.14). It is advisable to develop several assays to detect the active cells in the product (positive markers) as well as assays to identify possible contaminating cells (negative markers) (Carpenter 2017).

8.2.2 Potency Assays

Potency is defined as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended to effect a given result" (21 CFR 600.3(s)). Regulations require that "tests for potency shall consist of

either in vitro or in vivo tests, or both, which have been specifically designed for each product so as to indicate its potency in a manner adequate to satisfy the interpretation of potency given by the definition in § 600.3(s) of this chapter" (21 CFR 610.10).

Potency can be measured by biological assays (within a living biological system) or nonbiological analytical assays that consist of methods that measure:

(a) Immunochemical (e.g., quantitative flow cytometry, enzyme-linked immunosorbent assay (ELISA)),
(b) Molecular (e.g., reverse transcription polymerase chain reaction (RT-PCR), quantitative polymerase chain reaction (qPCR), microarray) or
(c) Biochemical (e.g., protein binding, enzymatic reactions) properties of the product outside of a living system.

Multiple assays (assay matrix) might consist of a combination of biological assays and analytical assays or analytical assays alone and could be used if some products have:

(a) Complex and/or not fully characterized mechanism of action (e.g., MSCs),
(b) Multiple active ingredients and/or multiple biological activities,
(c) Limited stability and/or
(d) The biological assay is not quantitative, not sufficiently robust, or lacks precision.

Potency assays should be designed to incorporate a sufficient number of replicates to allow for statistical analysis, use sample randomization to reduce biases, and include appropriate controls. Variability should be reduced wherever possible. When variability is unavoidable, it should be balanced, measured, and modeled. General principles for the reduction of variability are the use of qualified reagents, qualified and calibrated equipment, and adequately trained and qualified operators. Assay variability can also be substantially reduced by following detailed standard operating procedures (SOPs) and having appropriate controls in place (Guidance for Industry Potency Tests for Cellular and Gene Therapy Products 2011).

8.2.3 Reference Materials and Controls

FDA guidance for competitive generic therapy (CGT) products states that potency assays should include appropriate assay controls and comparison to an appropriate product-specific reference material when available. Manufacturers should develop their own "in-house" reference material(s) that may include well-characterized clinical lots or other well-characterized materials prepared "in-house" or by another resource. Other reference materials include fluorescent beads/antibodies and particle size standards or reference materials for various vectors. Consultation with the

Center for Biologics Evaluation and Research (CBER) review team is strongly recommended when developing or obtaining reference materials.

8.2.4 Genome Stability

Prolonged in vitro culture poses a risk of genome alterations; therefore, it is essential to detect changes that may occur. A fundamental test for the detection of chromosomal aberrations is karyotyping. A minimum of 20 metaphases should be analyzed, and in the case that an aberration is found, 20 more metaphases should be examined. The following criteria are generally accepted: no clonal aberration in any of the studied metaphases and nonclonal aberrations in no more than 10% of studies metaphases (2 of 20 metaphases) (Barkholt et al. 2013). Karyotypes can be examined by conventional methods, such as Giemsa banding (G-banding), 4′-6-diamidino-2-phenylindole (DAPI) banding, or quinacrine (QFQ) banding. Other methods used for the analysis of the genome include fluorescence in situ hybridization (FISH), spectral karyotyping (SKY), single nucleotide polymorphism (SNP) array, and microarray-based comparative genomic hybridization (aCGH), also called molecular karyotyping (Guadix et al. 2019).

8.3 Mesenchymal Stem Cell Characterization

We would like to emphasize that characterization methods and in-process and release criteria can vary upon the intended application of MSCs for cell therapy.

8.3.1 Identity

8.3.1.1 Morphology

MSCs are large spindle-shaped cells with large nuclei containing frequent large, dark granules when cultured in vitro. They form a uniform monolayer without aggregates.

8.3.1.2 Molecular Markers

In 2006, The International Society for Cellular Therapy provided a statement on minimal criteria for defining multipotent mesenchymal stromal cells (Table 8.1, Dominici et al. 2006). These criteria are broadly accepted and are considered a standard for MSC characterization. Guadix et al. defined less strict criteria: the minimal limit for positive markers measured by flow cytometry should be 90%, and the maximal limit for negative markers should be 5% (Guadix et al. 2019).

Aside from these criteria, other markers related to stemness have been proposed (Stro-1, SSEA-4, CD271, and CD146), but their use is problematic, as their expression can vary among different sources of MSCs. MSC marker expression is also dependent on culture conditions in vitro and physiological and pathological processes in vivo (Lv et al. 2014).

Table 8.1 Minimal criteria for defining multipotent MSCs

Culture	MSCs must be plastic-adherent when maintained under standard culture conditions
High expression (≥95%)	CD105 CD73 CD90
Lack of expression (≤2%)	CD45 CD34 CD14 or CD11b CD79a or CD19 HLA-DR
In vitro differentiation	Osteoblasts, adipocytes, chondroblasts (demonstrated by staining of in vitro cell culture)

The International Society for Cellular Therapy position statement (Dominici et al. 2006)

Although positive and negative markers for MSCs have been established, there is no sole marker that is truly MSC-specific because of the heterogeneity of MSC populations and various MSC sources. Therefore, multiple markers in combination with well-characterized MSC line(s) prepared by the manufacturer or another resource as a control should be used.

8.3.1.3 Multilineage Differentiation

The multipotency of MSCs is tested by their ability to differentiate into osteoblasts, chondrocytes, and adipocytes.

Osteogenesis can be induced by factors such as ascorbic acid, dexamethasone, FGF, BMP, and others. Osteogenic differentiation can be assessed by alkaline phosphatase activity and calcium deposition staining (von Kossa staining or alizarin staining) (Torre et al. 2014; Samsonraj et al. 2017).

Chondrogenesis can be stimulated by ascorbate, insulin, transferrin, selenic acid, and TGF-β. The ability to differentiate into chondrocytes can be demonstrated by culturing MSCs in commercial chondrogenic medium, which is followed by the creation of cellular aggregates floating freely in suspension culture. These aggregates, after inclusion in paraffin, are stained by alcian blue to prove the presence of proteoglycans (Torre et al. 2014; Samsonraj et al. 2017).

Adipogenesis can be induced by factors such as dexamethasone, indomethacin, insulin, and isobutylmethylxanthine. Adipogenic differentiation can be demonstrated by oil red O staining, which identifies the presence of lipid droplets (Torre et al. 2014; Samsonraj et al. 2017).

8.3.2 Purity

Final products should be free from residual proteins or peptides used to stimulate cells, reagents/components used during manufacture, such as cytokines, growth factors, antibodies, serum, unintended cellular phenotypes (e.g., cells expressing

CD14, CD34, and CD45), and nonviable cells (Bieback et al. 2019; Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs), b.r.). Concentrations of reagents are usually negligible, but it is necessary to demonstrate, by evidence or rationale, that the final product is free from contaminants (Robb et al. 2019).

8.3.3 Viability

Viability should be quantified, and the minimal acceptable viability should be established. Generally, the accepted lower limit for viability is 70% of living cells in the final product (Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs), b.r.). However, opinions on viability limits differ. Torre et al. established a 90% viability lower limit for the final batch of MSCs (Torre et al. 2014), while Robb et al. defined the minimum as 90% viability for final fresh cell products and 70% postthaw viability for cryopreserved products (Robb et al. 2019).

In the case that cells are cryopreserved, assays for viability, cell identity, and functionality should be performed and the results compared to values obtained before freezing (Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs), b.r.). Cryopreservation-induced delayed-onset cell death may occur after thawing, and it is advisable to distinguish early apoptotic MSCs by annexin V binding to phosphatidylserine, a marker of early apoptosis (Koopman et al. 1994; Robb et al. 2019). Viability is usually measured by the trypan blue exclusion test with the use of microscopy or by propidium iodide or 7-aminoactinomycin-D (7-AAD) exclusion with the use of flow cytometry (Torre et al. 2014). A combination of propidium iodide and annexin V seems to be a sufficient strategy to distinguish and quantify necrotic and early apoptotic cells by flow cytometry (Robb et al. 2019).

Alternatively, there are a number of automated cell counters that are suitable for use in manufacturing under current good manufacturing practices (cGMPs). Usually, they work on the principle of the trypan blue exclusion test and offer high precision and sample throughput but are quite expensive and vary in their ability to recognize cell debris or cell clusters (Robb et al. 2019).

8.3.4 Proliferation

Growth is traditionally measured according to the number of passages through culture. It is recommended that MSC cultures should not exceed three to seven passages (Guadix et al. 2019); other authors recommend four passages (Torre et al. 2014).

However, population doubling can better reflect the replicative aging of MSCs. The result can be presented as the cumulative population doubling (CPD) according to the equation log N/log 2, where N is the final number of confluent cells divided by the initial number of cells seeded. The CPD determines the number of divisions that a cell has undergone during a process (Guadix et al. 2019). However, the recommended number of CPDs has not yet been determined.

8.3.5 Potency

Potency assays should represent the mechanism of action. However, MSCs have complex and not fully characterized mechanisms of action. Nevertheless, maximal effort should be made to determine which attributes are the most relevant for the intended therapeutic purpose.

8.3.5.1 Immunoactivity

Responder cells used in assays should correspond to the target cells in the therapeutic setting. Immunomodulatory potency assays usually involve $CD3^+$ T cells, $CD4^+$ T cells, or peripheral blood mononuclear cells (PBMCs). PBMCs contain a mixture of lymphomyeloid cells that varies among humans and brings in more variability; therefore, the use of purified immune effector cells (e.g., T cells or their subtypes) that are involved in the pathogenesis of the treated disease is more informative. T cell subtypes can be examined with the use of flow cytometry, although it is good to take into account that different cell types may have overlapping phenotypes (de Wolf et al. 2017).

Types of assays that measure the immunomodulatory capabilities of MSCs include the following (de Wolf et al. 2017):

- **MSC activation assays** measure important surface or secreted factors involved in MSC functionality. MSCs (responder cells) react to proinflammatory cytokines (e.g., IFN-γ, TNF-α, and IL-1β) by the expression of intracellular and surface proteins (CD markers, such as CD200, and cytokine receptors, such as TNF-αR2 and IFN-γR1) and soluble factors (e.g., TGF-β, TNF-αR1, and PGE2).
- **Immune cell inhibition assays** measure the effect of MSCs on an important cell type (e.g., T cells). Responder cells (e.g., PBMCs, T cells, and murine antigen-specific T cell clones) react to the addition of MSCs by exhibiting (inhibited) cytokine production (e.g., IFN-γ, TNF-α, and IL-10) or (inhibited) surface marker expression (e.g., cytokine or chemokine receptors or CD markers) or (inhibited) proliferation.
- **Immune cell migration assays** measure the effect of MSCs on an important cell type (e.g., T cells, specifically their effector function). Responder cells (immune cells, e.g., T cells) react to added chemokines (e.g., CXCL10) by exhibiting (inhibited) chemotaxis after coculture with MSCs.
- **Suppressor cell induction assays** measure the effect of MSCs on an important cell type (e.g., T cells) in terms of augmenting their suppressive function.

Responder cells (immune cells, e.g., T cells) react to the addition of MSCs by the induction of regulatory T cells (Tregs) (e.g., through cell surface marker expression, cytokine production, and suppressive function).

8.3.5.2 Angiogenic Assays

In addition to their immunomodulatory and anti-inflammatory potency, MSCs have been shown to support angiogenesis. Angiogenic assays measure the amount of secreted angiogenic factors and demonstrate the potency of MSCs to support endothelial cells. Key proangiogenic factors include VEGF, CXCL5, IL-8, bFGF, PDGF, angiopoietin-1, and IL-6, and to determine their levels in medium, ELISA is usually used (Lehman et al. 2012; Tao et al. 2016).

An endothelial cell tube formation assay is a functional in vitro assay that is used to prove that endothelial cells, usually human umbilical vein endothelial cells (HUVECs), divide and migrate to form tubes in response to various compounds (in this case angiogenic factors). Conditioned medium from an MSC culture is used to promote HUVEC tube formation. Transwell migration assays can be used to prove that endothelial cells migrate through a semipermeable membrane in response to angiogenic factors.

The combination of secreted angiogenic factor measurement and endothelial cell tube formation assays was used to prove the potency of Multistem®, a large-scale expanded adherent multipotent progenitor cell population (MAPC) (Lehman et al. 2012). More recently, a combination of angiogenic factor measurement and functional assays was used for the characterization of Stempeucel®, an allogeneic pooled human bone marrow-derived mesenchymal stromal cell (phBMMSC) product (Thej et al. 2017).

8.4 Human Pluripotent Stem Cell Characterization

There are different types of hPSCs based on the source material and derivation method (such as human embryonic stem cells (hESCs), human induced pluripotent stem cells (hiPSCs), and human parthenogenetic embryonic stem cells (hpESCs)); however, these types of hPSCs are defined by the same properties and therefore should meet the same specifications. Nevertheless, genome testing might vary for cells from different sources, such as the evaluation of vector clearance in hiPSCs (Vanneaux 2019) or more thorough evaluation of mutations in hpESCs, as their homozygosity might further increase the inherent tumorigenic potential of hPSCs, as the loss of heterozygosity is a significant factor of carcinogenesis (Daughtry and Mitalipov 2014).

While the use of hPSCs themselves for therapy has been proposed (Xiang et al. 2019; Lu et al. 2019), the concept was abandoned due to their high tumorigenic potential. Instead, approaches using differentiated cells derived from hPSCs are now widely adopted. Considering the complexity of obtaining high-quality hPSC lines, there is great benefit in creating clinical-grade hPSC lines that can then be used in multiple applications. This places hPSCs in the role of a product for further

Table 8.2 Proposed mandatory criteria for the characterization of hPSCs for clinical usage

Characteristics	Methods	Release criteria
Differentiation	Embryoid bodies formation	Detection of endoderm, mesoderm, and ectoderm
Genetic stability	Karyotype analysis	Normal diploid (>20 metaphases)
Identity	Short tandem repeat (STR) analysis	hiPSCs: identical with donor and over time; hESCs: identical over time
Vector clearance[a]	PCR	Negative
Morphology	Photography	Normal morphology[b]
Pluripotency	Flow cytometry	>70% for at least 2 surface and 2 intracellular markers; <10% for SSEA1
Viability	Viability	>60%

[a]Only hiPSCs when applicable
[b]Colony: flat, clear borders with well-defined edges; cells: small, round with large nuclei and prominent nucleoli (Rehakova et al. 2020)

manufacturing instead of a final cell therapy product. Thorough characterization greatly enhances their applicability in derived cell therapy and facilitates the process of bringing such hPSCs into practice. A set of quality control tests including release criteria was previously suggested by our team (Table 8.2, Rehakova et al. 2020).

8.4.1 Identity

8.4.1.1 Morphology

The standard method of culture is adherent, albeit on synthetic matrices or a layer of feeder cells. Typically, colonies should be uniform and have sharp edges, and cells should be small with visible nuclei and little cytoplasm, but the exact morphology can vary among different culture conditions (Amit a Itskovitz-Eldor 2012; Chen et al. 2017; Orozco-Fuentes et al. 2019; Rodin et al. 2014).

8.4.1.2 Molecular Markers

Undifferentiated hPSCs express various markers, such as Oct3/4, Sox2, Nanog, SSEA3, SSEA4, Tra-1-60, and Tra-1-81, that can be assessed by immunocytochemistry or flow cytometry. Published protocols differ in the specific markers used, but at least two markers should be used. Positivity greater than 50% (Tannenbaum et al. 2012) or 70% (Baghbaderani et al. 2015) has been used for cGMP-grade hPSCs, while 80% positivity has been suggested (Sullivan et al. 2020). Flow cytometry is used for quantification, especially for surface markers. Negative markers can also be used, such as SSEA1 (De Sousa et al. 2016).

8.4.1.3 Multilineage Differentiation

The potential for differentiation is crucial for the application of hPSCs; therefore, the ability to differentiate into cells of all three germ layers must be demonstrated. Spontaneous differentiation is mostly used, but it can be replaced with directed differentiation if protocols for all three germ layers are implemented (Shafa et al. 2018; Sullivan et al. 2018).

Spontaneous differentiation is based either on embryoid body (EB) formation in vitro or on teratoma formation in mice in vivo. There are limited data showing the benefits of teratoma assays, while evident disadvantages include low comparability (Andrews et al. 2015), ethical concerns, and the long duration of the test (several weeks); therefore, it is not required (Sullivan et al. 2020).

For the reasons stated above, EB formation is usually used to prove the differential potential of hPSCs. EBs can be subsequently plated on coated coverslips or sectioned and then analyzed for the expression of differentiation markers, such as β-III tubulin for ectoderm, Sox17 or AFP for endoderm, and α-actin for mesoderm, mostly by immunocytochemistry (Baghbaderani et al. 2015; Tannenbaum et al. 2012; Ye et al. 2017). Alternatively, EBs can be analyzed by RT-PCR (Sullivan et al. 2018).

8.4.2 Viability

The dye exclusion test or flow cytometry can be used to assess viability (Sullivan et al. 2018). Postthaw survival can be measured, which provides more information about freezing tolerance but can be more variable (Andrews et al. 2015). Viability can also be measured after seeding and culturing cells for a few days, which better illustrates the ability of cells to attach and start growing in vitro. Both approaches are combined by some manufacturers (Baghbaderani et al. 2015).

8.4.3 Proliferation

Although hPSCs are highly proliferative and can be kept in culture for many passages/doublings, prolonged culture increases the risk of genome alteration. As such, the passage number (or even better, the number of CPDs) should be monitored, and cells from low-passage cultures should be used for further applications. Examples of passage numbers used as a working cell bank in published works are passage 10 (Main et al. 2020), passages 13 and 14 (Baghbaderani et al. 2015), or passages 16–33 (Tannenbaum et al. 2012).

8.4.4 Potency

As hPSCs are not intended for direct application in patients, potency tests are performed on specific subsequent products and depend on the application. An

example can be visual performance rescue in rats by hiPSC-derived retinal pigment epithelial cells (Zhang et al. 2021) or behavioral recovery in a nonhuman primate Parkinson's disease model after the transplantation of hpESC-derived NSCs (Gonzalez et al. 2016).

8.5 Neural Stem Cell Characterization

While specific guidelines for NSC characterization are lacking to date, there is a consensus about necessary testing in research areas and in published cGMP protocols and clinical trials.

The traditional source of human NSCs is embryonic tissue from donors after miscarriage (Wenning et al. 1997), which presents multiple hurdles, such as ethical concerns and rarity of the starting material (Irion et al. 2017). Other sources that help to overcome some of these problems are transdifferentiated somatic cells (Boese et al. 2018) or cells differentiated from hPSCs, including hiPSCs, hESCs, and hpESCs (Isaev et al. 2011; Tang et al. 2017).

Regardless of their origin, NSCs need to meet criteria ensuring their identification and safety. Additional testing might be necessary according to the complexity of the manufacturing process. For example, in the case of NSCs derived from hiPSCs, vector clearance should be tested by PCR. Interestingly, the NSC functionality test, which demonstrates their ability to differentiate into neurons, oligodendrocytes, and glia upon the removal of growth factors FGF2 and EGF, also indicates their safety (Gelati et al. 2013). As the abovementioned growth factors are absent in the implantation site, NSCs undergo differentiation and lose their proliferative capacity, reducing the risk of tumor formation.

8.5.1 Identity

8.5.1.1 Morphology

While morphology is a useful indicator that can be assessed continuously by trained personnel without influencing the culture, it only gives an overview of the culture state. NSC morphology depends on culture conditions (Conti et al. 2005). In adherent culture, NSCs form a combination of small bipolar cells and flat apolar cells (Sun et al. 2008). An important morphological factor is stability over time, as changes in morphology are an indicator of differentiation, which can be caused by suboptimal conditions or limited self-renewal ability (Rosati et al. 2018).

