CHAPTER 15

Mesenchymal Stromal Cell Therapies – The Next Frontiers

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INTRODUCTION

The emerging field of cell therapy has been considered by many to be the next revolution in medicine, promising unprecedented and previously unimaginable treatments for major life-altering conditions and degenerative diseases. The new discipline of regenerative medicine has cell therapy at its cornerstone, and there are probably few areas of biomedical research which have attracted so much attention and been surrounded by so much expectation and promise. Mesenchymal stromal cell (MSC) therapy has been at the centre of this effort and has garnered unrivalled attention. However, in this particular revolution, the wheels have turned slowly and a decade of concentrated and sustained efforts at clinical testing of MSCs in a broad spectrum of diseases gave results that were either mixed or disappointing (Trounson and McDonald, 2015; Nowbar et al., 2014). It appeared for a time that the field was unable to deliver on its promise.

However, the tide turned in recent years with several regulatory approvals of therapies consisting of administration of MSCs. For example, FCB Pharmicell received approval in South Korea in 2011 for HeartiCellgram-AMI, an autologous bone marrow-derived MSC treatment for acute myocardial infarction. Shortly thereafter, also in South Korea, Medipost and Anterogen received approvals, respectively, for Cartistem, an allogeneic umbilical cord-derived MSC treatment for osteoarthritis of the knee and Cupistem, an autologous MSC treatment for fistulating Crohn's disease. Also in 2012, Mesoblast's remestemcel-L received approval in Canada for the treatment of steroid-refractory acute graft versus host disease in children. Importantly, this was the first approval of a commercial cellular therapy for systemic administration.

The first approval in Europe in 2018 for an allogeneic MSC therapy was for Alofisel, an allogeneic adipose-derived MSC treatment for complex perianal fistulas in adult patients with nonactive or mildly active luminal Crohn's disease. This approval followed a positive opinion by the European Medicines Agency based on the results of the ADMIRE-CD phase 3 study (Panes et al., 2016) that reported that there was sustained efficacy in patients treated with the cell product compared to placebo. In the study, a total of 212 patients were enrolled and randomly assigned – 107 to the treatment group

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and 105 to the placebo group. Other allogeneic MSC protocols have also received approval or are very close to this point. For example, Mesoblast's TEMCELL HS, an allogeneic MSC therapy, is approved in Japan for the treatment of acute graft versus host disease in bone marrow transplant patients (Konishi et al., 2016). Stempeucel, marketed by Stempeutics, has received limited approval in India for the treatment of critical limb ischaemia (associated with Buerger's disease) (Stempeutics). These are now regarded as milestones in the field of MSC technology, reflecting many years of sustained research and clinical assessment, and are likely to be the forerunners of other approvals.

However, in addition to approvals listed above, many clinical studies have been completed which did not lead to a clear outcome or regulatory approval. The mixed clinical outcomes suggest several possibilities, such as the wrong clinical target, inadequate trial design, lack of understanding of the mechanism of action or uncertain quality of the cell product being tested. The latter two points have come into sharp focus in recent times, and there are legitimate questions about the slow pace of comprehension of the biological activity of the cells versus the fast pace of patient testing (Galipeau and Sensébé, 2018). There are still concerns that the biology of the in vivo MSC, the consistency and quality of culture expanded preparations, the nature of lineage commitment and the therapeutic mechanism of action have not been adequately addressed. For these reasons, MSCs may be regarded as a cellular 'poultice', used because we did not know any better and to be replaced by more refined therapies when these become available. Therefore, MSCs may be primarily a means to an end, that end becoming apparent with greater scientific comprehension.

MESENCHYMAL STROMAL CELLS

The first identification of marrow stromal cells or MSCs was reported over 50 years ago by Alexander Friedenstein (Friedenstein et al., 1966). He isolated adherent, fibroblastic cells from the stromal compartment of bone marrow and demonstrated that the cells were capable of differentiating into osteocytes. The cells were also capable of forming colony-forming unit fibroblastic in vitro (Galipeau and Sensébé, 2018; Friedenstein et al., 1970, 1974) and could give rise to different skeletal tissues in vivo.

