Stem Cells in Clinical Applications

Phuc Van Pham *Editor*

Stem Cell Processing

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Preface

The term "stem cell" appeared in the scientific literature as early as 1868 in the work of the eminent German biologist Ernst Haeckel. In this work, Haeckel used the term Stammzelle ("stem cell") to describe the ancestor unicellular organism from which he presumed all multicellular organisms evolved. Particularly, he also suggested fertilized oocytes as the source giving rise to all cells of the whole body. In 1892, Valentin Hacker described stem cells as the cells that later produce oocytes in the gonads. Then, this term becomes more popular with some experimental results in developmental biology. Some studies about nuclear programming in the 1900s showed that adult cells can become pluripotent stem cells, and pluripotent stem cells can differentiate into all specialized cells from three germ layers. The first successful study about epigenetic reprogramming was performed by John Gurdon in 1962 in the African clawed toad, *Xenopus laevis*. He could produce healthy and sexually mature fertile frogs by nuclei transplantation from differentiated cells. Therefore, he and Shinya Yamanaka shared a Nobel Prize in Medicine or Physiology in 2012. Besides pluripotent stem cells, the multipotent stem cells also were detected and isolated in the adult, so-called adult stem cells. Adult stem cells such as hematopoietic stem cells and mesenchymal stem cells are the essential source of stem cells in an adult that play the important roles in tissue homeostasis, wound healing, and tissue regeneration after injuries. These discoveries suggested that stem cell transplantation can help to regenerate the injured tissues. And stem cell therapy, as well as regenerative medicine, was formed from these observations.

The first autologous stem cell transplant was undergone by Dr. E. Donnall Thomas in 1957; he later received the Nobel Prize in Medicine in 1990 for this achievement. The clinical application of hematopoietic stem cells rapidly grew from the 1990s to date. From the 2000s, some other adult stem cells including mesenchymal stem cells, limbal stem cells, epidermal stem cells, and neural stem cells were used in the clinic. In recent years, embryonic stem cells, as well as pluripotent stem cells (induced pluripotent stem cells), also were permitted for use in some clinical trials.

The clinical application of stem cells broke out since the 2000s when some countries approved some stem cell-based therapies and stem cell-based products. To date, stem cells including both adult stem cells and pluripotent stem cells were clinically used in more than 50 different diseases and medical conditions. More than ten stem cell-based therapies, as well as stem cell-based products, were approved as routine treatments in some countries.

Therefore, the *Stem Cells in Clinical Applications* series brings some of the field's most renowned scientists and clinicians together with emerging talents and disseminates their cutting-edge clinical research to help shape future therapies. While each book tends to focus on regenerative medicine for an individual organ or system (e.g., the liver, lung, and heart, the brain and spinal cord, etc.), each volume also deals with topics like the safety of stem cell transplantation, evidence for clinical applications including effects and side effects, guidelines for clinical stem cell manipulation, and much more. Volumes also discuss mesenchymal stem cell transplantation in autoimmune disease treatment, stem cell gene therapy in preclinical and clinical contexts, clinical use of stem cells in degenerative neurological disease, and best practices for manufacturers in stem cell production. Later volumes will be devoted to safety, ethics and regulations, stem cell banking, and treatment of cancer and genetic disease.

This volume, *Stem Cell Processing*, presents some significant sources of stem cells for clinical applications. Mainly, this volume also introduces some new techniques to collect and expand stem cells with GMP guidelines so that these obtained cells can be used in the clinic.

In the first edition of this volume, ten chapters will focus on the recent hot topic about some accepted and approved clinical applications of stem cells (Chapter One) and some clinical trials and approved products from mesenchymal stem cells (Chapter Two). The techniques for isolation and expansion of mesenchymal stem cells are also provided in Chapters Six, Seven and Ten. In this volume, some effects of aging and senescence on mesenchymal stem cell properties are also suggested in Chapters Three and Four. Some recent efforts in clinical usages of pluripotent stem cells are discussed in Chapter Four, with some concerns covered in Chapter Nine.

Many people have contributed to making our involvement in this project possible. We are extremely thankful to all of the contributors to this book. Many people have had a hand in the preparation of this book. We thank our readers, who have made our hours putting together this volume worthwhile. We are indebted to the staff of Springer Science+Business Media that published this book.

Ho Chi Minh City, Vietnam Phuc Van Pham

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About the Editor

Phuc Van Pham received his Ph.D. in human physiology from Vietnam National University, Ho Chi Minh City, Vietnam. He is currently a professor of biology at Vietnam National University and acting director of the Laboratory of Stem Cell Research and Application. He is a long-standing lecturer and translational scientist at the university and is a member of several societies and journal editorial boards focused on stem cells.

Dr. Pham and his colleagues have established one of the first multidisciplinary stem cell centers in Vietnam, and he has successfully launched an array of technologies in stem cell isolations. His research interests include stem cell isolation, stem cell therapy, mesenchymal stem cells, cancer stem cells, immunotherapy, and regenerative medicine, and he has published extensively in these areas.

After many years of experience as an embryologist, cell biologist, and molecular biologist, collaborating with leading researchers in Singapore, Japan, and the United States, Dr. Pham is a student again, keen to reach beyond the traditional boundaries of biology.

Chapter 1
Stem Cell Therapy: Accepted Therapies, Managing the Hope of Society, and a Legal Perspective

W.M. Botes, M. Nöthling Slabbert, M. Alessandrini, and M.S. Pepper

1.1 Background

There is little doubt that stem cells hold great promise for the treatment and cure of many diseases. Along with this promise however comes great expectation, which should be managed cautiously if the true potential of the stem cell research is to be realized. Several misconceptions persist in society in general and to an extent among the medical fraternity. These misconceptions, coupled to irresponsible clinical practices, have resulted in exploitation of vulnerable patients. Guidelines and legislation are being developed globally with the aim of providing ethically sound reference material for patients, practitioners, and regulators. However, as with most developing frameworks, a lack of regulatory cohesiveness often results. An overview of the current global regulatory framework is provided, including anticipated legal developments and recommendations.

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1.1.1 Basic Principles of Stem Cell Biology

A stem cell has the unique ability to differentiate into the various cell types of the body, while simultaneously replicating to maintain a stem cell lineage. Different forms of stem cells exist, each with varying capacity or potency. Potency refers to the ability of a stem cell to replicate and differentiate into different cell types. When the female egg cell is fertilized by the male sperm, a zygote is created. The zygote is referred to as a totipotent stem cell, which by definition has the potential to develop into the entire human body, including the placental tissues required for the early developmental stages of the embryo and fetus. After several cycles of replication, the zygote develops into a blastocyst, which consists of an outer layer of cells and an inner cell mass. The outer layer develops into tissues of the placenta, while the inner cell mass develops into the embryo. Embryonic stem cells (ESCs) are derived from the inner cell mass and are referred to as pluripotent stem cells. These cells have the ability to form any cell type of the human body, with the exception of cells of the placenta. Adult stem cells are multipotent and are present in various adult tissues and organs. They possess a renewal and regenerative capacity which is generally limited to the tissue within which they reside. The best known examples of multipotent stem cells are (1) hematopoietic stem cells (HSCs), which give rise to all of the cellular components of the blood; (2) mesenchymal stem cells (MSCs), which are able to develop into the bone, cartilage, muscle, and fat; and (3) neural stem cells (NSCs), which develop into cells of the nervous system. There is a large research community investigating the biology and therapeutic potential of stem cells. However, with the exception of HSC transplantation, the clinical benefit of stem cell therapies is yet to be demonstrated.

1.1.2 Clinical Applications of Stem Cell Therapy

The only globally accepted form of stem cell therapy is the use of HSCs for transplantation purposes—a practice that is successfully being applied in nearly 80 countries around the world. Of the more than 60,000 HSC transplants that take place globally per annum, the vast majority (>80 %) are for treating hematological malignancies, namely, acute and chronic leukemia, both Hodgkin's and non-Hodgkin's lymphoma, and the plasma cell disorders (mostly multiple myeloma). Secondary to these indications is the treatment of solid tumors, while nonmalignant conditions include bone marrow failures, hemoglobinopathies, and primary immune disorders. In all cases, the HSCs are used to replenish blood cells that are depleted in a chemotherapy regimen administered prior to the transplantation. In addition to these indications, there is a movement toward using HSCs for regenerative purposes, such as for treating cerebral palsy, autism, and type I diabetes. These approaches are still experimental, and sufficient evidence for their routine use is yet to be provided.

The potential benefit of using MSCs to treat a variety of conditions has gained significant interest in recent times. The primary reasons for this trend include the fact that these cells (1) can easily be procured from sources such as the bone marrow, umbilical cord blood, and adipose tissue; (2) have the unique ability to home to the site of injury once administered; and (3) do not require genetic matching when obtained from a donor (as is the case with HSCs). A large number of indications are being treated with MSCs at present, and based on our investigations of the clinical trial landscape, it is possible to cite over 120 different indications that have been treated in this setting. These include cardiovascular diseases such as myocardial infarction, cardiomyopathy and heart failure, neurological disorders such as multiple sclerosis and amyotrophic lateral sclerosis, and musculoskeletal conditions such as fracture non-unions and cartilage defects. To date however, only one MSC product has successfully achieved market approval from regulatory authorities, namely, Prochymal (developed by Osiris Therapeutics and acquired by Mesoblast Ltd. in Australia), which was approved in Canada and New Zealand for the treatment of graft versus host disease, a complication of HSC transplantation.

1.2 Controversies and the Consequence of Providing Unproven Stem Cell Therapies

The stem cell controversies of the past two decades have originated from the use of ESCs for medical research. Given that a fertilized embryo is destroyed in order to derive these cells, albeit in the laboratory setting with donated embryos, such research is deemed unacceptable by many and understandably has resulted in ethical debate. More recently, however, the use of unproven stem cell therapies and the subsequent emergence of a "stem cell tourism" industry have become a controversial issue in its own right. In such cases, vulnerable patients are enticed to travel abroad to dubious stem cell clinics and are subjected to unproven stem cell therapies at their own expense. Given the unique properties of MSCs and the ease with which they can be prepared from fat tissue, they have become the most attractive product/service on offer at a large number of so-called stem cell clinics around the world. The most concerning aspect is the extensive list of diseases that these clinics claim to treat. Although over 100 indications are being treated in the clinical trial setting, clinical benefit has been demonstrated in very few (with the exception of the previously mentioned Prochymal preparation).

1.2.1 Reference to Pertinent Issues and Controversial Reports

Clinics offering many different kinds of stem cell treatments for a wide range of diseases and conditions available for direct purchase through online market places have been established around the world, not only in developed countries such as the

USA and various European countries but also in newly industrialized countries such as China, India, and Mexico (Lau et al. 2008). While it was thought that the majority of clinical trials involving stem cells take place in the USA, the majority of trials involving MSCs currently take place in East Asia and Europe (Clinicaltrials.gov 2015), but the official number of these trials may be skewed as a result of underreporting. It should further be noted that the entry of any clinical trial information into a register does not imply endorsement of the trial by the regulatory authority in the country or region in which the trial is taking place.

With increasing global stem cell activity, further increases in offers for stem cell treatments are inevitable, and with different regulatory regimes in each country, the marketing, administration of stem cell treatments, and general management of society's hope are causes for concern (Caulfield et al. 2012). Even though the fact that potentially fraudulent treatments are being offered for a large number of conditions and that this is receiving increased attention, marketing practices not only remain unchanged but claims have actually escalated (Ogbogu et al. 2013). This situation leads increasingly to so-called scienceploitation and stem cell tourism where evidence suggests that the majority of people, desperate after finding out that conventional medicine offers no available treatment, travel mostly from developed countries to developing countries with no, poor, or more liberal regulations regarding stem cells, in an effort to access stem cell treatments (Regenberg et al. 2009). Major destinations are China, India, Mexico, Germany, and the Dominican Republic, which primarily treat conditions such as blindness, paralysis, multiple sclerosis, cerebral palsy, and brain injuries (Levine and Wolf 2012). Most of these treatments are still unproven and unauthorized, lacking testing of efficacy and safety, and thus pose a threat to people's lives, health, and emotional and financial well-being.

Stem cell tourists spent an average of $20,000–$50,000, travel expenses excluded, on clinically unproven treatments in 2014 (IOM and NAS 2014). Some received stem cells from animals such as sheep or rabbits, and stem cells were injected subcutaneously, intramuscularly, intravenously, via lumbar puncture, or into the subdural space during spinal surgery (Pepper 2009). Complications involving stem cell treatments include tumor growth (Amariglio et al. 2009), multiple autoimmune diseases (Bohgaki et al. 2007), meningitis (Mendpara et al. 2002), angiomyeloproliferative lesions (Thirabanjasak et al. 2010), and bone fragments growing in a patient's eye after cosmetic surgery (Jabr 2012).

From 2002 until 2006, Biomark International defrauded individuals suffering from amyotrophic lateral sclerosis (Lou Gehrig's disease), Parkinson's disease, muscular dystrophy, multiple sclerosis, and other incurable diseases by making false representations "…that science had proven the therapeutic power of stem cells and that Biomark was simply making it available to the world." (*United States of America v. Laura Brown and Stephen Mark van Rooyen* 2006). Under these pretenses, every patient was injected with the same type and quantity of stem cells, regardless of the disease they were suffering from, and charged between $10,000.00 and $32,000.00, if not negotiated otherwise. In 2006 Laura Brown and Stephen Mark van Rooyen, the directors of Biomark, were criminally indicted. During their hearing, the court also found that Biomark's website and advertisements made

numerous false, misleading, and inaccurate statements and that the proffered information had no scientific credibility. It further found that the stem cell treatments were illegally administered without a biologics product license (Public Health Services Act, Section 262(a)(1) 2011) and that licensing was very unlikely as preclinical trials in this regard only involved nonhumans. None of the patients undergoing these treatments were cured and many even died during the course thereof (Mahomed and Nöthling Slabbert 2012).

Medical tourism in this context can broadly be divided into three categories (IOM and NAS 2014):

1.2.1.1 According to the Legal Status of the Treatment

Some treatments are illegal in the patient's home country but legal in the destination country, a medical tourism style known as *circumvention tourism*, which includes abortion, assisted suicide, and stem cell treatments. Sometimes stem cell treatments might not necessarily be illegal in a patient's home country, but simply unavailable due to the fact that they are not yet approved.

1.2.1.2 According to Who Is Paying for the Treatment

In some cases, patients are paying from their own funds, but increasingly large insurers in the USA and Australia pay for medical tourism packages to nationals who are looking for lower-cost options elsewhere in clinics with guaranteed safety and quality (Parnel 2013). However, insurers in the USA typically refuse payment for experimental or investigational treatments unless clinical safety and effectiveness have been proven.

1.2.1.3 According to Where Patients Are Traveling for Treatment

Patients may travel from one developed country to another developed country, from a developed country to a less developed country, or from a less developed country to a more developed country.

However, progressing from basic research to clinical research to eventual translation thereof is a long, laborious, and expensive process with an increase in the number of patients at every successive stage, which means a similar increase in costs and risks. However, the majority of stem cell clinical trials are in the early stages, enrolling only a small number of patients (Trouson et al. 2011). The translation of stem cell therapy will only be safely and effectively achieved through international collaboration, including the sharing of research information to improve global public health. Advances in science and technology will facilitate the development of safe and effective biological products, thereby advancing regulatory science and research and managing organizational excellence and accountability (CBER 2011).

Considering the global fluency of patients seeking stem cell treatments in countries with differing regulatory regimes, ethical beliefs, and societal values, it is important to consider the ethical, legal, and social issues which inform these regulatory frameworks and policies.

1.2.2 Ethical and Legal Concerns

The main ethical issues in somatic stem cell research concern the sources and sourcing of stem cells, moral status of the human embryo, informed consent, reproductive as opposed to therapeutic cloning, and the clinical use of stem cells.

1.2.2.1 Sources and Sourcing of Human Stem Cells

Although stem cells are usually sourced from noncontroversial sources such as the bone marrow, umbilical cord blood, and adipose tissue, to which the below-discussed ethical and legal issues do not necessarily apply, this section will specifically deal with the controversial sources producing ethical and legal debate.

Human ESCs are mainly sourced from embryos arising from infertility treatment cycles, embryos created specifically for research purposes, somatic cell nuclear transfer (SCNT), and cadaveric fetal tissue.

hESCs are derived from the inner cell mass of a human blastocyst which is formed 5–7 days after fertilization. The inner cell mass of the blastocyst is destined to become all of the tissues of a human body after the trophoblast of the blastocyst becomes placental tissue upon successful implantation (Patil 2014). The destruction of the human embryo during extraction of hESCs is therefore inevitable and therefore the source of much ethical and legal debate regarding the moral status and legal personhood of the embryo.

Moral and Legal Status of an Embryo

At the one end of the spectrum is the belief that an embryo is created by God from the moment of conception and from then constitutes a person with the same moral rights as any adult human (Doerflinger 1999), while alternative views share the belief that the embryo acquires complete personhood and its accompanying rights in gradual stages during the process of development from conception to birth (House of Lords Select Committee on Science and Technology 2002). The latter view has found general social, ethical, and legal favor. At around 14 days after fertilization, the primitive streak of an embryo is formed when a thickening of the surface, indicating the first organization of the embryo, takes place. This has been suggested as a key cutoff point after which research involving embryos is prohibited. Up to 14 days the blastocyst has no central nervous system, a further landmark for the

definition of life, since a nervous system implies the possibility of sensation (Fishbach and Fishbach 2004). The early embryo up to this point was termed the "pre-embryo" in 1985, and notwithstanding arguments that this term was merely invented to justify embryonic research, the 14-day limit has generally been accepted and adopted in various jurisdictions globally (Mulkay 1997).

Although the Universal Declaration of Human Rights (UDHR) stipulates that all human beings are born free and equal in dignity and rights (UDHR, Article 2), the term "born" was used to exclude the fetus and embryo from the human rights granted in this declaration. Arguments to amend the UDHR by deleting this term were proposed but rejected (Copelan et al. 2005). Even the Convention on the Rights of the Child (CRC, Article 6) only recognizes the right to life from birth (Copelan et al. 2005). Although the US Supreme Court has never ruled on the constitutional status of human embryos outside the body, it has ruled that fetuses are not persons within the meaning of the 14th Amendment and accordingly have no constitutional rights (Robertson 2010). This ruling will presumably also extend to embryos, but although the American Convention on Human Rights (ACHR 1969, Article 4) stipulates that every person's right to life must be respected, that this right must be protected from the moment of conception, and that no one shall be arbitrarily deprived of his life, the Inter-American Commission on Human Rights clarified that this right is not absolute (Center for Reproductive Rights undated). In the matter of *Paton v United Kingdom* (1980), the European Commission on Human Rights held that the language of Article 2(1) of the European Convention on Human Rights (ECHR) which provides that "Everyone's right to life shall be protected by law" (ECHR Article 2) does not include the unborn and acknowledged that the recognition of an absolute right to life before birth would be contrary to the object and purpose of the said convention. In *Vo v France* (2004), the European Court of Human Rights affirmed that the unborn child is not regarded as a "person" directly protected by Article 2 of the ECHR and that if the unborn child does have a right to life, such right is implicitly limited by the mother's rights and interests. However, these cases refer specifically to unborn children in utero as opposed to embryos outside the human body (*Roe v Wade* 1973; *Planned Parenthood v Casey* 1992).

However, on 18 December 2014 in the patent matter of *International Stem Cell Corporation v Comptroller General of Patents, Designs and Trade Marks* (2013), the Court of Justice of the European Union ruled that embryos created through parthenogenesis, being unable to develop into human beings and having only one set of DNA, do not qualify as a human embryo having regard to the definition thereof contained in the European Parliament and Council's Directive 98/44/EC (1999) regarding the legal protection of biotechnological inventions dated 6 July 1999, and were therefore patent eligible.

The debate about the moral status of the embryo is not regarded as an ethical or legal one only. There is an obligation to do everything possible to alleviate the suffering of existing human beings, and if hESC research is the method to such a means, there is a moral duty to pursue it (Nuffield Council on Bioethics 2000).

Embryos Arising from Infertility Treatment

Although an embryo created for a reproductive technology program has been created with the view to implantation in the uterus and a successful pregnancy, it has no further use or future if it is not implanted. Rather than being discarded, spare embryos can be donated and used to derive stem cells (Thompson 1995). In most jurisdictions it is illegal to incentivize embryo donations and for the donors to have any financial stake in any materials or procedures developed from such donation (Corrigan et al. 2006). The commercialization of embryos as well as cadaveric fetal tissues are also banned (European Group on Ethics in Science and New Technologies to the European Commission 2002). The opinion has also been held that with the establishment of immortal cell lines, the need for further embryos by means of donation may become unnecessary and that the ethical questions surrounding issues in this regard may only be "transitional" until such time as a sufficient supply of stem cells from such lines can be secured (Nuffield Council on Bioethics 2000).

Embryos Created Specifically for Research Purposes

Embryos found unsuitable for IVF and donated after completion of a reproductive technology program will routinely be discarded, and the view exists that the derivation of ESC from such embryos will thus not affect their final disposition. But embryos created through in vitro fertilization (IVF) with the sole purpose to produce cell lines are essentially treated as a means to an end, and this does not accord with the respect owed to a potential human life. However, schedule 2 of the UK's Human Fertilization and Embryology Act (HFEA 1990) permits the creation of embryos for the specific research purposes as set out therein if a research project cannot be carried out on donated embryos. This situation is kept under review in the UK as the opinion exists that while there are sufficient donated embryos from IVF treatments, there are no compelling reasons to allow the creation of additional embryos merely for the sake of increasing the number of embryos available for research purposes (Nuffield Council on Bioethics 2000). The Council on Human Rights and Biomedicine, on the other hand, prohibits the creation of human embryos for research purposes, but not hESC research based on supernumerary or excess IVF embryos (Council of Europe 1997). Embryos created deliberately for research purposes remain a contested issue, and this is also closely linked to funding regulations for stem cell research.

Sourcing of Oocytes for the Creation of Embryos

Donation of oocytes involves hyperstimulation of the ovaries by hormone injection followed by surgery to harvest the produced oocytes. This process is painful and carries significant risks. Donation of oocytes to help infertile couples is considered to be an altruistic gesture, and money paid in this regard is seen as compensation for

the inconvenience, discomfort, or incurred expenses relating to the donation, not as payment for the oocytes. It is interesting to note that the Human Fertilization and Embryology Authority now allows limited compensation to oocyte donors in respect of lost earnings (Human Fertilization and Embryology Authority 2005).

In February 2004, Dr Hwang Woo-suk and his South Korean colleagues reported on their successful cloning of 30 human embryos from which they extracted stem cell lines (Hwang et al. 2004). In May 2005 Dr Hwang and his colleagues announced that they managed to make 11 patient-specific cell lines by using donated eggs and DNA from people suffering from diabetes and spinal cord injuries (Hwang et al. 2005). Not only did these papers contain fraudulent data, necessitating the authors to retract the articles, but the sourcing of oocytes for research purposes also raised serious ethical concerns (Normile et al. 2006; Kennedy 2006). It transpired that many of the oocyte donors suffered hyperstimulation syndrome resulting from donation (Chong 2006) and that some of the eggs were donated by junior female researchers that were part of the research team, pointing to possible coercion by senior investigators in the same team (Normile et al. 2006). This incident again raised issues of informed consent and compensation for oocyte donation.

A suggested alternative solution to the shortage of ova and the potential ethical problems involving donation is to use ova from other species in the creation of stem cells by means of nuclear replacement (Holm 2002). This technique for the creation of so-called hybrids or chimeras, organisms with a mixture of cells from two or more genetically distinct species, has been patented by the American firm Advanced Cell Technology (1998), but has been received with skepticism (Marshall 1998). One of the main arguments in favor of the creation of chimeras or hybrids is that many necessary stem cell experiments are ethically and legally prohibited from being performed on humans. Experiments involving chimeras or hybrids are subject to ethical and legal guidelines involving the use of animals in research activities (Knowles 2010).

Cadaveric Fetal Tissue

The acceptability of using fetal tissue to derive embryonic germ cells (EGC) is closely tied to the ethical acceptability of abortion. Pluripotent cells, derived from the blastocyst, have the potential to develop into any of these cell types in the body. Because these cells are derived from the embryo, they are called embryonic stem cells (ESCs). If these cells are derived from the region destined to develop into sperm and eggs, known as primordial germ cells in the fetus, they are called EGCs. Although attempts to derive adult cells from EGCs in mice have led to abnormalities and research is currently focused on ESCs, the Polkinghorne Review suggested that consent for the use of donated fetal tissue for the purpose of deriving EGC be reconsidered (Nuffield Council in Bioethics 2000).

A major concern is that abortions would then be sought with the primary objective of donating cadaveric fetal tissue in return for possible therapeutic of financial

benefits, considering the scientific and commercial value of cell lines that could be created from EGCs. It was suggested that great care be taken in these circumstances to separate any decisions regarding the abortion and the subsequent use of the fetal tissue and to prohibit the donor from specifying how the donated fetal tissue must be used (Review of the Guidance on the Research Use of Fetuses and Fetal Material 1989). However, this suggestion to prohibit inappropriate incentives for fetal donation contradicts standard guidelines for obtaining informed consent which requires that a mother must be adequately informed, in a comprehensible manner, to enable her to make an informed decision whether or not to allow the fetus to be used for research purposes and the extent of the research to be conducted on the fetus. The Polkinghorne Review recognized that although women might be willing to consent to certain research activities, they might not consent to all (Review of the Guidance on the Research Use of Fetuses and Fetal Material 1989). The National Bioethics Advisory Commission's (NBAC) report recommended the prohibition of directed donation of cadaveric fetal tissue for EGC derivation, to prevent an expectation of inappropriate incentives and that no direct therapeutic benefits resulting from the production of such tissue or abortion are received (National Bioethics Advisory Commission 1999).

Although issues regarding incentives may be addressed sufficiently by separating decisions pertaining to abortion and the use of aborted fetal tissue, specific consent is still necessary when an immortal cell line is to be produced from the aborted and donated fetal tissue, as is the case with donated embryos (Nuffield Council on Bioethics 2000).

Somatic Cell Nuclear Transfer (SCNT)

Pluripotent cells produced by reprogramming the nucleus of a somatic cell have the potential to produce tissue that would allow autologous transplants of specific tissue types with the benefit of lowered risk of rejection of these tissues by the recipient's immune system (Tashibana et al. 2013). Producing cells via this method requires oocytes, which raises concerns similar to those mentioned above in section "Sourcing of Oocytes for Creation of Embryos."

1.2.2.2 Therapeutic Versus Reproductive Cloning

If implanted into a uterus, an embryo created by SCNT has the potential to develop into a human being and thus raises issues of human reproductive cloning. The outcome of using this technique is the production of a genetically identical copy of the person from whom the nucleus was taken and transferred into the somatic cell. This may lead to potential eugenic uses by creating offspring with desired genetic traits or enhanced characteristics, which invites a plethora of ethical and legal issues, including diminished human individuality and integrity, limitations on a person's

freedom, dignity and identity, and human reproductive autonomy. However, human cloning is currently banned globally (United Nations Declaration on Human Cloning 2005). If researchers plan to use SCNT, they should clearly distinguish between therapeutic and reproductive cloning when defining their research goals. Reproductive cloning currently has no therapeutic purpose.

Therapeutic cloning on the other hand promises the in vitro development of replacement cells and tissue for purposes of clinical treatment. Some of the greatest concerns involving therapeutic cloning are that researchers may attempt to circumvent the current global prohibition to clone human beings or the uncertainty whether or not a sufficient therapeutic effect will be realized through therapeutic cloning (Sui 2013).

1.2.2.3 Informed Consent in the Context of Embryos or Fetal Tissue

Informed consent is the foundation of all scientific research and protects donors of embryos or fetal tissue. As discussed in section "Cadaveric Fetal Tissue," issues of consent for abortion and the use of the donated fetal tissue for research purposes may be separated, but specific consent is still necessary when an immortal cell line is to be produced. In this regard the HFEA's Code of Practice for IVF clinics states in addition that when informed consent is sought for the use of embryos in stem cell research, donors must be appropriately informed, which includes being informed that they may withdraw their consent and that stem cell lines may continue indefinitely and be used in different research projects (Human Fertilization and Embryology Authority 2003). These guidelines recommend that IVF clinics obtain consent from donors regarding their wishes with regard to the fate of their surplus embryos. Criticism of this recommendation is that couples may not be in the best position to carefully assess the implications of donating their embryos due to physical and psychological stress brought about by the infertility treatment process itself and may feel pressurized to donate their embryos or feel overwhelmed by the informed consent process (Throsby 2002). In contrast, the American Society for Reproductive Medicine recommends that consent for embryo donation should only be sought once IVF treatment has ceased for whatever reason (Ethics Committee of the American Society for Reproductive Medicine 2002).

In an effort to standardize informed consent by creating a core set of information sheets and consent forms on stem cell research, the UK Stem Cell Coordinators have formed a national group, the human Embryonic Stem Cell Coordinators Organization (hESCCO), to inform and assist with obtaining formal informed consent (Franklin and Cornwell 2005).

Considering the traceability of immortal stem cell lines, the privacy and confidentiality of donor information and the reporting of incidental findings to the donor during research activities are complex issues that should also be addressed during the informed consent process (Aalto-Setälä et al. 2009).

1.2.2.4 Clinical Use

The translation of stem cell research has raised further ethical issues of which the most prominent is the patient's exposure to health safety risks. Inherent risks of stem cell treatments include risks related to stem cells differentiating into undesirable tissue types, such as tumorous tissue and the transmission of viruses or pollutants which can cause secondary disease (Prockop and Olson 2007).

1.2.3 Impact on Societal Perceptions

Patients may experience frustration at not being able to access potentially effective stem cell treatments, but at the same time governments are obligated to protect their nationals from unproven, ineffective, and possibly dangerous treatments and fraudulent advertising in this regard. An analysis of patients' online blogging reveals that patients often visit dubious stem cell clinics for therapeutic purposes because they feel that they have no other choice and that they cannot afford to wait for stem cell treatments to be approved through traditional regulatory processes (Ryan et al. 2010). A great concern is the lack of information given to patients regarding the reason for the existence of regulatory processes or the value thereof (Master et al. 2013). A proactive approach to the education of patients and innovative methods of communicating important information regarding stem cell research and translation are needed to properly educate the public to enable them to make informed choices, failing which the public will be infused with false hope based on yet to be proven scientific possibilities.

1.3 Stem Cell Therapy as Biological Medicine

1.3.1 Product Categorization and Registration Requirements

Although it is highly unlikely that *all* stem cell products will fit comfortably into a single category, stem cells, with their very unique qualities and abilities, could qualify as either drugs, biologics, devices, or combination products. It has been argued that stem cells could in some circumstances qualify as "drugs" in terms of the Federal Food, Drug, and Cosmetics Act (FD&C Act Sec. 201 2006), considering that stem cells might generate proteins in large quantities such as the protein interferon which was regarded as a drug by the court in the matter of *Moore v Regents of the University of California* (1990), or may be used as a "device" to deliver other products to the right place in a patient's body (Munzer 2012). In most jurisdictions, stem cell-based products are categorized as biological products or biologics, which are subject to regulatory approval and registration before they may be marketed

(Public Health Services Act, Section 262(i) 2011). Similar to vaccines, stem cells can be therapeutically used to reconstitute or strengthen a patient's immune system, and stem cells derived from umbilical cord blood also qualify as blood products (Munzer 2012). To use stem cells therapeutically also requires that cells be delivered to specific locations in the body which may require the use of a device to aid the cells in this regard, which yields another form of combination product (FDA 2010). It is clear that the categorization of stem cells is challenging and further complicated by external factors such as cryopreservation which raises concerns about their stability and requires safeguards pertaining to pre-freeze and post-thaw preservation (Center for Biologics Evaluation and Research (CBER) 2008). Stem cells can support the growth of pathogens and are unable to be sterilized. When delivered to sensitive areas such as the central nervous system, this may further complicate their categorization (Barinaga 2000).

In December 2014, the Food and Drug Administration (FDA)—the American regulatory authority—released two draft guidance documents (FDA 2014a, b), describing its view on the preparation of MSCs from fat and their use in patients. In essence, these draft guidelines recommend that MSCs are to be regarded as biological drugs, meaning that the provider/manufacturer will have to prove benefit in the clinical trial setting before they will be reviewed and considered for marketing approval by the FDA. If this recommendation informs the FDA's decisions in this regard, no clinic in the USA will be authorized to offer unproven MSC products. It is anticipated that regulatory authorities in other major markets will follow suit, particularly the European Union.

1.3.2 Minimal Manipulation Requirements

Many biotechnology companies try to fast-track stem cell-based treatments to market without the prescribed regulatory approval or registration by arguing that their stem cell-based products are minimally manipulated and do not fall within the ambit of such regulations. A similar argument was proffered in the Regenexx case in which the classification of stem cell therapy as biological medicine with regard to autologous stem cell therapy, where a patient's own stem cells are administered back to the same patient after having been processed, cultured, mixed with other therapeutic substances, stored, or even cryopreserved, was challenged (Jordaan 2012). In this case the FDA claimed that the autologous stem cell-based substance produced using the Regenexx procedure not only qualifies as a "biological product," but the offered cell treatments were more than minimally manipulated and therefore fall within the regulatory ambit of the FDA and need FDA approval and registration (United States of America v. Regenerative Sciences 2014). The FDA subsequently ordered its developers, Regenerative Sciences, to stop offering their unapproved biological drug product.

1.4 Global Legal Positions

Below is a summary of the regulatory approaches of some countries active in the field of stem cell research (Table 1.1).

Table 1.1 Stem cell research: regulatory approaches

Jurisdiction	Permit creation of human embryos for research, including SCNT	Prohibit creation of human embryos, including SCNT	Permit derivation of hESC lines from excess IVF embryos	Prohibit derivation of hESCs	Prohibit derivation of hESCs, but permit importation of hESC lines
Australia (Research Involving Embryos Act 2002)	√		√		
Austria (Reproductive Medicine Act of 2 June 1998)		√		√	
Belgium (Research on Embryos 2003)	√		√		
Brazil (Bio-Safety Law 2005)		√	√		
Bulgaria (Convention on Human Rights and Biomedicine 1997)		√			
Canada (Canadian Institutes of Health Research 2007)			√		
China (Ethical guiding principles on human embryonic stem cell research 2003)	√		√		
Costa Rica (Law 7739 of 1998)		√		√	

(continued)

Table 1.1 (continued)

Jurisdiction	Permit creation of human embryos for research, including SCNT	Prohibit creation of human embryos, including SCNT	Permit derivation of hESC lines from excess IVF embryos	Prohibit derivation of hESCs	Prohibit derivation of hESCs, but permit importation of hESC lines
Denmark (Medically Assisted Procreation in Connection with Medical Treatment, Diagnosis and Research 1997)		√	√		
Finland (Medical Research Act 1999)	[a]	[a]	√		
France (Law on Bioethics 2013)		√	√		
Germany (Protection of Embryos 2008)		√		√	√
Greece (Medically Assisted Human Reproduction Law 2002)		√	√		
Iceland (Artificial Fertilization Regulation 1997)		√	[a]		
India (Indian Council of Medical Research 2012)	√		√		
Iran (Ethical guidelines on human embryonic stem cell research and therapy 2011)	√		√		
Ireland (Irish Council of Bioethics 2008)		√		√	
Israel (Prohibition of Genetic Intervention Law 1999)	√		√		

(continued)

Table 1.1 (continued)

Jurisdiction	Permit creation of human embryos for research, including SCNT	Prohibit creation of human embryos, including SCNT	Permit derivation of hESC lines from excess IVF embryos	Prohibit derivation of hESCs	Prohibit derivation of hESCs, but permit importation of hESC lines
Italy (Regulation of Medically Assisted Human Reproduction Law 2004)		√		√	√
Japan (Guidelines for the derivation and utilization of human embryonic stem cells 2001)	√		√		
Latvia (European Commission 2005)			√		
Lithuania (Law on Ethics of Biomedical Research 2000)		√		√	
Mexico (General Health Act 1984)	√		√		
Netherlands (Embryos Law 2002)		√	√		
New Zealand (Guidelines for using cells from established human embryonic stem cell lines for research 2006)		√	√		
Norway (Application of Biotechnology in Human Medicine Act 2003)		√	√		
Poland (Law of 7 January 1993)				√	
Portugal (Procriação medicamente assistida de 26 de Joelho 2006)		√[b]			

(continued)

Table 1.1 (continued)

Jurisdiction	Permit creation of human embryos for research, including SCNT	Prohibit creation of human embryos, including SCNT	Permit derivation of hESC lines from excess IVF embryos	Prohibit derivation of hESCs	Prohibit derivation of hESCs, but permit importation of hESC lines
Russian Federation (Law on the Temporary Prohibition of Human Cloning 2002)	[a]	[a]			[a]
Saudi Arabia (Fatwa edict 2003)		√	√		
Singapore (Human Cloning and other Prohibited Practices Act 2004)	√		√		
Slovakia (Health Care Law 1994)		√[b]			
Slovenia (Law on Biomedically Assisted Fertilisation 2000)		√	√		
South Africa (National Health Act 2003)	√		√		
South Korea (Bioethics and Safety Act 2008)	√		√		
Spain (Biomedical Research Law 2007)	√		√		
Sweden (Genetic Integrity Act 2005)	√		√		
Switzerland (Stem Cell Research Act 2003)		√	√		
Taiwan (Department of Health. Policy instructions on the ethics of human embryo and embryonic stem cell research 2006)	√		√		

(continued)

Table 1.1 (continued)

Jurisdiction	Permit creation of human embryos for research, including SCNT	Prohibit creation of human embryos, including SCNT	Permit derivation of hESC lines from excess IVF embryos	Prohibit derivation of hESCs	Prohibit derivation of hESCs, but permit importation of hESC lines
Tunisia (Law 01-93 2001)		√		√	
United Kingdom (Human Fertilisation and Embryology Act 2008)	√		√		
USA (variation of laws within states)	No federal ban on hESC research, but restrictions on federal funding before 9 March 2009; federal funding only permitted for non-hESC research or those using hESC lines in existence prior to 9 August 2001. President Obama's Executive Order lifted these restrictions for research involving new hESC lines. No federal funding under 2009 NIH Guidelines for research that creates human embryo for research purposes or destroys a human embryo (human cloning) (National Academy of Sciences 2009)				
Arkansas (Arkansas Code 2010)		√			
California (Business and Professions Code)	√		√		
Connecticut (Public Act 2005)	√		√		
Illinois (Stem Cell Research and Human Cloning Prohibition Act 2007)	√		√		
Iowa (Iowa Code 2007)	√		√		
Maryland (Maryland Stem Cell Research Act 2006)	√		√		
Massachusetts (Acts of 2005)	√		√		
Michigan (Michigan State Constitution 1963)		√	√		
Montana (Montana Code Ann 2009)	√		√		

(continued)

Table 1.1 (continued)

Jurisdiction	Permit creation of human embryos for research, including SCNT	Prohibit creation of human embryos, including SCNT	Permit derivation of hESC lines from excess IVF embryos	Prohibit derivation of hESCs	Prohibit derivation of hESCs, but permit importation of hESC lines
New Jersey (New Jersey Revised Statutes Title 2C:11A-1)	√		√		
Oklahoma (Oklahoma Statute)		√		√	
Virginia (Virginia Code Ann 2001)		√[a]	√		

Source: Adapted from Nöthling Slabbert and Pepper (2015)
[a]Legal position unclear, e.g., whether permitted or prohibited
[b]No specific legislation regarding hESC research

1.5 International Harmonization Efforts

1.5.1 A Need for Harmonization

Globally, policies and legislation regulating stem cell research and therapy are complex and varied, aptly described by some authors as "a patchwork of patchworks" (Caulfield et al. 2009a, b), being shaped by different social, religious, cultural, economic, historical, ideological, and political factors informing national laws and policies. Despite these differences, which suggest that international binding instruments or policies may be difficult to achieve, the setting of enforceable ethical and professional norms and standards, as well as professional, quality, and safety standards, seems to be an achievable goal.

A range of international and regional instruments, guidelines, and regulations, directly or indirectly related to biomedical research, exist. The United Nations Educational, Scientific and Cultural Organization (UNESCO), the Council of Europe, and the European Union, for example, have all addressed aspects of stem cell research and its clinical applications through various reports, treaties, resolutions, declarations, and guidelines. In addition, nonbinding codes of practice, guidelines, and recommendations by international organizations, such as the Council for International Organizations of Medical Science (CIOMS 2012), the Hinxton Group (2015), the International Consortium of Stem Cell Networks (ICSCN 2015), the International Stem Cell Forum (ISCF 2015), and the International Society for Stem Cell Research (ISSCR 2015), provide a useful resource for national policy makers. Efforts to promote international cooperation, as well as harmonizing and standardizing processes for stem cell banking, include those by the International Stem Cell

Banking Initiative (ISCBI 2015) coordinated by the UK Stem Cell Bank, the ISSCR's Registry of Provenance of Human Embryonic Stem Cell Lines (Registry of Provenance of Human Embryonic Stem Cell Lines 2013), the International Stem Cell Registry (UMASS 2015) (ISCR 2015), and the European Human Pluripotent Stem Cell Registry (hESCreg 2015).

Reference is often made to the need for harmonized legal or ethical standards, without a clear understanding of the concept and objectives of harmonization. Harmonization, which strives to achieve compatibility, is often confused with standardization, which is aimed at unification. Harmonization may assist with producing consensus guidelines, or alternatively, with converging different regulatory approaches. In this regard, harmonization has resulted, among others, in informal cooperation agreements; common technical, safety, and quality standards and requirements; as well as mutual recognition agreements (MRAs). Some of the broad goals of harmonization include: (1) promoting and safeguarding global public health, (2) promoting and facilitating the availability of safe and effective products, (3) promoting the development of more effective product standards, and (4) minimizing inconsistent standards. Harmonization in the field of cellular therapy, in addition to that of stem cell research, is equally important, as cell therapy as an emerging product class poses difficult regulatory challenges, different regulatory frameworks do not display the same level of maturity, and experience in reviewing marketing applications for cell therapy globally is limited. Harmonization of technical requirements will be an important tool for strengthening the safe and effective use of cell therapy products.

The scope of this chapter does not permit an exhaustive discussion of harmonization efforts in this field. The focus will instead be on some of the key efforts, including the most significant trends that have emerged from these harmonization activities.

1.5.2 Consensus Principles

From existing guideline documents and codes of conduct relating to stem cell research and therapy, referred to above, consensus exists with regard to the following principles:

1. The prohibition of reproductive cloning
2. The prohibition of gene-editing research in human embryos (ISSCR 2015)
3. Respect for human autonomy (expressed, e.g., in the provision of free and informed consent by research participants, gamete or tissue donors, and patients)
4. Respect for human dignity, inviolability of the human person, privacy, and confidentiality (expressed, e.g., in prohibitions relating to research on embryos beyond 14 days after fertilization; measures aimed at the protection of donor identity and the traceability of cell lines)
5. The prevention of conflicts of interest (e.g., by separating informed consent for research donation from clinical treatment)

6. The need for independent peer review and approval of research protocols by research ethics boards or research ethics committees, including ongoing monitoring of the research
7. The need for observing professional codes of conduct; clinical, safety, technical, and quality assurance standards; as well as protecting and promoting scientific integrity generally (e.g., by complying with standards relevant to quality, derivation, and maintenance of cell lines)
8. The need for risk-benefit assessments
9. The non-commercialization of human tissues
10. The need to consider issues of social and distributive justice and beneficence (acknowledged in the need to improve and protect health and ensure equitable access to research and the benefits resulting from research)

1.5.3 Issues of Difference

Issues on which regulatory and policy approaches vary more clearly are the following:

1 The creation of human embryos for stem cell research.
2 The legal and moral status of the human embryo.
3 Creation of embryos via somatic cell nuclear transfer (SCNT).
4 The derivation of stem cell lines from excess IVF embryos.
5 Intellectual property issues (e.g., variance in legal approaches regarding the patenting of human embryonic stem cells across jurisdictions, notably between Europe, the USA, and Japan) (Mathews et al. 2013). Despite broad agreement on the principles referred to in Sect. 5.2 above, jurisdictional variation is evident with regard to a range of activities, such as requirements for research funding, research governance mechanisms, ethics review processes, quality assurance, and access to stem cell material and data (Caulfield et al. 2009a, b). There are also disparities with regard to donor eligibility requirements and the suitability of stem cell lines for use in clinical trials and subsequent commercialization (Feigal et al. 2014). The legal regulation of human tissue (governing the procurement, use, and disposal of human tissue) and tissue establishments across jurisdictions is also diverse. Harmonization attempts are furthermore hampered by ambiguity and uncertainty with regard to regulatory discrepancies, such as stem cell product classification (e.g., novel stem cell products may exhibit characteristics which could label these as either or both medicines and medical devices).

The promotion of ethical and responsible stem cell research requires a delicate balance between minimizing overregulation, while still at the same time ensuring adequate protection of research subjects (Isasi and Knoppers 2011). In this regard, the recommendations of the Hinxton Group (2010) regarding data and material sharing, as well as intellectual property issues, should be noted. The Group recommends the establishment of a centralized and comprehensive hub for accessing global stem cell registry and stem cell patent information, as well as incentives for data and materials sharing globally.

1.5.4 US Food and Drug Administration (FDA) and the European Medicines Agency (EMA)

Increased collaboration between the EMA and the FDA has led to concerted efforts to converge regulatory activities among international regulators in the field of cell therapy.

1.5.4.1 FDA-EMA-Health Canada ATMP Cluster

In 2008, the US FDA and EMA established the Advanced Therapy Medicinal Products (ATMP) Cluster, with Health Canada joining in June 2012. Its aim is to provide, among others, a platform for discussing different regulatory approaches and for sharing information on draft documents.

1.5.5 International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH and the International Medical Device Regulators Form (IMDRF), which replaced the Global Harmonization Task Force (GHTF), are two important international task forces formed specifically for the purpose of promoting global harmonization. The ICH is comprised of industry and regulatory agency representatives from the USA, Europe, and Japan (along with several nonvoting observers, such as Canada and Australia). It has developed common guidelines on safety, efficacy, quality, and multidisciplinary issues in the field of pharmaceutical regulation. The ICH has not formulated any guidelines specific to stem cell-based products, but its guidelines on biotechnology products provide some direction (e.g., Q5A (RI); Q5B; Q5D; Q6B; Q7, Q8, Q9 and Q11).

1.5.5.1 ICH (Regulators Forum Cell Therapy Group (RFCTG) and Regulators Forum Gene Therapy Group (RFGTG))

The Regulators Forum (RF) consists of the ICH members (US FDA, EMA, and Japan PMDA/MHLW), ICH observers (Canada, European Free Trade Association (EFTA), WHO), regional harmonization initiatives (APEC, Association of the Southeast Asian Nations (ASEAN), Southern African Development Community (SADC), Gulf Cooperation Council (GCC), Pan-American Network for Drug Regulatory Harmonization (PANDRH)), as well as individual drug regulatory

authorities (e.g., from Australia, Brazil, China, India, Russia, and Singapore). The purpose of the RF is to provide a platform for the sharing of regulatory and scientific expertise among regulatory authorities in order to enhance the availability of safe and effective products in the global market. In March 2011, members of the RF founded RF Cell Therapy Group (RFCTG) with the objective of mapping out potential areas for convergence in the regulation of cellular therapy products, informed by the view that an understanding of similarities and differences in global regulations and policies relating to cellular therapy products is critically important in identifying common ground for future regulatory convergence activities. The FDA proposed at the RF held in November 2010 in association with the ICH that the RF consider undertaking a preliminary assessment of potential topics of regulatory importance for cellular therapy products (Arcidiacono et al. 2012). The RF Gene Therapy Group (RFGTG) constitutes a forum for discussing novel technologies.

1.5.6 International Medical Device Regulators Forum (IMDRF)

Cognizance should also be taken of the role of the International Medical Device Regulators Forum (IMDRF), a voluntary group of medical device regulators from across the world, which replaced the Global Harmonization Task Force in 2011. Its aim is to accelerate international harmonization with regard to the regulation of medical devices (IMDRF 2015).

1.5.7 Asia-Pacific Economic Cooperation/Life Sciences Innovation Forum (APEC/LSIF)

APEC, established in 1989 to promote and facilitate trade among the Asian Pacific countries, founded the Life Sciences Innovation Forum (LSIF 2002) in 2002. The purpose of LSIF, a tripartite forum of the government, industry, and academia, is to create a policy environment for life sciences innovation (LSIF 2002). At the 2011 workshop on stem cell product quality assurance and control, APEC/LSIF initiated a process of identifying technical requirements for stem cell products for clinical use. One of the primary objectives of the APEC/LSIF Regulatory Harmonization Steering Committee is to identify international standards and guidelines to propose to APEC members. In 2012, this committee identified the promotion of regulatory convergence for the regulation of cell- and tissue-based therapies as a priority work area, starting with a harmonized understanding of cell- and tissue-based therapies via training programs.

1.5.8 Alliance for Harmonization of Cellular Therapy Accreditation (AHCTA) and the Foundation for the Accreditation of Cellular Therapy (FACT)

AHCTA and FACT both play an important role in developing harmonized standards with regard to cellular therapy. AHCTA commits itself to the harmonization of global standards with the aim of creating a single set of quality, safety, and professional requirements for cellular therapy, including hematopoietic stem cell (HSC) transplantation, addressing all aspects of the process, from assessment of donor eligibility to transplantation and clinical outcome. Represented by, among others, the American Association of Blood Banks (AABB), American Society for Blood and Marrow Transplantation (ASBMT), European Federation for Immunogenetics (EFI), European Group for Blood and Marrow Transplantation (EBMT), Foundation for the Accreditation of Cellular Therapy (FACT), International Society of Cellular Therapy (ISCT) (Europe), Joint Accreditation Committee ISCT and EBMT (JACIE), and the World Marrow Donor Association (WMDA), AHCTA released a position paper in 2006, revised in 2008, entitled "Towards a Global Standard for Donation, Procurement, Testing and Distribution of HSC and Related Cellular Therapies" (AHCTA 2008).