8.5.1.2 Molecular Markers

An essential marker of NSCs is nestin (Glass et al. 2012; Tirughana et al. 2018), but additional markers, such as Sox2, DACH1, PLZF, and Pax6, are used (Gonzalez et al. 2016; Vitillo et al. 2020). Immunocytochemistry or flow cytometry are the common methods of choice, but for quantification and higher comparability, RT-PCR might be advisable (Garitaonandia et al. 2016; Thomas et al. 2009). The

Table 8.3 Identification criteria for NSCs

High expression (≥93%)	Nestin
hPSC-derived NSCs: Lack of expression (0%)	Oct3/4 SSEA4
In vitro differentiation	Neurons (β-III tubulin) Astrocytes (GFAP) Oligodendrocytes (O1, NG2, and GALC)

reported minimal limits for positive markers are 90% (Gonzalez et al. 2016) and 93% (Thomas et al. 2009). Some include negative markers, such as differentiated neural cell markers or even endothelial and muscle markers (Glass et al. 2012).

In the case of hPSC-derived NSCs, the presence of residual undifferentiated cells should be assessed by hPSC markers, such as SSEA4 or Oct3/4, because of the tumorigenic potential of hPSCs (Gonzalez et al. 2016).

8.5.1.3 Multilineage Differentiation

Spontaneous differentiation is used as a crucial potency assay. Depletion of the growth factors EGF and FGF2 should lead to the differentiation of NSCs into a mixture of cell types, ensuring both functionality and safety. Differentiation is tested mostly by immunocytochemistry for the detection of expressed markers (Table 8.3). Widely used markers are β-III tubulin (also called Tuj1) for neurons, GFAP for astrocytes, and O1, NG2, and GALC for oligodendrocytes (Glass et al. 2012; Guo et al. 2010; Mazzini et al. 2015; Rosati et al. 2018). Depending on the intended application, directed differentiation might be required to ensure that a specific cell line has the ability to generate the intended cell type, such as motoneurons (Guo et al. 2010).

8.5.2 Viability

Postthaw viability is a good indicator of cell line performance. At least 60% or 70% postthaw viability is usually acceptable (Glass et al. 2012; Rosati et al. 2018).

8.5.3 Proliferation

As growth is one of the main features of stem cells, stable exponential growth over time in culture not only shows the quality of NSCs but also indicates the feasibility of the manufacturing process (Gelati et al. 2013; Pollock et al. 2006; Thomas et al. 2009). To our knowledge, no data are available on the recommended maximal time in culture for NSCs, contrary to other stem cell types.

8.5.4 Potency

The exact mode of action of NSCs is still being discussed, as not only direct tissue replacement but also modulatory properties play a certain role (Baker et al. 2019). Prior to human transplantation, the ability to integrate into host tissue, migrate, and differentiate should be verified in animal models, such as mouse models (Gelati et al. 2013; Rosati et al. 2018). If an appropriate animal model for the disease is available, it should be used to evaluate the positive impact of NSC transplantation on a given condition (Glass et al. 2012), such as delayed amyotrophic lateral sclerosis (ALS) onset and progression in the SOD1 G93A rat model (Xu et al. 2006) or improving behavior in a nonhuman primate model for Parkinson's disease (Gonzalez et al. 2016).

8.6 Hematopoietic Stem Cell Characterization

According to the FDA criteria, relevant biological characteristics of cells generally include the cellular properties that contribute to the cell function or functions in the donor. Relevant biological characteristics of hematopoietic stem/progenitor cells (HSCs/HPCs) include the ability to repopulate the bone marrow via self-renewal and differentiating along myeloid and lymphoid cell lines (Regulatory considerations for human cells, tissues, and cellular and tissue-based products: minimal manipulation and homologous use; Guidance for Industry and Food and Drug Administration Staff, b.r.).

HSC transplantation (HSCT) is a common type of transplantation in which multipotent HSCs (a component of bone marrow, peripheral blood, or umbilical cord blood (UCB)) are transplanted. Usually, HLA typing is performed to find donor-recipient matches, and the number of HSCs is established via the detection of $CD34^+$ cells (Khaddour et al. 2021).

8.6.1 Identity

8.6.1.1 Morphology

HSCs have a lymphoblastoid morphology that is very similar to other types of blood cells; therefore, molecular marker detection is necessary to identify HSCs with certainty (Mayani 2019).

8.6.1.2 Molecular Markers

The principal marker of HSCs is CD34. The Foundation for the Accreditation of Cellular Therapy and the Joint Accreditation Committee of ISCT-EBMT (FACT/JACIE) International Standards state that an assay measuring viable $CD34^+$ cells should be used for HPC products intended to restore hematopoiesis (FACT-JACIE International Standards for Hematopoietic Cellular Therapy: Collection, Processing, and Administration, seventh edition 7.0 2018). It was shown that the number of

$CD34^+$ cells has a significant impact on the outcome of HSCT. A threshold of 1×10^6 $CD34^+$ cells per kg has been proposed for both bone marrow (Singhal et al. 2000) and peripheral blood HSCs (Gandhi et al. 1999) transplantation. In contrast, a lower threshold of 1.7×10^5 $CD34^+$ cells per kg was proposed for transplantation of UCB (J. E. Wagner et al. 2002).

8.6.2 HLA Typing

In the case of allogenic HSCT, HLA typing of both donors and recipients must be performed to ensure histocompatibility, as differences in HLA systems can lead to transplant rejection and/or graft-versus-host disease (Guidance for Human Somatic Cell Therapy and Gene Therapy 1998; Khaddour et al. 2021). High-resolution molecular methods should be performed by an accredited laboratory (FACT-JACIE International Standards for Hematopoietic Cellular Therapy: Collection, Processing, and Administration, seventh edition 7.0 2018). HLA-A, B, DRB1, and HLA-C typing must be included (HLA-C typing can be omitted if the donor and the recipient are siblings). The level of similarity in the stated HLA types has a large impact on posttransplant survival (Lee et al. 2007).

8.6.3 Viability

According to the FACT/JACIE International Standards (7th edition), a total nucleated cell count and viability measurement should be performed (FACT-JACIE International Standards for Hematopoietic Cellular Therapy: Collection, Processing, and Administration, seventh edition 7.0 2018). Generally, viability over 70% is accepted for cellular products by the FDA (Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs), b.r.). It was shown that viability over 75% in cord blood units improves the chances of engraftment (Scaradavou et al. 2010).

8.6.4 Colony-Forming Cell Assays

HSCs differentiate into hematopoietic progenitor cells (colony-forming cells, CFCs) that can be detected with CFC assays. This clonogenic assay is able to detect and quantify various progenitors, such as colony-forming unit erythrocytic (CFU-E), blast-forming unit erythrocytic (BFU-E), colony-forming unit granulocytic and monocytic (CFU-GM), colony-forming unit granulocytic, erythrocytic, monocytic and megakaryocytic (CFU-GEMM), and highly proliferative progenitors (HPPs) (Morrison et al. 1997; Wognum et al. 2013). It is important to emphasize that CFC assays are not able to detect HSCs but can be a useful tool for detecting hematopoietic progenitor cells for specific clinical applications. It has been shown

that a higher CFC count is associated with better engraftment after cord blood transplantation (Castillo et al. 2015; Migliaccio et al. 2000).

Acknowledgments This work was supported by the European Regional Development Funds—Projects ENOCH (No. CZ.02.1.01/0.0/0.0/16_019/0000868) and MAGNET (No. CZ.02.1.01/0.0/0.0/15_003/0000492). Supported by Ministry of Health of the Czech Republic, grant nr. NV18-08-00412. All rights reserved. Supported by the European Regional Development Fund—Project CZECRIN_4 PACIENTY (No. CZ.02.1.01/0.0/0.0/16_013/0001826). Tereza Souralova is a Brno Ph.D. Talent Scholarship holder and is funded by the Brno City Municipality.

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9 Stem Cell Safety and Sterility Testing: A Promising Approach in Regenerative Medicine

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Abstract

Stem cells and their derivatives are considered as most sought-after and well-received therapeutic agents in the field of regenerative medicines. As we know, stem cells are naïve cells which have the ability to differentiate into multiple lineages, thus making them an ideal candidate for cell-based therapeutics. In the field of clinical research especially in transplantation studies, stem cells play a very crucial and specific role. The transplantation experiments/studies demand heavy use of immunosuppressive agents in the patient for appropriate grafting and acclimatization of the tissue into the recipient's body. If the transplanted cells/tissue/cell-derived product is contaminated, it can lead to the deterioration of the graft. Like other cells, stem cells also have issues regarding safety and sterility. If the sterility of stem cells is compromised at any stage of therapeutic application, it renders the entire process unfit to proceed. As the field of cell-based therapies is expanding with new laboratories and manufacturers budding every

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F. A. Khan (ed.), *Stem Cell Production*,
https://doi.org/10.1007/978-981-16-7589-8_9

day, it has become important that such industries and labs should have a thorough comprehension of the regulatory guidelines that govern the microbiological and non-microbiological testing of stem cell-based therapeutics. In this chapter, we are going to discuss the importance of stem cells as therapeutic agents with an overview of how to ensure safety, sterility, and efficacy of such therapies. We have attempted to highlight the importance of numerous regulations such as that of the Food and Drug Administration (FDA) and multifaceted areas like Good Manufacturing Practices (GMPs) and several other regulating bodies which have provided us with internationally acceptable laws to ensure the safe and efficient usage of stem cells as therapeutics.

Keywords

Stem cells · FDA guidelines · Stem cells safety · Stem cells sterility

9.1 Introduction

9.1.1 Stem Cells

The naive cells found in the embryonic, juvenile, and adult phases of life and can multiply into different types of differentiated cells, which further serve as the monads for tissues and organs, and are termed stem cells (SCs). The specific qualities that characterize a cell as stem cells are its potency, self-renewal, and clonality. These characteristics also serve as a mode of differentiation between different kinds of stem cells (Mummery et al. 2021). SCs are classified into broad categories—totipotent, pluripotent, multipotent, and unipotent according to their potential to spawn into a variety of cell lineages (da Silva Barcelos et al. 2021). Totipotent stem cells are the rudimentary and under-developed type, which are capable of forming embryo as well as the extra-embryonic layers (Zakrzewski et al. 2019). Post-fertilization of sperm and egg, the embryo undergoes subsequent divisions and on reaching the blastocyst stage it loses its totipotency and acquires pluripotency and gains the ability to differentiate into three embryonic germ layers (ectoderm, mesoderm, and endoderm) and the cells derived from this phase is known as embryonic stem cells (ESCs). ESCs are procured by destructing the inner cell mass resulting in damage to the embryo. The cells further continue to divide and attain multipotent stage, thus becoming adult stem cells which can only variegate into restricted cell types of specific tissues. Inside the organism, i.e., in-vivo, these adult stem cells exist in the fallow state, but are metabolically active, in tissues like bone marrow, skin, cornea, etc. (Clevers and Watt 2018). Multipotent cells give rise to specific cell lineages like hematopoietic stem cell (HSC), which further differentiates into numerous other kinds of blood cells (Chatterjee et al. 2021). The stem cells and their fate are represented in Fig. 9.1. A stem cell is oligopotent when it can self-renew and form two or more lineages within a specific tissue. Further, there are unipotent stem cells that are characterized by the least

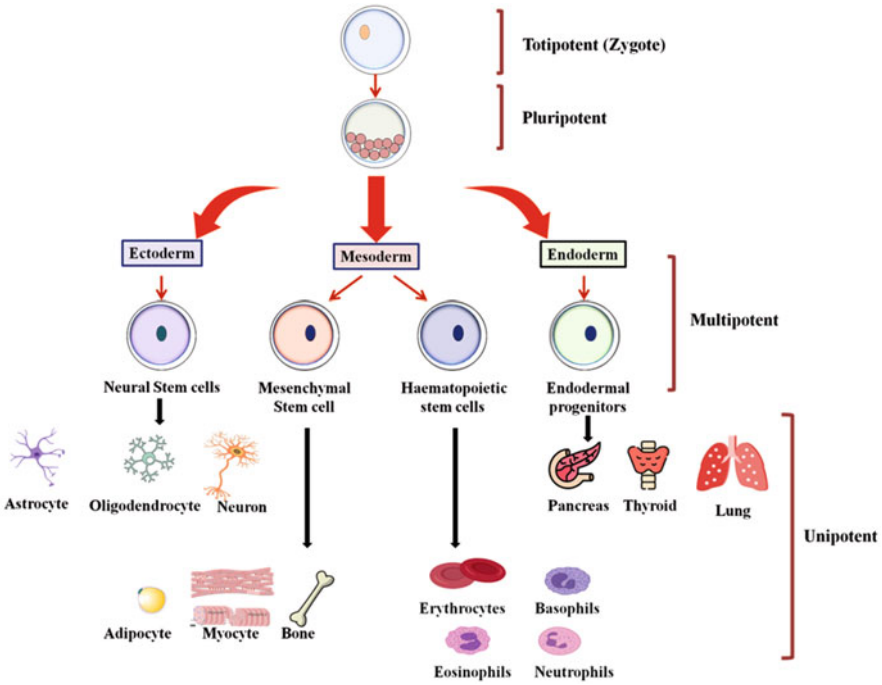

Fig. 9.1 The diagrammatic representation of stem cell classifications: the fate starts from the totipotent cells which differentiate into multipotent cells. The naïve cells further differentiate into three layers, i.e., ectoderm, mesoderm, and ectoderm. As per the specificity, each layer reaches to its destination. Here the cells are known as multipotent. Only some specific lineages can be differentiated from the multipotent cells and finally converts into unipotent cells. Therefore, stem cells here are converted into different lineages like neurons, blood cells, bone marrow, lungs, glands, etc.

differentiation potential and have the ability to divide repeatedly, thus making them an ideal candidate for therapeutic usage (Kolios and Moodley 2013). Various transcription factors such as OCT4, Nanog, KLF4, and SOX2, etc. regulate the potential of stemness and differentiation of any stem cell (Fatma and Siddique 2021). Restoration of these endogenous transcription factors in adult cells through genetic modulations, causing these to depict stem cell-like functions yield induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006; Yu et al. 2007). In the field of regenerative medicine, stem cell classification can be as follows: tissue-specific progenitor stem cells (TSPSCs), embryonic stem cells (ESCs), umbilical cord stem cells (UCSCs), mesenchymal stem cells (MSCs), iPSCs, and bone marrow stem cells (BMSCs) (Seetharaman et al. 2019). At the site of application, stem cells must endure, multiply and transdifferentiate within the host system, for successful outputs of the therapy.

9.2 Stem Cells as Therapeutic Agents

In the field of regenerative medicine, stem cell classification can be as follows based on their respective uses: embryonic stem cells, tissue-specific progenitor stem cells (TSPSCs), mesenchymal stem cells (MSCs), umbilical cord stem cells (UCSC), bone marrow stem cells (BMSCs), and iPSCs (Seetharaman et al. 2019). At the site of application, stem cells must endure, multiply and transdifferentiate within the host system, for successful outputs of the therapy (Mahla 2016).

Regenerative medicine is the future of health care sector as it offers treatment for some incurable diseases. It opens a window towards a wide array of treatments that are transformational and assuredly analeptic towards a few of the lethal morbidities (Takashima et al. 2021) Cell-based therapies are a recent approach towards regenerative medicine and are contemplated as one among many propitious tools for the treatment of remediless pathologies like Parkinson disease, Alzheimer disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), etc. (Aly 2020). Stem cell-based therapies have become more and more popular among clinicians and researchers due to advancements in methods of safe usage of SC and their derived products (Phelps et al. 2018). Stem cells provide a vast array of applications in the past and continue to do so. Stem cell-based therapies have exponentially advanced since the usage and importance of stem cells are discovered in clinical research (Lim 2021). Some major applications include treatment of tissue regeneration therapies like blood-related diseases, cardiovascular diseases, brain diseases, cell deficiency therapy, etc. Despite the many obstacles, the possibilities of stem cell applications have grown with each research study. Clinical therapies based on direct or indirect use of stem cells seem very progressing in providing cures for numerous incurable disorders (Badyra et al. 2020). Stem cell-based therapies offer contemporary ideas for immunotherapies and regenerative therapies. The complexity of preparation and biological sources of such therapeutics calls for stringent and full proof quality control criteria to avoid any sort of post-application complications (Joshi and Enver 2003).

For instance, microbial contamination of these therapeutics may cause severe complications in patients which is both ethically and scientifically unacceptable. Due to such fixes, the application of stem cells is restricted in clinical applications even though a lot of literature is available, which suggests that SCs can expedite various repair mechanisms and further promote clinical applicapabilities (Howell et al. 2015; Kwon et al. 2018). The importance of stem cells in clinical research is presented in Fig. 9.2.

9.3 Types of Stem Cells for Tissue Regeneration

Embryonic stem cells, discovered by JA Thomson in 1998, are derived from inner cell mass of gratsula and have pluripotent potential because of which it can give rise to more than 200 cell kinds. Two decades ago, he stated that "*these cell lines can be put to human use in the field of transplantation, regenerative medicine, etc.*"

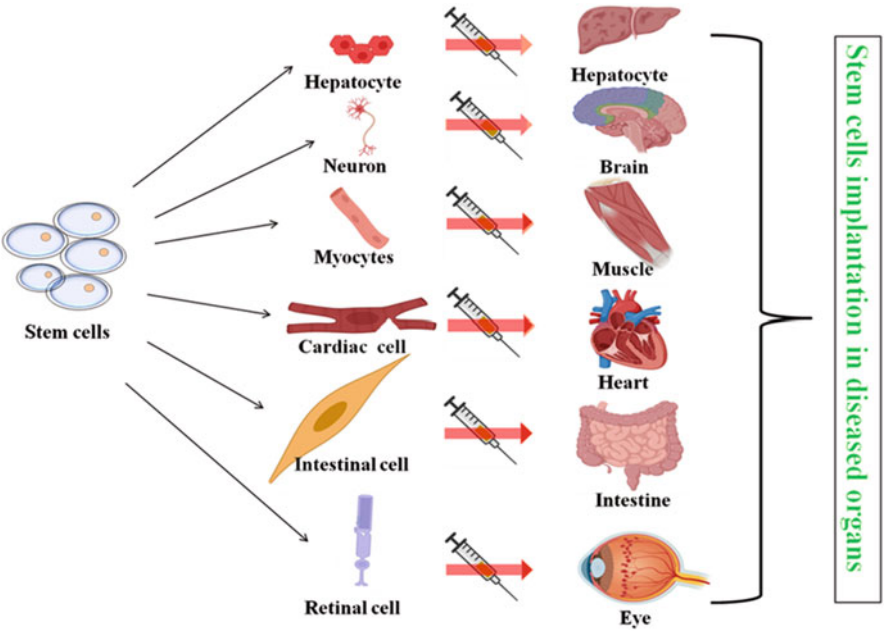

Fig. 9.2 Use of stem cells as therapeutic agent in clinical research: The property of plasticity and differentiation capacity of stem cells leads its usage in the field of clinical research. The stem cells can be converted into different types of cells, organelles and further into the organs (hepatocytes, brain, muscles, heart, intestine, and eye) when transplanted to the specific site of the cells (Hogan et al. 2014)

(Thomson et al. 1998). This has become a reality today that ESCs have been exploited to a great extent for translational uses such as diabetes, neuronal injuries, neurodegenerative disorders, cancer, muscular degeneration, therapies for macular degeneration, etc. (Yamanaka 2020; Petrus-Reurer et al. 2020). In patients with spinal cord injuries, ESC-based therapies have shown improvement in movement, balance, and sensation, where ESCs reside at the injury site and improve the condition (Shroff and Gupta 2015). In Age-related Macular Degeneration (AMD), application of ESC-based therapies have shown significant improvements (Khateb et al. 2021). Various research groups across the globe have demonstrated this by generating Retinal Pigment Epithelial (RPE) from ESCs which is very similar to the natural one both morphologically and functionally (Coco-Martin et al. 2021). The disintegration of RPE is the cause of AMD. Transplantation of ESCs derived from embryos which have been administered with *COCO* gene, develop into dedicated cone cells, which help in recovery from AMD (Choudhary et al. 2017; Li et al. 2019). ESCs can differentiate into cardiac cells as well. A study conducted by Menasche and group showed improvement within 3 months in a patient with heart failure after transplantation of ESC-derived cardiac progenitor cells (Menasche et al. 2018). ESC-derived cardiac cells like cardiac progenitor cells and cardiomyocytes can repair damaged heart tissues, improve cardiac rhythms, improve mechanical

functioning of the heart, etc. and yet do not generate any tumorigenic effect in most of the cases (Nakamura and Stempien-Otero 2020). Multiple studies have eventually been reported for the role of ESC-based therapies for regenerative medicine in fields like intrauterine adhesions, Parkinson's disease, amyotrophic lateral sclerosis, etc. (Yamanaka 2020) and it still has a lot of scope to be explored further. Umbilical cord blood stem cells are isolated from the usually considered as discard umbilical cord post child birth (Jahan et al. 2017). The umbilical cord is a rich source of hematopoietic stem cells and mesenchymal stem cells, both of which have huge therapeutic potential. Multiple tissues of the body such as adipose tissue, tonsils, umbilical cord, dental pulp, Wharton's jelly, and bone marrow are major sources of MSCs (Kashyap et al. 2019). MSCs exhibit fibroblast-like morphology and are positive for surface markers CD105, CD 90, and CD 73 (Lv et al. 2014; Jahan et al. 2017). MSCs can transdifferentiate into multiple cellular lineages like chondrocytes, adipocytes, osteocytes, neurons, etc. (Pittenger et al. 2019). MSCs are angiogenic and immunomodulatory capabilities and have been shown to work with cells of immune system and generate enhanced immune responses (Davies et al. 2017). They are also known to enhance angiogenesis (Guo et al. 2020). Due to these multiple advantages, MSCs have been considered an excellent candidate in regenerative medicine for neurodegenerative disorders, cardiovascular diseases like myocardial infractions and stroke, nephrological diseases like acute kidney injury (Togel and Westenfelder 2010), etc. MSC-based cell therapies are gaining popularity worldwide due to advancement in research methods (Alzahrani et al. 2020) but is still under question because of concrete evidence (Sahu et al. 2021).