Caplan first coined the phrase 'mesenchymal stem cells' (Caplan, 1991) to associate the 'stemness' shown by the capacity of the cells to form tissues of the mesenchymal lineage (Friedenstein et al., 1966). There has been considerable disagreement regarding the most accurate and acceptable terminology, and Bianco et al. (2008) proposed the term 'skeletal stem cells' to describe mesenchymal cells coexisting with haematopoietic stem cells in marrow.

Although there are tantalising glimmers of positivity and a small number of approved MSC products, as mentioned above, there are also many reported clinical studies that gave rise to failed or indifferent outcomes. The reasons given for these failures include the poor characterisation and lack of consistency of the cell product, an absence of rigorous quality standards and, as mentioned above, an incomplete understanding of the

therapeutic mechanism. Although it was first believed that MSCs exert their therapeutic effects by cell replacement activity, it gradually became apparent that this is not the case. Other, subtler and more complex, mechanisms have been proposed including the idea of 'paracrine' effects. Although widely discussed and evoked as a rationale for host repair responses, only a small number of paracrine molecules have been specifically identified and validated as essential transplanted cell-derived contributors to repair. Amongst the paracrine mediators that have been the discussed in this context are stem cell-derived factor-1 (Kanki et al., 2011), hepatocyte growth factor (Iso et al., 2007), vascular endothelial growth factor (Iso et al., 2007) and factors that act as immunomodulators, such as prostaglandin E2 and TNFa-stimulated gene/protein 6 (Prockop, 2013).

Some new and important insight has been gleaned into a recent series of studies that indicate that apoptosis of infused MSCs may be a central element in their capacity to act as immunomodulators. These studies also indicate that host monocytes appear to play a role as critical responder cells, acting in the clearance of delivered cells. Galleu et al. (2017) have shown that, in systemic infusion studies in mice, host cytotoxic cells induce a perforin-dependent apoptotic response by the infused MSCs, and that this is required to initiate MSC-induced immunosuppression. The same response was observed in patients receiving MSC treatment for graft versus host disease. The apoptotic MSCs were engulfed by recipient phagocytes, stimulating the latter to produce indoleamine 2,3-dioxygenase, necessary for effecting immunosuppression. This and other studies (Braza et al., 2016; Nemeth et al., 2009; de Witte et al., 2018) all suggest that phagocytosis is a central element in the host response to infusion of MSCs.

BEYOND MESENCHYMAL STROMAL CELLS

In the very recent past, new ideas have emerged that seem to provide a more illuminated path forward and are at last providing explanations for MSC-mediated therapy in precise molecular terms. This new understanding may indeed open the doors to new therapeutic modalities and define new products, so that ultimately the most effective treatments may be based on new *cell-inspired* or *cell-derived* principles rather than on uncertain or incompletely characterised whole cell populations. These new principles include exosomes, cell-conditioned expansion media, specifically validated cell-derived paracrine factors, immunomodulation driven by apoptosis of the delivered cells and gene-modified cells. In considering this next generation of therapies, those which are cell-derived – including conditioned media, exosomes and gene-modified cells – will still rely on cell manufacturing platforms. Cell-inspired products, discovered because of their association with MSCs, will ultimately be manufactured in a different, and perhaps simpler, setting. These include, for example, specific recombinant protein and miRNAs. The panel of next generation technologies derived from or inspired by MSC therapy is shown in Fig. 15.1. The discussion presented in this chapter is focused on some of those opportunities.

