Chapter 14
Advances in Lentiviral Vector-based Cell Therapy with Mesenchymal Stem Cells

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Abstract The field of possible application of mesenchymal stem cells in medicine and research expanded tremendously with the advent of improved Lentiviral-vectors capable of inserting stable copies of genes of interest and expressing proteins or biologically active RNA species ad libitum, performing delicate gene editing or active gene silencing or serving as advanced drug delivery systems utilized in ex vivo cell therapy. The combination of these two fields has created a number of new areas of research in the landscape of modern medicine which are now extensively studied and discussed here. These areas include tissue engineering, tissue repair, wound healing and tissue implants, anticancer therapies, angiogenesis, myocardial infarction and repair as well as understanding and treating acute lung damage and injury. In addition, genetically modified, tagged MSCs are being intensively deployed in research and therapeutic attempts of the various ailments of the central nervous system including Parkinson's disease, Alzheimer's disease, various phases of acute ischemia and trauma. The emergence of new and important data for type II diabetes research is being followed with treatment suggestions and studies of senescence to find novel applications for genetically engineered MSCs. We find in general that genetically modified MSCs are at the cusp of breaking through from basic research into the next phase of clinical trials.

Keywords Alzheimer's disease • Angiogenesis • ARDS • CD105(+), CD90(+), CD73(+), CD14(−), CD19(−) or CD79a (−), HLA-DR1 (−) • CD105 (+), CD90 (+), CD73 (+), CD34 (−), CD45 (−), CD11b (−) • Cell therapy • Cerebral ischemia • Chronic granulomatous disease • Cystic fibrosis • Diabetes • Drug delivery • Duchenne muscular dystrophy • Gene editing • Gene silencing • Gene therapy • Lentiviral vector

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14.1 MSCs as Targets for Lentiviral Vector

Stem cells, including the mesenchymal stem cells (MSCs), are very close manifestations of Plato's imagery of the shadows on the cave wall, since they are difficult to study outside their intimate interactions with their microenvironment [288]. Our observation methods change their responses and characteristics [9, 74, 82, 103, 145, 252], as in quantum physics, when the observation changes the observed. With that caveat, we can admire the rapid development in stem cell research. Alas, the difficulties in research are faithfully reflected in the confusion in the nomenclature used for describing and classifying stem cells, including the classes of stem cells of mesodermal origin. The recent definition of MSCs by Dominici states that MSCs are a stromal cell type, possessing the following characteristics and markers: plastic adherence in cell culture, specific surface antigen expression of CD105(+), CD90(+), CD73(+), CD34(−), CD45(−), CD11b(−) or CD14(−), CD19(−) or CD79a(−), HLA-DR1(−) and multi-lineage in vitro differentiation potential (osteogenic, chondrogenic, and adipogenic) [59]. However, this definition would neatly exclude CD34+ hematopoietic stem cells (HSCs), while one also could argue that the hematopoietic stem cells are just a specialized subclass of the mesenchymal progenitors [180]. Another subset of MSCs that express hyaluronan (CD44), an adhesion molecule important for stem cell homing [14, 125, 281], would also be excluded, but their perivascular equivalents could be considered to be true MPCs [180]. It becomes even more complicated if we include the results of (stem) cell reprogramming, when more or less differentiated cell types are regressed into less differentiated, pluripotent cell types [91, 185, 192, 239], providing us with a never ending stream of novel biomarkers, more often represented by whole proteome analysis [1, 203]. Time will tell, what are the biomarkers and criteria for properly characterizing the particular stem cell populations, but there is a functional definition lingering around as a firm conceptual handle on the idea of cell plasticity of which stem cells are prominent representatives [21, 22]. The plasticity indicates the ability of matured or not fully differentiated cells to differentiate into novel cell types, or more accurately, it describes the existence of cells specialized into becoming progenitors of differentiated cells while sustaining their own type and maturation level. Combining this with the embryology and origins of cell lineages from the three primordial "dermata" (ecto-, endo-, and mesoderm) provides us with a useful and generalized definition of MSCs being the pluripotent, self-renewing stromal cells of mesenchymal origin and allowing us to determine the specific biomarkers later, at our convenience, as the state of affairs in mesenchymal stem cell biology progresses and solidifies. There is no doubt that we will find the appropriate placement for the specialized subtypes as well as the proper and practical placement of some of the induced pluripotent cells (iPCs) in the realm of MSCs.

Regardless of the exactitude of the classification, the (omni-) presence of the mesenchymal cell lineages in all of the organs and tissues [11, 102, 116, 132, 180] renders them good candidates not only for general stem cell therapy [22, 73, 102, 235], but even more promising is the potential use of MSCs for gene therapy [35, 45, 176, 196], cell reprogramming [9, 36, 252], delivery of bioactive molecules [163, 196], and tissue engineering [4, 82, 153]. In addition, a number of new issues are arising from the results describing the importance of stem cells in inducing and sustaining the malignant phenotype and the potential therapeutic targeting of a wide range of the elusive cancer stem cell types [27, 183, 217, 244]. The genetic modification of MSCs and associated cell types with Lentiviral vectors opens their application beyond reliance upon the innate properties of the cells. Expression of proteins that can modulate their biology or therapeutic properties enormously expands their utility for therapy.

The lack of a crisp definition of all the stem cell types affects targeting of Lentiviral vectors to specific subsets of stem cells. However, recent successful efforts in the pseudotyping of Lentiviral vector is a step in the right direction. The use of the VSVg pseudotype expanded the tropism of the Lentiviral vectors and, as a result, practically any cell type can be targeted and the narrowing of the tropisms by developing novel vector-pseudotypes will be addressed. The emergence of single chain antibodies as pseudotype indicates that we can expect a rapid expansion of this technology in the near future and will result in a precise tool for studying cell lineages.

We focus and limit our review on the recent progress made in stem cell research using Lentiviral vector-based gene delivery, a method that is emerging as the safest and most effective way to modify (stem) cells permanently or temporarily, if using non-integrating versions of the novel generations of Lentiviral vectors, both of which have clear potential for a wide range of research application in preclinical studies as well as therapeutic applications.

14.1.1 Lentiviral Vectors: Overview of Design and Safety Issues

The most commonly used Lentiviral vector framework is HIV-1 based although HIV2, SIV (Simian Immunodeficiency virus), FIV (Feline immunodeficiency virus) have been successfully tested; see review by Dropulic [62]. The native HIV-1 is a human pathogen; but it had been modified to eliminate pathogenicity and increase safety before considering it as a broadly available tool for gene transfers.

Typically, Lentiviral vector are generated by trans complementation, a process that separates the essential components of HIV (the genes encoding Gag-Pol, Rev, and Env) into separate plasmids, which lack the packaging signal and, therefore, can never end up in a packaged vector unless they appear in a recombinant sequence [63]. The components (Tat, Vif, etc.) responsible for pathogenicity by upregulation of transcription [54] and export of genomic RNA to the cytoplasm have been successively removed from the constructs. The potential for a recombination event is minimized, and for all practical purposes avoided, by carefully editing the genes

and using codon degeneracy to reduce the chances of recombination with the wild-type virus. These separate plasmids are used to co-transfect a packaging cell, typically HEK293, along with a payload plasmid that carries the packaging signal necessary for starting the envelope formation and encapsidation of the mRNA that carries the payload gene (s) as well as the 5′ and 3′ long terminal repeats (LTRs) necessary for integration into the transcriptionally active regions of the host chromosomes. Packaging is a delicate process, which ensures that with the RNA, appropriate tRNAlys, protease, integrase, and reverse transcriptase enzymes are carried by the vector with the packaging elements necessary for successful cell entry, reverse transcription of vector RNA to DNA, transport of that DNA into the nucleus, and the permanent integration of the DNA into host chromosome. The ENV gene encodes the protein GP160 that is cleaved into trimer-forming GP120, which appears as spikes decorating the vector particle; and GP41, that carries a transmembrane region and a carboxy terminal sub-domain that interacts with the nucleocapsid within the envelope. The N-terminal domain has a fusogenic domain that facilitates cell entry by fusing the outer membrane of the vector with the cell membrane. A region further down to from the amino terminal region also binds to GP120 which in turn binds to primary HIV-1 receptors on the target CD4+ of T lymphocytes. This property if left unmodified would significantly limit the usability of the Lentiviral vector, as very few cells types can be directly infected by HIV-1. Pseudotyping overcomes this limitation and permits targeting to any mammalian cell.

Pseudotyping essentially replaces the original HIV ENV gene with a corresponding molecule from other viruses and carries over the cell-targeting specificity (i.e., the tropism of the virus) and obviates some of the safety concerns related to GP120 [258]. The list of successful pseudotypes and cell tropism is rather lengthy [18, 76–79, 105, 222] and growing. The most successful pseudotype so far uses the ENV from vesicular stomatitis virus (VSVg) that successfully broadens the tropism to cells in the brain, kidney, and liver amongst other. It extends to mesenchymal (stem) cells, even those in nondividing (resting) state [77, 136, 280]. Filovirus ENV pseudotypes shift the tropism to a more limited set of cells, airway epithelial and endothelial cells [130, 148]. Baculovirus GP64 and Hepatitis C virus E1 and E2 pseudotypes redirect the vectors toward liver cells targeting their respective receptor, CD81 (tetraspanin) [19]. Rabies virus ENV has been shown to efficiently retarget the vectors to neuronal cells [162, 262]. RD114 ENV pseudotyped Lentiviral vector show preference for hematopoietic cellular compartment [20, 56, 85, 115, 221, 268]. However, some applications require targeting a specific cell type, which is not necessarily covered by the available pseudotypes listed above. In those cases, new targeting methods have been developed to further tighten the tropism of Lentiviral vector by co-expressing cell-specific coreceptors that recognize one of the cell-type specific markers.

The payload plasmid components providing the backbone for the transfer vector in the early HIV vectors were composed of a 5′ LTR, followed by a major splice donor site, a packaging signal site encompassing the packaging signal components of the 5′ region of Gag (necessary for high efficiency packaging and high vector titer) and a deletion of the rest of the gag gene. Deletion of the U3 region from the 3′ LTR promoters became also possible by relaying on constructing genes with their

own promoter(s). The latest generation of Lentiviral vectors carry an additional safety element, the self-inactivating LTR (SIN Lentiviral vector, [114]) replacing the LTR with an HIV-independent promoter from Cytomegalovirus (CMV). In these vectors the LTRs are modified in a way that upon integration they lose their intrinsic promoter ability reducing genotoxic potential. In addition the irreversible changes that occur during integration diminish the ability to mobilize after integration and to recombine with other elements to form a full-fledged, replication capable virus [25, 279]. The formal proof of increased safety is still lacking, ironically, because of the inability to create and detect RCL capable viruses from Lentiviral vector-treated cells [25], indicating that this risk is mainly theoretical.

The removal of RRE and the associated splice donor and acceptor elements results in significant loss of transduction efficiency of the vector [127], while adding a 100 nucleotide central polypurine tract (central DNA flap) restores the transduction efficiency by improving the reverse transcription and nuclear transport efficiency [47, 283]. The woodchuck hepatitis virus transcriptional regulatory element (WPRE) is another widely used regulatory element added to the Lentiviral vector backbone to stabilize the transcription transgene mRNA levels and improve transgene expression [292]. However, an open reading frame of the oncogenic WHV-X element has been found within the native WPRE sequence [128], so the sequence has been modified to remove the translation start site [282].