The technological advancements in the methods of obtaining Induced pluripotent stem cells from various adult cells have enabled us to use these cells in regenerative applications (Liu et al. 2020). They are being used to create organoid models like that of kidney/brain/liver, which are very similar in function to that of natural systems (Pasteuning-Vuhman et al. 2020). The iPSCs are being used to create disease models for evaluation of drug efficacy, toxicity testing, disease progression, and many other applications (Smith et al. 2017). Before iPSCs were discovered, ESCs were the only source of pluripotent cells, which had their ethical concerns (Liu et al. 2020). But as iPSCs were discovered, they opened new opportunities and horizons in the field of regenerative medicine or cell-based therapies. Drug screening, toxicological investigations, and disease modeling are some of the additional biological uses of iPSCs (Shi et al. 2017). Procurement of iPSCs from various sources and their differentiation into desired cell lines has initiated a stir in the field of regenerative medicine and cell-based therapy human trials with the first reported clinical trial for AMD in which retinal pigment epithelial cells were developed from iPSCs (Mandai et al. 2017). The first clinical trial for Parkinson's disease was done at the Kyoto University of Japan where they transplanted dopaminergic neurons from iPSC in patients (Doi et al. 2020). Clinical studies for cancer immunotherapy to treat advanced solid tumors are also using iPSC-derived cells (Rivera et al. 2020). A clinical experiment for heart disorders employing iPSC-derived cardiomyocytes was just authorized (Cyranoski 2018). The biggest

advantage with iPSCs is that they can be patient-specific and thus reducing chances of graft rejection (Moradi et al. 2019) and also reduce ethical issues associated with ESCs.

The advancement in the field of stem cells offers great potential; however, there are still multiple challenges that we as researchers face in this field (Liu et al. 2020). It can be predicted that probably the coming decade will bring new advancements in this area and will enable us to create stem cell-based therapies with much greater efficacy. In the next section of this chapter, we will discuss about the challenges in stem cell therapies and the methods to overcome the hurdles. Therefore, we are inserting detailed information regarding stem cell safety and sterility in the coming part of the chapter.

9.4 Impediments and Ethical Concerns with Stem Cell-Based Therapies: How to Perpetuate Stem Cells Safety and Sterility

Ever since the dawn of the era of stem cell-based therapies, the most prevalent and persistent concern has been ethical considerations regarding the use of stem cells. ESCs, a great source for therapies, are surrounded by ethical controversies the most as they are acquired after the destruction of human embryos. In this direction iPSCs have come forward as a great alternative, nevertheless, these too have other ethical concerns (Volarevic et al. 2018). For instance, their unlimited regenerative potential is questioned when it comes to human use. Certain studies have shown that transplantation of iPSCs can induce tumor formations making them unfit for use in regenerative medicine. This calls for the development of internationally acclaimed and accepted protocols for the intended clinical use of iPSCs (Uhlin 2019).

9.5 Stem Cells Safety

Maintaining original cell line characteristics during cell culture expansion is the most crucial step for any cell-based therapy (Mathen and Dsouza 2021). Due to prolonged cell culture, the original functions of a cell might change or disappear resulting in varying or non-significant results (Lukomska et al. 2019). The reason for instability can be long-term culture, stress during passaging, and the nature and condition of the original cell line, for instance, MSCs or iPSCs can differentiate anonymously while they are being propagated under lab conditions due to various reasons like genetic drift and stress signaling (Vaskova et al. 2013). Cell lines can also get cross-contaminated with other cell lines if cultured carelessly in the same environment as HeLa. HeLa cells have uncontrolled and unlimited proliferative capacity. It originated from the cervical cancer tissue of an American woman in 1952 (Gey 1952). HeLa cells have an abnormal proliferation rate and can adapt to various growth conditions and culture media, like DMEM, MEM, RMPI1640, DMEM/F12, etc. and can be very easily cultured, thus making them one of the most important

sources of cell cross-contamination (Lin et al. 2019). Cell cultures are very often contaminated with bacteria, yeast, or *Mycoplasma* causing loss of cell lines entirely. Such conditions are not 100% inevitable but can be avoided to greater extents with some precautions and ethical and scientific misconducts can be avoided (Nelson-Rees et al. 1981; Stacey et al. 2013). In this part, we will discuss detailed information of contaminants which are a key cause to inhibit the safety of the cells.

9.5.1 Types and Sources of Contamination and Their Characteristics

Culturing animal-derived stem cells requires culture mediums that are highly rich in nutrients for appropriate growth of cells for therapeutic applications (D'Esposito et al. 2020). Such nutrient-rich mediums are a major attraction for microbes like bacteria, viruses, and fungi, thus causing severe contaminations which compromise with the quality of the therapy (Langdon 2004). In a study conducted at the University of Minnesota, 32 stem cell lines were analyzed for contaminants. Gram-positive cocci and *Mycoplasma* species were detected in 19 of the samples with more gram-negative rods and gram-positive rods (Catalina et al. 2007; Cobo et al. 2007). Some studies have also reported that patients were transfused with contaminated products causing some fatal accidents (Klein et al. 2006. Protozoans are the rarest contaminants in cell cultures. Culture media is rich in glucose, salts, micronutrients, etc. This serves as perfect growth conditions for microbes like *Mycoplasma*, *Escherichia coli*, *Saccharomyces cerevisiae*, etc. Good culture practices if not followed will lead to the growth of such microbes (dos Santos et al. 2021). The serums used in in vitro cultures are a major source of nutrients to microbes and can be transferred to media via the use of non-sterile culture wares and reagents or physical contact with the surface or skin. Non-living contaminants like molecules generated by the breakdown of media components like amino acids such as tryptophan, riboflavin, or buffers like HEPES under the influence of light contribute towards the degradation of media (Frigault et al. 2009). Fetal bovine serums or other kinds of serums are a major source of *Mycoplasma* and bacterial infections (Drexler and Uphoff 2002). *Mycoplasma* contamination is one of the hardest to eradicate because in most cases it is unnoticeable until several passages (Sharma et al. 2020). It can hamper the growth and morphology of cells. A set of experiments conducted with fibroblasts and keratinocytes and it was noticed 32.35% of cell cultures were contaminated, in which 17.65% accounted for *Mycoplasma*, 8.85% fungi, and 5.8% bacteria. Among fungi contaminations, *Aspergillus* is the most common one and from lab members, *M. orale* is most common. *Bacillus*, *Enterococcus* and *Staphylococcus* are other common bacterial contaminants (Mahmood and Ali 2017).

Stem cell culture systems are also infected by viral contaminants like retroviruses, hepatitis viruses, papillomaviruses, or herpes viruses (Barone et al. 2020). Other viral contaminants are porcine circovirus 1, bovine leukemia virus or bovine viral diarrhea virus (de Oliveira et al. 2013; Pinheiro de Oliveira et al. 2013) whose major

source is serums used in the preparation of culture media. Storage conditions of cells also determine the levels of contamination in stem cells. A study conducted on hematopoietic stem cells, bone marrow and bone marrow progenitor cells showed that seven (1.2%) of 583 thawed components were found to be contaminated with a variety of environmental or waterborne organisms, despite a meticulous protocol to prevent contamination during thawing (Fountain et al. 1997).

9.5.2 Microbial and Viral Contamination: Methods for Detection and Eradication

Various methods are known to identify the possible cell contaminants. The methods are influential for manufacturing GMP-complied cell-based products for use in regenerative medicine (Hawkins et al. 2017). On obtaining cell lines, copies of the original source should be cryopreserved and retained (Shaik et al. 2018). It helps in morphology check using a microscope if one finds any changes in the cell line being used (Shafa et al. 2020). The American National Standard Institute (ANSI) has issued guidelines for the use of Short Tandem Repeats profiling (STR profiling) as the authentication method for cell lines. The isolated DNA fragments run on a gel and analyzed are compared to public access database by which one can validate the cell line authenticity (Shafa et al. 2020). DNA is isolated at the exponential phase of the cell cycle for PCR amplification, followed by gel electrophoresis. This generates an STR profile which is compared to the database for authenticating cell lines (Masters et al. 2001). This method can be utilized for the identification of contaminant cell lines. To validate the authenticity of cell lines, several methods such as karyotyping, DNA barcoding, or isoenzyme analysis have been used. Iso-enzymatic analysis can be replaced by PCR-RFLP, which is less costly and time-consuming. It has been used to determine the origin species of 23 distinct animal cells and to detect interspecies cross-contamination.

Microbial contamination is much easier to detect over cellular contaminations. In cases of bacterial and yeast contamination, the culture medium turns turbid and a drop in pH is also observed (Mahmood and Ali 2017). Yeast appears like small oval particles floating in media. In cases of molds, pH remains constant in the initial stages but increases with time and results in turbid mediums. The *Mycoplasma* contamination is a bit tricky to detect as it is visible in high-density cultures and leads to culture deterioration. Sometimes *Mycoplasma* infections are difficult to treat and can stay in the culture environment for long and affect subsequent cultures. Effective methods to detect it are PCR, ELISA, and immunostaining (Drexler et al. 1994). As the numbers of viral infections are less, so are the ways to detect viral loads. Electron microscopy, PCR or ELISA or RT-PCR are some of the assays done to detect viral contamination. Other than detecting microbes, it should be taken utmost care of that Good Laboratory practices (GLP) and Good Manufacturing Practices (GMP) protocols are followed.

9.6 Sterility in Stem Cell Culture

The cells procured from various sources tend to adapt themselves as per the culture environment being provided to them in vitro. Hence, they can be easily affected by their surroundings like external pathogen or culture environment, thus compromising the potency and regeneration ability of stem cells (Yoshino et al. 2013). The inception of SCs based therapies is followed by culturing SCs in a controlled environment and maintaining sterile laboratory conditions.

When stem cells are used for therapeutic purposes, it is usually observed that they are maintained under enriched media without anti-microbial agents, which tends to increase the risk of contamination while using it for regenerative purposes thus rendering them useless sometimes. Thus, maintaining sterility while undertaking such therapies is a major concern for clinicians across the globe.

Microbes like bacteria, viruses, fungal infections or contamination in cell cultures meant for therapeutic uses can cause complete eradication of cells or complications post-therapy. In most therapies, the cells are grown in antibiotic-antimycotic free culture media, thus it becomes of utmost importance to maintain sterility throughout the process. Such pathogens can avert the results and can interfere with data reproducibility (Crook et al. 2010; Pamies et al. 2017). It is mandatory to check for any contaminates like mycoplasma, fungi, etc. before preserving and banking cells or using them in therapies (Geraghty et al. 2014). It applies to cell-derived products as well and it should be ensured that if the culture system or the product is found to be contaminated, it should be immediately relinquished before it comes in contact with the other systems (Gerő 2018). The disposal of such waste should be done under strict regulatory protocols and only those products should be retained which are entirely contamination-free.

Due to the favorable, efficient and targeted efficiency of stem cells and their product-based therapeutics in various pathologies, these are categorized under advanced therapeutic medicinal products (ATMPs) (Detela and Lodge 2019).

Sterility testing is an important aspect to clinch safe usage of cell-based products before incorporating it into the patient's system, as post-transfusion sterilization is impossible (Gebo and Lau 2020). With the increasing use of such products, it has become more and more important for clinicians to create a tight framework for the usage of such therapeutics.

9.6.1 Tests and Guidelines for Sterility Testing

Current Good Manufacturing Practices (cGMP) are rules and regulations enforced by the Food and Drug Administration (FDA), which regulate the quality of pharmaceuticals with full care and caution. One of the major concerns for the FDA is the stringent governance over the therapeutics meant for human use. cGMP monitors the quality, purity, efficacy, and adequate controlled measures for manufacturing of drugs for safer use to humans. cGMP looks out for strong quality management systems, procuring apposite quality raw materials, establishing

vigorous working protocols, recognizing and scrutinizing product quality deviations, and perpetuating trustworthy testing laboratories.

Clinical trials are increasing for stem cell-based therapies with each passing day which calls for strategies to encompass safety standards to ensure the efficacy of therapy and patient safety. In this direction, Food and Drug Administration (FDA) has released regulatory guidelines for this purpose (Mendicino et al. 2019). These guidelines ensure that the stem cells have been least possible manipulated and are subject to regulatory guidelines (Deasy et al. 2013). For clinical trials in 2014 in Japan, two laws were passed as regulatory reforms for approval of cell-based therapies which stated that at least ten patients' safety efficacy data is required for pre-clinical studies. This eliminated the chances of risks associated with therapies that were taken based on "fast track approvals" causing risky trials (Cyranoski 2019). For treatment of conditions like cardiac-related, spinal cord-related injuries, etc., conditional approvals are required because of the lack of standard rules and guidelines for safety protocols. This calls for increasing demand in single set guidelines which are internationally acceptable and include recent techniques like the use of serum-free or xeno-free media, growth factors, etc. in full compliance with cGMPs (Marks et al. 2017).

The FDA is the regulatory authority in the USA and it is responsible for looking over the regulatory guidelines in the laboratories which perform cGMP activities. Title 21 of the Code of Federal Regulations gives the FDA the authority to enforce it (CFR). The parts 210, 211, 600–680 of the CFR are responsible for cell-based therapies. GMP regulations first came into action in 1978 with 21CFR210 and 21CFR211 finalized back then and these keep getting revised for better refinement. The manufacturers or users of cell-based therapeutics should always abide by these guidelines to ensure the "*safety, integrity, strength, purity, and quality (SISPQ)*" of the products. The most important role played by these regulations is the protection of consumer/patient from products which are dangerous or lethal sometimes.

The Title 21 only provides a framework for compliance for pharmaceutical and biologics manufacturers but lacks in providing methods that control the implementation of these guidelines. Here the role of the FDA is pivotal, as it maintains and keeps updating these guidelines as per the current industrial usage and because of this, the GMP regulations are termed as cGMP, i.e., current good manufacturing practices. If one needs to remain updated with these rules and regulations, then the person should peruse the decrees for data archives maintained by the FDA and inspection reviews conducted by the FDA. To comply with the dynamic industrial patterns, some other organizations also have a pivotal role like Parenteral Drug Association [PDA], International Society for Pharmaceutical Engineering [ISPE], United States Pharmacopeia [USP] chapters, International Council for Harmonization [ICH] guidelines, or International Standards Organization [ISO] (Cook and Cai 2018).

All the sterility runs and microbial detection methods are to some extent well established. But the CFR also states the importance of an acquiescent facility where all these tests can be performed. The CFR requires that "adequate laboratory facilities for testing and approval (or rejection) of components, drug product

containers, closures, packaging materials, in-process materials, and drug products shall be available to the quality control unit." It also states that each operation has to be carried out in specific areas to prevent contamination and sort of mixing during the entire process. Although these set of guidelines provide a closely knitted framework to ensure the safest possible delivery of a product, but sometimes it becomes hard for laboratories that have open and mix operations to stay uptight with the cGMP compliances (FDA 2004, 2019a; Gebo and Lau 2020).

As we know that the stem cell-based therapy products are designed in high-end laboratory facilities, but it is also the responsibility of the manufacturer to ensure that that the sterility tests are also held in such high-risk-free zones, to comply with the cGMP guidelines. Some major requirements for a cGMP compliant facility are exclusive rooms for each function, HEPA filtered rooms, easy to clean surfaces like that made of epoxy floors, unidirectional airflow, etc. As per cGMP, it is entirely up to the laboratories performing clinical trials to conduct sterility testing within their facility or create an extra aseptic room apart from the manufacturing facility for sterility tests.

In a cGMP compliance lab, three rooms are required: staging, growing, and testing. In some cases, a fourth room might be allocated for functions like waste flow, and additional workflows. The rooms are designed in compliance with the ISO 14644-1 standard and the ISO 14644 series. These lay rules for the drafting, constructing, and functioning of these sterile rooms. For every laboratory setup along with space and users requirements, other things that are taken care of are: laboratory staff and their hierarchy in the organization, genres of tests to be conducted, contemplating the SOPs for volumes of chemicals and samples for numerous tests, designing the roadmap for laboratory chores including management of samples, workers and their suitable attires like PPEs, waste products, etc., laving of facilities, fulfillment of technical pre-requisites; all as per the set industrial criteria. All these fall under User Requirement Specification (USR) and it is the core requirement for the design, construction, certification, and ongoing operation of the facility (ISO 2015b; Lamkharbach et al. 2019). All the critical sterility-related workflows are required to be conducted in compliance with ISO class 5 which states to carry out such functions inside of a biosafety cabinet. For much lesser critical cases, manufacturers can follow ISO 7 or ISO 8 guidelines. Any testing facility whether cGMP compliant or not must ensure that its sterility standards meet up with the laid criteria and they should also ensure regular and continuous monitoring while the facility is in running. This can be kept in check by following the IOPQ process (installation, operation, and performance qualification). Installation Quotient or IQ is one such criterion. Any new cGMP facility should call for test runs to check with the IQ ensuring that all elements and facilities are successfully created and installed. The cGMP facility should also check for operation qualification (OQ) and performance qualification (PQ) which looks over the checks for operational corrections and intended use of laboratory, respectively. PQ is responsible for the maintenance of cleanliness, airflow in the facility under both functional and non-functional conditions. Airflow visualization surveys (smoke studies), non-viable and viable particle surveillance (environmental monitoring) all fall under PQ. All this

monitoring required multiple personnel and is time-consuming as well. This calls for designing a proper plan of execution that works adequately and also works effectively to reduce manpower labor and is time effective. The frequency for conducting IQ is one compared to multiple times for PQ which is responsible for sanitation and airflow pattern under non-operational situations as well as routine operational conditions (FDA 2011; Process Validation 2011). IOPQ process ensures the efficacy and safety of any laboratory and aids in monitoring the facility. It is also responsible for keeping the facility up to date with the cGMPs. Environmental monitoring (EM) of any cGMP facility is of utmost importance and thus it is important to keep a check on parameters, during operations or no operations, like airflow and pressure, viable microbes, temperature and humidity of the facility, and zero tolerance towards dust. As the cGMP facilities are designed in a way that they remain robust and functional for longer durations, it is important to keep a check on the aforementioned parameters (FDA 2019a; Gebo and Lau 2020). EM is an incessant process and is used to generate data that is used for assessing the controls and processes of the facility and can also be utilized to take significant decisions for the facility. EM protocols are designed keeping in mind the intended use of the facility, classification of rooms, size of the rooms, air flow, risks associated with any particular material or process being used, frequency of sample collection, alert and action levels (based on previously collected data evaluation), and frequent assessment of EM data to gauge trends (Moldenhauer 2014). EM protocols need to be documented and maintained throughout and should be readily available for inspections or surveys or audits (FDA 2019a; Gebo and Lau 2020; Roesti and Goverde 2019).