FACT, a nonprofit corporation cofounded in 1996 by the International Society for Cellular Therapy (ISCT) and the American Society of Blood and Marrow Transplantation (ASBMT) for the purposes of voluntary inspection and accreditation in the field of cellular therapy, focuses on developing standards for high-quality medical and laboratory practice in cellular therapies. These standards are developed by expert committees consisting of clinicians, scientists, technologists, and quality experts that cover the entire spectrum of cell manufacturing (FACT 2015).

1.5.9 Future Global Regulation

The rapidly evolving fields of stem cell research and therapy require regulators and policy makers to provide clear and unambiguous, yet flexible rules and guidelines which will ensure public safety on the one hand, yet not impede scientific innovation. Clear and harmonized standards and guidelines will, in addition to some of the benefits mentioned above, assist in curbing the proliferation of unsafe or potentially harmful experimental treatments offered to vulnerable patients, which are generally excluded from the scope of the regulation of medicines. However, with increasing attention on the creation of a harmonized global framework for stem cell research and therapy, care should be taken not to introduce too many uncoordinated harmonization activities, which may introduce unintended obstacles that will need to be navigated in an already uncertain terrain.

1.6 Conclusion

Stem cell treatments hold the promise of curing diseases for which no medical treatment currently exists, creating enormous public expectation. Unfortunately therapeutic misconceptions or the frustration of not being able to gain access to these life-saving treatments has resulted in fraudulent and often harmful practices, leading to medical tourism to countries where treatments are less regulated than the patients' home countries. In this regard the public should be educated concerning the reasons for and processes involved in clinical trials as well as the various complex ethical and legal issues which must be considered to ensure their safety and manage realistic hopes. National regulatory frameworks vary substantially due to differing religious, political, historical, cultural, and social values. In an effort to harmonize this "patchwork" of national regulations, various international organizations are combining efforts to create globally accepted standards, guidelines, and policies to enhance scientific cooperation through sharing of research materials and outcomes. Cohesion in the field of stem cell and related research is of utmost importance to establish a basis for public trust and scientific integrity to propel medical innovation forward.

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Chapter 2
Mesenchymal Stem Cells in Clinical Applications

Phuc Van Pham

2.1 Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate into a variety of cell types, e.g., osteoblasts (bone cells), chondrocytes (cartilage cells), and adipocytes (fat cells). MSCs were first discovered by Alexander Maximow, who identified a cell type within the mesenchyme with potential to develop into various types of blood cells. McCulloch and James later revealed the clonal nature of marrow cells in 1963 (Becker et al. 1963; Siminovitch et al. 1963). An ex vivo assay for examining the potential of multipotent marrow clonogenic cells was reported in the 1970s by Friedenstein and colleagues (Friedenstein et al. 1974, 1976). MSCs were determined based on three common characteristics: ability to adhere to culture vessels with a fibroblast-like shape; expression of characteristic markers Stro-1, CD133, CD29, CD44, CD90, CD105 (SH2), SH3, SH4 (CD73), c-kit, CD71, and CD106; and ability to differentiate into specialized cells, e.g., the bone, cartilage, and fat. To easily determine which stem cells are MSCs, in 2006 the International Society of Cellular Therapy defined MSCs with some minimal criteria (Dominici et al. 2006), including:

1. MSCs must be adherent to plastic under standard tissue culture conditions.
2. MSCs must express some specific markers such as CD73, CD90, and CD150 and lack expression of CD14, CD34, CD45 or CD11b, CD79 alpha or CD19, and HLA-DR.
3. MSCs must successfully differentiate into osteoblasts, adipocytes, and chondroblasts under in vitro conditions.

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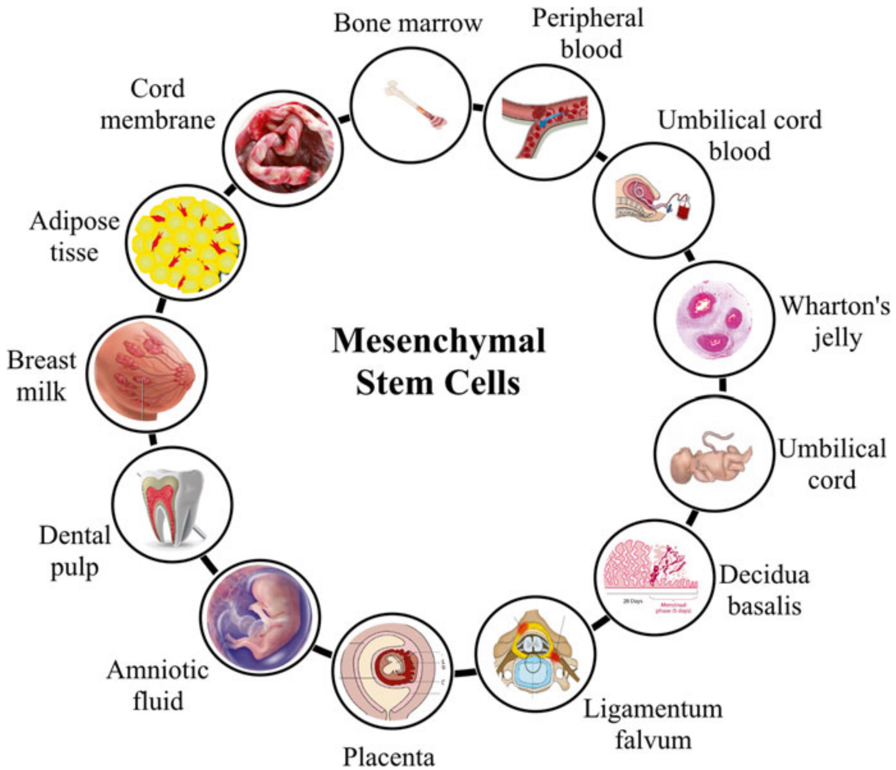

Fig. 2.1 Sources of MSCs. MSCs can be derived from several tissues in the adult or infant human body

The first identified source of MSCs was bone marrow. MSCs are currently isolated from many different tissues in the body, such as the adipose tissue, peripheral blood, umbilical cord blood, banked umbilical cord blood, umbilical cord, umbilical cord membrane, umbilical cord vein, Wharton's jelly of the umbilical cord, placenta, decidua basalis, ligamentum flavum, amniotic fluid, amniotic membrane, dental pulp, chorionic villi of the human placenta, fetal membranes, menstrual blood, breast milk, and urine (Fig. 2.1, Table 2.1).

2.2 How MSCs Can Treat Diseases?

Different than other stem cells, MSCs can be used to treat diseases by two different mechanisms, including tissue repair and immune modulation. While tissue repair is related to the differentiation of multipotent MSCs, immune modulation is a particular property of MSCs. Over the last decades, MSCs have been considered as a feasible source of stem cells for tissue regeneration. It hopes to open the new era of

Table 2.1 Human MSC sources, cell surface markers, and expansion media with serum supplements

Source	Method of isolation	Media	Serum supplement	Cell surface markers		References
				Positive	Negative	
Bone marrow	Ficoll density gradient method	DMEM	FBS	CD73, CD90, CD105, STRO-1	CD14, CD34, CD45, HLA-DR	Mamidi et al. (2012), Otsuru et al. (2013) and Stewart et al. (1999)
	Novel marrow filter device	DMEM-F12				
		ADMEM				
Adipose tissue	Digestion method	DMEM	FBS	CD73, CD090, CD29, CD44, CD71, CD105, CD13, CD166, STRO-1	CD14, CD31, CD34, CD45	Castrechini et al. (2012), El-Kheir et al. (2014) and Gronthos et al. (2001)
	Membrane filtration method	DMEM-LG	FCS			
Amniotic fluid and membrane	Density gradient method	α-MEM	FBS	CD29, CD44, CD90, CD105, CD, SH2, SH3, HLA-DR	CD10, CD14,CD34, HLA-DR	Cai et al. (2010), In 't Anker et al. (2003) and Tsai et al. (2004)
	Digestion method	DMEM/F12				
Dental tissues	Digestion method	α-MEM	FCS	CD29, CD44, CD90, CD105	CD14, CD34, CD45	Huang et al. (2009), Kadar et al. (2009) and Seifrtova et al. (2012)
		MEM	FBS			
Endometrium	Digestion method	DMEM-F12	FCS	CD73, CD90, CD105, CD146	CD34, CD45	Schuring et al. (2011)
Limb bud	Digestion method	DMEM-LG	FBS	CD13, CD29,CD90, CD105, CD106	CD3, CD4, CD14, CD15, CD34, CD45, HLA-DR	Jiao et al. (2012)
Peripheral blood	Ficoll density gradient	α-MEM	NBCS	CD44, CD90, CD105, HLA-ABC	CD45, CD133	Ab Kadir et al. (2012)
Placenta and fetal membrane	Digestion method	DMEM-LG	FBS	CD29, CD73, CD90, CD105	CD34, CD45	Raynaud et al. (2012)
Salivary gland	Digestion method (Ringer solution)	DMEM	FCS	CD13, CD29, CD44, CD90, STRO-1	CD34, CD45	Rotter et al. (2008)
Skin and foreskin	Digestion method	DMEM-HG DMEM DMEM-F12	FBS	CD44, CD73, CD90, CD105, CD166, SSEA-4, vimentin	CD34, CD45, HLA-DR	Bartsch et al. (2005) and Riekstina et al. (2008)

(continued)

Table 2.1 (continued)

Source	Method of isolation	Media	Serum supplement	Cell surface markers		References
				Positive	Negative	
Sub-amniotic umbilical cord lining membrane	Digestion method	DMEM-HG	FBS	CD29, CD44, CD73, CD90, CD105	CD34, CD45	Kita et al. (2010) and Moretti et al. (2010)
		DMEM				
		CMRL1660				
Synovial fluid	Ficoll density gradient method	α-MEM	FBS	CD44, CD90, CD105, CD147, STRO-1	CD31, CD34, CD45, CD106	Morito et al. (2008)
Wharton's jelly	Enzymatic digestion method	DMEM	FBS	CD73, CD90, CD105	CD14, CD34, CD45, CD79, HLA-DR	Hou et al. (2009)
Menstrual blood	Density gradient centrifugations	α-MEM	FBS	CD56, CD73, CD90, CD105 and CD146	CD14, CD45, HLA-DR	Rossignoli et al. (2013)
Human milk	Centrifugations	DMEM	UCBS	CD44, CD29, SCA-1	CD33, CD34, CD45, CD73	Patki et al. (2010)

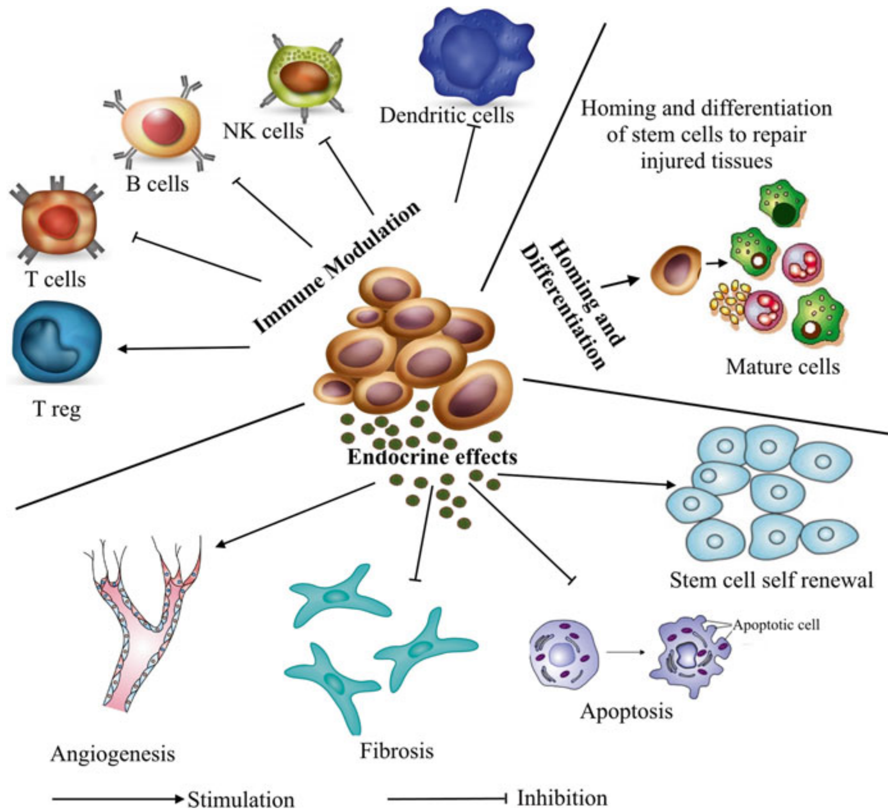

Fig. 2.2 Some mechanisms of MSCs in therapeutic application. MSCs are multipotent stem cells; therefore, they can differentiate into some specific cells that can replace some injured cells/damaged adult cells. In another strategy, MSCs can modulate the immune response via some cytokines

stem cell therapy for degenerative diseases. However, the immune modulation capacity of MSCs has been the subject of recent interest over the past several years. The first MSC drug, Prochymal produced by Osiris Therapeutics, was approved in 2012 and is used for immune modulation in graft-versus-host disease (GVHD) treatment (Fig. 2.2).

2.2.1 *Tissue Regeneration*

MSCs were shown to have differentiation potential into mesenchymal cells as well as endoderm and ectoderm cells. Based on this capacity, MSCs were considered as a suitable cell source for tissue regeneration from the bone, cartilage, adipose tissue, heart,

muscle, and skin. Using in vitro assays, MSCs have been successfully differentiated into osteoblasts (Castren et al. 2015; Glueck et al. 2015; Wang et al. 2015), chondroblasts (Ibrahim et al. 2015; Moghadam et al. 2014; Pustlauk et al. 2015), adipocytes (Li et al. 2015b; Mohammadi et al. 2015), neurons (Bagher et al. 2015; Kim et al. 2015; Nan et al. 2015), insulin-producing cells (Allahverdi et al. 2015; Balici et al. 2016; Ngoc et al. 2011; Van Pham et al. 2014), skeletal muscle (Xu et al. 2015), endothelial progenitor cells (Ikhapoh et al. 2015), cardiac progenitor cells (Li et al. 2015a; Pham et al. 2014; Yang et al. 2015c), and hepatocytes (Han et al. 2015; Sawitza et al. 2015; Ye et al. 2015).

Animal models showed that transplanted MSCs could differentiate in vivo into functional cells at injected sites and contribute to recovering tissue functions. In the minipig model with injured cartilage, Ha et al. (2015) showed that injected human umbilical cord blood-derived MSCs (UC-MSCs) could differentiate and regenerate the cartilage (Ha et al. 2015). Similarly, MSCs can also successfully differentiate into functional insulin-producing cells in vivo in diabetic mice (Yang et al. 2015b), hepatic cells (Hu and Li 2015; Zhong et al. 2015), and neurons (Taran et al. 2014). In animal models, MSCs from the bone marrow, umbilical cord blood, umbilical cord, and peripheral blood have been successfully used to treat several diseases, such as injured cartilage (Punwar and Khan 2011; Song et al. 2014), osteoarthritis (Ozeki et al. 2015; Wolfstadt et al. 2015; Xia et al. 2015), myocardial infarction (MI) (Chen et al. 2015), cornea damage (Guo et al. 2006; Ma et al. 2006), wound healing (Li et al. 2015d; Pelizzo et al. 2015), brain and spinal cord injury (Mannoji et al. 2014; Wu et al. 2015), lung failure (Liu et al. 2014a; Matthay et al. 2010), liver cirrhosis (Tang et al. 2015; Yang et al. 2015a), bone healing (Dehghan et al. 2015; Li et al. 2015c), and diabetes mellitus (DM) (Hao et al. 2013; Kong et al. 2014; Lian et al. 2014; Yaochite et al. 2015).

Based on these studies, MSCs have been clinically applied in disease treatment, especially for tissue injury and degenerative medicine. One popular application of MSCs in degenerative disease is in osteoarthritis as well as injured cartilage. Bornes et al. (2014) showed that MSC transplantation shows positive functional outcomes at 12–48 months postimplantation (Bornes et al. 2014). The first reported use of MSCs to repair cartilage damage in humans was conducted by Wakitani et al. in 1998 (Wakitani et al. 2004). To date, approximately 15 publications have reported the application of MSCs in cartilage regeneration (Bornes et al. 2014). The first MSC-based product (allogeneic umbilical cord blood MSC or CARTISTEM) was approved to treat injured cartilage in Korea in 2014. MSCs have also been clinically used in the treatment of wound healing (Falanga et al. 2007; Rasulov et al. 2005; Ravari et al. 2011; Vojtassak et al. 2006).

2.2.2 *Immune Modulation*

In comparison to other stem cells, MSCs exhibit a powerful capacity of regulating immune responses. Many studies showed that MSCs could regulate immune responses both in vitro and in vivo. The effects of MSCs on immune cells are summarized in Tables 2.2 and 2.3. MSCs can affect all kinds of immune cells including

Table 2.2 Immunomodulatory effects of MSCs on immune cells

Immune cell type	MSCs' effects
T lymphocyte	Suppress T-cell proliferation induced by cellular or nonspecific mitogenic stimuli (Di Nicola et al. 2002)
	Alter the cytokine secretion profile of naive and effector T cells (Aggarwal and Pittenger 2005)
	Promote the expansion and function of Treg cells losh (English et al. 2009)
B lymphocyte	Inhibit proliferation of B lymphocyte (Augello et al. 2005)
	Affect the chemotactic properties of B cells (Corcione et al. 2006)
	Suppress B-cell terminal differentiation (Asari et al. 2009)
NK cell	Alter the phenotype of NK cells and suppress proliferation, cytokine secretion, and cytotoxicity against HLA class I-expressing targets (Sotiropoulou et al. 2006; Spaggiari et al. 2006)
Dendritic cells (DCs)	Influence differentiation, maturation, and function of monocyte-derived dendritic cells (Zhang et al. 2004)
	Suppress dendritic cell migration, maturation, and antigen presentation (Chen et al. 2007)
	Induce mature DCs into a novel Jagged-2-dependent regulatory DC population (Zhang et al. 2009)

Table 2.3 Important bioactive molecules secreted by MSCs and their functions

Bioactive molecules	Functions
Prostaglandin E2 (PGE2)	Antiproliferative mediators (Bouffi et al. 2010)
	Anti-inflammation (Foraker et al. 2011)
Interleukin-10(IL-10)	Anti-inflammatory (Nemeth et al. 2009)
Transforming growth factorβ-1 (TGFβ1), hepatocyte growth factor (HGF)	Suppress T-lymphocyte proliferation (Di Nicola et al. 2002)
Interleukin-1 receptor antagonist	Anti-inflammatory (Ortiz et al. 2007)
Human leukocyte antigen G isoform (HLA-G5)	Antiproliferative for naive T cells (Selmani et al. 2008)
LL-37	Antimicrobial peptide and reduce inflammation (Krasnodembskaya et al. 2010)
Angiopoietin-1	Restore epithelial protein permeability (Fang et al. 2010)
MMP3, MMP9	Mediating neovascularization (Kim et al. 2007)
Keratinocyte growth factor	Alveolar epithelial fluid transport (Lee et al. 2009)
Endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placental growth factor (PlGF), and monocyte chemoattractant protein-1 (MCP-1)	Enhance proliferation of endothelial cells and smooth muscle cells (Kinnaird et al. 2004a, b)

T lymphocytes (Aggarwal and Pittenger 2005; Di Nicola et al. 2002; English et al. 2009), B lymphocytes (Asari et al. 2009; Augello et al. 2005; Corcione et al. 2006), natural killer cells (Sotiropoulou et al. 2006; Spaggiari et al. 2006), and dendritic cells (DCs) (Chen et al. 2007; Zhang et al. 2004). MSCs have thus been successfully applied in both preclinical and clinical treatments for some immune disorder-related diseases. For example, MSCs have been used to treat GVHD in patients transplanted with hematopoietic stem cells (Introna and Rambaldi 2015; von Dalowski et al. 2016; Zhao et al. 2015a), systemic lupus erythematosus (Gu et al. 2014; Wang et al. 2014a; Yan et al. 2013), Crohn's disease (Ciccocioppo et al. 2015; Liew et al. 2014), multiple system atrophy (Lee et al. 2012; Sunwoo et al. 2014), multiple sclerosis (Dulamea 2015; Gharibi et al. 2015), and amyotrophic lateral sclerosis (Hajivalili et al. 2016; Lewis and Suzuki 2014; Rushkevich et al. 2015). An allogeneic MSC-based product was approved as drug for GVHD treatment in Canada in 2015 (Prochymal, which is produced by Osiris Therapeutics). This represents the first approved stem cell drug.

2.3 Clinical Applications of MSCs

2.3.1 Approved MSC-Based Products

For the past 5 years, MSCs have been widely used in clinical applications mainly through two main approaches: approved MSC-based products and clinical trials. To date, approximately nine MSC-based products have been approved by several countries for the treatment of different diseases such as degenerative arthritis, post-acute MI, and GVHD (Table 2.4, Fig. 2.3). These products have been used in autologous and allogenous transplantation in several countries and have significantly contributed to the growth of MSC clinical applications.

CARTISTEM®, a combination of human UC-MSCs and sodium hyaluronate, is intended to be used as a single-dose therapeutic agent for cartilage regeneration in humans with cartilage defects of the knee as a result of aging, trauma, or degenerative diseases.

CardioRel® is an autologous product designed for early or planned intervention in patients of MI providing mononuclear and mesenchymal stem cells for cardiac regeneration.

Trinity® Evolution™ is an allograft of cancellous bone containing viable adult stem cells and osteoprogenitor cells within the matrix and a demineralized bone component. Trinity Evolution offers an ideal alternative to autograft and other bone grafting options (without their drawbacks).

Osteocel® Plus is an allograft cellular bone matrix that retains its native bone-forming cells, including MSCs and osteoprogenitors. Osteocel® Plus is intended for the repair, replacement, and reconstruction of skeletal defects.

Hearticellgram®-AMI are bone marrow-derived MSCs (BM-MSCs) used to treat acute MI through intracoronary injection. This study assessed the safety and efficacy of intracoronary autologous transplantation of BM-MSCs in patients with

Table 2.4 Allogeneic mesenchymal stem cell-based products approved by several countries

Names of products	Components	For diseases	Kind of transplantation	Company	Country
CARTISTEM	MSCs from umbilical cord blood	Degenerative arthritis	Allo	Medipost	Korea
MPC	Mesenchymal precursor cells	N/A	Allo	Mesoblast	Australia
Cupistem	MSC from adipose tissue	Anal fistula (Crohn's disease)	Auto	Anterogen	South Korea
Prochymal	Mesenchymal stem cells from bone marrow	GVHD	Allo	Osiris Therapeutics	Canada
AlloStem	Bone matrix+BM-MSC	Orthopedics	Allo	AlloSource	USA
Hearticellgram-AMI	BM-MSC	Post-acute myocardial infarction	Auto	FCB Pharmicell	South Korea
Osteocel Plus	BM-MSC	Orthopedics	Allo	NuVasive	USA
Trinity Evolution	Bone matrix with MSC	Orthopedics	Allo	Orthofix	USA
CardioRel	BM-MNC/ MSC	Post-acute myocardial infarction	Auto	Reliance Life Science	India

Fig. 2.3 Some approved MSC-based products in some countries. (**a**) CARTISTEM; (**b**) Trinity Evolution; (**c**) Osteocel; (**d**) Prochymal

acute MI. There were no adverse reactions or major cardiac events. There was an improvement in left ventricular (LV) ejection fraction, already evident 6 h after treatment, in acute myocardial function patients who underwent percutaneous transluminal coronary angiography within 72 h of chest pain onset.

AlloStem is partially demineralized allograft bone combined with adipose-derived MSCs (AD-MSCs). Suitable for general bone grafting applications, AlloStem is similar to autograft bone because it provides the three key properties necessary for bone formation: osteoconductive (partially demineralized allograft bone, the foundation for the AlloStem tissue, provides a natural scaffold for new bone formation), osteoinductive (naturally occurring growth factors present in allograft bone have been shown to encourage osteogenic activity), and osteogenic (AlloStem contains adult MSCs that naturally adhere to the bone substrate and may contribute to the formation of new bone).

Prochymal is the first stem cell therapy approved for use in Canada. It is also the first therapy approved in Canada for acute GVHD. It is an allogeneic stem therapy based on MSCs derived from the bone marrow of adult donors. MSCs are purified from the marrow and cultured and packaged, with up to 10,000 doses derived from a single donor. The doses are stored frozen until needed.

2.3.2 Clinical Trials of MSC-Based Therapy

In addition to approved MSC-based products, MSCs have been used in disease treatment through clinical trials. According to clinicaltrials.gov, approximately 542 registered clinical trials have used MSCs for treatment. The first clinical trial using in vitro expanded MSCs was performed in 1995, in which 15 patients were treated with autologous stem cells (Lazarus et al. 1995). According to clinicaltrials.gov, almost all of the current trials are in phase I, phase II, or phase I/II, and some of these trials are in phase II or phase II/III (Fig. 2.4, Table 2.5).

2.3.2.1 MSCs for Osteoarthritis

MSCs easily differentiate into osteoblasts as well as chondroblasts, and therefore they can be rapidly applied in treating several diseases related to bone and cartilage degeneration. MSCs from various sources have been clinically used in bone and cartilage regeneration (Table 2.6).

Autologous MSCs from bone marrow were used in osteoarthritis with good results (Orozco et al. 2013). Autologous in vitro expanded MSCs were also transplanted in cartilage defects (Wong et al. 2013). Allogeneic expanded MSCs from bone marrow were used to treat chronic knee. Vega et al. (2015) showed that allogeneic MSC therapy is simple, without requirement for surgery, and significantly improves cartilage quality (Vega et al. 2015). ADSCs are also used in cartilage

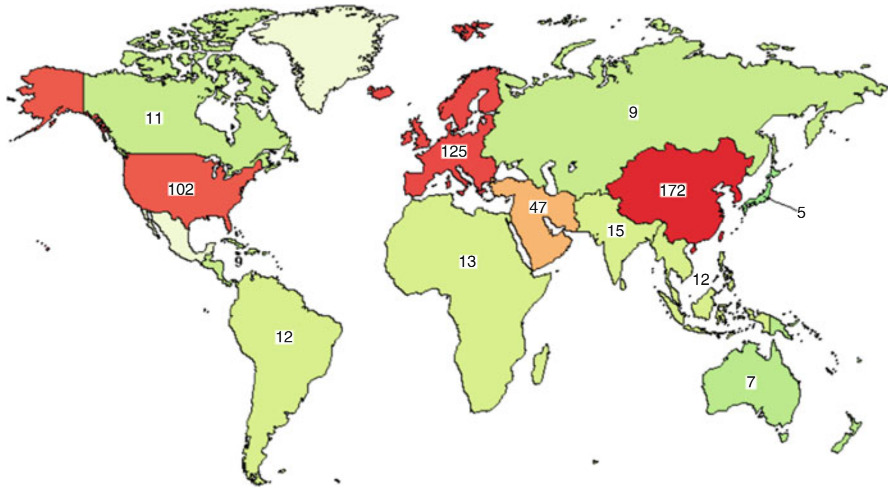

Fig. 2.4 Clinical trials using mesenchymal stem cells

Table 2.5 MSC-based clinical trials in a completed status

Pathology	Clinical status completed						
Overall	Phase I	Phase I/II	Phase II	Phase II/III	Phase III	Phase IV	ND
Hematological disease	1	2	1	0	0	0	0
GVHD	0	4	2	0	1	0	0
Diabetes	1	1	0	0	0	0	1
Liver disease	0	3	0	0	0	0	0
Kidney disease	0	0	0	0	0	0	1
Lung disease	3	0	1	0	0	0	0
Cardiovascular disease	2	11	4	1	0	0	1
Bone and cartilage disease	12	8	3	1	2	0	3
Neurological disease	9	8	2	0	0	0	1
Crohn's disease	0	1	1	1	0	0	1
Lupus erythematosus	0	0	0	0	0	0	0
Other	3	2	1	0	11	1	2
Overall	31	40	15	3	4	1	10

regeneration. Autologous ADSCs have been successfully applied in osteoarthritis treatment. Stromal vascular fraction as non-expanded ADSCs was injected to improve knee osteoarthritis for several years (Bui et al. 2014; Koh et al. 2013; Pak 2011). Almost all studies have shown that ADSC transplantation is safe, with no treatment-related adverse events. Intra-articular injection of ADSCs into the osteoarthritic knee improved function and pain of the knee joint and reduced cartilage

Table 2.6 Clinical trials using MSCs for intra-articular injection of cells

Study name; clinicaltrials.gov identifier	Cell type and source	Indication	Study phase; design
Articular Cartilage Resurfacing With Mesenchymal Stem Cells In Osteoarthritis Of Knee Joint; NCT01207661	MSC, autologous (source unspecified)	Knee OA	Phase I; open label
Adult Stem Cell Therapy for Repairing Articular Cartilage in Gonarthrosis; NCT01227694	MSC, autologous, bone marrow derived	Knee OA	Phase I/II; open label
Side Effects of Autologous Mesenchymal Stem Cell Transplantation in Ankle Joint Osteoarthritis; NCT01436058	MSC, autologous, bone marrow derived	Ankle joint OA	Phase I; open label
Stem Cell Transplantation for the Treatment of Knee Osteoarthritis; NCT00550524	MSC, autologous, bone marrow derived	Knee OA	Phase I; open label
Intra-Articular Autologous Bone Marrow Mesenchymal Stem Cells Transplantation to Treat Mild to Moderate Osteoarthritis; NCT01459640	MSC, autologous, bone marrow derived	Mild-to-moderate knee OA	Phase II; open label, active comparator: hyaluronic acid
Safety and Efficacy of Autologous Bone Marrow Stem Cells for Treating Osteoarthritis; NCT01152125	MSC, autologous, bone marrow derived	OA, KLG III–IV	Phase I/II; open label
Treatment of Knee Osteoarthritis With Autologous Mesenchymal Stem Cells (KDD&MSV); NCT01183728	MSC, autologous, bone marrow derived	Knee OA, KLG II–IV	Phase I/II; open label
Mesenchymal Stem Cell Transplantation in Osteoarthritis of Hip Joint; NCT01499056	MSC, autologous, bone marrow derived	Hip OA	Phase I; open label
The Effects of Intra-articular Injection of Mesenchymal Stem Cells in Knee Joint Osteoarthritis; NCT01504464	MSC, autologous, bone marrow derived	Knee OA	Phase II; double-blind RCT
Allogeneic Mesenchymal Stem Cells in Osteoarthritis; NCT01453738	MSC, allogeneic, source unspecified	Knee OA	Phase II; double-blind RCT

(continued)

Table 2.6 (continued)

Study name; clinicaltrials.gov identifier	Cell type and source	Indication	Study phase; design
Allogeneic Mesenchymal Stem Cells for Osteoarthritis; NCT01448434	MSC, allogeneic, source unspecified	Knee OA	Phase II; double-blind RCT
Treatment of Knee Osteoarthritis With Allogenic Mesenchymal Stem Cells (MSV_allo); NCT01586312	MSC, allogeneic, bone marrow derived	Knee OA	Phase II; double-blind RCT, active comparator: hyaluronic acid
A Phase I/II Study of Chondrogen Delivered by Intra-Articular Injection Following Meniscectomy; NCT00225095	MSC, allogeneic, source unspecified	Meniscectomy	Phase I/II; double-blind; randomized
Follow-up Study of Chondrogen® Delivered by Intra-Articular Injection Following Meniscectomy; NCT00702741	MSC, allogeneic, source unspecified	Partial medial meniscectomy	Phase II; double-blind RCT
Safety and Efficacy Study of MSB-CAR001 in Subjects 6 Weeks Post an Anterior Cruciate Ligament Reconstruction; NCT01088191	MSC, allogeneic, source unspecified	ACL reconstruction	Phase I/II; double-blind RCT, active control: hyaluronan
Autologous Adipose Tissue Derived Mesenchymal Stem Cells Transplantation in Patients With Degenerative Arthritis; NCT01300598	MSC, autologous, adipose tissue derived	Knee OA	Phase I/II; open label
ADIPOA - Clinical Study; NCT01585857	MSC, autologous, adipose tissue derived	Knee OA, moderate or severe	Phase I; open label
Autologous Adipose-Derived Stromal Cells Delivered Intra-articularly in Patients With Osteoarthritis; NCT01739504	MSC, autologous, adipose tissue derived	OA	Phase I/II; open label
Outcomes Data of Bone Marrow Stem Cells to Treat Hip and Knee Osteoarthritis; NCT01601951	Bone marrow concentrate, autologous	Hip and knee OA	Phase unspecified; prospective, observational
Peripheral Blood-derived Stem Cell Trial on Damaged Knee Cartilage (PBSC); NCT01076673	Peripheral blood stem cells (identity unspecified)	Damaged articular cartilage	Phase unspecified; open label

(continued)

Table 2.6 (continued)

Study name; clinicaltrials.gov identifier	Cell type and source	Indication	Study phase; design
Allogeneic Mesenchymal Stem Cells in Osteoarthritis; NCT01453738	MSC, source unspecified, allogeneic	Knee OA, KLG II–III	Phase II; double blind
Autologous Adipose Tissue Derived Mesenchymal Progenitor Cells Therapy for Patients With Knee Osteoarthritis; NCT01809769	Mesenchymal progenitor cells, autologous, adipose tissue derived	Knee OA	Phase I/II; double blind
Autologous Bone Marrow Mesenchymal Stem Cells Transplantation for Articular Cartilage Defects Repair; NCT01895413	MSC, bone marrow, autologous	Knee OA	Phase I/II; open label
Transplantation of Bone Marrow Derived mesenchymal Stem Cells in Affected Knee Osteoarthritis by Rheumatoid Arthritis (*sic*); NCT01873625	MSC, bone marrow, not stated whether autologous or allogeneic	Knee OA	Phase II/III; randomized, open label
Safety and Efficacy Study of MSB-CAR001 in Subjects 6 Weeks Post an Anterior Cruciate Ligament Reconstruction; NCT01088191	MSC, source unspecified	Knee, ACL injury	Phase I/II; double-blind RCT
Autologous Adipose Stem Cells and Platelet Rich Plasma Therapy for Patients With Knee Osteoarthritis; NCT02142842	SVFs (from autologous adipose tissue)	Knee, OA	Phase I/II; randomized, open label
Clinical Study of Umbilical Cord Tissue Mesenchymal Stem Cells (UC-MSC) for Treatment of Osteoarthritis; NCT02237846	MSCs from umbilical cord (allogenic)	Knee, OA	Phase I/II

defects by regeneration of hyaline-like articular cartilage (Jo et al. 2014). Intra-articular autologous activated peripheral blood stem cells also improved quality of life and regenerated articular cartilage in early osteoarthritic knee disease (Saw et al. 2011, 2013; Turajane et al. 2013).

2.3.2.2 Cardiovascular Diseases

Today, more than 40 clinical trials are listed with a majority of bone marrow, Wharton's jelly, and adipose stem cells (Chen et al. 2004; Gee et al. 2010; Hare et al. 2009; Trachtenberg et al. 2011). Both autologous and allogeneic MSCs have been used to treat MI. In 2012, Hare et al. (2012) compared allogeneic vs. autologous BM-MSCs delivered by transendocardial injection in patients with ischemic cardiomyopathy. The authors showed that there was no difference between allogeneic and autologous BM-MSC injection, and MSC injection favorably affected patient functional capacity, quality of life, and ventricular remodeling (Hare et al. 2012). Efficiency of MSCs or mononuclear cells (MNCs) derived from bone marrow was also compared in a recent study (Heldman et al. 2014). Although both MSCs and MNCs from bone marrow were safe by transendocardial injection in ischemic cardiomyopathy patients, improvements such as the 6-min walk distance score, infarct size as a percentage of LV mass, and regional myocardial function as peak Eulerian circumferential strain at the site of injection were only improved in MSC-injected patients (Heldman et al. 2014). Gao et al. (2015) intracoronary infused Wharton's jelly-derived MSCs (WJMSCs) to treat acute MI. After 18 months of follow-up, the absolute decreases in LV end-systolic volumes and end-diastolic volumes at 18 months in the WJMSC group were significantly greater than those in the placebo group (Gao et al. 2015). In another randomized placebo-controlled clinical trial, Musialek et al. (2015) showed that allogeneic transplantation of WJMSCs is safe and effective in MI patients (Musialek et al. 2015). However, the efficiency of treatment based on MSCs differs based on the age of patients. By transendocardial injection of expanded MSCs, Golpanian et al. (2015) showed that MSC injection improved the 6-min walk distance and quality of life using the Minnesota Living with Heart Failure Questionnaire score and reduces MI size in younger patients (younger than 60 years old); in older patients, these scores were not improved (Golpanian et al. 2015).

Other diseases related to cardiovascular diseases, especially hind limb ischemia, were studied for treatment with MSC injection. ADSCs were collected and expanded ex vivo to treat non-revascularizable critical limb ischemia (Bura et al. 2014). ADSCs were intramuscularly injected into the ischemic leg of patients; no complications were observed, transcutaneous oxygen pressure tended to increase in most patients, and ulcer evolution and wound healing were improved (Bura et al. 2014). Allogeneic MSCs also can improve critical limb ischemia (Gupta et al. 2013). However, different than MSCs, BM-MNCs injection was insufficient to treat critical lower limb ischemia (Moazzami et al. 2014).

2.3.2.3 MSCs for Chronic Inflammatory and Autoimmune Diseases

MSCs have a strong capacity of immune modulation that affects all kinds of immune cells. Several clinical studies have examined MSCs in refractory and severe systemic lupus erythematosus treatment. Some results showed that MSC transplantation

resulted in the induction of clinical remission and improvements in serological markers of organ dysfunction (Liang et al. 2010; Sun et al. 2009; Wang et al. 2013a). MSCs have also been used in treatment of Crohn's disease, which is a chronic inflammatory disorder of the gastrointestinal tract. Crohn's disease is currently treated by steroids, immunosuppressive agents, or anti-TNF therapy; however, the efficiency of these therapies is low. MSCs from various sources, such as the bone marrow, adipose tissue, and umbilical cord of both autologous and allogeneic forms, were tested to treat Crohn's disease. Autologous BM-MSCs were safe and beneficial in refractory fistulizing Crohn's disease (Ciccocioppo et al. 2011; Duijvestein et al. 2010). Molendijk et al. (2015) showed that local administration of allogeneic BM-MSCs was not associated with severe adverse events in patients with perianal fistulizing Crohn's disease and promoted healing of perianal fistulas (Molendijk et al. 2015). These results were consistent with the study by Forbes et al., in which administration of allogeneic MSCs reduced CDAI and CDEIS scores in patients (Forbes et al. 2014).

2.3.2.4 MSCs for Liver, Lung, and Kidney Disease

The numbers of MSC-based treatments for liver, lung, and kidney diseases have increased over the past several years. The lungs are susceptible to edema and endothelial permeability caused by traumatic injury and represent good targets for MSC-based cell therapy. Three kinds of pulmonary diseases are clinically treated by MSCs, including idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD), and severe acute respiratory distress syndrome (ARDS). Recent clinical trials have clearly assessed the safety and feasibility of MSCs for the treatment of IPF patients. Both MSCs from the placenta (Chambers et al. 2014) and adipose tissue (Tzouvelekis et al. 2013) were used to treat IPF. The first clinical study of MSC transplantation for COPD was performed in 2013 (Weiss et al. 2013). In this report, Weiss et al. (2013) used in vitro expanded allogeneic MSCs from bone marrow with good results, showing a significant decrease in levels of circulating C-reactive protein in patients treated with MSCs (Weiss et al. 2013). Both BM-MSCs and AD-MSCs were transplanted into ARDS patients. While the clinical results showed that this is a safe method, the disease did not significantly improve after treatment (Simonson et al. 2015; Zheng et al. 2014).

MSC transplantation also shows great promise for the treatment of impaired livers, especially advanced fibrosis. Several clinical studies have examined liver fibrosis treatment by MSC transplantation. Almost all these clinical studies (over ten studies) used BM-MSCs, while four studies used allogeneic MSCs, with three studies using UC-MSCs and one study using BM-MSCs (Shi et al. 2012; Wang et al. 2014b, 2013b; Zhang et al. 2012). Interestingly, allogeneic MSC infusion is clinically safe, without side effects, and improved liver function. Zhang et al. examined the safety and efficacy of UC-MSCs in patients affected by liver cirrhosis. The results showed significantly improved liver function in transplanted patients without side effects or complications (Zhang et al. 2012). UC-MSCs were also used to treat acute chronic liver failure patients. The results showed that UC-MSC transfusions

significantly increased the survival rates in acute chronic liver failure patients (Shi et al. 2012). In summary, these data demonstrated that MSC transfusions are safe and may serve as a novel therapeutic approach for liver diseases.

MSC transplantation is also considered as a promising therapy for kidney failure based on several results in animal models. To date, three phase I/II clinical trials have examined the use of MSCs for kidney failure treatment (Gaspari et al. 2010; Gooch et al. 2008; Togel and Westenfelder 2010). Some initial results showed that MSC infusion could prevent and treat acute renal failure patients (Togel and Westenfelder 2010). Preliminary data indicate that MSC infusion is safe and feasible and that it reduced the length of hospital stay and readmission rates by 40 % (Gooch et al. 2008; Togel and Westenfelder 2010). Gooch et al. indicated that the infusion of allogeneic MSCs seemed to prevent all complications in patients with post-cardiopulmonary bypass-induced acute kidney injury and promote kidney recovery (Gooch et al. 2008).

2.3.2.5 Diabetes Mellitus (DM)

Several clinical trials have examined the application of MSCs in T1DM patients. The first clinical trial was performed by Haller et al. (2008) to assess the safety and efficacy of using MSC-containing autologous cord blood infusion for DM in children (Haller et al. 2008). This study suggested that cord blood infusion was feasible and safe; there was an increase of peripheral regulatory T-cell level and reduced insulin requirement 6 months after cord blood infusion (Haller et al. 2008). Nevertheless, after 2 years, the therapeutic effect disappeared (Haller et al. 2011).

In another study, Hu et al. evaluated the long-term effects of injecting WJMSCs for new-onset T1DM patients (Hu et al. 2013). Treated T1DM patients had better glycemic control and increased C-peptide levels after 2 years of follow-up (Hu et al. 2013). Ten other clinical trials using MSCs for DM were registered in clinicaltrials.gov. In addition to autologous MSCs, some clinical trials used allogeneic and expanded MSCs for treatment. Prochymal was also evaluated for DM treatment. Some improvements were recorded in treated patients such as glycemic control in newly diagnosed T1DM patients (NCT00690066). Four kinds of MSCs have been used in the clinic, including MSCs from the umbilical cord blood, umbilical cord, adipose tissue, and bone marrow.

MSCs have also been used to treat T2DM. Although, the mechanism of MSCs in T2DM treatment is not yet clear, some clinical trials showed that MSC transplantation is promising. Kong et al. (2014) showed that UC-MSC transfusion was safe and well tolerated, effectively alleviated blood glucose, and increased the generation of C-peptide levels and Tregs in a subgroup of T2DM patients (Kong et al. 2014). This result was similar to another study (Liu et al. 2014b). Placenta-derived MSCs also showed huge potential for T2DM treatment. Transplanted T2DM patients had no fever, chills, liver damage, or other side effects. More importantly, renal function and cardiac function were improved after infusion (Jiang et al. 2011).

2.3.2.6 MSCs in Acute Brain Injury: Stroke

In recent years, clinical trials using MSC in stroke have increased dramatically. Since 2009, there were 22 clinical trials in phase I/II (Bang et al. 2005; De Keyser 2005; Smith and Gavins 2012). Bang et al. performed the first phase I study to assess safety of intravenous administration of 10^8 autologous MSCs in patients with severe neurological deficits due to subacute ischemic stroke. The results showed that intravenous cell infusion appeared safe and feasible. In 2010, Lee et al. transplanted MSCs in 16 patients with stroke. Some neurological recovery scores were improved in the MSC group compared with the placebo group (Lee et al. 2010). Both autologous and allogeneic MSCs have been used to treat stroke. All clinical studies showed that MSC transplantation for stroke is safe, with improvement of functional recovery such as neurological scores and size of infarct. These results suggest the potential therapeutic use for MSC in stroke management.

2.4 Safety of MSCs in Clinical Applications

Although the number of clinical applications of MSCs has increased over recent years, the safety of MSCs is still a focus for scientists and medical doctors. The highest risk for MSC transplantation is tumorigenesis in vivo after transplantation. Some hypothesis demonstrated tumorigenesis related to MSC characteristics and some modifications in MSCs during the in vitro expansion. Some studies showed that MSCs without in vitro expansion were safe in both preclinical and clinical applications. For this reason, in 2014, the FDA clarified minimal manipulation of cell/tissue products to be used in the clinic.

In regard to in vitro expanded MSC transplantation, some concerns about the genetic alterations of expanded MSCs were addressed with recent in vitro studies as well as several clinical trials using expanded MSCs. In vitro assays showed that three commonly used MSC types, including BM-MSCs, ADSCs, and UC-MSCs, maintained phenotype and genotype after extended culture. For example, Bernardo et al. showed that BM-MSCs can be cultured long-term in vitro without losing their morphologic, phenotypical, and functional characteristics. These cells can maintain normal karyotype after 44 weeks of culture (Bernardo et al. 2007). ADSCs also did not bypass senescence after 2 months of culture, with no evidence of transformation in vitro (Meza-Zepeda et al. 2008). Chen et al. reported that human UC-MSCs maintained their biological characteristics and function after long-term in vitro culturing and were not susceptible to malignant transformation (Chen et al. 2014). In this study, MSCs could be expanded up to the 25th passage without chromosomal changes by G-band (Chen et al. 2014).

The key obstacle of stem cell therapy is related to whether stem cells may undergo malignant transformation. Some previous studies have described spontaneous transformation of MSCs in vitro (Pan et al. 2014; Ren et al. 2011). However, almost all of these studies have been retracted owing to cross-contamination with cancer cells

(de la Fuente et al. 2010; Garcia et al. 2010; Rubio et al. 2005; Torsvik et al. 2010). Roemeling-van Rhijn et al. (2013) showed that ADSCs can form aneuploid cells during in vitro culture. However, they also confirmed that aneuploidy was not a predecessor of transformation or tumor formation (Roemeling-van Rhijn et al. 2013). In preclinical trials, all studies on NOD mice, NOD/SCID mice, guinea pigs, rabbits, and monkey models showed that upon the use of UC-MSCs from the master MSC bank (passage 2, P2) and culturing for an additional five passages (P7) or 11 passages (P13) with a dose of 1×10^7/mouse or 2.10^6 or 1.10^7 cells/kg body weight for monkeys, no tumor formation was observed after 2 months (Wang et al. 2012a, b).

Based on these results, in vitro or ex vivo expanded MSCs were accepted for use in clinical trials in various diseases (Table 2.7). Almost all trials were in phase II, and some were in phase II. All trials showed that expanded MSC transplantation was safe and exhibited good effects for disease improvement. Using both methods of delivery of MSCs, including intravenous infusion and local injection, MSC transplantation was shown to be safe. Performed a meta-analysis of clinical trials examining the safety of MSC transplantation, and the results confirmed the safety of MSC transplantation. A total of 2347 citations and 36 studies were reviewed, which included a total of 1012 participants with diseases such as ischemic stroke, Crohn's disease, cardiomyopathy, MI, GVHD, and healthy volunteers. The authors showed that there was no association between acute infusional toxicity, organ system complications, infection, death, and malignancy. These authors also showed that there was no difference in safety between autologous MSC and allogeneic MSCs, between matched allogeneic MSCs and unmatched allogeneic MSCs, between non-expanded MSCs and in vitro expanded MSCs, and between fresh MSCs and cryopreserved MSCs. However, there was a significant association between MSC transplantation and transient fever.

2.5 Conclusions

MSCs have become the most frequently applied stem cell type in the clinic. To date, multiple degenerative diseases and several immune-related diseases have been clinically treated by MSC transplantation. Several sources of MSCs include MSCs from the bone marrow, adipose tissue, umbilical cord blood, umbilical cord, and placenta, both with and without in vitro expansion. With useful characteristics about immune modulation, MSCs not only autologously injected into patients but allogeneic graft also was used. After over 10 years of MSC-based treatments, all reports have shown that MSC transplantation is safe. Many reports demonstrate some improvements in disease treatment using MSCs, and several MSC-based products have been approved as stem cell drugs for diseases such as GVHD and osteoarthritis. Together this demonstrates that MSC transplantation is a safe and promising therapy for disease treatment.

