Gustav Steinhoff *Editor*

Regenerative Medicine – from Protocol to Patient

4. Regenerative Therapies I

Third Edition

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Foreword: Regenerative Medicine: From Protocol to Patient

Third Edition

The vision to unravel and develop biological healing mechanisms based on evolving molecular and cellular technologies has led to a worldwide scientific endeavour to establish *Regenerative Medicine*. This field is involving interdisciplinary basic and (pre)clinical research and development on the repair, replacement, regrowth or regeneration of cells, tissues or organs in congenital or acquired disease. Stem cell science and regenerative biology is prompting the most fascinating and controversial medical development of the twenty-first century. It can be envisaged that this development will establish completely new molecular and cellular techniques for medical diagnosis and therapy. An early rush of scientific development was set up more than one hundred years ago by the physiology of blood regeneration (Hall and Eubanks, 1896) and successful vascular surgical techniques for organ transplantation (Carrel and Guthrie, 1905). However, the clinical realization of allogenic blood transfusion lasted until the discovery of the blood group antigens (Landsteiner and Levine, 1928) and successful routine allogenic organ and bone marrow transplantation even until the end of the last century.

Similar to the field of allogenic cell and organ transplantation, it seems that *Regenerative Medicine* again condenses mankind's visions, hopes and fears regarding medicine: hopes of eternal life and effective treatment of incurable disease as well as fears of misuse of technology and uncontrolled modifications of life are polarizing the scientific field. The development and public acceptance of new ethical and regulatory guidelines is a necessary process to support further clinical development. Nevertheless, the vision of a new medicine using the regenerative power of biology to treat disease and restructure the organism is setting the aim for scientific, technological and medical development. Viewing the great expectations to restructure and regenerate tissue, organs or organisms, the current attempts of scientist and physicians are still in an early phase of development.

The field of *Regenerative Medicine* has developed rapidly over the last 20 years with the advent of molecular and cellular techniques. This collection of volumes on *Regenerative Medicine: From Protocol to Patient* aims to explain the scientific knowledge and emerging technology as well as the clinical application in different organ systems and diseases. The international leading experts from four continents describe the latest scientific and clinical knowledge of the field of *Regenerative Medicine*. The process of translating science of *laboratory protocols into therapies* is explained in sections on basic science, technology development, and clinical translation including regulatory, ethical and industrial issues.

This collection is organized into five volumes: *(1) Biology of Tissue Regeneration, (2) Stem Cell Science and Technology, (3) Tissue Engineering, Biomaterials, and Nanotechnology, (4) Regenerative Therapies I, and (5) Regenerative Therapies II.*

Biology of Tissue Regeneration (Volume 1) focuses on regenerative biology with chapters on extracellular matrix, asymmetric stem cell division, stem cell niche regulation, (epi)genetics, immune signalling, and regenerative biology in organ systems and model species as axolotl or zebrafish.

Stem Cell Science and Technology (Volume 2) provides an overview as classification of stem cells and describes techniques for their derivation, programming and culture. Basic properties of differentiation states as well as function in human organism are illustrated, and areas of stem cell pathologies in cancer and therapeutic applications for these cells are discussed with emphasis on their possible use in *Regenerative Medicine*.

Tissue Engineering, Biomaterials and Nanotechnology (Volume 3) focuses the development of technologies, which enable an efficient transfer of therapeutic genes and drugs exclusively to target cells and potential bioactive materials for clinical use. Principles of tissue engineering, vector technology, multifunctionalized nanoparticles and nanostructured biomaterials are described with regard to the technological development of new clinical cell technology. Imaging and targeting technologies as well as biological aspects of tissue and organ engineering are depicted.

Regenerative Therapies I (Volume 4) gives a survey on history of Regenerative Medicine and clinical translation including regulation, ethics and preclinical development. Clinical state-of-the-art, disease-specific approaches of new therapies, application technology, clinical achievements and limitations are described for the central nervous system, head and respiratory system. *Regenerative Therapies II (Volume 5)* contains state-of-the-art knowledge and clinical translation of *Regenerative Medicine* in the cardiovascular, visceral and musculoskeletal systems.

These volumes aim to provide the student, the researcher, the health-care professional, the physician and the patient a complete survey on the current scientific basis, therapeutical protocols, clinical translation and practised therapies in *Regenerative Medicine*. On behalf of the sincere commitment of the international experts, we hope to increase your knowledge understanding, interest and support by reading the book.

After the successful introduction in 2011 with 41 chapters, this work has been actualized and extended for the third edition with into five volumes containing 60 chapters.

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Chapter 1
History of Regenerative Medicine

Raymund E. Horch, Laurentiu M. Popescu, and Elias Polykandriotis

Abstract Generation and regeneration as an answer to disease are far from being a new idea. Philosophers, naturalists and scientists were intrigued by the marvels of regeneration seen in nature. By the middle of the nineties life scientists thought we were only a few years away from bioartificial organs grown in a Petri dish. However, by the dawn of the new millennium it became clear that the mechanistic approach dictated by tissue engineering so far, had neglected issues of vascularization. Processes of angiogenesis were central to homeostasis, bioassimilation and biointegration of tissue engineered constructs. Furthermore, the field of tissue engineering had evolved into something vast, encompassing satellite technologies that were becoming separate science sectors. Advances in genetical engineering, stem cell biology, cloning, biomaterials and biomedical devices to name a few, would come to play a major role of their own – tissue engineering had become a part of a bigger whole. Regenerative medicine is the collective field to shelter these technologies "*…that seeks to develop functional cell, tissue, and organ substitutes to repair, replace or enhance biological function that has been lost due to congenital abnormalities, injury, disease, or aging"*.

Keywords Regeenratrive medicine • Tissue engienering • History • Cell culturey • Arterio-venous loop • 3d vascularization • Telocytes

"Those who cannot learn from history are doomed to repeat it", claimed the philosopher G. Santayana in his book "The life of reason" (Santayana 1905). Although this statement reminds somehow of a cliché and its essence is being constantly disputed through the ages, one could hardly find a better example to report upon, other than the case of regenerative medicine. It is widely admitted that the very term of "Regenerative medicine" was coined to express a need for reorientation

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Table 1.1 Scientometric data based on Thomson Reuters released information (August 2009)

	Tissue engineering	Regenerative medicine
Total No of PubMed Papers (starting in..)	14517 (since 1988)	2197 (since 2001)
Year of maximum	–	2008 (ca 600)
Most cited paper	2452 citations	1366 citations
Top 100 papers	At least 200 citations each	At least 40 citations each

(Table 1.1). By the end of the twentieth century, biotechnology firms had maneuvered themselves into a dead-end financially, as well as conceptually (Mason 2007). Furthermore, the field of tissue engineering had evolved into something vast, encompassing satellite technologies that were becoming separate science sectors. Advances in genetical engineering, stem cell biology, cloning, biomaterials and biomedical devices to name a few, would come to play a major role of their own – tissue engineering had become a part of a bigger whole. And it is undisputable that biologicals will be the future (Mason and Dunnill 2008). To quote Paul Kemp: "hype, hubris and hyperbole aside – regenerative medicine will make a real and positive difference…"(Kemp 2006). But where did it all start?

1.1 Regenerative Medicine in the Ancient World

In his *Theogony*, Hesiod (eighth century BC) introduces Prometheus (Fig. 1.1) having created man out of clay and providing him with fire as a source of knowledge. "Hear the sum of the whole matter in the compass of one brief word-- every art possessed by man comes from Prometheus." (Aeschylus 415 BC). By doing that, Prometheus had provoked the wrath of Zeus. He had Prometheus carried to Mount Caucasus (or the Carpathian mountains) where an eagle (often mistaken as a vulture) by the name of Ethon would pick at his liver; it would grow back each day and the eagle would eat it again. His torture lasted 30.000 years until he was freed by Hercules (Fig. 1.1). Interestingly enough, the liver is generally speaking the only of the human organs to regenerate itself spontaneously in the case of lesion.[1] The ancient Greeks were well aware of this, hence they named liver (Greek: hēpar, ἧπαρ) after hēpaomai (ηπάομαι), meaning to "repair oneself".

Later on, Aristotle devised two scripts dealing with generation and regeneration in the animal realm. In his "Generation of animals" he related early development with regenerative potential, whereas in "The history of animals" he made observations on regeneration on the limb of salamanders and deer antlers (Aristotle 1984). He propagated that biological form originates from undifferentiated matter and clearly favoured what would later be described as "epigenesis"(Fig. 1.2).

[1] Now the phenomenon of desquamation of the intestinal epithelium and the epidermis has been described. The intestinal epithelium is completely regenerated in 4–5 days. The total regeneration of the epidermis takes 4 weeks. This may mean that for a life expectancy of 77 years, the human epidermis is regenerated 1000 times.

Fig. 1.1 Prometheus. “Prometheus”, Gustave Moreau 1868 (Musée Gustave Moreau, Paris). According to some investigators, his torture held for 30.000 years. After having provoked the wrath of Zeus, the eagle Ethon, picked at his liver every night. During the day the liver would regenerate

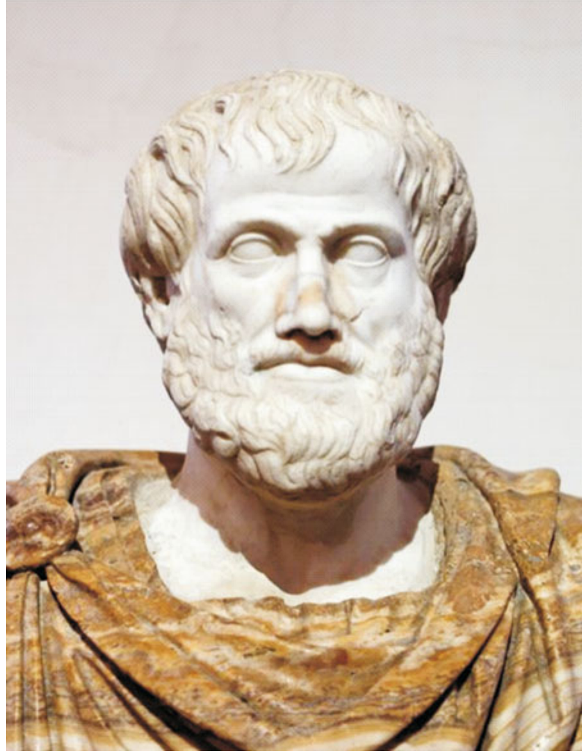

Fig. 1.2 Aristotleb. Aristotle 's bust. Roman copy from the bronze original by Lyssipos (fifth century B.C.).. (Ludovisi Collection). Aristotle wrote two major works on generation and regeneration in the animal realm. He related early development with regenerative potential and propagated that biological form originates from undifferentiated matter (epigenesis)

In the biblical tradition "the Lord God then built up into a woman the rib that he had taken from the man" (Wenin 2001). The quest for tissue replacement was even more graphically demonstrated in the tradition of Cosmas and Damian. Their practice of medicine and surgery in Asia Minor without fee (hence called 'Anagyroi,' without silver) and their martyrdom in Aegea, in Cilicia made a lasting impression upon the early Church. The grafting by these physician-surgeons of a moor's leg in replacement of a patient's diseased leg, and his surprise at finding himself possessed of two sound legs, his own white, and the other black, has been the subject of numerous paintings the majority of which depict the brothers in long robes, holding surgical instruments, boxes of salves, gallipots, or other medical appliances (Matthews 1968) (Fig. 1.3). Graveyards from the Paracas and Parachamac regions in Peru provide ample evidence that pre-Incan surgeons were performing trephination in great numbers as early as 3000 BC. A survey of more than 10,000 mummies from prehistoric Peru demonstrated that roughly 6% showed cranial trephination. There is strong evidence that the occasional cranioplasty was also performed. Trephined Incan skulls have been discovered adjacent to shells, gourds, and silver or gold plates (Asenjo 1963).

Fig. 1.3 Saints Cosmas and Damian. “Transplantation of a leg by Saints Cosmas and Damian, assisted by angels”, early sixteenth century (Stuttgart, Germany). According to the tradition of Cosmas and Damian these saints grafted a moors leg as a replacement of a patient’s diseased leg

1.2 Regeneration in Early Research

Until the middle of the eighteenth century the motive power of biological organisms was thought to be an abstract vital force. Descartes (1596–1650) in his L' Homme postulated that the body works like a machine and biological phenomena are void of a divine meaning but can be explained by means of their physical properties. Lavoisier (1743–1794) postulated further on, that function and viability of organisms depended on chemical processes that could be reproduced in the laboratory. During the same time phenomena of generation and regeneration intrigued scientists and divided them into two distinct camps. Preformationists supported that appendages to be regenerated and organisms to be born pre-existed as miniatures at the site of interest. So, at the base of a severed lizard tail, in their conception a miniature tail was preformed and waited to be "activated" by an amputation. Likewise, in the sperm or in the ovum of the human there existed a miniature "homunculus" that grew into a newborn infant. This theory prevailed until the middle of the eighteenth century being concordant with the mechanistic framework provided at the time and did not come into a direct conflict with the Christian beliefs about divine involvement in the processes of life. On the contrasting end, came the Aristotelean thesis that undifferentiated matter was able to give rise to life. This theory had been actually named "epigenesis" by William Harvey (1578–1657) in his work "on the generation of animals" grossly repeating on Aristotle's works.

In the eighteenth century the process of regeneration in amphibians was matter of intense study. Abraham Trembley (1710–1784) produced several publications on the regenerative phenomena on freshwater polyps. He managed to obtain a clone of 50 polyps from one organism that he had quartered. He performed sections at every conceivable plane, contradicting preformational beliefs of the time (Dinsmore 1991). The question was posed: If the animal soul was the organizing and unifying element of life, how could a newly regenerated form arise? Reaumer and Spallanzani reported about their studies on crustaceans and salamanders respectively (Dinsmore 1991). The latter, being a great methodologist, expanded his research on a number of different organisms including frogs, toads, slugs and snails. He published his findings in 1768 in his work "Prodromo". It was noted by Newth. "In 1768 the snails of France suffered an unprecedented assault. They were decapitated in their thousands by naturalists and others to find out whether or not it was true, as the Italian Spallanzani had recently claimed, that they would then equip themselves with new heads" (Newth 1958; Weaver and Garry 2008).

Until the end of the eighteenth century philosophical and religious debate linked to the science of regeneration was set aside, and epigenesis gained acceptance with the eventual ascendancy of epigenetic embryology.

The last years of the eighteenth century marked a new field of interest for regenerative medicine: organ transplantation. John Hunter (1728–1793) performed allograft transplantations between chickens as well as dental transplantation utilizing xenografts of human teeth to avian hosts. John Hunter was the most prominent

surgeon and anatomist of his time. According to his instructions, his corpse was used for an anatomical dissection by his medical students on the day after his death.

At the beginning of the nineteenth century – following the 1794 description by B.L. in The Gentlemen's Magazine in London of a forehead tissue transposition to restore the nose of a bullock cart driver named Cowasjee, that had been cut off as a punishment (BL 1794) – the English surgeon Carpue was the first surgeon to apply methods of nasal reconstructions known to Indian surgeons for centuries (Carpue 1981 [1816]). Dieffenbach described methods for reconstructions of several components of the face, as well as the anus and the urethra (Goldwyn 1968). Reverdin devised a method for transplantation of skin islets, similar to the later techniques for keratinocyte transplantation (Horch et al. 2001). Transplantational biology was investigated by experimental approaches: In 1824, Franz Riesinger attempted corneal transplants from rabbits to humans, which were not successful (Moffatt et al. 2005). In 1837 Samuel Bigger performed a corneal transplant from a lab gazelle to another Gazelle with full recovery. Later on, Schleiden and Schwann in 1838–1839 postulated the cell theory that was afterwards confirmed by Rudolf LK Virchow through microscopic observations. He stated in 1858 the famous "*omnis cellula ex cellula*". Ultimately, the idea of cells being the elementary units of life being able to replicate themselves by division was born (Coleman 1978; Stocum 2006).

The eminent German pathologist Julius Cohnheim postulated in 1867 what became known as the "Cohnheim hypothesis". He suggested that all of reparative cells taking part in the regeneration of wounds come from the bloodstream (and therefore from the bone marrow) (Wohlrab and Henoch 1988).

At the end of the nineteenth century, Barth observed that upon autologous bone transplantation in hounds the vast majority of cells die and leave a scaffolding behind to be slowly repopulated by new host cells and an adequate neovascular network (Barth 1893).

Another very important advance was new knowledge on descriptive embryology that elicited a revolution in developmental biology. Even Darwin considered embryology as key to providing a special insight into evolution of forms, as seen in his correspondence to his friend Asa Grey.

If the living cell is the key to the tissue engineering of implantable parts and devices, then the advent of mammalian cell culture technology, i.e. the growing of mammalian cells out of the body, represents an event which ultimately opened the door for this field. Modern cell culture dates back to the early part of this century when a French scientist, Alexis Carrel, working at the Rockefeller Research Institute in New York, started a culture from a small slice of heart muscle taken from a chick embryo (Leff 1983). This culture continued for several decades, although along the way the heart muscle cells died out and only fibroblast cells continued to proliferate. Carrel's historic chick-cell culture finally was allowed to expire 34 years after it was started–and 2 years after his own death. Anecdotally and in the retrospect, his "immortal" adult cells might have benefited from interventions from Carrel's staff to keep the cells going and their teacher happy (Witkowski 1980). Now it is known, that according to the "Hayflick limit", cells in culture are not able to replicate more

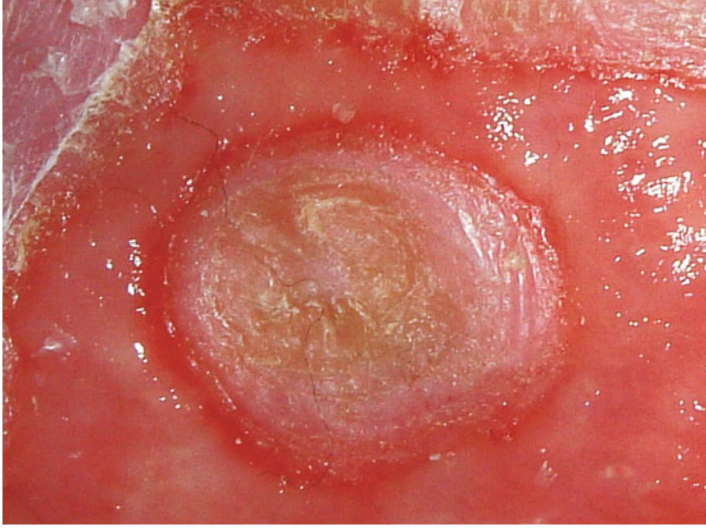

Fig. 1.4 Back from bench to bedside. Back from bench to bedside: small particles of skin inoculated into "biological" environment of wound showing expanding skin islet within a large wound 3 weeks after seeding

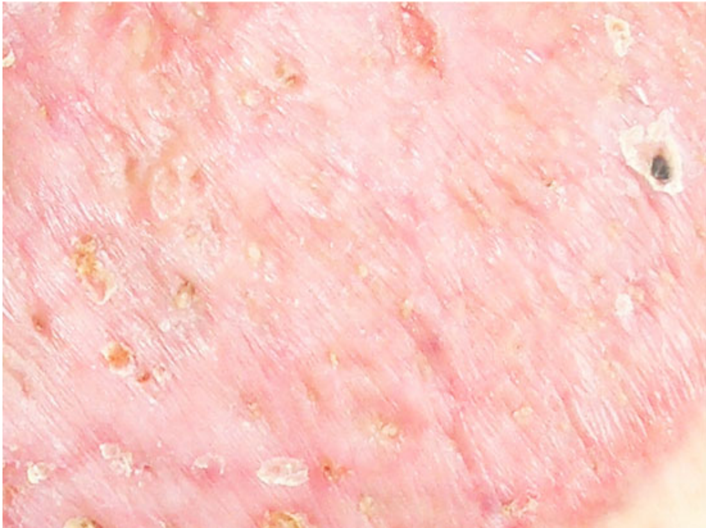

Fig. 1.5 Reepithelialisation. Eight weeks after skin particle seeding. Complete reepithelialisation is accomplished after "in situ culture" utilizing regenerative potentials of the human body

than 40–60 times and they are bound to display signs of senescence with successive passages (Hayflick 2007).