For any cGMP facility, keeping sterility in check is a major concern. This also includes micro to macro managing the entire system and hence calls for sturdy Quality Management Systems (QMS) along with qualified professionals to run the QMS most efficiently. For the Twenty-First Century Initiative, the FDA's Pharmaceutical cGMPs have solicited quality systems (QS) and quality risk management (QRM) modus operandi into the current GMP (FDA 2006; Patil and Pethe 2013). cGMP compliance laboratories that are involved in manufacturing or testing cell-based products need to apply a comprehensive approach that integrates calibrated attributes in their products rather than being dependent on the testing quality of their products. This will save both time and resources. Quality assurance (QA) and Quality control (QC) both are indigenous to the cGMPs. The role of QA is to review procedures and records, approve production and maintenance methods and evaluate trend analysis and its performance (FDA 2006; Hinz 2006). The QA department should have the authority to perform regulatory functions and its decisions should be supported by the management of the organization (FDA 2019a; Gebo and Lau 2020). The QA group should robustly manage the entire QMS operations and extend the support to the GMP functioning. The QMS must contain protocols and processes to inspect, emend, and supervise variations, disparities (also called "out of specifications"), complaints, and provide counteractive and deterrent actions (CAPA), and change control (EMA 2008; FDA 2006, 2019a, b; Gebo and Lau 2020; Hinz 2006). Since these regulations have been made a mere requirement, so

many laboratories do not strictly imply these. Here International standards like ISO 9000 and ISO 9001 and the FDA medical device quality system regulations (21CFR820) can offer ancillary counsel for the QMS accomplishment (ISO 2015a; Ong et al. 2015).

The regulation under section 361 of the Public Health Service Act (PHS Act) and Title 21 of the Code of Federal Regulations (CFR) Part 1271 provides the manufacturer with accurate advocacy with illustrations that enables the latter to comply with CFR 1271.350(b). This helps in regulating the manufacturing of "non-reproductive human cells, tissues, and cellular and tissue-based products (HCT/Ps)." These regulations investigate any kind of deviations at the manufacturer's end. Such deviations are delineated to FDA's Centre for Biologics Evaluation and Research (CBER) (FDA 2017).

Investigational New Drugs (INDs) or cell-based therapies have to comply with mandatory sterility testing under CFR Title 21, parts 210 and 211 for cGMP and parts 600–680 for additional biological product standards. For instance, to keep a strict check on *Mycoplasma* contamination in biological therapeutics Sections 610.12 and 610.30 are to be followed (Gebo and Lau 2020; Panch et al. 2019). For any IND applications, it is a must for the researcher/manufacturer to guarantee the constancy, efficacy, and well-being of the product (FDA 2018; Gee 2018). The number of times merchandise needs to pass the sterility test depends on the risk associated with its applications in regenerative medicine. The product with the closest resemblance to the final product developed needs to pass through sterility testing. If the potential candidate possesses a higher risk of contamination, like microbiological, then in between the manufacturing process the sterility tests can be conducted. This is permitted because the minimum time for sterility testing is 14 and 28 days for *Mycoplasma*, which can render the developed or isolated product functionless due to biological origin sources.

Due to massive growth in the field of cell-based therapies, the protocols for sterility testing are adapted from USP titles 71 and 63, which were formed to ensure large-scale product testing for any industry. USP 71 lays the guidelines for sterilized produce and testing, and 63 for *Mycoplasma* testing. USP 71 is considered the gold standard for sterility tests in industries (Lysák et al. 2016). It has been formed to warrant the safety of produce globally that is being used in the health care sector. Also, it has been made in synchronization with European Pharmacopeia and/or the Japanese Pharmacopeia.

USP 71 has defined the amount of in-process and/or final merchandise to be used for sterility tests and it depends upon the total quantity of a product and the total amount of articles (i.e., containers) in a consignment. An approximate volume as per the guidelines is inoculated directly into tryptic soy broth (TSB) on 20–25 °C and fluid thioglycolate medium (FTM) from 30 to 35 °C for 14 days (England et al. 2019). The 0.45-μm membrane filter is used for membrane filtration. The filter used is then incubated in TSB at 20–25 °C and FTM at 30–35 °C for at least 14 days. These culture broths are then observed for turbidity at various incubation intervals on days 3, 5, 7 and at the end of 14 days incubation (Variable from lab to lab) (Gebo and Lau 2020). The call for removing probable culture inhibitors from the

manufactured goods, expenses, and equipment sources determines that whether direct inoculation of the culture medium or membrane filtration will be put to use for sterility testing for the product under question.

Some other sterility testing methods include blood-based systems like Bactec by Becton Dickinson (Itani et al. 2005; Padley et al. 2007) and BacT/Alert by BioMérieux systems (Jacobs et al. 2017; Kim et al. 2019; Szabados et al. 2011). These methods are automatic and can be easily monitored for detecting microbial growth and are advantageous over USP71. The cell-based therapeutics tends to have a turbid effect in culture media which is often confused with bacterial turbidity. Here these automatic techniques help in differentiating microbial with natural turbidity of the product. As per the ISCT data of 2002, 86% of laboratories/manufacturers for minimally manipulated products and 56% for cultured and expanded produce were using modern methods for sterility testing over USP71. The National Agency for Medicines and Health Product Safety (ANSM) also depicted similar outputs stating more than 91% of labs have replaced traditional methods with the blood-based new quick ones. In 2012, chapter 2.6.27 of European Pharmacopeia has recognized the usage of aerobically and anaerobically augmented media as an alternative testing method for detection of contaminants in stem cell-based products (Gebo and Lau 2020). Earlier the FDA did not approve the use of blood-based sterility tests for the production of ATMPs, but in 2011 FDA approved RMMs by demonstrating the use of ATP bioluminescence technology (Rapid Milliflex detection system) for detection of microbes or CO_2 monitoring systems (Bactec and BacT/Alert). It declared these as suitable substitutes for turbidity detection as described in the Code of Federal Regulations Title 21, section 610.12 (21CFR610.12) and USP 71 (Parveen et al. 2011).

According to USP 71, comparative testing of at least six organisms (mentioned in USP 71 guidelines) is required for any test to be considered an internationally acclaimed standard. However, these microbes cannot be collective representatives of all the living contaminants as these have been found in limited places, and thus any method complying with these cannot be standardized. In 2019, 118 organisms (from previously positive samples) were screened using Bactec and BacT/Alert and USP71 compliant methods in a cGMP environment. When these competed beyond routine clinical bloodstream isolates and the six USP <71> reference organisms, the BacT/Alert system performed excellently and a relatively poorer demonstration was seen of Bactec FX. The results were contradictory to previously obtained ones from (Hocquet et al. 2014; Khuu et al. 2004; Lysák et al. 2016; Padley et al. 2007). This showed that more groups of organisms need to be tested other than six as mentioned in USP71 for evaluating the performance of any test for sterility testing. New England Compounding Centre founded in 1998, faced a critical situation of meningitis induced by fungus, as it sickened 798 individuals and resulted in the deaths of more than 100 people. This was followed by stringent re-evaluation and reforms in testing as 41 fungi species were tested for contamination using modifications like the use of dextrose agar plate along with Bactec and USP 71 systems.

Sources of contamination of *Mycoplasma* (size ranging from 0.2 to 0.8 μm) are media supplements like Fetal Bovine Serum (FBS) and other cell lines, donated

organ/blood, culture room air, and people working in the facility. *Mycoplasma fermentans*, *Mycoplasma hyorhinis*, *Mycoplasma arginini*, *Mycoplasma orale*, and *Acholeplasma laidlawii* are the most contaminating *Mycoplasma* species out of 120 identified. 4–35% of biological materials are contaminated with *Mycoplasma* even after sterile filtration (Barile 1981; Cobo et al. 2007; Drexler and Uphoff 2002; Mirjalili et al. 2005; Windsor et al. 2010). The cell cultures should be gratis of *Mycoplasma* contamination and should comply with regulations governing the safety of cell-based products. Very few percentages of cell cultures have been reported to be free of *Mycoplasma* due to the adding antibiotics to the media. The biggest disadvantage is that its contaminations hardly result in turbidity and hence can go unnoticed for several passages. It can change the cell morphology, that too sometimes (Gebo and Lau 2020; Windsor et al. 2010). As far as patient safety is concerned, the clinical pathologies in humans arising from *Mycoplasma* are very few like from *M. hominis*, *M. orale*, *M. genitalium*, and *M. pneumonia* (Dixit et al. 2017).

The USP 63 constitutes three methods to detect *Mycoplasma,* i.e., agar culture, broth culture, and inoculation of indicator Vero cell lines with fluorescence staining. The latter is for those species that cannot grow on agar plates and require eukaryotic cells for growth and proliferation. The most suitable media for *Mycoplasma* growth are the Hayflick medium, Frey medium, and/or Friis medium, especially for slowly proliferating *Mycoplasma*. The product under testing is taken (200 μL) and it is spread over the solid agar media plates and it is kept at humid 36 °C at CO_2 ranging from 5 to 10% and O_2 ranging from 2 to 10%. After 14 days of incubation, if *Mycoplasma* is there, it appears like a fried egg (Metwally et al. 2014). For growth in liquid broth, the test product is incubated and is checked for regular intervals ranging from 2, 4, 6/7, 13–15, and 19–21 days. For check at regular intervals, 200 μL inoculated broth is taken and spread upon agar plates and kept for 14 days as done in the previous method. For the Vero cell line based method, 1000 μL of the test sample is inoculated in a co-culture system and incubated for 3–5 days. As the cells reach 50% confluence, they are rinsed and fixed with bisbenzimide and examined for *Mycoplasma* under fluorescence microscopy. The contamination might appear like particles or filamentous structures which is a confirmation for *Mycoplasma* presence. The CFU range for a culture to be infected with *Mycoplasma* ranges from 3 to 10 CFU/10 mL for broth culture and 10–100 CFU/mL for Vero test (Dabrazhynetskaya et al. 2013; Netto et al. 2014, 2015; Simon 1989).

These three methods are time consuming and laborious as it takes around 28 days for them to yield results making it a disadvantage for USP 63. It also requires large quantities of test products, which might not be possible to provide every time as it has to be used for patient transfusion as well. Usually, the cell-based products are very unstable and might denature while in the middle for sterility tests thus rendering the entire test systems. These can be easily applicable at large-scale manufacturing industries/labs where there is ample amount of time and sample product but render useless where safety and efficacy of stem cell therapeutic strategy have to be assessed immediately and transferred to the patient.

To overcome these disadvantages, molecular level testing for *Mycoplasma* detection is very much in use these days called Rapid Microbial test Methods (RMMs).

The FDA and European medicines Agency have approved of these RMMs. The condition at which any RMM is considered is when these methods have proved efficient enough as USP63 and have undergone multiple rigorous experimentations. It should also take less time in yielding results and should be sensitive enough as well (Dreolini et al. 2020). Several industries have also developed commercial assays for *Mycoplasma* detection like MycoTOOL by Roche Pharma (Weber et al. 2021), MycoSEQ™ Kit by Applied Biosystems, Foster City, CA, USA in a 7500 FAST Real-Time PCR (D'Apolito et al. 2020) and VenorGeM *Mycoplasma* Detection Kit, developed by Minerva Biolabs GmbH (Blood 2004; Nielsen et al. 2020). These assays are easily available in ready-to-use formats from respective manufacturers and might require optimizations which can vary from lab to lab usage (EDQM 2012; Gebo and Lau 2020). Although the development of such tests has eased out the incurrence of labor and consumable charges, however, the data acquired varies from lab to lab which is why these cannot be accepted as on gold standard for *Mycoplasma* sterility testing. To overcome this, FDA and WHO came forward to design an international standard to detect *Mycoplasma* in 2015 which constituted *Mycoplasma fermentans* at 2.0×10^5 IU/mL for nucleic acid test assay development and validation (Nübling et al. 2015). But till 2019, as per the Quality Control for Molecular Diagnostics (QCMD), the test results based on this assay showed differences across laboratories and hence no standard is announced as yet. The findings varied from 57.1 to 100% depending on the testing laboratory, test method, organism, and organism concentration examined (Buckingham 2019). The availability of quicker *Mycoplasma* testing certainly enhances cell treatment safety by detecting contamination occurrences earlier. However, as previously stated, a suitable alternative RMM must be at least as good as the compendial technique. Several cases have been reported to have shown negative for *Mycoplasma* in culture but positive in PCR-based tests and such cases need to be dealt with stringent screening methods which have high sensitivity, so as to ensure patient's safety and efficient therapy (Fig. 9.3).

9.7 Impact of Contamination in Cell

Microbial contamination in cell cultures affects the growth and quality of culture which creates drifts from obtaining original hypothesized results. Such mishaps compromise the quality of culture and results and also affect the reproducibility of the experiments (Hirsch and Schildknecht 2019). The prevalence of *Mycoplasma* in cell culture systems is a serious concern to biosafety issues. They were first isolated in 1956 and since then many cell lines have been reported to be infected with *Mycoplasma* (Esber et al. 1973). The most commonly found species are *M. hominis* and *M. pneumonia* and can account for up to 96% of *Mycoplasma* contamination (Drexler and Uphoff 2002). It was believed that these species have adherent properties towards cells only but later it was found that these can form intracellular colonies too (de Oliveira et al. 2013). The infected cell lines can undergo morphological changes and their metabolism can be altered. It affects the

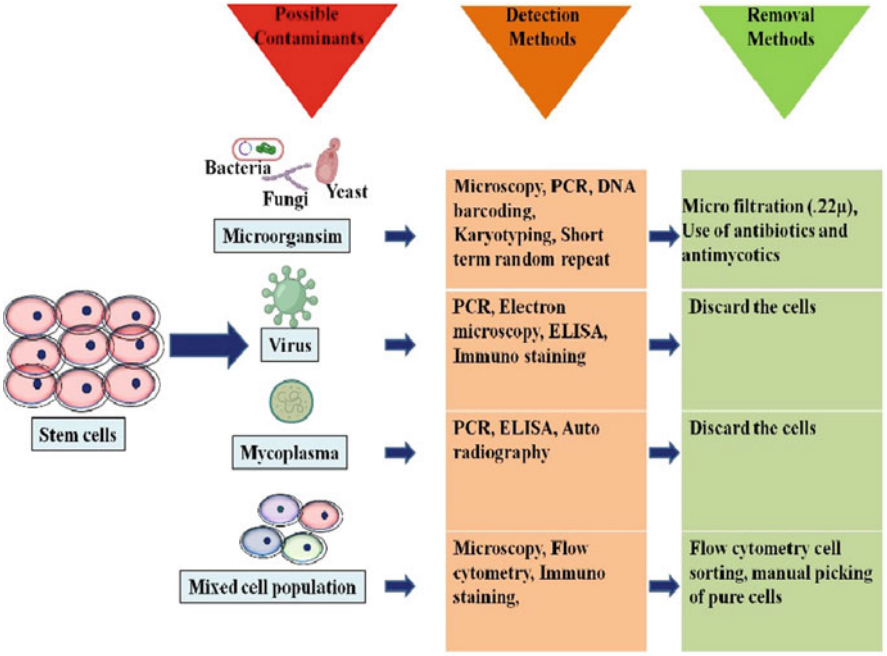

Fig. 9.3 Identification of stem cells contaminants: Stem cells safety and sterility is essential for successful research and transplantation. Microbial contaminants (bacteria, fungi, mycoplasma, and virus) and mixed cells populations are major threats to cells sterility and safety. The detection methods are including many techniques like microscopy, PCR, ELISA, autoradiography, immune staining, etc. for removal of contaminants we use antibiotics/antimycotics, 0.22 µm filters, cell sorting by flow cytometry and by discarding the contaminated cells

production of cellular proteins, DNA and RNA as well. Usually, changes in the cell membrane have been seen which can be due to alterations in the cell's metabolism. This affects adherence of cells, surface-antigen changes, slowing in the growth of cells. All of this ultimately leads to cell death and disruption of culture systems (Borchsenius et al. 2020).

The contamination from bacteria is marked by changes in pH and culture media appearing turbid. The cells initially grow normally but as the bacterial growth increases the normal cell growth is adversely affected causing cell death (Levy et al. 2018). Contamination with fungal spores or fungi appears to be floating in the media when observed under a microscope. This can even become prominent in later stages of contamination and can be seen as fuzzy patch-like structures and that can be easily seen by naked eyes. Unlike bacterial contamination, pH change or cell growth is not observed in fungal contaminations. It is only after prolonged contamination that such changes occur. This leads to challenges in cell survival and growth and eventually, the culture dies (Xu et al. 2020).

9.8 Methods to Maintain Sterility

Various kinds of contamination sources like biological, chemical, visible or invisible are there which lead to loss of culture systems. It causes compromises in the quality and efficacy of culture systems and has deleterious effects on experiments or therapies and even leads to false results (Geraghty et al. 2014). Living contamination like microbes or other cell lines affects the culture adversely but there is an almost equal contribution of non-living contaminants from water/storage containers/supplements in disruption of culture systems by causing undesirable effects (Mahmood and Ali 2017). Non-living contamination can be eliminated by being extra cautious and following all the guidelines laid down by cGMP (Ryan 1994).

To prevent contamination of stem cells and their derived products, it should be taken care of that ultrapure water is used for the preparation of culture media and rinsing of glassware meant for preparation and storage of media and supplements (Wiemer et al. 1998). All the culture components should be purchased from verified manufacturers who provide sterility certificates (Sukhanova and Zakharova 2018). Extra care and precautions should be taken while using serum-based production of therapeutics. As much as possible, xeno-free media should be used in the production of ATMPs meant for stem cell-based therapies (Agostini et al. 2018). Serum if being used should be tested for contamination prior to use and the same lot should be used so as to avoid discrepancies in outcomes (Baust et al. 2017). All the culture ware should be irradiated (Lavrentieva 2018) and must fulfill no leaching criterion. All the work should be done in biosafety cabinets of appropriate BioSafety Levels (BSL) (Bayot and King 2020) depending on the nature of work (Ta et al. 2019). Multiple tests, in vivo or in vitro should be run for sterility tests for all types of parameters for all kinds of contaminations. Human activity should be kept in check in the BSL areas and restricted personnel should be allowed to enter the area. If any facility has any sort of contamination, and it goes unnoticed, it might proliferate through aerosols and affect other cultures as well (Nikfarjam and Farzaneh 2012). To avoid this regular disinfection and sanitization of the facility should be done. The use of antibiotics and antimycotics should be done in compliance with regulations of FDA and cGMPs so as to avoid their deleterious effects on the patients receiving the therapy. Any culture system which is meant for cryopreservation should be checked before and after cryopreservation. The personnel working in stem cell facilities should be sincere and should avoid malpractices in the facility like eating, coughing, conversations, etc. Last but definitely not least, all incidences of contamination in any cell culture facility should be documented as per the internationally acclaimed compliances which in turn will help in easy detections of contaminations in the future.

9.9 Conclusion

Embryonic stem cells, tissue-specific progenitor stem cells (TSPSCs), mesenchymal stem cells (MSCs), umbilical cord stem cells (UCSCs), bone marrow stem cells (BMSCs), and induced pluripotent stem cells (iPSCs) are all types of stem cells used in regenerative medicine (Mahla 2016). For promising therapeutic outcomes, stem cells must persist, grow, and transdifferentiate inside the host system at the application site. Cell-based treatments are a relatively new approach to regenerative medicine, and they are being considered as one of several promising strategies for the treatment of irreversible illnesses such as Parkinson's, Alzheimer's, ALS, and MS, among others (Nooshabadi et al. 2018). Stem cell-based therapies have grown in popularity among doctors and researchers as approaches for safe use of SC and their related products have improved. Individuals with spinal cord injuries who received ESC-based therapies showed improvements in movement, balance, and sensation. ESCs remained at the injury site and improved the condition. In areas such as intrauterine adhesions, Parkinson's disease, and amyotrophic lateral sclerosis, among others, several studies have been published on the usefulness of ESC-based therapy in regenerative medicine. The most prominent and ongoing worry surrounding the use of stem cells has been ethical problems since the birth of the age of stem cell-based therapeutics. Because they are obtained from the destruction of human embryos, ESCs, a valuable source of medicines, are the subject of the greatest ethical debates. In this regard, iPSCs have emerged as a viable option; nonetheless, they, too, have ethical difficulties. The most important stage in any cell-based therapy is to maintain original cell line properties throughout cell culture growth. Due to prolonged cell culture, the original functions of a cell might change or disappear resulting in varying or non-significant results. Stem cell culture systems are also infected by viral contaminants like hepatitis viruses, retroviruses, herpesviruses, or papillomaviruses.