Table 2.7 List of completed clinical trials using ex vivo expanded MSCs

Clinical trial no.	Source of MSCS	Serum supplement	Disease treated	Dose	No. of treatment	Route of administration	Phase	Design	References
NCT00395200	Au-BM	FBS	Multiple sclerosis	1–2 × 10^6 cells/kg BW	Single	Intravenous	I and II	Non-randomized, safety/efficacy study, single group assignment, Open label	Connick et al. (2012) and Connick et al. (2011)
NCT00504803	Allo-BM	Irradiated FBS	Graft-versus-host disease	–	Single	Intravenous	II	Non-randomized, safety/efficacy study, single group assignment, open label	Baron et al. (2010)
NCT01087996	Au-BM Allo-BM	–	Ischemic cardiomyopathy	20/100/200 × 10^6 cells	Single	Transendocardial	I and II	Randomized, safety/efficacy study, parallel assignment, open label	Hare et al. (2012)
NCT00114452	Allo-BM	–	Myocardial infarction	0.5/1.6/5 × 10^6 cells/kg BW	Single	Intravenous	I	Randomized, safety study, parallel assignment, double blind (subject, caregiver, investigator, outcomes assessor)	Hare et al. (2009)
NCT00658073	Au-BM	–	Renal transplant rejection	1–2 × 10^6 cells/kg BW	Twice	Intravenous	–	Randomized, efficacy study, parallel assignment, open label	Tan et al. (2012)
NCT00734396	Au-BM	FBS	Renal transplant rejection	1 × 10^6 cells/kg BW	Twice	Intravenous	I and II	Non-randomized, safety/efficacy study, single group assignment, open label	Reinders et al. (2013)
NCT00883870	Allo-BM	–	Critical limb ischemia	2 × 10^6 cells/kg BW	Single	Intramuscular (gastrocnemius muscle)	I and II	Randomized, safety/efficacy study, parallel assignment, double blind (subject, caregiver, investigator)	Gupta et al. (2013)
NCT00823316	Allo-UCB	FBS	Graft rejection and graft-versus-host disease	1–5 × 10^6 cells/kg BW	Single	Intravenous	I and II	Randomized, safety/efficacy study, parallel assignment, open label	Lee et al. (2013)
NCT00911365	Au-BM	FBS	Multiple system atrophy	40 × 10^6 cells	Multiple	Intra-arterial (one time) Intravenous (three times)	II	Randomized, parallel assignment, single blind (subject)	Lee et al. (2012)
NCT01274975	Au-AD	FBS	Spinal cord injury	400 × 10^6 cells	Single	Intravenous	I	Randomized, safety study, single group assignment, open label	Ra et al. (2011)

NCT00683722	Allo-BM	–	Coronary obstructive pulmonary disorder	100×10^6 cells Multiple	Intravenous	II	Randomized, safety/efficacy study, parallel assignment, double blind (subject, caregiver, investigator, outcome assessor)	Weiss et al. (2013)
NCT00956891	Au-BM	FBS	Liver failure	$\approx 100 \times 10^6$ cells Single	Hepatic artery	–	Case control, retrospective	Peng et al. (2011)
NCT00187018	Allo-BM	FBS	Osteogenesis imperfecta	$0.68–2.75 \times 10^3$ cells/kg BW Single	Intravenous	–	Non-randomized, safety/efficacy study, single group assignment, open label	Otsuru et al. (2012)
NCT00816803	Au-BM	Serum-free	Spinal cord injury	2×10^6 cells/kg BW Multiple	Lumbar puncture	I and II	safety/efficacy study, parallel assignment, single blind (outcomes assessor)	El-Kheir et al. (2014)
	Allo-UC		Severe systolic heart failure		Injected into left coronary artery	I and II	Improve cardiac remodeling and cardiac function and reduce the mortality rate	Zhao et al. (2015b)
	Allo-WJ	FBS	Acute myocardial infarction	30×10^6 WJMSCs	IRA using a cell-delivery perfusion catheter	I	Safety	Musialek et al. (2015)
NCT01218464	Allo-UC	FBS	Acute-on-chronic liver failure	0.5×10^6 UC-MSCs, three times at 4 weeks intervals	Intravenous	I and II	Serum total bilirubin and alanine aminotransferase levels were significantly decreased UC-MSC transfusions are safe	Shi et al. (2012)
NCT01662973	Allo-UC		Primary biliary cirrhosis	0.5×10^6 cells/kg body weight	Intravenous	I and II	UC-MSC transfusion is feasible and well tolerated in patients with PBC who respond only partially to UDCA treatment	Wang et al. (2013b)
	Allo-AD-MSC		Lateral epicondylosis	$10^6–10^7$/patient	Local injection	I and II	Allo-ASC therapy was thus safe and effective in improving elbow pain, performance, and structural defects for 52 weeks	Lee et al. (2015)
NCT00260338	Auto-BM-MSC		Myocardial ischemia		Intramyocardial injections	I and II	Not only improve symptoms but also slow down disease progression	Mathiasen et al. (2013)
NCT01392105	Auto-BM-MSC	FBS	Acute myocardial infarction	$7.2 \pm 0.90 \times 10^7$ cells	Intracoronary injection	II/III	Tolerable and safe with modest improvement in LVEF at 6-month follow-up by SPECT	Lee et al. (2014)

(continued)

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Chapter 3
Ageing and Senescence in Mesenchymal Stem Cells

Hitesh D. Tailor, Yiannis Pengas, and Wasim S. Khan

Stem cells are often described as being cells that have pluripotent ability when grown in a desired culture environment. They are also described as being obtainable from multiple sites of the human body. Much of the focus in the literature tends to focus on the ideal growth factors or culture environment to induce growth towards specific cell lineages. However, the focus in this chapter is on how donor age and senescence of stem cells affect its usability and culture outcomes, particularly with regard to mesenchymal stem cells (MSCs) and established protocols for the chondrogenic, osteogenic and adipogenic differentiation of these cells.

3.1 Donor age

Looking at the process of osteoporosis, Stenderup et al. (2001) isolated MSCs from the iliac crest of volunteers in two distinct age groups: young (aged 22–44 years) and old (aged 58–83 years). The older group was further divided into those with known osteoporosis and those with normal bone architecture. They found that there was no significant difference in colony-forming efficiency of cells. Cell density per colony and percentage of alkaline phosphatase-positive (AP+) colonies between

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groups were also statistically indifferent. In another similar study, by Dexheimer and colleagues (Dexheimer et al. 2011), MSCs were isolated from iliac crest bone marrow aspirates and comparisons were made of culture outcomes of MSCs from donors ranging from 5 to 80 years of age. No significant correlation was found between age and number of colony-forming unit-fibroblasts (CFU-F). It is, however, suggested that the number of stem cells that can be aspirated per millilitre are hugely variable (Muschler et al. 1997), and therefore, CFU-F data may not be a reliable outcome variable in these studies. In addition, there are known stages in bone marrow stem cells numbers: there is continual bone growth until the age of 18 years (Whiting et al. 2004) then there is steady decline until the age of 40 years (D'Ippolito et al. 1999), followed by a plateau.

There are authors who have found difficulties in isolating and/or expanding stem cells from older aged donors. Bertram et al. (2005) cultured MSCs isolated from iliac crest of 21 donors (aged 11–76 years) and from 32 donors of cancellous bone grafting material (aged 18–34 years). The failure rate for culture was 55.6 % in donors over 60 years of age, whereas it was up to 22.2 % in donors under 60 years of age. Age, however, had no significant influence on specifically isolating MSCs amongst the different age groups. Shamsul et al. (2004) isolated MSC from the bone marrow of 38 donors aged 10–70 years. Fourteen of the 15 samples from donors over 40 years failed to proliferate. whereas only seven of the 23 samples from the younger donors failed. It is possible that these were anomalies as culturing of stem cells from older donors is generally possible by the vast majority of authors.

Where culturing is largely successful, it is observed that during each expansion of MSC populations, there is increased population doubling (PD) time as donor age increases. Passage 3 in one study (Dexheimer et al. 2011) had a positive correlation between age and PD time of $R^2=0.669$ ($p<0.001$). Stenderup et al. (2003) compared rates of PD between two groups of population ages. Again, using MSCs from bone marrow, the mean PD rate was 0.05/day compared to cells from younger donors at 0.09/day. Others have also examined proliferation rate and have observed a slower rate with cells from older donors (Mendes et al. 2002; Stolzing et al. 2008; Chen et al. 2011).

Another property to look at is the differentiation potential. Commonly, osteogenic potential has been investigated as mineralisation or AP+ cultures can be measured. De Girolamo and colleagues (de Girolamo et al. 2009) isolated adipose-derived MSCs from two groups of donors: those younger than 35 years and those older than 45 years. They found that the osteoblastic differentiation potential from older donors was significantly reduced, whereas there was little difference on adipogenic differentiation. Chen et al. (2011) extracted adipose-derived stem cells (ADSC) and MSCs from each of their patients of two groups: young (mean 36 years) and elderly with osteoporotic fractures (mean 71 years). Apart from ADSC displaying no changes in PD time with age, osteogenic differentiation measured from matrix mineralisation also displayed no significant difference between the two groups. However, BMSC from the elderly group showed much less mineralisation compared to the young donors. Others have also found that there is a loss of osteogenic potential with increased age (Roura et al. 2006).

3.2 Senescence

One important consideration in terms of defining ageing is whether it is the individual cells which have become aged or whether the biochemical influence of the older individual has influenced the cell's stem cell potential. Organismal ageing is beyond the scope of this chapter; however, it is widely known that stem cells are subject to altered function after extrinsic toxicity. For example, ultraviolet radiation on skin (Fuchs and Raghavan 2002) or chemotherapy on hemopoiesis (Richman et al. 1976) are well- known effects.

A recurrently cited theory has been suggested by Hayflick and Moorhead. In their investigations, they described senescence as being a state of irreversible cell division (Hayflick and Moorhead 1961). In vivo, stem cells on the whole clearly have no finite replicative capacity. Embryonic stem cells show no loss of proliferation potential (Rosenberger 1995); however, MSCs in vitro have shown replicative limit. Bruder et al. (1997) isolated MSCs from iliac crests of nine individuals. Cells were cultured and serially passaged, and it was found that from being spindle-like shaped cells, they became broad and flattened in morphology. Furthermore, cells averagely lost replicative capacity after 38 population doublings, that is, they became senescent.

Homing in on a cellular level, this area is somewhat fairly well documented. The cellular markers p21, p53 and reactive oxygen species (ROS) are all described as important markers of cellular stress. P53, commonly regarded as a tumour-suppressor, is found to be upregulated in aged stem cells (Stolzing et al. 2008). Levels of p21 and beta-galactosidase levels were found to be significantly less in ADSC compared to BMSCs in one study (Chen et al. 2011). Zhou et al. (2008) investigated expression of these senescence-related factors, isolating MSCs from bone marrow of donors aged 17–90 years. They found that samples from older donors were significantly more positive for senescence-related beta-galactosidase, as well as having increased expression of p53, and its related BAX and p21 genes.

Where DNA damage occurs, it is known that there would be increased expression of p16INK4A and p53 (Kim and Sharpless 2006). Upregulation of these divert the cells' fate to exit the cell cycle and induce senescence and/or apoptosis. This would also occur with regard to MSCs, suggesting the mechanisms by which these cells have shown replicative stress. The INK4a/ARF gene encodes two proteins p16INK4a and p14ARF. p16INK4a is known to inhibit the cyclin-dependent kinases, CDK4 and CDK6, which promote proliferation (Kim and Sharpless 2006). It also increases with age as well as is being involved in regulating age-dependant senescence (Zindy et al. 1997). The p14ARF protein, however, regulates cell cycle pathways involving p53 towards senescence/apoptosis. The role of ROS shows that it can be used to manipulate in vitro cellular fate. Previous studies have shown that it can limit proliferation (Meagher et al. 1988) and, furthermore, cause DNA damage inducing cellular senescence (Ko et al. 2011).

3.3 Tactics to Overcome Ageing

The advanced knowledge of how stem cells from aged donors will behave sets the scene for how to overcome the discussed difficulties in ex vivo culturing. There are known important genetic factors that influence cellular division, as discussed above. In a recent study (Lee et al. 2009), the authors introduced wild-type p53-inducible phosphatase-1 (Wip1) in culturing hMSCs, and it was found to lower p16INK4A expression leading to p38 mitogen-activated protein kinase (MAPK) inactivation. This successfully delayed cell growth arrest in prolonged culture. However, it failed to induce senescent MSCs back into a replicative cell cycle. This study does suggest that induction of p16-related senescence is an irreversible step in stem cell fate.

Simpler methods to help promote proliferative capacity in cells from older donors include the use of antioxidants in culture, as described by Ko et al. (2011) or using low oxygen tension (Fehrer et al. 2007). Cell density in culture has an impact on replicative capacity (Colter et al. 2009). hMSCs were shown proliferate at a greater rate if plated at reduced cellular densities of 1.5 or 3.0 cells/cm^2. In addition, single cell-derived MSC clones were able to be expanded up to 50 PDs in serial passages of low cellular density, whereas growth was seen to be a plateau after 15 passages when cultured at higher cellular densities (Colter et al. 2009).

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Chapter 4
New Trends in Clinical Applications of Induced Pluripotent Stem Cells

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4.1 History and Generation of iPSCs

Induced pluripotent stem cells (iPSCs) were established based on fundamental reprogramming backgrounds. The first significant finding was reported by John Gurdon in 1962 on the successful generation of tadpoles by transferring the nucleus of intestinal cells into enucleated oocytes (Gurdon 1962). In 1987, several key transcription factors were discovered, including Antennapedia, a *Drosophila* transcription factor (Schneuwly et al. 1987), and MyoD, a mammalian transcription factor (Davis et al. 1987). These studies led researchers to identify master cellular regulators. Mouse embryonic stem cell (ESC) generation was first reported by Evans and Martin in 1981 (Evans and Kaufman 1981; Martin 1981), followed by the eventual generation of human ESCs by Thomson (Thomson et al. 1998). These breakthroughs led to the hypothesis that there is a coordination of factors in either oocytes or ESCs that enable reprogramming of adult cells into an embryonic state.

The early publication in 2006 of the successful screening of a pool of 24 individual factors that have a vital role in reprogramming adult cells into pluripotent cells was accomplished by Yamanaka and Takahashi (Takahashi and Yamanaka 2006). The core factors essential in the reprogramming process included Otc4, Sox2, Klf-4, and c-Myc (Takahashi and Yamanaka 2006). The iPSCs were generated by selection of ESC-specific Fbxo15 activation that expresses markers of PSCs

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(Takahashi and Yamanaka 2006). However, compared with ESCs, these iPSCs expressed lower levels of several pluripotent genes and failed to produce viable chimeras or to contribute to the germline. These mouse-derived iPSCs only achieved reprogramming. The “gold standard” for PSCs, that the cells could generate viable chimeric models and contribute to the germline, was able to be reproduced and improved after this study. For example, the selection of Nanog instead of Fbxo15 could obtain ESCs closely functional to iPSCs (Maherali et al. 2007; Okita et al. 2007). Unfortunately, these cells still contain oncogenic c-Myc, and thus 20 % of the chimeric models develop cancer (Nakagawa et al. 2008; Okita et al. 2007). Yamanaka and others later succeeded in generating iPSCs without c-Myc (Nakagawa et al. 2008; Wernig et al. 2008). Thomson and Yu used two other factors, Nanog and LIN28, instead of Klf-4 and c-Myc (Yu et al. 2007), a milestone achieved in 2007, successfully generating human iPSCs, as well as that of Yamanaka and colleagues using four typical factors on the same day (Takahashi et al. 2007b). Since these earlier studies, iPSCs have been derived from a wide range of species, such as human (Gianotti-Sommer et al. 2008; Takahashi et al. 2007b; Warren et al. 2010; Yu et al. 2007), buffalo (Deng et al. 2012), cattle (Han et al. 2011), dog (Shimada et al. 2010), horse (Nagy et al. 2011), mouse (Heng et al. 2010; Nakagawa et al. 2008; Takahashi and Yamanaka 2006), pig (Esteban et al. 2009), goat (Sandmaier et al. 2015), rat (Chang et al. 2010), and rhesus monkey (Liu et al. 2008), using the four factors selected by Yamanaka’s group.

In recent years, a number of methods have been established to generate iPSCs (Fig. 4.1) (Kumar et al. 2015).

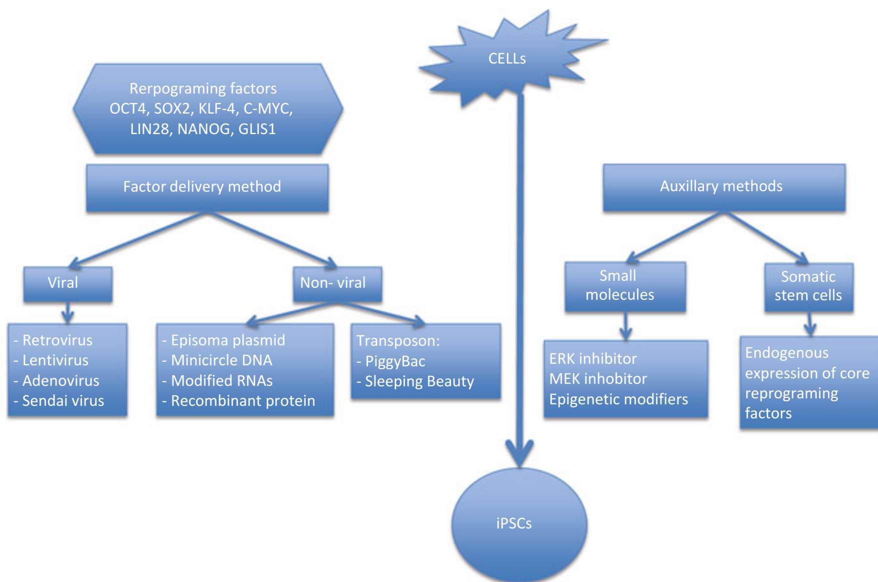

Fig. 4.1 Several approaches to produce induced pluripotent stem cells (iPSCs). iPSCs are currently generated by various strategies using different inducing factors, different donor cells, and different gene delivery methods

Several viral vector strategies were used to obtain iPSCs, including retrovirus, lentivirus, adenovirus, and Sendai virus approaches. The first iPSCs were established through the constitutively active retroviral transduction of c-Myc, Sox2, Klf4, and Oct4 (Takahashi and Yamanaka 2006). In this system, the active retroviral vector is stably integrated into the genome. Lentiviral vectors are more effective than retroviral vectors because of the ability to infect different somatic cell types; furthermore, lentiviruses can be used to express polycistronic cassettes encoding all four reprogramming factors, thus increasing reprogramming efficiency (Maherali et al. 2007). Optimization of retroviruses or lentiviruses has helped increase the efficiency of infection of mammalian cells (Kumar et al. 2015). The pantropic vesicular stomatitis virus G protein was used as a substitute for viral transduction of a number of derived cells (Kumar et al. 2015). However, the viral approach has three major disadvantages. First, this system has a limited cloning capacity with a maximum of 7 kb. The second limitation is the induction of immune response by the host upon infection. The third limitation involves potential genotoxic effects. Retroviruses and lentiviruses do not randomly integrate in the genome but show a significant preference for promoter and exonic regions, which may compromise the regulation of endogenous genes (Kumar et al. 2015). In the case of lentiviral vectors, the inert drug doxycycline can regulate expression, decrease continued transgene expression, and help select fully reprogrammed iPSCs, because cells that depend on the expression of exogenous factors promptly stop proliferating when doxycycline is removed (Brambrink et al. 2008). The iPSCs formed by retroviruses or lentiviruses had the absence of integration sites as an indispensable result, which indicates that insertional mutagenesis has a supportive function during iPSC production. To avoid insertional mutagenesis, viruses that do not integrate into the genome or that can be subsequently removed are required, such as adenovirus or Sendai virus (Ban et al. 2011).

The derived nonviral iPSCs have been highly recommended for regenerative medicine. Advantages of this approach include the ability to insert DNA into host cells without integration and no lack of limitation of DNA size in the transferring process. Nonviral methods include transposons, "minicircle" DNAs, episomal vectors, plasmid vectors, mRNAs, small molecules, transposons, and recombinant proteins such as Sleeping Beauty and piggyBac (Stadtfeld and Hochedlinger 2010). A recent study described nonintegrating episomal vector-derived human iPSCs. After episome removal, iPSCs are derived that are completely deficient of transgene sequences and vectors and resemble the proliferative and developmental potential of human ESCs. These results indicate that reprogramming human somatic cells does not require the continued presence of exogenous reprogramming factors or genomic integration and eliminates one obstacle to the clinical application of human iPSCs (Yu et al. 2009). The use of "minicircle" DNA (Chabot et al. 2013), a vector that is capable of high expression in cells and is free of bacterial DNA, was successful in producing transgene-free adult human adipose stem cell-derived iPSCs (Jia et al. 2010). One strategy that will augment reprogramming uses small molecules as core reprogramming factors. Two approaches were explored in determining conditions for replacing viral transduction of oncogenic transcription factors and

increasing reprogramming efficiency. One can apply fewer genetic manipulations to preprogram neural progenitor cells in comparison with previously reported somatic cells. On the other hand, some small molecules may be used as integration factors instead of certain viral particles. These molecules also promote the reprogramming process (Shi et al. 2008). These approaches can readily translate cell types directly into a clinical type; such has been successful in neurons and cardiac cells. This achievement is an essential contribution of chemical biology in stem cell research that elucidates a number of specific advantages in applying small molecules (Jung et al. 2014).

Transposon systems as viral-based methods have an interesting development in hyperactive transposase enzymes. PiggyBac, Sleeping Beauty, and Tol2 transposon systems are simple strategies that have been frequently used for various applications, and the system components can be separated on two plasmids. The first plasmid carries an expression cassette for the particular transposase enzyme and the second plasmid carries the transgene flanked by inverted terminal repeats (ITRs). Both plasmids are introduced into cells and the transposase is expressed, followed by the transposition of the ITR-flanked transgene into the genome. Importantly, the transgenes integrate by a cut-and-paste mechanism and all residual plasmid elements are eliminated by degradation. In general, transposon integrations occur randomly in the genome with no preference for gene-containing regions or promoter sites. The transposase can be introduced in trans for completely integrated transposon removal, resulting in safe and clean iPSCs (Kumar et al. 2015). For instance, the bovine-derived iPSCs that use the piggyBac and Sleeping Beauty transposon systems include a different group of reprogramming factors, each regulated by the chimeric CAGGS promoter and separated by self-cleaving peptide sequences. Another bovine iPSC line produced by a piggyBac vector that consists of six key reprogramming genes has been examined in detail, including alkaline phosphatase expression, morphology, and typical pluripotent hallmarks, such as pluripotency marker expression and mature teratoma formation in a mouse model. Furthermore, this iPSC line is able to transfer the Sleeping Beauty transposon in a second round. These achievements are promising for germline-competent bovine-derived iPSCs and provide a strategy for bovine genome genetic modification (Talluri et al. 2015).

One obstacle in reprogramming is the introduction of exogenous genetic modifications in host cells. This obstacle can be overcome by directly transferring reprogramming proteins into cells instead of requiring the transcription of inserted genes by the host cells. A fully somatic reprogramming study on murine fibroblasts reported a protein transduction method that directly transfers recombinant reprogramming proteins. This result served as a significant landmark in generating iPSCs and has several principal advantages over previous methods (Zhou et al. 2009). However, this method does suffer some disadvantages, including low efficiency and high costs because of the number of required protein factors.

Another trend in nonviral iPSCs is the use of RNA molecules. The major steps include synthesizing mRNAs encoding Oct4, Sox2, c-Myc, Klf-4, and SV40 large T cells and introducing these mRNAs into host cells (i.e., human fibroblasts) by electroporation. Transfecting fibroblasts with this mRNA mixture significantly

increased the expression of endogenous DNMT3β, Nanog, Oct4, Rex1, and Sall4. The cells were cultured in human ESC medium, and small aggregates positive for Oct4 protein and alkaline phosphatase activity were detected within 30 days (Plews et al. 2010). Another published approach is based on highly reproducible RNA that uses a single, synthetic self-replicating VEE-RF RNA replicon. The replicon consists of four essential factors (Klf-4, Oct4, and Sox2, with GLIS1 or c-Myc) with high-level expression before regulated RNA degradation. iPSCs were successfully generated from adult or newborn human fibroblasts by transfection of a single VEE-RF RNA. These transfected cells expressed all the hallmarks of stem cells, including global gene expression profiles, cell-surface markers, and in vivo pluripotency, with differentiation into all three germ layers (Yoshioka et al. 2013). These studies demonstrate that mRNA transfection is a promising approach to activate pluripotency genes in differentiated cells. However, one limitation of this approach is the short half-life of RNA.

Together, these studies show that several successful methods have been established for the generation of iPSCs. The final application or the required yield of iPSCs may help dictate the appropriate strategy.

4.2 Production of iPSCs with Clinical Grade

4.2.1 iPSCs Can Be Produced in Clinical Conditions

Differing from preclinical studies, clinical-grade iPSCs must be produced in a Good Manufacturing Practice (GMP)-compliant manner that minimizes the risk of viruses and infection as well as modifications during the iPSC production process. Three issues must be addressed to satisfy the clinical grade of iPSCs.

First, gene delivery vehicles must be improved to minimize genome instability of iPSCs. In the initial effort to produce iPSC, retroviruses vectors were used to carry transgenes to target cells (Kitamura et al. 2003; Takahashi et al. 2007a, b). Lentiviral vectors were subsequently used to increase the efficiency of infection compared with retrovirus vectors (Blelloch et al. 2007; Yu et al. 2007). Both retroviral and lentiviral vectors can cause genomic integration, and these integration events can activate oncogenesis in iPSC-derived cells (Okita et al. 2007). As a greater concern, the transgenes have the potential to interfere with functional genes. Therefore, some recent efforts have aimed to generate iPSCs without genomic insertions. Adenovirus vectors have been the subject of current focus as these vectors integrate into the genome of target cells at extremely low frequencies (Harui et al. 1999). A recent study used a Cre-deletable lentivirus system to produce iPSCs (Hanna et al. 2007). However, although these systems can avoid transgene reactivation, there is a risk of introduction of gene breaks near the insertion site (Nagy 2000). Finally, scientists have successfully developed a transgene system without gene disruption near the insertion site and reactivation of transgenes using the Sendai virus (Fusaki et al. 2009). Moreover, because the Sendai virus genome is negative-sense single-stranded

RNA, these strands are easily removed from infected cells using siRNA (Nishimura et al. 2011). Viral-free vectors were also recently developed to completely remove viral factors during iPSC production. In the first effort, episomal vectors were developed to generate iPSCs (Gonzalez et al. 2009; Jia et al. 2010). However, this method shows lower efficiency of successful reprogramming than that achieved using retrovirus. Another viral-free vector approach uses the piggyBac transposon. Although some authors have successfully produced iPSCs using the piggyBac transposon, this technique also generally shows extremely low efficiency (Kaji et al. 2009; Yusa et al. 2009). To avoid introducing genetic material, much attention has been recently focused on introducing reprogramming factors such as RNAs or proteins. For example, mRNAs of pluripotent factors (Warren et al. 2010) and microRNAs (Anokye-Danso et al. 2011) have been shown to successfully reprogram somatic cells to a pluripotent state. These RNA-based reprogramming methods avoid both breaks in existing genes and the reactivation of transgenes. Therefore, these methods hold much promise as novel iPSC generation methods for clinical use. More recently, recombinant proteins and small molecule drugs were also reported as successful means of gene introduction for generating iPSCs. Zhou et al. used recombinant Oct4, Sox2, Klf4, and c-Myc proteins that were designed with a poly-arginine (11R) protein transduction domain to aid in penetration into the cytoplasm (Zhou et al. 2009). Hou et al. (2013) reported that pluripotent stem cells can be generated from mouse somatic cells at a frequency as great as 0.2 % using a combination of seven small molecule compounds (Hou et al. 2013). With viral-free vectors, iPSCs can be used in clinical applications with a high degree of safety related to genome stability.

The second issue for clinical grade of iPSCs involves minimizing the invasiveness in obtaining donor cells for iPSC production. In early studies, iPSCs were successfully produced from human dermal fibroblasts. However, only small fragments of skin can be collected, and the collection of the skin dermal layer is relatively invasive. A technique with lower invasion was developed to produce iPSCs from keratinocytes (Aasen and Izpisua Belmonte 2010). Other cells can be obtained by less invasive techniques and were also considered as suitable sources for iPSC production, including dental stem cells (Yan et al. 2010) and mesenchymal stromal cells derived from human third molars (Oda et al. 2010), oral gingival cells (Egusa et al. 2010), and oral mucosa fibroblasts (Miyoshi et al. 2010). More recently, peripheral blood cells were successfully used to produce iPSCs (Brown et al. 2010; Loh et al. 2009). Collection of peripheral blood is less invasive, and therefore generating iPSCs from peripheral blood could be one of the most appropriate methods for establishing iPSCs. With some breakthroughs in techniques, iPSCs could also be successfully produced from fresh or frozen peripheral blood samples.

Third, animal composition-free culture systems must be implemented to remove risks related to xenogenic proteins. The highest risk in culture systems is related to fetal bovine serum supplementation in cell culture medium. The second highest risk involves the use of murine cells for the feeder layer. Some efforts to use human cells for feeder layers were initiated (Takahashi et al. 2009); however, these techniques were time consuming and complex. In other studies, Matrigel was used to replace

cell feeder layers (Totonchi et al. 2010; Xu et al. 2001). iPSCs show excellent growth in Matrigel. However, Matrigel is produced from Engelbreth–Holm–Swarm mouse sarcoma cells, and thus it needs to be replaced with other matrices from human or recombinant proteins. Other types of matrices have been developed to culture iPSCs, such as CellStart (Ausubel et al. 2011; Bergstrom et al. 2011), recombinant proteins (Chen et al. 2011; Rodin et al. 2010), and synthetic polymers (Lu et al. 2012; Mei et al. 2010). To remove the fetal bovine serum in culture medium, knockout serum replacement (KSR) has been established as a definitive material for maintaining human ESCs (Draper et al. 2004) and considered suitable to culture iPSCs (Ludwig et al. 2006). However, both KSR and mTeSR1 also contain animal-derived products. New media have been commercially developed as xeno-free media for maintaining human pluripotent stem cells and iPSCs, such as TeSR2 (Meng et al. 2012), NutriStem (Sugii et al. 2010), Essential E8 (Chen et al. 2011), and StemFit (Nakagawa et al. 2014) (Fig. 4.2).

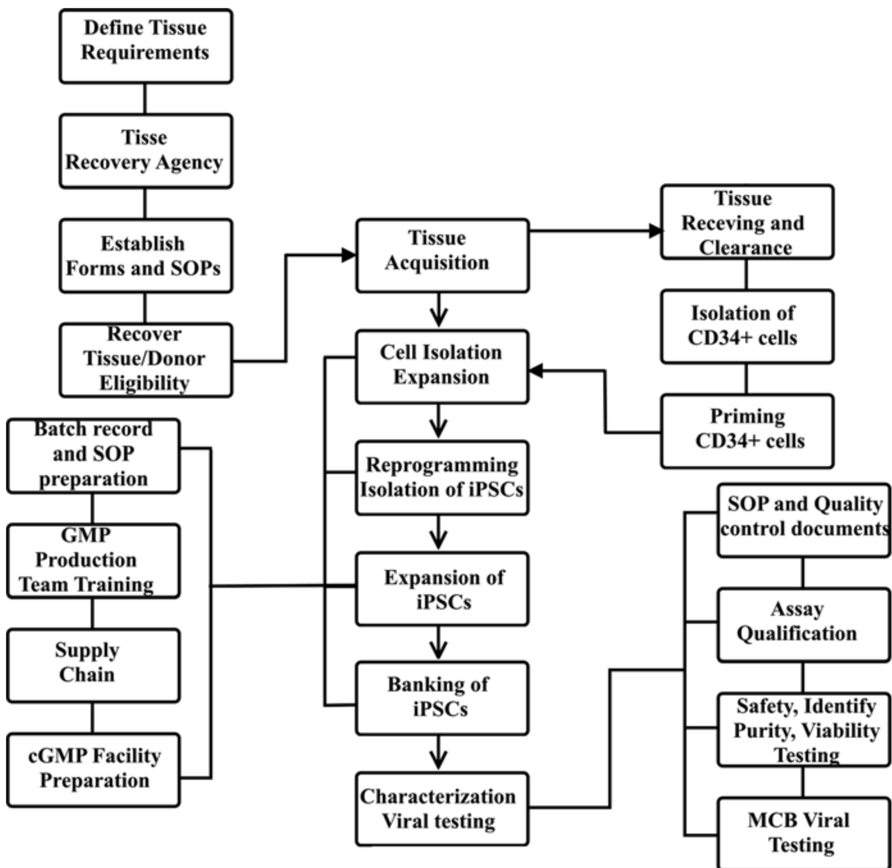

Fig. 4.2 Protocols for production of clinical-grade iPSCs. Clinical-grade iPSC production method requires virus-free systems, absence of animal-derived components, and less invasive techniques

4.2.2 Transferring the Manufacturing Process to the GMP Suite

To produce iPSCs for clinical application, the procedures from the preparation medium to banking the iPSCs should be compliant with GMP standards. GMP compliance procedures request that (1) the laboratory facilities should be designed with a GMP suite and (2) the procedure should be developed to standard operating procedures (SOPs).

A GMP laboratory for iPSC production must perform three phases: phase I, training runs; phase II, engineering runs; and phase III, manufacturing runs. The training runs are related to training SOPs at the laboratory; engineering runs are the final evaluation of all prerequisites related to iPSC manufacturing using SOPs. Manufacturing runs include the production process of iPSCs using the optimized iPSC manufacturing process established during phases I and II.

4.2.3 Quality Control Testing During iPSC Production

According to GMP-compliant production, quality control (QC) testing is always required during the production line. QC testing aims to establish the final product with the highest levels of identity, safety, purity, and viability of the final products. Multiple QC tests should be performed, including plasmid clearance, karyotype, STR, sterility, *Mycoplasma*, and endotoxin tests.

Upon completion of the GMP manufacturing process, a master cell bank of approximately 100 vials (each vial containing approximately 2×10^6 human iPSCs) should be produced and stored in liquid nitrogen following established protocols.

4.3 Applications of iPSCs in Clinic

4.3.1 Disease Modeling and Drug Screening

4.3.1.1 Disease Modeling

The use of animal models to mimic human disorders has been successfully practiced in scientific and clinical research for years. These models have furthered our understanding of the causes and mechanisms of diseases, and many therapeutic drugs have been developed from the results of these analyses. However, preclinical treatment effectiveness does not always guarantee successful human clinical trials. For example, congenital megakaryocytic thrombocytopenia (CAMT) is caused by mutation in the gene encoding MPL (thrombopoietin receptor). Although mutation of the MPL gene in humans results in significantly reduced platelets and

megakaryocytes, the mpl knockout mice did not present conventional CAMT human disease (Hirata et al. 2013). This finding and other research indicated that pathophysiological development processes might differ between humans and mice (Carver-Moore et al. 1996; Ebert et al. 2012). Therefore, this difference reduces direct translation from preclinical animal studies to clinical trials.

Since the early studies on iPSCs, iPSCs have been considered as a powerful tool for in vitro and in vivo modeling of genetic disorder diseases. iPSCs can be used to monitor the development of many diseases, such as hematopoietic, neurological, cardiovascular, hepatic, and other inherited diseases (Ebert et al. 2012; Juopperi et al. 2011). To date, several neurological disease models have been successfully generated using iPSCs, such as models for amyotrophic lateral sclerosis (Dimos et al. 2008), Down syndrome (Park et al. 2008), fragile X syndrome (Urbach et al. 2010), Huntington's disease (Park et al. 2008; Zhang et al. 2010), spinal muscular atrophy (Ebert et al. 2009), and Parkinson's disease (Soldner et al. 2009). Soldner and coworkers showed that human iPSCs become a more suitable cell source for human disease modeling when fibroblasts from Parkinson's disease patients can be efficiently reprogrammed and differentiated into dopaminergic neurons (Soldner et al. 2009). Patient-derived iPSCs could be also generated from other skin cells (Dimos et al. 2008; Takahashi et al. 2007b), neuronal cells (Dimos et al. 2008), hematopoietic cells (Brown et al. 2010), and other cell sources (Sun et al. 2009). The technology to establish human iPSC lines provides a basis to make clean the mechanism of cellular reprogramming. It also helps further our understanding of the safety and efficacy of iPSCs differentiated from humans for next-generation medicine.

The use of iPSCs for modeling disease has led to multiple benefits for the medical industry, especially for treatment of cancer and infectious diseases (Siller et al. 2013). iPSCs are now being used to delineate the molecular events involved in cancer and tumorigenicity, such as the mechanism of their oncogenic potential (Ghosh et al. 2011). Gore and colleges showed that human iPSC lines contain a majority of protein-coding point mutations in the regions sampled as nonsynonymous, nonsense, or splice variants. In addition, these mutations were causative effects in cancers (Gore et al. 2011). Yoshida and coworkers also suggested that iPSC-derived hepatocyte-like (iPSC-Hep) cells are an appropriate model for hepatitis C virus infection as they successfully used iPSCs generated from human hepatocyte-like cells to investigate the entry and genomic replication in iPSC-Hep cells (Yoshida et al. 2011).

Thus, iPSCs have become an important disease model that shows more advantages than other classical models and provides an unlimited source of proliferating cells for next-generation regenerative medicine (Fig. 4.3).

4.3.1.2 Drug Screening

Evaluation of human drug toxicity is a critical stage in the drug discovery process. When a new drug is invented, the prediction of toxicity is a critical issue during safety and efficacy testing (Rubin 2008). Functional cells differentiated from human

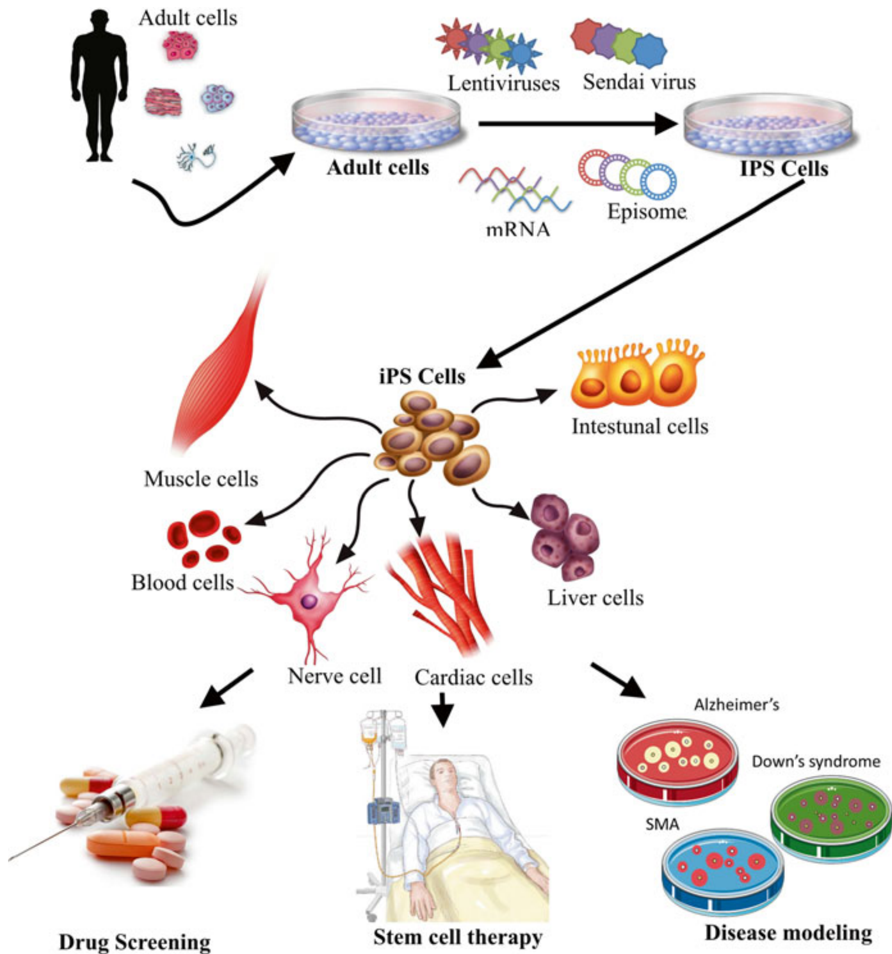

Fig. 4.3 Some strategies for iPSC production and application. IPSC could be produced from some adult cells by transfection with lentiviral viruses, Sendai viruses, mRNA, or episomes of some pluripotent factors. The obtained iPSCs could be used in drug screening, stem cell therapy, and disease modeling

iPSCs, such as hepatic cells, neuron cells, and myocardial cells, could be effectively used for drug screening because of recapitulating salient disease features in vitro (Chun et al. 2010).

A study by Mathur used iPSC-formed cardiomyocytes to construct 3D tissue to mimic a minimal organoid structure of the heart. The authors applied this system to test cardiac response to various drugs. The results showed that human iPSCs derived from the cardiac microphysiological system could significantly improve the ability to predict drug toxicity in vitro (Mathur et al. 2015).

Another study on drug screening used iPSCs to assess and control the activity of new drugs to treat some diseases, such as neurological diseases (Egawa et al. 2012; Xu and Zhong 2013) and liver disease (Choi et al. 2013). Many studies have suggested that patient-derived iPSCs may provide a useful tool for screening drug candidates.

Using iPSCs to prognosticate efficacy and toxicity could help to reduce the cost and duration of drug development processes. iPSCs may also help overcome many of the challenges and shortcomings associated with disease modeling and drug screening. The promise of iPSCs for regenerative medicine and drug screening is likely to be expanded in the near future.

4.3.2 Regenerative Medicine

The discovery of iPSCs has introduced a novel approach in regenerative medicine. Reprogramming of somatic cells has marvelous potential for clinical uses (Cherry and Daley 2013). Although adult stem cells have high potential for degenerative disease, these obstacles could be overcome by limitation of cells and restriction of differentiation capacity. Physiological profile matches in allogeneic stem cell transplantation are also a risk and restriction of adult stem cells. Scientists believe that iPSCs could be used in degenerative disease treatment by generating autologous cells (Cherry and Daley 2013), correcting gene mutation, differentiating into specific tissues, and transferring these to the patient (Cherry and Daley 2013). iPSCs represent an unlimited source of cells that can be used to form functional organ structures. In fact, iPSCs have potential for cell therapies because of their capacity for differentiation into a variety of cell types including neural cells, cardiomyocytes, islet cells, and hepatocytes (Hirschi et al. 2014). However, guidelines and safety issues of these cells should be considered to reduce side effects and increase therapeutic effects.

A number of publications on iPSC generation protocols are available, and many studies have focused on how to develop and enhance large-scale production of specific cell types from iPSCs or directed differentiation into specific lineages (Hirschi et al. 2014). Many factors have been discovered that contribute to differentiation of iPSCs into specific cell types, such as chemicals, growth factors, signaling inhibitors, cytokines, and biophysical stimulation. Furthermore, a number of culture methods have been explored to promote cell differentiation such as coculture with supporting cells and 3D cultures.

In addition, many approaches have been examined to direct reprogramming to reduce the risks of iPSCs. It is believed that direct reprogramming from somatic cells into a specific cell type would eradicate the generation of pluripotent cells (Hirschi et al. 2014).

Table 4.1 Chemicals and small molecules used for neural differentiation

Name/factor	Mechanism
Retinoic acid	Morphogen/agonist of the Sonic Hedgehog pathway
Epidermal growth factor (EGF)	Mitogen
Fibroblast growth factor (FGF-2, FGF-8, FGF-4)	Regulation of neural stem cell proliferation and self-renewal
Platelet-derived growth factor (PDGF)	Neural induction factor
Sonic Hedgehog (SHH)	Morphogen, induction factor
Noggin	Bone morphogenetic protein (BMP) antagonist
SB431542	Inhibition of the TGF-β/activin/nodal pathway/inhibition of SMAD
Dorsomorphin	Inhibition of BMP pathway/inhibition of SMAD
LDN193189	Inhibition of BMP pathway
Purmorphamine	Activation of the Hedgehog pathway

Source: Skalova et al. (2015)

4.3.2.1 iPSCs and Neural Regenerative Medicine

Among specific cell lineages, neural cells have been considered as the first lineage to be reliably attained from iPSCs (Tabar and Studer 2014). A previous report showed that neural lineage differentiation of iPSCs is similar to ESCs (Skalova et al. 2015). iPSCs can differentiate into neural stem cells and neural crest stem cells and subsequently into specific neural lineages (Hirschi et al. 2014). Biochemical factors, small molecules, and morphogen-based induction have been explored to generate neural cells from iPSCs (Table 4.1).

Embryoid body (EB) formation, coculture on feeder layers, and direct neural induction using growth factors have been introduced to derive neuronal subtypes from iPSCs (Tabar and Studer 2014). As an early protocol, EB formation was based on using selection medium for neural cell lineages. Because of similarities between iPSCs and ESCs, analogous factors can induce neural differentiation of these cells; however, iPSC generation is associated with significantly reduced efficiency and increased variability (Hu et al. 2010). For instance, retinoic acid has been effective with both ESCs and iPSCs (Yuan et al. 2013). One challenge to differentiate human iPSCs cells to Purkinje cells has recently been solved by using FGF2 and insulin in EB formation (Wang et al. 2015). Coculture on stroma-feeder has also been extensively applied for production of neural cells, including dopamine neuron-like cells, from iPSCs (Tabar and Studer 2014). Muratone and colleagues showed that coculture of iPSC-derived neurons with astrocytes increases neuronal maturity by day 40 (Muratore et al. 2014).

Direct induction protocols do not require EB formation and coculture induction; however, inhibition of TGF-β receptors (reviewed by Hirschi et al. 2014) and the SMAD signaling pathway (Chambers et al. 2009) is an important key in the differentiation of iPSCs into neural cells. Previous studies showed that inhibitors of TGF and BMP (dual SMAD inhibition) have extremely enhanced the efficiency and the

speed of neural induction. However, regardless of the specific neural induction strategy used, the main challenge during the past 10 years has been to determine how to combine these approaches and optimize the culture conditions with biochemical and biophysical factors to enrich a specific neural lineage.

Recently, direct transdifferentiation from somatic cells to multipotent neural stem cells has been explored. Fibroblasts and other types of cells have been transdifferentiated directly into neural lineages with combinations of transcription factors (Ambasudhan et al. 2011; Caiazzo et al. 2011; Son et al. 2011; Vierbuchen et al. 2010). This approach would also lower the risk of teratoma formation as a strategy to bypass the pluripotent stage (Ring et al. 2012). A single factor, Sox2, has been used to direct reprogramming of fibroblasts into multipotent neural stem cells (Ring et al. 2012).

4.3.2.2 iPSCs in Cardiac Regenerative Medicine

Mummery and colleagues explored a model of coculture of human ESCs with visceral-endoderm-like cells (END-2) to promote cardiomyogenesis of human ESCs (Mummery et al. 2003) (reviewed by (Acimovic et al. 2014; Sinnecker et al. 2014). Cardiac differentiation has been successful using the same protocol with iPSCs (Freund et al. 2010) (reviewed by Acimovic et al. 2014; Sinnecker et al. 2014). Cardiac differentiation using this protocol has been considered as one of the first protocols to direct iPSCs into cardiomyocytes.

EB using the hanging drop method is a common method for generating functional cardiomyocytes from iPSCs (Tabar and Studer 2014). Compared with ES cells, the efficiency of iPS differentiation into cardiomyocytes is lower (Zhang et al. 2009; Zwi et al. 2009). In this approach, several differentiation factors, such as activin A, BMP-4, AA/Nodal, Bmp4, Cerberus, and Wnt3a, have shown high efficiency in inducing iPSC differentiation into cardiomyocytes (Skalova et al. 2015). Recently, these growth factors have been combined with small molecules to promote the differentiation of iPSCs (Skalova et al. 2015). A large number of small molecules have been used to induce iPSC differentiation, including 5-azacytidine (Qian et al. 2012) (reviewed by Liu et al. 2013), RepSox (Ichida et al. 2009), valproic acid (Qian et al. 2012), KY02112 (Minami et al. 2012, Bay K8644 (Mehta et al. 2014), and dimethyl sulfoxide (DMSO) (Chetty et al. 2013). These small molecules are involved in specific signaling pathways and function as specific inhibitors (pluripotin, RepSox, valproic acid, KY02112), agonists (Bay K 8644), and regulators (DMSO) of the differentiation process.

Moreover, a model of 3D cell culture using biowire technology, collagen wires, and electrical stimulation has been explored for iPSC differentiation to cardiomyocytes (Nunes et al. 2013) (reviewed by Acimovic et al. 2014; Hirschi et al. 2014; Sinnecker et al. 2014). Furthermore, a model of coculture with OP9 cells showed that iPSCs could be differentiated into endothelial cells and hematopoietic progenitor cells (Choi et al. 2009).

Recently, direct transdifferentiation from somatic cells to cardiomyocytes has been introduced as a safety protocol with a low risk of tumorigenesis because of pluripotent stage elimination (Fu et al. 2013; Wada et al. 2013) (reviewed by Acimovic et al. 2014; Tabar and Studer 2014). Many kinds of growth factors and transcription factors have been discovered to induce direct reprogramming from somatic cells to cardiomyocytes. Overexpression of transcription factors including Gata4, Mef2, and Tbx5 caused the direct transdifferentiation of fibroblasts into cardiomyocytes (Xu 2012). Recently, the role of noncoding microRNAs including miR-1, miR-133, miR-208, and miR-499 in direct transdifferentiation into cardiomyocytes has been demonstrated (Piubelli et al. 2014; Xin et al. 2013; Xu 2012). Although several protocols have been explored, the translational potential of this approach needs to be determined. Further confirmation of maturation and function of transdifferentiation cells is required.

Further application of iPSC-derived cardiomyocytes in regenerative medicine requires a high-throughput method for cell purification. Although it is possible to purify cardiomyocytes from differentiated iPSCs by flow cytometry using several markers such as EMILIN2, SIRPA, and VCAM (reviewed in Sinnecker et al. 2014), specific markers for iPSC-derived cardiomyocytes should be explored.

Heart disease is currently considered as the most serious disease with a high death rate. Some researchers have proposed that cardiomyocyte transplantation may restore both structure and function of the heart (reviewed by Skalova et al. 2015). Therefore, cardiomyocytes from iPSCs may be a potential source of cells for therapeutic intervention in heart regenerative medicine.

4.3.2.3 iPSCs in Diabetic Mellitus and Liver Disease

Owing to the shortage of β-cells for transplantation, iPSCs may be a potential source of cells to treat diabetic mellitus. In early studies, a robust protocol to induce human iPSC differentiation into insulin-producing cells in vitro was published (Kunisada et al. 2012; Zaida et al. 2010). Later, Pagliuca and colleagues explored an efficiency differentiation protocol using polyhormonal (PH) cells. PH cells resemble fetal β-cells more than they do mature β-cells, and these cells functioned like primary β-cells in vitro and in vivo posttransplantation (Pagliuca et al. 2014). Another protocol based on 3D culture combined with forskolin, dexamethasone, Alk5 inhibitor II, and nicotinamide showed high efficiency in iPSC differentiation into pancreatic progenitor cells (Takeuchi et al. 2014). A recent study reported the induction of iPSCs into islet-like clusters via a four-step protocol using biochemical and growth factors [insulin, transferrin, selenium (ITS), N2, B27, fibroblast growth factor, and nicotinamide] (Shaer et al. 2015). Despite the promising results of the use of iPSCs in diabetic mellitus, these studies are still in the early stages. Because of the lack of monitoring of the safety and long-term efficacy of iPSCs, more research should be performed.