Cell culture has led to research which has paved the way for a number of important breakthroughs in the life sciences (Figs. 1.4 and 1.5). This includes the study of cellular processes, molecular biology and the ability to genetically manipulate cells, and the resulting development of new drugs, with much of recent drug-related research and product development being based on recombinant DNA technology (Nerem 1992). In the early 1970s, Dr. W.T. Green, a pediatric orthopaedic surgeon at Children's Hospital Boston, undertook a number of experiments to generate new cartilage using chondrocytes seeded onto spicules of bone and implanted in nude mice. Although unsuccessful, he correctly concluded that with the advent of innovative biocompatible materials it would be possible to generate new tissue by seeding viable cells onto appropriately configured scaffolds (Figs. 1.6 and 1.7). Several years later, Drs. John Burke, Massachusetts General Hospital, and Ioannis Yannas,

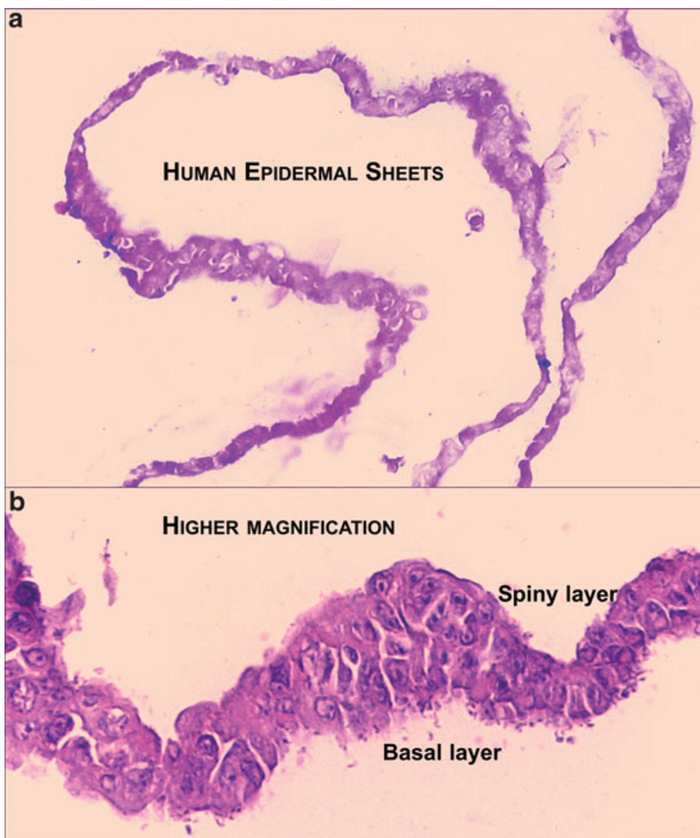

Fig. 1.6 Human epidermal sheets. Human epidermal sheets of autologous keratinocytes, obtained by cell culture (14 days). Light microscopy. (**a**) objective 20×. (**b**) objective 60× (Laboratory of Cellular and Molecular Medicine, Prof. L.M. Popescu, Bucharest)

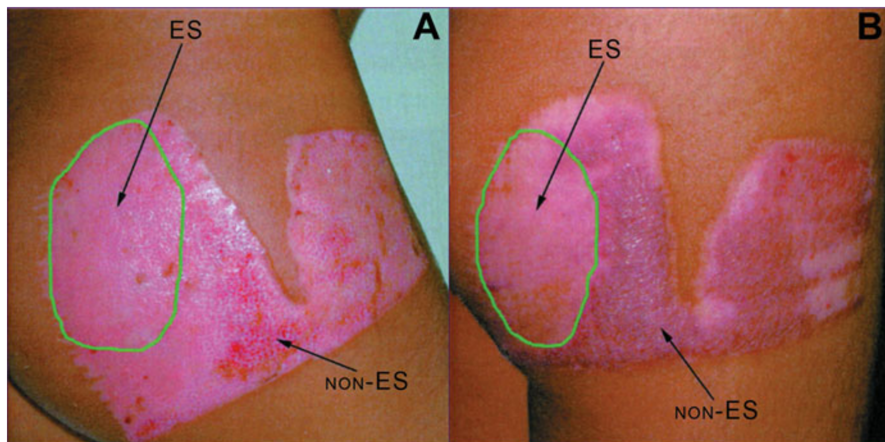

Fig. 1.7 Epidermal sheet treatment. Comparison between the regenerative processes of a donor area with (ES) or without (non-ES) epidermal sheet treatment. (**a**) Ten days after the application of the epidermal sheet. (**b**) Five weeks later (Courtesy of Prof. D. Enescu, Department of Plastic Surgery, Children's Hospital, Bucharest)

MIT, collaborated in studies in both the laboratory and in humans to generate a tissue-engineered skin substitute using a collagen matrix to support the growth of dermal fibroblasts. Dr. Howard Green later transferred sheets of keratinacytes onto burn patients. Dr. Eugene Bell seeded collagen gels with fibroblasts, referring to them as contracted collagen gels. All of these examples represent seeds of the new discipline now known as tissue engineering (Vacanti 2006).

Modern research on embryonic stem cells originates from studies on teratocarcinomas arising from the gonads of imbred mice. These neoplasias displayed a characteristic mixture of different tissues lined up next to each other randomly. By the end of the sixties it was established that they originated from germ cells that were able to give rise to a plethora of different tissues. So the concept of pluripotency of germinal cells was introduced (Kleinsmith and Pierce 1964). From its potential to generate a multitude of different cells, the tumour cell was named embryonal carcinoma stem cell (EC). Research with EC stem cells expanded considerably in the 70s. In a series of experiments, chimeric mice were produced by injecting EC s into early blastocysts (Papaioannou et al. 1975). Interestigly enough, in most of the cases, the tumour cells succumbed to the environment around the developing embryo and they contributed to a perfectly normal mouse pup. Hence it was shown that their genetic code could be "reprogrammed" according to the influence of the environment. Furthermore, the EC stem cells in culture could be constantly kept undifferentiated by frequent splitting or left to differentiate when the culture became too dense and they piled up. However, the EC stem cells were inherently flawed displaying chromosome abnormalities and were unable to differentiate into sperm and egg cells. Since ectopic blastocyst injections were also found to generate teratomas it became soon evident that pluripotent cells could also be derived from blastocysts directly (Damjanov 1993). Soon the next logical step was

undertaken, when Gail Martin (Martin 1981) in USA and Martin Evans (Evans and Kaufman 1981) in England generated in 1981 a stable diploid cell line that could generate every tissue of the adult body, including germ cells. Gail Martin referred to her cells as “embryonic stem cells” and gave them the nickname “ES cells”.

The same line of advances had to be repeated for human cells: Human EC stem cell lines could be isolated and cultured from a rare tumor of the male testes, after orchiectomy procedures (Andrews 1988). However, these cells are always aneuploid and usually lack the capacity to differentiate into somatic tissue (Pera et al. 1989). Human ES cells were not available at this time. What was available, were blastocyst-derived embryonic cells from primates including rhesus monkeys and marmosets (Thomson et al. 1996). These cells displayed all favourable characteristics: they were diploid and were able to give rise to all three types of germinal layers, including germ cells. Their phenotype resembled that of the human EC and were distinctly different from the mice ES. All major technological advances for cultivation and characterization of human ES was achieved by the late 90s – but their harvest was not yet possible.

In 1998 a major step was accomplished toward this direction marking the dawn of a new era. Couples undergoing treatment for extracorporeal fertilization donated a surplus of blastocysts for experimental purposes. James Thomson isolated and cultivated a human ES line from these blastocysts (Thomson et al. 1998).

Adult stem cells were also to enter the arena of biomedical research. The idea that bone marrow contained some kind of osteogenic precursor cells started in 1963 when Alexander Friedenstein (Petrakova et al. 1963) showed that by implanting pieces of bone marrow under the renal capsule, it was possible to obtain an osseous tissue. After this he and his co-workers revealed a series of in vivo studies in which the possible existence of stem cells in the bone marrow was shown. Almost 20 years later, Caplan gave these cells the name they have today, Mesenchymal Stem Cells. In 1994, the same author described that these cells, when placed in the adequate culture conditions, could be differentiated into cells with mesenchymal origin and eventually give origin to bone, cartilage, fat, muscle skin, tendon and other tissues of mesenchymal origin, through what he named “the mesengenic process”(Caplan 1994). Since then, a series of researchers have elaborated on the use of hES for the purposes of tissue engineering and regenerative medicine (Guillot et al. 2007).

In summary the idea of utilizing stem cell transplantation for tissue regeneration or even potential organ replacement is by itself fascinating and generates a huge amount of various experimental and hypothetical approaches within the last years. Especially – similar to the principal idea of tissue engineering – the implantation of adult human autologous or embryonic stem cells, which are expanded ex vivo, might circumvent some of the current problems associated with transplantation surgery, particularly in the elderly. This encompasses the hitherto naturally limited availability of organs or tissues as well as the numerous complications that are related to disease transmission and immune rejection.

This is especially true for the complex of so called musculoskeletal degeneration, that is closely associated with the aging process. However, to introduce adult MSC into clinical practice of substituting organs or tissues, it is necessary to vigor-

ously define the capacity of MSC to maintain growth potential and regulated differentiation of such cells into the desirable cell lineage. There is still not enough body of knowledge at the moment with regard to the physiological and pathophysiological parameters of MSC, including environmental conditions such as biomechanical forces, as to fully understand the potential influence on MSC to differentiate and grow into desired tissues, once extracted and cultured ex vivo (Cheung 2010). It is not known how MSC from young individuals behave versus cells harvested from the elderly. Nevertheless, many efforts are underways to gain more insight into the promising field of harnessing the power of stem cells for tissue and organ regeneration (Cheung 2010). Other issues that concern the ethical aspects of human embryonic stem cells need to be further addressed before research and clinical translation will make its break through.

1.3 Tissue Engineering

The origins of tissue engineering are generally traced to the beginning of the eighties in Boston. Funding was received by the Bell Laboratories in Massachusetts Institute of Technology (MIT) for preparing a cell based vascular scaffold. Prior to that, Eugene Bell had published on the use of "living skin equivalents" in Science as early as 1981 (Bell et al. 1981). Lysaght tracked a press release in 1982 stating that "Flow General", one of the funding firms based in Virginia, was pursuing research and development efforts in business segments including tissue engineering and "smart" computer systems (Lysaght and Crager 2009). E. Bell founded in 1985 "Organogenesis Inc." and later on, "Tissue Engineering International – (TEI) Biosciences Inc." both of which are renowned companies in the biotechnology landscape. During the same time, a few doors further in MIT, Joseph Vacanti of Children s Hospital approached Robert Langer with the idea to design custom made scaffolds for cell delivery. Thereupon, they started an extensive collaboration with studies on the properties of functional tissue equivalents (Vacanti 2006). In 1987 a special session was held at the US National Science Foundation meeting in Washington DC, where the denomination "Tissue engineering" (TE) was officially given to the field and organisation of the first conference with focus on "the engineering of living tissue" was initiated, mainly by Y.C. Fung (Nerem 1992). This conference took place in 1988, at Lake Tahoe, California and the first definition of tissue engineering was introduced by Robert Nerem:

> *Tissue engineering is the application of the principles and methods of engineering and the life sciences towards the fundamental understanding of structure/function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve functions.*

The proceedings of this meeting were published a year later as a book titled "Tissue Engineering" (Skalak and Fox 1989). The first peer reviewed article accessible through the NLM database with the term appeared in 1989. It was a report on a

biologically based vascular graft published by Tadashi Matsuda in ASAIO Transactions (Matsuda et al. 1989). Maybe the most cited early review on tissue engineering is a 1993 publication by JP Vacanti and R Langer in the journal Science where the definition is stated again in brief (Langer and Vacanti 1993).

1995 was a turning point of TE, since it was the year of the "auriculosaurus". Charles Vacanti, seeded a polymeric scaffold in the shape of a human ear with cartilage cells and implanted it subcutaneously on the back of a nude mouse. The pictures of this ear-formed bioartificial implant, filmed by a BBC crew, quickly made the round of the world and attracted a huge interest on the new biotechnology. It became a symbol for the emerging field of TE.

In 1996 the Tissue Engineering Society international (TESi) was officially founded by Joseph and Charles Vacanti, and the inaugural meeting took place at the Lake Buena Vista Hotel in Orlando, Florida the same year. The Asian tissue engineering societies were incorporated in TESi by 2000. By the turn of the century Raymund E Horch and G Björn Stark from Freiburg encouraged the foundation of the European branch of TESi the ETES, and they hosted in 2001 the TESi meeting in Germany.

In 1998, a clinical application of tissue engineering became popular by the media. Charles Vacanti, used a biogenic matrix out of coral seeded with osteoblasts, for reconstruction of the skeleton of a traumatized thumb (Vacanti 2006). The first tissue engineering products cleared FDA approval in the same year. Apligraf came from the E. Bell Laboratories and the firm Organogenesis as living skin equivalent. Epicel evolved from Greens laboratory whereas Yannas together with Integra Life Sciences Inc. brought in 2002 an acellular dermis regeneration scaffold by the name of Integra in the market (Kemp 2006). By the beginning of the twenty-first century, there was a wild media hype about these fascinating new technologies with unrealistic expectations both from the public and the biomedical society (Kratz and Huss 2003). Time magazine described with a cover story, tissue engineers as the "hottest job" for the future: "*With man-made skin already on the market and artificial cartilage not far behind, 25 years from now scientists expect to be pulling a pancreas out of a Petri dish*" (What will be the 10 hottest jobs? 2009). Just before 2001 there were over 3000 people working in the sector, with funding exceeding US $ 580 million (Kemp 2006; Lysaght and Hazlehurst 2004).

1.4 The Era of Regenerative Medicine

However, by the middle of the first decade of the twenty-first century, tissue engineering seemed to be going through a crisis. Lysaght noted very graphically in 2006 that "…such highly favourable media treatment has its benefits, but research-minded professionals increasingly recognized a disconnect with the realities. And such disconnects rarely lead to happy endings" or "…Although aggregate development costs exceed $4.5 billion, the field has yet to produce a single profitable product." (Lysaght and Hazlehurst 2004).

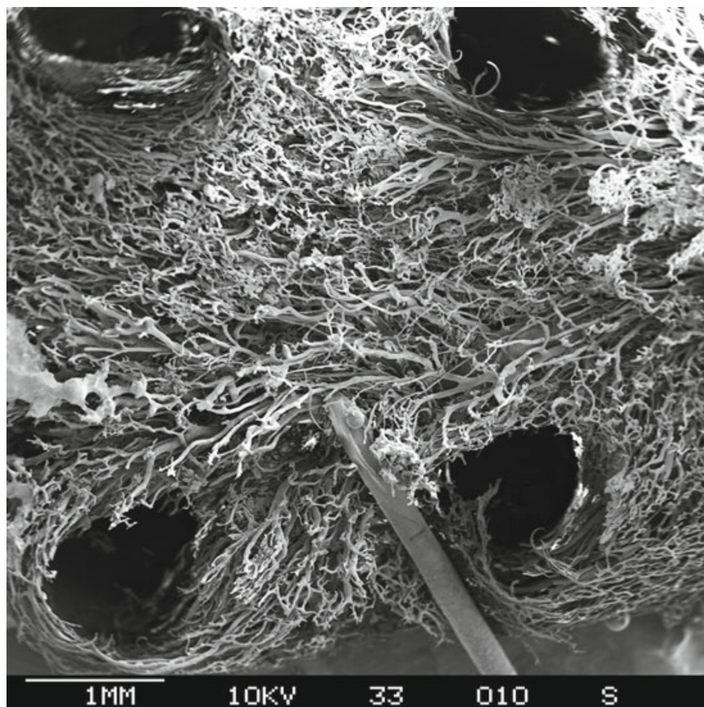

Fig. 1.8 Corrosion casting. A microvascular replica of a bioartificial organoid showing angiogenesis. The neovascular capillaries are "polarized" towards a maximum regenerative stimulus. All these capillaries were formed during the course of less than 1 week

Furthermore, tissue engineering had reached some biological limitations. The mechanistic approach dictated by biomaterial scientists, neglected issues of vascularization. It became clear that angiogenic processes were central to homeostasis, bioassimilation and biointegration of tissue engineered constructs (Vacanti et al. 1998; Mooney and Mikos 1999). Experimental activities were directed to encompass integrative strategies towards generation of autonomously vascularised bioartificial tissue elements (Polykandriotis et al. 2007, 2008; Weigand et al. 2015) (Figs. 1.6 and 1.7). In addition to that, emphasis was being given to cellular therapies, since the era of human embryonic stem cells had arrived. Other satellite technologies had acquired a momentum of themselves, with gene technology reaching the point where a whole mammal could be easily cloned (Wilmut et al. 1997) or genetically manipulated (the Monsanto swine case). Nanotechnology came also into play with generation of new biomaterials (Beier et al. 2009) (Figs. 1.8 and 1.9).

The whole field was consecutively renamed into "regenerative medicine". The terms tissue engineering and regenerative medicine were used in parallel and synonymously to each other, but it is widely accepted that the very change of the name epitomized the beginning of a new era.

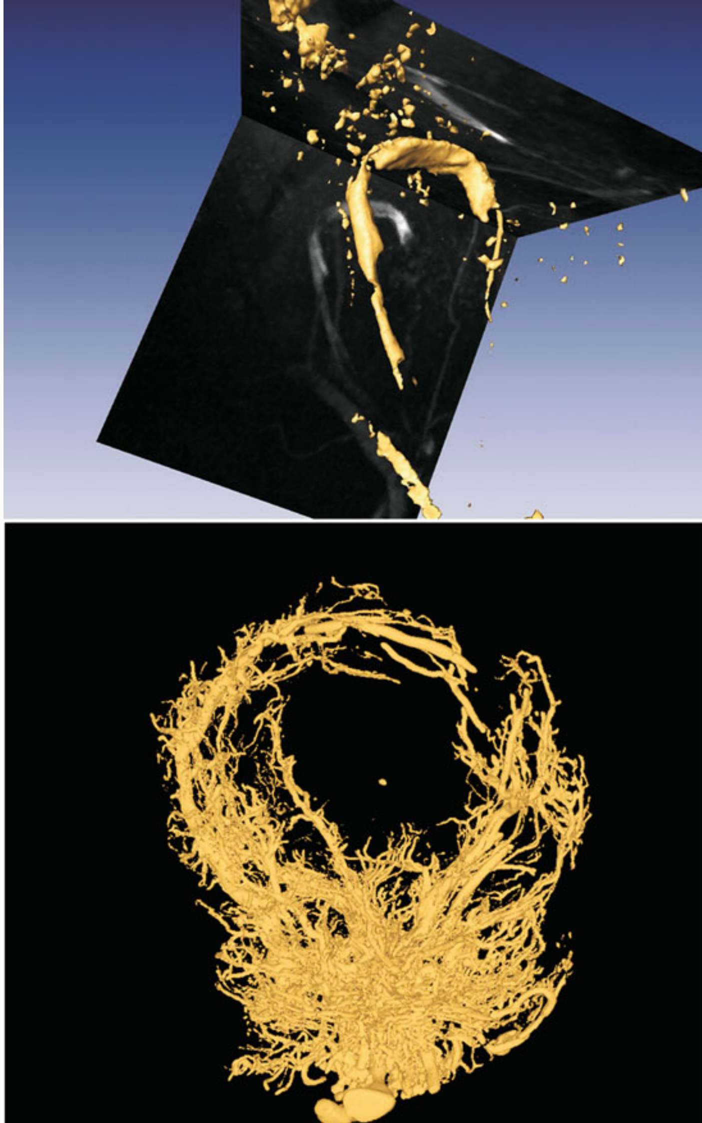

Fig. 1.9 Advanced imaging applications. *Above*: Micro magnetic resonance angiography of a bioartificial organoid grown in a rat. A 4,7 Tesla Brucker bioscan equipment has been used for in vivo monitoring of the nascent biological construct. *Below*: Ex vivo Micro CT study of the same organoid after injection of a contrast medium. Plasticity of the microvascular network produces a parenchyma – like circulation

The term “regenerative medicine” was popularized by William Hazeltine, the founding editor of Liebert’s first e-journal Regenerative Medicine and organizer of four conferences on this same field, in Washington, DC between 2000 and 2003 (Lysaght and Crager 2009). However, he was not the first to introduce it.