As the numbers of viral infections are less, so are the ways to detect viral loads. Electron microscopy, PCR or ELISA or RT-PCR are some of the assays done to detect viral contamination (Mahmood and Ali 2017). Other than detecting microbes, it should be taken utmost care of that Good Laboratory practices (GLP) and Good Manufacturing Practices (GMP) protocols are followed. When stem cells are employed for therapeutic purposes, they are often kept in an enriched medium without antimicrobial agents, which increases the danger of infection when employed for regenerative reasons, rendering them unusable in certain cases. Thus, maintaining sterility while undertaking such therapies is a major concern for clinicians across the globe. Current Good Manufacturing Practices (cGMP) are rules and regulations enforced by the Food and Drug Administration (FDA), which regulate the quality of pharmaceuticals with full care and caution. One of the major concerns for the FDA is the stringent governance over the therapeutics meant for human use. cGMP monitors the quality, purity, efficacy, and adequate controlled measures for manufacturing of drugs for safer use to humans. Strong quality management systems, the acquisition of acceptable quality raw materials, the establishment of robust operational procedures, the detection and investigation of

product quality violations, and the maintenance of dependable testing facilities are all part of cGMP. The FDA is the authority to implement Title 21 of the CFR, and also has regulatory jurisdiction above any laboratory conducting cGMP testing in the United States. Parts 210, 211, and 600–680 are particularly significant for pharmaceutical items and biologics (cell and gene treatments). Other elements of Title 21 (such as part 11 for electronic records and signatures) are, nonetheless, needed and frequently invoked during regulatory inspections. A successful cGMP testing facility should be certified for its anticipated use and should be monitored at regular intervals for ensuring that it remains a condition that is regulation-complied. A cGMP facility must be qualified before it can be used to guarantee that IQ, PQ, and OQ criteria are met and fulfilled to the best of their capabilities. The cGMP services are extremely specialized, requiring an intricate system of controls and inspections to provide "adequate control over air pressure, microbes, dust, humidity, and temperature" throughout the operations. The FDA's major instrument for enforcing the GMP regulations is set out in CFR Title 21, sections 210, 211, and 600–680 for on-site inspections. If a clinical laboratory decides to put up with GMP testing operations, then it must do so with a thorough grasp of all of the preceding issues, as well as the assumption that the laboratory will be inspected by the FDA.

Acknowledgments The author would like to thank the Deanship of Scientific Research at Majmaah University for supporting this work.

Funding Source NA

Declarations The authors of the manuscript declare no conflicts of interest/competing interests. All data and materials are available in the manuscript.

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Stem Cells Storage, Packaging, and Transportation

10

Ali Raza Ishaq, Shouwen Chen, Ayesha Noor, Rabia Batool, He Pengui, and Min Xiong

Abstract

Nature has several treasures to provide stem cells which help humans through reformation of lost organs. Stem cells are unspecialized cells, present in diverse parts of the human body that have capability to repair, regenerate, and reform the tissue of any organs. They have different types based on the level of differentiation and contribution in regeneration of tissue. They have unusual therapeutic potential to treat blood-related diseases like leukemia, lymphoma, neuroblastoma, and multiple myeloma. Thus, several experiments are going to adopt the best preservation method along with optimum conditions that can sustain the quality of stem cells. Scientists have found a tool based on freezing temperature for long-term preservation known as cryopreservation. For short-term preservation, chilled storage is a best technique, while ambient temperature pausing widely applies for the transportation from one region to another. Screw cap and cell freezing bags hermetically sealed containers that can hold a huge number of cellular products. Cell Seal and Crystal vials are the commercial products that are widely used for dealing or storage of stem cells.

Keywords

Stem cells storage · Stem cells packaging · Stem cells transportation

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F. A. Khan (ed.), *Stem Cell Production*,
https://doi.org/10.1007/978-981-16-7589-8_10

10.1 Introduction

In today's world, scientists must deal with monstrous task of perplexing medical circumstances. In this scenario stem cells have emerged as viable approach in the field of tissue regeneration, diagnosis of cardiovascular disorders, brain diseases, and blood diseases treatment. In last few decades, successful contributions of stem cells in organ transplantation has pushed scientists to take up stem cells to treat multiple ailments. Stem cell-based therapeutic approaches are radical hidden treasures for limitless scaffolds with versatile properties (Alison and Islam 2009; Hedayati et al. 2020). Stem cells are basic cells of body with unique property to develop into specialized cells. Stem cells are mothering cells of remarkable potential that give rise to specialized cells including nerve cells, heart muscles cells, and blood cells (Avasthi et al. 2008). Undifferentiated property of stem cells makes up valuable candidate bestowed with versatile pharmacological activities. Stem cell storage and transportation is one of key concern for successful applications of stem cells all around globe. To fill gap between tissue regeneration and implantation, and to transport biomaterials from one area to another area there is urgency for long run and convenient survival method. Despite significant advancements in biotechnology, scientists have identified new unsatisfactory challenges linked with the preservation of cells (Avasthi et al. 2008; Gordon et al. 2006; Jesson et al. 2016; Lerou and Daley 2005).

There are different types of stem cells ranging from embryonated stem cells to adult stem cells. Numbers of classifications are available for stem cells but mainly division is based on source or types of cells and cell potency. There are many different types of stem cells on the basis of cell potency named as totipotent cells, pluripotent cells, multipotent cells, and unipotent cells. Other cells are embryonated stem cells, adult stem cells, cancer stem cells, and induced pluripotent cells (Tuch 2006). Bodies repair system mainly constitutes stem cells. Artificially cultured stem cells are used in medical therapies on regular basis. Stem cells of various types help scientists to discover potential candidates of diverse characteristic to treat plethora of diseases including cancer, diabetes, Parkinson, Alzheimer, and many more. Stem cell research holds potential to treat abnormal development without directly studying human embryo which is imaginary vision of past (Jesson et al. 2016; Watt and Driskell 2010).

To meet increasing demand of patients there is need of efficient regulatory guidelines and technologies. Stem cells preservation and transportation are major platforms for stem cell-based therapies. However, despite of recent development data about future of stem cell-based therapies, their preservation and transportation are still sporadic. Three techniques which are used for preservation are cryopreservation, chilled storage, and ambient temperature pausing (Jesson et al. 2016). In packaging of biomaterial, main concern is cell viability throughout transportation process. Vials and bags use for transportation are mostly made up of polyvinyl chloride and ethylene vinyl acetate (Jesson et al. 2016; Zakrzewski et al. 2019).

This chapter suggests that stem cells are queen cells of versatile characteristics, but additional studies are required to determine their role to validate their potential

for treatment of multiple diseases. This chapter sheds light on stem cells, their storage, preservation, and packaging and challenges with the hope that it will serve as comprehensive summary for clinicians who treat multiple diseases utilizing stem cell-based therapies. Prospects will also be discussed in chapter. Novelty of this present study is mainly attributed to whole lot about stem cell in single piece of paper.

10.2 Classification of Stem Cells

Stem cells can be defined as the undifferentiated cells with unusual potency of differentiation to produce mature cells of a particular tissue. Each tissue arises from its pre-existing tissue stem cells like hemopoietic stem cells divide rigorously for the generation of hematolymphoid system (blood-immune forming system). In evolutionary perspective, stem cells are the ancient cell-lineage in all tissue of both embryonic and adult organisms. Stem cells hold the aptitude to repair tissues through cell therapy and tissue regeneration in the combat against disorders. The research of stem cells has identified as a prominent area of biomedicine in the twenty-first century.

Stem cells are classified based on unique level of differentiation and propensity of self-regeneration as shown in Fig. 10.1. Therefore, these cells have unlimited lifespan, as well as dividing potential. Human stem cells have been classified into two major categories based on source and dividing potential: embryonic stem cells and adult stem cells. Adult stem cells comprise the mesenchymal stem cells and hematopoietic stem cells. Upon fertilization, gametes cells (eggs or sperms) eventually mature into a whole individual having multiple tissues. Embryonic cells produced from a human embryo or fetus are also stem cells having significant differentiation potential. Adult stem cells are poorly differentiated cells occurring in the tissue or organs along with specialized cells. According to recent investigations, adult stem cells have not distinct potency of self-renewing or differentiation as compared to embryonic stem cells (Jopling et al. 2011).

Embryonic stem cells have ability to differentiation into all cell lineage that make them remarkable tool for cell therapy studies (Nishikawa et al. 2007). Embryonic stem cells have been used in regenerative medicine due to self-renewal and pluripotency properties. These cells have ability to regenerate functional progenitor cells for tissue restoration. The advancement of gene manipulation and tissue regeneration can lead to the development of therapeutic cells (Doğan 2018).

Adult stem cells are rare, undifferentiated cells, normally kept in quiescent, nondividing state, these cells proliferate and differentiate into new cells to replace and repair the injury. Due to proliferative nature, these cells have ability to regenerate the tissue, and have potency to treat variety of degenerative diseases (Cable et al. 2020; Dulak et al. 2015; Gurusamy et al. 2018).

Cancer stem cells comprise a small populace cells within a tumor cell (Nassar and Blanpain 2016). These cells have been recognized in many solid tumors, such as brain tumor, breast cancer, melanoma, colon cancer, and lung cancer. Cancer stem

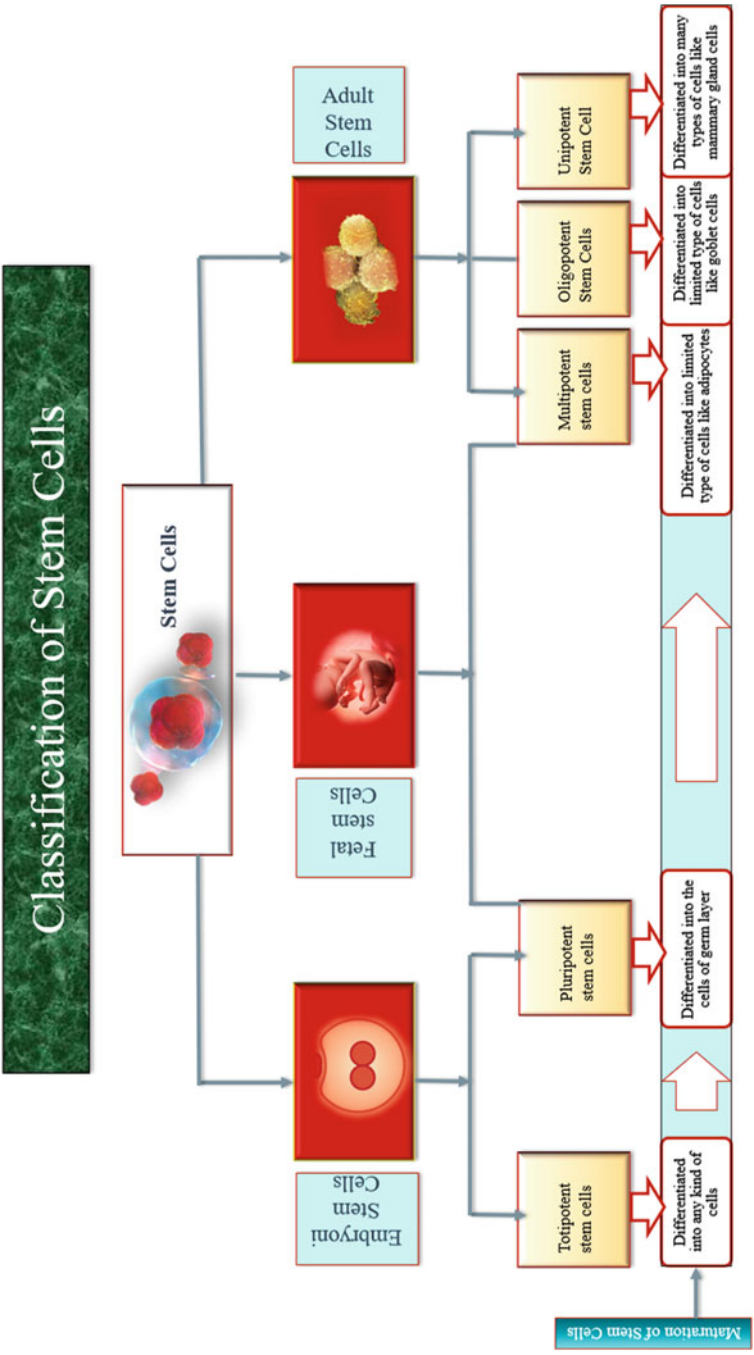

Fig. 10.1 Classification of stem cells and their fate

cells have capacity to self-renew, to produce many progenies, and to utilize the common signaling pathways. These stem cells might be the reservoir of tumor cells, the reason for the resistance to the chemo-therapeutic agents used to overcome the malignant tumor, as well as may be the source that gives rise to distant metastases (Dawood et al. 2014).

Multipotent cells are progenitor cells including hematopoietic cell and mesenchymal stem cells that give rise to a limited range of cells within a tissue type. Multipotent stem cells play a significant role in procedure of tissue repair, protection, and development (Khanlarkhani et al. 2016). These cells used to repair many aliments such as the spinal cord injury, bone fracture, and autoimmune disease (Sobhani et al. 2017).

Induced pluripotent stem cells are the type of pluripotent stem cells which is artificially derived from non-pluripotent cells such as adult somatic cells (Karagiannis et al. 2019). Totipotent are a group of cells that give rise to all human cells including brain, liver, heart, or blood cells. These cells can give rise to an entire functional organism (Baker and Pera 2018). While pluripotent are a group of embryonic stem cells that can give rise to all type of tissues but cannot give rise to an entire organism (Liu et al. 2020).

10.3 Storage and Biopreservation of Stem Cells

Biopreservation is defined as in which various chemicals or deviate temperature applied on cells for the sake of halting the metabolic activities. Biopreservation is an essential step to sustain the physiological properties of stem cells for cellular therapy. Cryoprotectant agent (dimethyl sulfoxide) and cooling are key factor in biopreservation of cells. Stem cell storage occurs frequently over the formulation of a cell therapy, from the initial donor cell through the manufacturing process ultimately transportation and inoculation into the hostl. Proper supervision of abovementioned steps plays crucial role for sustaining the efficacy and stability of cellular therapies. It can be difficult to synchronize the transportation and delivery of donor cells along with good quality. Biopreservation has removed this drawback in cellular therapies through giving an option of storage (Frey et al. 2006).

10.3.1 Cryopreservation

Cryopreservation is the method of preserving anatomically intact living cells and tissues through cooling them at low temperatures (Pegg 2007). Cryopreservation is the most widely used method for protecting cells and is already employed in medical technologies including Dermagraft and phase III clinical trials (Stroemer et al. 2009). Dr. Polge et al. (1949) discovered the concept of cryopreservation that was firstly employed on sperm preservation by using glycerol (Polge et al. 1949). Embryo (Mouse Embryo) preservation was not documented until 1972 due to elevated amount of water that increased the formation of ice in cells. First successful bovine embryo perseveration was reported in 1973 (Moore and Hasler 2017).

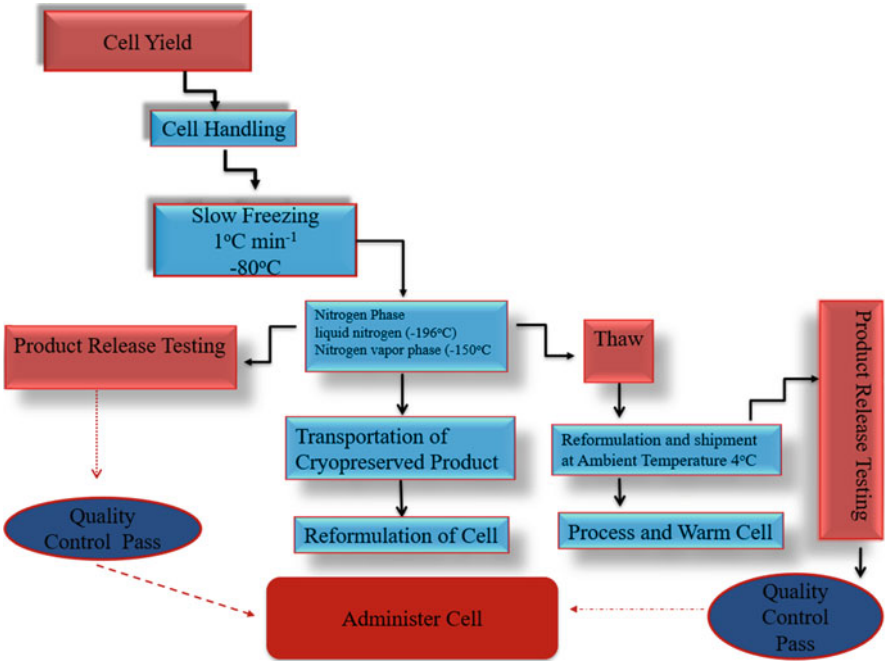

Fig. 10.2 Schematic representation of cryopreservation and rewarming of stem cells

In this process, cryogenic temperatures freeze all the water inside a cell to ice, ultimately silencing all chemical and metabolic processes. An efficient cryopreservation retains the 90% cell viability after freezing, but it is linked with the type of cells (Kleeberger et al. 1999; Kotobuki et al. 2005). For example, embryonic stem cells (ESCs) survive at low temperature because they are highly vulnerable to freezing which means that high cooling may lead to the death of cells. To overcome this problem, cryopreserved cells wash with anti-freezing agents which ultimately cause the further loss of cells. Thus, a highly controlled method is required to avert the loss of cells as well as to retain the physiological properties of cells (Alison and Islam 2009; Li et al. 2010; Xu et al. 2010).

Cryo-biologist has recommended the following highly controlled condition for cryopreservation; the process is done by gradually freezing the cells at −80 °C, afterward inserting them in liquid nitrogen (−196 °C) and finally in nitrogen vapor phase (−150 °C) (Polge et al. 1949). The abovementioned cooling rate has proven an ideal temperature for maintaining the structural and functional integrity during preservation of cells. An appropriate cooling rate is either slow enough to prevent the ice crystal formation inside cells and high to maintain the osmotic gradient as well as prohibit cell shrinkage (Mazur 1984).

To mitigate these complications, an ideal cooling rate 1 °C min^{-1} usually applied during the intermediate zone of temperature (15–16 °C) that stops the ice crystal formation inside the cells as well as averted the high osmotic gradient. Figure 10.2

represents the mechanism of preservation as well as their safe transportation. Dehydration and high osmotic gradient are the major factors that disrupt the core metabolism of cells as well as lipoprotein complexes of plasma membranes. Controlled rate freezer (CRF) and cryoprotectant agents (CPA) are alternative ways for sustaining the functional ability of preserved cells. CRF or freezing containers like Mr. Frosty or Cool Cell, are very useful for obtaining the ideal cooling rate temperature (1 °C min^{-1}) (Jesson et al. 2016; Scheinkönig et al. 2004; Van Buskirk et al. 2004).

Cryoprotectant agents (CPA) are also employed to modulate the rate of water transport and crystal formation, which aims to lessen the adverse impacts of freezing (Elliott et al. 2017). Sugars, glucose, trehalose, and hydroxyethyl starch, ethylene glycol and dimethyl sulfoxide are the documented cryoprotectant agents in the process of cryopreservation. The solvent dimethyl sulfoxide is the most widely prescribed CPA (DMSO) that lessen the ice crystal formation in sperm, oocytes, and embryos. DMSO is necessary evil for cryopreservation because it retains the metabolic properties of cells as well as reduces the loss of cells during cryopreservation. DMSO is a ubiquitous solvent effective for sustaining cell membranes in dynamic conditions, prohibiting the accumulation of intracellular ice crystals after freezing and the release of heat during transitions phase (Awan et al. 2020; Weng and Beauchesne 2020).