Many studies have demonstrated that iPSCs can differentiate into hepatocyte-like cells (Ghodsizadeh 2010; Forbes and Newsome 2012; Yu et al. 2012). Currently,

endoderm differentiation from iPSCs can be promoted by using activin A or a combination of activin A with hepatocyte growth factor and Wnt3a (Chen et al. 2012; Sullivan et al. 2010). iPSC-derived hepatocytes were generated by BMP-2/bFGF and HGF combined with low oxygen culture (Hirschi et al. 2014). Hepatocytes can also be obtained using direct reprogramming. By using Gata4, Hnf1α, and Foxa3 combined with p19 inactivation, Huang and colleagues successfully induced iPSCs into functional hepatocyte-like cells (Huang et al. 2011). Modification strategies have since been introduced using a variety of combinations of transcription factors (Hnf4α, Foxa1, Foxa2, or Foxa3) (Sekiya and Suzuki 2011; Takayama et al. 2012).

Another efficient differentiation protocol for generating functional hepatocyte-like cells from iPSCs uses a 3D microscale culture system (Zhang et al. 2014). By this promising approach, a large number of hepatocyte-like cells can be generated from iPSCs; this implies the potential of iPSCs in future industrial and clinical applications.

4.3.3 Clinical Applications of iPSCs

The first clinical application of iPSCs was reported in Japan in September 2014. A 70-year-old female patient suffering from exudative age-related macular degeneration was transplanted with a cell sheet derived from autologous iPSCs. The primary aim of this clinical research study was to demonstrate the safety of the transplantation of the iPSC-derived retinal pigment epithelium (RPE) sheets. Therefore, the patient would be monitored and evaluated for 1 year.

In this study, autologous iPSCs were produced from skin cells taken from a patient and then differentiated into RPE cells, and small monolayered sheets were produced. Before transplantation, RPE sheets went through a rigorous safety and quality check. Especially, RPE were investigated concerning cell shape and function and gene expression equivalent to in vivo RPE. The study also confirmed no traces of the plasmid used to initially insert genes to reprogram the skin cells and no undifferentiated cells. iPSC-derived RPE cells also did not show tumorigenicity in animals.

4.4 Conclusions

iPSCs are a potential therapeutic strategy for disease treatment. After approximately 10 years of technology development, iPSCs are now ready for clinical applications. Production of clinical-grade iPSCs has been confirmed with some breakthroughs including the complete removal of virus vectors to deliver

pluripotent factors, culture systems without animal cells or contamination, and iPSC production according to GMP standards. These improvements in iPSC technology will significantly contribute not only to drug discovery and drug screening but also to regenerative medicine.

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Chapter 5
The Effects of Ageing on Proliferation Potential, Differentiation Potential and Cell Surface Characterisation of Human Mesenchymal Stem Cells

Emma Fossett, Yiannis Pengas, and Wasim S. Khan

5.1 Introduction

Stem cells are an undifferentiated population that develop asymmetrically generating specialised cell types through cell differentiation and possess the ability to self-renew (Kanitkar et al. 2011). Stem cells are a slow cycling but highly clonogenic population. Stem cells have a varying differentiation potential depending on their hierarchical position. The earliest and most potent stem cell of the human body, the fertilised oocyte, is totipotent, having the potential to differentiate into all cell types of the embryo, as well as trophoblasts of the placenta. As differentiation of the oocyte into the blastocyst occurs, potency of the cells decreases. The inner cell mass (ICM) cells are pluripotent, with the capacity to differentiate into cells of the three germ layers: endoderm, mesoderm and ectoderm. As development of the foetus continues, cells become progressively more restricted in differentiation potential, with the production of multipotent, oligopotent and unipotent stem cells (Alison et al. 2002).

The two types of stem cells with a large research and clinical application interest are embryonic stem (ES) cells and adult stem cells. ES cells are derived from human embryos in the blastocyst stage of development. The blastocyst is formed at days 4–5 after fertilisation and is composed of an inner cell mass (ICM) and an outer cell mass. It is the ICM that is the source of these pluripotent embryonic stem cells that have the ability to differentiate into all three germ layers producing all specialised cells of the human body, except supporting tissues of the embryo (Alison et al. 2002).

Stem cells of a multipotential nature, mesenchymal stem cells (MSCs), have been discovered in adult tissue. As ethical issues surround the use of ES cells and permission of their use being limited in many countries, the focus has turned to investigating the

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potential of these adult MSCs. Their presence was first reported by the works of Friedenstein and colleagues in which stromal bone marrow tissue was found to contain colonies of cells with a spindle-like fibroblastic appearance in vitro (Friedenstein et al. 1968, 1974, 1970). These cells were found to adhere effectively to plastic in culture, enabling non-adherent cells that were not MSCs to be filtered out. These cells also showed that after a lag phase of 2–4 days, they have the ability to rapidly proliferate in vitro, demonstrating their potential to differentiate down mesenchymal lineages into osteoblasts, adipocytes and chrondrocytes in vitro and following heterotopic transplantation (Friedenstein et al. 1966; Friedenstein 1980). Further work by others confirmed the multipotential nature of mesenchymal stem cells from bone marrow as well as from many other tissue sites of the human body, such as from skeletal muscle, synovium, periosteum, adipose tissue and fat pad (Sakaguchi et al. 2005; Khan et al. 2012).

Adult mesenchymal stem cells are found in very low numbers in most adult tissues, for example, satellite cells make up only about 5 % of muscle tissue (Alison et al. 2002), and only one out of every 18,000 bone marrow nucleated cells are MSCs (Muschler et al. 2001). These adult MSCs lay quiescent in tissues until activated to replace local cells that have undergone alterations due to injury, ageing or normal tissue turnover, restoring natural function to tissues in vivo (Chen and Tuan 2008).

Numerous studies have commented on the ability of the use of MSCs in clinical applications and regenerative medicine due to their ability to adhere to plastic, proliferate and differentiate in vitro, and as they have a low or absent expression of HLA-2 markers on their cell surface, they are suitable for transplantation, as they have low immunogenic and high immunosuppressive properties (Le and Ringden 2005). One of the best qualities of stem cell therapy is tissue-directed differentiation. This enables stem cells from one tissue origin to differentiate into another cell type once transplanted. For example, Concejero and colleagues reported that they were able to osteogenically differentiate fat-derived stem cells and use them along with three-dimensional scaffolds to reconstruct rat palatal bone defects (Conejero et al. 2006). A phase one clinical trial reported that myocardial regeneration occurred in patients who had previously had an acute transmural myocardial infarction, as well as reporting an enhanced left ventricular function and improved tissue perfusion to infarcted areas after transplantation of bone marrow stem cells (Stamm et al. 2003).

Mesenchymal stem cells have demonstrated immense capabilities for their use in regenerative medicine and clinical applications. One question that poses doubt into their use is how they are affected by the ageing process. With an increase in the prevalence of cardiovascular, musculoskeletal and neurological disorders due to an ageing population, the use of MSCs in autologous transplantation to restore tissues to their normal function is becoming of greater importance.

5.2 Ageing

The complex progressive process of ageing causes changes in every cell of an organism, causing a decline in its bodily functions, such as an elongated wound and bone healing time, loss of lung elasticity and an increased susceptibility to

infections (Ho et al. 2005). Ageing is also seen as a reflection of the cumulative senescence of an organism's component cells (Clark 1999). During senescence, the replicative potential of stem cells halts, and the repairing and replacing process of damaged cells is prevented, interfering with the function of the tissue involved. Some studies have suggested that a decline in stem cell numbers throughout the body may contribute to ageing and the appearance of age-related disease such as osteoarthritis (Ho et al. 2005). Previous studies state that on average cells stop dividing after an average of 50 cumulative population doublings and can remain alive, unlike apoptotic cells, despite a derangement of function (Fossett and Khan 2012; Itahana et al. 2001).

Studies investigating the effect of ageing on MSCs have reported inconsistent results, with some studies finding age-related changes and many finding no difference. How ageing affects proliferation rate, differentiation potential and cell surface characterisation will be discussed here.

5.3 Ageing and Proliferation

It is important to research how age affects proliferation rate of MSCs to enable a decision to be made as to whether MSCs from older patients are suitable for expansion in vitro prior to autologous transplantation, as the need for a biological repair of age-related diseases is increasing with the ageing population. A mixed result from studies about the effect of age on proliferation rate has been published by numerous authors, using various MSC sources, although as with most MSCs studies, many results have been obtained using bone marrow-derived mesenchymal stem cell (BMSCs).

A study using BMSCs from three age groups (0–20, 21–40 and >40 years old) by Stolzing et al. (2008) looked at the effect of age on proliferation potential. They found that for the first 5 weeks in culture, growth patterns of all three groups were similar; however, after 5 weeks, growth of the MSCs from the group older than 40-year-olds began to decline, with the growth curve reaching a plateau compared to the other two groups. This was investigated for 4 months in culture with groups achieving 17–35 cumulative population doublings. It was also noted that significantly lower CFU-f numbers following MNC isolation was seen in the older donors compared to younger. This was confirmed using multi-parameter flow cytometry where the frequency of CD45lowD7-FIB + LNGFR+ cells was also found to show an age-related decline. Shamsul and colleagues also reported a declined proliferative ability of BMSCs, as 14 out of 15 samples from donors over 40 years old failed to proliferate compared to 16 out of 23 samples successfully proliferating from donors younger than 40 years old (Shamsul et al. 2004). A more recent study stated that the time taken for single-seeded BMSCs in 96-well plates to undergo one population doubling in passage 3 was more than double for MSCs from older patients than that of the younger patients (young 37.9 and old 89.4 h per population doublings), providing results that are similar to previous literature (Dexheimer et al. 2011). An age-related increase in hours per population doubling was consistently

higher for MSCs from older patients throughout passages 1–4. This is also supported by Zhou et al. (2008) and Mareschi et al. (2006) who both reported a doubling time for BMSCs of older donors that was almost double that of younger donor MSCs.

In comparison, many studies have reported no effect of age on proliferation of MSCs. Acquiring 98 samples of MSCs from the femoral shaft, Scharstuhl and colleagues found no correlation of age with proliferation, as well as MSC yield and cell size (Scharstuhl et al. 2007). A study extracting MSCs from the neck of femur showed the same results as from the femoral neck: that proliferation has no correlation with age of donor (Suva et al. 2004). No statistical relationship was found by Phinney and colleagues when growth rates of 22 samples of MSCs from the iliac crest were correlated against age (Phinney et al. 1999). One study using infrapatellar fat pad-derived MSCs measured cell numbers on five occasions (day 2, 4, 6, 8 and 10) using a haemocytometer for two age groups. There were no significant differences between cell numbers from each group at each time point or any difference between proliferation rates up to day 10 (Khan et al. 2009).

5.4 Ageing and Differentiation Potential

The ability of MSCs to differentiate in vitro makes them useful in cell-based therapies; however, there is no conclusive answer as to whether MSC differentiation potential is affected by age of donor. Most studies looking at the effect of age on differentiation have explored osteogenic differentiation potential of MSCs. Some work has suggested that osteogenic differentiation potential is the first to be lost by MSCs during ageing and that MSCs increase their adipogenic potential (Meunier et al. 1971). However, other work states that adipogenic differentiation is lost first (Muraglia et al. 2000).

In 2008, Zhou and colleagues investigated the effect of age on osteoblastogenesis of human BMSCs of 17–90-year-olds. When measuring alkaline phosphatase (AlkP) enzyme activity, they reported that there was a significant decrease of AlkP activity in older donors compared to younger. Similar trends inferring that osteogenic differentiation decreases with increasing age were confirmed when semi-quantitative RT-PCR analysis was carried out showing a significantly greater expression of osteoblast marker genes (Cbfa1/Runx2, osterix, AlkP, bone sialoprotein (BSP) and osteocalcin (OC)) from BMSCs of younger donors (Zhou et al. 2008). A study by Stolzing et al. (2008) investigated osteogenic, chondrogenic and adipogenic differentiation potential also using BMSCs, in three age groups: 7–18, 19–40 and >40 years old. Results showed that the "Aged" group (>40 years old) had the lowest AlkP activity, lowest amount of Oil Red O positive cells and lowest amount of GAG concentration compared to the "Young" (7–18 years old) and "Adult" (19–40 years old) groups, implying that BMSCs from older donors had a decreased differentiation potential down all three lineages. Interestingly, one study that also looked at mRNA expression by executing quantitative RT-PCR found a positive correlation of RANKL (osteoclast marker) and PPAR-gamma (adipocyte marker) with increasing age in BMSCs from females but not males (Jiang et al. 2008). They also reported an age-related decrease

in Cba1/Runx2 (osteoblast marker) but no significant relationship between age and SOX9 (adipocyte marker). Similarly with osteogenic differentiation, Roura et al. (2006) also found a strong negative correlation with donor age when comparing two groups with mean ages of 24 and 77. However, no significant differences with age were found when analysing adipogenic differentiation on CD105+ bone marrow MSCs.

On the other hand, investigating osteogenic differentiation of infrapatellar fat pad-derived MSCs found no differences with age of donor (Khan et al. 2009). Alizarin red staining and AlkP enzyme activity assays also showed no difference between the two age groups (mean ages 57 and 86 years old). Similarly, Evans et al. (1990) also noticed no change in osteogenic differentiation of MSCs obtained from trabecular bone as osteocalcin levels, and AlkP synthesis did not show any change with increasing donor age. When investigating chondrogenic differentiation, Scharstuhl et al. (2007) reported no age-dependant changes in differentiation after investigating with 98 MSC samples from the femur.

5.5 Ageing and Cell Surface Characterisation

Cell surface characterisation has been achieved by looking at cluster of differentiation (CD) markers and cell surface proteins via flow cytometry and cell surface staining. The origin of the cell and its differentiation potential can be shown by characterisation (Khan et al. 2009). The International Society for Cytotherapy has suggested that for a population of cells to qualify as MSCs, they should have positive expression for CD73, CD90 and CD105 and be negative for CD14, CD34, CD45 or CD11b, CD79a or CD19 and HLA-DR surface molecules (Dominici et al. 2006).

There is little literature on the effect of age on cell surface characterisation. Khan et al. (2009) found no differences of cell surface marker expression between two groups with mean ages of 57 and 86 when investigating the effect of age on cell surface characterisation using human fat pad-derived MSCs. Similar trends have also been noted by Mareschi and colleagues in a study using BMSCs. They reported that there was no significant difference in the expression of cell surface markers with age after analysing them throughout ten passages (77 days) (Mareschi et al. 2006). In comparison, Stolzing et al. (2008) reported that BMSCs were positive for CD13, CD44, CD90, CD105, STRO-1 and D7-Fib, and of those markers the majority (CD44, CD90, CD105 and STRO-1) were found to have significant age-related changes in expression when looking at three age groups (7–18 years old, 19–40 years old and >40 years old). It is important to determine how age affects cell surface characterisation, as consistently expressed markers can then be used to isolate MSC populations regardless of age of donor.

Although cell surface characterisation is important to identify cells, results between studies are not always reliable as the expression of markers can differ depending on the amount of time cells spent in culture, levels of foetal calf serum (FCS) in culture medium which inhibits expression of some surface markers (Garcia-Pacheco et al. 2001), antibody source and variability and donor variations (Fossett et al. 2012).

5.6 Conclusion

Although there are no consistent results as to whether MSCs are affected by ageing, of those studies that did find a relationship, there are again discrepancies in how MSCs are affected. Some reasons as to why inconsistent results have been obtained are due to studies using different cut-off points for age groups, and the inclusion of confounders such as gender, medical history and chronic illness is not accounted for but could all cause a difference in results. Also, the period of time spent proliferating in culture before differentiation media is added could affect the differentiation properties of MSCs as they begin to lose their characteristics when in ex vivo conditions for a long duration. In addition, the type of growth and differentiation media, characterisation methods, the passage of cells used and source of MSCs can all contribute to discrepancies in results.

There are various MSC age markers that are thought to be at an increased level in MSCs from older donors and interfere with the function of MSCs. Some literature has suggested that age-related changes in the surrounding environment of MSCs can affect them such as a reduction of proteoglycans and glycosaminoglycans. This change has been found to reduce viability and proliferation potential of MSCs in vivo (Bi et al. 2005). Zhou et al. investigated whether there are age-related intrinsic factors affecting MSCs. They found that there was four times as much senescence-associated β-galactosidase found in MSCs from older than younger donors (Zhou et al. 2008). This is supported by Stolzing et al. (2008) who also found an increase in senescence-associated β-galactosidase with age. A positive correlation relating age with levels of advanced glycation end products (AGEs) has also been suggested by Zhou et al. to inhibit proliferation by increasing reactive oxygen species production (ROS) and inducing apoptosis in MSCs. This is also linked to the age-related increase of RAGE, the receptor for uptake of AGEs (Stolzing et al. 2008). Zhou et al. also suggested that an age-related increase in p53 and its pathway genes, p21 and BAX, may be responsible for a reduction in proliferation ability and osteoblastogenesis. A tumour suppressor (p161NKa) that inhibits G1 cyclin-dependant kinases 4 and 6 prevents the cell cycle and therefore reduces proliferation (Zindy et al. 1997).

This chapter has focused on how ageing affects properties of MSCs: proliferation, differentiation and also cell surface characterisation. It can be concluded that there is a mixed view about the effects of ageing in the current literature. It is important to identify the relationship between ageing and MSCs to find out whether they can be used for autologous transplantation of older patients, as the prevalence of age-related disorders is increasing. Our described protocols for the isolation and expansion of these cells are applicable to cells from patients of all ages.

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Chapter 6
Production of Clinical-Grade Mesenchymal Stem Cells

Phuc Van Pham and Ngoc Bich Vu

6.1 Introduction

Stem cell transplantation is a novel treatment method for many diseases, especially degenerative diseases. There are reports of the clinical application of stem cells for more than 70 diseases. Mesenchymal stem cells (MSCs) have become popular for disease treatment in recent years via two approaches: personalized medicine (autologous transplantation) and as a stem cell drug (allogeneic transplantation) (Larsen and Lewis 2011; Squillaro et al. 2015).

According to clinicaltrials.gov (2015), there are currently about 560 clinical trials using MSCs from several sources (Fig. 6.1). Many clinical trials are being performed in East Asia (176/560) and North America (111/560) (Table 6.1). Although there are more than 20 diseases that can be treated by MSC transplantation, researches have focused on two groups of diseases: degenerative and immune system-related diseases. Unlike other kinds of stem cells, MSCs exhibit two therapeutic properties including a differentiation potential for specific cell types such as osteoblasts (Montespan et al. 2014; Shao et al. 2015), chondroblasts (Berninger et al. 2013; Perdisa et al. 2015), and adipocytes (Gruia et al. 2015; Lee et al. 2015) and immunomodulation of certain kinds of immune cells such as T cells, B cells,

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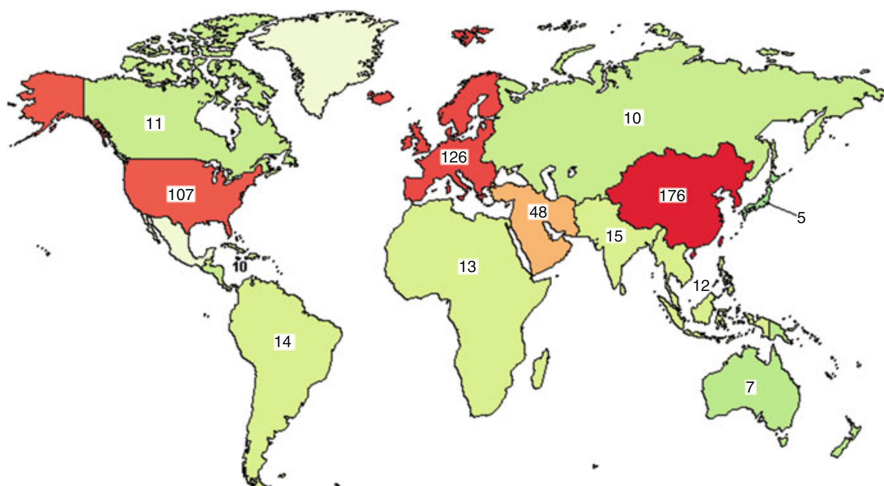

Fig. 6.1 Clinical trials using MSCs according to clinicaltrials.gov. There are more than 500 clinical trials registered at clinicaltrials.gov. Many clinical trials are being performed in East Asia and North America

Table 6.1 Distribution of clinical trials using MSCs worldwide (according to clinicatrial.gov, November 20, 2015)

Region name	Number of studies
Africa	13
Central America	10
East Asia	176
Japan	5
Europe	126
Middle East	48
North America	111
Canada	11
USA	107
North Asia	10
Pacifica	7
South America	14
South Asia	15
Southeast Asia	12
World	560

natural killer cells, dendritic cells, and T regulatory cells (Cardoso et al. 2012; Melief et al. 2013; Saeidi et al. 2013). Therefore, in earlier clinical studies, MSCs have differentiated into specific cells to recover the degenerated cells in injured tissues, whereas recent clinical studies have used the immunomodulation of MSCs to treat immune dysfunction.

Recent studies have shown that allogeneic MSCs can perform better immunomodulation than autologous MSCs. These results triggered the use of allogeneic MSCs in clinical applications. Commercialized MSC-based products have been developed and approved as stem cell drugs in some countries (Table 6.2). Osteocel (NuVasive), Trinity (Orthofix), and LiquidGen (Skye Orthobiologics) use allogeneic MSCs as the main component for bone regeneration and reduction of inflammation. MSC-based products have also been approved in Canada and Korea for certain diseases. Cartistem is stem cell drug containing umbilical cord blood-derived MSCs, which was approved in Korea as a drug for osteoarthritis. In 2012, Prochymal (Osiris Therapeutics), an allogeneic MSC-based product, was approved in Canada for graft-versus-host disease treatment. To date (2015), there are nine commercialized MSC-based products approved worldwide. Interestingly, most of them are allogeneic MSC-based products (Table 6.2).

Although MSCs are widely used in clinical treatments, there still are some issues related to the quality and safety of MSCs (Fig. 6.2). To maintain MSC quality and reduce the risks after MSC transplantation, MSCs should be produced in accordance with good manufacturing practice (GMP) guidelines.

Table 6.2 MSC products approved for clinical use

Names of products	Component of stem cells	Indications	Kind of transplan tation	Company	Country
Cartistem	MSCs from UCB	OA	Allo	Medipost	Korea
MPC	MSCs	N/A	Allo	Mesoblast	Australia
Cupistem	MSCs from AT	Fistulizing Crohn's disease	Auto	Anterogen	Korea
Prochymal	MSCs from BM	GVHD	Allo	Osiris Therapeutics	Canada
AlloStem	MSCs from BM	OA	Allo	AlloSource	America
Hearticellgram-AMI	MSCs from BM	Acute myocardial infarction	Auto	FCB Pharmicell	Korea
Osteocel Plus	MSCs from BM	OA	Allo	NuVasive	America
Trinity Evolution	MSCs from BM	OA	Allo	Orthofix	America
CardioRel	MSCs from BM	Acute myocardial infarction	Auto	Reliance Life Sciences	India

MSCs mesenchymal stem cells, *BM* bone marrow, *UCB* umbilical cord blood, *Auto* autologous, *Allo* allogeneic, *OA* osteoarthritis

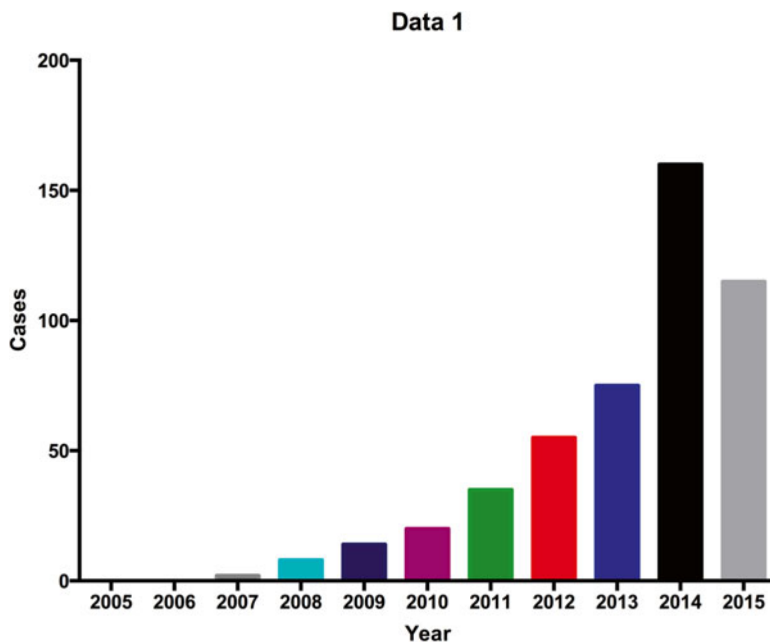

Fig. 6.2 Clinical trials using MSCs (clinicaltrials.gov). The number of clinical trials using MSCs dramatically increases from 2007 to date. In 2015, the number of clinical trials was recorded to June 2016

6.2 GMP Principles

First, it is necessary to mention that GMP guidelines are not instructions or procedures to produce or manufacture any specific product. In fact, GMP guidelines are a series of principles that must be fulfilled during the manufacturing process. The application of GMP guidelines during the manufacturing process will not only result in high-quality products but also maintain the quality from lot to lot. Similar to medical production, cell-based products are also regulated as medicines and must comply with GMP.

However, cell-based products have different properties compared with medicines as follows. (1) Cell-based products are live cells; (2) these products require special methods for storage and transportation; (3) it is difficult to decontaminate bacteria, fungi, and viruses; (4) product quality is difficult to control; and (5) it is difficult to scale up the manufacturing procedure. Some cell-based products are considered as personalized medicine produced to apply to only one patient. Therefore, stem cell-based products are divided into two groups: (1) personalized medicine in which isolated stem cells from patients are reinjected into themselves and (2) stem cell drugs developed from stem cells to treat diseases in groups of patients. Personalized medicine is autologous transplantation of stem cells, whereas stem cell drugs are allogeneic transplantation of stem cells.

Fig. 6.3 Five principles of GMP. There are five principles for GMP including (1) hygiene in production, (2) quality management, (3) suitable facilities and qualified personnel, (4) complaints and recall, and (5) traceability

To date, there are no complete GMP guidelines for cell-based products, although some main principles should be noted during the manufacturing process (Fig. 6.3).

- Materials: According to GMP guidelines, all raw materials must be controlled for quality. Popular materials produced under GMP should be chosen for further development. Otherwise, they should have documented quality with a certificate of analysis for each batch. For cell-based products, materials such as culture medium and kits for isolation should be clinical or in vitro diagnostics (IVD) grade. However, in some cases, there are no commercial products that satisfy these requirements, and non-clinical-grade materials are used. When using non-clinical-grade raw materials, it is important to note the endotoxin concentration.
- Manufacturing processes: all steps in the manufacturing process must be organized as standard operational procedures (SOPs). All batches should be documented from beginning the preparation of raw materials to storage of the final product. Any deviations from SOPs must be documented.
- Validation: National legislation usually has sets of recommended procedures for certain parts of the manufacturing process (e.g., required tests for bacterial contamination are described in pharmacopoeia). Validation is the comparison of alternative procedures to customary procedures and proof that these deviations from standard procedures bring desired outcomes.
- Standardization: Good management of internal quality controls is necessary. At present, there are many programs for external quality controls, which are performed by national authorities or commercial organizations. A set of standards has to be adopted for the release of product batches.

Requirements for cellular products are also mentioned in the *International Standards for Cellular Therapy Product Collection, Processing and Administration* (Fourth Edition, Version 4.1, April 2011) established by FACT-JACIE.

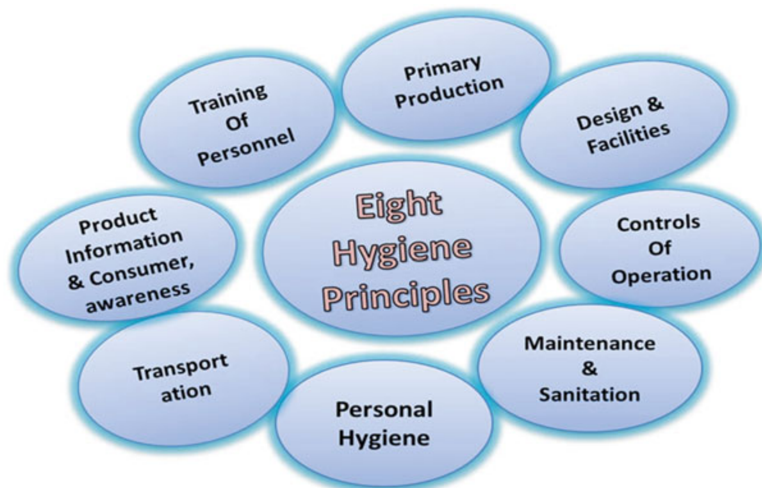

Fig. 6.4 Eight hygiene principles of GMP: (1) primary production, (2) design and facilities, (3) controls of operation, (4) maintenance and sanitation, (5) personal hygiene, (6) transportation, (7) product information and consumer awareness, (8) training of personnel

– Traceability: Records of manufacture to enable the complete history of a batch to be traced are retained. A system is available for recalling any batch of product from sale or supply.
– Training: Operators must be fully trained in SOPs.

GMP requirements are regulated by national and international legislature. For example, Europe has the European Medicines Agency (EMA) and the USA has the Food and Drug Administration (FDA). Australia, Canada, Japan, Singapore, and the UK have highly developed GMP requirements. In other countries, especially in the developing world, the World Health Organization (WHO) version of GMP is used by pharmaceutical regulators and the pharmaceutical industry (WHO-GMP) (Fig. 6.4).

6.3 GMP Facilities for Cellular Products

GMP facilities are a basic prerequisite for GMP preparation of medicinal and cellular products. The core of GMP facilities for cellular products is a clean room. They are designed to create the appropriate production environment to prevent product contamination by raw materials and cross contamination between batches and to ensure that SOPs are followed as intended.

A clean room should be designed to generally comply with International Standard ISO 14644—clean rooms and associated controlled environments. ISO 14644 consists of eight parts:

- ISO 14644-1: Classification of air cleanliness
- ISO 14644-2: Specifications for testing and monitoring to prove continued compliance with ISO 14644-1
- ISO 14644-3: Test methods
- ISO 14644-4: Design, construction, and start-up
- ISO 14644-5: Operation
- ISO 14644-6: Vocabulary
- ISO 14644-7: Separative devices (clean air hoods, glove boxes, isolators, and mini-environments)
- ISO 14644-8: Classification of airborne molecular contamination

There are two classes of clean room suggested for cellular production: classes 1000 and 10,000. However, clean room class 10,000 is very expensive with high initial investment and maintenance fees; therefore, most GMP facilities for cellular production are clean room class 1000. Specifications of the two kinds of clean room are listed in Table 6.3.

In addition to a clean room, GMP facility equipment for production procedures should also be considered. Because of limited funds, it is necessary to prioritize the specific needs of the facility. All equipment should be divided into three groups:

1. Essential: tissue culture cannot be performed reliably without this equipment.
2. Beneficial: culture would be performed better, more efficiently, quicker, or with less labor.
3. Useful: items that would improve working conditions, reduce fatigue, enable more sophisticated analyses to be made, or make the working environment more attractive.

Based on the budget, required equipment should be prioritized when purchasing instruments. However, there are three issues that should be considered before purchasing equipment for GMP facilities:

Table 6.3 Specifications and test results of two classes of clean room facility based on ISO 14644 standards

	ISO class 6 (class 1000)		ISO class 7 (class 10,000)	
Cleanroom classes specifications	Standard limit	Test result	Standard limit	Test result
Air change per hour	≥110	115	≥50	60
Particle count				
0.5 µm	35,200	12,849	352,000	169,969
5 µm	293	424[a]	2930	1977
Temperature (°C)	22 ± 3	21.5	22 ± 3	21
Noise (dB)	≤65	59	≤65	60
Air pressure (Pa)	≥37.5	38	≥12.5	15
Humidity (%)	30 ± 15	26	30 ± 15	22
Microbial count (CFU/m^3)	<5	3	<50	22

[a]Non-compliance with the specified ISO class. The tests were performed at-operation by cleanroom supplier

Table 6.4 Elements that should be carefully considered for cellular production in GMP facilities

No.	Elements	Specifics
1	Quality program	Formal quality program, all aspects of operations, assure GMP compliance
2	Organization and personnel	Personnel qualification, training
3	Procedures	SOPs for all significant manufacturing steps, authorizations of deviations
4	Facilities	Facility and equipment operations, cleaning, validation Equipment and environmental monitoring
5	Environmental control, monitoring	
6	Equipment	
7	Supplies and reagents	Requirements, qualification, control of materials
8	Process controls	Validation, control of manufacturing processes, process modifications Corrective action plan
9	Process changes	
10	Process validation	
11	Labeling controls	Controlled product labeling, prevention of mix-ups
12	Storage	Provisions for raw materials, product storage
13	Receipt and distribution	Record keeping, data management Tracking—from donor to recipient, recipient to donor Outcome analysis, deviation tracking, AE reporting
14	Records	
15	Tracking	
16	Complaint file	

– Different to research laboratories or facilities, GMP facilities for cellular production are only investigated when some technologies that used in these facilities were located. All suggested equipment should be arranged according to the stages of the production line.
– IVD- and FDA-approved equipment or research only equipment: IVD- and FDA-approved equipment is considered as priority machines to purchase for a GMP facility. However, not all machines for cellular production are compliant with IVD or FDA conditions. Moreover, IVD- and FDA-approved equipment will have higher prices than non-IVD- and FDA-approved equipment. Therefore, IVD- and FDA-approved equipment should be used at certain steps of production, especially at quality control steps with measurement equipment.
– All equipment (IVD/FDA approved or not) must be validated after installation and SOPs established for maintenance, calibration, and work instructions (Table 6.4).

6.4 Regulation of MSC Production

MSC production is regulated differently worldwide. Most regulations were established in Europe and the USA. In recent years, some developed countries such as Japan and Korea have also published regulations related to the production and usage of MSCs.

In Europe, MSCs are considered as somatic cell therapy products, referred to as advanced therapy medicinal products (ATMPs), which are under European Regulation No. 1394/2007. Regulation 1394/2007 contains rules for authorization, supervision, and technical requirements regarding the summary of product characteristics, labeling, and the package leaflet of ATMPs. In addition, the production and delivery of MSCs should be performed according to European GMP (Euralex).

In the USA, MSCs are considered as human cell- and tissue-based products (HCT/Ps). Hence, the production of MSCs must comply with Current Good Tissue Practice requirements under the Code of Federal Regulations (CFR) [FDA for facilities (Part 1271.190a and b); environmental control (Part 1271.195a); equipment (Part 1271.200a); supplies and reagents (Part 1271.210a and b); recovery (Part 1271.215); processing and process controls (Part 1271.220); labeling controls (Part 1271.250a and b); storage (Part 1271.260a–d); receipt, pre-distribution shipment, and distribution of an HCT/P (Part 1271.265a–d); and donor eligibility determination, screening, and testing (Parts 1271.50, 1271.75, 1271.80, and 1271.85)]. Under this regulation, MSCs and other cell culture production systems require the use of a class A cabinet operated in a B class room.

6.5 Production Processes for Non-expanded MSCs

Non-expanded MSCs are directly isolated or enriched from tissue. Studies have referred to MSCs in peripheral blood, umbilical cord blood, and bone marrow as mononuclear cells (MNCs). In fact, using centrifugation or other enrichment methods, MSCs can only be enriched in the MNC fraction. For solid tissues such as adipose tissue, non-expanded MSCs are enriched after removing contaminating cells such as adipocytes. Non-expanded MSCs from adipose tissue are the so-called stromal vascular fraction (SVF).

Although non-expanded MSCs are approved by some countries as a minimal manipulation of stem cells, non-expanded MSC processing should be performed by standardized protocols and procedures. In most countries, the stem cell process must be carried out using FDA-approved or IVD kits in combination with IVD equipment in GMP facilities.

6.5.1 Isolation of Non-expanded MSCs from Peripheral Blood, Bone Marrow, and Umbilical Cord Blood

MSCs exist at a low density in peripheral blood, bone marrow, and umbilical cord blood. Both MSCs and hemopoietic stem cells (HSCs) can be enriched by similar methods. Although some studies have purified MSCs from blood using kits based on certain markers, such as the CD117 MicroBead Kit for isolation of MSCs from bone marrow, non-expanded MSCs as MNCs are enriched from blood, amniotic

fluid (Guan et al. 2012). Traditionally, MNCs from peripheral blood, bone marrow, and umbilical cord blood can be isolated by Ficoll density centrifugation (Ardjomandi et al. 2015; Grisendi et al. 2010; Pierini et al. 2012).

6.5.2 Isolation of Non-expanded MSCs from Adipose Tissue

To date, systems or kits have been developed and commercialized worldwide to facilitate the application of MSCs from adipose tissue in the clinic. There are two types of systems for isolation based on enzymes or mechanical force. The following systems aim for automation of preparation by collagenase-based digestion, such as the AdiStem™ Small/Large Kit and AdiLight (AdiStem Pty. Ltd., China), Sepax 2 (Biosafe Group SA, Switzerland), Cellthera Kits I and II and Method for isolation of adipose tissue-derived stromal vascular fraction (Cellthera, s.r.o., Czech Republic), A-Stromal™ Kit (Cellular Biomedicine Group, Inc./Cellular Biomedicine Group HK, Ltd., USA), Celution® 800/CRS and 820/CRS (Cytori Therapeutics, Inc., USA), adipose-derived stem cell (ADSC) Extraction Kit (GeneWorld, Ho Chi Minh City, Vietnam), Sceldis® (ED Co. Ltd. and Pure Biotech Co., Ltd., South Korea/Medica Group, United Arab Emirates), automated systems and methods for isolating regenerative cells from adipose tissue (General Electric Company, USA), Cell Extraction Kit (Regenmedlab Co Ltd., Ho Chi Minh City, Vietnam), GID SVF-1™ (GID Group, Inc., USA), HuriCell (Hurim BioCell, Co., Ltd., South Korea), apparatus and methods for cell isolation (Ingeneron, Inc., USA), STEM-X™ (Medikan International Inc., USA), Beauty Cell (N-BIOTEK, Inc., South Korea), UNISTATION™ (NeoGenesis Co., Ltd., South Korea), CHA STATION™ and Multi-Station (PNC International Co., Ltd., South Korea/PNC North America Division Of Advanced Bio-Medical Equipment Co., Inc.), CID300 (SNJ Co., Ltd., South Korea/TOPMED CO., LTD., South Korea), Stempeutron™ (Stempeutics Research Pvt. Ltd., India), and Tissue Genesis Icellator Cell Isolation System and handheld micro-liposuction adipose harvester, processor, and cell concentrator (Tissue Genesis, Inc., USA) (Fig. 6.5; Table 6.5).

Other systems do not include enzymatic digestion but disrupt the adipose tissue by mechanical force, such as devices for harvesting and homogenizing adipose tissue containing autologous endothelial cells (Baxter International Inc., USA), Puregraft® (Bimini Technologies LLC, USA), Fastkit (Fastem) (CORIOS Soc. Coop., Italy), LipiVage™ (Genesis Biosystems, Inc., USA), Revolve™/GID 700™ (LifeCell Corporation, USA/GID Group, Inc., USA), Lipogems® (Lipogems International S.p.A., Italy), LipoKit GT (Medikan International Inc., USA), StromaCell™ (MicroAire Surgical Instruments, LLC, USA), and MyStem® (MyStem LLC, USA). Several other nonenzymatic isolation systems aim at isolation of adipose tissue-derived cells to obtain the pure SVF: method for isolating stromal vascular fraction (Agency Science, Tech & Res, China), procedure and device for separating adult stem cells from fatty tissue and device for separating adult stem cells (Human Med AG, Germany), ultrasonic cavitation-derived stromal or mesenchymal vascular extracts and cells derived therefrom obtained from adipose

Fig. 6.5 Commercialized kits for ADSC isolation. (**a**) Sepax 2, (**b**) Celution, (**c**) StromaCell, (**d**) Lipogems, (**e**) HuriCell, (**f**) MyStem

tissue and use thereof and isolation of stromal vascular fraction from vascular tissues (IntelliCell BioSciences Inc., USA), Cell Extraction Easy Kit (Regenmedlab Co., Ltd., Ho Chi Minh City, Vietnam); nonenzymatic method for isolating human adipose-derived stromal/stem cells (Pennington Biomedical Research Center, USA), isolation of stem cells from adipose tissue by ultrasonic cavitation, and methods of use (Rusty Property Holdings Pty. Ltd., Australia/Amberdale Enterprises Pty. Ltd., Australia/Tavid Pty., Australia), and selective lysing of cells using ultrasound (Solta Medical, Inc., USA) (Fig. 6.5; Table 6.6).

6.6 Ex Vivo-Expanded MSCs

6.6.1 Media

Popular media for MSC expansion are α-minimal essential medium or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum FCS. In recent studies, DMEM/F12 (1:1) has been used as basal medium for MSCs,

Table 6.5 Kits and isolation systems for adipose tissue based on enzymes currently patented, published, or commercially available

No.	Company	Device/method
1	ED Co. Ltd. and Pure Biotech Co., Ltd., South Korea/Medica Group, United Arab Emirates	Sceldis®
2	Cytori Therapeutics, Inc., USA	Celution® 800/CRS and 820/CRS
3	Cellular Biomedicine Group, Inc./ Cellular Biomedicine Group HK, Ltd., USA	A-Stromal™ Kit
4	Cellthera, s.r.o., Czech Republic	Cellthera Kits I and II and Method for isolation of adipose tissue-derived stromal vascular fraction
5	Biosafe Group SA, Switzerland	Sepax 2
6	AdiStem Pty. Ltd., China	AdiStem™ Small/Large Kit and AdiLight
7	General Electric Company, USA	Automated systems and methods for isolating regenerative cells from adipose tissue
8	GID Group, Inc., USA	GID SVF-1™
9	Hurim BioCell, Co., Ltd., South Korea	HuriCell
10	Medikan International Inc., USA	STEM-X™
11	Ingeneron, Inc., USA	Apparatus and methods for cell isolation
12	N-BIOTEK, Inc., South Korea	Beauty Cell
13	NeoGenesis Co., Ltd., South Korea	UNISTATION™
14	GeneWorld Co., Ltd, Vietnam	ADSC Extraction Kit
15	PNC International Co., Ltd., South Korea/PNC North America Division Of Advanced Bio-Medical Equipment Co., Inc.	CHA STATION™ and Multi-Station
16	SNJ Co., Ltd., South Korea/ TOPMED CO., LTD., South Korea	CID300
16	Regenmedlab Co., Ltd, Vietnam	Cell Extraction Kit
17	Stempeutics Research Pvt. Ltd., India	Stempeutron™

except for MSCs from umbilical cord blood, which use Iscove's modified Dulbecco's medium. Using these media, MSCs can be grown but with a long doubling time (more than 1 month) to achieve useful quantities. To stimulate MSC growth and reduce the doubling time, cytokines or growth factors (GFs) are added to the medium. Three GFs are commonly used: epidermal growth factor (EGF), basic fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) (Tarte et al. 2010). Although these media are simple, inexpensive, and convenient for ex vivo MSC culture, they contain a xenogeneic source of GFs and proteins (from

Table 6.6 Kits and isolation systems for adipose tissue based on nonenzymatic methods currently patented, published, or commercially available

No.	Company	Device/method
1	MicroAire Surgical Instruments, LLC, USA	StromaCell™
2	Medikan International Inc., USA	LipoKit GT
3	Lipogems International S.p.A., Italy	Lipogems®
4	LifeCell Corporation, USA/GID Group, Inc., USA	Revolve™/GID 700™
5	Genesis Biosystems, Inc., USA	LipiVage™
6	CORIOS Soc. Coop., Italy	Fastkit (Fastem)
7	Bimini Technologies LLC, USA	Puregraft®
8	Baxter International Inc., USA	Devices for harvesting and homogenizing adipose tissue containing autologous endothelial cells
9	MyStem LLC, USA	MyStem®
10	Regenm edlab Co. Ltd, Vietnam	Cell Extraction Easy Kit
11	Agency Science, Tech & Res, China	Method for isolating stromal vascular fraction
12	Human Med AG, Germany	Procedure and device for separating adult stem cells from fatty tissue and device for separating adult stem cells
13	IntelliCell BioSciences Inc., USA	Ultrasonic cavitation-derived stromal or mesenchymal vascular extracts and cells derived therefrom obtained from adipose tissue and use thereof and isolation of stromal vascular fraction from vascular tissues
14	Pennington Biomedical Research Center, USA	Nonenzymatic method for isolating human adipose-derived stromal/stem cells
15	Rusty Property Holdings Pty. Ltd., Australia/Amberdale Enterprises Pty. Ltd., Australia/ Tavid Pty., Australia	Isolation of stem cells from adipose tissue by ultrasonic cavitation and methods of use
16	Solta Medical, Inc., USA	Selective lysing of cells using ultrasound

FCS) with a high risk of disease transmission. Potential risks are also related to allergies against xenoproteins and transmission of prions and viruses.

In the next generation of culture media for MSCs, FCS has been replaced by human blood-derived products. Both autologous and xenogenic blood-derived products have been successfully used for ex vivo culture of MSCs. Autologous blood-based products are optimal for ex vivo culture of MSCs for clinical application. However, it is sometimes detrimental for patients to undergo blood

withdrawal. Therefore, allogeneic blood has been used as a replacement. Allogeneic samples must be subjected to serological and nucleic acid testing of blood-transmitted viruses, such as human immunodeficiency virus (HIV) and hepatitis C virus, with a supplemental step of virus inactivation before use as a supplement in culture medium.

There are two forms of blood-based products used in ex vivo culture: plasma platelet lysate (PL) and platelet-rich plasma (PRP). Recent reports show that PRP is the most reliable and used product to grow MSCs from diverse sources, such as bone marrow, adipose tissue, umbilical cord, and dental pulp tissue. Only 2–8 % PRP stimulates MSC proliferation with a higher efficacy than FCS (Fekete et al. 2012). In fact, PRP is a pool of many GFs, including EGF, acidic FGF, PDGF, transforming growth factor, keratocyte growth factor, hepatocyte growth factor, and insulin-like growth factor. These are human GFs and efficiently stimulate MSCs compared with bovine GFs in FCS (Fekete et al. 2012). Studies have shown that PL- or PRP-based media efficiently maintain the phenotype and genotype of cells in long-term culture. Furthermore, the self-renewal, differentiation potential, and surface marker expression of MSCs are preserved during long-term culture in PL- or PRP-supplemented medium.

Although in vitro-cultured MSCs in media based on PL or PRP are clinically used to treat diseases via local injection or intravenous transfusion, several independent reports show that PRP or PL can drive spontaneous differentiation of MSCs in vitro. For example, Kasten et al. showed that bone marrow-derived MSCs grown in medium supplemented with PL commit to an osteoblastic lineage (Kasten et al. 2008), whereas Van Pham et al. (2013) showed that PRP drives ADSC differentiation into chondroblasts (Van Pham et al. 2013). For this reason, depending on the application, medium supplemented with PL or PRP should be carefully evaluated before use in ex vivo culture.

The third generation of media is completely defined and lacks any biological products from animal or human origins. At least five companies have successfully developed this type of medium. To replace non-defined components such as FCS, PL, and PRP, GF cocktails have been used to supplement culture media. Some of these media are produced under GMP guidelines and have obtained FDA approval as medical devices. These media also maintain the phenotypic and functional characteristics of cultured MSCs (Chase et al. 2010). The most significant problem of these media is the use of a specific protein to ensure primary cell attachment. In FCS, PL, or PRP, there are proteins that facilitate MSC attachment to the surface of flasks or dishes. Conversely, for defined media, substrates must be coated to the flask/dish surface before plating MSCs to assist MSC attachment. Although xenogeneic proteins have been removed in this culture system, some coating substrates originate from animal or non-defined components.

6.6.2 Culture Platforms

To date, there are two platforms for ex vivo culture of MSCs: monolayer and suspension culture. In both platforms, MSCs must adhere to a surface. In fact, MSCs only grow in an adherent state. In monolayer culture, MSCs are plated in flasks or

dishes with a treated surface. In a clinical study, T75 or T125 flasks showed more advantages than T25 flasks. In suspension culture, MSCs adhere to microbeads suspended in media. Suspension culture of MSCs on microbeads is a new technique and easy to scale up to obtain MSCs in short-term culture (Hervy et al. 2014; Wise et al. 2014). However, most clinical applications of MSCs use monolayer culture.

Monolayer culture is the traditional technique to culture MSCs. This technique allows MSCs to proliferate for a long time without changes in MSC properties or genetic stability. Studies show that MSCs maintain their karyotype until the 25th passage (Chen et al. 2014b). In another study, aneuploidy has been detected by some studies when MSCs are cultured (Tarte et al. 2010). However, they also confirmed that these modifications did not cause tumorigenesis of MSCs (Chen et al. 2014a; Tarte et al. 2010; Wang et al. 2013). MSCs also maintain stemness after long-term culture with a stable phenotype, self-renewal, and differentiation potential (Wang et al. 2013). Although monolayer culture has a high risk of contamination by bacteria or fungi because of the many steps during culture depending on manipulators as well as the need for a class A cabinet, monolayer culture of MSCs is the standard for ex vivo expansion of MSCs. Most expanded MSCs used in clinical applications are cultured as a monolayer. Using this technique, the quality of the flask or dish is very important. In well-treated flask surfaces, MSCs develop better. Ventilated flasks are recommended for MSC monoculture.

Closed systems for MSC expansion have been developed in recent years. Closed culture systems are considered to be optimal for clinical applications of MSCs. They can significantly decrease the number of steps, exposure to the environment, and the risk of contamination. However, not all closed systems for ex vivo cell culture satisfy GMP requirements. The first generation of closed systems was multilayer, such as CellSTACK (Corning, Corning, NY, USA) or Cell Factory (Nunc, part of Thermo Fisher Scientific Inc., Waltham, MA, USA), which could be stacked in incubators. These systems increase the surface area for culture to enable expansion to one billion pure MSCs in 2–3 weeks (Tarte et al. 2010). However, there were many limitations because they were not completely closed systems and required a class A cabinet for each manipulation.

The second generation is a fully closed and automated bioreactor. The main advantages of bioreactors are a large surface area to volume ratio, a closed system, automated inoculation and harvesting, and automated control of culture parameters. Terumo (Somerset, NJ, USA) has developed a fully automated bioreactor based on hollow-fiber technology to allow large-scale expansion of MSCs in a GMP-compliant system (Rojewski et al. 2013). Although this system can provide optimal tools for delivering MSCs of clinical grade, which comply with GMP, the behavior or properties of MSCs can change in this platform (Guo et al. 2014). In a recent study, a low oxygen concentration was used to maintain the growth and genetic stability of MSCs cultured in suspension culture (Bigot et al. 2015; Estrada et al. 2012; Hung et al. 2012; Oliveira et al. 2012). In another report, three-dimensional culture increased the anti-inflammatory properties of MSCs (Bartosh et al. 2010; Hong et al. 2015).