Further optimization continues to improve the safety of Lentiviral vector, such as isolating the integrated vector DNA to prevent translation beyond the vector boundaries by adding isolating elements. However, the insulators have themselves proven to be genotoxic in some instances, and no proof has emerged that such isolators are truly needed [100]. Gene switches such as Tet-On and -Off have been added to subsequent generations of Lentiviral vector and proven to be highly functional, operating with very low leakage [194, 260]. The Cre-Lox system has also been successfully implemented in the Lentiviral context allowing high efficiency engineering and sophisticated, site-specific recombination techniques including the delivery and irreversible switching by small hairpin RNA (shRNA) expression [135], a tool extensively used in gene function analysis [37, 193].

A major concern regarding the safety of Lentiviral vectors has been the potential genotoxicity resulting in oncogenesis, as observed previously during clinical trials for treating X-linked severe combined immunodeficiency (X-SCID) with transplanted HSCs treated with murine oncoretroviral vectors carrying the gamma chain of IL2R genes [96–99]. The preferential insertion of oncoretroviral vectors in proximity of the LMO2 proto oncogene and the subsequent constitutive activation of the proto-oncogen driven by the enhancer element (LTR) in the vector resulted in uncontrolled cell proliferation. However, in a series of studies comparing oncoretroviruses and Lentiviral vector, it has been shown that while the oncoretroviral vectors trigger a dose dependent acceleration of cancer onset in a mouse transplantation model sensitive to cancer-triggering genetic changes (CDkna2−/−), Lentiviral vectors lacked such activity [172, 175] even though the vector integration rate was significantly higher. This important observation implying that a low level of insertional mutagenicity has been confirmed independently by several groups indicating

a favorable safety profile for Lentiviral vectors while emphasizing the importance of vector design and avoidance of strong enhancers in the vectors [32, 169, 171].

Recent clinical trials have supported the good safety profile of Lentiviral vectors. There have been no oncogenic effects reported in any of trials using Lentiviral vectors to date [30, 83, 123, 143, 161, 195, 210, 276].

14.2 Development of Gene Therapy Techniques Using Lentiviral Vectors

14.2.1 Gene Silencing with RNA Interference

Gene silencing by small interfering RNA (RNAi) is based on duplex formation between the mRNA and a short complementary micro RNA or small inhibitory RNA, each having the ability to interfere with the protein synthesis and downregulate the expression levels of the targeted protein. A major problem with the inhibitory RNA technologies is the short half-life and delivery of the RNAi. This can be resolved using Lentiviral vectors encoding artificial genes with appropriate micro RNA sequences that can be integrated into the host cell DNA and efficiently transcribed into primary micro RNA that utilizes the natural intracellular processing by microprocessor complex formation with Drosha to form small hairpin RNA (shRNA) in the nucleus. The exported miRNA is subsequently cleaved by Dicer and produces the complex forming inhibitory RNAi. The process is rather complex, but efficient to produce a significant blockade of protein expression that may be incomplete but readily achieves significant reduction, that is adequate for gaining insight into the function of the targeted protein and efficient enough for phase I and II clinical trials, though no therapeutic use has been approved by the FDA. It is interesting to note that MSCs are capable of secreting cholesterol-rich phospholipid microparticles encapsulating miRNA, and therefore have the potential to facilitate intercellular communication and act as regulatory agents in their microenvironment [38]. An excellent review has been published on the biogenesis and clinical applications of small RNA compounds by Davidson and McCray [49].

Hematopoietic or general pluripotent stem cells are often selected targets for RNA interference-based interventions and one of the promising efforts deal with creating artificial virus resistance genes and virus resistant somatic cells. Preventing HIV infection by reconstituting the immune system with such stem cell-derived virus-resistant progeny has been used as model system with significant clinical relevance [121]. The idea is that an efficient HIV infection requires virus entry through the CD4 surface antigen and one or more virus co-receptors, among which CCR5 has been shown to play an essential role in the case of R5 tropic viral strains involved in primary HIV infection. Clinical data indicate that CCR5 deficiency or certain mutations in this co-receptor protect the infected individuals from the onset of full blown AIDS, and the hope is that the artificial knockdown of CCR5 using gene

therapy and RNAi will achieve similar protection [8, 57, 121, 146, 233]. The relative inefficiency of the CCR5 suppression remains a significant issue, but major improvement and complete knockdown of CCR5 have been achieved with somewhat longer (28 base instead of 23) shRNA [7].

Mesenchymal stem cell research is taking full advantage of the shRNA techniques by characterizing the subtle, and not so subtle, changes induced by individual gene knockdowns. It is a long held view that mechanical stresses and mechanical characteristics of stem cells, as well as the microenvironment, can affect stem cell proliferation and differentiation. Lentiviral vectors are excellent and efficient targeting tools for these stem cells, even resting ones, and can deliver the shRNA without causing major changes and stress that would otherwise change the stem cells on its own. Chowdhury et al. studied the spreading response of MSCs and showed that myosin II, F-actin, Src, or CDC42 were essential for cell spreading and changes in the mechanical characteristics ("softening") of the stem cells led directly to the downregulation of the OCT3/4 gene. This indicates the possibility that small mechanical events may affect the embryo and developing tissues and even transplanted stem cells [41].

Another area of efficient use of Lentiviral vectors and RNAi technology in stem cell research is the production of transgenic embryos which carry knockdown genes. Production of transgenic embryos is highly efficient, and if the fertilized egg is transduced at a single cell stage, the entire germ line is affected, or partial chimerism can be achieved if multicellular embryos are treated with Lentiviral vectors. An example of such a study is that by Wang et al., in which they showed that the knockdown of RunX1 in embryonal tissues and MSCs by Lentiviral vector-delivered interfering RNA blocked chondrogenesis in limb buds [257]. The technique has been shown to be very efficient for transgenesis, as high as 44% average rate of germ-line transmission can be achieved [227], providing a new source of gene-modified MSCs. A recent comprehensive review of the use of naturally occurring regulatory miRNA technology in mesenchymal stem cell research has been written by Guo et al. [93], indicating that stem cells have discrete and distinct expression profiles that can account for intrinsic stem cell properties such as self-renewal and pluripotency, a property that cannot longer be overlooked by experts dealing with MSCs. The accumulating data indicate that the progenitors and terminally differentiated mesenchymal cells can be tracked and defined by function-related miRNAs in addition to the already established sets of surface markers. The miRNAs already identified affect osteogenic differentiation, chondric differentiation, adipogenic differentiation, myogenic differentiation, neuronal differentiation, wound healing, and replicative senescence. These advances open a wide array of possibilities to direct the differentiation patterns of the stem cell population temporarily by using non-integrating Lentiviral vectors that are automatically lost from dividing cell populations and lead to the natural disappearance of control signal after a few cell division but potentially giving a push to the original stem cell population to develop in a preferred direction.

Extensive progress has been made in regards to the elucidation of the Hedgehog signaling pathway in MSCs using RNA interference delivered with Lentiviral vectors. The data suggest that at least some of the elements indeed act through the

regulatory miRNA network, by downregulating the cellular miRNA levels. The data, however, also suggest significant off-target effects of the interfering RNA molecules and indicate that we are a long way from the potential clinical use of the elucidated networks [124].

An ingenious method was devised by Hu et al., to prepare the brain for traumatic interventions (surgery, extensive stem cell transplantations, etc.) by downregulating the cerebral Matrix Metalloproteinase 9 (MMP9) using Lentiviral vector and MMP-9 shRNA 2 weeks before the trauma. The knockdown of MMP-9 with the shRNA proved to be an effective way to preserve the blood–brain barrier, and they achieved significant reduction of brain infarction volumes, reduction of brain water content and evans blue/IgG extravasation (measure of edema formation) as well as a reduction in the neurobehavioral deficit in their rat brain trauma model [108] implying a potential for improved protocols for traumatic brain interventions needed for more extensive type of intracranial stem cell implantations.

14.2.2   Gene Transfer into MSCs

As mentioned earlier, Lentiviral vectors provide a very efficient method for generating transgenic embryos, significantly reducing the need for the generation of a high number of embryos to establish new sources of gene-modified stem cell lines, embryonic, or other [227]. The Lentiviral technology is able to deliver a payload of 6–8 kB very efficiently, but payloads of 10kB can be handled and delivery of 12–13 kB is possible, at a cost of lower efficiency. This payload-carrying capacity allows the delivery of very large genes such as the gene encoding blood clothing factor VIII, a 2,351 amino acid long protein together with its stabilizer, the von Willebrand factor (2,813 amino acids in its native form) simultaneously or, one may need to use domain-engineered and shortened version of both; similarly it can be used to deliver all three chains of an IgM molecule in a single, tri-cistronic complex. The implication is that the Lentiviral vector system has sufficient payload capacity to deliver a number of relevant genes together with several supporting molecules envisioned for highly complex gene therapy scenarios currently outside the scope of monogenic gene therapy as practiced today. It may be used to target diseases with multi-gene disorders such as high blood pressure, arthritis, or diabetes in the future.

14.2.3   Gene Editing Using Zinc Finger Nucleases Encoded Within Lentiviral Vectors

Zinc-finger nucleases (ZFNs) have the remarkable ability to (a) bind to a specific location in the double-stranded DNA; (b) break the double-stranded DNA at that specific location and, if an endogenous repair template is provided, (c) initiate homology-directed repair, restoring the integrity of the newly edited double-stranded DNA. As their name implies, there is a specific DNA-binding part of this class of

enzymes that consists of a tandem repeat of DNA-binding zinc-finger motifs, hence the DNA binding specificity and a catalytic domain, FokI. For DNA cleavage to occur, FokI has to dimerize, one on the sense and the other on the antisense strand, while the zinc-finger domains attach to the right target half site and the left target half site. Upon binding, a nick with a 5′ overhang is initiated by FokI between the target sites and the homology-directed DNA repair mechanism is activated. What makes this configuration useful is that the spacer between the two target half sites can be several hundreds or even thousands of base pairs long and by providing a template for the activated repair mechanism, a novel DNA sequence of equal length can be introduced into the DNA; see a recent reviews by Caroll [29] and others [50, 101, 117, 134, 246].

Fundamentally, two factors determine the efficacy of the DNA editing or repair that the technology allows. The first is the specificity of the zinc-finger binding, which also determines the length of the spacer and the proper specificity and uniqueness of the binding site and allows the minimization of the off-target effects that may be introduced by similar sites far away from the desired and targeted locus [101]. Huge efforts are being made to tailor the zinc-finger nucleases for particular applications and improving the selectivity by successfully engineering the DNA-binding specificity of the binding domain [3, 101, 158, 201, 220]. The second factor is the efficient delivery of the ZFNs and the template DNA by vectors. While the early attempts relied on retroviral vectors, adeno and adeno-associated vectors, and even baculovirus vectors, the recent advances in the field clearly indicate that the Lentiviral delivery system is considered to be a safer and more efficacious route. As high as 50% conversion rate can be achieved with lentiviral delivery in a variety of cell lines and human embryonic stem cells [154] as compared with the earlier best rates of 18% with other methods in human and other species [3, 101, 197, 198, 201, 220, 245, 264].

14.2.4   Using Lentiviral Vectors in Tissue Engineering and Repair

One of the many Holy Grails of medicine, the ability to replace diseased tissue or even entire organs, seems to be hovering at the not too distant horizon. There is rapid progress in a wide range of areas, but at the center of the solution is almost always biocompatible scaffolding that is populated with a wide variety of cells. The strategically positioned cells find their place within the 3D structure, propagate, differentiate, and fill the available space, while producing a structure that can replace or enhance the damaged tissue in the form of various implants or prosthetics. As for scaffolding, the options are quite numerous, including those obtained from cadavers or live organs (animal or human origin), by removing the cells while preserving the fibrous tissue that maintains the basic morphology of the organ. Alternatively, a scaffold can be printed with various 3D printers [126, 159, 214–216, 229, 255, 261]. Processed cartilage can also result in scaffold and it can be used to rebuild and regrow an implantable ear, nose, or cartilage for trachea reconstruction [174].