10.3.2 Chilled Storage

Chilled storage is an effective method for short-term preservations of stem cells especially during the transportation. In this method, hypothermic temperature (2–8 °C) is provided to the stored organ/cell before transplantation. Like, low temperature preservation is required for the storage of RBCs in donating purposes. Refrigeration is the best method to attain the conditions for chilled storage that can maintain the cell viability up to 42 days. US industry standard for current good manufacturing practices (cGMP) recommend +4 °C ideal temperature including storage and transportation (Collins et al. 1969; Hess 2012).

Stem cells are far more intricate than RBCs that is why they require some potent techniques for chilling them. For example, in the commercial hypothermic storage medium Hypothermosol Free Radical Solution, human mesenchymal stem cells (hMSCs) were adequately stored for up to 96 h at +4 °C for maintaining membrane integrity and metabolic activity (85% viability) (Mathew 2013). Similarly, human embryonic stem cells stored for 48 h at 4 °C in an optimum culture medium sustained 64% membrane viability. Additionally, by employing freshly isolated hematopoietic stem cells (HSCs) for transfusion can be packaged and delivered within a few hours; yet, cells still require to be proliferated at the manufacturing plant and shipped to the clinic, taking days to weeks for storage (Ginis et al. 2012; Heng et al. 2006).

The bulk of investigations provide scientific proof of effectively chilling mammalian cells for 24–48 h, with certain predicting recuperation after a week of chilling. Transportation of preserved products within Europe requires less than

24-h time approximately, although, custom checks and setbacks to surgery take additional time. The major challenges are maintaining the functional ability of stem cells for therapeutic purposes after exposure to refrigerated temperature. To ease the transportation of stem cells, we can employ the existing facilities and the current cold-supply chain equipment for maintaining cellular properties during freezing (Heng et al. 2006; Mathew et al. 2004).

Despite of cryopreservation, cell metabolism and cell cycle progression do not cease under hypothermic treatment. Cell pausing is an emergent technology to preserve the mammalian cells for transportation. In this technology, metabolic reactions and energy-dependent systems including protein formation and membrane transportation become lower due to decline in the temperature. Like, as per Q10 temperature coefficient, metabolic reactions lag by 50% for every 10 °C fall in temperature. With the progression of this temperature coefficient, there is no energy substrate inside cells to produce adenosine triphosphate (ATP) (Heng et al. 2006; Mathew 2013; Reyes et al. 2008). It can be rectified for a short time generating energy from the lactic acid cycle, but that is only transitory. There is no ice crystal formation and high osmotic gradient during chilling, but preserved cells face the hypothermic stress that ultimately damages them. To overcome this stress situation, hypothermia gives two alternative mechanisms for balancing the energy level: (a) reducing energy-dependent processes, (b) ATP-driven ion pumps (Mathew 2013). These mechanisms are incredibly useful to mitigate osmotic gradients and membrane permeability, and halt free radical production (ROS or RNS). Hypothermic retention promotes cellular swelling (Plesnila et al. 2000), amplification of cold-shock proteins (Fujita 1999), protein uncoupling (Tseng et al. 2011), and the unfolded protein response (Corwin et al. 2011). The continuous cold stress results prolonged pausing time that increased the risk of activation of apoptotic and necrotic pathways ultimately to cell death. Under normothermic temperatures, metabolic reactions are reamplified that cause higher production of ROS, egestion of waste and induced cell death signaling pathways. Rewarming or normothermic temperatures take many hours or even days for cell death and this is commonly referred to as delayed onset cell death and the term “delayed onset cell death” is being used to describe this situation. Abovementioned complications provide us knowledge about the significance of viability evaluation timing and recommend us to employ an efficient functional assay during chilling. Shelf life cannot be appropriately addressed without an adequate assay (Morris and Acton 2013).

10.3.3 Ambient Temperature Pausing

Ambient temperature pausing is a fascinating new weapon for transient cell storage or live cell shipping which does not require cryopreservation. It offers another approach for the storing and shipping of cells for SCTs. This hypothermic method optimizes the preserving process and reduces the requirement for specialist tools including dry shippers or liquid nitrogen vessels that are usually employed in cryopreservation. In contrast to cryogenic and chilled preservation procedures, the

research on ambient temperature preservation is limited, especially for therapeutic cell lines (Robinson et al. 2014).

Alginate gel entrapment and encapsulation in hermetic culture chambers like PetakaG3 are two of the finest tools for maintaining its ambient temperature pausing application (Chen et al. 2013; Jesson et al. 2016). A closed system is considered to insulate cells from cold-induced stress and trauma by establishing a moderate hypoxia that assists in cell cycle arrest at the Gap 1/Synthesis interface, in which cells are prepared to begin mitosis but are not proactively divided. The limitation of employing gel entrapment is that it involves a cell extraction step, which is likely to occur at the clinical site and escalate the processing time of systems as well as mutate cell viability. Ambient temperature storage is the best method for storage of hair follicles and mammalian cells that preserve at 4–24 °C under the diversity of culture vessels like microcentrifuge tubes, spinner flasks, and 3 L bioreactors (Hunt et al. 2005; Kim et al. 2002).

Ambient temperature pausing can be a useful approach for inter-process pooling/pausing, although the optimum temperature range needs to be decided initially. Cell pooling will always be needed through the production of cell products, and delays may happen due to supplementary processing faulty machinery, or operator issues. A precise insight of the optimum duration for halting cells yet remaining viable and functional following warming will be incredibly helpful in the cell fabrication and biotechnology industries because it would dictate the manufacturing design. The combined pausing time of the cells can also be accounted for in the ambient temperature freezing tool.

Abovementioned coefficient temperature about Q10 decreases the working of an energy-dependent system during the process of chilling, but in ambient temperature pausing, there is no significant effect of temperature decreasing on metabolic machinery of the cell. In mild to severe hypothermia, cells metabolize at a high enough rate to deplete nutrients and generate waste products such as lactic acid, which lowers pH and contributes to cellular damage over time (Fujita 1999; Scott et al. 2005). Storage at ambient temperature promotes osmotic-induced swelling and free radical formation, as well as altering the membrane lipid bilayer integrity and cytoskeletal structure, as it happens with cold cells (Scott et al. 2005). At 25–33 °C, the elevation of p53, WAF1, and cold-inducible RNA-binding proteins offer a defensive mechanism to help cells tolerate recurring stress (Matijasevic et al. 1998; Nishiyama et al. 1997; Ohnishi et al. 1998).

10.4 Stem Cell Transportation and Packaging

Stem cellss are totipotent, multipotent, unipotent, or pluripotent; these totipotent cells have potential to develop different type of cells including both embryonic and extra-embryonic tissues. The hematopoietic stem cells are derived from adult stem cells, used to develop all blood cells and immune system. The use of these hematopoietic stem cells for the treatment of hematologic malignancies is the pioneer of cellular therapy and translation research. Stem cell treatment faces various

challenges. Thus, it is difficult to cultivate, preserve, as well as to transport stem cells. Several synthetic analogs for the stem cells provide a new approach to destroy these problems and grip the potential to develop renewing drug (Luo et al. 2017).

Scientific studies of growing body have proved the therapeutic potential of different stem cells such as embryonic stem cell, skeletal myoblast, bone marrow derived mesenchymal stem cells, and cardiac stem cells in cardiac illnesses (Segers and Lee 2008). The examination of these different stem cells demonstrated that mesenchymal cells have greater potency to produce mesodermal as well as non-mesodermal tissues. The preclinical and clinical investigation exposed that the mesenchymal stem cells have been potential for repairing and regeneration of cardiac muscles, resulting low retention rate of myocardium (Squillaro et al. 2016; Toma et al. 2002)**.**

Mesenchymal stem cells have a key mechanism for therapeutic potential in myocardial functions and have broad spectrum to produce exosomes for restoring the heart failure (Gnecchi et al. 2008; Lai et al. 2010). In vivo and in vitro studies revealed that mesenchymal stem cells have direct transport for therapeutic potential of stem cells action of cardiovascular diseases (Angoulvant et al. 2011; Boomsma and Geenen 2012)**.**

Furthermore, in an experiment a single dose of cytokines such as vascular endothelial growth factor and insulin are tested for cardiac therapeutic (Baraniak and McDevitt 2010). It is remarkable that exosomes defend their stuffing from degradative enzyme or elements (Emanueli et al. 2015). Additionally, exosomes contain proteins and RNAs which have great potential for cardiac injury (Gray et al. 2015; Lai et al. 2011).

Hence, exosome-based therapeutics also face various problems such as standard isolation protocol, quick clearance, and wash away owing to their very small sizes. Poly lactic-*co*-glycolic acid, a bio-degradable and bio-compatible polymer, is emerging as a bulging element in drug delivery system due to its competence of defending cytokines from degradation although allowing for the constant proclamation of factors that trigger in specific organ or cell (Danhier et al. 2012; Tang et al. 2017). The schemetic protocol of mesenchymal stem cells packaging disccuss in (Fig. 10.3).

Moreover, Fang et al. described cancer cell-membrane layered by nanoparticles folded by coating the cancer cell membranes on to PLGA-loaded immunologic elements. The membrane bounded tumor linked antigens permit CCNPs to be efficiently transported to antigen offering cells to encourage anti-cancer immune response (Fang et al. 2014). Synthetic mesenchymal stem cells exposed a profile and surface antigens alike to those of genuine mesenchymal stem cells. Synthetic mesenchymal stem cells show cardiomyocyte role, cryopreservation and lyophilization stability. In another study, mouse model with acute myocardial infarction is directly injected by synthetic mesenchymal stem cells that promote angiogenesis and mitigated left ventricle remodeling (Luo et al. 2017).

Mesenchymal stem cells are heterogeneous group of cellular population containing various progenitors that modulate the immune and inflammatory responses, also play vital role in hematopoiesis (De Luca et al. 2017). Mesenchymal

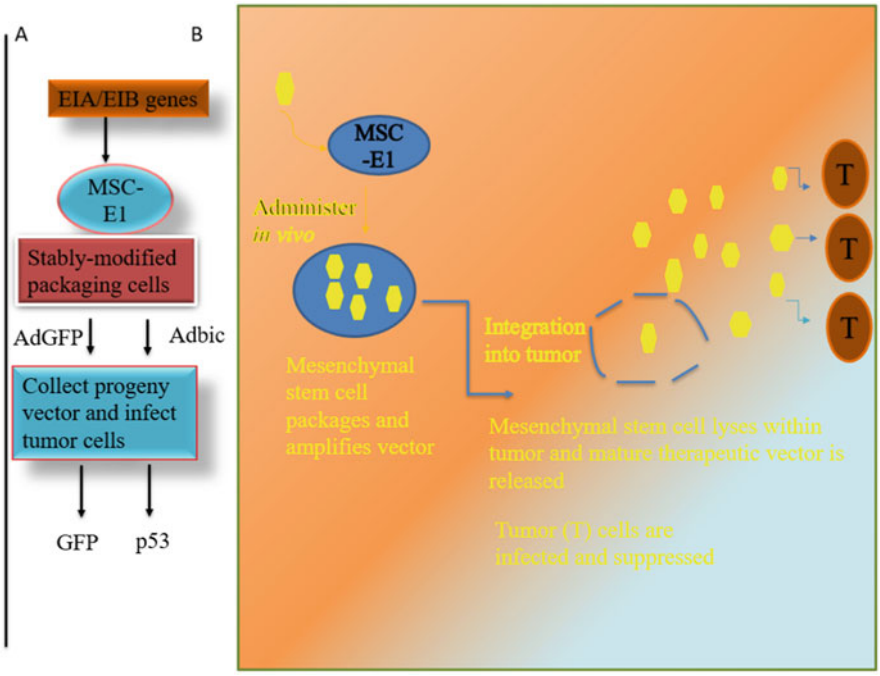

Fig. 10.3 Diagrammatic representation of packaging of mesenchymal stem cells in section (**A**) and (**B**)

stem cells are multipotent progenitor cells with several biological roles such as differentiation, immunosuppression, and tissue repair (Uccelli et al. 2008). Mesenchymal stem cells have been extensively working clinically and exposed vital role in cardiovascular ailments, type I diabetes mellitus, and in GvHD.

Extracellular vesicles are membranous bounded comprises different biomolecules, containing proteins, lipids, nucleic acid as well as carbohydrates (Maas et al. 2017). "Extracellular vesicles" is mainly used to define the two EV populations, such as macrovesicles that was originated from budding of plasma membrane, and the exosomes of endosomes of endosomal origin.

Extracellular vesicles are derived from mesenchymal stem cells that possess exosomal surface markers such as CD107, CD9, CD63, and CD81 (Bruno et al. 2015), and express the surface markers as CD29, CD73, CD105, and CD44 that are distinguished by their origin of cells (De Luca et al. 2016). Several experiments looked at the content of nucleic acid and proteins conveyed to target cells for immune regulation.

Tomasoni et al. proved that MSCs derived EVs containing numerous types of RNAs, transcripts that intricate to control the transcription, cell proliferation, and immune regulation responses (Tomasoni et al. 2013). Furthermore, MSC-derived EVs comprise microRNAs, for example miR-564, miR-223, and miR-451, that are

Table 10.1 Clinical studies of mesenchymal stem cells application to promote hematopoietic stem cell

Clinical studies	MSC source	No. of pts	Results	References
Breast cancer	Bone marrow	28	Rapid hematopoietic recovery	Wu et al. (2013a, b)
Hematological malignancy	Bone marrow	162	Improvement of early lymphocyte recovery	Wu et al. (2014)
Hematological disorders; UCBT	Bone marrow	8	Prompt hematopoietic recovery	Wu et al. (2013a)
Hematological disorders	Bone marrow	13	GvHD prevention	Macmillan et al. (2009)
Severe aplastic anemia	Umbilical cord blood	21	Sustained donor engraftment	Koç et al. (2000)

involved in multi-organ development, cell survival, differentiation, and immune system (Baglio et al. 2012; Bruno et al. 2015).

Hemopoietic stem cells (HSC) are used for the treatment of several diseases and providing a new vision to biology of cord blood transplantation. The clinical studies on HSCT are summarized in Table 10.1 as well as the response rate (De Luca et al. 2016). In an experiment it is revealed that the interaction among the UCB-CD34+ cells and bone marrow mesenchymal stem cells derived EVs. The EVs derived from miRNAs and piRNAs can influence the UC-CD34+ cell fate. The gene expression profile of UCB-CD34+ cells treated with EVs, showed that 100 downregulated genes are among those targeted by EV-derived miRNAs and piRNAs, indicating that EV content was able to modify gene expression profile of receiving cells (De Luca et al. 2016). In an in vitro study, mesenchymal derived cells extracellular vesicles comprising anti-inflammatory cytokines such as IL-10, TGF-β, and HLA-G on PB mono-nuclear cells as well as NK cells remote from one patient that were stirred with allo-genetic target cells (Kordelas et al. 2014); for example, a treatment led to in a diminished the release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ as shown in Fig. 10.4. Wang et al. exhibited that UCB-MSC-EVs might stop GvHD in a mouse model of allo-HSCT via modulating the immune responses (Wang et al. 2016).

In a microarray examination, MSC-EVs were transferred to THP-1/bone marrow derived macrophages, in which miR-27a-3p is efficiently expressed in microRNAs in THP-1 cells.

MSC-EVs endorsed M2 polarization that was repressed by lentiviral anti-miR-27a-3p transduction. MSCs work efficiently in the acute lung injury, as well uprose the miR-27a-3p levels in alveolar macrophages and increases the M2 macrophage polarization (Wang et al. 2020). Extracellular vesicles have a great influence on the phenotype and recipient cell function via transferring the bioactive particles from one cell to another cell. Therefor EVs act as biomarker of ailment progression or responding to therapies and have a great potential to work as therapeutic mediators in various hematological diseases (Lia et al. 2020).

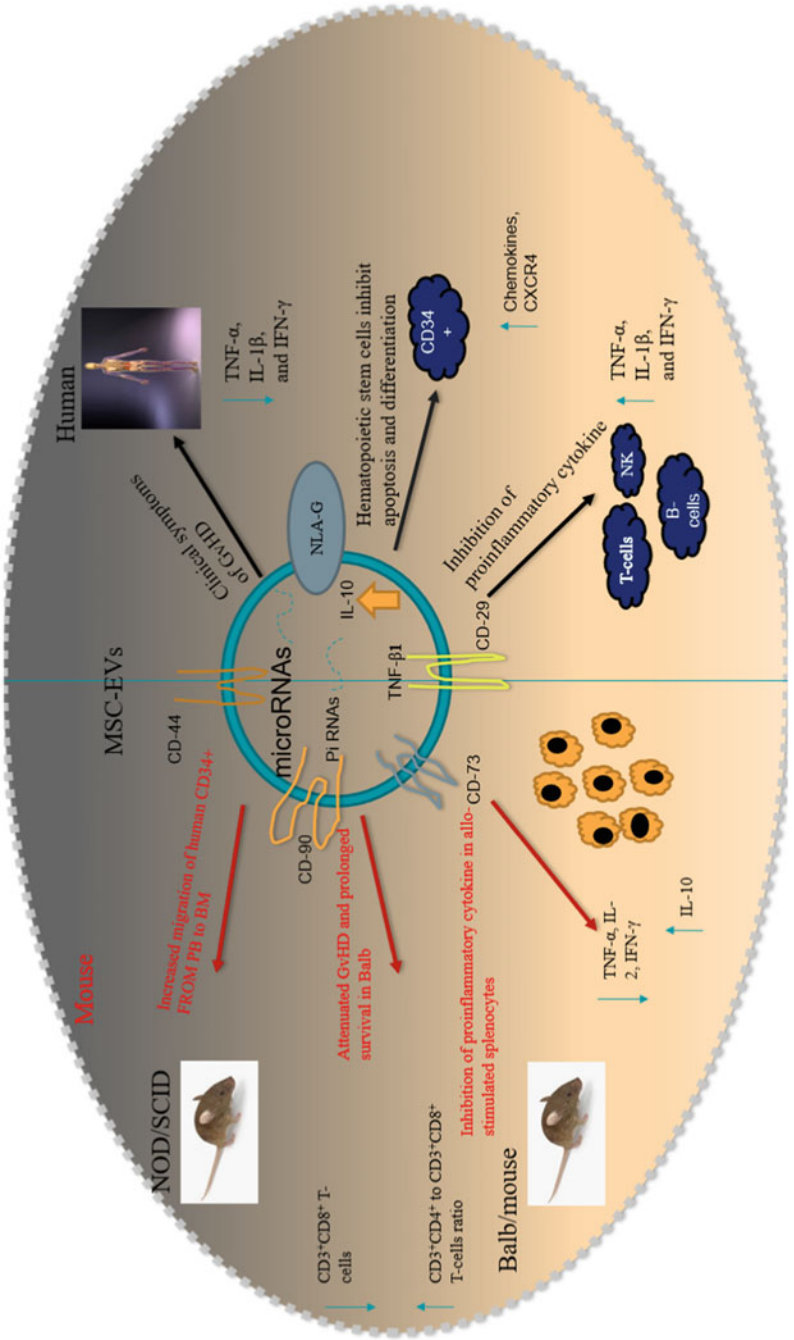

Fig. 10.4 Schematic illustration of the therapeutic potential of MSCs-EVs in HSCT and graft versus host disease (GvHD)

The effect of MSC-EVs on the immune-modulatory effect on adaptive lymphocytes, both the B cell to plasma cell ratio and the proliferation of T cells, appear to be less affected by human MSC-EVs than by intact MSCs both in vitro and in vivo study. The cell-to-cell contact may show a pivotal role in the immune-suppressive potential of MSCs derived from adipose tissue (Conforti et al. 2014; Gouveia de Andrade et al. 2015; Wang et al. 2016). Furthermore, the immune regulatory consequence of human bone marrow-MSC-EVs could differ depending on the framework and on the EV preparation (Kordelas et al. 2014). Additionally, the effect on adaptive immune-cells, MSC-EVs also effect on innate immune responses. Moreover, in vitro experiment exposed that bone marrow-MSC-EVs can indirectly effect on immune-tolerant phenotype in T and B cells by acting as an anti-inflammatory property (Harting et al. 2018).

Extracellular vesicles (EVs) extracted from human embryonic stem cells that show anti-inflammatory activity via M2-polarization, through activation of TLR-dependent signaling in human and murine monocytes model. The expression of anti-inflammatory IL-10 and TGF-β is enhanced due to M2 polarization and a decrease in the pro-inflammatory cytokine level such as IL-6, IL-1β, IL-12P40, and TNF-α response. M2 macrophages can also promote a Treg phenotype in $CD4^+$ T (Li et al. 2016; Ti et al. 2015; Zhang et al. 2014).