The term can be found in a 1992 article on hospital administration by Leland Kaiser. In the last paragraphs there was a brief account of future technologies about to influence the sector. Under the epigram “Regenerative Medicine” it was stated that “A new branch of medicine will develop that attempts to change the course of chronic disease an in many instances will regenerate tired and failing organ systems” (Kaiser 1992). Over the next few years, several reports used the expression in connection with the New Jersey Company Integra and it could also be found in scattered newspaper reports. In spring of 1998 Business Week brought an article on biotechnology with a special reference on “Regenerative Medicine” (Lysaght and Crager 2009; Arnst and Carey 1998). The term first appears in peer-reviewed citations found on PubMed in 2000 and was in widespread use by the following year.

At the early years of the new century a shattering process took place in USA. The new legislature had frozen most of federal granting for stem cell research. Robert Nicholas Klein II, a lawyer and real-estate developer from Palo Alto, whose son suffered from diabetes mellitus type I, and whose mother suffered from Alzheimer’s disease became the leader of a public effort to change policy of the government on stem cell research and regenerative medicine. He himself invested more that $3 million from his own money on this purpose and was crowned with success. On 2 November 2004, the proposition No71 passed through a public ballot initiative with a 59.05–40.95 % majority. As a response to that, the California Institute of Regenerative Medicine was established as a superintending instance for a huge funding of more than $3 billion over 10 years. It is worth noticing, that proposition 71 was unique in many ways. First, it used General obligation bonds, which are usually used to finance construction projects such as bridges or hospitals, to fund scientific research. Second, by funding scientific research on such a large scale, California assumed a role that is typically fulfilled by the Federal Government of the United States. And third, Proposition 71 established the state constitutional right to conduct stem cell research. This initiative also represented a unique instance where the public directly decided to fund scientific research (California Proposition 71 2004).

The NIH has currently adopted the following definition: “*Regenerative medicine/tissue engineering is a rapidly growing multidisciplinary field involving the life, physical and engineering sciences that seeks to develop functional cell, tissue, and organ substitutes to repair, replace or enhance biological function that has been lost due to congenital abnormalities, injury, disease, or aging”* (Daar and Greenwood 2007). The current FDA approved RM products cover a limited circumscribed range of markets in the 20–50 million scale (Kemp 2006). Still, the public and academic interest remains vivid, owing mostly to the fact that RM has the potential to tackle huge health challenges including cardiovascular disease, brain and spinal cord damage, as well as organ failure. These medical issues cost the community billions nowadays, and more often than not so, medical solutions devised so

far are unsatisfactory. When biotechnological advances permit it, these medical problems will be addressed and firms well placed in the field fashioned with infrastructure, skills and a corresponding logistic network will have a clear advantage (Kemp 2006).

Apart from haematopoietic stem cell transplantation for haematological disorders many stem cell-based therapies are still experimental. Nevertheless, meanwhile in various developed countries stem cell technology had been expected to assert significant added value in the commercialization and industrial application of stem cell technologies for regenerative purposes. Intermediate steps that make use of mesenchymal stem cells have been shown to be effective in a clinically relevant dimension already (Cheung 2010; Horch et al. 2014). Difficulties that have impeded the immediate clinical translation in this area have been attributed to the very small number of clinical trials and approved products for regenerative medicine or cell therapy so far. This includes also the difficulties which are to be encountered en route to clinical trials. Despite some drawbacks in translation and commercialization of stem cell therapies the experimental results of recent developments are still encouraging the acceleration of harnessing stem cells.

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Chapter 2
Regulatory Framework for Cell and Tissue Based Therapies in Europe

Gudrun Tiedemann and Sebastian Sethe

Abstract Whereas some basic therapies based on tissues and cells have been in clinical use for years, advances in regenerative medicine present new challenges and new types of products for regulation. This article focuses on cell therapies which can be classified as medicinal products across the European Union.

We describe the regulatory boundaries of these products by showing how European regulators have sought to developed rules to distinguish 'advanced' cell therapy products from their 'basic' predecessors and discuss the limits of European legislation for therapies which are not placed on the European market. We further chart the envisaged regulatory route of these products from pre-clinical data generation through manufacturing, clinical trials, and regulatory review into routine, closely monitored use. We conclude with a brief reflection on the low number of products that have travelled along this regulatory path to date in Europe.

Keywords Stem cell • Regulatory requirements • Regulatory organizations • Pharmaceutical quality • Potency • Toxicity

2.1 Introduction: Cell Therapies as Medicinal Products in the EU

This article focuses on the subset of regenerative medicine strategies that are cell therapies which can be classified as Medicinal Products ("MPs") for human use in the European Union (EU).

The essential aim of the European medicines legislation is to safeguard public and patient health and to ensure free movement of the products between the

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Members States of the EU without hindering research and innovation. European medicines legislation is developed by the European Commission (EC) and adopted by the European Parliament together with the Council of the EU. European Law distinguishes between Regulations and Directives. Whereas European Directives are considered to have direct effect, they first require implementation by national legislation in the individual Member State. In contrast, European Regulations are directly applicable (yet may still be in need of substantiation in a national context). Therefore, although European legislation is in principle harmonized, the interpretation and implementation of the regulatory requirements in individual Member States may differ.

Production, distribution, and use of MP's are controlled by specific regulatory provisions. Directive 2001/83/EC (the "Medicines Directive") applies to MPs which are intended to be placed on the EU market and are either prepared industrially or manufactured by a method involving an industrial process. A special 'sub type' of MP relevant for the cell therapies discussed in this volume are so-called "Advanced Therapy MPs" ("ATMPs").

MP's that fall within the scope of the "centralized authorisation procedure" are evaluated for quality, safety and efficacy by the European Medicines Agency (EMA), headquartered in London. Within the EMA, the Committee for Advanced Therapies (CAT) has a specialist role with regards to ATMP. Based on a recommendation by the CAT and EMA, the EC may issue a marketing authorization (MA) for the product in Europe. Medicines that do not fall within this scope are marketed in the individual EU Member States in accordance with national authorisation procedures not involving the EMA.

Thus, for many practical purposes and for the purposes of this chapter there are two main considerations: Firstly can the product be classified as an (AT)MP at all? If yes, is the ATMP "placed on the market" industrially and what are the implications?

2.2 Product Classification of Cell Therapies

At the distinction to the classification of an MP lie regulatory regimes applicable to medical devices, tissue and cell transplants, veterinary applications, foodstuffs and cosmetics.

With regards to devices, excluded from being an MP is any product made exclusively of non-viable materials which act primarily by physical means.

One of the most frequent borderline cases is the question whether a cell or tissue product should be regulated as an ATMP or as a 'basic' cell/tissue transplant. The first part of this sub-section will focus on this question.

In the second part, we consider further classification of a product that has been classified as an MP in the context of ATMPs.

2.2.1 Medicinal Product Or Cell Transplant?

Directive 2004/23/EC sets standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells and is supplemented by two technical Directives: 2006/17/EC & 2006/86/EC (collectively hereinafter "Tissue Directive").

The Tissue Directive applies to the various tissues and cells including bone-marrow-, adult- and embryonic stem cells as well as reproductive cells (eggs, sperm) and foetal- tissues and -cells. It covers hematopoietic peripheral blood and umbilical-cord (blood), but not blood and other blood products. Blood[1] and Organ[2] transplants are regulated separately.

Also excluded from the Tissue Directive are tissues and cells which are used as an autologous graft (tissues removed and transplanted back to the same individual), within the same surgical procedure and without being subjected to any banking process. The quality and safety considerations associated with this process are not harmonized within the EU.

In general, if there is no 'substantial manipulation' and the cells/tissues are administered to fulfil their 'same essential function' in the recipient as in the donor the product is not regarded as ATMP but classified as a tissue transplant. The Tissue Directive has been transposed into national law by the individual Member States. Particularly with regard to cell products with no substantial manipulation and homologous use the classification of the products may vary among Member States.

2.2.1.1 Substantial Manipulation

Annex I of the ATMP Regulation lists manipulations that are not considered 'substantial', (based on the equivalent test set out by the FDA in the United States). Most of the actions listed in Annex I would suggest that an isolated or concentrated cell population would not constitute 'substantial manipulation'. In a number of its classification decisions, the CAT has not suggested that extraction of sub-populations of stem cells for autologous use (e.g. CD133+ bone marrow cells) would constitute 'substantial manipulation'.[3]

However, regardless of the actions listed in Annex I, the test as set out in the definition of an 'engineered product' specifies that a cell will only have been substantially manipulated if the relevant biological characteristics, physiological functions, or structural properties have been changed.

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[1] Directive 2002/98/EC.

[2] Directive 2010/53/EU.

[3] EMA Scientific recommendation on classification of advanced therapy medicinal products; EMA/921674/2011; 24 November 2011.

2.2.1.2 Same Essential Function

The concept of 'same essential function or functions in the recipient as in the donor' is intentionally similar to the equivalent, 'homologous use' test in the United States, which refers to "the repair, reconstruction, replacement, or supplementation of a recipient's cells or tissues with an HCT/P that performs the same basic function or functions in the recipient as in the donor". The question is often difficult to decide: It has been argued that 'relevant' biological characteristics, physiological functions or structural properties are those that are relevant to the intended use of the cell and that as a result, the applicable test should be whether there is a change in the characteristics relevant to the intended use.[4] The alternative approach places on whether there is a change in , primary'or ,dominant' characteristics.

Controversially, even if a minimally manipulated cell-based product is administered in the same anatomical location it may fall under the definition of an ATMP on grounds that it is used for a different essential function. This can be encountered when the mode of action (MoA) of the cells is not identical to the one attributed to the origin cells. Examples are autologous bone marrow-derived progenitor cells intended for treatment of patients with myocardial infarction or even injection of concentrated bone marrow at the site of bone injury with the aim of healing a bone lesion can be considered as non-homologous use.

Similar questions arise with respect to cells derived from blood (blood products)[5]: the CAT suggests that cells derived from human blood that are substantially manipulated or used for a different essential function are classified as MP.

2.2.1.3 Procurement of Tissue Or Cells

Even if it is clear that the product is an MP, the Tissue Directive is relevant to developers as it covers the donation, procurement and testing of cell and tissue ,source materials'– whereas the further manufacturing steps are regulated by the Medicines Directive. Such use may not have been foreseen when the cellular source materials were initially obtained, especially in the case of gametes from which embryonic stems were derived. This may present particular challenges to ATMP developers.

2.2.2 Classification Within the Medicines Directive

European Medicines Regulation recognises a special 'class' of MP's that does not exist in this form for the other ICH partners: "Advanced Therapy Medicinal Products" (ATMP).

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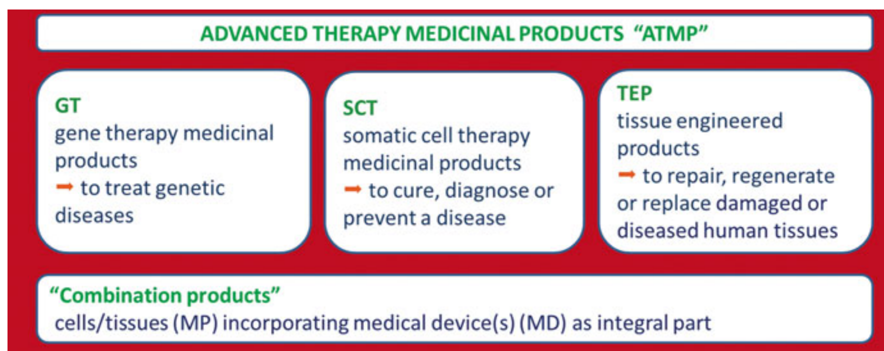
[4] Denoon A, Hitchcock J, Lawford Davies J; "The regulation of stem cells in the UK and the EU" in: Vertès, et al (eds) *Stem Cells in Regenerative Medicine: Science, Regulation and Business Strategies*, 2015 John Wiley.

[5] COM: report from the commission to the European parliament and the council Brussels 28.3.2014.

Table 2.1 Definition/Classification of ATMPs regarding Dir. 2009/120/EC amending Dir. 2001/83/EC



ATMP generally fall into one of the categories:

(A) Somatic cell therapy medicinal product (SCT): contain or consist of cells or tissues which (as we have seen above) are substantial manipulated and/or not intended to be used for the same essential function(s) as in their original position. SCT are used for treating, preventing or diagnosing a disease through the pharmacological, immunological or metabolic action of its cells or tissues.
(B) Tissue engineered product (TEP): contain or consist of cells or tissues (SCT) that are used to repair, regenerate or replace damaged or diseased human tissues. TEP may contain cells or tissues of human or animal origin, or both (viable or non-viable) and furthermore may contain additional substances, such as cellular products, bio-molecules, biomaterials, chemical substances, scaffolds or matrices.
(C) Gene therapy medicinal product: contain an active substance which contains or consists of a recombinant nucleic acid with a view to regulating, repairing, replacing, adding or deleting a genetic sequence. Its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence.
(D) Combined Advanced Therapy Medicinal Products: are products where the cells/tissues also incorporate Medical Device(s) (MD) as integral part. The cellular or tissue part of a Combination product must contain viable cells or tissues, or its cellular or tissue part containing non-viable cells or tissues must be liable to act upon the human body with action that can be considered as primary to that of the devices referred to (Table 2.1).

'Borderline cases' are classified according to the 'dominant characteristic' and the 'mechanism of Action' is important: is it for treatment, prevention or diagnosis, and exerts it its activity via a pharmacological, immunological or metabolic action, or is it intended for regeneration, repair or replacement of cells/tissues? The predominant mode claimed will affect whether it will be classified as somatic cell therapy or tissue-engineered product. If there is doubt about the category within the ATMPs there is a demarcation rule: GT persists over TEP and TEP persists over SCT.

To help classify products, the CAT offers a classification procedure which is optional and free of cost, under which it offers scientific recommendation to an applicant about the nature of the product under consideration. This advice is non-binding, but it is unlikely that the national Authorities will contradict the CAT classification. Until 2015 there were more than 100 products assessed by CAT, 90 % being classified as ATMPs.[6]

In essence however, the classification ‘label’ within the Medicines Directive by itself is of little practical relevance to developers. All (AT)MP’s are subject to the detailed technical requirements of medicines regulation and at a granular level the product-specific data review and Good Manufacturing Practice requirements apply largely irrespective of the broader classification label. With the exception of Combination Products, there are no fundamental differences according to product type for their placing on the market, discussed in the next section.

2.3 Placing Cell Therapy Products on the Market

As we have seen, the Medicines Directive applies only to products “placed on the market”. There is some uncertainty about what the definition entails for cell therapies. In the context of medical devices, ‘placing on the market’ means the first ‘making available in return for payment or free of charge … with a view to distribution and/or use in the community market’.[7] The 2008 Market Surveillance Regulation defines it as “any supply of a product for distribution, consumption or use on the Community market in the course of a commercial activity, whether in return for payment or free of charge”. Controversy surrounds the question of whether ‘placing on the market’ requires some kind of ‘transfer’ to occur.

In any event, there are certain recognized scenarios where products are *not* placed on the market. These are discussed in the first sub-section, with a particular emphasis on the so-called ‘hospital exemption’. In the second part, the steps involved in placing a product on the market are briefly discussed.

2.3.1 Not on the Market: Trials, Specials and the ‘Hospital Exemption’

2.3.1.1 Clinical Trials

Clinical Trials in pursuit of a Marketing Authorization are, by necessity, exempt from the requirement to have a marketing authorisation in place. However, it should be pointed out that the general requirement for a separate ‘manufacturing authorisation’ discussed later in this chapter still applies even to investigational ATMP.

[6] EMA/CAT/600280/2010 rev.1.

[7] Directive, 93/42/EEC Article 1(2)(h); compare: Interpretative Document of the Commission’s Services: Placing on the Market of Medical Devices. SANCO/B/2/PBE/pdw Ares(2010) 332016.

From 2016, a new Regulation applies to all clinical trials conducted within the EU.[8] It distinguishes between observational "clinical studies" and interventional "clinical trials". Low-intervention clinical trials are trials that use authorised (rather than investigational) medicinal products and that do not pose a substantial additional risk compared with clinical practice.

The application dossier for clinical trials comprises two parts. Part I – which deals mainly with the type of clinical trial, risk-benefit analysis, and compliance with technical requirements – is assessed by the so-called "reporting Member State" and is valid for the entire EU. Part II deals with specifically national aspects, such as informed consent which are assessed by each state separately in a procedure that also involves ethical committees. For gene therapies the timeline period available to Ethics Committees and Competent Authorities to review clinical trial applications is extended. Controversially, there is a blanket prohibition on gene therapy trials that result in modifications to the subject's germ line genetic identity.

EMA has published 'detailed guidelines on good clinical practice specific to advanced therapy medicinal products'[9] and a general reflection paper[10] which should be read alongside the general rules for good clinical practice.[11]

An EU database will contain a summary of the results of all trials as well as a summary for laypersons, which the sponsor is required to submit regardless of the trial outcome. All the information on the EU database will be accessible to the public, with the exception of: personal data; confidential commercial information (unless there is an overriding public interest in disclosure); confidential communications between Member States relating to the assessment report; information whose disclosure would jeopardise effective supervision of the conduct of a trial by Member States.

2.3.1.2 Individual Patient Treatment

Distinct from alternative "compassionate use" provisions,[12] Article 5 of the Medicines Directive allows Member States to exclude individual MPs from the requirements to obtain a marketing authorisation where the medicinal product is needed to fulfil 'special needs' provided that such MP's must only be supplied in response to a bona fide unsolicited order formulated in accordance with the specifications of an authorised healthcare professional for use by an individual patient under the healthcare professional's direct personal responsibility. Generally, a 'special need' requires that no equivalent licensed MP is already on the market.[13]

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[8] Regulation (EU) No 536/2014.

[9] Brussels, 03/12/2009 ENTR/F/2/SF/dn D(2009) 35810.

[10] EMA/CAT/571134/2009 (16/3/2010).

[11] Directive 2005/28/EC.

[12] Regulation (EC) 726/2004 Article 83.

[13] MHRA Guidance Note 14 "The supply of unlicensed medicinal products ("specials")" 2014 edition.

While the Art.5 'specials' exemption is widely used for certain regenerative medicine therapies in some Member States, the more specific provision is the so-called "Hospital Exemption" of Art.3 Medicines Directive which only applies to custom-made ATMPs in the absence of a Marketing Authorisation prepared on a non-routine basis used for an individual patient under the exclusive professional responsibility of a medical practitioner. While the requirements of Art. 3 and 5 are similar, there are many significant differences, and the cross-applicability of the provisions is interpreted and implemented differently in different Member States.

Here, by means of example, we briefly consider 'Hospital Exemption' provisions in Germany:

2.3.1.3 "Hospital Exemption" in Germany

The German approval and authorisation requirements are laid down in the German MP-Act the 'Arzneimittelgesetz' (AMG). The Competent Authority responsible for cell and tissue products is the Paul-Ehrlich-Institut (PEI – Federal Institute for Vaccines und Biomedical Drugs). In addition, there are local authorities with specific functions in particular manufacturing.

The national provisions for the "Hospital Exemption" are laid down in §4b (1 and 2) AMG and apply to ATMPs, which are prescribed by a physician for an individual patient as a custom-made preparation, applied under the responsibility of a physician in a specialised health care unit and manufactured on a non-routine basis according to specific quality standards.

Here 'manufactured on a non-routine basis' means in particular ATMPs which are manufactured on a small scale and where – on the basis of routine production – the product has to be individually modified because of a medical indication for a single patient, or products not yet manufactured in a sufficient number to lay down the necessary results for a comprehensive evaluation.

The German provisions for these 'non-routine' ATMPs require that quality standards for production have to be assured by a manufacturing authorisation ('Herstellerlaubnis') regarding §13 AMG via local authority and PEI (same authorisation as for 'routine ATMPs').