6.6.3 Harvesting Adherent Cells

MSCs must be cultured as adherent cells in both monolayer and suspension culture. After expansion, MSCs should be harvested by an enzyme. Trypsin/EDTA solution is popularly used to detach MSCs from the surfaces of culture dishes/flasks or microbeads. However, trypsin is usually derived from porcine and not optimal for GMP production of MSCs. Some recombinant enzymes produced under GMP compliancy can replace trypsin/EDTA, such as TrypLE (Invitrogen, Thermo) and TrypZean (Sigma-Aldrich, St. Louis, MO).

These second-generation enzymes are gradually being used to harvest MSCs for clinical use. Mechanical detachment using cell scrapers has also been suggested to harvest cells cultured in dishes or flasks. Although a cell scrapper-based method is simple, the percentage of live detached cells can be affected. Recently, a new de-attachment method with GMP compliancy combining EDTA and chilling was patented.

6.6.4 Cryopreservation of Cellular Products

There are two forms of MSC cryopreservation. Commonly, 1.5- or 2.0-mL cryotubes are used to store MSCs in cryopreservation medium. However, a vial only holds about 1×10^7 cells which is insufficient for transplantation. In fact, for MSC transplantation, 1×10^6 cells per kg of weight are required. Therefore, similar to HSC cryopreservation, some studies have used bags for MSC cryopreservation. However, the protocols for MSC cryopreservation may be different to HSC cryopreservation. Prochymal is a commercial product containing MSCs cryopreserved in a bag, whereas Cartistem contains MSCs cryopreserved in penicillin vials.

Cryopreservation media significantly affects the quality of MSCs after thawing. They not only directly affect MSC viability but also factors affecting clinical usage. Traditionally, culture media with serum and 10 % DMSO have been used in most studies. DMSO is a popular cryoprotectant. However, it also has some limitations, especially because it damages cells when present at high concentrations during the thawing procedure. Moreover, if DMSO is not completely removed from the cryopreserved cells, it can cause adverse reactions in patients, such as nausea, vomiting, tachycardia, bradycardia, and hypotension. Therefore, in recent years, a second generation of cryopreservation medium with other kinds of cryoprotectants has been developed, such as methylcellulose, sucrose, trehalose, glycerol, hydroxyethyl starch, polyvinylpyrrolidone, and various combinations of these cryoprotectants. However, reports show that none of these cryoprotectants are superior to DMSO. Hence, recent studies have tried to reduce the percentage of DMSO to 5 or 2 %. In addition to DMSO, the serum in medium also affects MSC quality. MSCs can be well preserved in 10 % DMSO and 90 % FCS. However, the high ratio of animal serum can cause some adverse effects in patients. Therefore, in recent studies, FCS has been reduced to 10 % or replaced with human serum. However, cryopreservation medium containing

serum also has risks related to viral transmission or xenoprotein-related reactions. Auto-serum is suitable to replace animal serum or allogeneic serum. Currently, defined, serum-free, and animal component-free freezing media have been developed and commercialized, such as CryoStor™ CS10 (STEMCELL Technologies), Plasma-Lyte-A (Baxter), and Synth-a-Freeze (Gibco, Thermo).

There are two methods for freezing cryotubes for MSC cryopreservation, controlled-rate freezing and uncontrolled freezing (three-step freezing). In the controlled-rate freezing method, a rate of 10 °C per minute has been applied with good results of viable thawed cells. The three-step freezing method involves the cells passing through three temperatures, (1) 4 °C for 30–60 min, (2) −20 °C for 60–120 min, and (3) −85 °C overnight, and then storage in nitrogen liquid. Although controlled-rate freezing is clearly better than uncontrolled freezing, the most significant limitation of controlled-rate freezing is the high cost of controlled-rate freezing systems. At present, cryopreservation boxes have been developed. Using these boxes, the freezing rate is controlled but fixed at a specific rate. These boxes are inexpensive and can be used for MSC cryopreservation. After cryopreservation, the thawing method significantly contributes to MSC quality, especially cell viability. Commonly, MSCs are rapidly thawed by incubating the vials in a 37 °C water bath for 1–2 min. The cells are then centrifuged to remove DMSO/cryoprotectants and cryopreservation medium.

6.6.5 Control of MSC Quality and Safety

Although a standard for MSC expansion has not been published or is in agreement, there are two issues that need to be controlled before using expanded MSCs for clinical application: quality and safety.

6.6.5.1 MSC Quality Control

The first issue relates to MSC characteristics. Expanded MSCs should maintain their phenotypes in long-term culture. Spontaneous differentiation of MSCs always occurs during in vitro or ex vivo culture because of a heterogeneous population of MSCs. This process will proceed quickly or slowly depending on the culture conditions, especially the culture medium. Some studies have added GFs to inhibit spontaneous differentiation of MSCs. However, before application to patients, MSC characteristics must be checked.

Similar to other types of stem cells, MSCs have two important properties, self-renewal and a differentiation potential. Self-renewal is evaluated by a clonogenicity assay. This test involves seeding cells at densities of 1.5, 3, 5, and 10 cells/cm^2 in a 100-mm Petri dish. It is simple, inexpensive, and highly reproducible. However, the time needed for this assay is longer than the shelf-life of the final product. Therefore, this assay should be performed during evaluation of the production procedure.

Although MSCs exhibit self-renewal, they also undergo senescence after long-term culture. MSCs typically proliferate for 20–50 doublings, depending on the cell source and culture conditions (Cholewa et al. 2011; Izadpanah et al. 2006; Suchanek et al. 2007). Senescent cells display aneuploidy without transformation and exhibit mutations in certain genes, such as the p53 gene (Tarte et al. 2010); upregulation of hyaluronan and proteoglycan link protein 1, keratin 18, brain-derived neurotrophic factor, or renal tumor antigen; and downregulation of pleiotrophin (Schallmoser et al. 2010). To date, senescence is easy to evaluate by a β-galactosidase staining assay.

Differentiation is also an important characteristic of MSCs. According to Dominici et al., MSCs must be able to differentiate into three kinds of mesodermal cells, namely, osteoblasts, adipocytes, and chondroblasts (2006). This suggestion has been used as a guideline to evaluate MSCs. Some reports show that senescent MSCs have a reduced differentiation potential for only osteoblasts. Differentiation assays are easy to perform with commercial differentiation kits. When cultured in inducing medium for 14–21 days, MSCs differentiate into adipocytes, osteoblasts, or chondroblasts depending on the media. Similar to self-renewal testing, differentiation potential tests are also performed for 2–3 weeks. Therefore, this test is usually applied during evaluation of the production procedure.

To evaluate MSC quality before transplantation, there are two popular indicators, surface marker expression and cell viability. Assessment of both can be carried out by flow cytometry. For cell viability, collected MSCs are stained with 7-aminoactinomycin D (7-AAD), and dead cells are identified based on the signal of 7-AAD. Although there is no guideline or regulation concerning the percentage of live MSCs for clinical grafts, most studies only use MSC samples with more than 95 % live cells. In terms of surface markers for MSCs, according to Dominici et al. (2006), there are two groups of markers used to confirm MSCs: positive markers (CD13, CD44, CD73, CD90, and CD105) and negative markers (CD14, CD34, CD45, and HLA-DR). Profile marker kits for these have been commercialized to confirm MSC phenotypes (Dominici et al. 2006) (Fig. 6.6).

6.6.5.2 MSC Safety

The first safety issue relates to contamination by viruses, including hepatitis B and C, HIV, human T-cell leukemia virus type 1, and syphilis. This issue must be addressed at donor or sample collection. All donors must be carefully checked according to blood bank guidelines. Only viral negative samples are used in further processes.

The second safety issue relates to bacterial and fungal contamination. Both bacterial and fungal contamination of classical pharmaceutical products are excluded by standardized tests, as set, for example, in Europe [Pharmacopoeia (EP), Chap. 2.6.1] or in the USA [Pharmacopoeia (USP), Chap. 71]. These growth promotion tests involve two cultivation media, fluid thioglycolate and soybean casein digest media, and two temperatures, 22.5 and 32.5 °C, for growth of each tested sample. However, this test requires 14 days and is clearly unsuitable for products with a short shelf-life. There are instructions on validation of rapid microbiological tests in

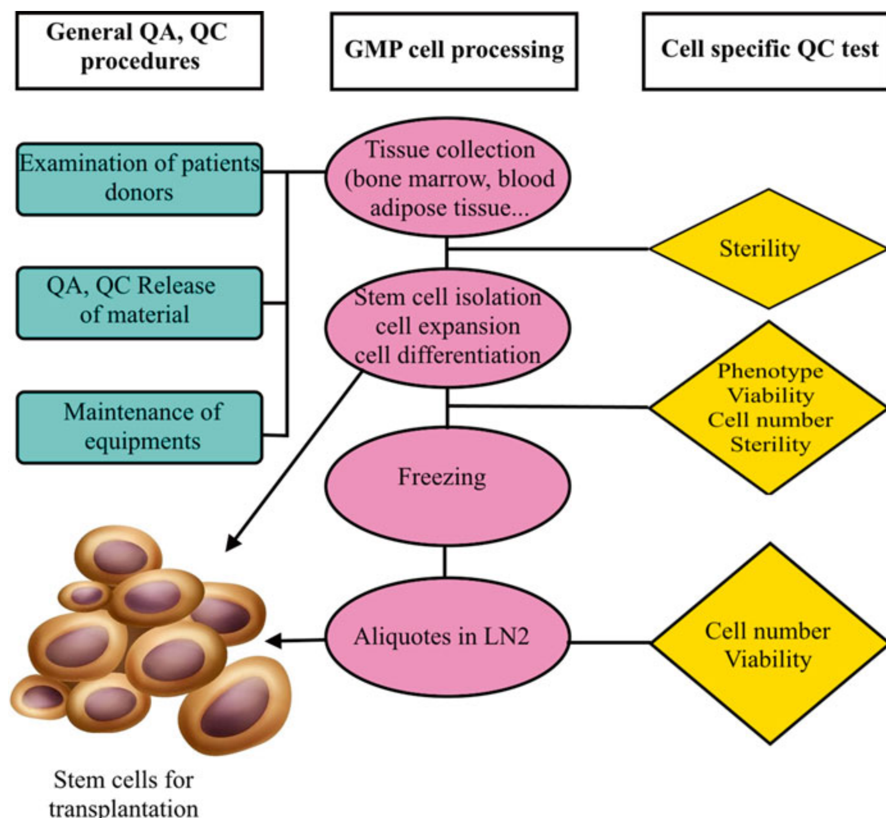

Fig. 6.6 Flowchart of GMP-compliant production of MSCs for clinical application. All steps from donor selection to storage and delivery should be controlled and recorded

both USP (ch. 1223) and EP (Sect. 5.1.6). At present, there are some solutions for rapid microbiological testing, but all have advantages and disadvantages. The most comparable to pharmacopoeial methods are cultivation methods based on CO_2 detection (BACTEC™, Becton Dickinson, and BacT/ALERT®, bioMérieux). Other kits using DNA detection tests (e.g., LightCycler® SeptiFast Test, Roche) may be more challenging for validation, because they may not detect all possible contaminating organisms. Fluorescent cytometry tests (Scan RDI®, AES Chemunex) provide ultrarapid detection of microorganisms (90 min), but are very expensive and typically used by large pharmacological companies.

Mycoplasmas are microorganisms without a cell wall, which can pass through 0.2-μm filters used for sterilization. Mycoplasma detection is required for cell culture according to European, US, and Japanese pharmacopoeias. Although there are kits to detect mycoplasma based on DNA, standard tests are still used to confirm mycoplasma contamination. There are two types of tests to confirm mycoplasma contamination. The first is inoculation of cell culture samples on solid agar or in

liquid enrichment medium, from which mycoplasma cultures are transferred onto agar after several days. The second is coculture of samples with permissive cell lines (usually Vero cells) and then staining with fluorescent DNA-binding dyes (DAPI or Hoechst). Recently, commercialized kits to detect mycoplasma DNA have been validated and applied to cell production, such as MycoTOOL™ (Roche Diagnostics) (a test that amplifies a region of the 16S rRNA of mycoplasma) that has been validated with European Pharmacopoeia tests (Chap. 2.6.7) and the MycoSensor QPCR assay kit developed by Stratagene, which is acceptable in preclinical regulatory validation of amniotic MSC manufacturing protocol.

Another test relating to safety issues is endotoxin testing. Endotoxins are lipopolysaccharides from Gram-negative bacteria. They are the most common cause of toxic reactions resulting in serious health problems, such as diarrhea, septic shock, and marrow necrosis. Therefore, testing for endotoxins is standard in cellular and gene therapy products. The acceptable level of endotoxin in these products is usually 5.0 EU/kg/dose. Endotoxin is generally tested by the Limulus amebocyte lysate method. Commercialized kits for endotoxin detection and quantitation have been developed to facilitate endotoxin measurement.

The last safety issue is tumorigenicity of expanded MSCs. This is the most concerning safety issue using expanded MSCs for clinical applications. This risk originated from observations of spontaneous transformation of human MSCs in culture (Rosland et al. 2009; Rubio et al. 2005; Wang et al. 2005). However, most studies have been retracted because of cross contamination with cultures of exogenous tumor cell lines (de la Fuente et al. 2010; Torsvik et al. 2010). There are no reports of tumorigenicity after transplantation of MSCs into patients. Because of these results, tumorigenicity testing has not been suggested to evaluate MSCs before clinical application.

6.7 Conclusion

MSC production with GMP compliancy appears to be a compelling condition to use MSCs in clinical application. GMP will maintain the quality and safety of MSCs. Clinical-grade MSCs are only produced by application of regulations as well as the requirements or elements of GMP. However, all procedures should originate from clinical demands. GMP is not a standard but a set of guidelines or rules for production procedures with the highest quality and safety.

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Chapter 7
Isolation and Characterization of Adipose-Derived Stromal Cells

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7.1 Introduction

A stem cell is defined by its ability to self-renew and to differentiate along multiple lineage pathways. Stem cells have the potential to develop into different cell types in the body during their lifespan. Even after long periods of quiescence, stem cells retain the ability to divide, and the two daughter cells have the potential to either remain a stem cell within the stem cell niche or to develop into a more specialized cell with specific functions. Stem cells can broadly be classified into two categories, namely adult stem cells and pluripotent stem cells. Pluripotent stem cells include embryonic and induced pluripotent stem cells and will not be discussed further in this chapter. Adult stem cells are further subdivided into two categories, namely, haematopoietic stem cells (HSCs) and mesenchymal stromal cells (MSCs). The latter contains a small population of stem cells. MSCs are present in most tissues in the body and are required to restore normal tissue function via repair and regeneration mechanisms (Jones et al. 2002). The bone marrow, Wharton's jelly present in the umbilical cord and adipose tissue are the most common sources used to isolate MSCs.

The clinical translation of MSC research remains a major challenge. Research groups around the world are working on various strategies to apply MSCs in a variety of settings. There is currently a need for consensus in standardization regarding isolation and expansion procedures, characterization of the cells, evaluation of multipotency, including tissue lineage induction, the best route of administration and monitoring of engraftment success.

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Stem cells should meet various criteria before being considered for regenerative medicine purposes. The cells should (1) be present in abundant quantities in the tissue of interest, (2) require minimally invasive harvesting procedures, (3) differentiate along multiple cell lineage pathways that are reproducible and can be regulated/controlled, (4) be safe and effective when transplanted in an autologous or allogeneic manner and (5) be manufactured according to GMP guidelines (Gimble et al. 2007).

In 2013, the International Society for Cellular Therapy (ISCT) reviewed the nomenclature and characterization guidelines for adipose-derived stromal cells (ASCs) and in a joint statement with the International Society for Cellular Therapy (ISCT) deemed it important to distinguish between the adipose-derived stromal vascular fraction (AD-SVF) and culture expanded ASCs (Bourin et al. 2013). SVF refers to the cellular component present in adipose tissue after mature adipocytes and peripheral blood cells have been removed during the isolation procedure. AD-SVF consists of a heterogeneous cell population which includes preadipocytes, MSCs, endothelial cells, leukocytes, adipose tissue-associated macrophages and other cell types. ASCs refer to the adherent multipotent cell population within the AD-SVF. It remains to be established whether AD-SVF cells or culture expanded ASCs are better suited for clinical applications.

This chapter will discuss AD-SVF isolation procedures, ASC expansion conditions, phenotypic characterization and qualitative and quantitative techniques used to assess the differentiation capacities of ASCs into the adipogenic, osteogenic, chondrogenic and myogenic lineages.

7.2 Isolation Methods

MSCs have been successfully isolated from nearly all postnatal organs and tissues. To date, bone marrow-derived MSCs (BM-MSCs) are the best described, particularly in the clinical setting. These cells serve as the gold standard to which other sources of MSCs are compared (Kern et al. 2006). Adipose tissue, like the bone marrow, derives from the embryonic mesodermal layer (Zuk et al. 2001). Interestingly, adipose tissue is one of the tissues in the human body with the highest number of stem cells, containing between 100- and 1000-fold more multipotent cells per volume unit when compared to the bone marrow. It has been shown that about 3.5×10^4 preadipocytes can be isolated from 1 g of adipose tissue (Fournier and Otteni 1983; Ersek and Salisbury 1995; von Heimburg et al. 2004; Strem et al. 2005). Fraser and colleagues (2006) also demonstrated that adipose tissue yields a 500-fold greater number of ASCs when compared to the MSC yield obtained from the bone marrow. Liposuction procedures can yield anywhere from 100 ml to >3 l of lipoaspirate, which is routinely discarded as biological waste. A recent review mentioned that 20 mg of lipoaspirate should yield $>1 \times 10^7$ SVF cells (Banyard et al. 2015). This translates to an average expected yield of 1×10^6 ASCs, as it is estimated that 15–30 % of the AD-SVF consists of ASCs (Bourin et al. 2013). The ease with which these cells can be harvested and abundant cell yields render adipose tissue a preferred stromal/stem cell source (Fraser et al. 2006).

The isolation of ASCs from adipose tissue was first pioneered in the 1960s. Minced rat fat pads were extensively washed to remove contaminating HSCs, then incubated with collagenase and centrifuged to obtain an AD-SVF pellet. The selection for plastic-adherent fibroblast-like cells from the AD-SVF concluded this isolation process (Rodbell 1966; Rodbell and Jones 1966). ASCs, at that time referred to as resident MSCs in human adipose tissue, were first described by Zuk and colleagues in 2001 (Zuk et al. 2001). The initial procedure of mincing human adipose tissue by hand was simplified by the development of liposuction surgery.

7.2.1 Manual Isolation Procedure

In 2006, Dominici and colleagues (Dominici et al. 2006) suggested that the standard isolation protocol developed by Zuk and colleagues (2001, 2002) should be accepted as an established methodology to obtain the AD-SVF from lipoaspirate. In 2010, Estes and colleagues published a detailed method, adapted from the method described by Zuk and colleagues (Zuk et al. 2001), to isolate ASCs (Estes et al. 2010). This commonly accepted approach involves five basic steps, namely, (1) the removal of contaminating peripheral blood through washing steps, (2) enzymatic digestion of the adipose tissue, (3) separation of SVF from mature adipocytes, (4) lysis of red blood cells present in SVF and (5) selective isolation of the adherent cell component present in AD-SVF. Isolation procedures may differ concerning the enzymatic digestion step, type and composition of culture medium, approaches used to lyse red blood cells and cell seeding density. Data comparisons between research groups become problematic due to these different approaches. Other factors that may influence the quality of a cell therapy product include the donor's age, the location (subcutaneous vs. visceral) of the adipose tissue and the surgical procedures used for tissue harvesting (Gimble et al. 2007; Mizuno 2009; Fossett et al. 2012). These factors should be considered when developing an isolation protocol in order to obtain a reliable source of cells that are safe, free from contamination and are of a high quality for application in the clinic. Please refer to the supplementary material for a brief description of the manual isolation procedure.

7.2.1.1 Seeding

Seeding refers to the cells being placed in a culture flask to allow proliferation (expansion). Seeding density is the number of cells seeded onto a culture surface to ensure a standardized concentration of cells per culture and is reported as the number of cells per cm^2. The initial seeding density of the AD-SVF is higher than the subsequent seeding densities that will be used during the expansion phase. This ensures that a sufficient number (15–30 % of AD-SVF) of stromal cells are introduced into the culture flasks. Approximately 5×10^5 cells/cm^2 of the AD-SVF is usually used and decreased to 5×10^3 cells/cm^2 during the ASC expansion phase. Accurate cell quantification is important to ensure that the correct number of cells have been seeded in the culture flask.

Cell counts may be obtained by using either a manual method such as the trypan blue (0.4 %) dye exclusion method or automated cell counting devices, including benchtop flow cytometers. Benchtop flow cytometers are usually not volumetric and provide an absolute count (cells/μl) when a known concentration of counting beads is added to the sample. The Accuri benchtop flow cytometer (BD Biosciences, San Jose, USA) is an exception; it is a volumetric system that allows for direct determination of an absolute count without the addition of counting beads.

Various flow cytometry counting beads are available commercially, including Flow Count™ counting beads (Beckman Coulter, Miami, USA) and CountBright™ absolute counting beads (Invitrogen/Molecular Probes, Life Technologies, Eugene, USA). Counting beads are commonly referred to as fluorospheres with a broad excitation/emission range. Certain flow cytometers, such as the FC500 and Navios flow cytometers (Beckman Coulter, Miami, USA), have algorithms built into the instrument software that will perform the absolute count calculations in the background and report the absolute cell counts as the number of cells/μl. It is crucial in these flow cytometric systems that the volume of beads to the volume of cell suspension is the same (1:1; vol/vol) to ensure the reporting of an accurate absolute count. The absolute count can also be calculated manually by using Eqs. (7.1) or (7.2) below.

Equation (7.1) is used to manually calculate the absolute count when the ratio of beads to cells is 1:1 (vol/vol):

$$\text{Absolute cell count}\left(\text{cells / μl}\right) = \left[\left(\frac{\text{number of events in area of interest}}{\text{number of bead events}}\right) \times \text{calibration factor}\left(\text{known bead concentration}\right)\right] \tag{7.1}$$

Equation (7.2) is used in situations where the ratio of sample volume to bead suspension volume is not equal [Eq. (7.2)]:

$$\text{Absolute cell count}\left(\text{cells / μl}\right) = \left[\left(\frac{\text{number of events in area of interest}}{\text{number of bead events}}\right) \times \left(\frac{\text{bead concentration assigned to specific lot}}{\text{volume of sample in ul}}\right)\right] \tag{7.2}$$

Please refer to the supplementary material for a brief description of the trypan blue (0.4 %) dye exclusion assay as well as an example of how an absolute count is obtained using a benchtop flow cytometer.

7.2.1.2 Expansion of ASCs In Vitro

Several investigators suggest that seeding density may affect cell proliferation. There is once again no consensus among investigators regarding the seeding densities used, although most investigators use a seeding density of 5×10^3 cells/cm^2 during the ASC expansion phase. The effect of seeding density on MSC proliferation was demonstrated with BM-MSCs that were seeded at 1×10^2 and 5×10^3 cells/cm^2

(Both et al. 2007). The cells seeded at the lower density reached the target of 2×10^8 cells in total in a shorter time period compared to cells seeded at 5×10^3 cells/cm^2. The cells seeded at the higher density took approximately 4 days longer to achieve the same target. Similar results were observed by Lode and colleagues in 2008 using synovial fat pad-derived MSCs seeded on three-dimensional scaffolds (Lode et al. 2008) and by Fossett and colleagues (2012) who showed that low seeding densities increase the proliferation capacity in vitro.

Cell Expansion for Research Purposes

The in vitro expansion of primary cells such as ASCs to reach therapeutic numbers requires that growth conditions mimic the physiological environment in vivo. Such an environment is achieved in vitro through the use of a synthetic basal culture medium enriched with growth factors, hormones and other necessary nutrients (Freshney 2006; Freshney 2010). In addition, ASC proliferation may be stimulated by several exogenous supplements including fibroblast growth factor 2 (FGF-2), sphingosylphosphorylcholine, platelet-derived growth factors and others (Song et al. 2005; Kang et al. 2005; Chiou et al. 2006; Jeon et al. 2006; Mizuno 2009).

Traditionally, ASCs are expanded and maintained in culture medium (DMEM or α-MEM) containing serum. The use of serum during ASC expansion plays a vital role in ASC attachment, longevity and proliferation as it contains essential components such as amino acids, growth factors, hormones, lipids, vitamins, adhesion factors, binding proteins, spreading factors and other trace elements (Lennon et al. 1995; Lennon et al. 1996; Van Der Valk et al. 2004, 2010). Foetal bovine serum (FBS) is widely accepted as the standard serum supplement for in vitro studies in the research and experimental setting (Sotiropoulou et al. 2006; Chen et al. 2009). Many disadvantages such as batch-to-batch variation and xeno-immunization are however associated with the use of FBS. Another associated risk is the transmission of zoonotic disease through contamination with *Mycoplasma*, viruses and prions (Lennon et al. 1995, 1996; Van Der Valk et al. 2004; Kocaoemer et al. 2007; Van Der Valk et al. 2010; Chieregato et al. 2011; Kølle et al. 2013; Kyllonen et al. 2013).

Preparation of cellular therapy products under GMP conditions requires the minimal use of animal-derived products in the manufacturing process (Müller et al. 2006; Crespo-Diaz et al. 2011). Consequently, several human and chemical alternatives are being explored in GMP manufacturing of ASC cell therapy products. Human-derived alternatives such as human serum (Stute et al. 2004), platelet lysate (Schallmoser et al. 2007) and platelet-rich plasma (Doucet et al. 2005) (Table 7.1) may potentially result in an enhanced culture environment that more accurately mimics the in vivo environment (Azouna et al. 2012). However, their relative efficacy is still largely debated, and there is currently no consensus on which of the alternatives is the best to replace FBS (Baer et al. 2010; Bieback et al. 2012; Patrikoski et al. 2013; Koellensperger et al. 2014).

Table 7.1 A comparison of the most commonly used human alternatives to FBS

Human alternatives	
Serum	
Definition	Liquid portion of blood that has been allowed to clot in the absence of an anticoagulant. Contains low levels of intact platelets
Advantages	• May originate from autologous or allogeneic sources • Cells grown in serum have a greater proliferative capacity, cell cycle prolongation and differentiation when compared to FBS • Suggested to be slightly better than PRP
Disadvantages	• The availability of autologous serum is limited and shows significant variation between patients • There is a lack or rigorous testing when using autologous serum
Human platelet lysate (HPL)	
Definition	Concentrated portion of plasma, consisting of platelet growth factors which are obtained by lysing platelets by temperature shock
Advantages	• Contains a higher concentration of growth factors than other human alternatives • Cells grown in HPL maintain their classic immunophenotype, differentiation capacity, clonogenic efficiency, cell purity and cell viability when compared to FBS
Disadvantages	Demonstrates a large variation between individuals
Platelet-rich plasma (PRP)	
Definition	Plasma component of peripheral blood. Contains high levels of platelets and some growth factors
Advantages	• Cells grown in PRP maintain a classic ASC phenotype and morphology and demonstrate increased proliferative capacity when compared to FBS
Disadvantages	• Studies suggest that osteogenic differentiation is less efficient in PRP • PRP is a poorly defined supplement due to its high biologic variability • Large quantities of whole blood are needed to yield enough PRP for experimental purposes

Source: Bernardo et al. (2006, 2011); Bieback et al. (2009); Chieregato et al. (2011); Doucet et al. (2005); Kocaoemer et al. (2007); Schallmoser et al. (2010); Stedman (2006)

Xeno- and Serum-Free Cell Expansion for Clinical Application

Synthetic, chemically defined serum-free medium has been recommended as a potential replacement to animal-derived serum as it contains the necessary growth factors required to sustain ASC expansion (Van Der Valk et al. 2004, 2010). Nonetheless, many studies have revealed that when using serum-free medium, ASCs are incapable of adhering to plastic without the addition of coating agents. In addition, ready-to-use serum-free medium is expensive and might therefore be a less cost-effective alternative for the replacement of FBS in the clinical setting (Lennon et al. 1995; Lund et al. 2009; Baer et al. 2010; Rajala et al. 2010; Yang et al. 2012; Kyllonen et al. 2013; Patrikoski et al. 2013).

7.2.2 Isolation Using Automated Closed Systems

An automated closed system allows for minimal handling of a sample by the operator, resulting in a potentially safer, less labour-intensive isolation procedure. Contamination is a highly undesirable event in the clinical setting and is limited through strict control of standard operating procedures (SOPs) and processing according to GMP (Gimble et al. 2011). Although the manual isolation procedure is relatively easy to reproduce and is carried out in a sterile environment, there is a higher contamination risk when compared to a closed system. The introduction of contaminating particles into the culture system is significantly reduced using closed systems. Hicok and colleagues suggest that in order to comply with the regulations involved in a clinical trial, an automated system for cell isolation should be used (Hicok and Hedrick 2011). Commercial companies such as Cytori Therapeutics Inc (San Diego, USA) and Biosafe Ltd (Lake Geneva, Switzerland) have designed state-of-the art cell-processing devices that allow for the isolation of the AD-SVF from lipoaspirate in automated closed systems. It has been suggested that automated systems may contribute to the advancement of clinical research and the translation thereof into clinical trials.

The Sepax® cell-processing system (Biosafe Ltd, Eysins, Switzerland) was developed to isolate and concentrate nucleated cells from the umbilical cord blood, peripheral blood or bone marrow (Zingsem et al. 2003; Aktas et al. 2008; Zinno et al. 2011). The successful application of the system in the HSC field resulted in the system also being validated for the isolation of AD-SVF cells from human adipose tissue (Guven et al. 2012). The Sepax® system automates the cell washing and concentration steps during adipose tissue processing. The device is comprised of the main Sepax® sample processing unit, single-use sterile disposable sample processing kits and ready-to-use application-specific software protocols.

In Asia, Australia and Europe, but not in the USA, the Cytori's Celution® sample processing system has been granted regulatory approval for the use in adipose tissue processing and AD-SVF cell isolation. Multiple clinical trials are ongoing in several countries using the Celution® system (Akita et al. 2012; Houtgraaf et al. 2012). This automated system was specifically designed for fat processing and to mimic the modified manual isolation steps from the method described by Zuk and colleagues (Zuk et al. 2001, 2002). The Celution® system is a fully closed automated system, which uses a single-use sterile disposable kit comprised of a processing canister, connection tubes and waste bags. The Celase® enzyme is used for adipose tissue digestion instead of collagenase. One of the advantages of the system is that it performs several quality control processes before the automated sample processing is initiated. For example, upon insertion of the sample processing kit into the device, the Celution® system performs a wet test to validate the integrity of the kit by checking for any leakages in the system and consequently ensures that the sample will be processed in a fully closed manner. After the lipoaspirate suspension is introduced into the processing canister, the system automatically weighs the sample and performs rinsing steps with Ringer's lactate solution to remove blood cells and other

contaminants. The Celution® system automatically calculates the amount of Celase® enzyme required for proper digestion of the volume of adipose tissue processed. The instrument will indicate the volume of Celase® enzyme which needs to be added manually. Enzymatic digestion takes place automatically through constant agitation. Following digestion and separation, the fraction of cells released from the adipose tissue matrix is pumped into the centrifuge chamber where it is washed and concentrated into the output chambers on either end of the centrifuge chamber. The final AD-SVF cell product is then aspirated from the output chambers (~5 ml).

Evaluation of the different automated systems needs to take into account factors such as the ability to process a large variety of lipoaspirate volumes and the degree of automation it provides. Practical considerations include the cost of the device and the consumables required. Sample processing time (which is influenced by the volume of lipoaspirate introduced) may also be a consideration. It should be noted that the Sepax® system is not fully automated as adipose tissue digestion needs to be carried out manually. However, an automated adipose tissue protocol has been developed for the isolation of the AD-SVF following digestion. In comparison, the Celution® system is a fully automated system validated for the processing of adipose tissue and requires minimal intervention by the operator.

7.3 In Vitro Characterization of ASCs

In 2006, the ISCT released a position statement defining the minimal criteria required to identify ASCs (Dominici et al. 2006). According to these guidelines, ASCs and BM-MSCs share the same characteristics, namely, that they are multipotent, plastic-adherent cells that express specific surface antigens and have the capacity to differentiate into adipocytes, chondrocytes and osteoblasts. These criteria became the gold standard for all in vitro studies involving ASCs. However, in 2013 a new set of criteria was suggested by the IFATS and the ISCT. The overall criteria remained essentially unchanged, except that the report suggested a repertoire of protein surface markers that will allow investigators to distinguish between AD-SVF, ASCs and BM-MSCs (Bourin et al. 2013).

7.3.1 Immunophenotypic Characterization of ASCs

The challenge in phenotyping ASCs is that none of the surface expression markers used is specific to ASCs (Table 7.2). Therefore, to be more confident in the phenotypic assessment of ASCs, it is advisable to follow a multicolour flow cytometric approach, where multiple surface protein markers are simultaneously stained with various fluorochrome-conjugated antibodies to provide a more accurate co-expression profile of the cells (Zimmerlin et al. 2013; Bourin et al. 2013; Baer 2014; Donnenberg et al. 2015). According to the IFATS criteria, more than 80 % of the

Table 7.2 A summary of marker expression profiles and known physiological functions of the more common surface antigen markers used to phenotype adipose-derived stromal cells[a]

Surface protein marker	Alternative name(s)	Cells which express the protein	Physiological functions
CD13	Aminopeptidase N (APN)	• Granulocytes • Interdigitating dendritic cells • Large granular lymphocyte subpopulation • Macrophages • Mast cells • Monocytes • Myelomonocytes • Osteoclasts • Others	• Play a role in enzymatic degradation processes
CD29	• Glycoprotein IIa (GP IIa) • Integrin beta-1 (ITGB1) • Fibronectin receptor beta subunit • VLA (CD49) beta chain	• Fibroblasts • Platelets • T cells • Monocytes • Granulocytes (low expression level) • Mast cells • Endothelial cells • Myoepithelium • Others	• Receptor for collagen and laminin • Play a role in cell-to-cell adhesion • Play an important role in lymphocyte trafficking and transvascular migration
CD44	• CD44s (standard isoform) • CD44H (lacks all alternative exons)	• Expressed by the majority of mammalian cell types	• A glycoprotein that plays a role in cell-cell interactions, cell adhesion and cell migration • Plays a role in the polar orientation of cells • Involved in leukocyte attachment and rolling on endothelial cells
CD73	Ecto-5'-nucleotidase	• B- and T-cell subpopulations • Endothelial cells • Follicular dendritic cells • Epithelial cells	• Acts as a catalyst in the dephosphorylation of ribo- and deoxyribonucleotides to their corresponding nucleosides • Plays a role in lymphocyte adhesion to dendritic cells and endothelium
CD90	Thy-1	• Immature haematopoietic stem cells • Neurons • Activated endothelial cells • Fibroblasts	• Potentially mediates the differentiation of haematopoietic stem cells • Plays a role in the adhesion of various leukocytes to activated endothelial cells
CD105	Endoglin	• Activated monocytes • Erythroid precursors in the bone marrow • Trophoblasts	• Acts as a regulatory component of the TGF-beta receptor complex • Mediates cellular response to TGF-beta 1

[a]Information obtained from the PathologyOutlines.com website—http://www.pathologyoutlines.com/cdmarkers.html

cells should co-express the following markers: CD13, CD29, CD36, CD44, CD73, CD90 and CD105 (Bourin et al. 2013). There should be less than 2 % haematopoietic cells as well as endothelial cells present in an ASC isolate. Consequently less than 2 % of the cells should stain positive for CD31 (endothelial cells), CD45 (leukocytes) and CD235a (erythrocytes) (Bourin et al. 2013). Simultaneous staining of cell surface markers allows for better discrimination of ASCs from other cells that may be present in the isolation, but do not adhere to the above-mentioned co-expression profile.

7.3.2 In Vitro Differentiation Assessments and Limitations

It is well established that ASCs or subpopulations thereof have the ability to differentiate into adipocytes, osteoblasts, chondrocytes and myocytes. The differentiation capacity of ASCs suggests a role in the turnover and maintenance of adult mesenchymal tissues (Caplan 2009). In vitro differentiation of ASCs into these lineages (adipocytes, chondrocytes, osteoblasts and myocytes) is therefore traditionally used to confirm the multipotent characteristics of the isolated ASCs. However, true stem cell plasticity entails the ability of these cells to form tissues from different germ layers. Investigators have shown that under appropriate culture conditions, ASC subpopulations display stem cell plasticity by differentiating into non-mesodermal tissues such as neurons (Ning et al. 2006) and epithelial cells (Brzoska et al. 2005). However, a significant amount of research is still needed to confirm the purported plasticity of ASCs. Effective clinical application of ASCs will rely on understanding the biological effectors that are responsible for maintaining a specific differentiation state. In addition to the criteria set out by the IFATS and ISCT above, it has been suggested that histochemical staining techniques used to establish differentiation are qualitative and that a quantitative approach, such as quantitative reverse transcription polymerase chain reaction (RT-qPCR), is required. The minimum number of genes required to confirm the differentiation of ASCs into adipocytes, osteoblasts, chondrocytes and myocytes, respectively, is summarized in Table 7.3.

7.3.2.1 Adipogenesis

High-calorie intake without energy expenditure promotes adipocyte hyperplasia. Signalling factors are responsible for this increase in adipocytes by inducing the conversion of ASCs to preadipocytes and finally differentiation into mature adipocytes (Tang and Lane 2012). ASCs become committed to preadipocytes by bone morphogenetic protein (BMP) -2 and -4 (Huang et al. 2009) and Wnt signalling (Bowers and Lane 2008), causing them to go into growth arrest (Fig. 7.1). Differentiation inducers such as hormones and mitogens (insulin, glucocorticoids and cAMP) then initiate mitotic clonal expansion where the cells' fibroblastic morphology is lost and the accumulation of cytoplasmic triglycerides occurs, eventually

Table 7.3 Minimum gene expression requirements to confirm in vitro adipogenic, osteogenic, chondrogenic and myogenic differentiation

Lineage	Gene		Reference(s)
Adipogenic	Adiponectin		Bourin et al. (2013)
	C/EBPα		
	FABP4		
	Leptin		
	PPAR-γ		
Osteogenic	Alkaline phosphatase		Bourin et al. (2013)
	Bone sialoprotein		
	Osteocalcin		
	Osterix		
	Runx2		
Chondrogenic	Aggrecan		Bourin et al. (2013)
	Collagen type II		
	Sox 9		
Myogenic	Striated[a]	Desmin	Beier et al. (2011) Carvalho et al. (2013)
		Myogenic differentiation factor 1 (MyoD1)	
		Myogenin	
		Myogenic enhancer factor 2 (MEF2)	
		Myosin heavy chain (MHC)	
	Smooth[a]	Alpha-smooth muscle actin	
	Cardiac[a]	Sarcomeric alpha-actinin	
		Connexin 43	

[a]Muscle type

forming mature adipocytes (Tang et al. 2004). Peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer-binding proteins (C/EBP) are key regulators of adipogenesis (Kang et al. 2007).

In the laboratory, in vitro adipogenic differentiation takes 2–3 weeks after initiating differentiation following exposure of the cells to the appropriate induction media. The composition of the adipogenic induction medium is provided in the supplementary material. Adipogenesis is associated with the accumulation of lipid droplets in the cytoplasm of the adipocytes. The number and size of lipid droplets increase as the ASCs differentiate from preadipocytes to mature adipocytes. The lipid droplets are highly organized organelles, consisting of an outer layer of amphipathic lipids such as phospholipids and a core of neutral lipids including triacylglyceride and sterol esters (Guo et al. 2009; Krahmer et al. 2009). Studies using immortalized preadipocytes, such as the mouse 3 T3-L1 cell line, revealed that combinations of 3-isobutyl-methylxanthine with dexamethasone or insulin induce intracellular lipid accumulation. Therefore, to promote the effective differentiation of ASCs into adipocytes, the induction medium is supplemented with IBMX, insulin and dexamethasone. 3-Isobutyl-methylxanthine (IBMX), a cAMP-phosphodiesterase inhibitor, has been shown to inhibit soluble cyclic nucleotide

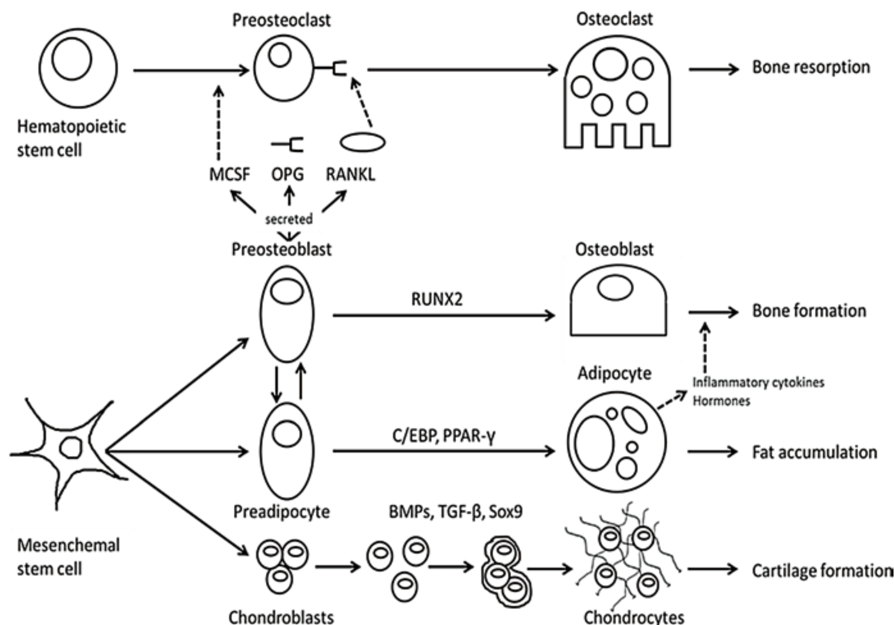

Fig. 7.1 Regulation of ASC differentiation. Fat accumulation, cartilage formation, bone formation and bone resorption are closely related. Adipocytes, osteoblasts and chondrocytes have a common origin, the mesenchymal stem cell, while osteoclasts originate from the haematopoietic stem cell. Osteoblasts regulate bone resorption through the RANKL/RANK/OPG pathway. Osteoblast differentiation is regulated by RUNX2. Key regulators of adipogenesis are PPAR-γ and C/EBP. Adipocytes secrete inflammatory cytokines and hormones which are capable of modulating bone formation. Chondrogenesis is mainly regulated by BMPs, TGF-β and Sox9. Modified from Fig. 7.1 in Cao (2011) and Rosen and Bouxsein (2006). *RUNX2* runt-related transcription factor 2, *RANK/RANKL* receptor activator of nuclear factor-kB ligand, *OPG* osteoprotegerin, *PPAR-γ* peroxisome proliferator-activated receptor-γ, *C/EBP* CCAAT/enhancer-binding proteins, *BMP* bone morphogenetic protein, *TGF* transforming growth factor, *Sox* sex-determining region Y-type high mobility group box (Cao 2011; Rosen and Bouxsein 2006)

phosphodiesterase activity causing an increase in intracellular cAMP levels by stimulating the cAMP-dependent protein kinase pathway. This increase in cAMP levels is subsequently responsible for the synthesis of lipogenic enzymes during adipogenesis (Spiegelman and Green 1981; Tang and Lane 2012). Ali and colleagues have shown that the removal of IBMX from the induction medium almost completely blocks the normally observed increase in intracellular lipid accumulation (Ali et al. 2006). Insulin acts as a potent adipogenic hormone by triggering the induction of a series of transcription factor, which facilitate differentiation of preadipocytes into mature adipocytes via the insulin-like growth factor 1 (IGF-1) receptor (Ntambi and Young-Cheul 2000). Dexamethasone is a synthetic glucocorticoid agonist used to stimulate the glucocorticoid receptor pathway. Although each agent is able to initiate adipogenesis, an additive effect is observed when the factors are combined (Russell and Ho 1976; Rubin et al. 1978). Please refer to the supplementary material for a brief description of the method used to induce adipogenesis in vitro.

Assessment of Adipogenic Differentiation

Adipogenic differentiation may be measured using both quantitative and qualitative (qPCR) assays. The minimum number of genes required to confirm the differentiation of ASCs into adipocytes is summarized in Table 7.3. Most of the histological, qualitative assays make use of lipophilic dyes which mainly stain the triacylglycerides present in the lipid droplet core. IFATS and ISCT jointly recommend using the lipid-specific stains Oil Red O and Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) as histological determinants of adipogenic differentiation (Bourin et al. 2013).

Quantitative Adipogenesis Assessment Using Oil Red O

Oil Red O is a nonfluorescent hydrophobic stain with a high affinity for neutral lipids, such as triacylglycerides, present in lipid droplets (Fig. 7.2). The stain can then be extracted from the cells using isopropanol and quantified by measuring the absorbance values spectrophotometrically, but this method of quantification lacks specificity due to non-specific Oil Red O staining (Ramírez-Zacarías et al. 1992). Please refer to the supplementary material for a brief description of the qualitative method used to evaluate adipogenesis differentiation using Oil Red O in vitro.

Quantitative Adipogenesis Assessment Using Nile Red

Lipid droplet formation during adipocyte differentiation may also be visualized using fluorescent microscopy techniques (Fig. 7.3). Nile Red and Bodipy 493/503 are currently the most common lipid-specific dyes used during fluorescence microscopy assessment of lipid droplet formation (McNeil et al. 1991; Smyth and Wharton 1992; Brasaemle et al. 2000; Lo Surdo et al. 2013; Aldridge et al. 2013).

Quantitative Adipogenesis Assessment Using Flow Cytometry

Flow cytometry provides a more quantitative measurement of both the proportion of cells containing intracellular lipid droplets and the degree of lipid accumulation within each cell (Fig. 7.4) (Fink et al. 2004; Schaedlich et al. 2010; Chazenbalk et al. 2011; Aldridge et al. 2013; Ceppo et al. 2014). Most investigators make use of the hydrophobic dye, Nile Red (Fink et al. 2004; Lu et al. 2010; Menssen et al. 2011; Lo Surdo et al. 2013; Aldridge et al. 2013). Nile Red is a solvatochromatic dye, meaning that it can change fluorescent colour in different polar environments (Fowler and Greenspan 1985; Greenspan et al. 1985). Nile Red emits yellow-gold fluorescence (emission >528 nm) when dissolved in neutral lipids such as triglycerides. The fluorescence emission spectrum of Nile Red shifts to the deep-red spectrum (>590 nm) when it dissolves in an amphipathic lipid environment such as phospholipids (Greenspan et al. 1985). However, when dissolved in lipid droplets, the yellow-gold fluorescence is more easily visualized than the deep-red fluorescence. This may be contributed to by the ratio of neutral lipids to phospholipids present in a lipid droplet.

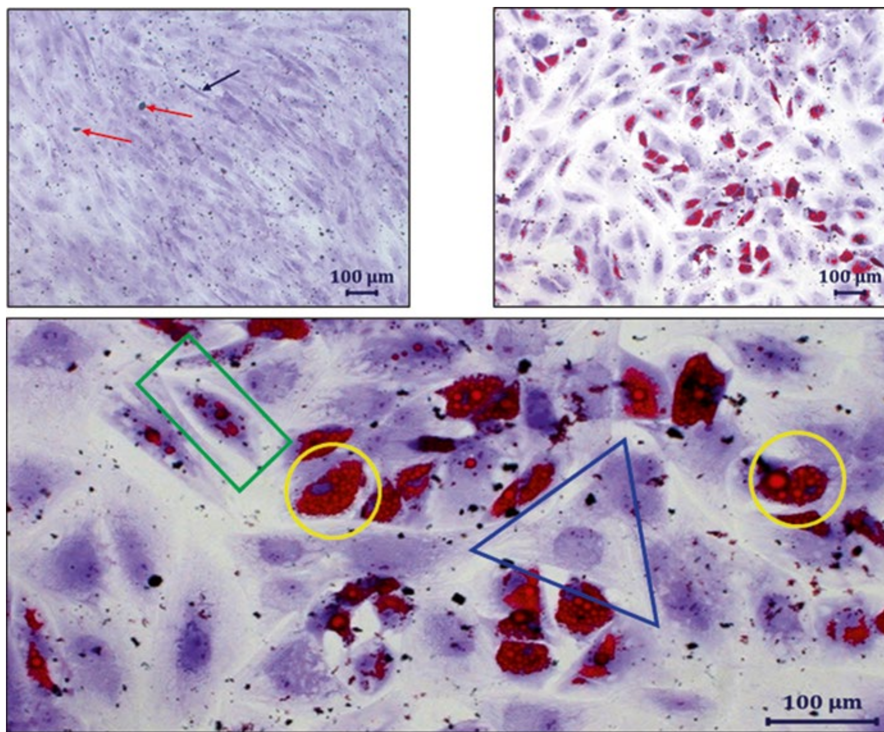

Fig. 7.2 Adipogenic lineage microscopy analysis. (**a**) Oil Red O-stained and 1 % Toluidine Blue-counterstained non-induced culture. The *red arrow* indicates Oil Red O residue overlaying the culture. The cells are fibroblast-like, small, slender and elongated as indicated by the *blue arrow* and conform to describe ASC morphology. (**b**) Oil Red O-stained and 1 % Toluidine Blue-counterstained adipogenic induced culture. Visible Oil Red O droplets confirm differentiation into the adipogenic linage. (**c**) A magnification snapshot of (**b**). The *red arrow* indicates the presence Oil Red O residue overlaying the cell. *Yellow circles* demonstrate adipocytes (cytoplasm completely filled with lipid droplets and also fusion of the droplets with associated lipid vacuole formation). *Green rectangle* demonstrates a more mature preadipocyte with incomplete lipid droplet formation. *Blue triangle* demonstrates a preadipocyte with an enlarged *triangular shaped* cell that conforms to the morphology of adipocytes but does not contain any visual Oil Red O-stained lipid vacuoles

There are also several other hydrophobic fluorescent dyes available that may be useful in the quantitative assessment of adipogenesis, such as Bodipy 493/503. There are reports that Bodipy 493/503 is more specific than Nile Red, but further studies are needed to validate this statement (Gocze and Freeman 1994). A definite advantage of Bodipy 493/503 is that it has a more specific fluorescent emission spectrum range. This allows for easier combination of cell surface markers with the lipid-specific dyes to study the phenotypic characteristics of adipocytes in more detail.