In an in vitro experiment bone marrow mesenchymal stem cells-extracellular vesicles in of type 1 diabetes patient show the reduction in expression of CD80, CCR7, and CD8. These monocyte-derived dendritic cells (moDCs) yield high levels of IL-10, TGF-β, IL-6, and PGE_2, thus they contribute great potential to produce an immune suppressant for T cells (Favaro et al. 2016). Bone marrow derived MSC-EVs have ability to change the expression of CD45RA on $CD4^+$ and $CD8^+$ T cells. Moreover, MSC-EVs have potential to increase the IFN-γ production level via $CD4^+$ TE and TEM (Kordelas et al. 2014).

Extracellular vesicles derived from bone marrow mesenchymal stromal cells, the inhibition of EV release led to the accumulation of intracellular miR-10a, that inhibit the cell proliferation, and induced apoptosis in multiple myeloma bone marrow mesenchymal stromal cells (MM-BMSCs) (Umezu et al. 2019).

The conversion of glucose into pyruvate in the cytosol via glycolysis, followed by pyruvate oxidation play an important role in maintenance and differentiation of stem cells. Intestinal organoids are treated with mitochondrial pyruvate carrier inhibitor, as result upsurging the level of proliferation, although due to overexpression of mitochondrial pyruvate carrier it may lead to reduction of stem cell proliferation (Schell et al. 2017).

In another study, protein cargo of extracellular vesicle to their parent mesenchymal stem cells in pig's metabolic syndrome (MetS) and lean controls. The proteomics analysis shows 6690 and 6790 distinct proteins in lean- and metabolic syndrome-EVs, respectively. Differential analysis exposed those 146 proteins were upregulated and 273 were downregulated in lean-EVs versus lean-MSCs, while as 787 proteins were upregulated and 273 downregulated in MetS-EVs versus MetS-MSCs (Eirin et al. 2019).

10.5 Cryopreservation

10.5.1 Advantage

For long run storage of biological constructs for future cryopreservation is oldest and reliable way. Cryopreservation is well-established scientific progress which facilitates reliable storage and transportation of stem cells for recipients (Pegg 2009). This is especially concerning with banking and therapeutic purposes of cord blood. Several purposed advantages of cryopreservation include feasible banking of cells for organ transplantation, enough time for cells storage and supplying search sources all around globe to facilitate scientific research (Gao and Critser 2000; Jang et al. 2017; Mazur 1970). For long-term storage of stem cells, cryopreservation is undoubtedly finest method.

10.5.2 Disadvantage

To search effective theme for stem cell preservation is still face off task for researchers. Low temperature facilitates very low metabolism which has inescapable side effects on osmotic conditions and genetics of cells that could result in reduction in cellular activity (Jenkins et al. 2012). A trained individual is requiring performing process because every step has ability to harm cells if not gone well. There is a possibility of cell damage at each step due to cryopreservation agents (Yong et al. 2017). Contaminations and infections could result in unimaginable results like chromosomal alternations and tumor formation (Hanna and Hubel 2009).

10.5.3 Demand

To develop effective method for stem cell preservation is worthwhile for their versatile lead in near future. The ability of stem cells to treat a variety of diseases necessitates standardization and optimization of process for future usage.

10.6 Chilled Storage

10.6.1 Advantage

For short-term storage an alternative option is chilled storage. Hypothermic temperature is used to store organs until being transplanted and red blood cells are also preserved before transfusion. Chilling allows cells to prepare for transportation by giving them enough time to arrive in clinical destination. In contrast to cryopreservation, cell metabolism slows rather than stops named cell pausing (Jesson et al. 2016). Unlike cryopreservation, these cells do not develop ice crystals or accumulate hazardous solutes. As a result, chilled storage allows for less harmful stem cell

transfer than cryopreservation. Chilled storage does not require complex manipulation skills for management (Jesson et al. 2016; Rafiq et al. 2017).

10.6.2 Disadvantage

One of major issue with chilled storage is cell deterioration due to low metabolic rate. In state of pausing cell begin to produce toxic substances. Different medium additives and stabilizers demand for appropriate facilitation and trained staff. Contamination risk need to be measured before clinical applications (Jesson et al. 2016).

10.6.3 Demand

Before application of chilled storage in practical life post pause cell viability should be strictly checked by train staff for successful transplantation and engraftment.

10.7 Ambient Temperature Pausing

10.7.1 Advantage

Another method of stem cell preservation is ambient temperature pausing. In this technique, cells are assumed to be protected from cold-induced stress owing to closed system. This is admirable technique for pausing cells in manufacturing of cell products (Jesson et al. 2016). Ambient temperature pausing could be potential future technique especially for cells pooling. In cell product manufacturing, cell pooling offers multiple benefits in machine failure and operator difficulties as it is easy to reconsider cells after permissible time. It enables researchers to store cells for days instead of discarding, saving both time and money (Scott et al. 2005).

10.7.2 Disadvantage

The main drawback with this technique is ambient temperature control. Effect of atmospheric CO_2 and temperature must be taken into account as it would result in unsuccessful recovery if not weighed. Anchorage dependence becomes problematic concerning ambient temperature (Jesson et al. 2016).

10.7.3 Demand

Remarkable limitations of preservation protocol demand for quality control examination and standardization and optimization of methods for clinical applications.

10.8 Prospects

In last few decades, stem cells therapeutic solutions have garnered increase amount of attention of researchers. For treatment of deliberating diseases, stem cells have most exciting prospects and especially the hope they offered has evoked life changing expectations in scientific research. Future hold of these remarkable cells is promising and efficient breakthrough in medicine. Regenerative strategies of stem cells shed light on treatment of multiple ailments by making tissue regeneration a reality. Stem cells hold great potential in tissue engineering and replacement therapy and remain an excellent tool for disease modeling. Scope of stem cells research is not only limited to regenerative field but also extends to drug toxicity, biosafety, and metabolic effects of chemicals. Recognizing the growing aspects of stem cells, they can be applied to developmental toxicity, an emerging field of science. Growing burden of diseases direly demands stem cells-based therapies to get reliable and futuristic solutions. Futuristic approaches of stem cells have led to a paradigm shift to produce favorable and high-quality outcomes to reduce burden of diseases. It is not false to say that stem cells are going to produce impressive number of precious scaffolds in future, but it also warrants furthering clarifying long-term safety issues to evaluate efficacy. In future stem cell-based therapies, it will be able to meet our unrealistic expectations.

10.9 Challenges

Although in last few decades considerable advances have been made in field of stem cells therapy but it still awaits further studies to remove practical hurdles prior to their use in clinical settings. As a controversial issue stem cell-based research has provoked significant clinical enthusiasm. Controversial issues of stem cell research not only encompass medical scientific challenges but also include ethical, social, political, economic, and legal challenges. Stem cells research is multidisciplinary field of science with multiple players and fraught which pose serious challenges to their applications. Major part of this debate about stem cell research has centered on public perception. So, to successfully make use of therapeutic potentials of stem cell-based research moral and social issues must engage. For biomedical applications of stem cells serious attention must pay to ethical concerns because policies become failed due to social and ethical concerns. To make use of stem cell research successfully, scientific communities must justify clinical promise with ethical concerns.

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Stem Cell Clinical Trials and Stem Cell Market

11

Arindam Mitra

Abstract

Stem cell therapy requires the usage of stem cells for prevention or treatment of a disease or condition. Stem cell therapies are under investigation in various clinical trials because of multipotent nature of stem cells, an ability of stem cells to mature into cells of diverse types with different functions. Stem cells are useful in therapies for many diseases, many of which have yielded promising results, but only very few got approval from regulatory agencies. More than hundreds of clinical trials are currently in progress targeting a wide variety of disease conditions such as spinal cord injury, cardiovascular disease, blood disorders, diabetes, muscular dystrophy, Alzheimer's disease and many others. Because of an increasing number of clinical trials around the world, there has been a significant growth of stem cell market over the years and the anticipation is that the field will continue to grow in the future. Several key drivers that shape the stem cell market include advances in regenerative medicine, awareness among patient groups, government and industry initiatives to support stem cell research and increase in number of chronic diseases. However, accessibility and use of stem cell therapy should be continuously under vigilance to prevent any misuse of this treatment.

Keywords

Clinical trials · Stem cell market · Stem cell therapy

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F. A. Khan (ed.), *Stem Cell Production*,
https://doi.org/10.1007/978-981-16-7589-8_11

11.1 Introduction

Clinical trials evaluate the effectiveness of a new therapeutic agent. Clinical trials also determine if an existing therapy implemented in a new way is more effective than the previous mode of treatment. In clinical trials, the safety of a new therapeutic agent and method of treatment are also under evaluation. As stem cells regenerate damaged tissues and also provide extracellular growth factors for cellular nutrition and rescue, they are under evaluation for several diseases or conditions in various clinical trials. These trials are opening up the possibility of the emergence of new treatments in the market, particularly where efficacious treatment for a disease or condition is lacking.

Researchers can find information about ongoing, withdrawn and completed stem cell clinical trials at clinicaltrials.gov by National Institutes of Health and also from the corresponding registry of other countries. Many of these trials undergo completion, many are in progress, many withdrawn, and few progressed into more extensive studies. A search for stem cell clinical trials returned 5882 hits in NIH clinical trials website at the time of writing this chapter. Figure 11.1 shows the clinical trials on stem cells as listed in clinical trials. USA conducts highest number of stem cell clinical trials in the world, followed by Europe and East Asia.

Stem cells hold promise to ease or cure the untreatable medical conditions. This demand has increased the need for stem cell clinical trials in the world over the years. Regulatory agencies, clinicians and patients require robust evidence to sustain the disease treatment by stem cells. Randomized controlled trials (RCT) design is

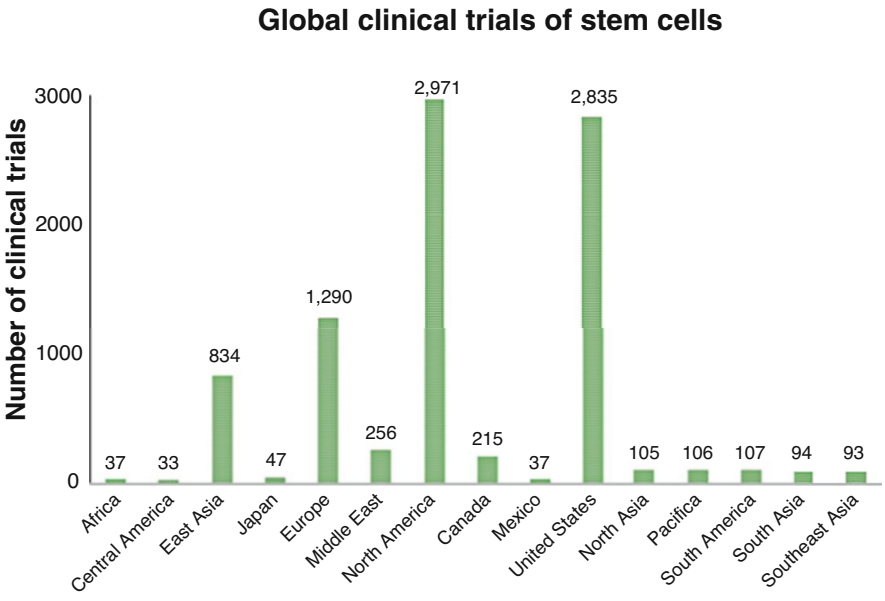

Fig. 11.1 Global clinical trials on stem cells

usually the choice for assessing effectiveness of health care. Such trials usually require a complex, multi-disciplinary approach and are also large scale besides being expensive. Clear guidelines, time period for assessment of subjects and suitable end points are indispensable for design of clinical trials. Stakeholders should thoroughly know the regulations as laid down by regulatory agencies from time to time. Some guidelines restrict the testing of any unproven stem cells outside the controlled clinical trials. All clinical trials must register with registry of clinical trials as applicable for a country.

Before moving into clinical trials, it also required that stem cells go through preclinical studies to address any safety and proof of concept as per regulatory requirements. Such studies involve experiments in vitro and or in suitable animal models. Proper characterization of stem cells is a requirement in preclinical studies and should be like the one to be used in clinical trials. Clinical trials can start only after the clearing safety, efficacy, proof of concept and regulatory approval requirements.

Clinical trials, when done correctly, can identify whether a particular treatment is efficacious. It required a combination of public and private funding to sustain stem cell research and clinical trials. Development of approved stem cell products would also require a substantial time frame as robust evidence and approval requires validation at various steps during the process. Results of few clinical trials have resulted in a tremendous impact in various diseases. Till date, well-established and approved stem cell treatment is bone marrow transplant for blood diseases. Other recent addition in clinical treatment by stem cells includes corneal regeneration and burns. As an example, in case of a junctional epidermolysis bullosa, a lethal genetic disease which can lead to skin cancer exhibited improvement post treatment with culture of epidermal cells (Hirsch et al. 2017). Despite tremendous allocation of resources, time, and limited successes, most trials do not receive approval from regulatory agencies to enter market as stem cell therapy. The approved stem cells for therapies include haematopoietic stem cells, mesenchymal stem cells and limbal stem cells. Figure 11.2 shows the distribution of the stem cells in clinical trials. There has been a significant increase of stem cell clinical trials using MSC, followed by HSC, neural stem cells, bone marrow-derived stem cells and others include limbal stem cells.

11.2 Haematopoietic Stem Cells

Haematopoietic stem cells (HSC) are populations of stem cells that differentiate into blood-forming cells and develop and regenerate blood-forming tissues. HSC are the first stem cells derived from the bone marrow used to build blood and immune systems. These stem cells are the first and only approved stem cell therapy by FDA, USA. Studies on animal models and clinical trials made HSC a practical solution for treatment against various blood disorders since 1945. Stem cell therapy has become the standard of care for bone marrow transplantation and haematological cancers. Currently, over 50,000 HSC transplantation takes place every year, of which

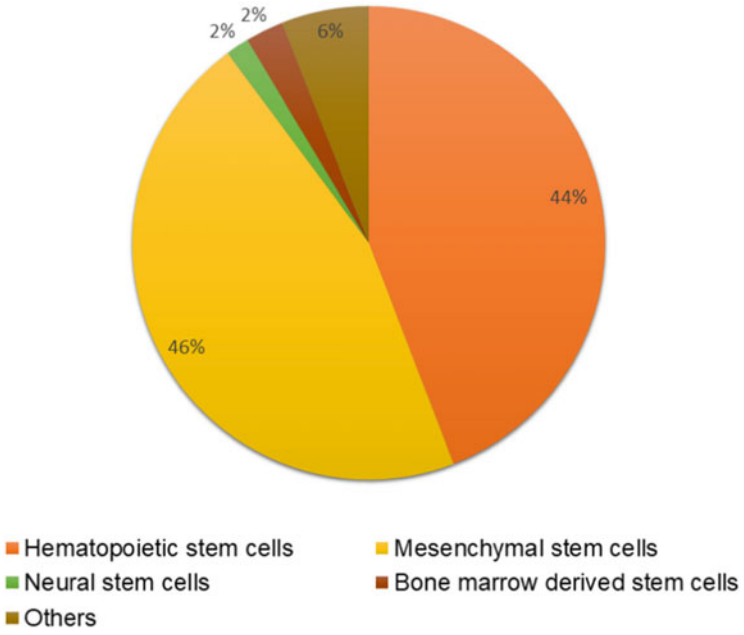

Fig. 11.2 Distribution of stem cells used in clinical trials

one-third are allogenic and two-thirds are autologous. The allogenic procedure is risky and associated with a significant treatment-related mortality and may not be the choice of transplant in most cases. Hosts undergoing allogenic transplants require chemotherapy and/or radiotherapy treatment to suppress the immune system. The graft can additionally result in graft vs. host disease complications as HSC generate immune system cells. Novel techniques for cord blood expansion and other protocols show reduction in graft failure rates in clinical trials (Mehta et al. 2015). Newer approved HSC products rely on cord blood due to lower risk of GvHD, greater tolerance for mismatch of HLA and easier accessibility as compared to allogenic HSCT.

Haematopoietic stem cells can differentiate into any types of red cells, white blood cells, or platelets. These stem cells are easy to isolate from the body either through blood or through adult bone marrow or umbilical cord of the new-borns. Besides, ease of identification, manipulation in the lab and administration of the cells to the patients via injection makes these cells quite popular. HSC can migrate to other locations such as liver, spleen and bone marrow which can benefit disorders other than blood. Today, clinical trials with HSC focus on two objectives. These aims are to reconstruct haematopoietic system and/or to affect other cells and tissues such as heart, liver or nerves. Most of the trials performed in the Europe and the USA focus on reconstitution of the haematopoietic system. Countries other than Europe and North America focus on the second aim of reconstruction of non-haematopoietic

system (Muller et al. 2016). Several newer clinical trials are investigating the use of lentivirus vector to treat blood disorders such as sickle cell disease and thalassemia.

11.3 Mesenchymal Stem Cells (MSC)

Mesenchymal stem cells (MSC) also mesenchymal stromal cells are adult stem cells which find application in various clinical trials (Holan et al. 2021). MSC are capable of regeneration and self-renewal. MSC are multipotent stromal cells, isolation of which can be from various sources, including umbilical cord, placenta, bone marrow, adipose tissue and others. Expansion of MSC in vitro generates high number of MSC cells. These cells are the choice for many transplants as they bypass the ethical issues related to the use of embryonic stem cells. Evaluation of MSC to treat human diseases or conditions relies on improvement of outcomes in animal studies. MSC differentiates into a definite cell type, facilitates survival of cells by release of certain factors, and also by modulation of effector cells (Shammaa et al. 2020). Most widely used source for mesenchymal cells in clinical trials is bone marrow. Other sources of MSC in clinical trials are from adipose tissue and umbilical cord. Clinical trials have established safety profile of MSC over the years. Challenges of using MSC in clinical trials include generation of heterogeneous MSC populations from various sources, variable protocols and passages for preparation of cells. Standardization of these parameters can facilitate comparisons between different clinical trials and help progress use of MSC in various diseases. MSC therapy treats a variety of diseases including graft-versus-host diseases (GVHD), cardiovascular diseases, inflammatory bowel diseases and others. Based on the mechanism of action, the approved MSC products work in two major ways, namely tissue repair and immunomodulation. As MSC are multipotent and can differentiate to cell types such as adipocytes, osteoblasts and others, they find application in approved therapy for trauma and degenerative disease. In fact, a significant number of clinical trials investigate tissue regeneration or repair potential of MSC focusing majorly on degenerative diseases. While several trials currently investigate the role of MSC in repairing cartilage to treat osteoarthritis, other trials investigate the safety and effectiveness of MSC in the treatment of Alzheimer's disease, Parkinson's disease, disc degeneration and others. As immunomodulators, MSC can regulate immune response in a variety of conditions and are useful in autoimmune disease, transplant-related diseases and infectious diseases. Many approved MSC products are currently under investigation in clinical trials for other conditions such as liver disease and acute respiratory distress syndrome caused by SARS-CoV-2. A majority of clinical trials with MSC are investigation of allogenic transplants. While most MSC trials are in phases 1 or 2, few even moved to phases 3 or 4. Recent MSC clinical trials investigate degenerative diseases, cancer and autoimmune disorders (Wang et al. 2021) .

11.4 Limbal Stem Cells (LSC)

Limbal stem cells (LSC) repair aged or impaired corneal epithelial cells and renew epithelial cells of cornea. Attributes of self-renewing ability and generation of transient amplifying cells by LSC maintains a constant number of corneal cells. Damage to the limbal stem cells results in impairment of epithelial cells of cornea and delays wound healing, resulting in a condition, limbal stem cell deficiency (LSCD) (Sacchetti et al. 2018; Barut Selver et al. 2017). While some LSCD attributes to genetics, others are because of burns, injuries or even surgeries affecting cornea. Limbal stem cell transplantation repairs and regenerates the corneal cells and improves vision recovery. Allogenic limbal stem cell transplantation, however, can cause immune reactions and may not be possible because of a shortage of donors. Novel regenerative approaches of different stem cells such as MSC, ESC or iPSC aim to restore vision. Oral mucosal cells are useful for treatment of LSCD and several clinical trials evaluate the effectiveness of this treatment (Oliva et al. 2020).