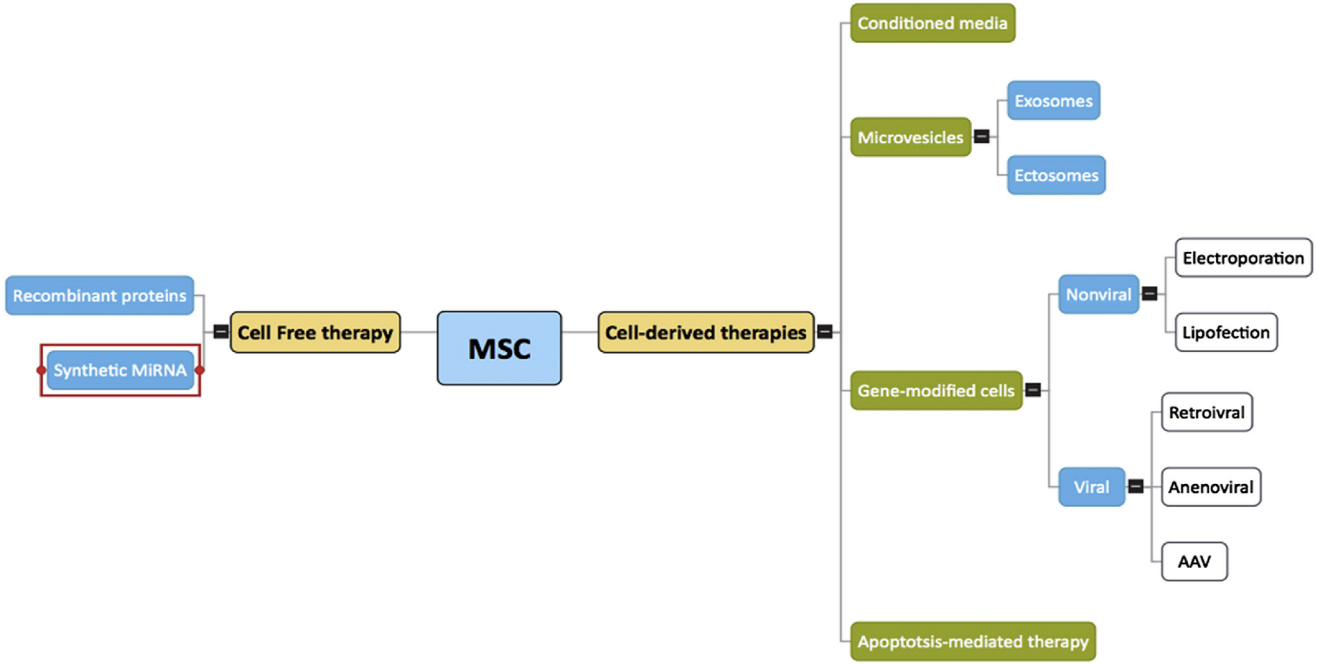

Figure 15.1 Next generation therapies arising from or inspired by conventional MSC treatments. Cell-derived therapeutics include conditioned media, extracellular vesicles and gene-modified cells. Cell-inspired strategies include identified recombinant proteins and synthetic miRNAs.

Exosomal Therapy

The extracellular vesicle (EV) is a subcellular structure enclosed within a spherical lipid bilayer of varying size ranging from a few nanometres to a few micrometres. It is now evident that EVs are produced by virtually every cell type in the body and play a major role in cell signalling and in directing cell-mediated physiological responses. EVs were first identified as platelet-derived particles in blood plasma (Chargaff and West, 1946; Wolf, 1967), then associated with cartilage calcification (Anderson, 1969) and subsequently with many other cell types, including sperm cells, erythrocytes, lymphocytes and multiple tumour cells (reviewed in Yáñez-Mó et al., 2015a,b). An understanding of the critical roles that EVs play in cell signalling began to emerge with the observations that they are involved in antigen presentation by B lymphocytes (Raposo et al., 1996) and the induction of tolerance in vivo (Karlsson et al., 2001). Further critical insight emerged with the observation that exosomes from mast cells carry a cargo of RNA, both mRNAs and microRNAs (Valadi et al., 2007), and it was found that the RNA present in the exosomes could be transferred to recipient cells and translated to give rise to new proteins. Thus, a major new cell–cell signalling modality was identified whereby the originator cell could impart genomic changes, influence gene expression and modify the proteome of recipient cells. This was a major milestone in cell communication. A comprehensive and illuminating review of exosome biology, origin, structure and function has recently been published (Colombo et al., 2014).

The biogenesis of EVs has been studied in detail and is somewhat well understood. Three pathways are important that relate to EVs of differing subcellular origin: (1) exosomes, (2) ectosomes and (3) apoptotic bodies. Exosomes (diameter 40–100 nm) have an endosomal origin (Fig. 15.2), and they arise by inward budding of the early endosomal membrane (Yáñez-Mó et al., 2015a,b). A number of these can accumulate within the lumen of intracellular structures referred to as multivesicular bodies (MVBs). It is apparent that the role of MVBs is to sequester intracellular molecules that are targeted either for degradation or for transfer to the extracellular environment to participate in cell communication. For degradation, the MVBs undergo fusion with lysosomes, and their molecular cargo is exposed to the proteases and other degradative enzymes present within that compartment. For release, they are trafficked to the plasma membrane where they fuse and discharge their contents to the extracellular space. The endosomal sorting complex required for transport (ESCRT), a complex of some 30 proteins, is a major component in this trafficking.