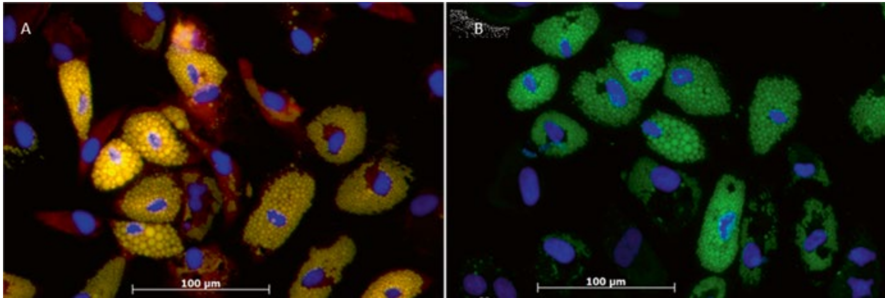

Fig. 7.3 Visualization of adipocyte formation following adipocyte differentiation using fluorescence microscopy. (**a**) ASCs were stained with Nile Red (2 µg/ml) and DAPI. (**b**) ASCs were stained with Bodipy 493/503 (20 µg/ml) and DAPI. Both images were captured at Day 21 after adipocyte differentiation was induced

7.3.2.2 Osteogenesis

There appears to be a complex relationship between adipogenesis and osteogenesis. Both adipocytes and osteoblasts are derived from a common multipotent MSC, thus leading one to believe that the two pathways are interconnected (Fig. 7.1). Obesity increases adipocyte differentiation and fat accumulation and is believed to decrease osteoblast differentiation and bone formation. Several recent investigators have reviewed the complex relationship between adipogenesis and osteogenesis (Cao 2011; Liao 2014; Nuttall et al. 2014; Romagnoli and Brandi 2014; Atashi et al. 2015).

The osteogenic maturation pathway involves cell proliferation, differentiation and matrix deposition followed by mineralization. Differentiation of ASCs into osteoblasts in vitro involves incubating a monolayer of ASCs with ascorbic acid, β-glycerophosphate and dexamethasone for 3 weeks. The composition of the osteogenic induction medium is provided in the supplementary material. Ascorbic acid acts as a cofactor for collagen synthesis through the hydroxylation of proline and lysine residues in collagen and induces extracellular matrix (ECM) production through the synthesis of non-collagenous bone matrix proteins. β-Glycerophosphate is needed for calcification and mineralization of the ECM. Dexamethasone regulates osteoblastic gene expression (Fiorentini et al. 2011). Dexamethasone treatment has been shown to increase alkaline phosphatase activity in vitro which is required for matrix mineralization and morphological transformation to cuboidal-shaped cells (Cheng et al. 1994). Alizarin Red S staining is commonly used for identifying calcific deposition during matrix mineralization in osteogenic differentiation cultures which is an early marker for differentiation (Fig. 7.5). Please refer to the supplementary material for a brief description of the qualitative method used to evaluate osteogenic induction in vitro.

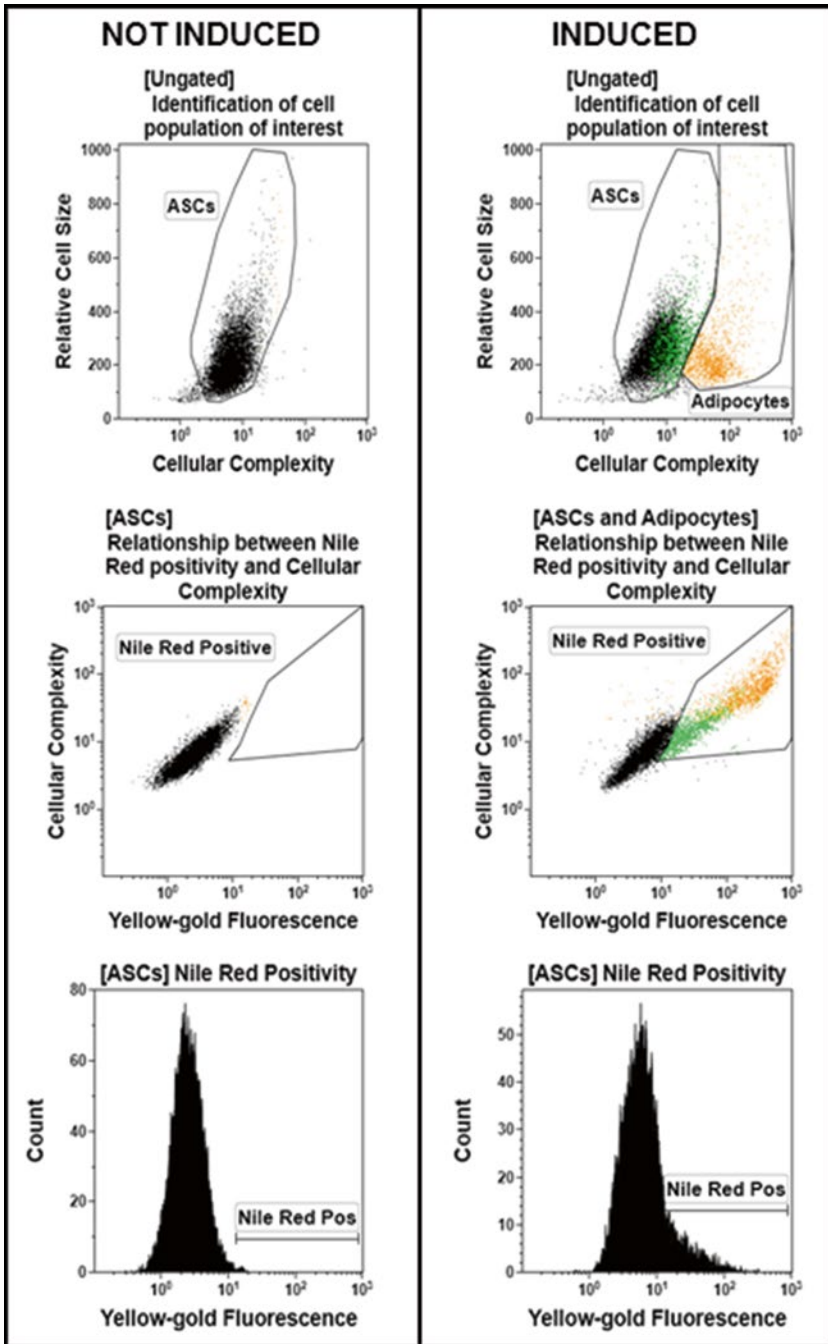

Fig. 7.4 Flow cytometric analysis of adipocyte differentiation of ASCs after staining with Nile Red (20 ng/ml final concentration). Adipocyte differentiation is associated with the intracellular accumulation with lipid droplets, which consequently results in an increase in cellular complexity. *Green-coloured* events indicate early stages of intracellular lipid accumulation. *Orange-coloured* events are indicative of cells with higher levels of intracellular neutral lipid content due to the accumulation of intracellular lipid droplets (increase in cellular complexity). Flow cytometric analysis was performed on a Beckman Coulter 3-laser, 10-colour Gallios flow cytometer, after 21 days of incubation of ASCs in adipogenic medium

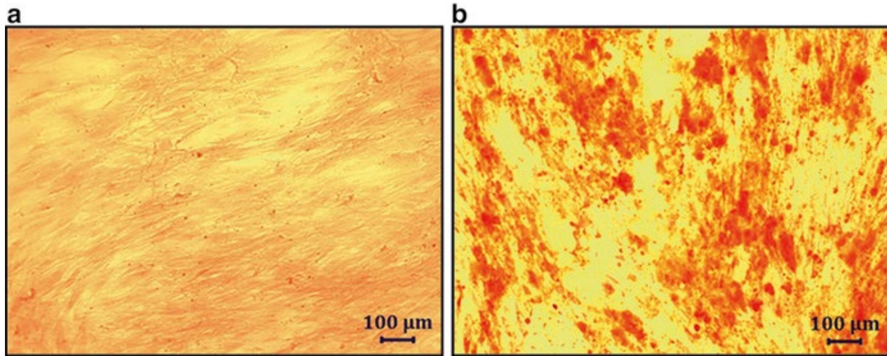

Fig. 7.5 Light microscopy analysis of osteogenesis. (**a**) Alizarin Red S-stained non-induced culture (osteogenesis control) showing no visible calcium depositions. (**b**) Alizarin Red S-stained osteogenic induced culture showing the red calcium deposits within the bone matrix characteristic of bone formation

7.3.2.3 Chondrogenesis

Cartilage is a specialized connective tissue which consists of chondrocytes surrounded by an ECM. Chondrocytes produce and maintain an ECM which is rich in glycosaminoglycans and proteoglycans and interact with collagen and elastic fibres. The cartilage matrix is composed mainly of type II collagen, providing tensile strength, and aggrecan, which provides compressive strength. Cartilage is classified according to its composition. Hyaline cartilage consists mainly of type II collagen fibres, elastic cartilage of type II collagen and elastic fibres and fibrocartilage of type I collagen fibres (Naumann et al. 2002). Chondrogenesis involves the recruitment and condensation of MSCs followed by differentiation into chondroblasts, chondrocyte maturation, and ECM production.

In vitro chondrogenesis is induced by artificially condensing the cells by centrifugation followed by addition of TGF-β3, dexamethasone and BMP-2 to the medium. The composition of the chondrogenic induction medium is provided in the supplementary material. Johnstone and colleagues (1998) developed a culture system to facilitate chondrogenic differentiation for BM-MSCs (Johnstone et al. 1998). This pellet culture system allows for cell-cell interaction similar to those that occur during embryonic development (Fell 1925). It has been shown that a defined medium, to which dexamethasone and TGF-β1 have been added, is required (Johnstone et al. 1998). Dexamethasone induces a metachromatic staining pattern in aggregated cells. This indicates the synthesis of a cartilaginous matrix which was confirmed by collagen type II immunohistochemistry. The use of TGF-β1 either alone or in combination with dexamethasone induces chondrogenesis in aggregated cells. It was later found that the addition of BMP-6 further increased chondrogenesis (Sekiya et al. 2001). Critical roles are played by BMPs in compaction of MSCs and for the shaping of the condensations (Zuscik et al. 2008). Comparisons between

BMP-2, -4, and -6 with regard to their ability to enhance in vitro formation of cartilage demonstrated that BMP-2 was the most effective, resulting in increased pellet weight along with more proteoglycan and collagen type II production (Sekiya et al. 2005). However, the addition of BMPs to cartilage induction medium is not necessarily required. Please refer to the supplementary material for a brief description of the method used to induce chondrogenesis in vitro.

More detailed strategies for the assessment of chondrogenic differentiation in vitro include techniques such as light microscopy, transmission electron microscopy and immunohistochemistry (Ichinose et al. 2005). Please refer to the supplementary material for a brief description of the qualitative method used to evaluate the induction of chondrogenesis in vitro. Other studies have shown that after a week in induction medium, cell pellets consist of three layers: the superficial zone, containing fibroblast-like cells; the middle zone, containing apoptotic cells; and the deep zone, containing matrix-producing chondrocyte-like cells. After 14 days, the middle zone disappears, and the deep zone dominates after an induction period of 21 days (Ichinose et al. 2005). Biochemical analysis of chondrogenesis includes methods that quantify the total glycosaminoglycan content in pellets (Naumann et al. 2002; Estes et al. 2010).

7.3.2.4 Myogenesis

The in vivo myogenic differentiation capacity of stem cells is highly sought after in regenerative medicine, as this property brings hope that these cells may restore regenerative capacity in muscular and neuromuscular disorders, myoskeletal tissue trauma, sport injuries and urologic incontinence. Benefits associated with this type of intervention include faster recovery time and a reduced risk of graft rejection, and preclinical evidence supports the application of MSCs across allogeneic barriers (Joo et al. 2014). Several lines of evidence exist for successful in vitro myogenic differentiation of MSCs from various origins (Gang et al. 2004). However, successful clinical translation remains to be convincingly demonstrated.

ASCs display superior myogenic differentiation potential (Stern-Straeter et al. 2014; Zych et al. 2013) and might be an ideal candidate for application in muscle tissue engineering of fibrotic muscle (Choi et al. 2012). ASCs can also be induced to differentiate into cardiomyocytes following induction by 5-azacytidine (Cao et al. 2004; Carvalho et al. 2013). Co-culturing of ASCs with primary myoblasts, in addition to stimulation with dexamethasone and FGF-2, promotes differentiation up to multinucleated myotubes (Beier et al. 2011; Bitto et al. 2013). Although no minimal criteria have been established for the myogenic differentiation potential of ASCs, the molecular markers in Table 7.3 have been used for quantitative assessment of myogenic differentiation.

7.3.3 Experimental Animal Models

In order to reach the long-term aim of clinical translation of cell-based therapy, preclinical safety and efficacy need to be demonstrated in animal models. Transplantation of in vitro expanded ASCs in the appropriate experimental animal model is therefore an important step in the development of cell therapy products. Sensitive cell-tracking techniques are needed in order to determine the most optimal route of administration as well as the homing ability of the transplanted ASCs. Transduced ASCs containing GFP lentiviral vectors could offer a feasible in vivo tracking system as it has been shown that transduction of ASCs with a GFP-expressing lentiviral vector does not affect their phenotypic expression or their differentiation potential (van Vollenstee et al. 2016).

7.4 Conclusion

Although manual isolation procedures are less costly than closed automated systems, the risk of contamination associated with manual procedures makes them a less attractive option for clinical applications. GMP isolation and expansion procedures require the minimal use of xenogeneic components, and only cell therapy products that are free of animal-derived products will lead to successful translation into the clinic. ASCs are regarded as non-minimally manipulated cells and are classified as a drug by the Food and Drug Administration (FDA). However, it is currently unclear whether in vitro expanded ASCs hold any significant benefit over AD-SVF, particularly in the clinical setting.

One of the major pitfalls in the rapidly expanding field of ASCs is that no inclusive panel of either cellular or molecular markers exists that specifically characterize these cells. The ISCT and IFATS regularly publish updated guidelines for the classification of ASCs. However, the current guidelines are still not specific and are based on the ability of the cells to adhere to plastic, to express a set of non-specific cellular markers and to differentiate into adipogenic, osteogenic, chondrogenic and myogenic lineages.

For example, although classical stains (Oil Red O, Alizarin Red S and Toluidine Blue) confirm lineage differentiation in vitro, it is unclear if all induced ASCs have differentiated into the respective lineage. The translation of in vitro assays to the patient is also unclear, and well-characterized in vivo experimental models are needed to validate the engrafting, homing and differentiation potential of the cell therapy in question. The availability of reliable and reproducible in vivo experimental models will therefore contribute to a more confident and potentially more rapid translation of ASC research to the clinical setting.

Appendix: Brief Description of Current Methods

Manual Isolation Procedure

The isolation protocol is adapted from the procedures described by Zuk et al. (2001), Bunnell et al. (2008) and Estes et al. (2010) (Fig. A.1).

The method requires the transfer of lipoaspirate material into 50 ml tubes (30 ml lipoaspirate/tube), followed by the addition of 20 ml phosphate buffer saline (PBS), supplemented with antibiotics (Fig. A.1). Depending on the future application of the adipose-derived stromal cell (ASC) isolates, culture medium may contain either FBS or human derivatives and antibiotics, usually penicillin and streptomycin (pen/strep). The adipose tissue is separated from peripheral blood contaminants by centrifugation at 1660 g for 3 min. The top oil layer is aspirated with a suction-assisted glass pipette system, and the compact lipoaspirate is carefully transferred to a sterile 50 ml tube. It is recommended that the washing steps are repeated until the compacted lipoaspirate material is golden yellow in colour without any visible evidence of peripheral blood contamination. The volume of the compacted lipoaspirate material should be recorded once most of the blood contamination has been removed.

To release resident ASCs in adipose tissue from the fibrous network, the lipoaspirate is enzymatically digested in culture plates. The most popular approach makes use of the enzyme, collagenase type I. Other enzymatic alternatives include dispase and trypsin. A filter-sterilized 0.1 % collagenase type I solution is prepared using PBS supplemented with 2 % antibiotics. The volume of collagen solution required is dependent on the volume of the compacted adipose

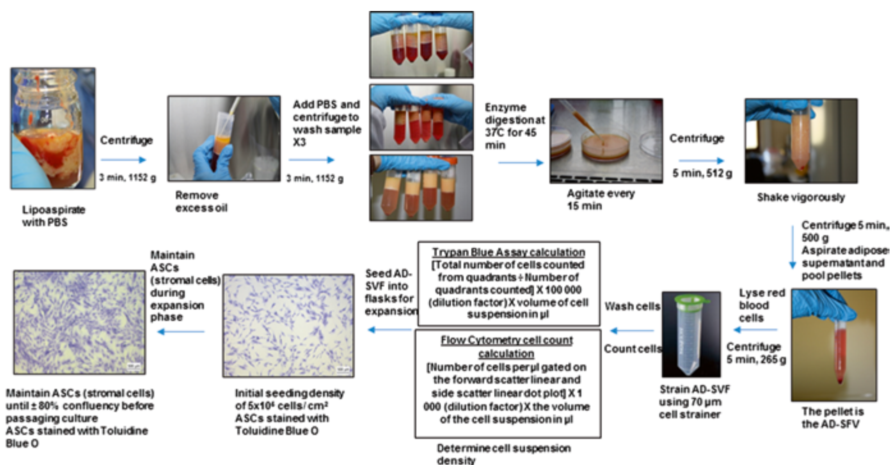

Fig. A.1 Outline of the procedure used for isolating adipose-derived stromal cells. Lipoaspirate samples are processed by enzymatic digestion, and the stromal vascular fraction (SVF) is collected. Adipose-derived stromal cells from the SVF adhere to the plastic culture dish, and non-adherent cells are washed away after 24 h

tissue previously recorded. The ratio of compacted adipose tissue volume to the volume of collagen solution should be at least 2:1, meaning that the final volume of collagen digesting solution added to the adipose tissue in the culture plates should be half that of the washed compacted adipose tissue volume. The ratio of collagenase to adipose tissue should be optimized by each laboratory as well as for each isolation technique used. A sterile plastic pipette is used to mix the adipose tissue well, before incubation at 37 °C, 5 % CO_2 for 45 min. The sample may either be continuously agitated using an automated rotating system or agitated manually every 15 min with a plastic pipette to aid the mechanical breakdown of the fibrous tissue.

The collagenase-digested sample is then transferred to sterile 50 ml tubes. The tubes are shaken vigorously and centrifuged at 500 g for 5 min, resulting in the SVF pellet settling to the bottom of the tube. The compacted adipose tissue and collagenase solution supernatants are carefully aspirated, and the collagenase activity is neutralized by adding 2 ml of stromal medium to the AD-SVF pellet. Stromal medium may consist of either Dulbecco's Modified Eagle Medium (DMEM) containing GlutaMax™, 4.5 g/L D-glucose and pyruvate or alpha-Modified Eagle Medium (α-MEM) containing GlutaMax™, supplemented with 10 % serum and 1 % antibiotics.

The AD-SVF pellets are pooled into a single tube (15 or 50 ml) and centrifuged at 265 g for 5 min, followed by aspiration of the supernatant. Red blood cells present in the AD-SVF pellet are lysed either by the addition of an ammonium chloride-based lysing solution or an enzymatic-based lysing solution like VersaLyse™ (Beckman Coulter, Miami, USA). After a 10–15 min incubation period at room temperature, the lysing reaction is stopped by filling the tube with PBS supplemented with antibiotics and centrifuged at 184 g for 5 min. The supernatant is removed and the pellet resuspended in stromal medium before filtering the cellular suspension through a 70 μm cell strainer to remove any larger, undigested fragments.

In order to seed the cells at the correct seeding density, an absolute cell count should be performed. Cell counts may be performed by using either a manual approach in which the cells are counted using a haemocytometer or an automated cell counting device such as a flow cytometer. Details are provided below.

The AD-SVF is seeded at an initial seeding density of 5×10^5 cells per cm^2. In order to determine the volume of cell suspension required for initial seeding, the following formula should be used:

$$\text{Volume}\left(\mu\text{l}\right) = \left(\frac{\text{initial seeding density} \times \text{seeding surface area of tissue culture flask or well}}{\text{total number of viable cells}}\right) \times \text{cell suspension volume in } \mu\text{l}$$

After the cells are seeded, the cultures flasks are swirled gently to ensure uniform distribution. The culture flasks are maintained in an incubator under standard cell culture conditions (humidity, 5 % CO_2 and 37 °C). The cultures are washed twice after 24 h with PBS supplemented with antibiotics to remove non-adherent cells, cellular debris and non-viable cells. Fresh stromal medium is added to the culture flasks and incubated under standard conditions until cells are 80–90 % confluent, implying that cells cover

80–90 % of the culture flask surface area. In order to determine the volume of cell suspension required for reseeding after thawing frozen samples, the following formula should be used:

$$\text{Total number of viable cells in cell suspension} \div \left(\text{reseeding density} \times \text{reseeding surface area of tissue culture flask or well}\right)$$

Methods Used to Obtain Absolute Cell Counts

Trypan Blue (0.4 %) Dye Exclusion Method

Trypan blue is a non-membrane permeable vital stain that is used to assess the viability of cells. Trypan blue is not able to cross the cell membrane of intact, viable cells and therefore only stains cells with compromised cell membranes. Dead cells display a distinct blue colour after staining, due to the accumulation of trypan blue in the cytoplasm of cells with compromised cell membranes The recommended procedure is as follows: (1) prepare the Neubauer counting chamber (haemocytometer) by carefully placing the cover slip on the counting grid; (2) carefully mix 80 µl of a 0.4 % trypan blue with 100 µl PBS and 20 µl of the suspension; (3) carefully load 10 µl of the solution onto both sides of the Neubauer counting chamber (haemocytometer); and (4) count the viable (unstained) cells as well as dead cells (stained) using a microscope (10 times objective lens). The following formula is used to determine the absolute cell concentration (Fig. A.1):

Absolute cell concentration (cells/ml) $= \left(\frac{Q1 + Q2 + Q3 + \ldots + Q8}{8}\right) \times 10 \times 10{,}000\,\text{cells}/\text{cm}^2$, where $Q1$–$Q8$ refer to eight quadrants on the haemocytometer. The factor 10 is to correct for the dilution of the sample with the 0.4 % trypan blue solution.

There are several commercial automated counting devices on the market that make use of trypan blue (0.4 %) dye exclusion assay principles. Examples of such devices are the Vi-Cell XR™ automated cell counter (Beckman Coulter, Miami, USA), Countess™ automated cell counter (Invitrogen, Carlsbad, USA) and TC20™ automated cell counter (Bio-Rad, Hercules, USA).

Absolute Cell Count Determination Using a Benchtop Flow Cytometer (Beckman Coulter Flow Cytometers)

An example of the strategy that is followed to obtain an absolute count on a benchtop flow cytometer is illustrated in Fig. A.2. The cell population of interest is identified by using a side scatter linear (SS lin) and a forward scatter linear (FS lin) histogram by placing a region around the cell population of interest only, excluding

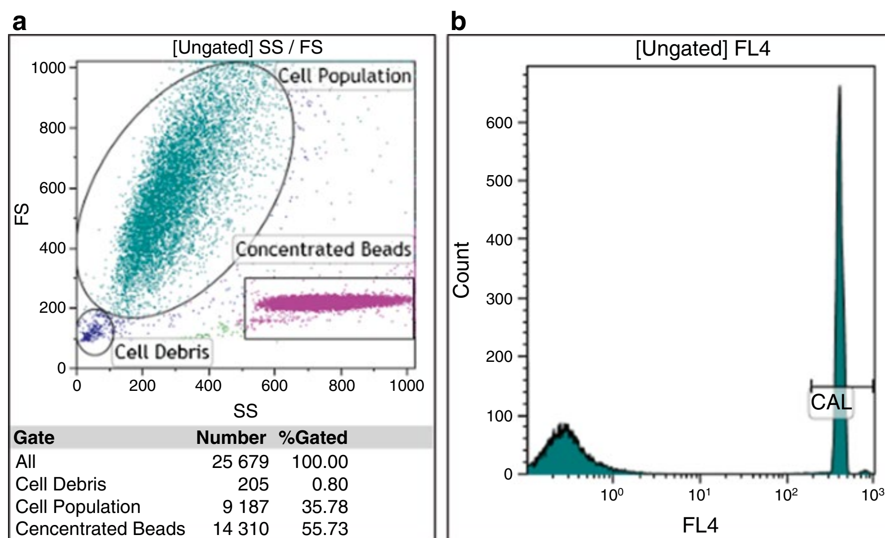

Fig. A.2 (**a**) A side scatter linear and forward scatter linear dot histogram displaying all the events measured by the flow cytometer. The flow beads (*pink*) and a gate were used to encircle the cell population that was counted until the CAL factor was reached. The gate-labelled cell population displays the cell population count that was expressed as the number of cells per μl cell suspension. (**b**) A histogram displaying the flow beads with a region of interest placed over the peak of the flow cytometry counting beads labelled as CAL. In this example, the specific calibration factor (assayed bead concentration) was 986

the counting beads and debris. The counting beads are enumerated in the CAL region. In order to obtain an accurate absolute count, it is important to count a sufficient number of cells as well as counting beads (>1000 events if possible) as well as to perform the count as soon as possible after the counting beads are mixed with the sample.

Induction of Adipogenesis In Vitro

ASCs are seeded at a density of 5×10^3 cells/cm^2 in a six-well plate and maintained under standard culturing conditions (37 °C and 5 % CO_2) with complete stromal medium (α-MEM or DMEM, containing 10 % FBS and 1 % pen/strep). When 70–80 % confluency is achieved, the stromal medium is removed and replaced with adipogenic induction medium (Table A.1) in three of the wells of the 6-well plate. DMEM supplemented with serum and antibiotics is added to the remaining three wells. These wells serve as non-induced controls. The cultures are maintained for 21 days under standard culturing conditions of 37 °C, 5 % CO_2. During this period the induction and control media are replaced every second day.

Table A.1 Composition of induction media for adipogenic, osteogenic, chondrogenic and myogenic differentiation in vitro

Lineage	Chemical	Media	Final concentration	Stock solution	Volume/mass of stock used[a]
Adipogenic differentiation	3-Isobutyl-methylxanthine	Complete DMEM[b]	0.5 M	Neat (powder)	0.011 g
	Indomethacin		200 μM	Neat (powder)	0.007156 g
	Insulin		10 μg/ml	4 mg/ml	250 μl
	Dexamethasone		1 μM	0.01 %	392.46 μl
Osteogenic differentiation	Ascorbate-2-phosphate	Complete DMEM[b]	50 μM	Neat (powder)	0.0016 g
	β-Glycerophosphate		10 mM	Neat (powder)	0.306 g
	Dexamethasone		1 μM	0.01 %	392.46 μl
Chondrogenic differentiation	Ascorbate-2-phosphate	DMEM	0.155 mM	Neat (powder)	0.005 g
	TGF-β_3		10 ng/ml	5 μg/5 ml	1 ml
	Proline		0.35 mM	Powder	0.004 g
	Pyruvate		0.9086 mM	Powder	0.010 g
	Dexamethasone		1 μM	0.01 %	392.46 μl
	ITS Premix		1 %	100 %	1 ml

[a]The exact volume or mass of chemicals used to produce a total volume 100 ml of induction medium

[b]DMEM culture medium supplemented with 10 % serum and 1 % antibiotics

Qualitative Assessment of Adipogenesis In Vitro Using Oil Red O

After 21 days of induction, the 6-well plates containing both induced and non-induced cultures are fixed by adding a 4 % formaldehyde solution for 1 h. A 0.5 % Oil Red O stock solution is prepared in isopropanol. An Oil Red O working solution is prepared from the stock solution by mixing three parts of the stock solution with two parts of double-distilled water (ddH_2O) (volume/volume). After removal (aspiration) of the fixative solution, the cultures are allowed to dry at room temperature before adding 1 ml of Oil Red O working solution to both the adipogenic induced and non-induced cultures, followed by a 20 min incubation at room temperature. The stain is removed, and the wells are thoroughly washed with ddH_2O until no pink discoloration of the freshly added ddH_2O is visible to the naked eye.

The cultures are then counterstained with Toluidine Blue O. A 0.01 % Toluidine Blue O counterstain solution is prepared (pH 11) by adding 0.005 g Toluidine Blue O and 0.005 g Na_2CO_3 to 50 ml ddH_2O. The Toluidine Blue O counterstain is added to the Oil Red O-stained cultures for 5 min at room temperature, after which the excess stain is washed away with ddH_2O. 1 ml ddH_2O is added to each well before microscopy analysis.

Induction of Osteogenesis In Vitro

Immunophenotyped ASCs are seeded at a density of 5×10^3 cells/cm^2 in a six-well plate and maintained under standard culture conditions (37 °C and 5 % CO_2) with stromal medium (α-MEM, containing 10 % FBS and 1 % pen/strep) until about 60–70 % confluency. Osteogenic induction medium (Table A.1) is introduced to half the wells and DMEM supplemented with serum and antibiotics to the other half to serve as non-induced (control) cultures. The cultures are maintained for 21 days under standard culture conditions of 37 °C, 5 % CO_2. The induction and non-induction media are replaced every second day.

Qualitative Assessment of Osteogenesis In Vitro

Twenty-one days after induction of osteogenic differentiation, the cells are fixed by addition of a 4 % formaldehyde fixative solution for 1 h. A 2 % Alizarin Red S classical stain is used to detect the calcium in the mineral matrix from mature osteocytes. An alizarin stock solution is prepared by adding 2 g Alizarin Red S powder to 100 ml of ddH_2O. The solution is mixed thoroughly using a magnetic stirrer until solutes are dissolved before filtering through filter paper.

The induced and non-induced cultures are pre-washed with 2 ml PBS at pH 4.2 for 5 min before introducing 2 ml of the 2 % Alizarin Red S stain and incubating the cultures for 10 min at room temperature. The cultures are washed thoroughly with ddH_2O to remove the excess stain. 1 ml ddH_2O is added to each well before microscopy analysis.

Induction of Chondrogenesis In Vitro

A suspension culture technique is usually used for the differentiation of ASCs into chondrocytes. ASCs are seeded in a T25 flask at a density of 5×10^3 cells/cm^2 and maintained under standard culture conditions until about 60 % confluence. The cells are enzymatically removed from the flask (0.25 % trypsin-EDTA) followed by the neutralization of the enzymatic action with the addition of stromal medium (α-MEM, containing serum and antibiotics).

The cell suspension is transferred into a 15 ml tube, and the sample is centrifuged for 5 min at 400 g. The substrate is carefully aspirated until only the ASC pellet remains in the tube. The ASC pellet is suspended in chondrogenic induction medium (Table A.1), for the chondrogenic induced cultures or DMEM supplemented with serum only and for the non-induced cultures, and centrifuged at 400 g for 10 min. The tubes are carefully placed into the incubator without disrupting the pellet. The tube caps are slightly loosened to allow for gas exchange to occur. Cultures are incubated under standard conditions of 37 °C, 5 % CO_2 for 21 days. The induction and control media (0.5 ml) are replaced every second day. After 24 h, the ASC pellets contract into a sphere. The cells that have not been incorporated into the sphere after 48 h are removed from the suspension cultures during medium replacement.

Qualitative Assessment of Chondrogenesis In Vitro

Each induced and non-induced chondrocyte sphere is serially dehydrated in 30, 50, 70 and 90 % ethanol, followed by three changes of absolute ethanol for 15 min per dehydration step. The dehydrated chondrocyte spheres are infiltrated with 50 % LR White Resin in absolute ethanol for 1 h, followed by an infiltration in a 100 % LR White Resin overnight. To embed the tissue spheres, they are transferred into resin capsules with 100 % LR White Resin and polymerized for 24 h at 60 °C.

Ten to 15 serial transverse sections of between 0.5 and 1.0 μm (optimal 0.5 μm) are prepared using a microtome, and the sections are stained with 1 % Toluidine Blue O stain for 30 s. The glass slides containing stained sections are gently rinsed with ddH_2O. One drop of ddH_2O is added on the glass plate and covered using a cover slip before microscopy analysis is performed.

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Chapter 8
Cord Blood Stem Cell Banking

Helen C. Steel, Marco Alessandrini, Juanita Mellet, Carla Dessels, Ahmed K. Oloyo, and Michael S. Pepper

Abbreviations

AABB	American Association of Blood Banks
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
EBMT	European Society for Blood and Bone Marrow Transplantation
GCSF	Granulocyte colony-stimulating factor
GVHD	Graft-versus-host disease
HIE	Hypoxic-ischemic encephalopathy
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
MSC	Mesenchymal stem cell
MUD	Matched unrelated donor
PBSC	Peripheral blood stem cell
UC	Umbilical cord
UCB	Umbilical cord blood
UCB SCB	Umbilical cord blood stem cell bank

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8.1 Introduction

The potential use of umbilical cord blood (UCB) was first proposed in 1982 by Edward Boyse, whereafter the first successful human leukocyte antigen (HLA)-identical UCB transplant was performed in 1988 by Gluckman and colleagues on a 5-year-old patient with Fanconi's anemia (1989). The first unrelated UCB transplant was performed in 1993 by Kurtzberg and Wagner (Kurtzberg et al. 1996; Wagner et al. 1996). Since then, UCB, previously considered a biological waste product, has been used as a source of hematopoietic stem cells (HSCs) and progenitor cells for HSC transplantation to treat individuals with sibling, related or unrelated, donor cells for a number of malignant and nonmalignant disorders as well as immune deficiency and genetic disorders.

This chapter provides a critical overview of UCB stem cell banking. The advantages and disadvantages of using UCB stem cells over the more traditionally used bone marrow or mobilized peripheral blood stem cells (PBSC) will be covered. The controversial debate surrounding public versus private UCB stem cell banks (UCB SCBs) will also be addressed. In addition, this chapter will focus on the ethical and regulatory issues surrounding UCB SCBs, the establishment of UCB SCBs in developed versus developing countries, and the use of UCB stem cells in transplantation and regenerative medicine.

8.1.1 Umbilical Cord Blood Versus Bone Marrow or Mobilized Stem Cells for Transplantation

Hematopoietic stem cell transplantation (HSCT) involves the transfer of immunocompetent hematopoietic stem and progenitor cells from donors to recipients to reconstitute the marrow and restore immune function in the treatment of high-risk acquired and inherited hematologic malignancies as well as nonmalignant hematopoietic and immunological diseases. However, the availability of an adequate HLA-matched sibling remains only 25–30 % (Gragert et al. 2014), and patients rely heavily on the worldwide network of bone marrow registries to find a suitable donor. This, in itself, has limitations due to the vast majority of registered donors being Caucasian, making it difficult to obtain matched unrelated donors (MUDs) of other races.

HSCT can be used following myeloablative or reduced-intensity chemotherapy regimens. Myeloablative treatment involves the administration of high doses of chemotherapy, which destroys cancer cells and normal cells within the bone marrow prior to transplantation, while reduced-intensity conditioning involves treatment with lower doses of chemotherapy agents such as busulfan and cyclophosphamide. There are two types of HSCT, autologous and allogeneic. In autologous transplants, the donor and recipient are the same individual. In the case of allogeneic transplants, the donor and recipient may be genetically related or unrelated; however, the donor and recipient are HLA matched as closely as possible. The rate of graft failure is higher in unrelated

transplants that mismatch at one or two alleles compared to a fully HLA-matched transplant (Kanda et al. 2014). Two distinct classes of stem cells are used in HSCT. These include bone marrow or mobilized PBSC and UCB stem cells.

The bone marrow is located within long and flat bones and is the site at which virtually all blood stem cells reside, constituting what is defined as the stem cell niche. Bone marrow-derived HSCs can either be harvested by inserting a needle into the marrow cavity of the iliac crest or by a process known as apheresis, which enables the collection of mobilized PBSC. The growth factor granulocyte colony-stimulating factor (GCSF) facilitates the mobilization of stem cells from the bone marrow into the bloodstream. The mobilized stem cells can then be obtained from the peripheral blood, which is a less invasive procedure than acquiring stem cells from the bone and is currently the most frequently used source of HSCs. UCB is also successfully used in HSCT and is easily accessible as it is harvested from the placenta through the umbilical vein. The blood from the umbilical cord/placenta is a rich source of stem cells (Gluckman et al. 1989), and due to the immaturity of the immune cells in UCB, HLA typing is only performed for HLA-A, HLA-B (antigen level), and HLA-DRB1 (allele level) (Eapen et al. 2007), and a 4/6 to a 6/6 match is adequate for unrelated donors (Barker et al. 2010; Eapen et al. 2007). Recent studies suggest that it would be optimal to perform high-resolution (allele-level) typing for four HLA loci (HLA-A, HLA-B, HLA-C, and HLA-DRB1) for a single unit to minimize the risk of mortality after UCB transplantations (Eapen et al. 2011, 2014). Matching of the class I alleles is vital, since mismatching at these alleles is reported to increase the risk of graft failure (Petersdorf et al. 2001). Although UCB-derived stem cells have several advantages, the number of cells obtained from a single UCB unit is limited, as a result of which pediatric patients remain the primary focus. A minimum of $2–5 \times 10^7$ nucleated cells are required per kilogram body weight to be confident of a successful transplant (Welte et al. 2010). Nonetheless, the application of UCB in HSCT is being extended to treat adult patients through the use of single- or double-unit transplants.

UCB has sufficient numbers of hematopoietic progenitor cells to ensure long-term engraftment (Broxmeyer et al. 1989), and the rapid proliferative capacity of these cells makes it possible to reconstitute the entire bone marrow (Gluckman et al. 1997). Clinical observations have shown that the risk and severity of graft-versus-host disease (GVHD) is decreased in patients receiving UCB stem cells compared to cells from the bone marrow or peripheral blood. UCB stem cells differ from bone marrow and peripheral blood HSCs, in that UCB stem cells are "immunologically naive" (Wagner and Gluckman 2010). In addition, UCB T cells are phenotypically and functionally immature and are less responsive to stimulation compared to their adult counterparts, which has been suggested as a possible reason for the lower incidence of GVHD (Harris et al. 1992). UCB also contains increased numbers of natural killer cells and lower cytotoxic T-cell activity (Bensussan et al. 1994; Berthou et al. 1995). Consequently, UCB transplantations result in delayed engraftment of neutrophils and platelets and aberrant immune reconstitution.

In addition to its use in transplantation, UCB is a valuable source of cells for cellular therapies associated with tissue repair, replacement, and regeneration aimed at restoring impaired function resulting from congenital defects, disease, and trauma. The therapeutic potential of stem cells obtained from UCB is currently being investigated in over a hundred clinical trials for a wide range of disorders, including autism, diabetes, cerebral palsy, and spinal cord injury. This will be discussed in more detail in Sect. 8.1.4.

8.1.2 Umbilical Cord Blood Stem Cell Banks

The successful use of UCB in HSCT has led to the establishment of UCB SCBs worldwide with various options being available for banking/storage. An UCB SCB is a facility in which donated UCB stem cells are stored for future use (Ballen et al. 2008). These UCB units are retrieved upon request from a recipient for transplantation or regenerative treatment purposes. There are a variety of UCB SCBs which are either public or privately financed organizations (Butler and Menitove 2011). More recently, hybrid UCB SCBs have come into existence, where a combination of private and publicly funded units are banked (Guilcher et al. 2014).

8.1.2.1 Public Cord Blood Banks

Public UCB SCBs typically receive anonymous non-remunerated altruistic donations from willing donor families. These UCB units are subsequently made available for any histocompatible patient requiring a HSCT (Ballen et al. 2008; Brown et al. 2011; Wilson et al. 2011). Once the UCB unit is banked, it is anonymized where neither the donor nor the donor's family may retrieve it for personal use. Only in the prearranged instance of directed donation may the UCB unit be retrieved by the donor family to treat a family member (Ecker and Greene 2005; Ballen et al. 2008).

To ensure the safety of the donation, the UCB unit undergoes a series of tests prior to being banked. Should it pass and adhere to the stringent regulations and requirements (Table 8.1) set out by the American Association of Blood Banks (AABB) and NetCord Foundation for the Accreditation of Cellular Therapy (NetCord-FACT), the UCB unit is then banked and made accessible to the public (Butler and Menitove 2011). Should the unit not be eligible for banking, it is either discarded or used for research purposes (Sugarman et al. 1997; Ballen et al. 2008).

In the case of public UCB SCBs, the units are donated without any cost to the donor family. However, should a unit be retrieved by a recipient, the costs accrued for the banking, storage, and further preparation/testing required for release of the unit will be covered by the recipient. Even though these banks work on cost-recovery basis (not for profit), a major point of concern surrounding this type of UCB banking is the financial sustainability (Allan et al. 2013). The costs involved

Table 8.1 Benefits and limitations of UCB SCBs (Abdullah 2011; Ballen et al. 2008; Butler and Menitove 2011; Guilcher et al. 2014; Sugarman et al. 1997; Wagner et al. 2013; Yoder 2014)

Type of bank	Public	Hybrid	Private
Cost involved	Assumed by the bank and partially recovered from the recipient	A combination of public and private, with cross subsidization of the former by the latter	Borne by the donor
Cord blood owner	Bank	Bank or private donor	Private donor
Recipient	Unrelated transplant patients and researchers	Unrelated transplant patients, researchers, and private donors (donor or relative)	Donor or relative
Uses	Unrelated transplant and regenerative medicine (clinical trials and research)	Unrelated transplant and regenerative medicine (clinical trials, experimental treatments, and research)	Related transplant and regenerative medicine (experimental treatments)
Advantages	Unit available globally for a matching donor	Families can store units for personal use; may be made available to the public	Family has control over the stored unit
	Stringent quality control of units	Publically donated units available globally for a matching donor	
	No cost implications for the donor	Private section subsidizes the public costs	
	Low CBU attrition rate		
Disadvantages	Opportunity to donate is not universal due to stringent quality and testing criteria	Transferring the financial cost from public subsidies like philanthropists and government to the private client is seen as a conflict of interests	Stored unit not available to public
	High operating costs are difficult to manage		Donors often misinformed about the indications and efficacy of use
	Product is not available for family		May be less stringent quality control
			Low retrieval rate
			Substantial financial cost to families

include the collection, testing, and processing of the units for storage, the preservation of the UCB units, and the man power needed to maintain the facility (Ballen et al. 2008). As a result, this obstacle has prevented the establishment of many public banks and remains a reality for those currently in operation. Public UCB SCBs are funded in several ways which include federal/government funding, revenue generated through the sale of UCB units, grants, and private/philanthropic investors (Abdullah 2011; Allan et al. 2013).

8.1.2.2 Private Cord Blood Banks

In a private UCB SCB, the bank receives payment from families who wish to store their UCB stem cells for autologous use or for use by next of kin (Ballen et al. 2008; Jordaan et al. 2009; Butler and Menitove 2011). Therefore, the units are stored at the cost of the donor family and also retrieved at the donor family's expense. Private banking is expensive and operates on a for-profit basis with shareholder requirements. There is an ongoing and constant debate about private banking (Sullivan 2008; Hollands and McCauley 2009; Ballen 2010). Arguments in favor of private banking include the following facts: (a) there are no medical or ethical issues related to collection (assuming that the third stage of labor proceeds unhindered) with limited risk to mother and child around material that would otherwise have been discarded—the same would be true for public banking; (b) one should have the right to exercise control over one's own body and the bank should have the economic freedom to run its own business; and (c) private banking is practiced in many countries where the demand is high, and if prohibited locally, cells would be sent to another country where banking is allowed. None of these arguments speak in favor of the potential medical benefits that could potentially be derived from the stored cells. This is one of the major issues around which arguments against private banking are constructed.

Arguments against public banking include the fact that (a) the recall rate of the stored cells is limited, albeit far greater than in private banks; (b) other sources of stem cells are adequate; (c) the indications for use of autologous UCB stem cells for transplantation are limited although their use in regenerative medicine may be easier to justify but difficult to quantify; (d) the volume of UCB/number of stem cells limits use to pediatric patients (or requires more than one unit in adults); (e) parents are driven by subjective (emotional) factors to store their children's stem cells due to an overestimation of the perceived benefit of private banking; and (f) private banking is elitist, i.e., it is not accessible to all (due to cost). Other arguments such as the fact that private banks deprive public banks of material, that there is inadequate informed consent, or that there is less stringent quality control than in public banks may apply to some private banks but certainly not to all. Several international professional bodies have expressed their views on the question of stem cell banking (European Group on Ethics in Science and New Technologies to the European Commission 2004; American Academy of Pediatrics 2007; ACOG 2008; South African Society of Obstetricians & Gynaecologists 2014). Some of these arguments will be explored in more detail below.

With regard to the limited likelihood that a stored unit will be used, it is universally accepted that the recall rate on privately stored UCB stem cells remains very low

(Sullivan 2008). This is because the current applications of stem cell therapy are limited mainly to HSCT, for which the use of autologous UCB stem cells is limited. Marketing often overestimates the immediate benefits of stem cell therapy. It is accepted that one cannot ignore the real promise that stem cell therapy might hold in the future, but at present this remains difficult to quantify (Sullivan 2008; Ballen 2010).

With regard to the volume of UCB/number of stem cells required for a successful transplant ($2–5 \times 10^7$ nucleated cells or 2×10^5 CD34+ cells per kilogram body weight (Welte et al. 2010)), there is a direct correlation between the success of HSC engraftment following transplantation and the number of cells used to treat the patient. With UCB stem cells, there is a limitation to the size of the patient that can be treated which is dependent on the number of stem cells recovered after thawing. This limitation may be overcome when stem cell expansion becomes a routine procedure in the future.

With regard to the availability of other types of stem cells, there are a number of other sources which include (a) adult stem cells—HSCs (bone marrow, peripheral blood) and mesenchymal stem cells (MSCs; from a variety of sources including the bone marrow and adipose tissue)—and (b) pluripotent stem cells (induced pluripotent stem (iPS) cells and embryonic stem (ES) cells derived by various techniques). While the therapeutic potential of pluripotent stem cells remains to be demonstrated, the value of adult stem cells (and in particular HSCs) is beyond doubt.

With regard to informed consent, not only must individuals be empowered with the necessary knowledge to make decisions for themselves, but an individual's autonomy to make decisions must be respected. Informed consent and all communication in printed and electronic media should include the current statistics of the chances of a newborn or its family ever needing the banked stem cells. In addition, provision could be made for a cooling-off period after birth during which the stem cell banking contract must be confirmed by the parents. It is therefore important for regulatory authorities to enforce a high standard of informed consent.

With regard to marketing, perhaps one of the biggest marketing inaccuracies in the private banking business is to list the great potential of stem cells and then to infer that this is what can be done with autologous UCB. While much of the potential of autologous UCB may be realized at some point in the future, at present this is not the case and is difficult to quantify. Support for private stem cell banking is therefore often based on an overestimation of the benefits of stem cell therapy. The argument that the public may be exploited by unrealistic promises about stem cell therapy is certainly valid. It remains, however, that this is a period of emotional vulnerability and that despite adequate informed consent, prospective parents may not make decisions that are entirely rational. It has been argued that the enforcement of a high standard of informed consent could partially rectify this problem. However, to ignore the real promise that stem cell therapy holds would also be dishonest.

With regard to the elitist nature of private banking in which the service remains inaccessible to many because of the cost factor, it should also be appreciated that equality will not be achieved by denying everyone a benefit because it is currently only available to some. Objections to private stem cell banking based on elitism would be better addressed by thinking of constructive ways to increase access by the entire population to stem cell banking and related therapies, as in the case of public or hybrid banking (Jordaan et al. 2009).

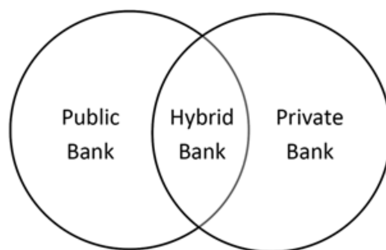

Fig. 8.1 Representation of hybrid banking

8.1.2.3 Hybrid Cord Blood Banks

Hybrid UCB SCBs are an amalgamation of public and private UCB SCBs (Fig. 8.1). In this setting, a UCB unit is stored and can be retrieved for personal or public use (Guilcher et al. 2014). There are currently two modes of storing UCB units in hybrid UCB SCBs, which are either sequential or splitting. The sequential mode is when a family stores the UCB unit for private future use, but if required can be used by someone else, with the family's consent. In the splitting mode, units are divided in two, where one part is stored for private purposes and the other is made available to the public (Wagner et al. 2013). Hybrid UCB SCBs, therefore, leverage funds obtained from the private section to subsidize the public section of the bank (Guilcher et al. 2014).

8.1.2.4 Global Policies and Legislation

The objectives of regional, national, or international policies and legislation are (a) to protect the individual from harmful and unethical practices and (b) to respect the individual's right to determine how to use her/his own stem cells. Provision should be made for all who might benefit from stem cells for therapeutic purposes, and everyone should be given an equal opportunity to benefit from the advances in medical science. In addition, policy and legislation should not be unduly restrictive so as to avoid stifling basic and clinical research and biotechnological innovation.

Several general recommendations have been put forward by a number of professional bodies which include working and research groups, healthcare providers, and UCB SCB representatives for consideration with the banking and retrieval of UCB units (Armson 2005; Ballen et al. 2008; Petrini 2013). These can be summarized as follows:

1. Balanced and accurate information must be provided on the advantages and disadvantages of UCB banking including the remote chance that the unit will ever be used.
2. Perinatal healthcare providers should be informed about the clinical potential and the indications that can effectively be treated with UCB stem cells based on scientific evidence. UCB donation should be discouraged when UCB stored in

a bank is to be directed for later personal or family use, because most conditions that might be helped by UCB stem cells already exist in the infants' UCB (i.e., premalignant changes in stem cells).