The culture, expansion, and differentiation of human MSCs into artificial tissues represent a very complex series of events and Lentiviral vectors often serve as excellent research tools for marking, visualizing, and tracking the process [253], or modifying the gene or protein expression patterns [68]. A number of tissue engineering attempts have reached the clinic and Lentiviral vector have played various roles in the advancement of the technology. A very promising technology is the use of these scaffolded artificial tissues employing MSCs and Lentiviral vectors for delivering biologics for prolonged times.

Van Damme succinctly described the potential of these artifical tissues built on scaffolds and providing artificial implants for drug delivery. Lentiviral vectors were used to transduce mesenchymal cells to express green fluorescent protein (GFP) or FVIII. Expression was superior compared to oncoretroviral transduction, showing consistently higher transduction rates and expression remained high for several months post-transduction. The transduced cells retained their stem/progenitor cell properties, and they were still capable of differentiating along adipogenic and osteogenic lineages in vitro, while maintaining high GFP and FVIII expression levels. Implantation of Lentiviral vector-transduced human bone marrow mesenchymal cells using collagen scaffolds into immunodeficient mice resulted in efficient engraftment of gene-engineered cells and provided sites for transgene-expression in vivo. In addition to the bone marrow-derived stem cells, adipose tissue-derived mesenchymal stem cells have been shown to be amenable to populate implantable scaffolds and retain the potential to differentiate into osteogenic cells. Some of these scaffolds have been engineered for use in reconstructing craniofacial bone defects. Lentiviral vector have been used to deliver fluorescent proteins to track cells during manipulation such as osteogenic differentiation. The GFP-marked stem cells and their progeny remained fluorescent over the 8 weeks of the study period. The GFP-marked stem cells were successfully induced into osteogenic cells both in monolayers and three-dimensional scaffolds. Quantification showed no decrease in staining of the osteoinduced stem cells indicating the efficiency and durability of the labeling [256].

14.3   Aortic Implants

Tissue engineered vascular grafts built on bilayered elastomeric poly (ester-urethane) urea scaffolds and seeded with pericytes have shown promise in the past. However, in vitro endothelialization is still an issue for the use of these types of grafts. Doebis et al. reported in 2006 enhanced endothelialization using allogeneic endothelial cells or their precursors, expressing recombinant anti-alpha-MHC I single chain antibody to prevent rejection. The recombinant antibody was delivered efficiently ex vivo using Lentiviral vector, and has significantly reduced the MHC-1 expression levels as well as the killing of allogeneic cells by MHC-1 specific CD*+T cells [58]. The results suggest that these allogeneic cells may provide a suitable alternative supply for the lining of vascular prostheses.

Endothelial cells and their precursors are attractive targets for gene therapy, both for the treatment of cardiovascular disease and for the systemic delivery of recombinant gene products directly into the circulation. There have been a few reports which show Lentiviral vector-mediated gene transfer efficiency. Sacoda and colleagues compared the effectiveness of Lentiviral vector compared to adeno and oncoretroviral vectors. Bovine aortic endothelial cells (BAECs) were infected, in vitro, with these viral vectors. Transduction efficiency of beta-Gal gene transfer in BAECs by adenovirus, Lentiviral vector, or retrovirus at a multiplicity of infection (MOI) of 10 (determined on HeLa cells) was 69 ± 11, 33 ± 8, or $22 \pm 6\%$ respectively. At higher MOI [50] both adenovirus and Lentiviral vectors achieved an almost 100% transduction rate. However, retroviral vectors showed only $48 \pm 6\%$ at MOI 50 and no increase at MOI 100. The percentage of beta-Gal positive cells decreased rapidly at longer passage of cells after being transduced by adenovirus. In contrast, Lentiviral vector and retrovirus vectors mediated transductions showed sustained higher percentage of positive cells. Furthermore, the transductions by Lentiviral vectors had no significant effect on viability of BAECs suggesting that for long-term cell therapy the Lentiviral vectors have overall the best features [219]. Expressing IL10 in similar settings in the early, initiation phase, also inhibited and delayed the onset of the rejection process [287].

One of such cases in which the performance of the endothelial cells may need to be boosted is to increase the resistance to ischemia–reperfusion injury of the vascularized transplants and implants or normal tissues undergoing prolonged surgery. This is a condition which occurs too frequently and is responsible for devastating tissue injury caused by systemic activation of the complement system. Lentiviral vectors can be used to force the over-expression of the anti-apoptotic gene, Bcl-xL and indeed, it has shown significant protection from early apoptotic loss of vascular endothelial cells [286].

14.4   Periodontal Stem Cells

Recently, tooth tissue engineering has attracted more and more attention. Stem cell-based tissue engineering is thought to be a promising way to replace a missing tooth. The potential MSCs for tooth regeneration mainly include stem cells from human exfoliated deciduous teeth (SHEDs), adult dental pulp stem cells (DPSCs), stem cells from the apical part of the papilla (SCAPs), stem cells from the dental follicle (DFSCs), periodontal ligament stem cells (PDLSCs), and bone marrow-derived MSCs (BMSCs). A recent review by Peng et al. shows promising progress [190]. However, in practice, tissues other than bone marrow can serve as stem cell donors, including adipose tissue, periodontal ligament, and pulp for oral tissue regeneration [206]. The experimental data suggest that not only the stem cells ex vivo, but cells in the osteogenic tissue are amenable to direct transduction by Lentiviral vector [259]. This opens up the periodontal reconstruction interventions to the beneficial effects of gene therapy enhancing the wound healing and improving

engraftment by expressing growth promoters at low and slowly decreasing concentrations. Estrela published an excellent review on the potential of MSCs in regeneration of dental tissues [68] and Rodrigues-Loza reviewed the mesenchymal cell types recovered from dental tissues [208]. Other data clearly show that the primary osteogenic cells are efficiently transduced by Lentiviral vector, and that their infusion into the mandible is a feasible method for locally delivering DNA to primary osteogenic and bone cells in rat models [259], indicating that future applications in vivo dental implant enhancement, using dental scaffolding, bone healing, and tooth regeneration may be feasible. Recent efforts extend toward engineering dental repair by changing the expression of growth factors and bone morphogenic proteins leading to dentin formation, as discussed in a 2011 review by Casagrande [31] and which seem to be amenable to cell therapy efforts with non-integrating Lentiviral vector.

14.5   Wound Healing

One of the tissues that is often injured but that presents difficulties when it comes to healing and repairs is the tendon. Enhancing the healing process by in situ overexpression of helper factors such as IL10 could reduce recuperation time and perhaps improve the quality of the repair. Richetti et al. reported promising results in a murine model of patellar tendon injury after direct injection of an IL10 transgene using Lentiviral vector. Although the tendons showed no obvious histological difference, the IL-10-treated groups had superior mechanical characteristics by day 42 [205]. Although the mechanism of wound healing in tendons is not yet understood, the involvement of MSCs is suspected and delivery of additional factors that partake in healing process is discussed by Meyerose and Ashlan [13, 163].

Recent findings by Shamis and colleagues [230] demonstrated that embryonic stem cells could be directed to specified and alternative mesenchymal cell fates whose function could be distinguished in engineered human skin equivalents. Lentiviral shRNA-mediated knockdown of hepatocyte growth factor (HGF) resulted in a dramatic decrease of HGF secretion from cell lines (EDK cells) that led to a marked reduction in their ability to promote keratinocyte proliferation and re-epithelialization of cutaneous wounds. In contrast, H9-MSCs demonstrated features of MSCs but not those of dermal fibroblasts, as they underwent multilineage differentiation in monolayer culture, but were unable to support epithelial tissue development and repair and produced significantly lower levels of HGF. Characterization of these induced mesenchymal cells in 3D, engineered human skin equivalents demonstrated the utility of this tissue platform to predict the functional properties of stem cell-derived fibroblasts before their therapeutic use in reconstructive skin transplantation and wound healing.

Inhibition of hyper-keratinization by expressing a mutant form of TCGf Beta3 that has lost its binding site for latency-associated peptide, reduced the re-epithelialization density and fibroblast/myofibroblast trans-differentiation within the wound

area [251] in a mouse skin wounding model. The expression of this mutated gene was achieved by injecting Lentiviral vectors encoding the mutTCGF Beta3, into the regenerating tissue and the changes induced by this intervention predict a significant decrease in keloid formation and provide a potential model for preventing the painful disfigurement that follows the abnormally strong skin remodeling and scar tissue formation that oftentimes accompanies wound healing. The data indicate that future stem cell therapy with carefully designed interventions for patients prone to scar tissue formation could find wide spread application.

14.6   Corpus Cavernosum

One of the causes of erectile dysfunction is the damaged penile cavernous smooth muscle cells (SMCs) and sinus endothelial cells. Song reports that it may be feasible to restore these cells by applying MSCs to penile cavernous ECs or SMCs. For this purpose immortalized (via Lentiviral vector encoding v-myc) human bone marrow mesenchymal stem cell line B10 cells were transplanted into the cavernosum of Sprague–Dawley rats and harvested 2 weeks later. The expression of CD31, von Willebrand factor (vWF), smooth muscle cell actin (SMA), calponin, and desmin was determined immunohistochemically in rat penile cavernosum. Multipotency of B10 to adipogenic, osteogenic, or chondrogenic differentiation was found. Expression of endothelial cell-specific markers (CD31 or vWF protein) and expression of smooth muscle cell-specific markers (calponin, SMA, or desmin protein) were demonstrated in grafted B10 cells indicating that human MSCs may be a good candidates in the treatment of penile cavernosum injury [238].

14.7   Rules of Attraction: Angiogenesis and the Mesenchymal Stem Cell Migration

Angiogenesis requires the presence and active involvement of MSCs and therefore MSCs are ready to be recruited into the areas when there is a need for novel blood vessels: the inflamed, hypoxic, tumor infested locations. Gehmert et al. described an interesting model to study the migration of MSCs. In their work, immunodeficient mice were engrafted with human breast cancer cells (4T1) in the left mammary pad. A day later, the mice were injected IP with luciferase-labeled adipose tissue-derived MSCs (using Lentiviral vector technology). The MSCs were found to rapidly migrate into the tumor, confirming the previous observations that MSCs can be found within the tumor stroma and vasculature, even if the inflammation is not present, as the immunodeficient mice lacked the inflammation signaling pathway. Based on this result, it can be suggested that MSCs can be attracted solely by the cytokines produced by the tumor. However, the power of inflammation has been clearly

demonstrated in control animals, which received *E. coli* injections at contralateral locations and attracted all the MSCs leaving the tumor implant MSC-free [84]. Elucidating the migratory mechanisms of the MSCs seems to be an important step toward finding a delivery system to inflammatory sites and finding the conditions for clear migration into established tumors. Even the simple marking of tumor tissue with fluorescent proteins (such as GFP) holds important promise for surgeons, as delineating a breast cancer in situ during surgery would be possible by applying UV light and tracing the contours of the tumor. The technique already allows sophisticated molecular imaging combined with stem cell therapy [254].