Membrane vesicles that shed directly from the cell membrane are referred to as ectosomes (Sadallah et al., 2011), and these have a larger diameter (100–1000 nm) compared to exosomes. Different cell types produce ectosomes in response to different stimuli. For example, the production of ectosomes is stimulated in endothelial cells, monocytes and platelets by exposure to complement (Pilzer et al., 2005) lipopolysaccharides and thrombin (Freyssinet, 2003), respectively. Finally, apoptotic bodies arise from cells that are

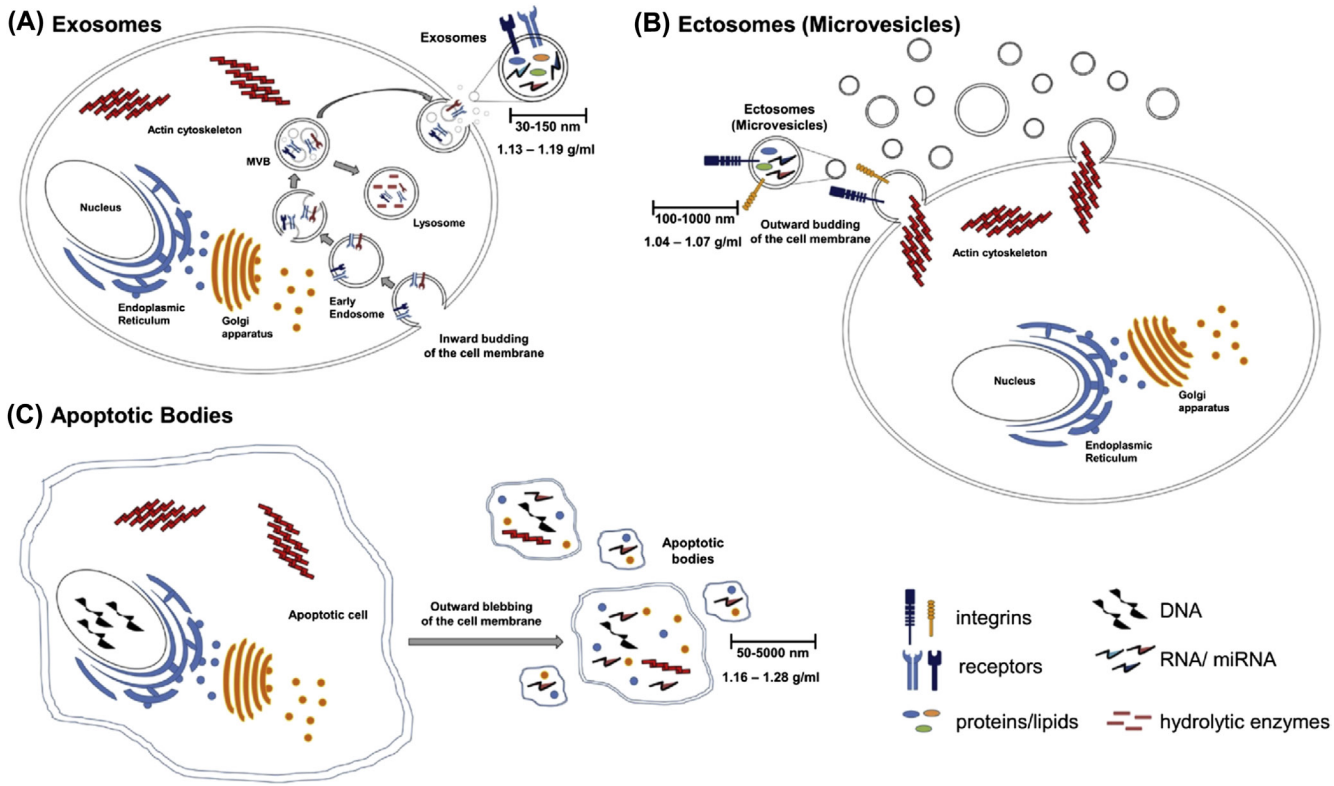

Figure 15.2 Biogenesis of extracellular vesicles *((From Cabral et al., 2018, with permission).)*

undergoing apoptosis by outward fission of the membrane. The major function of this appears to be to allow the apoptotic bodies to be phagocytosed.