Although there is no need for the European MA according (§21 AMG) (as there is no placing on the market (§43 AMG)) ATMPs which are "handed over to others" need an authorisation through 'Genehmigung' (§21a(2–8)AMG) via PEI. This requires specific quality standards whereby the template for authorisation corresponds in general with the Common Technical Document (CTD) for approval but the 5 CTD-modules for quality, preclinical and clinical data, summaries and registration are abbreviated versions. If the manufacturer is the user (this is sometimes the case in hospitals, especially with "point-of-care-manufacturing") no permission for application is required. Traceability and pharmacovigilance are overseen by the competent local authority and/or PEI and have to be equivalent to the rules on EU level.

If there is doubt whether an ATMP falls under the provision 'Genehmigung' or not, the relevant local competent authority is responsible to decide this on request of the applicant and after consultation with the PEI.

Authorisations are time-limited and will be withdrawn when the prerequisites for the exemption are not or no longer fulfilled. The holder of the authorisation has to report to the PEI about the scale of production and the consolidated findings for the evaluation of the MP.

2.3.2 Marketing Authorisation

All ATMP are subject to a centralised Marketing Authorisation at European Level: Following a formal application for Marketing Authorisation, the period for issuing or rejecting an application is 210 days at EMA but for follow-on request for supplementary information, additional 'clock- stops' may be applied.

The general requirements for approval of ATMP are not different compared to any other (biological) medicine: through the scientific assessment of quality, efficacy, and safety, it has to be demonstrated that the benefits of marketing the product outweigh the risks.

Further specific technical requirements are laid down according to guidelines and provisions for quality and preclinical/clinical data and specific provisions are given for products that combine biological materials and chemical structures. Part IV, Annex I of the Directive 2001/83/EC lays down detailed scientific and technical requirements regarding the testing of medicinal products for human use and describes the format requirements (Modules 1–5) for MA. The regulations specify a number of requirements. A few examples:

Cell sources: Information on donation, procurement and testing shall be provided. Animal cells or tissues are expressly not excluded but specific acceptance criteria must be provided. If 'non-healthy' cells or tissues are used as starting materials, their use shall be justified. Problematically, if allogeneic cell populations are being pooled, the pooling strategies and measures to ensure traceability shall be described.

Pre-clinical development: The Regulations suggest that 'The use of homologous models (e.g. mouse cells analysed in mice) or disease mimicking models shall be considered, especially for immunogenicity and immunotoxicity studies'. Different scientific opinions exist on the value of such studies. The regulations state that 'conventional pharmacokinetic studies to investigate absorption, distribution, metabolism and excretion shall not be required'. However, parameters such as viability, longevity, distribution, growth, differentiation and migration of cells shall be investigated, unless otherwise duly justified. Given the reported difficulties in cell tracking, this requirement alone may provide a significant barrier to development.

Risk analysis: Risk factors that may be considered include: the origin of the cells (autologous, allogeneic, xenogeneic), the ability to proliferate and/or differentiate and to initiate an immune response, the level of cell manipulation, the combination

of cells with bioactive molecules or structural materials, the long-time functionality, the risk of oncogenicity and the mode of administration or use.

The manufacturing process involves the emulation of the concept of a 'production batch' used in the context of mainstream pharmaceuticals. Manufacturing must be validated to ensure "batch consistency" and "the proper differentiation state and the cell function with additional substances throughout the manufacture" – this would seem to place significant technical requirements on manufacturers in handling an inherently heterogeneous product. The regulation suggests that normally, the functional integrity of the cells should be tested at the moment of application/administration, but specify that if certain release tests cannot be performed on the active substance or finished product, but only on key intermediates and/or as in-process testing, this needs to be justified.

For combined ATMPs there is the additional requirement to comply with Devices regulation, i.e. to seek 'CE marking' for the device component of the product in collaboration with a Notified Body. The combined product is subject to final evaluation by the CAT (MP-route EMA). A collaboration group between the CAT and the Notified Bodies exists.

2.3.2.1 Manufacturing

EU Member States are obliged to "take all appropriate measures to ensure that the manufacture of the medicinal products within their territory is subject to the holding of an authorization". This manufacturing authorization is required irrespective of whether the medicinal products manufactured are intended only for export, partial manufacture or manufacture only for the purposes of a clinical trial.

Holders of manufacturing licences must comply with the principles and guidelines of good manufacturing practice (GMP) for medicinal products and use as starting materials only active substances, which have been manufactured in accordance with the detailed guidelines on GMP for starting materials. The EC's Directorate General of Health and Consumers (DG SANCO) publishes these GMP guidelines as Volume 4 of the "Rules Governing Medicinal Products in the European Union (EudraLex)". Volume 4 is in three parts. The first concerns the basic GMP requirements for medicinal products; the second concerns the basic GMP requirements for 'active substances used as starting materials'; and the third comprises a set of GMP-related documents. The volume includes annexes providing detailed rules on specific matters. The most relevant rules for ATMPs as medicinal products are located at Annex 2 entitled "Manufacture of Biological Active Substances and Medicinal Products for Human Use". Part A of this Annex 2 sets out general considerations on the manufacture of biological active substances and medicinal products. It provides detailed directions on personnel, premises and equipment, documentation requirements, production and starting and raw materials, appropriate seed lot and bank systems, operating principles and quality control. Part B includes consideration of the specific 'types' of ATMP discussed above.

2.3.2.2 Certification

Uniquely specific to ATMP's and only available to small and medium enterprises, a 'certification' regime exists under which EMA can 'certify' data as being of sufficiently high quality for regulatory consideration.[14] The scope of the evaluation is to certify relevant quality and, where available, non-clinical data as compliant with the relevant scientific and technical requirements set out modules 3 and 4 of Annex I of the Medicines Directive.[15] The certification procedure covers only a scientific evaluation of experimental data already generated. Advice for further development will have to be obtained by the Scientific Advice procedure. The certification procedure is independent from a future application for Marketing Authorisation, but it could facilitate the evaluation of any future application for clinical trial authorisation or a marketing authorisation application, provided that these applications are based on the same data. It is expected that successful certification may facilitate the raising of capital for further R&D. A certificate is not binding with regard to any future regulatory procedure. The certificate cannot conclude on the adequacy of the studies submitted to be further developed in a clinical trial. This is under the responsibility of the National Competent Authorities where the clinical trial will be conducted. Perhaps for these reasons, the take-up of the certification procedure has been extremely limited.

2.3.2.3 Post-Marketing

Applicants for Marketing Authorisation must present for review and commit to a detailed description of their post-marketing pharmacovigilance system and a separate risk management system, which involves, inter alia, an efficacy follow-up plan.

EMA has issued ATMP-specific guidelines.[16] Many ATMP's present particular indications that may merit long term- even lifetime follow-up. While privacy considerations must not be neglected, establishing registries of patients having undergone cell and tissue therapies[17] may be highly relevant for long-term safety and efficacy follow-up. The EMA is currently engaged in a 'pilot phase'[18] project in this regard.

A number of options exist for management of various post-authorisation commitments including letters of commitment; conditional approvals or approvals

[14] Regulation (EC) No 668/2009.

[15] Committee for advanced therapies (CAT) "Procedural advice on the certification of quality and nonclinical data for small and medium sized enterprises developing advanced therapy medicinal products" EMA/CAT/418458/2008; 15 October 2010.

[16] EMEA/149995/2008.

[17] Nesteruk J, Steinhoff G; "Stem cell registry program for cardiac patients: what benefits does it derive?" (submitted to Cell Transplant).

[18] EMA; "Initiative for patient registries – Strategy and pilot phase" EMA/176050/2014; 15 September 2015.

under exceptional circumstances with specific obligations. The Regulators also have discretion to stipulate particular reporting obligations. Used responsibly, this remit to place a strong emphasis on post-marketing authorisation studies combined with a regime that expedites access to the market, especially for treatments that address a significant unmet medical need.[19]

2.3.2.4 Regulatory Exclusivity

Competitors have to wait a minimum of 8 years before they can use the applicant's original data in support of an application, but generic manufacturers are still restricted from market access for two, or –in some cases– three further years. This rule for ‚regulatory exclusivity' of data applies to all MP's but Article 10(4), of the Medicines Directive clarifies that establishing in respect of biological medicinal products (of which ATMPs are subset) the competitor must justify "differences relating to raw materials or differences in manufacturing processes" through separate clinical studies. The difficulty in establishing ‚similarity' of complex product such as cell therapies will often mean that generic competitors have to engage in significant research, development and regulatory efforts themselves.

2.3.2.5 Reimbursement

Marketing Authorisation only establishes that product may be sold in principle. To get a product established on the market commercially, the benefit of the new therapy in comparison to existing therapies has to be demonstrated to the payers. Owing to different healthcare systems, reimbursement procedures for medicines are not harmonised across the EU.

2.4 Outlook

ATMP products are of high complexity raising challenges and issues for development, manufacturing and analytics which are very different from those of "small molecule" and even "complex biological" medicinal products. Experience has also shown that it is difficult to establish safety, purity, and potency of the products.

The regulatory requirements are often seen as burdensome by developers. This is reflected in the fact that although over 150 classification procedures have been undertaken by CAT, only 14 applications for Marketing Authorisation have been submitted of which half have been issued a positive opinion by CAT and only 3 are on the market today. Also, over 250 scientific advice procedures were undertaken,

[19] Ali RR1, Hollander AP, Kemp P, Webster A, Wilkins MR. "Regulating cell-based regenerative medicine: the challenges ahead" in: Regen Med. 2014 Jan;9(1):81–7.

referring to over 150 different products. This is against a background of many hundreds of cell therapy clinical trials conducted in Europe.[20]

It has been observed that for many ATMP products, especially cell-based and patient-specific treatments, the pharmaceutical industry has limited involvement[21] in playing its 'usual' role of financing development and of acting as a sponsor in clinical trials. Many ATMP are manufactured very differently from mainstream medicines and require highly specialised, often tacit, local expertise that is difficult to transfer and standardise. Moreover, due to the difficulties in scale-up, it is often not possible to conduct trials on a large patient population.

The fact that many innovators are *not* aiming to "place a product on the market" but are instead content to establish a treatment only locally[22] may explain the comparatively low number of Marketing Authorisation applications as well as the continued interest in seeking to 'avoid' the established pathway to market in favour of regulation under a 'tissues and cells' framework or under the 'specials' and 'hospital exemption' that were discussed in this chapter.

From a policy perspective, appreciation of the specific scientific, technical, and economic drivers coupled with a sensitivity to acute medical need which may be particular to each treatment in regenerative medicine, may be required in favour of 'one size fits all' attempts to harmonise. A shift in emphasis from pre- to post-marketing attention in the risk-benefit balance may be necessary, not just to facilitate actual translation "from protocol to patient" but also to establish greater familiarity across the health sector with the complex therapeutic strategies involved in regenerative medicine.

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[20] Li MD, Atkins H, Bubela T. "The global landscape of stem cell clinical trials" in: Regen Med. 2014 Jan;9(1):27–39

[21] Maciulaitis, R. et al. (2012) "Clinical Development of Advanced Therapy Medicinal Products in Europe: Evidence That Regulators Must Be Proactive" in: Molecular Therapy 20: 479–482.

[22] Pirnay JP1, Vanderkelen A, De Vos D, Draye JP, Rose T, Ceulemans C, Ectors N, Huys I, Jennes S, Verbeken G. "Business oriented EU human cell and tissue product legislation will adversely impact Member States' health care systems" in: Cell Tissue Bank. 2013 Dec;14(4):525–60.

Chapter 3
Ethics and Law in Regenerative Medicine

Nikolaus Knoepffler, Tade Matthias Spranger, Nikolai Münch, and Martin O'Malley

Abstract Regenerative medicine is the scientific approach to medical treatment by means of replacing, regenerating, or engineering human tissue. RM goals present no inherent ethical difficulty and this paper addresses legal and ethical issues presented by potential RM methods. These methods include the creation and use of artificial gametes and tissues for organ replacement using various genetic technologies.

The present ethical and legal discussion builds upon the framework of human dignity and human rights, as well as international conventions such as the Declaration of Helsinki, and guidelines such as those from the World Health Organization. We argue that RM presents great therapeutic potential that can be achieved within clear ethical and legal frameworks. These frameworks must protect research subjects, clarify the status of research embryos if human embryonic stem cells (hESC) are used, and to deal with societal impact.

Keywords Stem cell research • Human dignity • Ethics • Law • Organ replacement • Gene therapy • Gene editing

3.1 Point of Departure

Rapid scientific, medical and social developments necessitate a structure of ethical reflection and legal regulation to deal with the hard cases that push established moral norms and practice into conflict. RM is such a case. It covers a vast area of medical research and intended applications. Advances in RM using stem cells for organ growth and replacement provide some hope for people with failing organs.

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One aim of research with adult stem cells is to solve this very urgent problem. Instead of transplanting a heart researchers have used adult stem cells (aSC) aiming at providing a therapy to restore the former function of the heart, which was damaged by a heart attack for example (Schade et al. 2014; Gaebel et al. 2011). This kind of research raises classical ethical and legal research questions: how to do an ethically acceptable research on animals, how to protect the research subjects including patients? This protection is not only protection of health, but also protection of personality rights like the ownership of the tissue used in research. Similar questions arise when induced pluripotent stem cells (iPSC) instead of aSC are harvested. Since Yamanaka and his team have discovered a method to retrieve these stem cells (Kazutoshi and Yamanaka 2006; Kazutoshi et al. 2007), the advances are remarkable. iPSC were extensively used to create tissue specific cells, e.g. hepatic cells (Berger et al. 2015), cardiomyocytes (Kim et al. 2015; Sharma et al. 2015), chondrocytes (Nejadnik et al. 2015; Umeda et al. 2016), bone cells (Wang et al. 2015), and even neurons (Du et al. 2015; Nieweg et al. 2015).

Much more contested is this kind of research, if human embryonic stem cells (hESC) are utilized whose use is still the "gold standard" of research in this field. This use might lead to conflict if the embryo has the moral status of a born human being, because the embryo would have a right to life and corporal integrity. Here the ethical positions and regulation vary very much.

There are more ethical and legal issues involved if the purpose of research in RM is not restricted to restoring the health in the usual way. Progress in cell-reprogramming and in understanding of cell-differentiation processes has made the creation of artificial human gametes possible (Hendriks et al. 2015). The possible benefits of this development for research as well as for clinical application are considerable. Millions of people suffering infertility might get the option to have genetically related offspring. The depth of intervention in "natural" (non-artificial) reproductive processes is qualitatively new. This challenges current ethical considerations concerning the moral status of human embryos. Do they apply to human embryos created (even partly) from artificial gametes? Does artificiality make a difference in ethical evaluation? The social consequences of reproductive use of artificial gametes may change the fabric of rules in our society. Genetic parenthood could be shared by same-sex-couples as well as by more than two persons. These developments challenge current ethical considerations concerning the scope of reproductive autonomy, of the role and rights of parents, and even some basic ontological assumptions regarding human nature.

Two ethically and legally highly important controversial scientific steps were the development of a mitochondrial replacement therapy (Wolf et al. 2015; Amato et al. 2014) and the method of gene editing in non-viable embryos (Liang et al. 2015). These new technologies raise central questions in respect to our conception of the human person and of our understanding of society.

In February 2015 the House of Lords approved an amendment to the Human Fertilisation and Embryology Act, which had been passed by the House of Commons giving way to legally allowing mitochondrial replacement therapy

creating so called “three-parents-babies.” In the course of in vitro fertilization (IVF), it is possible to use different techniques (pronuclear transfer, maternal spindle transfer) to substitute mutated mitochondrial DNA (mtDNA) in an egg which causes serious inheritable diseases with healthy mtDNA from a donor’s egg. Children created this way carry DNA material from three persons – one father and two mothers. This challenges our ideas of genetic descent and parenthood, generating intense discussions in the UK (Nuffield Council on Bioethics 2012; Baylis 2013). New reproductive options potentially available through artificial gametes are the real challenge for our ideas of parenthood: The (genetic) reproduction of same-sex-couples, or more than two persons might transcend the hitherto insurmountable biological boundaries using one native gamete and one artificial gamete.[1]

Furthermore these mitochondrial replacement technologies are the first officially allowed genetic therapies on human gametes worldwide. This gave rise to some critics who see a slippery slope towards human genetic engineering (Sparrow 2014). These sceptics might be further alarmed by a second development: as mentioned above Chinese researchers have shown that early embryos can be altered genetically. The data shows the complexity of the procedure, highlights safety concerns and caused leading scientists to call for a moratorium on human genome editing (Lanphier et al. 2015; Baltimore et al. 2015). Pluripotent stem cell derived gametes (PSCDG, artificial gametes) might even foster human genetic engineering by providing “a quantitatively unprecedented supply of human eggs and sperm” (Palacios-González et al. 2014: 757) or by combining both methods. These artificial gametes could be altered genetically, too. So a thorough ethical and legal deliberation on these new technological possibilities is necessary. They may change our understanding of family, heritage, and society.

Before I am going to start with an ethical evaluation and some legal observations of the topics mentioned, I have to disclose the ethical and legal framework I depart from. This is necessary, because there are different ethical approaches, e. g. the four-principle approach of Beauchamp and Childress (Beauchamp and Childress 2013), utilitarian medical ethics (Harris 1985) or medical ethics in religious traditions (Engelhardt 2000), and different legal systems.

[1] Cf. http://learn.genetics.utah.edu/content/epigenetics/imprinting/. Whether it is possible to reproduce a single individual is highly controversial. Could IPS-derived gametes develop into a normal embryo as they shouldn’t have proper maternal and paternal imprinting patterns? In a correspondence with Albrecht Müller asking this question McLaughlin answered: “Unless the cells or one group of them go through the opposite (has to be female as you need start with XY) gender germ line there will be an imprinting imbalance.” Therefore, to reproduce a single individual parthenogenetic or androgenetic seems not to be possible, at least at the moment. The same is true for producing an individual using two different artificial gametes.

3.2 Ethical and Legal Framework

RM's fundamental purpose, as a branch of medicine, is providing a specific form of therapy to foster human health. This self-evident point must be kept in mind, because it is ultimately the measure of effective normative (ethical and legal) rules – even as we recognize that healthcare is only one aspect of the "common good" among many other social goods (such as safety, education, leisure, family life, and so on). The "good" of the common good is fostered by moral ideals, principles and practices that order all of social life – medicine included. Despite the fact that there are contemporary debates about some aspects of moral ideals, principles and practices, those debates should not mask the broad social consensus on fundamental moral matters that undergird social action. Human dignity and human rights represent important consensus principles, especially as articulated in the Universal Declaration of Human Rights (1948) and in subsequent international developments of those rights in UN and international conventions (regarding: minority groups, refugees, women, disabled persons, and others). The human dignity principle complements and supports these rights by representing the inestimable value of every human being. Because humans have dignity, they must be considered as subjects themselves – and not merely instruments for some purpose, including the common good. The dignity principle safeguards the fundamental equality of all human beings, with an implied right to at least basic access to adequate healthcare.

The Constitution of the World Health Organization, the Code of Nuremberg and the Declaration of Helsinki have specified this broad consensus for medical research and practice (Knoepffler 2008, 2011):

- A research subject/patients must be treated only with his clear informed consent (Principle of autonomy)
- Research subjects/patients have a right to appropriate medical treatment that does not impose unreasonable harm (Principle of non-maleficence).
- Research subjects/patients have a right to be treated with care (Principle of beneficence).

Most legal systems have integrated these three principles. In Germany ethical commissions oversee that research projects do not put human beings to more than minimal risk. In the US institutional review boards (IRBs) are responsible for protecting research subjects.

Interestingly so, all three international agreements do not stress another fundamental right: the principle of freedom of research of physicians and researchers as well, which is a fundamental right according to the German Basic Law, and for which some traces can already be found in the Hippocratic Oath. The oath begins with an invocation to the gods, and then the oath recognizes the great value of the teacher of medicine and the respect due to the teacher of medicine. The rule-finding process has to take into account the self-interests of medical professionals. They have to be treated with respect and compensated fairly. Hippocrates knew well that

worries about livelihood compromised care for patients. Ethics and jurisprudence therefore systematically considers the subjective interests of physicians, doctors, nurses, and other medical officials and care-givers, whereby these interests involve much more than simply financial, status and personal health matters.