Wang and colleagues used the ability of MSCs to differentiate into endothelial cells in vivo to establish whether the differentiated MSCs persist in vivo and to determine if this potential persistence contributes to functional improvement after experimental myocardial infarction. They generated a Lentiviral vector encoding two distinct reporter genes, one driven by a constitutive murine stem cell virus promoter and the other driven by an endothelial-specific Tie-2 promoter. The endothelial specificity of the Lentiviral vector was validated by its expression in endothelial cells but not in undifferentiated stem cells. The Lentivirus-transduced MSCs were injected into peri-infarct areas of the hearts of severe combined immune-deficient mice. Persistence of injected cells was tracked by bioluminescence imaging (BLI) and verified by immunohistochemical staining. The BLI signal from the endothelial-specific reporter revealed that the stem cells differentiated into endothelial cells 48 h after injection. However, both the constitutive and endothelial-specific signals disappeared by day 50. Nonetheless, the improvement in left ventricle ejection fraction with therapy persisted for up to 6 months. Immunohistochemical staining showed that stem cell-derived endothelial cells integrated into endogenous CD31+ vessels. Furthermore, stem cell-transplanted hearts had more CD31+ vessels and a lesser degree of cardiac fibrosis compared with the controls at 6 months. Increased angiogenesis and decreased fibrosis were associated with cardiac functional improvement. Similarly MSCs double-marked with GFP-Lentiviral vector and superparamagnetic iron oxide could be followed by MRI for up to 8 months in a porcine model of infraction and revascularization [274].

14.8   Myocardial Infarction

Endothelial cells respond to mild injurious stimuli by upregulating anti-apoptotic gene expression to maintain endothelial integrity. EC dysfunction and apoptosis resulting from ischemia/reperfusion injury may contribute to chronic allograft rejection. Under optimized conditions for Lentiviral vector transduction of rat aortic endothelial cells (RAEC) the delivery of the anti-apoptotic gene, Bcl-xL, via Lentiviral vector, protects RAEC from apoptotic death. The authors confirmed the damaging effect of the reperfusion phase. Endogenous Bax expression increased with I/R injury, whereas endogenous Bcl-xL remained constant. RAEC transduced with Lentiviral vector expressing Bcl-xL were protected from early apoptosis caused

by I/R injury, correlating with reduced cytochrome c release into the cytosol. This protective effect may be attributed to altering the balance of pro- and anti-apoptotic proteins, resulting in sequestration of the harmful Bax protein, and may open up new strategies for controlling chronic allograft rejection [286].

Inhibition of Na+/H+ exchanger 1 (NHE1) reduces cardiac ischemia-reperfusion (I/R) injury as well as cardiac hypertrophy and cardiac failure. Although the mechanisms underlying these NHE1-mediated effects suggest delay of mitochondrial permeability transition pore (MPTP) opening, and reduction of mitochondrial-derived superoxide production, the possibility of NHE1 blockade targeting mitochondria has been incompletely explored. A short-hairpin RNA sequence mediating specific knock down of NHE1 expression was incorporated into a Lentiviral vector (shRNA-NHE1) and transduced into the rat myocardium. NHE1 expression of mitochondrial lysates revealed that shRNA-NHE1 transductions reduced mitochondrial NHE1 (mNHE1) by approximately 60%, supporting the expression of NHE1 in mitochondria membranes. Electron microscopy studies corroborate the presence of NHE1 in heart mitochondria. Immunostaining of rat cardiomyocytes also suggests colocalization of NHE1 with the mitochondrial marker cytochrome c oxidase. To examine the functional role of mNHE1, mitochondrial suspensions were exposed to increasing concentrations of $CaCl_2$ to induce MPTP opening and consequently, rat heart mitochondrial swelling. shRNA-NHE1 transduction reduced the $CaCl_2$-induced mitochondrial swelling by $64 \pm 4\%$. Whereas the NHE1 inhibitor HOE-642 (10 μM) decreased mitochondrial Ca^{2+}-induced swelling by only 37 ± 6. Because mitochondria from rats injected with shRNA-NHE1 present a high threshold for MPTP formation, the beneficial effects of NHE1 inhibition in I/R resulting from mitochondrial targeting should be considered as a future target for cell therapy [250]

Oxidative stress is important in a number of pathologies, including cardiovascular diseases, such as atherosclerosis and cardiac ischemia–reperfusion injury. An important mechanism for adaptation to oxidative stress is the induction of genes through the antioxidant response element (ARE) which regulates the expression of antioxidant and cryoprotective genes via the transcription factor Nrf2 (nuclear factor E2-related factor 2). As Nrf2-regulated genes are induced during oxidant stress, occurring for example in reperfusion after ischemia, Hurttila et al. took a novel approach to exploit ARE for the development of oxidative stress-inducible gene therapy vectors. To this end, one, two, or three ARE-containing regions from human NAD(P)H: quinone oxidoreductase-1, glutamate-cysteine ligase modifier subunit and mouse heme oxygenase-1 were cloned into a vector expressing luciferase under a minimal SV40 promoter. The construct, which was the most responsive to ARE-inducing agents, was chosen for further studies in which a Lentiviral vector was produced for an efficient transfer to endothelial cells. Heme oxygenase-1 (HO-1), which has well-characterized anti-inflammatory properties, was used as the therapeutic transgene. In human endothelial cells, ARE-driven HO-1 overexpression inhibited nuclear factor-kappa B activation and subsequent vascular cell adhesion molecule-1 expression induced by tumor necrosis factor-alpha. They concluded that the ARE element is a promising alternative for the development of oxidative stress-inducible gene therapy vectors [111].

Progenitor cell therapy is a potential new treatment option for ischemic conditions in the myocardium and skeletal muscles. However, it remains unclear whether umbilical cord blood (UCB)-derived progenitor cells can be therapeutic in ischemic muscles and if yes, whether the ex vivo gene transfer can be used for improving the effect. The use of Lentiviral vector led to efficient transduction of both UCB-derived HSCs and MSCs resulting in long-term transgene expression. Moreover, it did not alter the differentiation potential of either HSCs or MSCs. In addition, the therapeutic potential of CD133+ and MSC progenitor cells transduced ex vivo with Lentiviral vector encoding the mature form of vascular endothelial growth factor D (VEGF-D) or the enhanced green fluorescent protein (eGFP) marker gene achieved permanent gene expression. The transplantation of the progenitor cells into nude mice serving as mouse model of skeletal muscle ischemia enhanced the regeneration of ischemic muscles, but notably, without a detectable long-term engraftment of either CD133+ or MSC progenitor cells. The results show that rather than directly participating in angiogenesis or skeletal myogenesis, the UCB-derived progenitor cells indirectly enhance the regenerative capacity of skeletal muscle after acute ischemic injury. However, rather counter-intuitively, the VEGF-D gene transfer into the progenitor cells did not improve the therapeutic effect in ischemic muscles [131].

Another cell type with improved adult stem cell functions has been discovered and cells have been isolated from the peripheral blood of young children. This clonally expandable, telomerase expressing progenitor cell type is distinct from hematopoietic or mesenchymal stromal cells and resembles that of embryonic multipotent mesoangioblasts. Cell numbers and the proliferative capacity correlate with donor age, and express the pluripotency markers Klf4, c-Myc, as well as low levels of Oct3/4, but lack Sox2. Overexpression of Sox2 by Lentiviral transduction of Sox2 (Sox-MABs) enhances pluripotency and facilitates differentiation to cardiovascular lineages. Furthermore, the number of smooth muscle actin positive cells was higher in Sox-MABs. In addition, pluripotency of Sox-MABs was shown in a mouse model by demonstrating the generation of endodermal and ectodermal progenies and injection of Sox-MABs into nude mice after acute myocardial infarction resulted in improved cardiac function compared to mice treated with control cells (cMABs). Furthermore, cell therapy with Sox-MABs resulted in an increased number of differentiated cardiomyocytes, endothelial cells, and smooth muscle cells in vivo [133].

14.9   Lung Damage and Lung Repair

Mesenchymal stem cell therapy emerges as a viable therapy in the context of acute lung injury/acute respiratory distress syndrome and chronic disorders, such as lung fibrosis and chronic obstructive pulmonary disease. There is evidence for beneficial effects of MSCs on lung development, repair, and remodeling. The engraftment in

the injured lung does not occur easily, but several studies report that paracrine factors can be effective in reducing inflammation and promoting tissue repair. MSCs release several growth factors and anti-inflammatory cytokines that regulate endothelial and epithelial permeability and reduce the severity of inflammation, as reviewed by Arboreau et al. [2], suggesting that carefully controlled expression of these factors using transduced stem cells could enhance the beneficial effects of the mesenchymal stem cell therapy. This may be a risky proposal, however, since constitutive expression of TGF beta/TGF alpha in epithelial MSCs generated breast cancer stem cells [12]. Acute respiratory distress syndrome (ARDS) is a crippling disease with no effective therapy, and characterized by progressive lung damage followed by dyspnea. MSCs have been proposed as a new therapeutic modality for ARDS because the stem cells can attenuate inflammation and repair the damaged tissue by differentiating into several cell types. The beneficial effect of the stem cells is still a minor mystery, as it is known that macrophages participate in the development of ARDS and that MSCs can only weekly modulate macrophage function. The chemokine CCL2 is a potent inducer of macrophage recruitment and activation, and its expression is elevated in patients with ARDS. A set of MSCs have been generated by transducing the cells with a Lentiviral vector expressing 7ND, a dominant-negative inhibitor of CCL2, expecting enhanced therapeutic function of the MSCs if the hypothesis is valid. The transduction was effective, and the stem cells produced a large amount of 7ND. After inducing lung injury by bleomycin treatment, the iv-injected MSCs readily migrated into the site of injury as confirmed by immunostaining 24 h postinjection. This finding suggests that MSCs could work as a drug delivery tool. Mice treated with 7ND-expressing MSCs showed significantly milder weight loss, suffered less severe lung injury, lower collagen content, lesser accumulation of inflammatory cells and inflammatory mediators, and ultimately showed significant gains in survival [218]. No evidence of 7ND-mesencymal stem cell-induced toxicity was observed during or after treatment. Thus, inhibiting the effects of macrophages may greatly enhance the ability of MSCs to affect lung repair in ARDS.

Direct transduction of lung tissues for gene therapy has always been an attractive proposal. The reoccurring problem, however, is that the airways are far less accessible to vector particles than hoped for and the depth of penetration of inhaled substrate ends in the branches which are larger than 100 µM in diameter [48, 263]. An attractive alternative delivery of gene therapy components could be the intrapleural injection of MSCs. To enable tracking, the cells were labeled with green fluorescent protein (GFP) using a Lentiviral vector, and were found readily attached to the pleura of Sprague–Dawley rats. The isolated and recovered cells preserved the typical mesenchymal stem cell phenotype and could differentiate into adipocytes, osteoblasts, and chondroblasts in vitro. The highest number of the labeled cells was found to be adhered to the mediastinal pleura, but no labeled cells were detected in the lung parenchyma or other tissues/organs, such as the liver, kidney, spleen, and mesenterium, a remarkable compartmentalization of a stem cell transplant [200].

14.10 Neurological Disorders

14.10.1 Alzheimer's Disease

Alzheimer's disease (AD) is one of the most devastating conditions and its prevalence is still rising paralleling the increase of average life expectancy. A hallmark of the disease is the accumulation of amyloid plaques and extensive neurodegeneration in the context of an intracerebral inflammation, leading to progressive dementia. Over the years, a tripartite set of goals crystallized, when the potential treatments of AD were considered: (a) stop the progression of the disease by reducing/reversing the plaque formation; (b) stop the neurodegeneration that seems to be a consequence of both internal changes (neurofibrillary tangle formation and related issues) and changes external to the cells, related to plaque formation and degeneration of the neuronal microenvironment; and (c) recover neurological function by replenishing the lost neuronal compartment [71, 81, 94, 122, 152, 188, 291]. Interestingly, MSCs and stem cell therapy are increasingly considered a potentially important part of the toolset to achieve these goals.