In addition to the ESCRT, other proteins associated with exosomes include the tetraspanins CD9, CD63 and CD81 as well as tumour susceptibility gene 101 (TSG101), heat shock protein 70 (HSP70) and programmed cell death 6 interacting protein (Alix). In addition, specific lectins appear to be associated with a conserved glycan signature for EVs (Batista et al., 2011). An innovative and resourceful database, Vesiclepedia (Microvesicles), is available as an open source for information relating to molecular data (lipid, RNA and protein) identified in EVs.

Given the importance of EVs in signalling and in the processing of cellular components and response to stimuli, it became important to assess whether they were part of the therapeutic machinery in MSCs. One of the early and important studies which showed that MSCs produce vesicles with intrinsic and measurable therapeutic activity was that of Bruno et al. (Bruno et al., 2009) who showed that exosomes obtained from human MSCs have a potent therapeutic effect in a mouse model of acute kidney injury. This study indicated that the vesicles themselves exerted proliferative and antiapoptotic effects on tubular endothelial cells, and that they were capable of effecting the transfer of their mRNA content to the target cells. Furthermore, it appeared that this transfer of RNA was a central element in the therapeutic effects associated with the vesicles, as treatment with RNase abrogated the effects (Karlsson et al., 2001). Many other studies have supported the observation that, in a variety of disease models, the therapeutic activity associated with cell transplantation could be recapitulated or improved when only an exosomal or microvesicle fraction of the cells was delivered (Lai et al., 2010). This represented a step forward in understanding the therapeutic mechanism of action of MSCs and gave substance to the many descriptions of potent but poorly defined paracrine effects. It also offered some explanation, yet to be confirmed in precisely managed in vivo studies, of the exceptionally low levels of engraftment and persistence of MSC following delivery.

Other examples of MSC EV-mediated therapeutic outcomes abound (Akyurekli et al., 2015) and include myocardial infarction (Arslan et al., 2013; Zhang et al., 2012), liver fibrosis (Li et al., 2013), pulmonary hypertension (Lee et al., 2012) and articular cartilage (Cosenza et al., 2017; Zhang et al., 2018).

A picture thus begins to emerge of several dynamic steps in the therapeutic pathway of MSCs in situ, comprising cell transplantation, rapid formation of exosomes with a cargo of protein and RNA, shedding of the exosomes, clearance of the whole cells, transfer of the cargo to host recipient cells and evocation of a repair response. This pathway is speculative and lacks specific information regarding the nature of the active cargo, the identity of the target cells and the nature of the target cell response. Nonetheless, it offers a direction for future investigation and asks some questions which are potentially answerable.

The idea of a new and effective therapeutic modality involving the transplantation, not of whole living cells but of a fraction of cell-derived exosomes, has substantial appeal. It takes us a further step towards understanding the mechanism of action and provides new possibilities with regard to manufacturing the therapeutic product. It also offers interesting approaches for generating engineered exosomes for enhanced therapeutic application. To understand the effects of exosomes on target cells, high resolution RNA, proteomic and lipidomic analyses are needed. One example of such an analysis shows that MSC-derived exosomes may contain several thousand protein and lipid molecules (Haraszti et al., 2016). The RNA cargo of vesicles has also been characterised and catalogued in the database mentioned previously (Raposo et al., 1996).

The concept of exosome engineering offers many opportunities for tailored therapeutic effect and for targeted delivery. There are several approaches that can be tested in developing methods for engineering exosomes to carry a customised cargo. For example, gene delivery can be achieved using both viral and nonviral methods, and this often entails genetic modification of the originator cell. Viral methods may involve retroviral vectors which are capable of efficient and durable transduction of cells but carry some risks. Alternatively, adenoviral vectors allow for safer but more transient transduction. Many nonviral methods have also been used, including electroporation and lipofection, but generally with less efficiency compared to viral methods. These approaches are reviewed in detail by Gilligan and Dwyer (Gilligan and Dwyer, 2017).