With this background, the essential task of ethics and jurisprudence is to participate in structuring rules that will effectively force people acting in ways consistent with these fundamental medical norms.

3.3 Ethical Consensus in Regenerative Medicine

International consensus regarding ethical research practice is necessary for researchers to enjoy sufficient freedom for achievement. Consensus principles include informed consent for research subjects, a right that the undertaken research promises them more benefits than harm in case of a so called experimental treatment, a right that the undertaken research burdens research subjects with minimal risks only where there is no benefit for them, and the research meets high standards of reasonableness and good methodology. Most countries have sufficient regulatory measures in place to protect these consensus principles and the lack of such leads to considerable risk for research subjects or patients in experimental treatments. Data collection has to follow certain rules as well. A present lack relevant to RM is regards property rights of tissue used for developing adult stem cells (aSCs) or iPSCs.

Deeply contested ethical questions remain, however, such as research on embryos and data protection. This will be dealt with below.

3.4 Embryo Research and the Use of hESC

There is no overlapping ethical and legal consensus regarding the ontological and moral status of embryos. Essentially three positions are advocated:

- Position 1 Human dignity exists from conception, namely with the union of genetic material from the egg and sperm cells
- Position 2 Human dignity for the embryo begins with the formation of the primitive streak
- Position 3 Human dignity begins at later time: at viability, or at the latest at birth

Representatives of the first position cannot allow any adverse treatment of the embryo, whether it is for research on the embryo or hESC research. The embryo from the moment of conception is a human being due every element of dignity that is due to a grown person. If one grants an embryo the status of born human, then it is tantamount to applying human dignity to the human embryo. It would be prohibited to use embryos for research or to develop hESC (Knoepffler 2004a).

The German “Act ensuring protection of embryos in connection with the importation and utilization of human embryonic stem cells” (in German the abbreviation is StZG) seems to be in line with this kind of reasoning prohibiting all kind of research with human embryos. But at the same time the StZG makes a remarkable exception. It is allowed to import hESC if they were created earlier than 2008, if three conditions are fulfilled:

1. High-level scientific research
2. Primary treatment
3. Experiments cannot be done with other stem cells exclusively (aSC, iPSC)

This kind of exception was criticized, e. g. by the Congregation of Faith of the Roman Catholic Church in its “Instructio Dignitas Personae”: “The criterion of independence” is not sufficient to avoid a contradiction in the attitude of the person who says that he does not approve of the injustice perpetrated by others, but at the same time accepts for his own work the “biological material” which the others have obtained by means of that injustice. When the illicit action is endorsed by the laws which regulate healthcare and scientific research, it is necessary to distance oneself from the evil aspects of that system in order not to give the impression of a certain toleration or tacit acceptance of actions which are gravely unjust“(Congregation of Faith 2008). But in the same Instructio the Vatican does not rule out the possibility of use of illicit hESC: “Grave reasons may be morally proportionate to justify the use of such “biological material”. Thus, for example, danger to the health of children could permit parents to use a vaccine which was developed using cell lines of illicit origin, while keeping in mind that everyone has the duty to make known their disagreement and to ask that their healthcare system make other types of vaccines available” (ibid.). So even the Vatican seems to open the door for research with hESC a little bit (Knoepffler 2012), but on the other hand the Commission of the Bishops’ Conferences of the European Community is demanding to scratch the embryonic stem cell research off the list on the research program Horizon 2020 (Comece 2011) together with other opponents of research with hESC. They feel encouraged by the recent European Court of Justice’s jurisdiction on biopatents, where it has formulated a definition of the embryo and a ban on the patentability of hESC research. Even a moratorium on research with induced pluripotent stem cells is being discussed, because these cells may gain totipotency. The German Ethics Council “sees the need for greater clarity of the definition of the word ‘embryo’” and the demand, that the “definition of totipotency should also be made more precise and its normative significance be clarified within the new context of stem cell research” (German Ethics Council 2014).

Representatives of the second and third positions do not see the life of the embryo as a full-moral-status human being. Therefore, the embryo has no human dignity, but may be protected in some way. This position seeks to balance the embryo’s protection with the good to be gained from embryo research (Knoepffler 2004b). According to these positions it seems problematic to equate the moral and legal status of a born human and the early embryo. A born human even at very early life is an actualized individual with a personal history shaped by, among other things,

her very own decisions. Somewhere in the totipotency of the developmental horizon of a cell lays the thread that can be spun to one exemplar of a human individuum (homo sapiens). Bridging this range from a single cell to a grown human being with one normative notion is difficult and requires a progressive account to adapt or incorporate advances in medical technology and embryonic stem cell research in particular.

3.5 RM as Organ Replacement Therapy Or as Neurological Therapy

While human cloning for reproductive purposes is the more spectacular issue, the use (and abuse) of stem cell technology to create parts of humans, be it tissue, blood or organs, is closer at hand and less covered by existing medical ethics research.

There are different ways to develop stem cell lines for organs: creation via hESC (1), via SCNT (somatic cell nuclear transfer) (2), via iPSC (induced pluripotent stem cells), and (3) via aSC. As shown above the use of hESC is highly controversial, ethically as well as legally. The use of the technic of SCNT is more controversial in the respect that it may prepare the way to reproductive cloning. Here fundamental ethical questions regard the societal risks introducing cloning techniques. The use of iPSC may induce cancer. A lot of research is needed to secure a safe practice. From an ethical and legal point of view the mentioned ethical principles and rules of securing the rights of research subjects and patients are in place. The use of aSC is less problematic, because the risk to trigger cancer is smaller. Especially the use of aSC in improving the performance of a damaged heart seems to be uncontroversial.

Nevertheless, some ethical problems arise: take for example the case of patients with amyotrophic lateral sclerosis (ALS). The last years have seen some articles mentioning some good results using hESC (Karussis et al. 2010). But the results are not convincing and seem to induce false hopes in the patients. From a medical point of view stem cell therapies for this disease will not succeed in a near future. When the disease becomes diagnosed, the damage to the neurons is practically not reversible. Even if stem cells could substitute the non-working motor neurons, the regenerative process proceeds with less speed than the deteriorating process.

One last ethical and legal question has to be mentioned here: the value of genetic information becomes more prominent, once stem cell applications emerge (blood therapy, tissue engineering, minimal-organs …). It is not a surprise that bodies like the EU are concerned with biobanking. The German Ethics Council has published an "Opinion" in 2010 (German Ethics Council 2010; cf. Simon 2010) suggesting a strengthening of the fundamental rights of the donor and restricting the use by third parties. Five important pillars according to the German Ethics Council are:

- Biobank secrecy
- Defining permissible use
- Involving ethics commissions
- Quality assurance
- Transparency

3.6 IPSC and the Use for the Creation of Artificial Gametes

In 2014 the German Ethics Council underlined in its ad-hoc recommendation on recent advances in stem cell research and the ban on human cloning that ethical attention is especially needed concerning “the relationship of generations to one another as well as the significance of the natural and the artificial at the beginning of a human life […] and discussed at a level far beyond the past controversies in reproductive medicine” (German Ethics Council 2014). The main reason for this ad-hoc recommendation are new developments in the field of stem cell research, especially the creation of artificial gametes (Hendriks et al. 2015), more precisely named as pluripotent stem cell-derived gametes (PSCDG).

Human gametes seem to have no special moral status (Jordan and Winter 2007). If PSCDG is acquired from hESC it implies the destruction of human embryos and will raise therefore the objections of those who grant human embryos full moral status. If PSCDG is acquired from iPSC an ethical evaluation of the purpose of research on artificial gametes is needed. This method does not imply the destruction of human embryos. New ethical and legal questions arise: How can the informed consent of people donating somatic cells for the creation of artificial gametes can be assured, if the iPS-method is used? How carefully is this kind of human tissue to be dealt with?

Given the assumption that embryos created with one artificial gamete and one natural gamete are functionally equivalent to their natural counterparts there seems to be prima facie good reason to treat this kind of embryo as a natural embryo. But could the ascription of ethical value to human embryos depend on the naturalness of their coming into being (Advena-Regnery 2015). This calls for a reevaluation of philosophical grounds of the value of human embryos, which integrates the new bio-technological developments.

Embryos created partially from artificial gametes could also be used for purposes of human reproduction. This would dramatically increase the options provided by assisted reproduction technologies (ART) – far beyond today’s possibilities (e.g. IVF, ICSI, PGD). The use of artificial gametes could open up the possibility of having genetically-related offspring for people currently lacking this option. Heuristically, one could differentiate the use of artificial gametes

- to restore “natural fertility”
- to expand fertility beyond existing biological limits (Palacios-González et al. 2014)

Natural fertility would help individuals and couples to have genetically related offspring who could be normally expected to be fertile relative to their age, sex and health and save from inherited or acquired deficits. The groups of persons in question here are post-puberty men who are unable to produce viable sperm, women who have undergone premature menopause, and those lacking healthy gonads due to injury or as consequence of a cancer treatment. While these uses of artificial gametes are therapeutic, there are possible uses that would revolutionize human reproduction. In the literature seven of these possible options are named [ibid.]:

- Cell lines
- Embryos, fetuses and children
- Deceased individuals
- Postmenopausal women
- Single individuals
- Same-sex couples
- Multiplex genetic parenting

Especially these possible uses highlight the new quality of intervention in natural process which will go along with the reproductive use of artificial gametes. Until now ARTs have more or less mimicked natural processes in vitro. The use PSCDG as ART would dramatically change this. Through cell-reprogramming and deriving gametes from somatic cells mimetic ARTs will be left behind and biological boundaries could be transcended. The new quality of technological intervention in biological processes of human reproduction is illustrated by these new possibilities. The possible reproductive use of artificial gametes creates ethical complexities which “need to be considered and discussed at a level far beyond the past controversies in reproductive medicine”, as the German Ethics Council pointed out recently (German Ethics Council 2014).

Restoring natural fertility via PSCDG poses the following questions regarding the ethical uncontroversial principles:

- Will there be any harm to the future child (violation of the principle of non-maleficence)?
- Will there be a violation of the future autonomy of the child conceived in this special way?

But there are other questions involved Do ARTs represent an “industrialization” of human procreation? Critics argue that this could have negative implications for the relation between parents and children. Others oppose the substitution of natural processes by artificial ones and some see a slippery slope towards eugenics. Behind this debate about the natural and the artificial at the beginning of human life there are controversies about the conception of the human person as well as diverging ideas about society. Therefore, from an ethical perspective these uses of PSCDG for purposes of human reproduction pose questions on three different levels:

3.7 Individual

Some argue (Palacios-González et al. 2014) that the transcendence of natural boundaries in human reproduction can be described as an expansion of reproductive autonomy. Hereby reproductive autonomy is understood as an individual negative right and is bounded exclusively or at least primarily to genetic parenthood. The desire for genetic parenthood as part of the human condition justifies this expansion Others do not agree, most famously Jürgen Habermas (Habermas 2001). They understand modern forms of reproduction, especially PGD as a form of violation of the right of autonomy of the future child. Logically Habermas would condemn PSCDG with similar arguments.

From the individual perspective of the child there are ethical concerns. Often one recognizes a right to know one's own genetic descent. This knowledge is claimed to be important for the formation of identity. Is the welfare of the child threatened because the matrix of genealogy is blurred or might it be even enhanced because social and genetic parenthood will (unlike in other ARTs) go along with one another by the use of artificial gametes?

3.8 Societal

On the societal level there is the suggestion (Bleisch et al. 2015) ART should be more adequately dealt with within a framework of a family ethics The traditional ideal of the family follows the natural biological precepts of two different sexes and the genetic parenthood of two people. This family conception is no longer a consensus view. Nevertheless, other family constellations are becoming commonplace. The wish to create own biological offspring stresses biological parenthood. What consequences do these developments may have for the family as social institution? What is the dialectic of nature and culture within the framework of an up-to-date family ethics? Classical medical ethics is far from answering these questions.

Possible clinical uses of artificial gametes may also have consequences for the human self-understanding. Habermas argued in context of human enhancement that it would be vital for us to regard ourselves as "grown" rather than "made" and to ascribe our origins to a beginning which eludes human disposal (Habermas 2001). With the new depth of intervention in natural processes at the beginning of human life, Habermas' question arises again. Also in need of clarification stands the possible consequence on human-self-understanding of relinquishing the involvement of two different sexes in reproduction and the possible dispense of genetic parenthood by two people.

3.9 Legal

The current versions of the embryo protection act and the stem cell act in Germany provide very limited answers to challenges rose in context of extraction and use of artificial gametes and at least partly from embryos formed in this particular way. Legal issues are:

Is the extraction and the use of artificial gametes and derived embryos permissible (e.g. with regard to special use/processing) in the light of the actual German embryo protection act and the stem cell act? Do they require amendments? In this context a juridical gap is evident regarding aforementioned constitutional appraisal especially, as well as relevant elements of international soft law (e.g. Biomedicine convention and amendment, UNESCO-Declarations etc.). Furthermore, the effects taken by the current development on family law have to be considered. The central question is insofar, which technological capabilities would require which modification of the established family law. In this respect there are questions to answer, like how for example some constitutional considerations could influence the immigration of people "with three parents" into federal territory.

Until now there was no concluding discussion about the impacts on inheritance law when the biological and social mother are not identical. The social and biological mother can diverge in many cases, for example when children are adopted or have both a genetic mother and a social mother as a result of egg- donation. So far egg donations are anonymous, one should not forget that it might be contrary to the right of every human being to know about his genetic ancestry. Even the Federal Constitutional Court held in its earlier decision (BVerfG, Urteil vom 31. 01. 1989,1 BvL 17/87) that a children's desire of clarification of its ancestry is an inalienable constituent of the Allgemeine Persönlichkeitsrecht which is established by law in Art. 2 I i.V.m. Art. 1 I GG. This fundamental right is extremely important for the formation and quest of one's personal identity. In certain circumstances the right to know about the genetic begetter might be even important for health-relevant facts. Taking account of this jurisdiction, one could think about possible inheritance claims or in general challenges for the hereditary right as the German civil law in §§ 1925, 1591 BGB constitutes that only the woman who has born the child is inheritable by law (see below further statements concerning legal and biological motherhood).

A challenge concerning hereditary right could also be the specific key subject "triparenthood" which means that children have after a so-called mitochondria replacement therapy (in which some or most of the environmentally aged or pathologically damaged mitochondria of an egg have been replaced with those from undamaged ooplasm) three genetic parents; a father contributing nuclear and two mothers, one contributing nuclear DNA and the other contributing healthy mtDNA (mitochondrial DNA). It could be conceivable to link to the jurisdiction of the Oberlandesgericht Hamm with its sperm donation- case (OLG Hamm 14 U 7/12) in which the Court held, that the physician has to provide information about the identity of the sperm donator. Thereby the risk for eventual inheritance and maintenance

claims has been increased. As the child has the right to know his or her biological father, it has presumably also the right to know his or her genetic parents. The use of artificial gametes for IVF, e.g. using a normal gamete and an artificial gamete to create a child, will make the issue more pressing.

The crucial question is, whether the civil law, concerning inheritance questions with regard to §§ 1925, 1591 BGB, only considers the genetic descent of the child or also considers the legal one. It is approved that the law also accepts the motherhood in the legal sense that means that the legal motherhood in some cases might not be conform to the biological motherhood. So it remains an open question whether the German Embryo protection act should be modified in order to allow mitochondrial replacement therapy or the production of artificial gametes for the purpose of creating a human embryo.

3.10 Gene Editing

There has been heated ethical debate about possible germline modification in human embryos (Sorgner and Jovanovic 2013), before it became a real possibility. Chinese researchers have modified the genome of human embryos for the first time in order to correct the genetic disorder for beta-thalessemia (Liang et al. 2015). This method could be used for other purposes as well. So human enhancement is a possibility, not only human therapy. The possibility of artificially creating human gametes could lead to "a quantitatively unprecedented supply of human eggs and sperm" (Palacios-González et al. 2014). Progress in the research on artificial gametes could thereby foster human genetic engineering.

The experiment of the Chinese group with non-viable embryos has shown that the success rate is very low. The main reasons famous researchers in Nature (Lanphier et al. 2015) and the International Society for Stem Cell Research (ISSCR 2015) call for a moratorium "of applying genome editing to the human germ line in clinical practice" are "significant ethical, societal and safety considerations".

They mention "unintended genome damage" as well as "unknown consequences" for future generations. At the moment all medical ethical positions converge that this method is too risky to not violating the principle of non-maleficence.

Furthermore, there is no consensus where therapeutic use of gene editing ends and where the use for genetic enhancement begins.

From a legal point of view regarding the risks of gene editing an approach in this topic could be the analysis of the jurisdiction of the German Federal Court of Justice referring to "a child as damage". The case was about whether the parents get a compensation for their disabled child, because the blamable physician was responsible for birth defects, which lead to extensive disabilities. Crucial in that decision was the determination of the term "damage" which is the main precondition for a compensation claim. Important was to work out the connecting factor for that term. Can one consider the child itself as the damage connected with the more or less ethical discussion if a human being can be a "damage" at all? Or is rather than its very

existence the demand of broad maintenance and care caused by its disability the damage? The Court held that not the child itself is the damage (with regard to human dignity in Art 1 I GG) but the economic burden for the parents caused by its disability. In another decision (BGHZ 98,95 (105)) the Federal Court held, that the physician who did not recognize the disability of the nasciturus, is liable for all financial costs such as additional expenses as a consequence of the disability.

3.11 Conclusion

RM is a new and fascinating field of research and therapy that seems likely to provide substantial medical benefits. RM can proceed within existing ethical and legal frameworks, with the qualification that it is subject to continuing ethical discussions on the moral status of embryos, property rights of human tissue used for therapy, and limitations on genetic science, for example. Here human dignity and human rights, especially the right of self-determination (principle of autonomy), the right of life and corporeal integrity (principles of non-maleficence and beneficence) provide the foundation for an ethical framework. The legal challenge is to provide a reasonable and sustainable framework that enjoys public consensus and legitimacy. This framework prevents misuse of the new technologies and encourages research and therapeutic success.

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Chapter 4
Guidelines for Preclinical Development

Edward Spack

Abstract Preclinical development encompasses the set of activities required to initiate testing for safety and efficacy in humans. These requirements are defined by national and international regulations, and are generally focused on definition of drug composition and safety as well as scientific rationale and establishing a proposed dose regimen. The preclinical regulatory requirements for cell therapies broadly parallel those for small molecules and proteins, but the complexity of cells can bring additional challenges to the definition of potency, composition, immunogenicity, and toxicity. As a consequence of their pluripotent origins, stem cell therapies require additional preclinical considerations related to potential tumorigenicity and genetic/epigenetic stability. The first FDA approved stem cell therapy study initiated clinical trial in 2010. Thus, the field is young and there are few completed studies to provide precedents for planned preclinical development of novel stem cell approaches. Rather, the field must draw practical lessons from other somatic cell therapies. The evolution and harmonization of regulatory guidances for stem cells must also contend with a growing number of unregulated sites around the world that offer unproven stem cell treatments. It takes time for any new technology to identify safety and efficacy barriers to clinical application, and to develop strategies to overcome them. As current research and clinical experience guide the next generation of stem cell therapies in preclinical development, the establishment of validated assays based on cells differentiated from human stem cells may revolutionize the preclinical safety testing for all classes of drugs.