3. UCB storage for personal use should only be considered by a family where a sibling or parent possesses a disorder or disease that can be treated with the autologous HLA-matched UCB—directed donation.
4. UCB should not be stored for personal use if an allogeneic transplantation is the treatment of choice for a child or family member that does not have an HLA-identical sibling or a well-matched family member.
5. Allogeneic UCB should be considered in adolescents and young adults with hematologic malignancies because of the advantage of the graft-versus-leukemia effect.
6. Donation of UCB for altruistic purposes to a public UCB SCB and subsequent allogeneic transplantation should be encouraged when UCB banking is being considered by expecting families and their healthcare providers.
7. Because there is limited scientific data at the present time to support autologous UCB SCB and given the difficulty of making an accurate estimate of the need for autologous transplantation and the ready availability of allogeneic transplantation, private storage of UCB as "biological insurance" should be discouraged.
8. Public, hybrid, and private UCB SCBs should strictly adhere to the regulations and requirements indicated for the safety and efficacy of the UCB units.
9. Recruitment of UCB donors should be fair and noncoercive.
10. Testing for maternal infectious and genetic diseases must be discussed.
11. Private UCB SCBs should be regulated to ensure that promotional marketing and financial costs are fair.
12. Parents and healthcare providers must understand and acknowledge the differences between autologous and allogeneic donations and the differences between private and public UCB SCBs.

Standards and regulations should be developed by perinatal facilities to educate the expecting family regarding the need for UCB in the public and private UCB SCB industry.

8.1.3 Cord Blood Banking in Developed Versus Developing Countries

Due to the high costs involved in establishing and maintaining UCB SCBs, it is in the developed nations of the world that the collection, banking, and utilization of UCB are most prevalent. Countries primarily involved in UCB banking include the United States, the United Kingdom, as well as those in Western Europe and Australasia.

The first public UCB SCB was established in New York, USA, in 1992, and the first private UCB SCB in 1995, also in the United States. Since then numerous pub-

lic and privately owned UCB SCBs have been established that are actively involved around the world in collecting, processing, testing, and cryopreserving UCB for potential future use.

With the increased interest in UCB-related therapeutics and the need for effective and reliable banking come the attending problems of regulation, standardization, and the protection of donors, recipients, and the public as a whole. Therefore registries and regulatory bodies are formed to establish standard protocols and provide guidelines for standard and good practice in all that pertains to UCB collection, banking, and usage. These national regulatory agencies and transplant centers are aware of the need for global standards whose major objective is to promote quality throughout all phases of UCB SCB with the goal of achieving consistent production of high-quality units for transplantation. This covers collection of UCB stem cells, regardless of the methodology or site of collection; screening, testing, and eligibility determination of the maternal and infant donor according to applicable laws; and all phases of processing and storage including qualitative testing and characterization of the unit.

Considering the rigors and financial implications involved in the establishment of registries and regulatory bodies, as would be expected, all the well-known registries and regulatory bodies are domiciled in developed countries, although some have member UCB SCBs in developing countries (Brazil, Iran, Saudi Arabia, and the United Arab Emirates).

Establishment and maintenance of an UCB SCB is financially intensive with costs including tissue typing, infectious disease testing, and also the annual cost of cryopreservation. However, these costs have in no way reduced the growth of UCB SCBs as there are over 150 public and 200 private UCB SCBs worldwide; however, the majority of these are found in the developed countries.

Hemoglobinopathies are inherited disorders which result in life-threatening noncommunicable diseases in children. The most common of these are β-thalassemias and sickle cell disease which are often associated with many of the developing countries of the world such as sub-Saharan Africa, the Indian subcontinent, Bangladesh, Myanmar, and Southeast Asia (Weatherall 2010; Faulkner et al. 2013).

HSCT is the only recognized cure for thalassemia and sickle cell anemia and is increasingly becoming more cost-effective as the cost of a transplant is comparable to a few years of supportive care for these individuals (Leelahavarong et al. 2010). Although unrelated HSCT has been used successfully, most patients with these hemoglobinopathies belong to ethnic groups that are underrepresented in donor registries. It is therefore unlikely that these individuals will be able to find a suitable donor and often cannot proceed with the transplant (Faulkner et al. 2013).

Information reported to date regarding UCB banking in developing countries is limited. However, the establishment of not-for-profit public UCB SCBs in these countries would service a large unmet need in increasing patients' chances of finding suitable donors as well as supplying a source of stem cells for applications in regenerative medicine that could potentially be used toward improving health in these countries.

Developing countries often have to prioritize providing basic healthcare needs to their populations while also addressing epidemic rates of communicable and non-communicable diseases and other health issues. Although there is an increase in the need for HSC transplants, most developing countries have a limited number of transplantation centers which also limit the use of UCB units in these countries. This shortage of transplantation centers needs to be addressed in parallel with the establishment of UCB banks. The cost of UCB unit processing can escalate in countries where there is an increased burden of microbial and viral infections. Evidence of microbial infection or positive serological tests prevents a UCB unit from being eligible for storage. Despite these costs, it remains critical that UCB SCBs meet global accreditation or quality standards as outlined by international organizations such as NetCord-FACT and AABB to ensure high and uniform quality of all UCB units available to patients requiring HSCT.

Although public UCB SCBs generally find it challenging to maintain financial viability, some developing countries have successfully managed to fund the operation of UCB SCBs with help from university-affiliated medical centers, charitable institutes, regional governments, or national support as well as revenue from exporting UCB units to transplant centers (Roh et al. 2014). The establishment of UCB SCBs should be supported in developing countries as they would service a large unmet need in these countries as well as the corresponding diaspora.

8.1.4 Cord Blood Stem Cells in Transplantation and Regenerative Medicine

HSCT is a globally accepted form of therapy for the treatment of malignant and nonmalignant hematological conditions. These therapies generally aim to reconstitute the hematopoietic system in patients who have undergone chemotherapy. Despite its benefits, the use of UCB is mostly used as a last resort when no HLA-matched bone marrow donors are available. Having said this, over 35,000 UCB

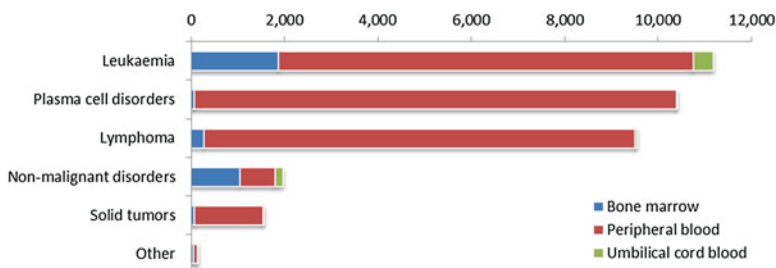

Fig. 8.2 Number of patients receiving hematopoietic stem cell transplantations in Europe in 2013

transplants have been performed to date, and there are over 700,000 and four million units stored globally in public and private UCB SCBs, respectively.

The most recent report by the European Society for Blood and Bone Marrow Transplantation (EBMT) indicates that nearly 35,000 patients received HSCTs (bone marrow, PBSC, and UCB) in European and affiliated centers in 2013 (Passweg et al. 2015), which is approximately half of all HSCTs performed globally (Niederwieser and Baldomero 2014). The hematological malignancies continue to be the most frequently treated indications, accounting for 90 % of all HSCTs (Fig. 8.2).

Figure 8.2 further indicates the source of HSCs used for each patient treated, which reveals that the minority were collection from UCB (2 %, $n=673$). Additionally, these UCB-derived HSCs were all predominantly used to treat patients with leukemia—mostly acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). The nonmalignant conditions treated mostly with UCB transplantations are primary immune disorders, inherited disorders of metabolism, and severe aplastic anemia. Importantly, of the 737 UCB units transplanted in 2013, 90 % were from unrelated allogeneic donors, while the remainder were either from HLA-identical or nonidentical family members (also allogeneic, $n=69$) and autologous banked units ($n=2$).

Given the low volume and hence limited cell dose obtained from an UCB unit, the use of these cells is generally limited to the pediatric setting. Adults are indeed treated with UCB units, but it is often the case that a second or third unit is required, which is cost prohibitive in most cases. However, with the increase in use of haploidentical donors for both pediatric and adult indications, there has been a notable decrease in the use of UCB transplantations over the last 2–3 years (Passweg et al. 2015). The clinical benefit of using haploidentical units over UCB is, however, still to be demonstrated.

Recently, there has been an increase in the use of UCB units for the treatment of a variety of indications that are of non-hematopoietic origin and regenerative in nature. The utility extends beyond using a traditional preparation of mononuclear cells derived from UCB and further includes the use of ex vivo expanded MSCs from either UCB or Wharton's jelly/umbilical cord (UC). Given the current limitation of UCB related to cell dose, the option to expand MSCs from UCB/UC is both feasible and an attractive solution for UCB SCBs. It is well recognized however that the spectrum of diseases that can be treated using these two sources of stem cells is quite different and, in the case of MSCs, still needs to be established from clinical trials.

In light of this, a list of currently registered non-hematopoietic- and regenerative-type clinical studies is provided in Table 8.2 to illustrate the scope of alternative indications being explored. The table is further structured to illustrate indication grouping and the cell therapy used. According to this registry (derived from ClinicalTrials.gov), 91 clinical trials have been registered to date, of which more than half make use of UC-derived MSCs (UC-MSCs). Forty different indications have been targeted for treatment, which can be grouped into over 15 different specialties. The broad range of conditions include, among others, cardiomyopathy, muscular dystrophy, spinal cord injury, autism, liver cirrhosis, and HIV/AIDS.

Table 8.2 Number of registered clinical studies using UCB and UC-derived cells for non-hematopoietic and regenerative medicine indications (Abdullah 2011; Ballen et al. 2008; Butler and Menitove 2011; Guilcher et al. 2014; Sugarman et al. 1997; Wagner et al. 2013)

Indication grouping	Indication	UCB	UC-MSC	UCB-MSC	Total
Autoimmune	Autoimmune hepatitis	–	1	–	1
	Epidermolysis bullosa	3	–	–	3
	Lupus erythematosus	1	2	–	3
Cardiovascular	Cardiomyopathy	–	2	1	3
	Hypoplastic left heart syndrome	1	–	–	1
	Myocardial infarction	–	1	–	1
Circulatory	Critical limb ischemia	2	1	–	3
	Ischemic stroke	2	–	–	2
Dermatological	Wounds (burn)	–	1	–	1
Endocrine	Diabetes—type 1	3	3	–	6
	Diabetes—type 2	–	2	–	2
Gastrointestinal	Ulcerative colitis	–	1	–	1
Hematological	Aplastic anemia	–	1	–	1
Hepatic	Liver cirrhosis	–	9	–	9
	Liver failure	–	2	–	2
Immunological	Graft-versus-host disease	–	2	1	3
	Organ transplantation (kidney)	–	1	–	1
Infectious diseases	HIV/AIDS	–	1	–	1
Musculoskeletal	Duchenne muscular dystrophy	–	1	–	1
	Osteoporosis	3	–	–	3
	Cartilage defects/injuries	–	–	1	1
Neurological	Alzheimer's disease	–	1	1	2
	Amyotrophic lateral sclerosis	–	1	–	1
	Ataxia	–	2	–	2
	Autism	2	2	–	3
	Cerebral palsy	9	1	–	10
	Global developmental delay	1	–	–	1
	Hypoxic-ischemic encephalopathy	3	1	–	4
	Multiple sclerosis/neuromyelitis optica	–	1	–	1
	Spinal cord injury	2	2	–	4
	Traumatic brain injury	1	–	–	1
Oncology	Myelodysplastic syndrome	–	1	–	1
	Leukemia	–	–	1	1
Reproductive	Premature ovarian failure	–	1	–	1
Respiratory	Bronchopulmonary dysplasia	–	1	2	3
Rheumatic	Ankylosing spondylitis	–	1	–	1
	Osteoarthritis	–	–	1	1
	Rheumatoid arthritis	–	2	–	2
Other	Hearing loss	1	–	–	1
	Preterm neonates	1	–	–	1
TOTAL		35	48	8	91

UCB umbilical cord blood, refers to the number of studies using a traditional mononuclear cell preparation, *UC-MSC* umbilical cord-derived mesenchymal stem cells, *UCB-MSC* umbilical cord blood-derived mesenchymal stem cells

The treatment of neurological diseases is by far the most active area of research (Iafolla et al. 2014). The rationale for this interest stems from the fact that UCB is known to contain a unique combination of stem and progenitor cells, including MSCs (Kang et al. 2006), embryonic-like stem cells (Zhao et al. 2006), endothelial progenitor cells (Hildbrand et al. 2004), and unrestricted somatic stem cells (Kogler et al. 2004). Additionally, the beneficial effects of these cells have been demonstrated in the preclinical setting, which indicate enhanced tissue repair and cognitive improvement (Geissler et al. 2011), as well as a stimulation of neural stem cell production (Wang et al. 2012).

Cerebral palsy and hypoxic-ischemic encephalopathy (HIE) are the indications being explored most, for which 12 clinical trials making use of a traditional UCB preparation (red cell depleted, mononuclear cells) have been registered to date. Of these studies, nine are still active and/or currently recruiting, with three having been completed. Important to note is that of the 12 registered studies, six make use of autologous therapies and hence make a case for privately banked UCB units. Sun et al. (2010) reported on the safety of using autologous UCB units in 184 children with neurological disorders (140 with cerebral palsy) and found that 1.5 % experienced hypersensitivity reactions during the autologous UCB infusion. Furthermore, no additional adverse events have been reported in these patients in 3 years of follow-up, indicating a favorable safety profile. The authors indicated that the quality of UCB units recalled from private UCB SCBs was inferior to the publicly banked units that were accessed—a situation that would need to be improved if autologous UCB therapies are to become a reality. In a separate study on children with cerebral palsy, significantly improved cognitive and motor function was reported (vs. a control group) when UCB and erythropoietin were administered (Min et al. 2013). With regard to HIE, a recent report of a Phase I study demonstrated safety of autologous UCB infusion in critically ill neonates, as well as positive preliminary data with regard to functional improvements and survival. Data from each of these early phase studies are promising, and sufficient evidence of safety is provided. The next steps are thus to further demonstrate efficacy in larger Phase II and III studies before these therapies are to become accessible in routine practice.

Promising findings have also been reported in studies on liver cirrhosis (Zhang et al. 2012; Xue et al. 2015). In contrast to the treatment of cerebral palsy and HIE, the experimental therapies recorded for liver cirrhosis make use of UC-MSCs exclusively. Large-scale pivotal studies are similarly required to demonstrate evidence of benefit. Contrary to the positive reports discussed above, little to no benefit from the use of UCB in type 1 diabetes has been reported. This was the case in two independent studies, both of which made use of autologous UCB transplantation in pediatric patients with type 1 diabetes (Haller et al. 2011; Giannopoulou et al. 2014).

There is no doubt that there will be a continued interest and investment in this area of research, which may result in approved UC and UCB-derived cellular therapies for non-hematopoietic and regenerative purposes. A further broadening of the scope of treatment is also anticipated, particularly given the potential clinical benefits of ex vivo expanded MSCs. However, if these experimental therapies are ever to become part of routine clinical practice, careful study design

based on rational principles will be essential. Notably, each and every indication and cell therapy will require specific consideration with regard to the cell source, preparation conditions, cell dose, and route of administration. Given the rise of haploidentical transplantation practices and in light of the fact that there are nearly five million UCB units stored globally (public and private combined), the industry and its stakeholders are watching this space with great anticipation.

8.2 Summary

The characteristics of UCB make it a suitable alternative to bone marrow and peripheral blood-derived stem cells for cell-based therapies. UCB is harvested at birth and stored in public, private, or hybrid facilities for future use. There are a number of unresolved ethical debates regarding the storage options of UCB, mainly due to the extremely low probability of the cells being retrieved for use from private banks. The majority of published sources have recommended that storage should primarily take place in a public UCB SCB, with the exception of a directed donation.

Most of the UCB SCBs worldwide are in developed countries due to the substantial costs involved in establishing and maintaining such facilities. Despite the costs, developing countries could benefit from establishing UCB SCBs as they could service an unmet need for donor-recipient matched units both in local populations and in the diaspora. With the ever-increasing number of clinical trials aimed at using UC and UCB-derived cellular therapies for non-hematopoietic and regenerative medicine, the need for readily available UCB units is likely to increase globally.

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Chapter 9
Human Embryonic Stem Cells and Associated Clinical Concerns

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9.1 Introduction

Stem cells are undifferentiated cells with the capacity to self-renew. Various different types of stem cells have been recognised depending on where in the body or what stage of development they come from (see Table 9.1). When exposed to the right signals under the right conditions, they can differentiate into any desired cell type found in the body such as heart, muscle and skin cells (Department of Health and Human Services 2001). With the potential to produce mature specialised cells with specific functions, stem cells are of great interest for stem cell-based therapy. Using stem cells, multiple therapeutically useful cell types can be made and then used to treat numerous diseases including cancer, Parkinson's, Alzheimer's, diabetes and heart failure (Mimeault et al. 2007).

With their unique characteristics, stem cells can be used in cell therapy and drug discovery and to expand our knowledge of the human body. Cell therapy makes use of stem cells, or cells grown from stem cells, to regenerate damaged tissue. As an alternative, researchers are now looking for drugs which can stimulate the body's own stem cells to repair an injury rather than laboriously growing cells in vitro and transplanting them. It is thought that many of the body's tissues contain stem cells capable of dividing to make new tissue, but this does not happen naturally and the cells need to be prompted (Pearson 2006). Alongside cell therapy, the use of stem cells can transform drug discovery and validity. Accurate human disease models, representative of diseases, can be made and used to fully understand underlying mechanisms. Moreover, disease-specific ES cells obtained from patients can be made to differentiate into various cell types affected in the disease; these can then

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Table 9.1 Different types of stem cells. Summary of different types of stem cells found in the body and their potential use

Type of stem cells	Where do they come from?	What can they do?
Embryonic stem cells (ES cells)	Inner cell mass of a blastocyst (early-stage embryo)	Differentiate to form all the different cell types found in the body
Tissue stem cells (TS cells)	Adult body tissues (e.g. skin, blood)	Form only the cells found in their tissue type, e.g. skin stem cells will only differentiate to form types of skin cells and not blood cells
Mesenchymal stem cells (MS cells)	Bone marrow	Form cells of the skeletal tissues (bone, cartilage and fat) Assist blood stem cells to make new blood cells
Umbilical cord blood stem cells	Umbilical cord after childbirth	Form different cell types found in the blood
Induced pluripotent stem cells (iPS cells)	Made in the lab from differentiated adult cells, e.g. skin cells	Differentiate to form all the different cell types found in the body (similar to ESCs)

be used to carry out more accurate drug discovery and toxicity studies on human models (Rubin 2008). Stem cells help to overcome the limited availability of patients and diseased tissues for study. Researchers and doctors are also using stem cells as human models to augment their understanding of how the body works and further why diseases and conditions develop.

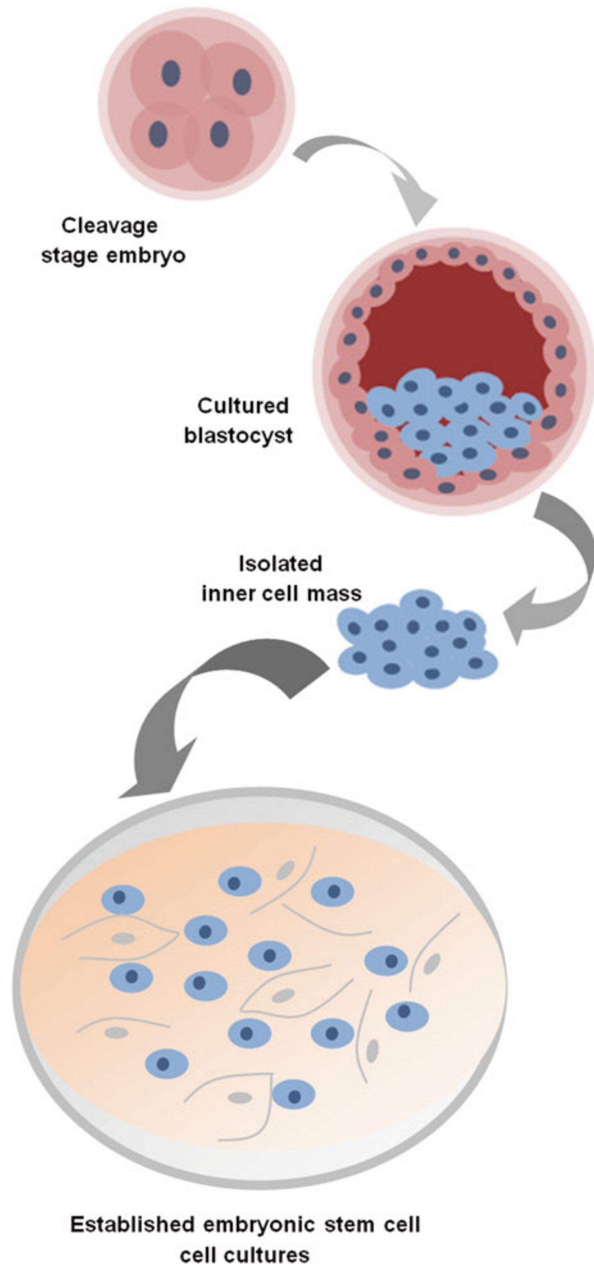
Embryonic stem (ES) cells are one of the most discussed biomedical issues of the day. These pluripotent cells have tremendous clinical potential in tissue repair and represent potential to cure many common diseases; transplantation of human ES cell-derived equivalents to damaged tissues in patients can restore normal function. However, the use of human ES cells raises many ethical concerns as they are derived from human preimplantation embryos which are then destroyed (see Sect. 9.3.1). Further, understanding the diversity amongst human ES cell lines and overcoming associated technical constraints, such as successfully isolating lines with robust differentiation potential, stand in the way of their full potential being realised.

9.2 Human ES Cells

9.2.1 Sources

ES cells are cells derived from the inner cell mass (ICM) of the blastocyst (see Fig. 9.1) which develops within 5 days of fertilisation of the oocyte (Thomson et al. 1998). They are capable of unlimited and undifferentiated proliferation in vitro as

Fig. 9.1 Generating ES cell lines from human blastocysts



they have increased expression of a gene called telomerase. High levels of telomerase maintain the telomere ends of the chromosomes after every cell division giving these cells the ability to divide indefinitely (Thomson et al. 1998). Even though ES cells possess the proven ability to form derivatives of all three germ layers, both

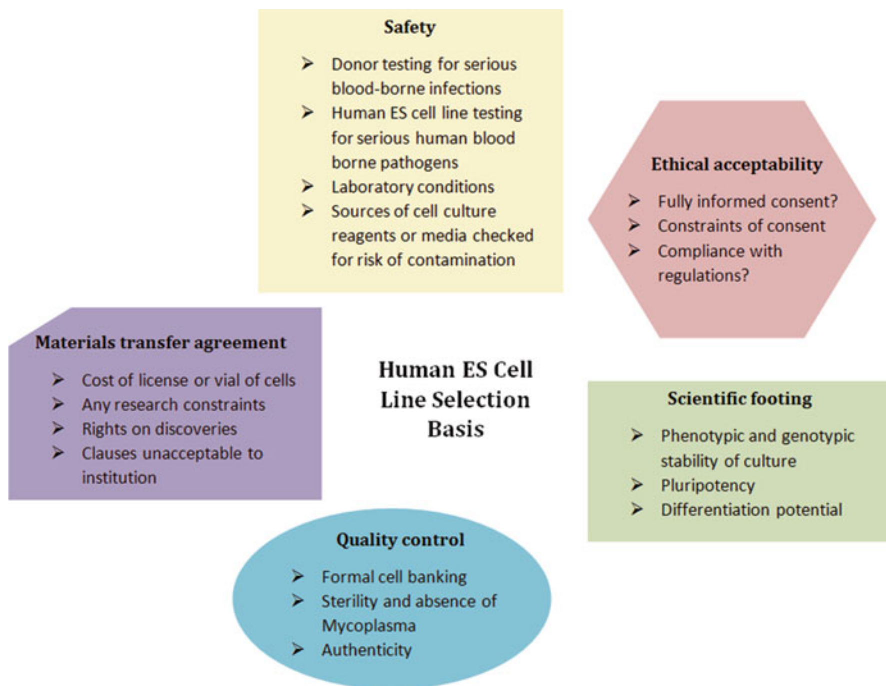


Fig. 9.2 Selection criteria followed when obtaining human ES cell lines

in vitro and in vivo (Chiu and Rao 2003), they cannot give rise to extraembryonic tissues such as the placenta and membranes necessary for complete development and so cannot form a whole new individual (Wert and Mummery 2003).

In most cases to date, spare IVF embryos have been used for ES cell isolation, although in some occasions IVF embryos were specially created for this purpose (Lanzendorf et al. 2001). Embryo quality is a major factor considered in the derivation of human ES cell lines (Turksen 2012). The best source would be from embryos created via nuclear transfer, also known as therapeutic cloning (see Sect. 9.4.1), especially to obtain ES cells. As summarised in Fig. 9.2, a strict regime is followed when selecting human ES cell lines for clinical application. Failure to apply the appropriate selection procedure could have many consequences, some of which include negative publicity of stem cell research, contamination of human ES cells, considerable waste of time and resources, loss of rights on discoveries and withdrawal of publications.

9.2.2 Isolation

Initially, most of the ES cells in humans were obtained by the isolation of the ICM from the trophectodermal (TE) cells of the blastocyst using immunosurgery, a two-step cytotoxicity procedure for selective killing of TE cells by pre-incubation with

antiserum followed by separate exposure to complement (Solter and Knowles 1975). However, immunosurgery uses substances, like antihuman antiserum and guinea pig complement, that are animal based and may carry pathogens and/or molecules that can contaminate the ICM and thus the ES cells derived from them (Turetsky et al. 2008). There is also the risk of damaging ICM cells, thus decreasing the chances of deriving new ES cell lines from these cells (Turksen 2012). Therefore, to avoid immunosurgery, alternative isolation methods, mainly mechanical or chemical dissolution of the TE layer, were developed, but still problems remained. Firstly, mechanical dissection is a crude method relying heavily on the operator's technical skills. Secondly, the use of acid tyrode for chemical dissolution of the TE layer can lead to acidification of the medium resulting in damage to the cell of the ICM (Turetsky et al. 2008). Thus, a better approach is to use a non-contact laser, both for dissection of the ICM and assisted hatching, facilitating isolation without the use of animal products or causing damage to cells (Turetsky et al. 2008). In addition, a large fraction of human ES cell lines have also been established from whole plated embryos on feeder cells; in some cases good-quality embryos are used, but mostly poor-quality embryos lacking a clear ICM are used. However, this method can lead to the development of TE stem cell lines as TE cells proliferate much faster than the cells of ICM and can suppress their growth (Turksen 2012). The best method to isolate ICM from the blastocyst, in order to generate ES cell lines, has not yet been consolidated as shown in Fig. 9.3. Although, over the years, new methods have been developed, all methods continue to be used. The method used for isolation depends on the availability of laser and micromanipulation equipment along with the morphology and quality of the embryo.

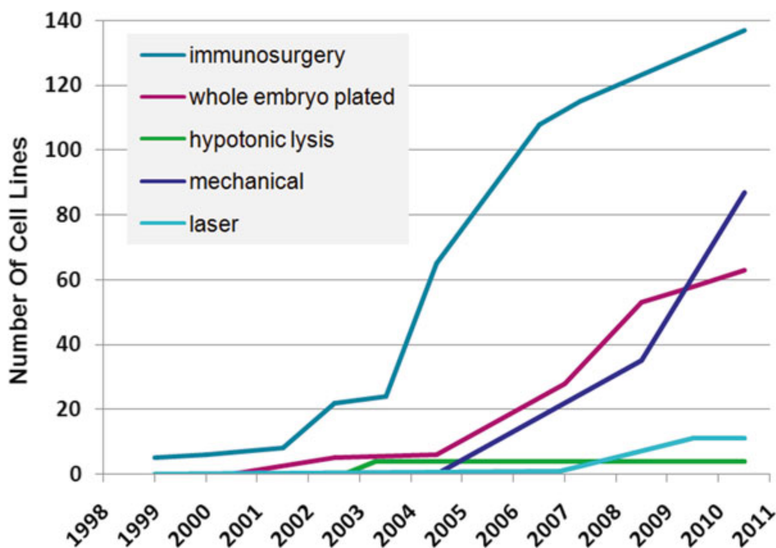


Fig. 9.3 Number of ES cell lines derived over the years, based on the method employed for extraction

9.2.3 Culture

Traditionally, human ES cells were cultured on mouse embryonic fibroblast (MEF) feeder layers, which maintain continuous proliferation in an undifferentiated state. The growth of ES cells and MEF together needs meticulous care and is often expensive (Amit et al. 2003). The dual growth of these cells induces risks of pathogen transfer and viral infection to ES cells. Moreover, the preparation of these feeder layers is labour intensive, and variations between different batches of fibroblasts make the experiments complex and difficult to define (Amit et al. 2000). For such reasons human ES cells grown on animal feeder layers are not suitable for application in humans (Lee et al. 2004).

One of the initial developments made to the original culture methods came about with the growth of human ES cells under serum-free conditions, using basic fibroblast growth factor (bFGF) instead of serum (Amit et al. 2003). Along with compounds beneficial to human ES cells, serum contains harmful compounds detrimental to stem cell survival as well, suggested by constantly low cloning efficiency in the presence of serum in the medium. The use of serum induces further variability to experiments as each serum batch shows different capability to support vigorous undifferentiated proliferation of ES cells. It is unknown though whether the effects of bFGF on undifferentiated human ES cell growth in such culture conditions are mediated through the fibroblasts, the ES cells or both (Amit et al. 2000). Serum replacement with defined components should reduce variability of experiments and permit more carefully defined differentiation studies (Amit et al. 2000). Further, identifying the factors produced by MEF cells responsible for promoting human ES cell renewal can be used to culture ES cells without the use of animal products.

Further improvement was made when Xu et al. (2001) were able to culture human ES cells under serum- and/or feeder-free culture conditions. In such feeder-free conditions, a cell adhesion coating supportive of the proliferation and maintenance of pluripotency of ES cells is necessary. They began to culture human ES cells on Matrigel or laminin in the medium conditioned by MEF (MEF-CM); these ES cells grown in feeder-free conditions were reported to have maintained a normal karyotype, stable proliferation rate and high telomerase activity (Xu et al. 2001). However, the growth of MEF for the production of the conditioned medium (MEF-CM) was still required, and Matrigel is also an animal product (Lee et al. 2004).

These xenosupport systems are associated with the risk of transferring animal pathogens from the animal feeder, matrix or conditioned medium to the human ES cells making them clinically inapplicable. Thus, a culture system based on a human feeder layer was developed. Richards et al. (2002) reported that 'human foetal and adult fibroblast feeders support prolonged undifferentiated human ES cell growth of existing cell lines' (Richards et al. 2002). These ES cells cultured with human feeder layers in the medium supplemented with human serum showed features similar to cells grown on MEF, including pluripotency, morphology and expression of cell-surface markers. Amit et al. (2003) were able to culture ES cells on foreskin

feeders and a serum-free medium, defining a totally animal-free culture system (Amit et al. 2003). Further, Lee et al. (2004) showed that human adult uterine endometrial cells, adult breast parenchymal cells and embryonic fibroblasts can be used as feeder cells for the growth of human ES cells (Lee et al. 2004). By comparing different types of feeders, more effective human feeder cells should be selected based on the expression of various factors such as extracellular matrices, growth factors and cytokines that induce proliferation and inhibit differentiation of human ES cells. These selective human feeder cells will aid the progress of cell-based therapies. Several chemically defined media systems have been reported since then for the maintenance and proliferation of pluripotent stem cells to try and overcome culture-induced variability.

Subsequently, feeder-independent human ES cell cultures were developed comprising of protein components derived exclusively from recombinant sources or purified from human material (Ludwig et al. 2006). Braam et al. (2008) demonstrated that human ES cells express integrin receptors for laminin, fibronectin, collagen and vitronectin which are all functional in facilitating adhesion. They found recombinant vitronectin as a suitable and functional alternative to Matrigel as it supported human ES cell growth just as well (Braam et al. 2008). More recently, Baxter et al. (2009) derived a feeder-/serum-free culture system containing fibroblast growth factor 2 (FGF2), activin A, neurotrophin 4 (NT4) and the N2 and B27 supplements along with a human fibronectin substrate. This culture system was reported to support the long-term proliferation of several euploid human ES cells without the need for manual propagation and showing little or no spontaneous differentiation. These components were explained to have discrete functions: both FGF2 and activin A were necessary to maintain ES cells in an undifferentiated state while NT4 to promote cell survival and the fibronectin substrate to sustain a rapid rate of ES cell culture expansion (Baxter et al. 2009). The cell-substrate interface provided by the adsorbed fibronectin that promotes human ES cell interactions is quite specific. This can be used to develop the therapeutic potential of human ES cells if the fibronectin layer could be characterised such that it could be reproduced synthetically (Kalaskar et al. 2013). Figure 9.4 shows the development of culture since the first human ES cells were cultured.

9.2.4 Differentiation

When removed from feeder layers and transferred to suspension culture, ES cells begin to differentiate and form embryoid bodies (EB) which are multicellular groups of differentiated and undifferentiated cells. In vitro differentiation is consistently disorganised and often variable between different EBs even within the same culture (Odorico et al. 2001). Initially, as human ES cell lines were not clonally derived, pluripotency was only demonstrated by populations of cells and not individual cells, so within a colony there were subpopulations of cells, and no individual cell showed the ability of differentiating into cells of all three germ layers

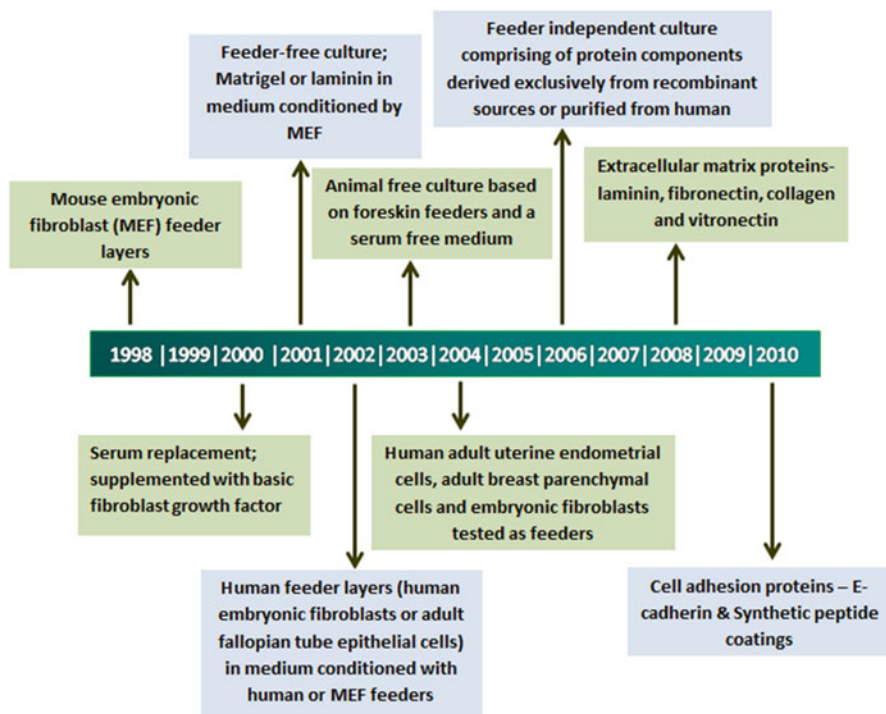


Fig. 9.4 Timeline of the development of human ES cell culture over the years

(Odorico et al. 2001). Later, clonally derived human ES cells were produced, as reported by Amit et al. (2000), that demonstrate pluripotency, maintained over an extended period of culture, at the level of a single cell that can self-renew for long periods of time (Amit et al. 2000).

Schuldiner et al. (2000) showed that both undifferentiated ES cells and EBs express receptors for different growth factors which effect the differentiation into cells with different epithelial or mesenchymal morphologies (Schuldiner et al. 2000). The overall effects of these factors were described in three categories: (1) growth factors mainly responsible for inducing mesodermal cells, (2) factors that activate ectodermal and mesodermal markers and (3) factors that direct differentiation into all three embryonic germ layers, including endoderm. However, none of these growth factors were found to direct differentiation solely to one cell type. For the success of ES cell-based therapy in treating human diseases, we must be able to direct human ES cells differentiation towards a particular cell type of interest and be able to obtain this lineage from the mixed population (Amit et al. 2000). This remains a challenge as hardly ever have specific growth factors or culture conditions resulted in cultures containing a single-cell type, and significant culture-to-culture variability remains even when identical growth factor and conditions are maintained (Amit et al. 2000). Various human cell types may be developed in vitro by using

specific factors, but this requires better understanding of the events regulating cell lineage commitment and differentiation. Even though in vitro differentiation can generate multiple cell types, in vivo differentiation, such as following injection into a host blastocyst, demonstrates the full developmental potential of undifferentiated ES cell lines (Amit et al. 2000). In vivo many of the normal features of tissue architecture are replicated, for example, cell epithelium exhibits polarity, is enveloped by a basement membrane and is surrounded by mesenchyme (Amit et al. 2000).

9.3 Challenges Faced

9.3.1 Ethical Dilemma

The use of human ES cells raises serious ethical, religious and political controversies. The main ethical issue concerns the derivation of ES cells from embryos which are then destroyed. Controversies arise due to varying views on the personhood of an embryo. For those who believe that life begins at conception, destroying a human embryo is wrong, while others argue that even though an embryo is potential for life, it cannot be regarded as a person, and it is more unethical to stop research (Buxton 2009). An intermediate position on the status accorded to the embryo attributes a special status to the embryo that is less than human life but deserving of respect that imposes limits on its ethical use (Chiu and Rao 2003). A second objection raised is that it is wrong to create blastocysts for research purposes, whether through fertilisation or nuclear transfer, with the intention of destroying it. Some fear that the use of nuclear transfer to derive human ES cells could eventually lead to its use to produce a child. Further concerns are shown with regard to research involving donor oocytes, which could result in the exploitation of women. In addition, some people are worried about the mixing of human and nonhuman cells for research purposes (Medicine and Council 2005). Other methods of obtaining stem cells such as induced pluripotent stem (iPS) cells avoid ethical problems specific to embryonic stem cells raising fewer concerns. However, as with any human stem cell research, there are difficult dilemmas, like consent to donate materials for research, early clinical trials of therapies and oversight of research (Lo and Parham 2009).

9.3.2 Technical Challenges

Therapeutic use of ES cells is yet to become a successful reality for treating diseases owing to the technical challenges that come with it. If these challenges are overcome, stem cell therapy can revolutionise medicine. However, results have shown that the approaches proposed so far do not permit clinically safe, reliable and cost-effective procedures (Di Nardo et al. 2012).

Even after successful isolation and expansion, the major obstacle of differentiation into the specific cell type remains, and once transplanted these cells must effectively integrate with the patient's body systems and function together with other cells. Following transplantation cell survival and the correct desired function must be ensured (Buxton 2009). Most importantly, serious side effects such as cancer or infection must be prevented. Since ES cells can proliferate indefinitely, they could result in both quantity and compatibility issues (Brunt et al. 2012). The challenge is to strike a balance between directing cell growth and differentiation into specialised tissues that can replace damaged ones and ensuring that cells do not excessively grow becoming cancerous. Following transplantation, the issue of tissue compatibility still remains one of the biggest challenges, and recipients usually have to take strong immunosuppressive drugs to minimise the risk of rejection; however, these drugs make the patient vulnerable to any other infections.

Another issue with ES cell-based therapies is timing: when to transplant these cells into the patient? Stem cells go through many intermediate stages before they become fully specialised; deciding when to transplant these cells remains an open question, and the answer is different for different diseases (Bor 2004).

Good Manufacturing Practice (GMP) guidelines are set up to ensure that the manufacturing of medicinal products is under quality standards; these must be employed in order to produce clinical grade cells with defined quality and safe usability in patients. Unlike a conventional pill, ES cell-based therapies involve living cells and so cannot be standardised easily. Before ES cells can be used in patients, firstly several components of the culture need to be developed according to GMP standards. As explained by Unger et al. (2008), 'the feeder cells or the culture matrix, all the components of the culture and cryopreservation media, and all the processes involved' have to be described, validated and standardised according to the GMP quality system. GMP adaptation for differentiation to other different cell types might prove to be even more difficult. Re-evaluation of GMP-verified human ES cell culture conditions must be carried out as these ES cell-derived cells are used to treat humans, so all safety precautions must be taken (Unger et al. 2008). As shown in Fig. 9.5, controlling, monitoring and achieving these vital standards is a complex and expensive process and remains a major constraint.

Human ES cell research should be conducted in accordance with all applicable laws and guidelines relating to recombinant DNA research and animal care. The federal government sets up protocols regarding the management of laboratories where products that might ultimately be used in humans (as in a clinical trial) are being developed. FDA's Good Laboratory Practice (GLP) regulations establish principles for nonclinical laboratory studies which include in vitro or in vivo experiments, determining the test articles, an activity that would be necessary in the preclinical phase of human ES cell research. Failure to abide by GLP regulations would leave human ES cells less useful in the future if they were considered for clinical trials of tissue transplantation or other cell-based therapies (Medicine and Council 2005).

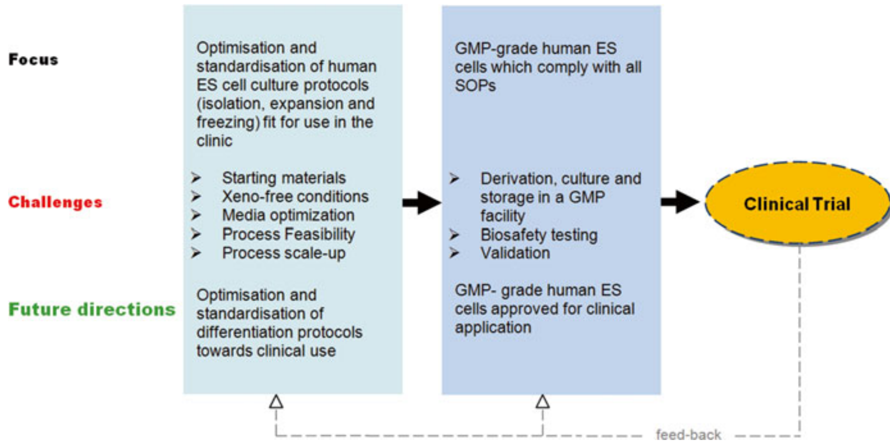


Fig. 9.5 Overview of current optimization possibilities and GMP processing as well as future developments that need to be made before ES cell can enter clinical trials

9.4 Overcoming Challenges

9.4.1 Somatic Cell Nuclear Transfer

The two major challenges faced with the use of pluripotent human ES cells include immunorejection by the recipient and the ethical concerns regarding destruction of human embryos. The best approach to overcome these drawbacks is to reprogramme a fully differentiated somatic cell to a level of pluripotency suitable for clinical use. To generate pluripotent ES cells, adult stem cells can be obtained from the patient by biopsy; the best source of cells in humans is still not known and can be reprogrammed to form an early embryo (Lovell-Badge 2002). This is done by somatic cell nuclear transfer where the somatic cell nucleus is injected into an unfertilised enucleated oocyte (see Fig. 9.6). Cytoplasmic factors present in mature metaphase II-arrested oocytes have a unique ability to reprogramme the transplanted somatic cell nuclei to an embryonic state (Tachibana et al. 2013). This would be cultured in vitro to the blastocyst stage and the cells from the inner mass used to derive ES cells. Since these techniques use unfertilised eggs, these do not present the same ethical concerns regarding destruction of human embryos. Making patient-specific cells and transplanting them back in the patient should overcome problems of immune rejection (Guha et al. 2013) as the nuclear genomes of the resulting human ES cells would be identical to those of the donors of the somatic cells and thus are purported to be the optimal medical use of human ES therapy (Medicine and Council 2005).

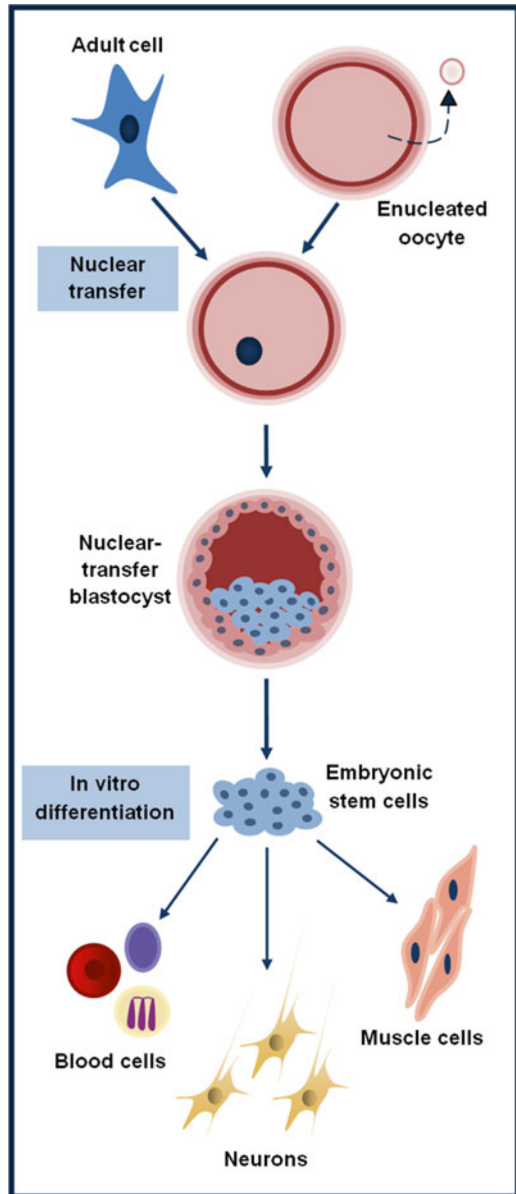


Fig. 9.6 Somatic cell nuclear transfer used to generate a line of ES cells that can potentially differentiate in vitro into any type of cell for therapeutic purposes

9.4.2 Induced Pluripotent Stem (iPS) Cells

Further, pluripotent cells exhibiting similar functionality to ES cells can be directly generated from adult cells. The reprogrammed cells, called induced pluripotent stem (iPS) cells, were first established in 2006 by Takahashi and Yamanaka; they

successfully generated ES-like cell lines from mouse embryonic fibroblasts and skin fibroblasts by simply expressing four transcription factor genes encoding Oct4, Sox2, Klf4 and c-Myc. These iPS cell lines showed similar morphology and growth properties as ES cells and express ES cell marker genes (Takahashi and Yamanaka 2006). In the same way, human iPS cells which resemble human ES cells have been generated (Lee et al. 2012). Generation of patient-specific iPS cells from somatic cells from any individual largely solves the two major obstacles mentioned earlier. However, the efficiency of generation of iPS cells is still a limiting factor in therapy due to the use of genetic manipulations for delivery of reprogramming factors, low efficiency of this process, slow kinetics of direct reprogramming and potential tumorous growth; this may be improved by replacement of genetic reprogramming factors with small molecules or other factors (Nakhaei-Rad et al. 2012).

9.4.3 Immune Isolation

Problems caused by limited donor supplies and permanent immunosuppressant use can be solved by using immune-isolation technology such as encapsulated islets (see Fig. 9.7) for treating severe diabetes as shown by Sakata et al. (2012). With the use of encapsulated islets or bioartificial pancreas, transplanted islets can be protected from the immune system by coating with semipermeable membranes composed of high polymer. Encapsulating semipermeable membranes shields the inner islets from the recipient's immune system along with mechanical stress while allowing diffusion of glucose, oxygen, nutrients, hormone and wastes. Islets are taken from the donor pancreas, obtained from heart- or brain-dead donors or from living donors (for islet autotransplantation), by islet isolation. Islets are isolated by digestion of the pancreas, preserved in cold preservation solution and then purified. The materials used for encapsulation must have two key properties: firstly, they must provide protection from the recipient's immune system; secondly, they must permit bidirectional diffusion of small molecules. Some suitable materials for encapsulation include alginate, polysulfone and polyvinyl alcohol. In conclusion, encapsulated islets could enable successful allogenic or xenotransplantation with large animals, such as pigs, overcoming limited donor supplies for islet transplantation without the need for chronic immunosuppressant administration, thus preventing adverse side effects induced by immunosuppressants (Sakata et al. 2012).

9.4.4 Bioactive Scaffold and Lentivirus

Developing tissue constructs from ES cells with matrix composition and biochemical properties fit for tissue replacement requires extensive in vitro manipulation involving large amounts of growth factor proteins which is expensive and unstable. The need for in vitro culture is eliminated if cell differentiation can be directed in vivo in the absence of exogenous growth factors. In a study carried out by Brunger

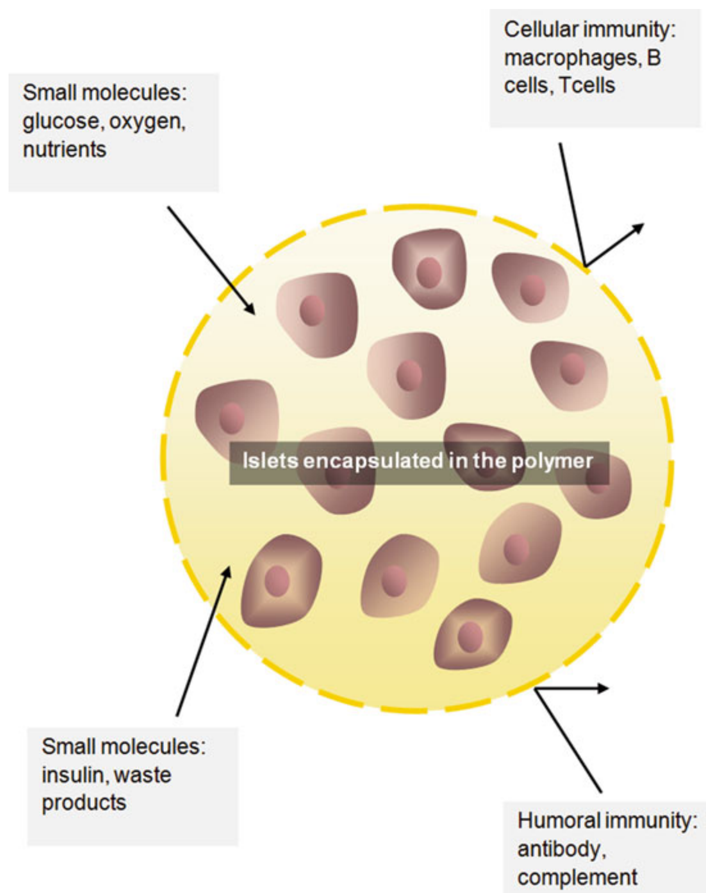


Fig. 9.7 Schematic representation of the mechanism for encapsulated islets

et al. (2014), a bioactive scaffold capable of mediating cell differentiation and formation of an extracellular matrix that mimics the mechanical properties of native tissues was developed for replacement of musculoskeletal tissue. *Lentiviruses* (LV) were used to deliver gene therapy to the stem cells, directing their differentiation, into a synthetic material, the scaffold, which serves as a template for tissue growth. LV drove overexpression of TGF-β3-inducing chondrogenesis, and poly-L-lysine (PLL) was used to functionalise polycaprolactone with LV, as PLL-coated biomaterials effectively immobilise viruses within and enable efficient transduction of cells. The results obtained demonstrate that scaffold-mediated transduction of human mesenchymal stem cells using LV vectors, causing expression of TGF-β3, leads to chondrocyte differentiation and formation of a cartilage like extracellular matrix. Using such an approach solves the challenges faced in expansion and conditioning of cell ex vivo by allowing the host's own cells to recapitulate functional tissues after penetrating the bioactive scaffold (Brunger et al. 2014).