11.5 Neural Stem Cells

Neural stem cells are useful in several applications in clinical trials, primarily to repair the CNS that is damaged. However, the correct use of neural stem cells may depend on the applications for a treatment against a disease condition. These cells can be a source of neuron cells or astrocyst or chondrocyst (Trounson and McDonald 2015). Clinical trials can evaluate the safety and efficacy of these stem cells. A recent clinical trial has shown that human neural stem cells are safe and show a temporal progression in the treatment of amyotrophic lateral sclerosis (Mazzini et al. 2019).

11.6 Stem Cell Clinical Trials for Diverse Indications

Figure 11.3 highlights stem cell treatment for diverse indications. These indications are cancer, infectious diseases, and autoimmune diseases, non-autoimmune inflammatory diseases, cardiovascular diseases, transplant-related diseases, blood disorders, degenerative disorders and others. As seen in the figure, a significant number of clinical trials investigates the safety and efficacy of stem cells in the treatment of cancer and degenerative diseases. The next section focuses on stem cells clinical trials for treating various diseases or conditions and highlights the progresses made due to these clinical trials.

11.7 Cancer

Haematopoietic stem cell transplantation or bone marrow transplantation is the most common cell transplantation done in the world today. Most aim to treat blood cancers such as leukaemia and lymphoma or genetic condition related to disorders

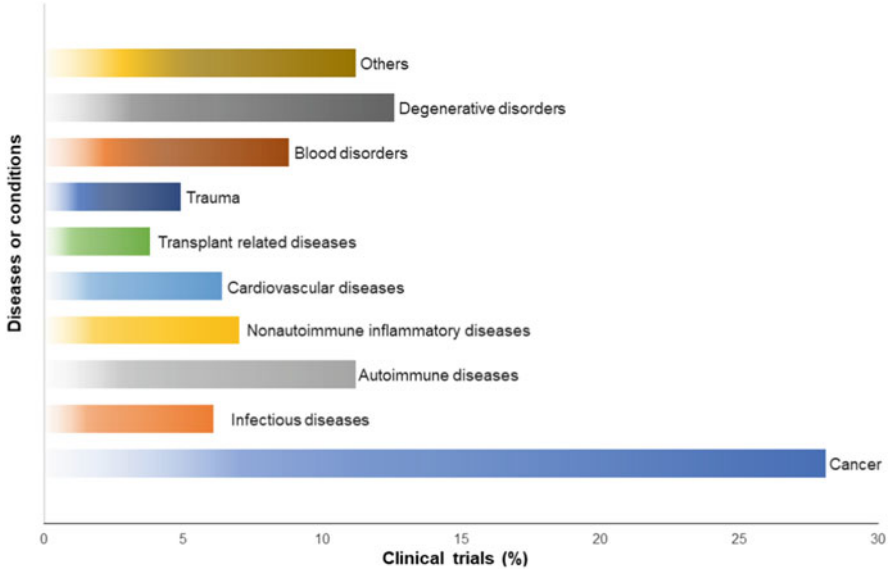

Fig. 11.3 Stem cell clinical trials based on indications

in the haematopoietic system. HSCT is the only stem cell therapy that got approval from FDA. Chemotherapy or radiation post treatment of cancers can damage blood-forming stem cells. Stem cells restore the ability of patients to produce stem cells post radiation or chemotherapy. In a direct manner, stem cells can also induce graft vs. tumour in which patients own white blood cells target cancer cells, improving the outcome. As a standard practice, HSCT can restore this condition in the treatment of various cancers such as leukaemia, lymphoma and myeloma post radiation or chemotherapy. Several trials are investigating the role of HSCT in the treatment of other cancers such as sarcoma, breast cancer and tumour of the brain. However, allogenic transplants in such cases would require the use of anti-rejection drugs to prevent GvHD. MSC infusion can facilitate maintenance and proliferative state of HSC. Recent clinical trials show safety and effectiveness of MSC infusion along with HSCT for treatment of cancers. Many clinical trials have also shown the immunomodulatory roles of MSC in controlling GvHD and adverse effects when combined with HSCT (Chu et al. 2020). Several preclinical trials on MSC show promise to treat cancer (Gomes et al. 2017). Many clinical trials investigate the direct role of MSC in treating cancer. Other trials show excellent safety profile of MSC to treat cancer in children (Melen et al. 2016). Cancer stem cells (CSC) are a subpopulation of cancer cells that are thought to be responsible for tumour formation. Clinical trials are currently investigating the safety and effectiveness of targeting CSC by chemotherapeutic agents. However, further clinical trials will ensure the safety and efficacy of these modalities in cancer treatment.

11.8 Clinical Trials in Heart Diseases

Heart diseases and heart failure are a significant cause of mortality and morbidity around the world. Many clinical trials investigate the use of stem cell therapy to treat heart ailments. Earlier belief was that stem cells could differentiate into cardiomyocyte and possibly repair the damaged heart tissues. However, results showed allogenic transplants could not persist for more than a week within the host. Current thinking is that the beneficial effects of the transplantation result from the paracrine action of the grafted cells, which aid in reducing the inflammatory response (Baraniak and McDevitt 2010). Using newer differentiation protocols for making cardiomyocyte from pluripotent stem cells has significantly improved such transplantation outcomes. The first clinical trial held in France developed cardiac progenitors from human embryonic stem cells for heart failure. In this trial, cells are embedded in a fibrin patch and grafted into the patient's heart near the infarct region (Menasche et al. 2015). Bone marrow-derived stem cells are used for the regeneration of damaged heart vessels and tissues. A systematic review and meta-analyses summarize efficacy of bone marrow-derived stem cell therapy for short term and long term. The authors analysed over 43 randomized clinical trials with 2635 patients and reviewed the effectiveness of the treatment at 6 months, 1 year, 3 years and 5 years post administration of bone marrow cells in acute myocardial infarction patients. A minor improvement in left ventricular ejection fraction is seen at 6 months but not at 3 or 5 year post administration as compared to control groups. However, mortality of the treated patients is reduced at 3- or 5-year period post treatment as compared to a control group (Lee et al. 2016). Induced pluripotent stem cells is also a promising source of cardiac cells because of its ability to contract and form junctions, but they may not survive in the damaged myocardium environment for long and hence may not confer long-term benefits (Seki et al. 2014; Gai et al. 2009).

11.9 Clinical Trials to Treat Neurodegenerative Diseases

Amyotrophic lateral sclerosis (ALS) is a debilitating disease characterized by progressive neurodegeneration affecting brain and the spinal cord, leading to paralysis, respiratory distress and death within 3–5 years post onset (Zinman and Cudkowicz 2011). Stem cell transplantation by MSC, human embryonic stem cells and induced pluripotent stem cells can be most beneficial to treat ALS. Analysis from clinical trials suggests that MSC and neural stem cells are promising treatment for ALS.

Alzheimer's disease (AD) is a progressive neurodegenerative disease of elderly people characterized by memory loss and failure to learn or remember. However, stem cells replacement for treatment of AD can be challenging as the pathology of AD is quite extensive. However, neural stem cell transplantation has shown promise to treat AD and showed reduced loss of neurons, improved cognition and synaptic plasticity in animal studies. Preclinical studies of stem cells usage for AD treatment

had been encouraging. Several clinical trials are currently investigating the possibility of using embryonic stem cells and iPSC derived neural stem cells to treat AD.

11.10 Clinical Trials in Spinal Cord Injury

Spinal cord injury (SCI) is a damage affecting any part of spinal cord and usually result from trauma. Damage to the spinal cord can cause temporary or permanent loss of motor, autonomic and sensory functions of the spinal cord with severe neurological deficiency or disability (Ahuja et al. 2017). Regenerative approaches using stem cells might restore motor functions and transformative for patients in these conditions. Clinical trials for therapy against spinal cord injury or amyotrophic lateral sclerosis are under investigation. Early phase clinical trials investigate the safety of such treatments in SCI patients. Mesenchymal stem cells are often the choice for treatment of SCI because of its paracrine activities. Secretion of several bioactive molecules such as VEGF, HGF and GM-CSF from MSC support persistence of damaged neurons and regeneration of axons. Besides MSC also prevent inflammatory response and thus reduce further damage to the existing tissues (Liau et al. 2020). Bone marrow, umbilical cord and adipose tissues are the choice of tissues for isolation of MSC. Clinical trials of treatment of MSC derived from bone marrow failed to improve the neurological functions of the patients, but improved some sensory and motor functions in these trials. Other trials investigate the delivery of MSC by scaffolds or combination with immunotherapy. A trial on adipose derived MSC is exploring the safety and effectiveness of the autologous MSC derived from adipose tissue with advanced SCI. This trial known as CELLTOP investigates the autologous mesenchymal stem cells derived from adipose tissue to treat paralysis because of SCI (Bydon et al. 2020). The intrathecal administration of adipose derived MSC seems to be safe and exhibits improvement in motor and sensory scores, as per preliminary results of the trial. However, this outcome warrants further validation.

11.11 Clinical Trials in Cerebral Palsy

Cerebral palsy (CP) is one of the devastating diseases affecting children in early life. Children and individuals with CP usually have motor impairments, which vary according to levels of severity, of which physical disability is the most common problem. Stem cell therapy could be an excellent choice in CP as in this disease, brain damage is restricted to a specific area and is not progressive and seen in only a few cell types. Stem cell treatment can potentially regenerate, replace or repair brain cells and differentiate them into new microglial cells or astrocytes. Stem cells might also induce an anti-inflammatory response which can also reduce the damage to white matter. It can also promote cell survival by releasing neurotrophic factors and forming new blood vessels (Novak et al. 2016). Stem cells used in clinical trials to treat this disease are usually human umbilical cord blood or umbilical cord or bone

marrow cells. The source of stem cells can be autologous or allogeneic for the treatment of CP. Bone marrow stem cells are considered more effective in treating cerebral palsy than bone marrow mononuclear stem cells. While neural stem cells, regarded as ideal stem cells for the treatment of CP, are used in few trials, these cells have specific ethical concerns. Peripheral blood cells have also shown promising results in one of the studies. More clinical trials are under investigation to determine the most optimal stem cell treatment regimen for CP (Lv et al. 2021). Routes of administration of these stem cells vary from intranasal routes, the least invasive, while the most intrusive being intracranial with side effects. Intrathecal and intravenous routes are also in use for stem cell administration. A lack of a suitable animal model in CP treatment remains one of the challenges in stem cell trials. However, stem cell therapy for CP is promising, and many clinical trials are currently in progress. Typical outcomes of stem cell treatments in CP determine any significant improvements in gross motor functions and cognitive functions.

11.12 Clinical Trials of Stem Cells for Treatment of Diabetes

Type I diabetic patients are offered islet transplantation, in which islet cells are isolated from the pancreas of deceased donors and introduced into the diabetic patient via the portal vein. Several of these cells can survive in the liver and can function in the long term. As these are allogenic transplants, patients should also take anti-rejection drugs to prevent any autoimmune response from the host. This technique was quite successful in the clinical scenario and resulted in significant improvement in the quality of life of diabetic patients, particularly following Edmonton protocol. This protocol, developed by James Shapiro and his colleagues in 2000, introduced steroid-free anti-rejection drugs, use of xeno-free protein media while isolation of islets, and rapid transplantation of islets from donors. While this protocol improved the insulin independence of all patients for a year, only 11% of those exhibited insulin independence for more than 5 years. Modification of this protocol reduced the adverse reactions post transplantation, restoration of hypoglycaemia awareness in T1DM and severe hypoglycemic events; however, only 25% of the patients with islet transplantation do not require insulin after 5 years of treatment. Survival of cells, rejection of allogenic grafts and lifelong immunosuppression are challenges in islet transplantation.

Several trials also explore the possibility of mesenchymal stem cells to serve as a protector and source of beta cells. These cells test the ability of MSC to form functional mature beta cells or organoids; however, the validation of in vitro and in vivo was not substantial. Beta cells derived from human embryonic stem cells can reduce the dependency of cadaveric islets and can also serve as an economical alternative to Insulin. These cells have moved into phase I and phase II clinical trials. However, as they are allografts, immunosuppression would still be required. The challenge of being on lifelong immunosuppression drugs was addressed by encapsulating the graft preventing the immune cells or antibodies while allowing the

glucose, insulin as well as oxygen and nutrients. Such restricted permeability allows grafts to survive and function without being damaged.

A clinical trial completed by researchers at the University of Miami using a combination therapy of intrapancreatic Autologous Stem Cell (ASC) infusion with Hyperbaric Oxygen Treatment (HBO) has shown a reduction in plasma glucose and Hb1Ac with an increase in C-peptide release. Several clinical trials are also studying diabetes by somatic cell nuclear transfer. Here, the DNA of the patient is transferred to a stem cell, which develops to become an insulin-producing cell. Such insulin-producing cells can study development, malfunctioning, and how beta cells work at a molecular level and knowledge of this can develop cures for diabetes (Yamada et al. 2014).

11.13 Clinical Trials of Stem Cells for Treatment of Eye Disorders

Allogenic corneal grafts from cadavers are employed to improve or restore vision in patients with corneal damage. Over 100,000 corneal grafts every year improve the quality of life for patients. Currently, in a technique known as CALEC (cultivated autologous limbal epithelial cell transplantation), patients with corneal burns in one eye undergoes successful treatment with limbal stem cells from the cornea in another eye. CALEC enables patients to see in both eyes, dramatically improving the quality of life in these individuals (Nurkovic et al. 2020). A clinical trial investigates the safety and the feasibility of CALEC transplantation to treat limbal stem cell deficiency (Jurkunas 2016). Another trial explores the efficacy of corneal stem cell transplantation to treat dry eyes (Rush 2019). Holoclar, an autologous limbal stem cell therapy to treat ocular burns, received an approval in the European Union only after bone marrow transplantation (Dolgin 2015). However, in case of damage to both eyes, allogenic transplantation would be the only choice with immunosuppressive therapy.

11.14 Clinical Trials of Stem Cells for Infectious Diseases

Infectious diseases are one of the leading causes of morbidity and mortality around the world. Stem cells, particularly MSC, are being explored as a treatment against infectious diseases including HIV, influenza and hepatitis. MSC modulate the immune responses of diverse cells of immune system and immunomodulatory role of MSC is currently under investigation in multiple clinical trials as a potential treatment for many infectious diseases with immune triggers (Harrell et al. 2019). MSC was useful for treatment of GvHD following an allogenic transplant because of its role as modulator of immune response. Besides, MSC possess anti-microbial properties and they secrete molecules with microbial inhibitory activities. Stem cell therapy against COVID-19 was initiated in China and later received FDA emergency approval. There were 25 reported clinical trials which investigated the efficacy of MSC to treat various infectious diseases, of which 13 were explored for the treatment

of viral diseases from 2010 to 2020 (Sleem and Saleh 2020). Allogenic transplantation of HSC lacking CCR5, a chemokine receptor used by HIV for entry, has shown to have positive outcome in the treatment of HIV (Gupta et al. 2019).

11.15 Challenges of Stem Cell Clinical Trials and Therapy

Adequate stem cells can be difficult to isolate in case where rare stem cells are used. Allogenic transplantation can be a source of risk of mismatch between donor and the recipient in contrast to autologous transplantation. Immunosuppressive therapies are used in case of allogenic transplant as body's own immune cells can target non-self-cells with different HLA marker. Immunosuppression for prolonged periods of time can have undesirable side effects to the patients. Toxic effects of the immunosuppressive drugs can also eventually cause loss of function of allogenic grafts. However, induced pluripotent stem cells and personalized stem cells can bypass immunosuppressive strategies during allogenic transplant.

For organ transplantation in clinical trials, sometimes there could be a dearth of donors and this may limit the treatments to only those who receive it. Depending upon the phase of clinical trial, a patient may or may not receive the actual therapy, as they could fall under a placebo group or might receive a meagre amount of the treatment in phase I clinical trials, which may not provide any therapeutic effect at all. Patient claims of apparent effects might alter the perception of the actual effect after stem cell therapy.

Clinical trials can be expensive, involving many animals and human subjects. It usually requires collaboration and extensive networking among scientists, industry, government and investors. Besides, stem cells are physiologically heterogeneous, and the route of administration or the mode of delivery is undefined most times. Regulatory approvals, criterion for cell therapy production and release, agreement of regulations between ethics committee and agencies are other challenges for a rapidly developing country like India, Brazil, China and others (Viswanathan et al. 2013). Other challenges include ethical concern during isolation of embryonic stem cells in the lab. The isolation of ESC could lead to destruction of embryos, which might develop into human. Tumours might develop post stem cell therapy and tumorigenicity of a treatment should be one of the safety criteria. These challenges and uncertainties are bottlenecks in stem cell clinical trials.

11.16 Stem Cell Market

Stem cell market has been growing over the years (Syed and Evans 2013). The increase in growth is because of the increasing number of preclinical studies and clinical trials being conducted around the world. The United States conducts the most significant number of clinical trials globally and represents the fastest-growing markets in North America. The Asia Pacific represents the highest growth rate. In 2020, the global market for stem cells was more than USD 10 billion and expected to

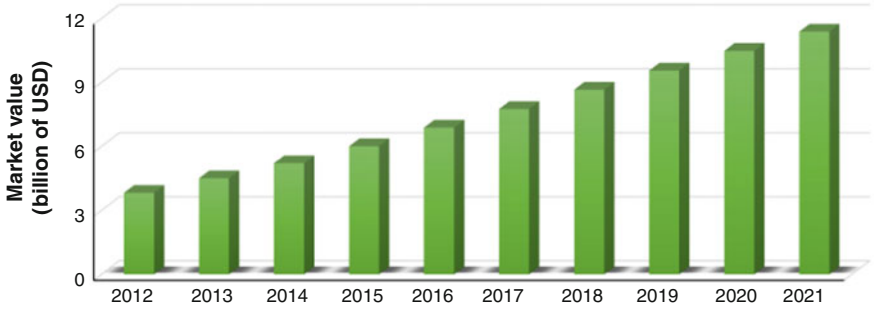

Fig. 11.4 Growth of global stem cells market (2012–2021)

exceed USD 27 billion by 2028. The stem cells market value in the USA alone is approximately USD 5.66 billion. The US market is expected to grow to USD 15 billion by 2025. The compound annual growth rate (CAGR) is expected to be around 12%. The steady growth of global stem cells market during 2012–2021 period is shown in Fig. 11.4.

Several key drivers control the growth of stem cell market. These include increase in degenerative disease in elderly patients, increased demand for biologic, increased use of allogenic stem cells for the treatment for leukaemia and other disorders, worldwide increase of trauma and burn cases, increased availability of stem cells in the clinics and availability of research funding. Clinical translation of stem cells is increasing due to pressure from the patient groups to find a cure for many untreatable diseases. Other stakeholders such as industrialists and policymakers are also keen on receiving their return of significant investments on stem cell research and trials. Besides, researchers aim to find a novel treatment for many currently untreatable diseases or conditions.

The usage of stem cells in the market is increasing as stem cell therapy has the potential to be transformative in many situations where a proven medical treatment is lacking. A significant concern for the stem cell market is the lack or failed governance to control unauthorized use of stem cells as the market continues to grow. But this promise of stem cells also gave rise to stem cell tourism, where a potential customer can receive unapproved stem cells of unproven efficacy via online mode from unregulated stem cell clinics (Miller 2011). Many profit-making stem cell clinics are taking advantage of the potential surrounding the stem cells and sells unproven stem cell-based intervention to consumers. Such unproven stem cells usage can have a negative outcome and poses risks to patients most times (Veceric-Haler et al. 2021; Julian et al. 2020; Brown 2012). Because of this emerging concern, the International Society for Stem Cell Research (ISSCR) has recently revised guidelines for stem cell research and clinical translation to ensure safety, ethical and regulatory standards required of stem cell research (Lovell-Badge et al. 2021). Growing sectors within stem cell market are regenerative medicine, particularly dermatology and also in cancer treatment. Stem cell banks are also growing due to support from local government. Few stem cell-based products,

such as Holoclar, are already in the market after receiving approval from the regulatory agencies (Pellegrini et al. 2018). Based on the results of clinical trials, few more will gain eventual approval and enter the stem cell market.

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