Keywords Preclinical • Toxicology • Stability • Regulatory • Immunogenicity

Abbreviations

ADMET	absorption distribution, metabolism, excretion, toxicity
AM	ancillary material
ASCs	adult stem cells

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ATM	ataxia telangiectasia mutated
ATMP	Advanced Therapy Medicinal Products
BDNF	brain derived neurotrophic factor
BSE	bovine spongiform encephalitis
CAT	Committee on Advanced Therapies
CBER	Center for Biologics Evaluation & Research
CDER	Center for Drug Evaluation & Research
CFR	Code of Federal Regulations
CGMP	Current Good Manufacturing Practice
CGT	Cellular and Gene Therapy
CGTP	Current Good Tissue Practice
CM	cardiomyocyte
CMC	Chemistry Manufacturing, and Control
CNS	central nervous system
CRO	contract research organization
CTA	Clinical Trial Authorisation
CTP	cellular therapy products
CYP450	cytochrome P450
DCEPT	Division of Clinical Evaluation and Pharmacology/Toxicology
DCGT	Division of Cellular and Gene Therapies
DHHS	Department of Health and Human Services
DHT	Division of Human Tissues
ECVAM	European Center for the Validation of Alternative Methods
EMA	European Medicines Agency
ESNATS	embryonic stem cell-derived novel alternative test systems
ESC	embryonic stem cells
FCS	fetal calf serum
FDA	Food and Drug Administration
FETAX	frog embryo teratogenesis assay
FGF	fibroblast growth factor
FPD	field potential duration
GDNF	glial cell-derived neurotrophic factor
GFP	green fluorescent protein
GVHD	graft versus host disease
HCT/P	human cells tissues, and cellular and tissue-based products
hERG	human Ether-a-go-go-Related Gene
hESCs	human embryonic stem cells
hiPSC	human induced pluripotent stem cells
hiPSC-CMs	human induced stem cell-derived cardiomyocytes
HSCs	hematopoietic stem cells
HLA	human leukocyte antigens
IBMIR	instant blood mediated inflammatory reaction
ICH	International Conference on Harmonisation
iPSC	induced pluripotent stem cells
iPSC-HCs	induced pluripotent stem cell-derived hepatocytes

IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon-gamma
IND	Investigational New Drug
ISCT	International Society for Cellular Therapy
LC-MS	liquid chromatography- mass spectrometry
MEA	microelectrode array
mEST	mouse embryonic stem cell test
MHC	major histocompatibility complex
MM	micromass
MOA	mechanism of action
MSC	mesenchymal stem cell
NDA	New Drug Application
NSC	neural stem cell
OCTGT	Office of Cellular Tissue & Gene Therapy
OKSM	OCT4, SOX2, KLF4, MYC
PHH	primary human hepatocytes
PHSA	Public Health Safety Act
PK/PD	pharmacokinetics / pharmacodynamics
PSC	pluripotent stem cell
ROS	reactive oxygen species
SCDI	stearoyl-CoA desaturase-1
SCID	severe combined immunodeficiency
SR-GVHD	steroid-refractory acute graft versus host disease
TdP	Torsade de Pointes
TGF-β	transforming growth factor beta
WEC	whole embryo culture

4.1 Preclinical Regulatory Requirements

4.1.1 Overview

Preclinical development can be defined as the set of activities that characterize a therapeutic candidate in preparation for clinical testing in patients. For small molecules and proteins, these activities are clearly defined by regulatory authorities, and primarily focus on demonstrating the physical characteristics, pharmacology, and safety of the drug candidate (Steinmetz and Spack 2009). Cellular therapeutics also require definition of composition and safety, but the complexity of cells and the challenges of defining potency add to the set of preclinical development issues. Stem cell therapies add yet another layer of complexity, as pluripotency raises safety issues including potential tumorigenicity, immunogenicity, and genetic and epigenetic stability. Certain stem cell populations, such as hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) have an established regulatory and clinical history. The advent of induced pluripotent stem cells (iPSCs) and growing

number of trials testing adult stem cells and cells differentiated from embryonic stem cells (ESCs) and iPSCs present new regulatory challenges. As the scientific and regulatory communities labor to define the preclinical path to clinical trial, a growing number of clinics and private offices around the world are promoting "stem cell tourism", administering stem cell-based therapies without any prior formal proof of safety and efficacy (Lau et al. 2008; Knoepfler 2013; Turner 2015). The pace of scientific advance and the pressures of patient expectations will likely drive a rapidly evolving regulatory landscape.

4.1.2 US Stem Cell Regulatory Organizations and Requirements

In the United States, the Food and Drug Administration (FDA), a federal agency of the Department of Health and Human Services (DHHS), has regulatory responsibility over clinical testing of experimental therapeutics and approval for sale and marketing. The FDA has two centers responsible for regulating therapeutics testing and approval: the Center for Drug Evaluation & Research (CDER) and the Center for Biologics Evaluation & Research (CBER). The Office of Cellular, Tissue & Gene Therapy (OCTGT) within CBER is responsible for:

- Stem cell and stem cell-derived products (e.g. hematopoietic, mesenchymal, cord blood, embryonic, iPSCs)
- Somatic cell therapies (e.g. pancreatic islets, chondrocytes, myoblasts, keratinocytes, hepatocytes)
- Gene therapies (e.g. genetically modified cells, plasmids, viral vectors, bacterial vectors)
- Therapeutic vaccines and other antigen-specific active immunotherapies

The OCTGT is composed of three divisions: the Division of Cellular and Gene Therapies, (DCGT), the Division of Clinical Evaluation and Pharmacology/Toxicology (DCEPT), and the Division of Human Tissues (DHT).

In the US, products derived from stem cells are regulated as biologics and considered a subclass of somatic cellular therapies. An exception to this classification is made for those cases in which a stem cell population contains a gene introduced through manipulation. In such cases it is considered a gene therapy, as it is a "medical intervention based on modification of the genetic material of living cells" (Halme and Kessler 2006). In 2013, the U.S. Court of Appeals for the District of Columbia Circuit upheld a 2012 ruling that a patient's stem cells applied for therapeutic use fell under the jurisdiction of FDA regulations (Grens 2014). Requirements for the processing of cell-based therapeutics are codified under the Good Tissue Practice (GTP) specified in 21 CFR Part 1271. The Public Health and Safety Act (PHSA) divides regulations for human cells, tissues, and cellular and tissue-based products (HCT/Ps) into sections 361 and 351, based on manipulation and intended use (George 2011). Traditional blood and bone marrow progenitors and tissues for

transplantation are considered "361 HCT/Ps" and must adhere to GTP requirements and submit to donor screening for relevant communicable diseases as the sole regulatory requirements. Cell therapies classified as "351 HCT/Ps" require additional regulatory oversight. Any cell-based product, including those of stem cell origin, that contains cells or tissues that are more than minimally manipulated, are used for other than their normal function, are combined with non-tissue components, or are used for metabolic purposes are subject to the Public Health Safety Act (PHSA), Section 351 that regulates the licensing of biologic products and requires submission of an Investigational New Drug (IND) application prior to initiating clinical studies (Halme and Kessler 2006; George 2011; Lysaght et al. 2013). For example, bone marrow extracted for the reconstitution of bone marrow function is considered "homologous use", classified as section 361 HCT/P, and regulated as a medical practice rather than a medical product (Ährlund-Richter et al. 2009). In contrast, the use of bone marrow samples for cardiac repair would be considered a "nonhomologous use" and classified as a section 351 HCT/P.

An IND application provides a scientific rationale and clinical study protocol, previous human experience, and sections summarizing preclinical studies defining therapeutic drug candidate composition (Chemistry, Manufacturing, and Control-CMC) and safety (Pharmacology and Toxicology). The content and format of information required in an IND submission is outlined in the Code of Federal Regulations (CFR) section 21 CFR Part 312.23. The FDA releases Guidances for Industry that provide additional instruction for preclinical activities leading to an IND application, including several that are specific to human cells, tissues, and cellular and tissue-based products (HCT/Ps):

- Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products, November 2013
- Guidance for Industry: Current Good Tissue Practice (cGTP) and Additional Requirements for Manufacture of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps), December 2011
- Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products, January 2011
- Guidance for Industry: Regulation of HCT/Ps- Small Entity Compliance Guide, August 2007
- Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps) from Adipose Tissue: Regulatory Considerations; Draft Guidance for Industry, December 2014
- Draft Guidance for Industry: Same Surgical Procedure Exception under 21 CFR 1271.15(b): Questions and Answers Regarding the Scope of the Exception

Stem cell therapies are evolving rapidly, and the FDA generally assesses preclinical testing programs on a case-by-case approach, as there are no universal requirements. FDA Guidances recommend that relevant animal models should be used, for example testing a stem cell therapy in an appropriate disease or injury model rather than exclusively in healthy animals. In some cases, FDA may ask for more than one animal species and may ask for large animal models. Given the

diversity and complexity of stem cell approaches, FDA recommends early and ongoing communication with OCTGT Pharmacology/ Toxicology staff during product development.

4.1.3 EU Stem Cell Regulatory Organizations and Requirements

In the EU, stem cell therapy falls under the Advanced Therapy Medicinal Products (ATMP) regulatory framework (Deal 2009). Under this authority the Committee on Advanced Therapies (CAT) was established to assess quality, safety, and efficacy of ATMPs. A Clinical Trial Authorisation (CTA) is required to conduct clinical trials; these are approved at the national level rather than through the European Medicines Agency (EMA). In contrast to the US distinction between 351 HCT/Ps and 361 HCT/Ps, in the EU there is no regulatory distinction between homologous and nonhomologous treatments. Rather, the level of regulation is set proportionally to the perceived degree of risk, including consideration of the extent of manipulation and the potential for pathogen contamination (Lysaght et al. 2013). Legislation on cell therapy in the EU is based on several key directives (Liras 2010; George 2011), including:

- EU 2003/63/EC: defines cell therapy products as clinical products and includes their specific requirements
- EU 2003/94/EC: defines Good Manufacturing Practice for Medicinal Products
- EU 2001/20/EC: emphasizes that clinical trials are mandatory for cell therapy products and describes the special requirements for approval of such trials, implements Good Clinical Practice
- EU2004/23/EC: establishes the standards for quality, donation safety, harvesting, tests, processing, preservation, storage, and distribution of human cells and tissues

The main regulation of stem cell therapeutics is encoded in EC No 1394/2007 on Advanced Therapy Medicinal Products. However, although the ATMP is the primary regulation for stem cells, member states can implement decisions at a national level and there is no overall consensus, adding complexity to the EU regulatory landscape (Deal 2009).

4.2 Pharmaceutical Quality /CMC

4.2.1 Overview

Much of the activity during preclinical development and IND submission of a small molecule or biologic is focused on documenting the composition, consistency, and stability of the drug candidate. This is also important for HCT/Ps, including stem cells. Each lot of an investigational HCT/P used in preclinical *in vitro* and *in vivo*

studies should be characterized to the appropriate degree consistent with the stage of development, as outlined in Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs) (Guidance for Industry 2008). Preclinical stem cell candidate therapies classified HCT/P 351 require CGTP/CGMP practices and additional regulatory oversight. Experience in the release criteria and potency definition learned from multiple clinical trials of mesenchymal stem cells (MSCs) can inform preclinical activities with other ESCs and iPSCs. Several recent reviews have summarized these issues and approaches for MSCs (Weber 2006; Galipeau and Krampera 2015; Wuchter et al. 2015).

4.2.2 Identity and Stability

Among the significant advantages of stem cells is their ability to scale up and differentiate in culture. However, a period of expansion in culture media raises concerns about media components, culture contamination, and genomic and epigenomic stability. Reports of stability during long term culture and scale up vary. There are reports of karyotypic stability in human embryonic stem cells lines cultured *in vitro* for over 1 year (Amit et al. 2005), but other studies have reported aberrations in copy number, mitochondrial DNA sequence, and gene promoter methylation in the long-term passage of human embryonic stem cells (Maitra et al. 2005; Hovatta et al. 2010; Närvä et al. 2010; Lund et al. 2012; Sverdlov and Mineev 2013). Scale-up aberrations are most common on chromosomes 1, 12, 17, and 20, but it is unclear whether particular genes or gene combinations are responsible and may be identified through screening (Draper et al. 2004; Ben-David et al. 2011; Hyka-Nouspikel et al. 2012; Heslop et al. 2015). Some of these genomic and epigenomic stability concerns may be allayed by recent non-integrative reprogramming techniques for generating hiPSCs which cause less genomic disruption or incomplete promoter silencing than in traditional techniques (Heslop et al. 2015). For example, an alternate approach has replaced the potentially oncogenic OSKM (OCT4, SOX2, KLF4, MYC) reprogramming factors with Sall4, Nanog, Esrrb, and Lin28, factors that are considered less efficient but generate higher quality iPSCs with reduced levels of genomic aberrations in histone variant 2A.X, a key determinant of iPSC/ESC quality and developmental potential (Wu et al. 2014). The susceptibility to chromosomal aberrations and mutations may vary amongst reprogrammed cells. Reprogramming of cord blood cells reduced the number of DNA mutations compared with reprogrammed dermal fibroblasts, suggesting that reprogramming from neonatal or more stem-like cells may be theoretically safer, despite the higher challenge (Su et al. 2013).

Given the potential for genetic sequence drift, it is prudent to set a limit to the number of passages permitted for a thawed batch of cells. This may necessitate the establishment of master cell banks and working cell banks. Freezing and thawing of stem cells may add to the challenges of maintaining cell identity and stability.

Standardization of cryopreservation and thawing procedures have improved the quality of peripheral blood mononuclear cells used in vaccine trials and of mesenchymal stem cells, and similar rigor is needed for the handling of ESCs and iPSCs and their differentiated progeny.

4.2.3 Purity and Ancillary Materials

Like other cells, culture and manipulation of stem cells carries the risk of contamination, and therefore current good tissue practice (CGTP) is required to prevent transmission of communicable diseases (Halme and Kessler 2006). Guidance for these considerations and practices is outlined in Section 21 of the Code of Federal Regulations (CFR) Part 1271 subpart D. Activities for preparing stem cells can include derivation, expansion, manipulation, banking, and characterization. Each of these steps requires a consistent protocol and may be vulnerable to contamination. In addition to potential viral contamination, stem cell preparations should be evaluated for contamination with undesirable cell populations. These cellular contaminants may include residual stem cells, cells that have differentiated into undesirable phenotypes, or contamination with feeder layer cells.

Non-cellular contaminants are also a concern. Ancillary materials (AMs) are components of cell culture or differentiation that are not intended as part of the final cell product (Atouf et al. 2013). These AMs (also known as ancillary products, ancillary reagents, or process reagents) are often critical for the survival, growth, and/or differentiation of a population or lineage, but may cause unintended consequences if introduced into the patient along with the proposed stem cell product. Stem cell AMs may include serum derivatives, antibiotics, cytokines, growth factors, culture media, antibodies, and enzymes. Therefore, evaluation of stem cell product quality includes evaluation of AM removal. Many culture conditions include nonhuman serum. If fetal calf serum (FCS) is included in culture media, FDA requires that it originates from a country certified free of bovine spongiform encephalitis (BSE) virus (Halme and Kessler 2006). Furthermore, FCS and other media containing xenoantigens may enhance the intrinsic immunogenicity of HLA-mismatched MSCs. Expansion of MSCs in media containing FCS may have contributed to problems in the Prochymal MSC product (Galipeau 2013). Culture in defined media that is free of xenoantigens or with blood component-derived culture supplements such as platelet lysate (Fekete et al. 2012) may circumvent problems associated with FCS and other foreign components. Media is not the only source of AMs. Some culture protocols use non-human feeder cells. This practice raises additional concerns that such cells could contain adventitious agents or cause inflammatory responses if they contaminate injected stem cells. ICH Q7 and Q10 quality guidelines and CBER guidances describe quality systems for examining any materials that come into contact with therapeutic product, including AMs (CBER/CDER/CVM/ORA 2006; CBER 2008).

4.2.4 Potency

The pharmacological definition of drug potency is a quantitative measure of activity expressed in terms of the amount required to produce an effect of a given strength. For small molecules and proteins, potency is often determined by the affinity of the drug for its receptor and its efficacy, the relationship between receptor occupancy and biological response. Although these definitions may have relevance in the characterization of certain stem cell therapies, as when a secreted product such as insulin is produced by a differentiated stem cell, many times stem cell biological activities are complex and challenging to standardize. The definition of potency can be further complicated for cases in which the cells differentiate or undergo major functional alterations following transplantation. In some cases, tracking potency may require testing of a cell lot in an appropriate animal model. Ideally, measures of cell potency should represent a product's mechanism of action (MOA) (Pritchett and Little 2012). Potency assays play several important roles in preclinical development of cellular therapy products (CTPs). Validated potency assays provide standards for monitoring the manufacturing process (e.g. induction, culture, purification) for cellular quality, consistency, and stability (Bravery et al. 2013). This standard is especially important for defining release specifications and the effect of any changes in the manufacturing process (e.g. donors, site, protocols, materials). In other words, potency assays provide a key indication of comparability between sources, between batches, and in a single batch over time in storage. For many HCT/Ps, including stem cells, it may not be possible to design a single assay that fully characterizes the product's MOA and strength. Often, HCT/Ps require a matrix of assays to define potency. The International Conference on Harmonisation (ICH), EMA, and FDA all reference the need for potency assays in their regulatory guidelines. Relevant to preclinical development of stem cells, the most specific guidance on potency for HCT/Ps is the FDA Guidance for Industry: Potency tests for cellular and gene therapy products. Although consistent potency is an important principle in CMC characterization, defining and monitoring potency for stem cell therapies will likely vary from case to case and require early discussion with appropriate regulatory authorities.

4.2.5 Lessons from Mesenchymal Stem Cell INDs

Recent experience in the therapeutic administration of mesenchymal stem cells (MSCs) provides important lessons for the wider preclinical development and testing of stem cell populations. Responding to inconsistent definitions of MSCs, the International Society for Cellular Therapy (ISCT) proposed a set of cell surface markers and other criteria to characterize MSCs for clinical trials. The committee's three recommendations for MSC characterization were: (1) plastic adherence under standard culture conditions; (2) ≥95 % expression of CD105, CD73, and CD90 and

lack (≤2 %+) CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface expression; and (3) able to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*. (Horwitz et al. 2005; Dominici et al. 2006). These recommendations have not been universally adopted. An FDA Commissioner's survey of 66 IND submissions between 2006 and 2012 revealed considerable diversity in the characterization and culture of MSCs (Medicino et al. 2014). Of 20 common markers used to characterize MSCs during preclinical and clinical production, none were utilized universally, ranging from 91 % of MSCs in trials screened for CD45 to 12 % screened for CD200. The percentages for the nine markers recommended by the ISCT screened in the surveyed trials were: CD45 (91 %), CD105 (73 %), CD90 (61 %), CD73 (52 %), CD34 (48 %), CD14 (47 %), HLS class II (44 %), CD19 (21 %), and CD11b (14 %). Adding to the confusion, consensus MSC markers do not correlate with functional heterogeneity, donor, or time in tissue culture and may not be sufficiently distinctive to define an MSC-based product (Medicino et al. 2014). A strict marker-based definition is not an FDA requirement, but a consensus on the definition of MSCs would greatly support interpretation of clinical outcomes and comparisons of separate trials.

The FDA survey also noted variability in cell culture conditions. A majority of the MSC products submitted to IND over this period (>80 %) were grown in fetal calf serum (ranging between 2 and 20 %, generally 10 % FCS). Along with this predominant use of FCS, 25 % of MSC preparations included growth factors (e.g. FGF, platelet lysate) as additional AMs. It is unclear how much this variability may have contributed to variability in MSC characteristics and potency amongst these trials – there is an ongoing debate about the merits of different MSC expansion protocols, which may differ in use of fetal calf serum, human platelet lysate, growth factor supplemented defined media, culture in normoxic vs hypoxic conditions (Bernardo et al. 2011). In addition to culture conditions, the number of passages is likely to affect MSC characteristics. Clinical data from the Karolinska Institute suggests that MSCs drawn from later passages survive longer in recipient graft versus host disease (GVHD) patients than MSCs from early passage (von Bahr et al. 2012). On the other hand, replicative senescence is observed in both mouse and human MSCs subjected to prolonged culture and expansion (Digirolamo et al. 1999; Bork 2010). The failed phase III MSC trial (NCT00366145) sponsored by Osiris Therapeutics produced 10,000 doses from a single volunteer donor, whereas virtually all the successful European phase II studies of steroid-resistant GVHD generated at most 5–10 doses from a single donor, and therefore underwent significantly fewer replication rounds (Martin et al. 2010; Galipeau 2013). These differences in passage outcome and clinical outcome suggest that expansion may be an important variable in MSC preparation.