The symptoms that are collectively categorized as AD often have different backgrounds, some of which seem to have roots implying genetic causes, such as improper processing of beta amyloid peptide. Consequently, a disease-modifying therapeutic approach in Alzheimer's disease aims to reduce the accumulation of neurotoxic beta amyloid aggregation peptides. Habish et al. report new findings for a potential autologous stem cell-based strategy for delivery of enzymatic activities against beta amyloid formation in the brain. F-spondin and neprilysin (CD10), genes expressed in adult MSCs, are known to be involved in the formation and degradation of beta amyloid peptides, respectively. Coincubation of the converted MSCs with HEK-293 cells stably expressing amyloid precursor protein (APP) lead to a significant cell dose-dependent decrease of amyloid peptide release and deposition, indicating that MSCs might be useful for delivering anti-amyloid activity to treat AD [95]. This direction of research is gaining new momentum from the discovery of a new beta amyloid secretase and the tremendous progress gained in recent years in the field of amyloid formation, its contribution to neurodegenerative diseases [122] and allowing new gene therapies to be conceived and tested.

One effort has utilized human umbilical cord blood-derived MSCs (hUCB-MSCs) which were transplanted into amyloid precursor protein and presenilin1 double-transgenic mice. This experiment resulted in significantly improved spatial learning and a decrease in memory decline. Furthermore, beta amyloid peptide deposition, beta-secretase 1 (BACE-1) levels, and the hyper-phosphorylation of the Tau proteins were dramatically reduced in hUCB-MSC transplanted APP/PS1 mice. Interestingly, these effects were associated with reversal of disease-associated microglial neuroinflammation, as evidenced by decreased microglia-induced pro-inflammatory cytokines, reduction in the number of alternatively activated

microglia, and decrease in anti-inflammatory cytokines. Combining these findings with the potential cell therapy targeting, these MSCs are expected to produce a sustained neuroprotective effect by establishing a feed-forward loop engaging the alternative activation of microglia, thereby ameliorating disease pathophysiology and reversing the cognitive decline associated with amyloid deposition [139]. Peng and colleagues report additional details on the use of Lentivirus-expressed siRNA as a method to ameliorate Alzheimer disease neuropathology in APP transgenic mice by reducing the levels of beta-site APP cleaving enzyme 1, or BACE1 [189].

A series of experiments demonstrated the potential of neural stem cells transduced by a multigenic Lentiviral vector stably expressing recombinant human nerve growth factor in relevant amounts to exploit their ability for therapeutic applications. The multigenic Lentiviral vector contained a tricistronic cassette to express simultaneously up to three independent genes: (1) rhNGF (beta subunit); (2) EGFP (enhanced green fluorescent protein); and (3) Neo (R) (neomycin antibiotic resistance gene). Lentiviral vectors were released in culture media and subsequently used to transduce mouse stem cells. Remarkably, the subsequent test revealed that engineered NSCs were all positive for EGFP and after 30 passages in vitro engineered cells maintained their multipotentiality to differentiate into neurons, astrocytes, and oligodendrocytes. Furthermore, it was found that rhNGF-stem cell-derived neurons expressed choline acetyltransferase and displayed an enhanced axonal growth. The stem cells showed an altered sphere forming frequency either in rhNGF-NSC or in both groups of control NSC. Lentivirus-mediated rhNGF gene transfer into NSC was achieved without changes in the expression of neural differentiation markers, like microtubule-associated protein 2 (MAP2) (a/b), glial fibrillary acidic protein (GFAP) and chondroitin sulfate proteoglycan [34]. Secreted rhNGF increased axonal sprouting by rhNGF-NSC-derived neurons, which was associated with ChAT expression. rhNGF-NSCs may prospectively be a good candidate for the treatment of neurodegenerative diseases.

A protein that has been shown to promote APP accumulation is beta-secretase (beta-site APP cleaving enzyme 1, or BACE1). Typically, a marked increase in the level of BACE1 is found in the cerebrospinal fluid of those affected with Alzheimer's disease. Through in vivo studies using APP transgenic mice, it has been demonstrated that decreasing the expression of BACE1 via Lentiviral vector delivery of BACE1 siRNA has the potential for significantly reducing the cleavage of APP, the accumulation of these products, and the consequent neurodegeneration. As such, Lentiviral-expressed siRNA against BACE1 is a therapeutic possibility in the treatment of AD.

Neprilysin has recently been implicated as a major extracellular beta amyloid degrading enzyme in the brain. A unilateral intracerebral injection of a Lentiviral vector expressing human neprilysin (Lenti-Nep) was tested in transgenic mouse models of amyloidosis reduced amyloid-beta deposits by half relative to untreated mice, indicating that neprilysin may have a role in Alzheimer's disease treatment. That said, a more efficient delivery system is likely required, a property that a neprilysin expressing stem cell could potentially provide [160].

14.10.2   Parkinson's Disease

Gene transfer to the central nervous system provides a powerful methodology for the study of gene function and gene–environment interactions in vivo, in addition to a vehicle for the delivery of therapeutic transgenes for gene therapy. Research has been significantly aided by successfully targeting specific regions of brain, and for Parkinson's disease, the substantia nigra. The key to success is the ease of pseudo-typing Lentiviral vectors, which makes it possible to change the patterns of tropism. Cannon et al. used Isogenic Lentiviral vector particles encoding a GFP reporter and pseudotyped with envelope glycoproteins derived from vesicular stomatitis virus (VSV), Mokola virus (MV), lymphocytic choriomeningitis virus (LCMV), or Moloney murine leukemia virus (MuLV). Adult, male Lewis rats were injected uni-laterally with stereotactic infusions of vector into the substantia nigra. Three weeks later, patterns of viral transduction were determined by immunohistological detec-tion of GFP. Different pseudotypes gave rise to different sites of transgene expres-sion. VSV and MV pseudotypes transduced midbrain neurons, including a subset of nigral dopaminergic neurons. In contrast, LCMV- and MuLV-pseudotyped Lentiviral vector resulted in transgene expression exclusively in astrocytes. The restricted transduction of astroglial cells was not explained by the cellular distribution of receptors previously shown to mediate entry of LCMV or MuLV. The availability of neuronal and astrocyte-targeting vectors will allow dissociation of cell autonomous and cell nonautonomous functions of key gene products in vivo. Similar tissue and cell-specific patterns can be achieved in stem cells using cell/tissue-specific promot-ers and miRNA [43, 79, 86, 177, 186, 199, 213, 232, 242, 266, 290].

Multipotent mesenchymal stromal cells have raised great interest for brain cell therapy due to their ease of isolation from bone marrow, their immunomodulatory and tissue repair capacities, their ability to differentiate into neuronal-like cells, and for their ability to secrete a variety of growth factors and chemokines. A subpopulation of human MSCs, the marrow-isolated adult multilineage inducible (MIAMI) cells, when combined with pharmacologically active microcarriers (PAMs) have shown great promise in a rat model of Parkinson's disease. PAMs are biodegradable and non-cyto-toxic poly (lactic-co-glycolic acid) microspheres, coated by a biomimetic surface and releasing a therapeutic protein, which acts on the cells conveyed on their surface and on their microenvironment. In this study, PAMs were coated with laminin and designed to release neurotrophin 3, which stimulate the neuronal-like differentiation of MIAMI cells and promotes neuronal survival. After adhesion of dopaminergic-induced (DI)-MIAMI cells to PAMs in vitro, the complexes were grafted in the partially dopaminer-gic-deafferented striatum of rats, which led to a strong reduction of the amphetamine-induced rotational behavior together with protection/repair of the nigros-triatal pathway. These effects were correlated with the increased survival of DI-MIAMI cells that secreted a wide range of growth factors and chemokines. Moreover, the observed increased expression of tyrosine hydroxylase by cells transplanted with PAMs may contribute to this functional recovery [52] and provide an excellent new delivery system for genetically modified/enhanced cells into substantia nigra.

Lewy body disease is a heterogeneous group of neurodegenerative disorders characterized by alpha-synuclein accumulation and includes gradually worsening dementia with Lewy bodies (DLB) accumulating in neurons followed by advanced Parkinson's Disease (PD). Recent evidence suggests that impairment of the lysosomal pathways (i.e., autophagy) involved in alpha-synuclein clearance might play an important role. For this reason, the expression levels of members of the autophagy pathway in brains of patients with DLB and Alzheimer's disease and in alpha-synuclein transgenic mice were examined by immunoblot analysis. In DLB cases, the levels of mTor were elevated and Atg7 were reduced compared to controls and AD. Levels of other components of the autophagy pathway such as Atg5, Atg10, Atg12, and Beclin-1 were not different in DLB compared to controls. In DLB brains, mTor was more abundant in neurons displaying alpha-synuclein accumulation. These neurons also showed abnormal expression of lysosomal markers such as LC3, and ultrastructural analysis revealed the presence of malformed autophagosomes in abundance. Similar alterations were observed in the brains of alpha-synuclein transgenic mice. Intracerebral infusion of rapamycin, an inhibitor of mTor, or injection of a Lentiviral vector expressing Atg7 resulted in reduced accumulation of alpha-synuclein in transgenic mice and amelioration of associated neurodegenerative alterations supporting the notion that defects in the autophagy pathway, and more specifically in mTor and Atg7, are associated with neurodegeneration. This supports the possibility that modulators of the autophagy pathway might have potential therapeutic effects using genetically altered stem cells [44, 270].

Although the advances in Parkinson's disease research to date are significant, the lack of clinical use of genetically modified cells is a bit surprising and may indicate an oversight and underuse of the advanced tools provided by the combination of stem cells and Lentiviral vectors.

14.10.3 Focal Ischemia, Disruption of Blood–Brain Barrier, and Neuronal Damage

Lasting cerebral ischemia is a frequent (~80%) consequence of stroke and, as a result, most of the stroke research is focusing on ameliorating the devastating consequences of ischemic events: endothelial damage, neurodegeneration, and breakdown of the blood–brain barrier (BBB) leading to difficult-to-treat cerebral edema [108]. Data indicate that transplantation of human umbilical cord stem cells helps to protect ischemic brain [149], and the protection is partially attributed to cytokines and protective factors produced by these stem cells [10, 149]. Another promising finding is that the mesenchymal and neuronal stem cells preserve their ability to differentiate into glial and neuronal cells [51, 119, 149, 231, 249, 275]. Various studies on focal cerebral ischemic models have implicated the direct activation and expression of matrix metalloproteinases (MMPs), especially MMP-9, as a key orchestrator of BBB disruption. Moreover, studies have shown that MMP-9 siRNA

can protect the BBB from ischemia/reperfusion injury. One study investigated the neuroprotective role of a Lentiviral vector-mediated MMP-9 shRNA following focal cerebral ischemia [108], indicating that it is possible to deliver MMP-9 inhibitors by genetically enhanced stem cells. This study also showed the ability to deliver the target deeper into the affected area normally not accessible by direct Lentiviral vector infusion. The forerunner of such interventions is a study testing the hypothesis that transplantation of human neurotrophin-3 (hNT-3) over-expressing neural stem cells into rat striatum after a severe focal ischemia would promote functional recovery. The rat neural stem cells were transduced with a Flag-tagged hNT-3 gene in a Lentiviral vector. The stem cells were transplanted into the striatum ipsilateral to the injury of adult rats 7 days after 2 h occlusion of the middle cerebral artery from 3 days to 2 weeks after transplantation. The modified cells (NSCs-hNT3, as defined by Flag immunofluorescence staining) that survived the transplantation procedures could secrete significantly higher levels of neurotrophin-3 protein in the graft sites than controls ($P < 0.001$). Furthermore, the rats that accepted NSCs-hNT3 exhibited enhanced functional recovery on neurological and behavioral tests, compared with control animals transplanted with saline or untransduced stem cells, indicating that they might have value for enhancing functional recovery after stroke [285].