9.4.5 Pro-survival Molecules

Work with ES cells is limited as they are very difficult to culture and usually die when stem cells are stripped off their cell colony; these result in problems associated with rapid expansion and genetic manipulation of human ES cells. Xu et al. (2010) identified two molecules, Thiazovivin and Pyrintegrin, through high-throughput chemical screening, which enhance ES cell survival more than 30-fold. Thiazovivin and Pyrintegrin were seen to have a dramatic impact on cell attachment even within just a few hours. However, they hardly effect cell proliferation, suggesting that the survival-promoting effect may be mainly due to increased cell adhesion following cell dissociation and the seeding processes. They found out that ES cells are sensitive to single-cell dissociation as e-cadherin, a surface protein that mediates interactions between cells, and the extracellular matrix is essential for ES cell survival, and renewal is disrupted as cells are stripped from their colony (Xu et al. 2010).

9.5 Conclusion and Future Perspectives

Even though stem cell research is on the cutting edge of biological science today, much is still to be known and experimented before stem cells therapy can be made a reality. In a report 'Stem Cells and the Future of Regenerative Medicine' published by Medicine and Council (2002), recommendations were made to help advance stem cell research. Firstly, the need to expand our knowledge of the biology of different types of stem cells was highlighted as there are significant differences between ES cells and adult stem cells and even within adult stem cells from different tissues in the body. Further studies on both embryonic and adult human stem cells must be pursued to progress the scientific and therapeutic potential of ES cells in regenerative medicine (Medicine and Council 2002).

Secondly, advances towards developing medical therapies are difficult without public funding for basic ES stem cell research. There is lack of high-quality, publicly funded research which is at the heart of any medical breakthrough. Publicly funded research carried out in accordance with established standards of open scientific exchange, peer review and public oversight provides the most promising future of stem cell use in regenerative medical therapies. If restrictions and guidelines to conduct controversial research involving embryonic stem cells are developed, human ES cell research will be scientifically validated and scrutinised for compliance with federally authorised ethical guidelines (Medicine and Council 2002).

Another future direction would be to study either combinations of adjuvants and autologous or allogeneic ES cell sources. With the help of pharmacology, bioengineering or gene therapy, the therapeutic utility of ES cells for expansion and function can be enhanced. For clinical use a system of standardised protocols for combining stem cells and adjuvant therapy could also be developed based on the specific needs of individual patients. IPS cells can solve problems of limited donor supply and ethical concerns raised with the use of ES cells. However, ensuring the safety and efficiency of inducing pluripotency in cells from aged and diseased

patients within limited time will require extensive research. A better approach would be to obtain and store allogeneic ES cells beforehand. A cell bank of allogeneic stem cells having all the variation of human histocompatibility is attractive and not entirely impossible. Such allogeneic sources in the future can help overcome quantity, age and disease limitations in stem cell therapy (Brunt et al. 2012).

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Chapter 10
Harvesting and Collection of Adipose Tissue for the Isolation of Adipose-Derived Stromal/Stem Cells

Fiona A. van Vollenstee, Danie Hoffmann, and Michael S. Pepper

10.1 Introduction

A stem cell is defined by its characteristics to self-renew and differentiate along one or more lineage pathways. These unspecialized cells have the potential to develop into many different cell types in the body during their lifespan, serving as an internal repair system. Even after long periods in quiescence, stem cells retain the ability to divide, and the two daughter cells have the potential to either remain a stem cell within the stem cell niche or to develop into a more specialized cell with specific cell functions.

Adipose tissue, like bone marrow, is derived from the embryonic mesoderm and contains a heterogeneous population of cells (Zuk et al. 2001). Adult mesenchymal stromal/stem cells (MSCs), present in many tissues in small numbers, are required to restore normal tissue function via repair and regeneration mechanisms (Jones et al. 2002). Mesenchymal stromal/stem cells have been successfully isolated from many postnatal organs and tissues, namely, the bone marrow (Friedenstein et al. 1968), placenta (Takahashi et al. 2004), umbilical cord blood (Kern et al. 2006), dermis, dental pulp (deciduous teeth, wisdom teeth, or permanent teeth) (Gronthos et al. 2000), hair follicles, pericytes, trabecular bone, infrapatellar fat pad, articular cartilage, Wharton's jelly within the umbilical cord (Fong et al. 2007), fetal liver (Fukuchi et al. 2004), and also from adipose tissue (Zuk et al. 2001). The cells derived from these sources share similar and distinct properties (Dominici et al. 2006; Sarugaser et al. 2009; Si et al. 2010). However, despite various successful

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isolation reports in the literature, protocols for the isolation of cells from these tissues and in particular from adipose tissue are the subject of much debate.

Bridging the gap between the surgical, scientific, therapeutic, and technical fields requires standardized protocols to be established for adipose-derived stromal/stem cell (ASC) processing and should address preharvesting requirements, the process of tissue harvesting (volumes and sites), isolation and expansion methods, as well as post expansion manipulation in order to achieve successful transplantation.

10.2 Factors Influencing ASC Characteristics

10.2.1 Adipose Tissue Type and Anatomical Location

Three types of adipose tissue are found: (1) white adipose (WAT) tissue, (2) brown adipose tissue (BAT), and (3) beige or brite adipose tissue. Adipose tissue can further be classified according to macroscopic tissue type, anatomical location, and structural/functional characteristics.

Although both WAT and BAT are of mesodermal origin, they are believed to originate from different mesenchymal stem cell lineages. Brown adipose tissue, anatomically located around the major organs in a neonate and known to dissipate with age, contains large numbers of intracellular mitochondria that release heat via oxidation of fatty acids mediated by BAT-specific uncoupling protein-1 (UCP1). Classical BAT derived from a myf-5 (myogenic marker)-positive cellular lineage is specialized to dissipate chemical energy serving a protective function against hypothermia (Giralt and Villarroya 2013; Wu et al. 2012).

Emerging from a myf-5-negative cellular lineage and developing in multiple anatomical sites, WAT stores chemical energy and demonstrates known structural, functional, metabolic, and endocrine differences between different WAT deposits (Gimble et al. 2007; Giralt and Villarroya 2013; Sbarbati et al. 2010; Wu et al. 2012).

Beige or brite adipose tissue has morphological and molecular characteristics of classical thermogenic brown adipocytes by responding to cyclic AMP stimulation with high UCP1 expression and respiration rates. Although beige adipose tissue emerges in WAT from a myf-5-negative cellular lineage and has low basal expression of UCP1, beige adipose cells demonstrate a distinct pattern of gene expression (Giralt and Villarroya 2013; Wu et al. 2012). It was demonstrated that peroxisome proliferator-activated receptor gamma (PPAR-γ) ligands require full agonism to induce a beige/brite adipocyte gene program in WAT, by activating the co-regulator PRDM16 and B cell factor-2, recruiting PPAR-γ to BAT-selective genes (Giralt and Villarroya 2013; Ohno et al. 2012).

Macroscopically distinct WAT can further be classified according to functionality and anatomical location, which include (1) bone marrow, (2) mammary, and (3) mechanical (Gimble et al. 2007). Bone marrow adipose tissue passively occupies space no longer required for hematopoiesis and actively serves as an energy reservoir

and cytokine source for osteogenic and hematopoietic events. During lactation, energy and some nutrients are provided by mammary adipose tissue, regulated in part by pregnancy-associated hormones (Gimble et al. 2007). It was suggested by Hamosh and colleagues that lipoprotein lipase activity from mammary adipose tissue diverts dietary lipid from storage in adipose tissue to mammary gland for milk formation (Hamosh et al. 1970). Mechanical adipose tissue offers support to critical structures in the body such as the retro-orbital fat pads, which provide support to the eye (Gimble et al. 2007).

Structural and anatomical location also play a role in WAT classification. Deposit WAT can be found in the periumbilical area, demonstrating tightly packed cells with weak isolated collagen fibers and few blood vessels. Structural WAT located in the iliac and femoral areas demonstrates well-represented stroma and good vascularization. Fibrous WAT occurs in areas experiencing great mechanical stress, as evidenced by the individual fibrous shell of every adipocyte (Sbarbati et al. 2010).

Over the past decade, it has become clear that adipose tissue must be regarded as a complex organ with metabolic functions that extend beyond the classical role of thermoregulation and storage of free fatty acids (FFA) after food intake, as well as the release of FFA during periods of fasting to ensure a sufficient and constant source of energy (Hajer et al. 2008; Harwood 2012). Recent studies have described adipose tissue as a metabolic and endocrine organ producing various substances including adipocyte-derived hormones such as leptin and adipsin; bioactive peptides known as adipokines such as adiponectin, visfatin, omentin, and resistin to name a few; as well as cortisol and various sex and steroid hormones. These substances act both locally (paracrine/autocrine) and systemically (endocrine), exerting various physiological effects (Gimble et al. 2007; Harwood 2012; Kershaw and Flier 2004).

It is well established that adipose tissue plays a critical role in the maintenance of energy homeostasis through secretion of a large number of adipokines that interact with peripheral and central organs such as the brain, vasculature, liver, pancreas, and skeletal muscle to control diverse processes. These processes include feeding behavior, blood coagulation, carbohydrate metabolism, lipid metabolism, inflammation, and energy expenditure (Chu et al. 2001; Ran et al. 2006; Yamauchi et al. 2001). It has also been demonstrated in humans that the anatomical location of adipose tissue has an impact on metabolic function. Visceral adipocytes have been shown to be more resistant to the antilipolytic effects of insulin and are more sensitive to the stimulation of lipolysis by catecholamines when compared to subcutaneous adipocytes (Bjorntorp 2000).

Prunet-Marcassus and co-workers (2006) demonstrated the complex nature of adipose tissue by showing different antigenic features and differentiation potentials in subcutaneous versus internal WAT and BAT in a murine model and also different ASC subsets depending on the anatomical location of the fat pads. BAT displayed a reduced plasticity and fewer ASC numbers when compared to WAT. Furthermore, subcutaneous and internal/deep WAT demonstrated discrete differences in the phenotype of their cell populations (Prunet-Marcassus et al. 2006). This raises the question as to whether the anatomical location of WAT could affect the functional capabilities of ASCs.

Loss of function studies has indicated that PPAR-γ is required for adipogenesis both in vitro and in vivo. Thiazolidinediones (TZDs) act by binding to PPAR-γ, thereby activating the protein cascade that affects metabolism as well as differentiation capacity. The latter occurs by increasing adipogenesis and lipid accumulation (Kelly et al. 1999; Schipper et al. 2008). It was demonstrated by Tchkonia and colleagues (2002) that preadipocytes isolated from subcutaneous adipose tissue had the highest PPAR-γ activity, displayed the greatest effects of TZDs on differentiation, and had the lowest amount of apoptosis compared to omental and visceral abdominal adipose deposits. These results suggested that ASCs isolated from subcutaneous adipose tissue may be more suited to differentiate into mature adipocytes than visceral or omental adipose tissue (Tchkonia et al. 2002).

Schipper and co-workers (2008) compared the functional variability between different anatomically located subcutaneous adipose tissue deposits. Apoptosis susceptibility was lowest in abdominal deposits, while arm deposits showed consistent expression of PPAR-γ-2 without the addition of ciglitazone (a TZD). Although the addition of TZDs can cause more extensive differentiation and lipid accumulation in subcutaneous compared to visceral adipose depositions, the expression of PPAR-γ was not found to be different between the different sites (Schipper et al. 2008).

The yield and growth characteristics of ASCs isolated from different donor sites were evaluated by Oedayrajsingh-Varma (2006). No significant difference in terms of the yield or viability of ASCs obtained from the abdomen, hip, or thigh donor areas was observed (Oedayrajsingh-Varma et al. 2006). In contrast, Jurgens and co-workers (2008) found that the yield of ASCs from the stromal vascular fraction (SVF) is dependent on the specific tissue-harvesting site. The abdominal area yielded significantly more ASCs when compared to the hip and thigh regions, although no difference was found in the total number of nucleated cells per volume or the ASC proliferation and differentiation capacity. When cultured, ASCs from both regions displayed homogeneous cell populations with similar growth kinetics and phenotype (Jurgens et al. 2008). Hauner and Entenmann (1991) also observed differences in the adipogenic differentiation potential between SVF cells harvested from abdominal and femoral adipose tissue (Hauner and Entenmann 1991).

Recently it was confirmed by Iyyanki and co-workers (2015) that the SVF and ASC yield from the abdominal area is significantly higher than from the axilla and flank areas (Iyyanki et al 2015). Taranto and co-workers (2015) explored abdominal adipose tissue further by comparing the stromal tissue compound yield, stemness, and multipotency of cells isolated from the superficial and deep adipose abdominal layers. They demonstrated that ASCs from the superficial adipose tissue layer displayed increased SVF cell yield, increase surface expression of CD105, multipotency (POU5F1, vascular endothelial growth factor (VEGF-A)) gene expression, and tri-lineage differentiation capacity, as well as stemness (Nanog and Sox2) gene expression (Taranto et al. 2015).

Identifying variation in ASCs isolated from different anatomical sites could help to identify an ASC population better suited for specific structural and functional requirements in tissue engineering (Rinkinen et al. 2015). These findings highlight the importance of the specific anatomical location as a source of ASCs.

10.2.2 Patient Age and Gender

Marked differences have been observed between genders in both the metabolism and endocrine function of adipose tissue. Women are known to have a higher percentage of body fat and mainly store adipose tissue in the gluteal-femoral region. Adiposity in this region is associated with larger fat cell size with increased stimulated lipolysis and triglyceride synthesis. Adipose tissue storage in men is primarily in the visceral and abdominal regions. Obesity in men is associated with increased lipoprotein lipase activity and with decreased stimulated lipolysis and triglycerides synthesis (Blaak 2001; Edens et al. 1993; Fried et al. 1993). Several studies have shown that the differences in visceral adipocyte metabolism between genders disappear with menopause. It was further suggested that the female sex hormones may play a role in this gender-specific adipose deposition; this includes, for example, weight gain in the abdominal region of postmenopausal women as well as associated metabolic changes (Rebuffe-Scrive et al. 1989; Trujillo and Scherer 2006).

Age and gender are also important factors to consider when isolating MSCs from adipose tissue. In the publication by Schipper and colleagues (2008), the authors stratified their study into different ages and compared the characteristics of ASCs isolated from the following age groups: 25–30, 40–45, and 55–60 years. The younger patients demonstrated significantly higher cell proliferation rates and higher lipolysis activity, with increased PPAR-γ expression in all of the subcutaneous deposits compared to the other two groups. Interestingly, with the addition of TZDs during adipogenic induction in vitro, the 40–45-year group showed statistically increased adipogenesis when compared to the other groups. When considering the site of isolation, only the upper arm deposits maintained a high lipolytic activity, regardless of the patient's age, when compared to the other sites (medial thigh, trochanteric, and both superficial and deep abdominal adipose deposits) (Schipper et al. 2008). There is still controversy with regard to what causes aging of MSCs, whether it is related to intrinsic or extrinsic factors, but in all likelihood, both. It was suggested by Zhou et al. (2008) that intrinsic factors such as senescence-associated β-galactosidase together with increased expression of p53 and its pathway genes (p21 and BAX) may be responsible for mediating reduced proliferation in MSCs from older patients by inducing senescence (Zhou et al. 2008). In contrast, extrinsic factors such as a reduced synthesis of proteoglycans and glycosaminoglycans in the microenvironment reduce cell proliferation and viability in vivo. In addition, the accumulation of advanced glycosylated end products inhibits proliferation of MSCs by activating apoptosis and reactive oxygen species production (Bi et al. 2005; Kume et al. 2005). This clearly illustrates the variability of ASCs isolated from patients from different age groups.

In contrast, no age-related or gender significant differences in cell surface marker expression (CD34, CD44, CD54, CD73, CD80, CD90, CD105, CD106, CD166, and STRO-1) from MSCs isolated from synovial fat pads were observed (Fossett et al. 2012). Also, the general trends observed with age-related decline in population doublings at low seeding densities and age-related increase in population

doublings at higher seeding densities were not statistically significant. The older patients however had a smaller regression coefficient than younger patients, demonstrating less change in population doublings with increasing seeding densities (Fossett et al. 2012). Fossett *and* colleagues (2012) further demonstrated that synovial fat pad-derived ASCs plated at a density of 50 cells/cm^2 showed a 980-fold increase in ASC proliferation for females and a 367-fold increase for male patients over a period of 21 days. In addition, the investigated cell surface markers demonstrated little difference between genders except for STRO-1 which was expressing at higher levels in female relative to male patients. Based on the fact that estrogens upregulate receptor expression on embryonic stem cells and the previously suggested notion that androgens have inhibitory effects and estrogens a stimulatory effect on MSCs, the possibility was suggested that gender may account for the variability observed (Fossett et al. 2012; Ray et al. 2008).

10.2.3 Harvesting Techniques

The ideal source of stem cells used for regenerative medical applications should (1) be easily obtainable, with minimal discomfort to the patient via a minimally invasive procedure, (2) yield sufficient numbers of cells for extensive cell culturing, (3) be able to differentiate along multilineage pathways in a controlled and reproducible manner, (4) be transplantable to either autologous or allogeneic hosts safely and effectively, and (5) be able to be manufactured in accordance with GMP guidelines (Mizuno 2009). Adipose tissue-derived stromal/stem cells can be considered to fulfill all these criteria. With regard to harvesting, raw lipoaspirate can easily be obtained by suction-assisted lipectomy also known as liposuction, lipoplasty, or simply fat suctioning. This method in comparison to other tissue-harvesting techniques has minimal ethical considerations and limited pain and discomfort to the patient and demonstrates an increased cellular yield and viability (Dominici et al. 2006).

10.2.3.1 Liposuction Versus Biopsy/Resection

Oedayrajsingh-Varma and co-workers (2006) evaluated the yield and growth characteristics of isolated ASCs using different harvesting techniques. Their findings demonstrated that adipose tissue harvested by both resection (biopsy) and wet/tumescent liposuction (described below) techniques provided high yields of rapidly growing ASCs, whereas adipose tissue obtained by ultrasound-assisted liposuction provided a low yield of ASCs exhibiting a low proliferative capacity. More than 80 % of the cells exhibited an ASC phenotype, irrespective of the operative procedure performed (Oedayrajsingh-Varma et al. 2006).

A comparative study evaluating the viability of ASCs from excised versus aspirated adipose tissue showed a significant loss in viability within the excised

cultures, isolated both at 1 h and at 24 h. In addition, these results clearly demonstrated that adipose tissue extraction by suction does not damage the SVF. The group went further and suggested that liposuction is the better method for harvesting ASCs (von Heimburg et al. 2004).

A recent side-by-side comparative study of bone marrow-derived MSCs with ASCs obtained through liposuction or from resection showed increased proliferation and differentiation capacity of ASCs obtained from liposuction, inferring that liposuction produces a more homogenous population of stem cells than the ASCs obtained from resection. Interestingly, although the ASCs obtained from liposuction and resection were collected from the same patient at the same donor site, differences in gene expression profiles were observed (Gnanasegaran et al. 2014). Gene expression in ASCs (liposuction) showed an endoderm propensity with the expression of SOX17 and ISL1 (roles in beta cells) and GFAP (role in neurogenesis). ASCs obtained from resection demonstrated distinct gene expression tending toward mesoderm and ectoderm lineages. ASCs obtained via resection distinctly expressed OLIG2, which is related to oligodendrocyte formation and regulation of ventral neuroectodermal progenitor cell fate (Gnanasegaran et al. 2014).

The procedure of performing liposuction has become common practice among plastic and reconstructive surgeons. A 2014 survey indicated that 13,728,901 liposuctions are performed annually by approximately 35,000 plastic surgeons worldwide (International Survey on Aesthetic/Cosmetic Procedures Performed). Liposuction was also scored at 14.2 % of the total surgical procedures performed by plastic surgeons, indicating that this is one of the most common surgical procedures in this field (http://www.isaps.org). The current trends in liposuction and other fat-removal techniques in the United States were also surveyed by the American Society for Aesthetic Plastic Surgery (ASAPS). The number of liposuctions performed in the Unites States from 1997 to 2014 has increased by 51.6 %, and in 2014 liposuction was the most popular cosmetic surgical procedure with a total of 342,494 procedures (http://www.surgery.org). Multiple factors such as genetic, epigenetic, and behavioral factors contribute to the increasing global obesity epidemic. This epidemic favors adipose tissue as a stem cell source for regenerative medicine, as subcutaneous adipose tissue is abundant and readily accessible (Katz et al. 1999).

Adipose tissue is the richest source of stem cells in the human body, containing 100- to 1000-fold more multipotent cells per unit volume compared to the bone marrow (Tjabringa et al. 2008). It was demonstrated that about 3.5×10^4 preadipocytes can be isolated from 1 g of adipose tissue (Ersek and Salisbury 1995; Fournier and Otteni 1983; von Heimburg et al. 2004). Fraser and colleagues (2006) demonstrated that 1 g of adipose tissue yields a 500-fold greater number of ASCs than the number of MSCs derived from 1 g of bone marrow. Liposuction can yield anywhere from 100 ml to >3 L of lipoaspirate, which is then routinely discarded. This data highlights one of the ideal characteristic of a stem cell source, namely, that it is easily obtainable in large quantities (Fraser et al. 2006).

10.2.3.2 Background on Liposuction

The surgical technique of removing fat through a small incision using suction was developed by Dr. Giorgio Fischer, a gynecologist from Rome, Italy, in 1974. His instrument, the planatome, contained an electric curette that would cut the fat before suctioning the tissue. Liposuction was popularized with a lipoplasty technique demonstration by the French surgeon, Dr Yves-Gerard Illouz, at the 1982 annual meeting of the ASAPS. The Illouz method involved suction-assisted lipolysis after infusing fluid into tissues using blunt cannulas and high-vacuum suction generated by a mechanical pump system (Illouz 1983).

Classical liposuction consists of two types of techniques as described in the literature, namely, the wet or tumescent and the dry technique. Both techniques are currently being used in clinical practice. The wet/tumescent technique was developed to reduce excessive bleeding to <1 % compared to the 30 % observed with the dry technique and was introduced by Klein in 1978 (Agostini et al. 2012). Better known as the tumescent technique, this type of liposuction involves infusion of a saline solution containing a local anesthetic agent and/or epinephrine (adrenaline) into the subcutaneous tissue that allows for regional anesthesia and vasoconstriction before removing both the liquid and tissue using suction. This technique improves on the safety of large-volume liposuction (>1500 ml of adipose tissue) by decreasing blood loss at donor site (Klein 1993). The initial dry technique only involves suction or assisted suction (by mechanical pump) of adipose tissue without prior infusion of the tumescent or Klein solution and is therefore known to harvest virgin lipoaspirate (Gimble et al. 2007; Herold et al. 2011; Klein 1987; Coleman 2001; Tommaso et al. 2012). Finely minced tissue fragments are produced by both techniques, where the size of the fragments is dependent on the dimensions of the cannula used (Gimble et al. 2007).

Over the past three decades, liposuction techniques have become more refined with improved patient safety. The emergence of new technologies however demands critical evaluation of basic science and clinical outcomes of these modalities, and these include the super-wet technique, ultrasound-assisted liposuction, power-assisted liposuction, laser-assisted liposuction, and water-assisted liposuction (Ahmad et al. 2011). Most ASAPS members currently in practice, with experience in different types of liposuction techniques, prefer suction-assisted liposuction to ultrasound-assisted liposuction and power-assisted liposuction (Ahmad et al. 2011). The difference between a wet and super-wet technique is in the amount of wetting solution injected prior to lipoaspiration. Fodor expanded the wet/tumescent technique concept to the super-wet technique, by introducing large volumes of wetting solution in a 1:1 infiltration-to-aspiration ratio, prior to aspiration (Fodor 1995).

It was confirmed that laser-assisted liposuction negatively impacts the biology of ASCs and is therefore not preferable for tissue engineering purposes with suction-assisted liposuction being the preferable technique (Chung et al. 2013). Recently it was shown that water-jet-assisted liposuction yields more viable ASCs in the SVF compared to tumescent- and ultrasound-assisted liposuction, but less viable ASCs compared to the Coleman harvesting technique (Meyer et al. 2015).

The introduction of a syringe instead of a machine to aspirate the fat was introduced by Fournier and later optimized by Coleman for the purpose of fat grafting in clinical practice (Coleman 2002, 2004; Fischer 1975, 1976; Fischer and Fischer 1977; Fournier 1988a, b). The Coleman technique tolerates local, regional, epidural, or general anesthesia depending on the patient's preference and the anesthetic risk. With local anesthesia, lidocaine and epinephrine are used, and during an epidural and general anesthesia, a solution of epinephrine and Ringer's lactate help to maintain homeostasis. After a small puncture incision, a blunt LAMIS™ infiltration cannula is used to introduce and infiltrate the respective wetting solution into the donor area with an estimated ratio of 1 ml solution per cm^3 fat to be harvested. Through the same puncture incision, a blunt tip harvesting cannula with two distal openings in a shape reminiscent of a bucket handle is inserted. The cannula is connected to a 10 ml Luer Lock syringe, which creates minimal negative pressure as the plunger is drawn out, while the cannula is advanced and retracted through the harvest site (Coleman 2002).

A lipofilling study by Witort and co-workers (2007) evaluated the effects of different harvesting techniques on adipocytes. The results indicated that the gentle Coleman technique was less traumatic than the mechanical aspirator (680 mmHg vacuum) which uses power-assisted aspiration (Witort et al. 2007). These results were supported by Herold and co-workers (2011) who compared the fat graft viability of adipocytes using the Coleman technique to the Shippert technique. The Coleman technique involves manual aspiration using a syringe and centrifugation, while the Shippert technique uses automatic liposuction (suction assistance from a mechanical pump system) and no centrifugation of adipose tissue. It was demonstrated using a WST-8 test (cell proliferation assay) and annexin V/IP FACS analysis (apoptotic assay) that the Coleman technique was superior with significantly increased fat graft viability (Herold et al. 2011). An important observation however was that ASCs in the harvested adipose tissue were more resistant to handling and ischemia than mature adipocytes, which are more fragile cells with a shorter lifespan once harvested (Tommaso et al. 2012).

Iyyanki and colleagues (2015) recently compared various yields of SVF cells and ASCs from different harvesting techniques including resection, mechanical-/suction-assisted liposuction, Coleman technique with centrifugation, and Coleman technique without centrifugation. The study revealed that the highest yields of SVF cells and ASCs were obtained from the resection samples and the Coleman technique with centrifugation.

A prospective, randomized, comparative study demonstrated that the maximum negative pressures of 10, 20, and 60 ml syringes were 275, 394, and 549 mmHg, respectively, and no significant differences in ASC integrity and viability were observed (Charles-de-Sá et al. 2015).

Using a wetting solution reduces the risk of excessive bleeding during liposuction procedures, and the patient will have less post-procedural bruising, although if liposuction is performed for the purpose of harvesting ASCs, it is questioned whether anesthetics used in the wetting solution could have an effect on the ASCs. Tommaso and colleagues (2012) demonstrated through histological evidence and

cell viability assessments that there was no substantial difference in cell phenotype using wet and dry liposuction techniques (Tommaso et al. 2012). Keck and colleagues (2010) however noted that local anesthetics have a marked influence on the quality and quantity of viable preadipocytes and ASCs. After the SVF was cultured for 24–48 h and nonadherent cells washed off, the ASCs were trypsinized and exposed for 30 min to different anesthetics before being analyzed by flow cytometry. It was demonstrated that articaine/epinephrine and lidocaine strongly reduced ASC viability, while bupivacaine had no effect. These exposed cells were then induced to differentiate into adipocytes for 12 days, and the expression of adiponectin was measured using quantitative real-time polymerase chain reaction. All the anesthetics tested, namely, bupivacaine, mepivacaine, ropivacaine, articaine/epinephrine, and lidocaine significantly decreased adiponectin expression and adipogenic differentiation capacity compared to saline controls. Interestingly, anesthetic-exposed ASC cultures, except those exposed to articaine/epinephrine, showed a similar phenotypic appearance to that of control cultures not exposed to anesthetics. All local anesthetic-exposed ASCs demonstrated impaired adipocyte differentiation as determined by adiponectin expression. The ASCs from the articaine-/epinephrine-exposed cultures appeared smaller in size, while a similar percentage of cells demonstrated lipid droplet formation (Keck et al. 2010).

10.2.3.3 Suggested ASC Harvesting Technique

The Coleman technique is a dry needle aspiration procedure and is commonly used to obtain virgin adipose tissue. No pharmacological substances, e.g., saline and/or lignocaine (wetting solution), are injected into the donor area before the procedure. The aim is to induce minimal trauma to the adipocytes and preadipocytes during collection compared to other harvesting techniques using high negative pressure.

Our group has employed the Coleman technique and restricted all adipose tissue collections to the use of a 10 ml syringe for the purpose of creating and maintaining a low negative pressure system. The negative pressure created by a 10 ml syringe has previously been measured with an aneroid vacuum meter. It was shown to be 510 mmHg when the plunger was withdrawn to a maximum and gradually decreased as the syringe was filled with harvested lipoaspirate (Novaes et al. 1998). General anesthesia is administered for the respective cosmetic procedures, and antiseptic cleaning of the donor area with chlorhexidine is performed by the theater nurse prior to the initiation of surgery. Sterility is maintained throughout the procedure. A small puncture wound (~10 mm) is made with a no. 15 scalpel blade through the epidermis in the donor areas, i.e., in the infraumbilical and/or flank areas (supralateral pelvic area). A Coleman blunt tip harvesting cannula with two distal openings, which give the tip a shape reminiscent of a bucket handle (Fig. 10.1) attached to a 10 ml Luer Lock syringe (syringe), is used to allow for the collection of strands of adipose tissue. Dry needle aspiration is performed with a harvesting cannula with dimensions of 150 mm in length, an outer diameter of 4 mm, and inner diameter of 2.5 mm (Johnson & Johnson, Biron 02-331).

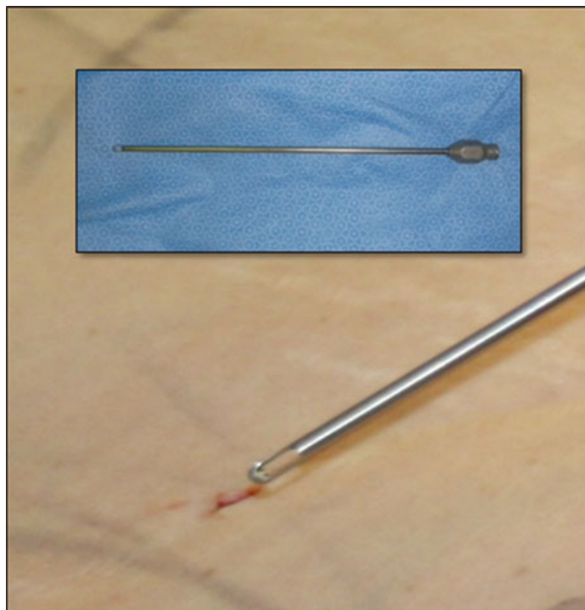

Fig. 10.1 Harvesting cannula with a blunt tip in the shape of a bucket handle. The Coleman harvesting cannula dimensions are 150 mm in length, 4 mm external diameter, and 2.5 mm internal diameter. The proximal end of the harvesting cannula is shaped to fit securely into a 10 ml Luer Lock syringe

The harvesting cannula is inserted through the puncture wound into the subcutaneous adipose layer of the abdominal donor site (Fig. 10.2). The plunger of the syringe is withdrawn 1–3 ml at a time to create the low negative pressure vacuum within the barrel of the harvesting cannula and syringe. The surgeon holds the harvesting cannula attached to the syringe in his dominant hand while gently grasping the skin of the abdominal area with his nondominant hand in order to lift the subcutis from the underlying structures. The fat is aspirated using a meticulous, atraumatic technique by smoothly advancing and retracting the harvesting cannula through the subcutaneous adipose layer. This movement is done very quickly and forcefully through the donor area, in order to avoid blood contamination of the collected adipose tissue sample. The harvesting cannula is repeatedly advanced in a transverse direction while slowly progressing in a circular pattern until the needle points in an inferior direction from the puncture wound. This directional movement in the liposuction technique is called the fan formation. The harvesting fan formation is repeated on the opposite side. This fan formation of fat aspiration is used to avoid blood contamination of the sample during the harvesting process.

As the syringe fills with lipoaspirate, the negative pressure decreases until the suction vacuum is insufficient to allow for further harvesting of adipose tissue (Fig. 10.3). The harvesting cannula is then extracted from the puncture wound and removed from the patient. The plunger is withdrawn from the syringe and the aspirate is decanted into a sterile bottle (Fig. 10.4). The sterile bottle contains phosphate-buffered solution at a pH of 7.4 (PBS) as well as 5 % penicillin and streptomycin (pen/strep). The harvesting process is repeated until a reasonable amount of virgin fat has been collected. The process is terminated when excessive

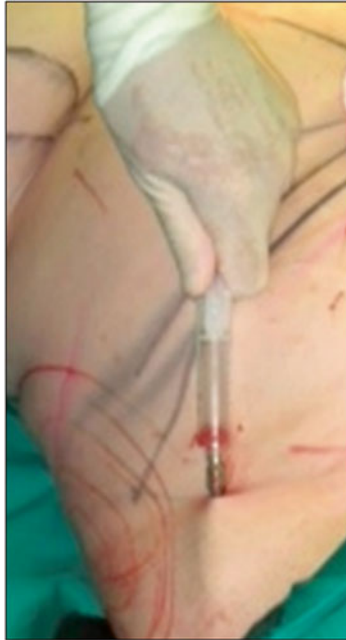

Fig. 10.2 A puncture wound made with a no. 15 scalpel blade in an aseptically cleaned donor area. The harvesting cannula is inserted through the puncture wound in the donor area. The harvesting cannula is advanced within the adipose layer of the donor area, and the plunger is withdrawn 1–3 ml at a time to create a low negative pressure within the barrel of the syringe. The negative pressure decreases within the suction system as the barrel of the syringe fills with adipose tissue. The plunger is then drawn again creating a vacuum to allow more adipose tissue to be suctioned through the harvesting cannula into the barrel of the syringe. The surgeon grips the donor area with the nondominant hand, while easily manipulating the 10 ml Luer Lock syringe to maintain a low negative pressure during harvesting. The surgeon advances and retracts the harvesting cannula quickly and forcefully through the adipose layer

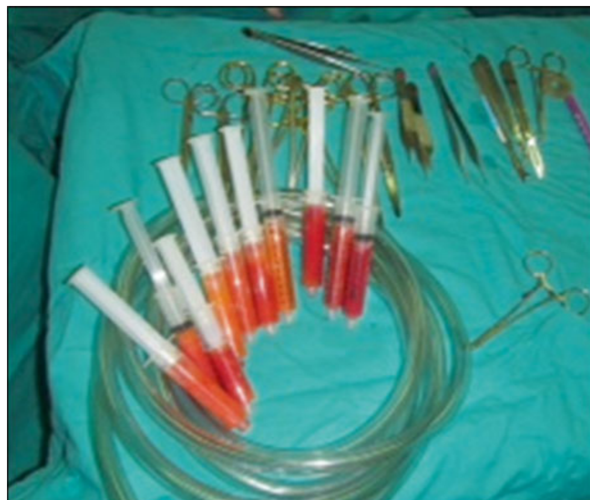

Fig. 10.3 Lipoaspirate fills the syringe during the harvesting process due to the negative suction pressure. Because a dry needle aspiration technique is used to obtain virgin lipoaspirate, the samples appear bloody. These blood cell contaminants will be removed during the isolation process

Fig. 10.4 The plunger is withdrawn completely from the syringe barrel and the lipoaspirate is decanted into a sterile bottle containing PBS

blood contaminants appear in the syringe. This method is successful in obtaining large volumes of ASCs from the SVF and does not negatively affect cellular expansion experiments.

Using previous literature recommendations, we formulated a harvesting technique, unique to our purposes. We have isolated, expanded, and differentiated ASCs successfully, harvesting tissue using the Coleman dry needle aspiration technique with an average of 1,019,129 cells/ml. Based on what is reported in the literature, we propose the abovementioned harvesting technique to be the preferred technique for isolation of adipose-derived SVF and ASCs. According to the literature, it is understood that this technique will be less invasive than resection while still producing high yields of SVF and ASCs with multipotency, stemness, and genetic profiling not influenced by local anesthetics.

10.2.4 Factors Influencing ASC Isolation and Expansion

The initial method for isolating ASCs from adipose tissue was pioneered in the 1960s. Minced rat fat pads were extensively washed to remove contaminating hematopoietic cells, incubated with collagenase and centrifuged to obtain a pellet of SVF containing a heterogeneous population of cells. The selection for plastic adherent fibroblastic like cells from the SVF concluded this isolation process (Rodbell 1966a, b; Rodbell and Jones 1966). Mesenchymal stem cells resident in human adipose tissue were first described by Zuk and co-workers in 2001. The initial procedure of mincing human adipose tissue by hand was simplified by the development of liposuction surgery. Many stem cell laboratories have developed methods to isolate and expand MSCs from various tissue sources including adipose tissue. Although most of these methods share similarities, there are some that differ significantly which leads to the following very important unanswered question within the stem cell research community. If these different tissue sources and methodologies are used for the preparation of MSCs, are these MSCs sufficiently similar to allow

for direct comparison of reported biological properties and experimental outcomes, especially in the context of cell-based therapy (Dominici et al. 2006)?

Dominici and colleagues suggested in 2006 that the standard isolation protocol developed by Zuk and co-workers (2001, 2002) should be accepted as an established methodology to obtain SVF from raw lipoaspirate (Dominici et al. 2006). Most research groups however make adaptations to this methodology, and this complicates the comparison of results between groups. Previous studies have suggested that ASCs exhibit an average population doubling time of 60 h or generally 2–4 days, depending on the donor's age, the type (white or brown) and location (subcutaneous or visceral) of the adipose tissue, the type of surgical procedure, culture conditions, growth factors, plating or seeding densities, passage number, and media formulations (Fossett et al. 2012; Gimble et al. 2007; Mizuno 2009). This again highlights the many factors to consider when developing standardized isolation protocols.

Different fat processing techniques have also been evaluated. A prospective cross-sectional study evaluated three widely used fat processing techniques in plastic surgery for the viability and number of adipocytes and ASCs isolated from collected lipoaspirate (Condé-Green et al. 2010). All samples were collected using the established Coleman technique under regional anesthesia. The aspirate was processed using three different techniques, namely, (1) decantation, (2) washing, and (3) centrifugation. The three basic layers, the superior oily liquid supernatant, the firmer white-yellow tissue, and the inferior layer consisting mostly of blood contaminants including the infiltration and washing liquids, were identified with all three techniques. A fourth layer, the pellet, was identified with centrifugation only. Significant differences were observed with regard to viable adipocytes in the middle firm tissue layer between various processing techniques ($p=0.0075$), where centrifugation rendered adipocytes nonviable compared to decantation and washing techniques.

Flow cytometric analysis has revealed various differences in ASCs, hematopoietic cells (blood contaminants), and endothelial cells, comparing the middle firm tissue layers of all three different processing techniques and the pellet of the centrifuged samples (Condé-Green et al. 2010). The firm tissue layer of the decantation process contained large amounts of blood contaminants and very few ASCs and endothelial cells. The firm tissue layer of the washing process contained few blood contaminants and more endothelial cells and ASCs, compared to the decantation process. The firm tissue layer of the centrifuged samples contained the least number of ASCs, blood contaminants, and endothelial cells, whereas the pellet of the centrifuged samples contained the greatest number of ASCs, blood contaminants, and endothelial cells. In addition, the firm tissue layer from the centrifuged samples did not expand and proliferate in vitro, while the pellet of the centrifuged samples demonstrated extensive proliferation and expansion (Condé-Green et al. 2010). A recent study confirmed this finding by comparing centrifuged and non-centrifuged samples collected from subcutaneous adipose tissue in the abdominal area using the Coleman technique and revealed that the centrifuged samples contained a significantly greater SVF and ASC yield (Iyyanki et al. 2015).

The results of this study also confirmed the proposition made by Tommaso and co-wokers (2012) that ASCs are sturdier cells than adipocytes and can withstand centrifugal forces of up to 3000 rpm (Condé-Green et al. 2010). The oil floating material layer seen in centrifuged samples was previously analyzed by Novaes and co-workers (1998). They used gas chromatography to examine the nature of this floating oil material and identified the substances as lauric acid, stearic acid, palmitic acid, and araquidic acid, where the highest volume was occupied by palmitic acid (Novaes et al. 1998) indicating contamination, which supports the practice of removal of the oily supernatant.

Various aspects surrounding the centrifugation process during the isolation procedure can influence the isolation yield. Baschert and co-workers suggested that centrifugation forces greater than 100 *g* are not appropriate for autologous fat transplantation as they observed an increased quantity of oil possibly due to adipocyte destruction (Baschert et al. 2002). In contrast, Kurita and colleagues found that more than a 100 *g* centrifugal force could be used for autologous fat grafting, since the increased oil portion does not necessarily mean an increase in adipocyte destruction, but rather an increase in the separation of oil from the adipose portion (Kurita et al. 2008). Centrifugation of adipose tissue separates fat cells from lipid, blood cells, water, and water-soluble ingredients such as proteases and lipases, but does not shift ASCs between the adipose and fluid portions, possibly due to the strong adherence to adipose tissue or since they are resident within the adipose tissue. It was also shown that increased centrifugal forces compacted the adipose portion more and therefore concentrated the red blood cells within the adipose portion rather than shifting the red blood cells into the fluid portion. In contrast to mature adipocytes, it was found that the yield of ASCs in culture for 1 week was consistent up to 3000 *g* but decreased with centrifugal forces of more than 3000 *g* (Kurita et al. 2008). Dickens and co-workers demonstrated that gentle centrifugation produced the highest cell viability, whereas long periods of centrifugation resulted in the selection of the most proliferative ASC subpopulation (Dickens et al. 2009).

Another factor to consider in the isolation process is the effect of seeding density on cell proliferation. Fossett and colleagues (2012) showed that low seeding densities increase the proliferation capacity in vitro. The effect of seeding density on MSC proliferation was demonstrated with bone marrow-derived MSCs that were seeded at 100 cells/cm^2 and reached their target of 200×10^6 cells 4.1 days faster than cells seeded at 5000 cells/cm^2 (Both et al. 2007). Similar results were observed by Lode and co-workers in 2008 using synovial fat pad MSCs seeded on three-dimensional scaffolds (Lode et al. 2008). Witzeneder et al. applied different ASC seeding densities for expansion (3200 cells/cm^2) and lineage induction experiments (7000 cells/cm^2), while Lindroos et al. seeded cells at 5000 cells/cm^2 for ASC expansion purposes. Krähenbühl et al. found good cellular expansion with seeding densities of 325, 750, 1500, and 3000 cells/cm^2 but in contrast to Fossett and colleagues found increasing yields with higher densities (Krähenbühl et al. 2015; Lindroos et al. 2009; Witzeneder et al. 2013). Fink and co-workers found that ASC expansion is optimal between 100 and 200 cells/cm^2 with a range of 50, 100, 200, and 800 cells/cm^2 (Fink et al. 2011). The literature therefore does not provide

consensus on opinions on the seeding density required for optimal expansion of ASCs, and laboratories currently appear to be following protocols based on in-house evaluations.

The proliferation of ASCs can be stimulated by several exogenous supplements including fibroblast growth factor 2 (FGF-2) via the FGF-2 receptor, sphingosylphosphorylcholine via activation of c-Jun N-terminal kinase (JNK), platelet-derived growth factors via the activation of JNK and oncostatin M via the activation of the microtubule-associated protein kinase or extracellular-regulated kinase, and the Janis kinase 3 or signal transducers and activators of transcription factors type 1 pathway (Chiou et al. 2006; Jeon et al. 2006; Kang et al. 2005; Mizuno 2009; Song et al. 2005). On the contrary, it was suggested by Zhang and co-workers (2010) that low-intensity and intermittent negative pressure treatment, e.g., creating a negative pressure (vacuum) environment within the processing cabinet, could inhibit MSC proliferation, promote cellular apoptosis, and enhance osteogenic activity. Inhibition of proliferation could be attributed to temporal hypoxia, caused by the negative pressure, which could cause hypoxia-inducible factor 1 (HIF-1) upregulation. The HIF-1 heterodimer is composed of hypoxia-inducible factor 1-alpha (HIF-1α), which is acutely regulated in response to hypoxia, and hypoxia-inducible factor 1-beta (HIF-β), which is insensitive to fluctuations in O_2 availability and allows for cellular adaptation to hypoxia (Zhang et al. 2010). This again highlights the importance of environmental factors to be included in standardized protocols.

ASCs are responsive to hypoxia, which promotes the secretion of the angiogenic growth factor VEGF (Thangarajah et al. 2009). Some studies however suggest that hypoxia reduces ASC proliferation and attenuates adipogenic, chondrogenic, and osteogenic differentiation (Lee and Kemp 2006), but the literature on hypoxia and ASCs has advanced considerably since 2006. Fotia and co-workers confirmed that hypoxia increases ASC proliferation while decreasing cell surface expression of CD184 (CXCR4) and CD34 and preserves NANOG and SOX2 gene expression. In addition to promoting proliferation and stemness, hypoxia and osteogenic stimuli (induction media) accelerates the cell differentiation and mineralization process (Fotia et al. 2015).

Recent studies have demonstrated multiple hypoxia-responsive pathways involving angiogenesis in superficial and deep abdominal adipose tissue. Rinkinen and colleagues have demonstrated that mRNA levels of angiogenic chemokines (VEGF-A, VEGF-B) and transcription factor HIF-1α significantly increase in deep abdominal tissue, in response to hypoxic culturing conditions, compared to superficial abdominal adipose tissue (Chung et al. 2012; Rinkinen et al. 2015). In addition, increased protein expression levels (VEGF-A and protein nuclear factor $_{\kappa}$B) were found within the ASCs derived from deep subcutaneous adipose tissue (Rinkinen et al. 2015). Although notable variations in ASCs from deep and superficial subcutaneous adipose tissue are ignored during tissue harvesting, an ASC population could be identified more suited for specified functionality in tissue engineering (Rinkinen et al. 2015).

It was observed by Amos and colleagues (2008) that harvesting techniques not only affect the viability of ASCs but also their level of adhesiveness to key adhesion

proteins (Amos et al. 2008). Thus they demonstrated that the technique of ASC extraction (liposuction versus lipectomy) impacts on the adhesion potential of these cells to proteins in the extracellular matrix and the proteins expressed by activated vascular endothelium, as well as their response to hypoxic culture. ASCs were able to firmly adhere to type I collagen, fibronectin, vascular adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) substrates but not to any of the selectins (P-selectin, E-selectin, L-selectin). With hypoxia pretreatment, ASCs extracted by liposuction showed an increased ability to adhere to VCAM-1 and ICAM-1, whereas ASCs extracted by lipectomy did not (Amos et al. 2008). They also showed that prolonged (>48 h) exposure to hypoxic conditions enhances the secretory, differentiation, and proliferative capacity of ASCs, in addition to their ability to firmly adhere, making this a viable approach for cell activation prior to therapeutic delivery. In clinical practice, adipose tissue-harvesting techniques could have an effect on the homing mechanisms of ASCs, by aiding in the mobilization and trafficking of both tissue-resident and therapeutically delivered cells in a setting where interaction with inflamed or injured tissue is necessary.

10.3 Conclusion

The development of standardized protocols for the harvesting and isolation of ASCs from adipose tissue has become critical in the rapidly expanding field of regenerative medicine. Researchers rely on accurate comparisons between groups to advance the field into clinical application. Many factors can however influence the behavior as well as the yield of ASCs and need to be considered. The Coleman technique, applied during routine liposuction procedures for harvesting of abdominal adipose tissue, is currently recommended. Further investigation with regard to post-harvesting processing techniques as well as culturing requirements is necessary to optimize these standardized protocols.

Current recommendations that will support optimal ASC yield, proliferation, and plasticity include (1) harvesting of subcutaneous abdominal adipose tissue using the Coleman technique associated with dry needle aspiration, collecting virgin lipoaspirate; (2) reducing trauma to the cells by decanting the lipoaspirate rather than ejecting or pipetting the tissue samples, reducing unnecessary mechanical pressure on the ASCs; and (3) using low centrifugal forces for short intervals during the isolation process.

Careful planning is required for clinical application. Various factors need to be taken into consideration and these include (1) the anatomical location, (2) the donor and the recipient (autologous or allogeneic), (3) the donor's age and gender, (4) the tissue-harvesting technique, (5) cell isolation procedures, (6) maintenance of cells under good tissue manufacturing practices, (7) possible cellular manipulations, and (8) mode of transplantation. The success of clinical applications will depend on the unity of a team of surgeons, physicians, scientists, and technicians.

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