Large-scale manufacturing of exosomes will undoubtedly be a challenge, and the prospect of cost-effective, reproducible and scalable systems remains distant. However, some principles can be discussed at this time, which apply equally to the production of unmodified and to gene-modified exosomes. In every case, the manufacturing strategy will require expansion of the donor cells, and this already presents problems in terms of consistency and quality, at least for MSCs. Traditional cell expansion methods for MSC production under Good Manufacturing Practice (GMP) guidelines are required. In addition, several further processing steps will be needed, including collection of the cell-conditioned media and fractionation of the exosomes by centrifugation, size exclusion chromatography, tangential flow filtration or precipitation (Phan et al., 2018). In addition, sensitive and accurate quality control tests will be required to confirm the identity of the exosomal product and ensure it is free from *Mycoplasma* and bacterial and viral contaminants. A GMP-grade protocol for the production of human MSC-derived EVs has recently been described. A further challenge is that the isolated exosome populations are not uniform: in one study it was shown that MSCs can secrete three different types of vesicles with varying lipid, protein and RNA content (Lai et al., 2016). It is also the case that the nature of the exosome population will depend on the health and conditioning of the cells from which they are derived (Pegtel et al., 2014; Yáñez-Mó et al., 2015a,b; Xin et al., 2014) and are likely to vary also from donor to donor.

Conditioned Media

Much has been written about the so-called paracrine activity of MSCs and the role that these secreted factors may play in the therapeutic mechanism. The interest in paracrine mediators arose when it became clear that the repair responses elicited following delivery of MSCs were less likely to involve directed differentiation of the delivered cells and more likely to involve contact and noncontact interactions with host cells. The first observations regarding the secretory activity of MSCs in vitro were made by Haynesworth (Haynesworth et al., 1996), and this gave rise to the idea that cell-conditioned media could potentially have therapeutic activity. Furthermore, in early studies of MSC treatment for osteoarthritis, it was apparent that a significant repair response was associated with delivery of cells, but retention and engraftment were noticeably low (Murphy et al., 2003), suggesting a trophic effect rather than a direct cell effect. Several studies have shown that the therapeutic response associated with cell delivery can be replicated using cell-conditioned media only, for example, in tendon (Sevivas et al., 2018) and bone (Hwang et al., 2018) repair, myocardial infraction (Timmers et al., 2007), lung (Ionescu et al., 2012) and osteoarthritis (Hassan et al., 2017). The application of the MSC secretome is comprehensively reviewed by Vizoso et al. (2017). In one illustrious example of the application of cell-conditioned media in lung repair in the mouse (Zhang et al., 2018), 15 mL of serum-free medium, conditioned for 24 h in the presence of 5×10^6 cells, was concentrated to 600 and 30 μL of this was delivered, representing a cell-equivalent of 250,000. Studies such as this highlight an alternative way forward where the product is no longer a living cell. At the very least, storage, manipulation, shipping and delivery of the conditioned concentrate will represent significant logistical advantages.

The obvious next step in developing conditioned media for therapeutic use is to replace the cell-conditioned media with fully defined synthetic formulations containing only recombinant or synthetic factors. This approach is much more aligned with the conventional practice of large pharmaceutical companies but will require an exhaustive understanding of components of conditioned media. Several steps have been taken in this direction. For example, in the aforementioned lung study, it was demonstrated that IGF-1 partially reproduced the protective effect of the conditioned media.

The use of conditioned media as a medicinal product presents clinical and manufacturing advantages because it is not a live cell product. The advantages include ease of delivery, storage and a more robust and quantifiable potency evaluation. From a manufacturing perspective, the cells may become discarded waste after conditioning. However, a manufacturing model such as this will still pose substantial upstream and downstream challenges, not especially different from the live cell model. It will require advanced cell manufacturing platforms, scalable bioreactors, the

establishment and maintenance of master cell banks and working cell banks, as well as a similar range of quality tests. It seems more likely that there will be two simultaneous product streams – the live cell product and the media conditioned by the live cells. In this case, the manufacturing challenges will be even more substantial, requiring protocols for expansion of cells and for processing and harvesting both cells and media (Fig. 15.3). Additional in-process and quality control testing will be needed for both product streams.