Recovery from ischemic events is slow and rather unpredictable. However, there seem to be new therapeutical opportunities that could enhance the process such as using VEGF-induction therapy [16]. There is accumulating evidence indicating that VEGF has direct neuroprotective effects on various cultured neurons of the central nervous system. Interestingly, in vivo VEGF controls the correct migration of facial branchiomotor neurons in the developing hindbrain and stimulates the proliferation of neural stem cells in enriched environments and after cerebral ischemia. On the other hand, transgenic mice expressing reduced levels of VEGF develop late-onset motor neuron degeneration, reminiscent of amyotrophic lateral sclerosis (ALS). Also, reduced levels of VEGF have been implicated in a polyglutamine-induced model of motor neuron degeneration. Intracerebroventricular delivery of recombinant VEGF protein delays disease onset and prolongs survival of ALS rats, whereas intramuscular administration of a VEGF-expressing Lentiviral vector increases the life expectancy of ALS mice by as much as 30%. Deciphering the precise role of VEGF at the neurovascular interface promises to uncover new insights into the development and pathology of the nervous system and should be helpful to the design of novel strategies to treat (motor) neurodegenerative disorders [137]. VEGF-expressing MSCs have also been found beneficial in Parkinson's disease [16, 271]. The development of Lentiviral particles engineered for macrolide-responsive human vascular endothelial growth factor 121 (VEGF121) expression will bring closer the in vivo use of inducible growth factor cell therapies, expressing the factors only in ischemic conditions using hypoxia-inducible erythropoietin promoter [6]. Alternatively, the inducible VEGF121 promoter system also compared favorably with isogenic streptogramin- and tetracycline-responsive configurations and showed excellent growth-factor fine-tuning following transduction into a variety of mammalian cell lines and different human primary cells. Chicken embryos transduced for macrolide-controlled VEGF121 production can be fine-tuned to prime a dose-dependent neovascularization [168].

Expression of survivin (SVV) using an SIN Lentiviral vector carrying vascular endothelial growth factor further improved the expression of VEGF and basic fibroblast growth factor in male Sprague–Dawley rats under hypoxic conditions. The in vivo experiment that produced this observation consisted of three groups of rats, one receiving intravenous injection of 500 μL of phosphate-buffered saline without cells (control group) and two groups administered the same volume solution with either three million GFP-MSCs (group GFP) or SVV/GFP-MSCs (group SVV). All animals were submitted to 2 h middle cerebral artery occlusion followed by reperfusion. Modification with SVV further increased secretion of both factors. The survival of the transplanted cells in the SVV group was 1.3-fold higher at 4 days after transplantation and 3.4-fold higher at 14 days after transplantation, respectively, when compared with group GFP and reduced the cerebral infarct volume by 5.2% at 4 days after stroke and improved post-stroke neurological function at 14 days after transplantation. Modification with SVV could further enhance the therapeutic effects of MSCs possibly through improving the MSCs survival capacity and upregulating the expression of the protective cytokines in the ischemic tissue [151].

The identification of the genes differentially regulated by ischemia will lead to an improved understanding of cell death pathways such as those involved in the neuronal loss observed following a stroke. Furthermore, the characterization of such pathways could facilitate the identification of novel targets for stroke therapy. One such novel approach was the amplification of the differential gene expression patterns in a primary neuronal model of stroke, by employing a Lentiviral vector system to specifically bias the transcriptional activation of hypoxically regulated genes. Over-expression of the hypoxia-induced transcription factor subunits HIF-1 alpha and HIF-2 alpha elevated hypoxia-mediated transcription of many known HIF-regulated genes well above control levels. Furthermore, many potentially novel HIF-regulated genes were discovered that were not previously identified as hypoxically regulated. Most of the identified novel genes were activated by a combination of HIF-2 alpha over-expression and hypoxic insult. These included several genes with particular importance in cell survival pathways and of potential therapeutic value. Hypoxic induction of HIF-2 alpha may therefore be a critical factor in mediating protective responses against ischemic injury. Further investigation of the genes identified in this study may provide increased understanding of the neuronal response to hypoxia and may uncover novel therapeutic targets for the treatment of cerebral ischemia [202] and the genes need to be considered as useful targets in future mesenchymal stem cell therapies. However, the use of hypoxia-induced gene therapy has to be evaluated carefully in the light of recent provocative observations indicating that the hypoxic phenotype contributes to appearance of highly malignant cancer forms from the initial epithelial–mesenchymal transition to the ultimate organotropic colonization, and that can potentially be regulated by hypoxia, suggesting a master regulator role of hypoxia and HIFs in metastasis [6, 155]. Furthermore, modulation of cancer stem cell self-renewal by HIFs may also contribute to the hypoxia-regulated metastasis program. The hypoxia-induced metastatic phenotype may be one of the reasons for the modest efficacy of anti-angiogenic therapies and may well explain the provocative findings that anti-angiogenic therapy increased metastasis in preclinical models [155].

14.10.4   Traumatic Spine Injury

The image of a wheelchair-bound superman exemplified for all of us the tragedy that affects many of the victims of traumatic spinal cord injury and motivated research into protecting and restoring spinal-cord functionality beyond and above the usual efforts. The results are promising on many fronts [236]. On one hand, the intervertebral disk, cartilage, and bone injuries that threaten the integrity of the spinal cord can be almost completely healed and the healing can be facilitated and enhanced by stem cell therapies in most of the experimental models. The treatment often includes stem cells engineered with Lentiviral gene transfer for enhancing and promoting wound healing and tissue restoration [13, 87, 89, 109, 247]. Significant success has been achieved by expressing bone morphogenic proteins in the injured tissue [80] and observations that mechanical stimulation has a multiplying effect in bone regeneration will hopefully carry the research into clinical trials [140] sooner than later. Probably, the first trials will be done in well-designed spinal surgery, allowing even risky interventions, currently not practiced [13, 88, 163].

The progress is significantly slower when it comes to restoring the functionality of severed spinal cord, but successful demonstration that MSCs migrate into the site of injury and differentiate into proper cell types needed for the healing [224] predicts potential breakthroughs. In this set of experiment, mesenchymal cells were labeled with green fluorescent protein using Lentiviral vector, were injected into the subarachnoid space, and their migration and differentiation was observed. Cells were found on the surface of the injured spinal cord parenchyma, in deeper area of the perivascular spaces and some of them had been found deeply integrated into the parenchyma. Immunostaining for nestin demonstrated that some GFP-positive cells differentiated into neural stem cells and mature neurons or glial cells in situ. Lentiviral vectors pseudotyped with rabies env were successfully used to deliver genes into spinal cord and site of injury and showed successful retrograde transfer into deeper areas, indicating that gene therapy is possible and factors necessary for further differentiation of stem cells can be delivered [224, 241]. Further advances in pseudotyping with Rabies virus glycoprotein has a promise for more efficient motor neuro-specific delivery of transgenes and restoration of neuronal functions.

14.11   Drug Delivery by MSCs

As the examples indicate above, MSCs have been recognized as promising delivery vehicles for gene therapy in the CNS. A particularly unmet need is delivery of compounds that could help patients suffering from a particularly aggressive form of cancer, gliomas. A glimpse into a possible future can be gained from experiments in which stem cells were used to evaluate the antitumor effect of cytosine deaminase (CD) in a rat C6 glioma model. Lentiviral vectors expressing CD and enhanced green fluorescent protein (eGFP) were constructed and transduced into rat MSCs

which were intracranially injected alone or in combination with C6 glioma cells supported by unlabeled parental MSCs. The presence and effect of the engineered stem cells were then correlated with the possible effects on tumor growth, tumor cell apoptosis, tumor size, and rat survival in the presence of 5-fluorocytosine (5-FC). Fei et al. found that the CD/eGFP cells were largely localized at the junction of the tumor with normal tissue. The mean survival time of rats co-injected with C6 glioma cells and MSCs-CD/eGFP cells was significantly extended to 45.9 days with tumor size reduction when compared with rats injected with C6 glioma cells alone surviving an average of 15.3 days, or those co-injected with C6 glioma cells and parental cells surviving only for 16.0 days. In addition, data suggest that MSC-CD/eGFP-mediated gene therapy promoted tumor cell apoptosis in rat C6 gliomas [72].

Without going into detail, hypoxia-induced genes seem to play an important role in the fate of MSCs and require further studies, as modifying and preconditioning as well as changing their effects temporarily by gene therapy indicates a plethora of important insights into the potential use of this complicated class of stem cells in tumor therapy [142, 147, 150, 155, 267, 272], and we expect rapid progress in this area in the near future. The rational is that tumor cells have significantly altered metabolism with a shift toward the anaerobic pathway and changes in the respective gene expression patterns providing novel targets and delivery methods for cancer therapy.

Transplantation of HSCs to correct a series of lysosomal storage diseases and peroxisomal disorders has almost 25 years of history and involves over 20 diseases [23]. However, the success was limited to only a small subclass of diseases such as Hurler syndrome, X-ALD, and infantile Krabbe disease. Detailed studies are now available suggesting that hematopoietic stem cells are suitable only for a carefully selected cases, leaving open the field for a more versatile mesenchymal stem cell therapy, especially those instances having neurological symptoms [69]. Bone marrow-derived MSCs are another promising platform for cell- and gene-based treatment of inherited and acquired disorders including a whole range of lysosomal storage diseases. Several animal models exist to run preclinical studies [164]. Human MSCs distribute widely in a murine xenotransplantation model, and the human stem cells are amenable to Lentiviral vector-mediated transduction to obtain expression of therapeutic levels of enzyme in xenotransplantation models of human disease (non-obese diabetic severe combined immunodeficient mucopolysaccharidosis type VII [NOD-SCID MPSVII]) [164]. Transduced MSCs persisted in the animals that underwent transplantation and comparable numbers of donor MSCs were detected at 2 and 4 months after transplantation. The level of circulating enzymes were sufficient to normalize the secondary elevation of other lysosomal enzymes and reduce lysosomal distention in several tissues providing additional evidence that transduced human MSCs retain their normal trafficking ability in vivo and persist for at least 4 months, while able to deliver therapeutic levels of proteins in an authentic xenotransplantation model of human disease.

Similar results have been reported by Muller and colleagues, who were able to restore aryl sulfatase and beta galactosidase levels in genetically deficient bone marrow MSCs, and showed that untransduced cells from patients with metachromatic leukodystrophy,

who are ASA deficient, took up a substantial amount of ASA that was released into the media from MSCs [173], an important milestone for future attempts to try stem cell therapy of metachromatic leukodystrophy. GM1 ganglyosiosys was successfully treated with MSCs in a mouse beta-galactosidase knockout model indicating that autologous transplantation may be feasible using Lentiviral-transduced MSCs [228].

Fabry disease affects an estimated 1 in 40,000–60,000 males, and far less frequently females. It is an inherited lysosomal disorder caused by a deficiency of alpha-galactosidase A (alpha-gal A). The systemic accumulation of globotriaosyl-ceramide (Gb3) results in gradual tissue deterioration leading to organ failure. There is a limited mouse model of the disease showing Gb3 accumulation in an alpha-gal A-deficient mouse model. However, most of the important clinical manifestations are absent and the lack of relevant large animal model hinders the development of proper cell therapy. When compared to the human alpha-gal A, the porcine alpha-gal A showed a high level of homology in the coding regions. Cell lysate and supernatants from Fabry patient-derived fibroblasts transduced with a Lentiviral vector carrying the porcine alpha-gal A cDNA (LV/porcine alpha-gal A) showed high levels of alpha-gal A activity, and its enzymological stability was similar to that of human alpha-gal A. Even more importantly, uptake of secreted porcine alpha-gal A by non-transduced cells was observed. Furthermore, Gb3 accumulation was reduced in Fabry patient-derived fibroblasts transduced with the LV/porcine alpha-gal A. The finding that the porcine version of the gene is also X-linked (X22q) provides hope that a large animal (porcine) model of Fabry disease can be constructed in the near future for use in testing a novel application of cell therapy using MSCs [278]. The success of such model and eventually the feasibility of the treatment depends on the "bystander phenomenon," i.e., the transduced mesenchymal cells intended for delivering the enzyme secrete the enzyme in abundance, but the defective cells in their microenvironment also must be able to take up the enzyme and utilize it. To facilitate the uptake, a fusion protein between Gb3 and HIV Tat protein has been made [104]. If successful, the range of enzyme replacement therapy approach could widen significantly. The data published by Higuchi et al. indicate that indeed the Tat's ability to penetrate the cell membrane was maintained in the recombinant fusion protein and it enhanced the enzyme uptake, as expected. Since the different manifestations of the disease produce problems in different organs (brain, kidney, and heart), it seems to imply that MSCs will be the best candidates for this enzyme replacement therapy as the earlier attempts to perform enzyme replacement therapy in mouse model showed insufficient efficiency [277].