Cryopreservation and thawing conditions are also important variables. Most MSC-based therapies rely on cryobanked cells, and studies indicate that freeze-thawed MSCs may have impaired therapeutic properties. For example, MSCs delivered to patients by infusion trigger an innate immune response, termed an instant blood mediated inflammatory reaction (IBMIR), in which MSCs and other stromal cells are lysed (Moll et al. 2012). Freeze-thawed MSCs trigger more IBMIR and complement-mediated cell lysis *in vitro* compared to fresh cells. IBMIR also com-

promises survival, engraftment, and function of other HCT/Ps, including systemically administered islet cells and hepatocytes (Nilsson et al. 2010). A survey of a limited sample of clinical trial results shows a trend toward better clinical outcome in patients treated with low passage MSCs compared to patients treated with freeze-thawed cells at higher passage (Moll et al. 2012, 2014). Studies of MSC treatment of graft versus host disease (GVHD) also suggest functional differences affected by freeze/thaw protocols. Freshly purified MSCs can suppress T cell proliferation through induction of indoleamine 2,3-dioxygenase (IDO) (Meisel et al. 2004; Ren et al. 2009). This property may be helpful for the treatment of autoimmune disorders and suppression of steroid-resistant GVHD (Le et al. 2008), as well as the GVHD that occurs when allogeneic stem cells are transplanted in a stem cell therapy. However, infusions with MSCs have failed to suppress GVHD in some cases, including a Phase III clinical trial of HLA-mismatched MSCs (Allison 2009). The variable results in GVHD studies may, at least in part, be related to differences in MSC freezing and thawing protocols, as well as time and conditions in culture (Francois et al. 2012a, b). In summary, experience with MSC cells may provide important considerations for the preclinical development of stem cell therapies in general, including the need for careful attention to cell freezing, thawing, and expansion protocols.

4.3 ADMET and Immunogenicity

4.3.1 Overview

Although the ultimate goal of clinical testing is proof of efficacy, a major focus of preclinical development and throughout clinical trial is the demonstration of safety. An IND includes a pharmacology and toxicology section (21 CFR 312.23 (a)(8)) detailing relevant studies establishing the safety of the drug candidate. For a small molecule or protein, this generally includes analysis of the absorption, distribution, metabolism and excretion of the compound as well as toxicology measurements to establish the maximum tolerated dose and adverse effects profile, collectively referred to as ADMET. General principles of pharmacology and toxicology apply to HCT/Ps, including stem cell preclinical candidates, but specific terms like ADME may not always apply in the way they do for small molecules or biologics (Guidance 2013). The complexity of stem cell products and the implications of pluripotency and potential for tumorigenicity bring unique challenges to the preclinical determination of safety.

4.3.2 Pharmacokinetics and Pharmacodistribution (PK/PD)

Proper dosing of stem cells requires an understanding of migration, distribution, engraftment, and long term survival, as well as the effect that route of administration and any accompanying scaffold has on these factors. Following administration,

depending on the route as many as 90 % of transplanted stem cells may be lost due to a combination of physical stress, inflammation, hypoxia, cell death following detachment, or immunogenic rejection (Nguyen et al. 2010; Zvibel et al. 2002). The half-life of transplanted cells is an important consideration- a short survival time may require multiple treatments for a chronic indication or delivery of a large number of stem cells. Infusion of high cell numbers or a long survival time in an undifferentiated state may increase the risk of teratoma formation. For example, in an animal model of spinal cord injury, GFP-labeled neural stem cells (NSCs) transplanted into the spine were detected 10 weeks later growing ectopically in distal locations in the spinal cord and brainstem (Steward et al. 2014). For some therapeutic applications (e.g. insulin production) the site of cell delivery is not critical (Shapiro et al. 2000). In such cases, systemic administration is a simple option. However, intravenous administration of stem cells can sometimes result in entrapment of cells in the lung or microvasculature, causing pulmonary emboli (Jung et al. 2013). Other approaches may require direct injection or homing to a specific site (e.g. replacing damaged dopaminergic neurons in Parkinson's disease). Direct transplantation to a target organ may reduce risks of inappropriate biodistribution, but may require challenging surgery for proper placement, introducing another risk. Optimization of delivery and longevity are important components of preclinical optimization and demonstration of safety. During preclinical PK/PD studies, tagging cell populations (e.g. staining cells with green fluorescent protein or detection of unique surface antigens such as HLA allele) can determine the location and longevity of administered cells.

4.3.3 Immunogenicity

Immunogenicity is a significant concern in the preclinical development of many therapeutic protein products, requiring assessment of potential induction of a neutralizing immune response as part of preclinical qualification (Guidance for Industry: Immunogenicity Assessment 2014). For most protein products, the primary concern is the preexistence or induction of neutralizing antibodies. In contrast, the main concern for cell therapies is the induction of a cellular immune response that could kill the transplanted cells and/or affect the recipient. The primary barrier to cell therapy is the major histocompatibility complex (MHC), a set of membrane proteins that bind antigens, forming a complex that can be recognized by specific T cell receptors. Human MHC genes are termed human leukocyte antigens (HLA), and include a set of "class I" molecules (HLA-A, -B, and C) expressed on virtually all nucleated cells and a set of "class II" molecules (HLA-DR, -DP, and DQ) expressed primarily on antigen-presenting cells such as macrophages and dendritic cells. These HLA molecules are highly polymorphic, i.e. there are many alleles for each gene. In cases where donor and recipient is the same person, the HLA alleles are matched and the transplant is termed autologous. In cases where the donor tissue is

derived from a different individual the HLA alleles are not all identical and the transplant is termed allogeneic. Other mismatches between donor and recipient can include ABO blood group antigens and differences in antigens that bind to donor and recipient HLA molecules. Allogeneic tissue grafts are rejected through T lymphocyte mediated recognition of the non-identical (foreign) HLA molecules. This may lead to rejection of stem cells or derivatives sourced from one individual and delivered into a different individual. If an allogeneic transplant contains immune cells it can similarly attack the host recipient, a phenomenon termed graft versus host disease (GVHD). Immunogenicity and GVHD are significant safety concerns for allogeneic stem cell therapies, and potentially for some autologous stem cell treatments as well. Preclinical development of stem cell therapies must therefore pay close attention to the factors that can contribute to immunogenicity. Stem cell protein expression, and hence their antigenic profile, changes during differentiation in culture (Draper et al. 2002). Therefore, the examination of stem cell immunogenicity is especially focused on their differentiated product, including the impact of culture conditions on potential rejection.

Several recent reviews have summarized stem cell immunogenicity issues (Boyd et al. 2012; Focosi et al. 2014; Heslop et al. 2015). The immunogenic potential of stem cells varies with the source and extent of differentiation. Allogeneic hematopoietic stem cells (HSCs) have been used for the treatment of hematological malignancies, but are susceptible to rejection (Locatelli et al. 2014). Furthermore, allogeneic T lymphocytes from the HSC transplant can infiltrate surrounding tissue and cause damage through a GVHD response; approximately 15 % of allogeneic HSC transplants in the past have resulted in fatalities (Blazar et al. 2012). Mesenchymal stem cells, on the other hand, are relatively unique amongst adult stem cells in that they can modulate the host immune response through effects on inflammatory cytokine release (Bartholomew et al. 2002; Aggarwal and Pittenger 2005). Furthermore, MSCs express low levels of HLA class I molecules and no extracellular HLA class II molecules or auxiliary costimulatory molecules that synergize with HLA to activate B- and T lymphocytes (Tse et al. 2003). In contrast to the immunogenicity concerns for other stem cells, MSCs have actually been employed for the treatment of GVHD (Zheng et al. 2013; LeBlanc et al. 2004), and have been proposed as an adjunct to other stem cell treatments to potentially ameliorate both GVHD and graft rejection (Kim et al. 2013). Cryopreserved allogeneic MSCs are widely used in Europe to treat GVHD, but the failure of a U.S. clinical trial (NCT00366145) of MSCs for GVHD to meet its clinical endpoint suggests a closer examination of MSC product variables, including immunogenicity, is warranted (Galipeau 2013). Cytokine components of inflammatory responses (e.g. IFN-γ and TGF-β), upregulate expression of MHC class I molecules and induces MHC class II expression in mouse and human MSCs (Romieu-Mourez et al. 2007). Thus, the immunogenicity of MSCs may be altered by host physiological conditions. Repeated administration of allogeneic MSCs in mice induced alloimmunization and mice were refractory to subsequent MSC transfusions (Nauta et al. 2006; Eliopulos et al. 2005). These observations caution that immunogenicity, either as an inherent

consequence of HLA mismatch or induced by cell culture or host conditions, may pose a significant preclinical and clinical hurdle for many proposed stem cell applications.

Studies of immunogenicity in mouse iPSCs have provided important insights, and some controversy, that should inform key decisions in the preclinical development of hiPSCs. A study by Zhao and colleagues reported that teratomas derived from murine iPSCs provoked an immune response in syngeneic (i.e. in genetically identical) inbred host mice (Zhao et al. 2011). The iPSCs in this study were derived with retroviruses or integration-free episomal plasmids. Retroviral vectors preferentially integrate into transcriptionally active sites, and can occasionally cause leakage of transgenes or activation of neighboring genes proximal to the integration site, causing aberrant transgene expression and production of an immunogenic protein. The teratomas in the syngeneic hosts had abnormal protein expression (e.g. over-expression of tumor related genes, such as *HORMAD* and *Zg16*), T cell infiltration, and elicited immune rejection. These characteristics may be related to the integration approach used to establish the iPSCs, as strong tissue rejection was observed with retrovirally-derived iPSCs, but not with integration free-iPSCs (Zhao et al. 2011). A different experiment with terminally differentiated endothelial cells, hepatocytes, and neuronal cells from mouse ESCs and iPSCs showed no T cell response to undifferentiated syngeneic iPSCs or their differentiated progeny (Guha et al. 2013). This study used plasmids and lentiviruses to generate iPSCs. Lentiviral vectors are thought to incur less risk of aberrant transgene expression. Inducing iPSCs with a polycistronic lentiviral vector encoding all required transcription factors may offer an alternative, less immunogenic approach to stem cell induction, as they would reduce the number of insertions into the genome (Chang et al. 2009; Papapetrou and Sadelain 2011). Based on the observed variations in immunogenicity of autologous iPSCs in animal experiments, retroviruses are not recommended for deriving human iPSCs, and alternatives are gaining favor.

As mentioned above, culture conditions can also contribute to stem cell immunogenicity. For example, the immunogenicity of stem cells can be altered by media containing FCS or the sialic acid derivative Neu5G from mouse feeder layers (Horwitz et al. 2002; Martin et al. 2005). For this reason, growth in serum free media or human growth factors may be preferable for stem cell expansion. Encapsulation of cells prior to transplantation may reduce interactions with immune cells and the risk of rejection, a potential strategy if the goal of the stem cell transplant is to provide secreted product(s). Studies in non-human primates seem to confirm the immunogenicity data from mouse ESC and iPSC experiments. Autologous iPSCs transplanted into nonhuman primates induced minimal immunological response, whereas allogeneic iPSCs were immunogenic, suggesting that autologous iPSCs are tolerated (Morizane et al. 2013). However, for many therapeutic applications the use of autologous iPSCs is not practical. In such cases, co-administration of immunosuppressive drugs may be required, introducing increased vulnerability to pathogens and cancer. Induction of tolerance is a holy grail for immunologists, and lessons from autoimmunity research may provide alternative approaches to reducing the immunogenicity of stem cells and their differentiated

therapeutic candidates. In the meantime, several groups are building cell banks of clinical-grade iPSC lines from HLA homologous donors in an attempt to provide close matches for prospective recipients (Okita et al. 2011; Taylor et al. 2012; Kaneko and Yamakaka 2013). For example, the Center for iPS Cell Research and Applications (CiRA, Kyoto University) aims to establish an iPSC bank that would cover ~90 % of the Japanese population. This banking approach, if established in accordance to international CGTP principles, may provide a means to address immunogenicity concerns while further research examines new approaches to reducing allogenicity in donor stem cells and inducing tolerance in recipients.

4.3.4 Tumorigenicity

Pluripotency and tumorigenicity are closely related (Knoepler 2009), and several clinical observations over the past decade emphasize that stem cell safety concerns are more than hypothetical. Beginning in 2001, a young boy with ataxia telangiectasia (a rare disorder caused by mutation of the ATM gene affecting brain regions controlling speech and movement) received several intracerebellar and intrathecal injections of human fetal neural stem cells from multiple sources. Four years after the initial treatment tumors were detected on his brain and spinal cord (Amariglio et al. 2009). Cytogenetic analysis of the glioneuronal neoplasm removed from his spinal cord revealed the tumors were of non-host origin; in addition to two normal copies of the ATM gene the tumor cells expressed different HLA alleles indicative of two different donor sources. The tumors were well differentiated, slow growing, and relatively benign. Nevertheless, this episode raises concerns about the tumorigenicity of at least some stem cell stages. In another example, in 2006 a lupus nephritis patient in Thailand received an infusion of autologous HSCs injected into her kidneys and subsequently developed cellular masses in her kidney, liver and adrenal gland (Cyranoski 2010; Thirabanjasak et al. 2010). In other cases, stem cell treatments of spinal cord injuries have resulted in spinal cysts. A pilot study reported the relative safety and potential benefit of olfactory mucosal cells, a proposed source of stem-like progenitor cells for neural repair, administered to seven spinal injury patients in 2001–2003 (Lima et al. 2006). Eight years after the cell transplantation one of the paraplegic patients developed mucosal-like masses, composed primarily of cysts lined with respiratory epithelium, submucosal glands with goblet cells, and nerve twigs, at the transplantation site (Dlouhy et al. 2014). Geron's human ESC-derived neuroprogenitor cell product GRMOPC1 administered to spinal cord injury patients resulted in cysts developing at regenerating tissue sites, leading to a 1 year clinical trial moratorium (DeFrancesco 2009). The trial was subsequently renewed and no other serious adverse events were reported.

Studies in animal models have provided insights into the safety risks of pluripotent stem cells and differentiated cells derived from cultured ESCs and iPSCs. In rodent studies, implantation of pluripotent ESC or ESC-derived precursor cells frequently leads to the development of teratomas or teratocarcinomas (Bjorklund et al. 2002;

Erdo et al. 2003). However, differentiation of ESCs *in vitro* prior to implantation greatly reduces the tumorigenic potential of these cells (Brustle et al. 1999; Arnhold et al. 2000). Injection of human ESC-derived dopaminergic neurons into the brains of a primate model of Parkinson's disease also resulted in tumors (Doi et al. 2012). However, these tumors appeared to result from residual undifferentiated ESC cells in the preparation of differentiated cells; in contrast, differentiated cells matured for by 42 days of culture with neurotrophic growth factors BDNF and GDNF did not form tumors.

The gene networks responsible for induction and maintenance of pluripotency in ESCs and iPSCs share many genes and pathways with networks involved in oncogenesis (reviewed in Lee et al. 2013). Specifically, the Myc transcription factor and other core pluripotency circuits, including Nanog, Oct4, and Sox2, that are instrumental in inducing pluripotency are shared by PSCs and many cancers and are crucial to both for proliferation and maintenance of pluripotency. High-resolution SNP genotyping of a large number of hESCs, hiPSCs, somatic stem cells, primary cells, and tissues revealed a higher frequency of copy number variations in pluripotent samples, and numerous deletions of tumor-suppressor genes in iPSCs upon pluripotency induction (Laurent et al. 2011). An additional concern stems from the observation that prolonged PSC culture can produce genomic abnormalities, and these confer a selective growth advantage through loss of tumor suppressor genes and/or gain of proliferation genes that could increase tumorigenic potential over time (Mayshar et al. 2010; Lee et al. 2013). Indeed, many of the genetic abnormalities found in ESCs and germ cell cancers accumulate in iPSCs during adaptation to growth in culture (Mayshar et al. 2010).

These experimental and clinical observations underscore the need to assess the tumorigenic potential of any proposed stem cell therapy during preclinical development to characterize and mitigate risk. One of the most common assays to demonstrate pluripotency is the induction of teratomas in immune compromised mice; this is often seen as a surrogate test for tumorigenicity (Knoepfler 2009; Buta et al. 2013). In this *in vivo* assay, stem cells are implanted into SCID or otherwise immunodeficient mice and their ability to form teratomas, with differentiated cells derived from all three germ layers (ectoderm, mesoderm, and endoderm), is assessed (Brivanlou et al. 2003). It is a useful experimental model for insights into tissue development, and was originally conceived as a tumor assay. However, although the teratoma assay is a frequent criterion for proving pluripotency in stem cell research and banking, there is no current standard for site of injection, number of cells implanted, number of passages, or collection and culture conditions of the stem cells. For example, the number of cells injected in teratoma assays as reported in the literature can range from 200 to 5,000,000 (Müller et al. 2010). In addition to the lack of validation, the teratoma assay is time, cost, and labor intensive. Also, because teratoma assays are traditionally performed in immunodeficient animals they do not often reflect the influence of the host immune system and other host factors. A healthy immune system may reduce the risk of tumorigenicity, but it may also reject allogeneic (and in some cases autologous) stem cells. The safety considerations for stem cell therapies extend beyond teratoma potential- treatments must also avoid forming malignant

tumors of more restricted tissues or even benign cell overgrowth. Thus, the teratoma assay is likely to provide a component of safety testing, but other analyses should be considered as well. The tumorigenic potential of mouse iPSCs has been measured in chimeric mice which contain a subset of tissues derived from the iPSCs (Knoepfler 2009). This approach has shown malignant tumors developing in up to 20 % of chimeric mice containing iPSC derived with exogenous Myc, whereas chimerics containing iPSCs generated without Myc did not form tumors (Okita et al. 2007; Nakagwa et al. 2008; Wernig et al. 2008). Although this is a useful research tool for mouse IPCs, the chimeric assay does not reflect how iPSCs would be used in a clinical application, and for ethical reasons it is not acceptable to combine hiPSCs with mouse cells to form a mouse-human hybrid embryo.

Tumorigenic potential is a safety concern for differentiated stem cells as well as for pluripotent ESCs and iPSCs. As few as 0.2 % of SSEA-1-positive pluripotent cells can give rise to teratomas, indicating that even at high levels of differentiated cell purity the potential for teratoma formation by "contaminating" undifferentiated pluripotent cells is a significant risk (Fujikawa et al. 2005). Tumors have been reported in immunodeficient mice injected with cell totals as low as 20 pluripotent mouse stem cells (Lawrenz et al. 2004) and 245 human ESCs (Hentze et al. 2009). Although treatments are often administered to immune-competent human patients, especially in cases of allogeneic cell therapies involving immunosupression, the tumorigenic potential of a small set of pluripotent stem cells merits consideration. Unfortunately, no current analytical technique is sufficiently sensitive to assure removal of all pluripotent cells (Nguyen et al. 2010). As an additional safety measure, some are considering incorporating suicide genes or stem cell specific toxins to mitigate the risk of tumors from malignant stem cell growth (Knoepfler 2009). One suggested approach to preventing contamination of a differentiated cell population with undifferentiated cells is to treat with small molecules targeting stearoyl-CoA desaturase-1 (SCDI), which selectively causes cell death in undifferentiated iPSC/ESCs (Ben-David et al. 2013). Safety is a primary focus of preclinical development for all drug applications. The potential for tumor development, either by contamination with undifferentiated pluripotent cells or genomic or epigenomic instability of a proposed HCT/P, will therefore dominate the attention of stem cell clinicians and regulators and challenge the implementation of additional safeguards and proof of risk reduction.

4.4 Application of Stem Cells for Toxicity Screening: Current Uses and Future Prospects

4.4.1 Overview

Demonstration of safety is a paramount goal of all preclinical drug development. Many of the current requirements for toxicity testing of small molecules and biologics use *in vitro* and *in vivo* models that are cumbersome, expensive, and/or imperfectly predictive of adverse effects in patients. The earliest impact of stem cells may

be in establishing new *in vitro* models of human tissue that provide sensitive and accurate predictions of drug safety. Many such systems are under development and several stem cell assays are joining the set of toxicity tests offered by preclinical contract organizations.