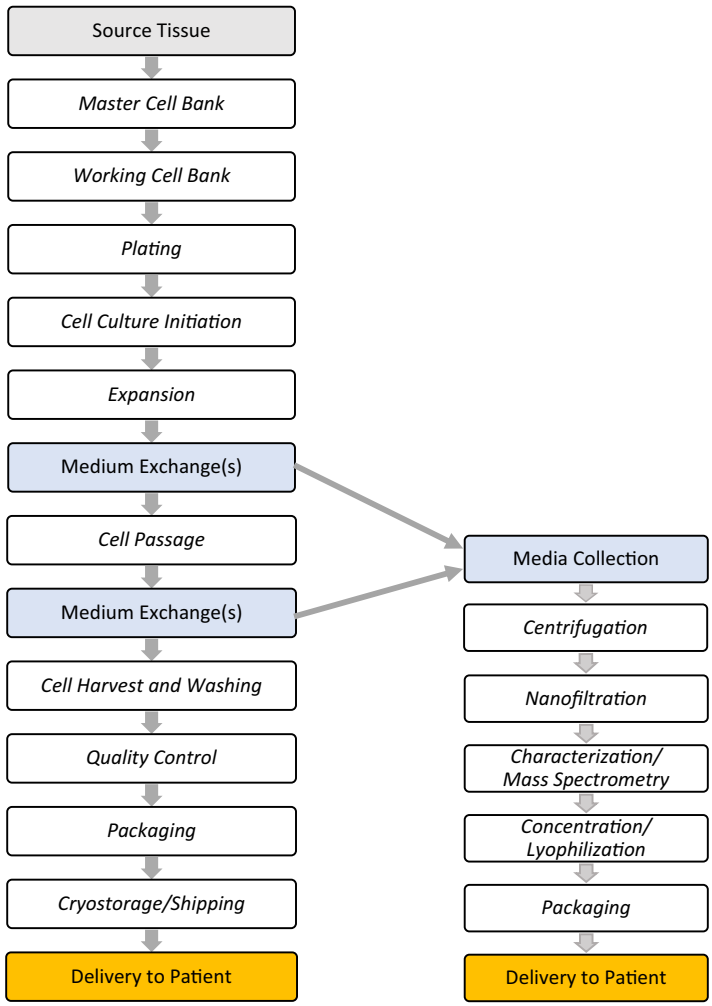

Figure 15.3 Flow diagram depicting the step-wise process for manufacture of allogeneic cell product (left) and additional or alternative steps required (right) for manufacture of a conditioned media stream.

Gene-Modified Cells

Without doubt, gene therapy provides exciting approaches for tissue repair and treatment of diseases. The use of gene-modified MSCs has received considerable attention in regenerative medicine and oncology, and this approach may possibly provide advantages over direct viral plasmid delivery. A variety of methods of gene modification of MSCs has been addressed, including viral transduction using adenovirus (Kang et al., 2004), adeno-associated virus (Stender et al., 2007) and *lentivirus* (Kallifatidis et al., 2008). A protocol has also been described for the utilisation of *Sendai virus* for MSC gene modification (Miere et al., 2014). Nonviral approaches have included electroporation, magnetofection and microinjection (reviewed in Mellott et al., 2013) and membrane permeabilising techniques (Medepalli et al., 2013; O'Dea et al., 2017). The feasibility of these approaches has been demonstrated in many studies, but the effectiveness of gene-modified MSCs has yet to be tested in patient studies.

Current mechanistic insights into the limited tissue engraftment, trafficking and apoptosis of infused MSCs will undoubtedly influence strategies for their use as gene delivery vehicles, and it seems more likely that, with the exception of potential oncology applications, these approaches will rely on local, highly targeted, rather than systemic, delivery. There are some intriguing examples that illustrate the potential of these approaches. For example, Ren et al. (2013) showed that GDNF-expressing MSCs delivered to the substantia nigra and striatum in cynomolgus monkeys offered protection in a model of Parkinson's disease. Other studies have suggested that MSCs expressing TRAIL may have application in cancer therapy and notably in glioblastoma. It will remain to be seen whether genetically modified MSCs represent an optimal cell choice for such applications.

PERSPECTIVES

It seems clear that, after several years of indifferent or modest results, there is now a revival of interest in MSCs as advanced therapeutics for complex conditions. It is also clear that there is no room for complacency regarding the characterisation of the cell product or the consistency or reliability of manufacturing protocols. There is still a long way to go to address mechanism of action and potency. It seems that the next generation products discussed here will be developed simultaneously with improvements in the parent technology.

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