14.12   Diabetes and the Hope for a Breakthrough in Mesenchymal Cell Transplantation

The enormity of the problems posed by diabetes is reflected by the statistics published on the NIH website (http://diabetes.niddk.nih.gov/dm/pubs/statistics/#dd). By the age of 65, almost one in four Americans suffers from diabetes. The at-risk population of

prediabetics is 37% of the population older than 20 years. The sheer number of patients indicates that restoring glucose metabolism by pancreas or pancreatic islet transplantation, even in the most severe cases, is just impractical if not impossible. The low engraftment rate makes the prospects of such treatment even worse, especially, as there never will be a sufficient number of donors. That leaves the stem cell technology as the major source of hope for solving the relevant issues in recovering regulated insulin production and glucose regulation functionality in diabetes. A large number of clinical trials using MSCs are under way [75, 106, 120], and a rather confusing sets of stem cell markers are listed in these studies indicating that there is a plurality of stem cells residing in different tissues, all of which have the potential to help pancreatic tissue regeneration. Not surprisingly, the most obvious source of these stem cells could be the pancreas itself, from which the resting stem cells can be isolated, reactivated, and expanded by variety of stimulants. The data are still being evaluated, and need further confirmation, reproduction, and lineage tracing. The currently available datasets could not firmly substantiate the claims when using different markers (*Carbonic anhydrase II* vs. *hepatocyte nuclear factor 1 beta*) [61, 113, 237, 243]. Since then, neurogenin 3 also was considered as a marker for endocrine type differentiation of proto beta cells [273], leaving the subject as to whether well-defined adult beta islet cell progenitors truly exist in significant numbers rather murky. The phenomenon of in vitro trans-differentiation of the acinar cells into beta cells upon exposure to EGF, LIF, notch1-inhibitors [15] looks promising, and recently Zhou and colleagues added a more extensive study on in vivo reprogramming of adult pancreatic exocrine cells into beta cells [289]. However, the reported efficiency was low and the progenitor cells remained elusive. This left the field searching for other sources, including MSCs from bone marrow, liver, intestine, and neural tissue (reviewed by Efrat [64–66] and Jones [118]), that are capable of trans-differentiating into insulin-producing beta cells. With the available results, their ultimate hope was that these cells could be used to seed the pancreas with new sets of insulin producing islands. Since lineage tracing was often omitted and the reproducibility of the results remained unsettled, the field, despite its high importance, seems to be somewhat in shambles [106, 107], ready for deployment of the novel, Lentiviral vector supported techniques.

Szabat et al. report a significant set of results on beta-cell maturation using Lentiviral vector-based lineage-study examining a novel Pdx1/Ins1 dual fluorescent reporter vector. They confirmed that individual adult human and mouse beta-cells exist in at least two differentiation states, distinguishable by the activation of the Ins1 promoter. They performed real-time imaging of the maturation of individual cultured beta-cells and followed the kinetics of the maturation process in primary human and mouse beta-cells and collected gene expression profiling data as well. The gene expression profiling of FACS purified immature Pdx1+/Ins1 (low) cells and mature Pdx1 (high)/Ins1 (high) cells from cultures of human islets, mouse islets, and MIN6 cells revealed that Pdx1+/Ins1 (low) cells are enriched for expression of multiple genes associated with beta-cell development/progenitor cells, proliferation, apoptosis, as well as genes coding for other islet cell hormones such as glucagon [240]. It turns out that trans-differentiation can be successfully performed using MafA. MafA is a leucine zipper transcription factor from the Maf family that can be

activated by p38 MAP kinase. This protein is a known pancreatic transcriptional factor controlling the beta-cell-specific transcription of the insulin gene [40]. Expressing it using Lentiviral vectors in placenta-derived multipotent stem cells (PDMSCs) that constitutively expressed Oct-4 and Nanog resulted in significantly upregulated expression of a series of pancreatic development-related genes (Sox17, Foxa2, Pdx1, and Ngn3), similar to that of native pancreas and islet tissues. MafA increased the expression levels of the mRNAs of NKx2.2, Glut2, insulin, glucagons, and somatostatin, and further facilitated the differentiation of PDMSCs into insulin + cells. Importantly, the expression of MafA in PDMSCs xenotransplanted into immunocompromised mice improved the restoration of blood insulin levels to control values and greatly prolonged the survival of graft cells in immunocompromised mice with STZ-induced diabetes [40].

Another successful lineage analysis and monitoring the induced trans-differentiation was reported by Cheng et al., in which a relatively abundant epithelial cell source, fetal human pancreas, was used to assess the proliferation potential, changes in lineage markers during culture, and capacity for generating insulin-expressing beta cells from fetal epithelial cells. The fetal epithelial cells readily formed primary pancreatic progenitor cultures, although their replication capacity was rather limited. This was overcome by introduction and expression of hTERT (human Telomerase Reverse Transcriptase) which greatly enhanced cellular replication in vitro. However, during culture the hTERT-modified pancreatic progenitor cells switched their phenotype gaining additional mesodermal properties. This phenotypic switching was inhibited when a pancreas-duodenal homeobox (Pdx)-1 transgene was expressed with a Lentiviral vector, along with inductive signaling through activin A and serum deprivation. This restored endocrine properties of hTERT-modified cells in vitro and were able to express insulin in vivo in immunodeficient mouse model [39]. The complexities of these result indicate that a sophisticated multi-gene cell therapies may be needed to solve the issues of proper modulation of transdifferentiation pathways.

Other strategies using a Lentiviral vector-based approach to achieve beta-cell proliferation through the beta-cell-specific activation of the hepatocyte growth factor (HGF)/cMet signaling pathway are also being explored. One of these methodologies is based on the beta-cell-specific expression of a ligand-inducible, chimeric receptor (F36Vcmet), under transcriptional control of the promoter from the human insulin gene, and its ability to induce HGF/cmet signaling in the presence of a synthetic ligand (AP20187) and result in specific proliferation of human pancreatic beta-cells [182]. The selective, regulated beta-cell expansion may help to increase the availability of cells for transplantation in patients with advanced diabetes.

These recent studies show that rapid progress may be achieved in this field and Lentiviral vectors may provide the necessary tools to analyze the issues. However, some of the notable efforts are made to avoid stem cell therapy altogether in certain types of diabetes. Instead, choosing a more direct route, applying in vivo gene therapy for expressing insulin gene in cell types other than beta cells. Ren et al. successfully restored near normal insulin levels for 500 days by expressing insulin in resting liver cells transduced with Lentiviral vector using a rat diabetes model [204].

14.13   Other Monogenic Diseases

Although monogenic disease appears to be the most obvious human diseases to treat with gene therapy, since they are caused by a single-gene defect, the progress in clinical studies has thus far been rather limited. Explanations for the lack of success include inefficiency of transductions in vivo, dangers posed by vectors, the failure to permanently correct the gene defect in sufficient number of cells, or the rapid turnover of cells. Alternative approaches therefore involve the search for and use of stem cell populations and depleting the active stem cell compartments ablation using cytostatic drugs to give chance if increased engraftment by transplanted stem cells. Combining the versatility and availability of MSCs, their ability to engraft, the use of autologous instead of allogeneic sources for safe transplantation, and the fact that the stem cell population can be expanded in vitro allows highly efficient ex vivo gene therapy relying on latest generation of Lentiviral vectors.

14.13.1   Cystic Fibrosis

Cystic fibrosis (CF) is caused by a mutation in the gene for the cystic fibrosis transmembrane conductance regulator protein (CFTR). The mutant form of the protein causes severe defect in mucus metabolism in the lungs and intestinal track that deteriorates into a life-long, deadly disease. CF is theoretically amenable to gene therapy. In spite of intensive research and a large number of clinical trials in the last 18 years, little practical success can be shown for treating cystic fibrosis [191]. The explanations include the fact that the deeper regions in the inner surface of the lung are not accessible to direct inhalation and direct treatment [48, 263]. In light of this finding, stem cells remain the most promising delivery vehicles. Castellani et al. reviewed the recent attempts to identify lung- or bone marrow-derived populations of stem cells or progenitor cells and application of such cells, allogenic or gene-corrected autologous cells, to colonize the airways, while differentiating into functional respiratory columnar epithelial cells [33]. When the reporter gene expression was analyzed in trachea-lungs and bronchoalveolar lavage, 0.4–5.5% of stem cells survived in injured airways, but no stem cells survived in control, healthy airway, or in the epithelial lining fluid [138]. The most successful approaches thus far appear to be obtained with bone marrow-derived MSCs, although the trans-differentiation rate thus far has been limited to below 10–14% [26]. As an alternative, the proven multipotent nature of bronchoalveolar stem cells isolated from lung tissue may provide another promising approach for stem cell therapy. Some additional improvement is expected from more efficient targeting of Lentiviral vectors. Mitomo and colleagues built a Sendai virus Env-pseudotyped SIV Lentiviral vector that can be manufactured at high enough titer and is capable of transducing respiratory epithelium of the murine nose in vivo at levels that may be relevant for achieving clinical benefit to cystic fibrosis patients [167]. Availability of novel cystic fibrosis gene-carrying

stem cell lines derived from placental mesenchymal cells certainly will help to speed up the research [53]. However, much more needs to be known about the normal differentiation and functioning of the airway's basal cells and the differentiation and lineages of stem cells to have more efficient treatment options both for gene therapy and for stem cell therapy [207]. We expect that the intensity of research and push for clinical trials will remain high as the outline of directions will become clearer. Also the methods to derive respiratory cell types from stem cells will remain a critical piece [181].

14.13.2 Duchenne Muscular Dystrophy

This disease is an X-linked recessive disorder caused by a mutation in the dystrophin gene that destabilizes muscle cell membranes and causes muscle dystrophy in approximately 1 of 3,600 boys. The musculoskeletal abnormalities deteriorate to a fatal level and the average life expectancy is no more than 25 years, even with high quality care. The research is facilitated by the availability of dystrophin-deficient transgenic mice (mdx-mice) and double knockout (utrophin/dystrophin-deficient mice) that can be used as experimental disease models [144, 269]. Human immortalized pluripotent cell lines expressing the mutant dystrophin gene are also available [187]. The ability of MSCs to differentiate into muscle cells places them on the top of the list of candidates that could be used to treat Duchenne muscular dystrophy [157].

Lentiviral vectors have been used in this field for conditional immortalization of human cells for basic biologic studies. Cudre-Maroux et al. demonstrated that the Lentiviral vector-mediated transduction of immortalizing genes into human primary cells is an efficient method for obtaining such cell lines. For Duchenne muscular dystrophy, the muscle satellite cell model was used to examine the impact of the transduced genes on the genotypic and phenotypic characteristics of the immortalized cells. The most commonly used immortalizing gene, the SV40 large T antigen (T-Ag), was extremely efficient at inducing the continuous growth of primary myoblasts, but the resulting cells rapidly accumulated major chromosomal aberrations and exhibited profound phenotypic changes. In contrast, the constitutive expression of telomerase and Bmi-1 in satellite cells from a control individual and from a patient suffering from Duchenne's muscular dystrophy yielded cell lines that remained diploid and conserved their growth factor dependence for proliferation. However, despite the absence of detectable cytogenetic abnormalities, clones derived from satellite cells of a control individual exhibited a differentiation block in vitro. In contrast, a Duchenne-derived cell line exhibited all the phenotypic characteristics of its primary parent, including an ability to differentiate fully into myotubes when placed in proper culture conditions. This cell line should constitute a useful reagent for a wide range of studies aimed at this disease [46]. A realistic source of stem cells would be the adipose tissue-derived stem cells that can be enhanced for muscle repair. Forced expression of MyoD using Lentiviral vector

in vitro strongly induced myogenic differentiation, while the adipogenic differentiation was inhibited. Moreover, MyoD-expressing human multipotent adipose-derived stem cells had the capacity to fuse with DMD myoblasts and can restore dystrophin expression. Importantly, transplantation of these modified human, multipotent, adipose-derived stem cells into injured muscles of immuno-depressed Rag2(−/−)gammaC(−/−) mice resulted in a substantial increase in the number of human multipotent adipose-derived stem cell-derived fibers [92]. Goncalves and colleagues went a step further and devised a technique to monitor the fusion events necessary for myoblast formation by using an elaborate bipartite genetic switch that relays on recombinase-inducible genetic switch that is activated after two cell types, one of which expresses Cre and the other the rest of the elements with LoxP1 sites, that switch on only upon fusion. This provides a sensitive tool to study the lineages and process of myocyte fusion in transgenic system [90].

Ikemoto et al. used high transduction-efficiency Lentiviral vector-mediated gene transfer into freshly isolated autologous satellite cells. Freshly isolated cells have better myogenic capability than satellite cell-derived myoblasts, and expansion of the satellite cells does not affect their regenerative potential. The transduced cells successfully regenerated the targeted muscle groups in mdx mice [112]. However, the VSVg pseudotyped Lentiviral vector are inferior in transducing nondividing murine cells, and Shunchang et al. demonstrated that by pseudotyping with feline immunodeficiency virus ENV better transduction rates can be achieved [234].

14.13.3   Chronic Granulomatous Disease

We include this disease because of the challenges researchers faced in attempts to use cell-based therapies. Granulomatous disease is a rather rare X-linked immunodeficiency disorder caused by mutations in the CYBB gene encoding the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase catalytic subunit gp91(phox). Earlier attempts to restore the gene function with oncoretroviral vectors failed due to (a) gene silencing common for retroviral inserts, (b) high risk of genotoxicity that these oncoretroviral vectors pose [165], and (c) low transduction efficiency and inability to target appropriate cell lineage and practice differentiation-restricted gene expression [17]. The solution for the compound problem seems to lie in using a safer and more efficiently targeting Lentiviral vector system [17, 223].

It has been demonstrated and repeatedly confirmed that by using Lentiviral vector it is possible to transduce HSCs as well as differentiated neutrophiles from patients with X-linked chronic granulomatous disease (X-CGD) and correct the X-CGD-phenotype in the NOD/SCID model. The Lentivector was a VSV-G-pseudotyped, third-generation, self-inactivating (SIN) Lentivector encoding gp91 (phox). Lentiviral vector efficiently transduced CD34+ peripheral blood stem cells under ex vivo conditions nonpermissive for cell division and resulted in 54% of the cells expressing gp91 (phox). Lentivector also achieved significant correction of

differentiated human X-CGD neutrophils arising in vivo in NOD/SCID mice that underwent transplantation (20% and 2.4%, respectively). Thus, third-generation SIN Lentivector-gp91 (phox) performs well as assessed in human X-CGD neutrophils differentiating in vivo, and the studies suggest that the NOD/SCID model is generally applicable for in vivo study of therapies evaluated in human blood cells expressing a specific disease phenotype [165, 209, 226]. However, long-term solution can be expected from transducing HSCs, and not the fully differentiated neutrophils that have only limited lifespan left and the lack of genotoxicity safety of the third-generation SIN v Lentiviral vector seem to address these requirements perfectly.

14.13.4   Wilson's Disease

Wilson's disease is a genetic disease caused by a spectrum of mutations in the ATP7B gene, whose product is a liver transporter protein responsible for coordinated copper export into bile and blood. Zhang and colleagues reported in 2011 an attempt to restore the normal phenotype by directed hepatocyte differentiation from human-induced pluripotent stem cells. The phenotype correction was achieved by chaperone drug curcumin that can reverse the functional defect in vitro in the case of the R778L Chinese hotspot mutation in the ATP7B gene. They propose this model system for correcting the gene using Lentiviral technology [284]. ATP7B gene seems to be relevant for the wider mesenchymal stem cell field as over-expressing it protects MSCs form copper toxicity. This in turn could be used as a selection advantage of transduced MSCs over the non-transduced ones in copper-rich environment for enriching the transduced mesenchymal cell compartment before transplantation [225].

14.14   Senescence, the Associated Diseases and the Role of Lentiviral vector-Modified MSCs

The image of the "fountain of youth" represents a mirage deeply engraved in the human psyche and expresses our fear and resentment of one of the inevitabilities of life: if we are lucky, we will get old, decrepit, suffer a lot from series of painful, chronic diseases, and finally succumb. The irony is that those who we consider unlucky, die young, but are saved from the long lasting predicaments of aging. Certainly, the intricacies of the factors leading to longevity, or the lack thereof, keep generations of stem cell researchers awake and busy and for good reasons. A model for aging has been found in the condition known as Progeria, or more precisely the Hutchinson–Gilford Progeria Syndrome, a rare disease affecting children of both sexes and which is caused by a mutant prelaminin A gene, encoding the lamin A-processing enzyme. Prelaminin A that retains a Farnesyl group, subsequently expressing its abnormal form, Progerin. Progerin in turn is anchored to the nuclear

membrane and destabilizes the nucleus, limiting the ability of cells to divide and leading to premature cell death. Unlike other accelerated aging diseases affecting DNA repair (Werner's and Cockayne's syndrome), progerin may play role in normal aging process [211] and its production is slowly turned on in cells that have uncapped chromosomes, i.e., have truncated telomeres, resulting in premature depletion of stem cell compartments. See the popular UCSF website for details: http://www.ucsf.edu/news/2011/10/10766/aging-disease-children-sheds-light-normal-aging.

Additional upregulation of multiple genes in major inflammatory pathways indicated an activated inflammatory response in progeria patients. This response has also been associated with normal aging, emphasizing the importance of studying progeria to increase the understanding of the normal aging process [178]. The progressing disease shows a pattern of tissue and organ degeneration that correlates with the depletion of a variety of stem cell compartments, a correlation first pointed out by Favreau [70]. The insight into the role of stem cell depletion in Progeria accumulated rapidly in the last couple of years [170, 178, 179, 211]. This leads to the establishment of an animal model by creating Zmpste24 knockout mice [67] in 2008, which showed premature senescence and progeroid symptoms. With the role of stem cells in aging and in Progeria, the doors opened for studying stem cell renewal via dedifferentiation. Autologous or heterologous transfer of native or Lentivector-enhanced cells [129, 184, 212, 265] are being actively considered as a possible interventions to slow down progeria as well as natural aging [28]. However, in both cases, the changes are systemic and murine gene therapy data indicate that the therapy in lysosomal storage disease models, affecting large segments of the body, is more efficient if done at an earliest possible age [28, 129]. This may have something to do with the limited availability of the microenvironment for the modified or transplanted stem cells. For this, the preexisting ones, even when "old" and malfunctioning, already occupy the microenvironment appropriate for stem cells. We already know that wound sites create new sites and attract MSCs [5]. Also, it is possible that cancer growth is able to generate and maintain an appropriate microenvironment for cancer stem cells [24] as well as MSCs [42] (potentially for use as anticancer agents) but the normal tissue, even in aging, seems to be resilient in accepting externally provided stem cells. Experiments are under way to create artificial microenvironments using nanotechnology to deliver stem cells that produce therapeutical factors [52], and 3D scaffolding mimicking bone marrow niches are being designed for similar purpose [55] and Lentiviral vector are often used to deliver the genes of interest [60, 141, 166, 248].

14.15 Clinical Trials Using Lentiviral Vectors and MSCs

VIRxSYS pioneered the use of lentiviral vectors in Phase I clinical trials to deliver antisense HIV genes as an Antisense RNA therapy for AIDS [110, 156]. This established an initial safety profile for the ex vivo use of Lentiviral vectors (see http://

ClinicaTrials.gov: identifier VRX496-USA-05-002). This Phase I trial demonstrated the safety and tolerability of a single dose of approximately ten billion autologous HIV infected CD4+ T cells transduced with the Lentivector VRX496 carrying a 937-base antisense targeting the HIV envelope. These encouraging results have led to design of a Phase II clinical trial to evaluate the safety, tolerability, and biological activity of four or eight repeated infusions of five to ten billion autologous VRX496-modified HIV+, CD4+ T cells. A major obstacle to completing this Phase II trial was manufacturing enough cells to administer multiple infusions in patients. For this study the safety issues were cleared successfully, opening the way for more extensive use of Lentiviral vectors in clinical trials.

Currently, 16 lentiviral clinical trials are listed at the ClinicalTrials.gov website. All of these trials are in early stage, Phase I or II. Three of these trials have not yet started and 11 trials are still recruiting patients. Most of the clinical trials are focused on hematopoietic stem cells, which are outside of the scope of this work. One trial targets Netherton Syndrome (ClinicalTrials.gov: identifier: NCT01545323) and attempts to restore LEKTI serine protease levels in an affected 5 cm^2 skin area, a proof of principle study that has the potential to utilize mesenchymal stem cells in the future.

14.16   Conclusions and Future Directions

We witnessed a tremendous progress in characterizing and understanding stem cells, the factors needed for maintaining the stem cell phenotype as well as changing it in a predictable mode forcing the mesenchymal stem cells into various differentiation pathways. This progress provides a test bed for a higher level of bioengineering when the genetic buildup of the stem cells is changed to achieve well-defined therapeutical goals. The overview of the recent literature presents a long list of "proof of concept experiments" in which tantalizing possibilities are validated as things that can be accomplished in a wide range of fields representing different pathologies: from the debilitating Alzheimer's, Parkinson's diseases; various traumatic neuronal injuries, diabetes, neuronal and cardiac ischemia; to age-related tissue degeneration and tissue engineering or delivery of biologics for therapeutical purposes. In parallel, Lentiviral vectors are becoming highly valued tools in this tedious work as they are highly efficient vehicles for gene delivery to mark cells, express genes of interest, proteins, and various inhibitory RNA species in stem cells. Consequently these stem cells, especially the various forms of MSCs, have been shown to be highly effective in delivering the targeted genes to difficult-to-reach tissues, including the CNS. Manipulating genes and gene expression, gene transfer has been made safe and efficient by the recent progress in Lentivector technology and merged successfully with the stem cell technology. This field has reached an advanced stage, at which it has become feasible to use them safely in a clinical environment. More and more researchers as well as clinicians are becoming familiar with the power of these technologies both for ex vivo and in vivo cell therapy. It

does not take a prophet to predict that advanced stem cell therapy has gaining a strong foothold, and even though a tremendous amount of work is needed to be done for it to become a everyday intervention, it is here to stay and will become a routine treatment for the next generation of patients.

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