4.4.2 Cardiotoxicity Screening

Drug-induced heart irregularities such as arrhythmia are a common cause of drug failure during clinical trial (Kola and Landis 2004). Cardiotoxic effects can include the induction of reactive oxygen species (ROS), apoptosis, altered contraction, arrhythmia, and changes in gene expression. Of these debilitating and potentially fatal effects, arrhythmia is a particular concern. In the decade between 1990 and 2001, 8 drugs developed for non-cardiovascular indications were withdrawn due to adverse cv effects including delayed cardiac repolarization, prolonged QT interval, and Torsade de Pointes (TdP), a potentially fatal ventricular tachyarrhythmia (Kola and Landis 2004). ICH S7B nonclinical guidance and E14 clinical guidance for Europe, Japan, and North America do not directly assess ventricular proarrhythmia (i.e. TdP), the common clinical concern. Instead, current *in vitro* screens for cardiotoxicity test drug candidates with stably transfected cell lines expressing the hKv11.1 potassium channel, also known as hERG (human Ether-a-go-go-Related Gene), employing high-throughput patch clamping to measure changes in the QT interval (Kannankeril et al. 2010). An odds ratio of 1.93 is reported for the association between anti-hERG activity and the risk for serious ventricular arrhythmias and sudden death (De Bruin et al. 2005), leading to regulatory requirements to include a hERG patch clamp assay prior to clinical trial (Guidances UCM07496). However, there are limitations to the reliability of hERG as an indicator of cardiotoxicity. There is a 30 % discordance between blockade of hERG and induction of serious arrhythmia *in vivo* (Kramer et al. 2013). The QT interval is the net result of several independent ion channels, and therefore *ex vivo* testing of rabbit and/or canine Purkinke fibers, a common follow up assay to *in vitro* hERG, evaluates drug effects on the cardiac action potential via intracellular microelectrode recording. However, these assays are expensive, low throughput and hampered by species differences in sensitivity to some cardiotoxic drugs. A more comprehensive and simpler system of monitoring for cardiac safety is needed.

In vitro cultured cardiac muscle cells (cardiomyocytes, CMs) differentiated from human stem cells have significant potential for enhancing or replacing current cardiotoxicity screening approaches as they express a set of ion channels similar to primary CMs but are more stable in culture, can be scaled to greater numbers, and express human genes, avoiding potential species differences in drug sensitivity (Ebert et al. 2012; Mordwinkin et al. 2013). *In vitro* studies of hESC-CMs treated with drugs associated with QT prolongation and/or TdP (including quinidine, sotalol, cisapride, and terfenadine) demonstrated dose-dependent effects on both

intracellular action potential duration (Peng et al. 2010; Jonsson et al. 2010) and extracellular field potential duration (FPD, Braam et al. 2010) consistent with known effects of the reference compounds. These results are encouraging indications that drug interactions with stem cell-derived cardiomyocytes could correlate with adverse cv effects *in vivo*, and suggest that validated stem cell assays could provide alternative tests for cardiotoxic drugs (Jonsson et al. 2010).

Recent studies have shown similar promise with hiPSC derived cells. hiPSC-CMs express virtually all cardiac ion channels and subunits found in adult human ventricular tissues, in spite of their immature morphology and high rate of spontaneous beating (Ma et al. 2011). Patch clamp cardiac action potential measurement of hiPSC-CMs showed AP prolongation upon exposure to hERG channel blockers terfenadine, quinidine, cisarpide, sotalol, E-4031, and verapamil as measured by patch clamp, and these stem cells were more sensitive to cardiotoxic drugs than standard patch clamp assays of rabbit or canine Purkinje fibers (Ma et al. 2011). In addition to patch clamp measurements, multiple electrode array (MEA) assays can measure multiple CM electrophysiological parameters, including beat frequency and field potential duration that correlate with CM action potential durations measured by patch-clamping, as well as with heart rates and QT intervals obtained by clinical EKGs (Guo et al. 2011). MEA/hiPSC-CM assays are sensitive to known ion channel blockers that affect specific phases of cardiac function, including: Vmax and FP amplitude in phase 0, affected by sodium channel blockers lidocaine and quinidine; AP plateau (phase 2), affected by L-type calcium channel blockers nifedipine and verapamil; Phase 3 repolarization, affected by class III antiarrhythmic hERG blockers (Navarrette et al. 2014). Studies of known cardiotoxins in MEA/hiPSC-CM correlates with detection of drug-induced arrhythmias in animal models, especially in canines (Navarrete et al. 2014). Thus, hiPSC-CM based assays may gain use as an early screen for cardiac toxicity, and may eventually complement or replace current incomplete and cumbersome methods.

Although at an early stage, measurements of arrhythmia and impedance in hiPSC-CMs are increasingly being used in pharmaceutical companies and offered as commercial services by contract research organizations (CROs) (Jonsson et al. 2011; Guo et al. 2011; Abassi et al. 2012). For instance, ChanTest, a Cleveland-based CRO laboratory within Charles River Labs, has developed several hiPSC-CM assays to analyze drug effects. One such assay uses the Axion Biosystems MEA (Atlanta, GA) to measure drug effects on Field Potential duration (related to QT interval prolongation), Na^+ spike (related to QRS interval), and hERG and Ca^{2+} modulation. ChanTest has also developed an assay to monitor effects on iPSC-CM spontaneous contractile activity reflected in transient changes in impedance using the ACEA. Biosciences xCELLigence (San Diego, CA) instrument to measure twitch behavior of a CM monolayer plated over gold electrodes. The assay can monitor cellular toxicity, hERG and Ca^{2+} channel modulation, and effects that increase over time exposure to a drug, such as inhibition of hERG trafficking to the cell surface. Availability of validated hiPSC-CM lines and validated assays should provide new standards for preclinical cardiotoxicity screening.

4.4.3 Reprotoxicity Screening

Three percent of humans are born with congenital abnormalities, and approximately 3–10 % of these are the result of toxic chemical effects (Tandon and Jyoti 2012). Therefore, screening for adverse drug effects on embryonic development is a critical component of safety testing. The gestational period between days 15–60 of embryonic development are especially critical for the development of multiple organs, but individual organs differ in the periods of gestational development sensitivity to chemical and environmental insult: heart weeks 3–4, external genitalia 8–9 weeks, brain and skeletal system 3 weeks to birth (Tandon and Jyoti 2012). The ICH guidelines for preclinical reproductivity testing of drugs focuses on three segments: fertility (production and release of gametes, fertilization, and implantation; embryotoxicity/teratogenicity (fetal development); and prenatal/postnatal toxicity (growth of neonate to sexual maturation). The FDA also requires several animal studies to assess the level of reproductive and embryo/fetal toxicity (Bailey et al. 2009). These studies are generally conducted in the later stages of clinical development, i.e. post-Phase I. Unfortunately, discovery of reproductive/developmental toxicity at this stage can cause very expensive delays or terminate drug development. Current testing relies on *in vivo* tests that are slow, expensive, and technically demanding. Three assays that are based on ontogenesis have been validated by an international study: the micromass (MM) system, employing dissociated rat embryo limb bud and brain cells; the whole frog embryo teratogenesis assay (FETAX)); the rat whole embryo culture (WEC) test. However, attempts to test for toxicity on whole embryo cultures have not gained widespread use due to predictive values of 70–80 % at best (Tandon and Jyoti 2012).

The human reproductive cycle is complex and likely cannot be modeled by a single *in vitro* system. Assessing effects on each phase of the reproductive and developmental cycle is critical, as some drug candidates affect a narrow phase while others can have effects gametes and embryos or on more than one stage of development. Culture of human stem cells may afford an opportunity to model discrete stages of gamete and embryonic development and to assay the effects of drugs on each stage (Speilmann 2005; Krtolica et al. 2009; Scott et al. 2013). A set of assays for teratogenic risk based on mouse embryonic cells was pioneered by Hans Spielmann and colleagues (Spielmann et al. 1997). This set of assays using mouse ESCs and other cell lines, termed the mouse embryonic stem cell test (mEST), was validated by the European Center for the Validation of Alternative Methods (ECVAM). Prediction of embryonic toxicity was based on an algorithm integrating the results of three assays in a 10 day screening: 50 % inhibition of cardiomyocyte differentiation in D3 cells, 50 % growth inhibition of D3 ESCs, and 50 % growth inhibition of 3 T3 fibroblasts (Spielmann et al. 1997; Genschow et al. 2002; Seiler and Spielmann 2011). Based on results of the mEST, the ECVAM correctly identified 78 % of teratogens and non-teratogens (Genschow et al. 2002). The mEST was transferred to labs in the EU and US, and validation showed very high reproducibility with a set of embryotoxic compounds (Tandon and Jyoti 2012). The mEST

has been adopted and used routinely by many pharmaceutical companies (Paquette et al. 2008). It was included in a set of *in vitro* assays that ranked the predicted embryonic toxicity of five conazole fungicides known to inhibit members of the CYP450 family which antagonize steroid hormone receptors such as the androgen and estrogen receptors and induce fetal abnormalities. A rough prediction of relative toxicity was shown, suggesting that *in vitro* EST assays could provide insights on likely toxicological outcomes in animal studies, aiding *in vivo* study design (Dreisig et al. 2013).

Although the EST assay was a promising start, there were several drawbacks. The scoring of beating embryoid bodies was qualitative, and the assays were labor-intensive. The statistical prediction model had moderate accuracy (Marx-Stoelting et al. 2009). Additionally, there are documented species differences in sensitivity to chemical effects on embryonic development (Shuey and Kim 2011). For example, thalidomide and isotretinoin do not manifest toxic effects in mice but induce severe malformations in human embryos. Therefore, use of hESCs or hiPSCs may detect toxic effects that could be missed by *in vitro* or *in vivo* tests of other species. Several attempts to improve upon the mEST have added additional differentiated cells or profiled transcription or metabolomic markers, including studies initiated in pharmaceutical companies. For example, the Discovery Toxicology team at Bristol-Myers Squibb screened changes induced in a set of 12 developmentally regulated gene targets in a 4-day version of the mEST, and used a decision tree model to define teratogens based on the IC_{50} concentrations and number of genes (Panzica-Kelly et al. 2013). An LC-MS analysis of hESC cells exposed to eight drugs with known teratogenicity correctly predicted the teratogenicity of 7/8 based on >10 % changes in the ratio of arginine to asymmetric dimethylarginine levels, demonstrating that metabolomic markers may be useful in identifying developmental toxins (West et al. 2010). Recently, a streamlined hiPSC assay was developed by scientists at Hoffmann-La Roche based on mesoendodermal cells which shortens stem cell differentiation three-fold and quantitates protein expression of a single lineage marker, SOX17 (Kameoka et al. 2014). In this assay, a threshold of 5 μM demonstrated a 94 % predictive assay (97 % sensitivity and 92 % specificity) in a screen of 71 drug-like compounds with known *in vivo* teratogenic effects. These encouraging developments point the way to new models of reproductive toxicity testing that may be useful for preclinical development as well as for screening chemical hazards.

4.4.4 Neurotoxicity Screening

The CNS is one of the most common targets of systemic toxicity, and the developing nervous system is especially susceptible (van Thriel et al. 2012). However, developmental neurotoxicity is one of the least studied forms of adverse effects (Makris et al. 2009), and several challenges have slowed progress toward developing alternatives to *in vivo* neurotoxicity testing. For example, some cases of

developmental neurotoxicity are not associated with cell death, but rather may be related to changes in the proportion, position, and/or connectivity of neural cells, complicating attempts to screen for toxic effects on neurons *in vitro* (Kuegler et al. 2010). The FDA estimates there are thousands of neuroactive chemicals requiring FDA regulation, and identifying methods for assessing neurotoxicity is a priority. In the EU, OECD TG 426 requires exposure of animals to test drug during gestation and lactation, with analysis of gross histology and behavior in offspring, an expensive and laborious process. These studies give indirect indications of toxicity, such as increased frequency of embryo/fetal death, changes in fetal weight, or anatomical/behavioral abnormalities. Stem cells may offer an alternative or supplement to indirect and cumbersome *in vivo* studies.

Neurotoxic chemicals can affect specific developmental stages and cell types, and these effects may occur through acute and/or chronic exposure. Therefore, iPSCs may offer the advantage of testing deleterious effects on both neuroprogenitors and fully differentiated nervous system cells. The choice of multiple neural lineage cells is likely critical for neurotoxicity testing, as non-neural cells do not always predict neurotoxicity. For example, the mEST cardiotoxicity assay mentioned above identified the majority of known embryotoxic agents tested, but failed to identify methylmercury, a known neurotoxin. A comparison of hESC-derived neurons and rodent primary neuronal cultures exposed differences in reproducibility, dynamic range, and sensitivity to neurite outgrowth inhibitors, underscoring the need for further hESC based neurotoxicity assay development (Harrill et al. 2011). Adapting the principles of the mEST to test hESCs undergoing neuronal differentiation identified methylmercury toxicity and found that early developing neural precursors were sensitive to this neurotoxin than were maturing neuronal cells (Stummann et al. 2009). To improve neurological testing and streamline it for planned increased screening of current chemicals, several groups are developing human ESC-based recapitulations of neuronal development, studying the effect of known neurotoxins and novel drugs on formation of neural ectodermal progenitor cells (Chambers et al. 2009; Balmer et al. 2012), neural tube formation based on the formation of neural rosettes (Stummann et al. 2009), or transition of neural precursor cells to mature neurons (Scholz et al. 2011; Stiegler et al. 2011). The human embryonic stem cell-derived novel alternative test systems (ESNATS) European commission research project has supported a consortium of researchers to establish a set of toxicity tests based on defined differentiation protocols of hESCs. This collaborative effort recently demonstrated that the ESNATS assay battery could classify developmental neurotoxins and reproductive toxins such as valproic acid and methylmercury based on their transcriptome profiles (Krug et al. 2013).

The European Union 7th Framework Programme "Innovative Medicine Initiative" called for human iPSC-based toxicity testing. Several iPSC–based neuron and glial cell lines have been derived from patients with neurodegenerative diseases to study disease progression, but the establishment of neurotoxicity screens using iPSC cells is not as advanced as testing of hiPSC-CMs. In an early study of a human neural stem cell line derived from umbilical cord blood, several stages of neural commitment and differentiation were established and less differentiated cells were

shown to be more sensitive to neurotoxins (Buzanska et al. 2009). Changes in intracellular calcium may also provide a useful indication of neurotoxicity, offering an alternative to PC12 cells and other immortalized cell lines that display a partial neuronal phenotype and/or are derived from non-human species (Deshmukh et al. 2012). With these encouraging developments, the next challenge is to standardize protocols for neurotoxicology testing with iPSC derived progenitors and differentiated cells.

4.4.5 Hepatotoxicity Screening

The liver has a central role in drug metabolism and is a frequent target organ for adverse drug effects. Liver toxicity is associated with over a third of acute liver failures in the US (Gunawan and Kaplowitz 2007; Stine and Lewis 2011), and is one of the major causes of drug failure in preclinical and clinical testing (Corsini et al. 2012). Furthermore, liver toxicity is the most frequently cited basis for the withdrawal of approved drugs (Lee 2003a, b). Given this critical role in drug metabolism and safety, early and predictive studies of potential liver toxicity are critical to the safe and efficient screening of drug candidates. Liver mediated drug metabolism occurs primarily in hepatocytes, and cytochrome P450 (CYP) enzymes in hepatocytes are the most common pathways affecting drug clearance. Immortalized cell lines such as Fa2N-4, HepG2, or HepaRG are widely used to test for hepatotoxicity and problematic CYP interactions *in vitro*, as are cultures of human, rodent or canine primary hepatocytes, but some of the CYP enzymes may be under-expressed in these cell lines. Primary human hepatocytes (PHHs) have become the gold standard for CYP induction studies. However, current *in vitro* tests of hepatotoxicity based on isolated PHHs are imperfectly predictive due to phenotypic variability and instability in culture (Mann 2015). In addition, PHH availability is limited and the quality of these cell preps is variable (Ware et al. 2015). Other models have attempted to preserve liver architecture and cell diversity by drug testing on liver slices (Wormser and Ben-Zakine 1990; Elferink et al. 2011), but this approach is likely impractical for high throughput screening.

Inter-individual differences in hepatic metabolism are primarily the result of genetic polymorphisms, and result in considerable variation in individual drug responses and adverse reactions (Takayama et al. 2014). Ideally, hepatotoxicity screening should therefore screen against cells from multiple individuals. This is currently impractical with PHHs, but may be achievable with induced pluripotent stem cell-derived hepatocytes (iPSC-HCs). Despite several encouraging recent studies, it should be noted that iPSC-HCs are not functionally equivalent to PHHs, expressing a somewhat different gene expression profile, including mixed immature and mature markers (Mann 2015). Takayama and colleagues recently established a hepatocyte differentiation method based on stage specific transient overexpression of hepatocyte-related transcription factors and a three-dimensional spheroid culture system, generating a more mature hepatocyte-like cell than previous stem cell

protocols (Takayama et al. 2013). Initial studies showed a close correlation between iPSC-HC and PHH cytochrome P450 metabolism, and showed that the inter-individual differences seen in PHHs were also seen in iPSC-HCs (Takayama et al. 2014). A recent multiparameter analysis of hepatotoxicity used high-content imaging to characterize multiple phenotypic readouts in iPSC-derived hepatocytes exposed to 240 known toxic and nontoxic drugs, including cell shape, cell adhesion, cytoskeleton integrity, accumulation of lipids, and short- and long-term changes in mitochondrial potential (Sirenko et al. 2014). The results of this study suggest that a multiparametric approach has promise, but other assays in addition to high-content imaging are likely needed to improve assay precision, robustness, and reliable prediction of liver toxicity. These recent studies give promise that in the future iPSC-HCs from multiple individuals may yield improved early prediction of hepatotoxicity, and may even provide key exclusion criteria and guide individually tailored therapies.

4.5 Conclusions

The advent of a new technology like stem cell therapy is accompanied by great initial enthusiasm and inevitable setbacks. So it was with monoclonal antibodies. Following on the first publication of hybridoma technology (Köhler and Milstein 1975), 22 years elapsed until the approval of Rituxan, the first chimeric antibody in 1997. Before clinical success, the field had to solve the problem of mouse antibody immunogenicity, first with chimeric mouse-human antibody constructs and later with humanized and fully human antibodies. Stem cell therapy is at the early threshold of a similarly challenging preclinical and clinical development path. Much can be learned from other cell therapies. Preclinical development of stem cell therapeutics will benefit from evolving regulatory guidance, preferably with harmonization of regulations amongst the various national and multinational agencies. Further experience should clarify the factors defining optimal cell culture, storage, and genetic and epigenetic stability. Autologous stem cells offer the hope of an individualized therapy, but will face challenges of cost and batch certification. Allogeneic stem cell therapies must solve the challenge of immunogenicity and the dual threats of rejection and GVHD. Interrelated issues of pluripotency and tumorigenicity pose serious safety concerns that will require improved characterization, selection, and control. In the near term, differentiated stem cells that predict toxicity rapidly, inexpensively, and accurately *in vitro* could revolutionize preclinical safety testing for all drug classes. As the understanding of preclinical development for stem cell therapies matures, the hype of this new technology should be increasingly countered with hope.

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Chapter 5
Regenerative Medicine in the Central Nervous System: Stem Cell-Based Cell- and Gene-Therapy

Seung U. Kim

Abstract Human neurological diseases such as Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), multiple sclerosis (MS), stroke and spinal cord injury are caused by a loss of neurons and glial cells in the brain or spinal cord. Cell replacement therapy and gene transfer to the diseased or injured brain have provided the basis for the development of potentially powerful new therapeutic strategies for a broad spectrum of human neurological diseases. However, the paucity of suitable cell types for cell replacement therapy in patients suffering from neurological disorders has hampered the development of this promising therapeutic approach. In recent years, neurons and glial cells have successfully been generated from stem cells such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs) and neural stem cells (NSCs), and extensive efforts by investigators to develop stem cell-based brain transplantation therapies have been carried out. I review here notable experimental and pre-clinical studies previously published involving stem cell-based cell- and gene-therapies for PD, HD, ALS, AD, MS and stroke, and discuss for future prospect for the stem cell therapy of neurological disorders in clinical setting. There are still many obstacles to be overcome before clinical application of cell- and gene-therapy in neurological disease patients is adopted: (i) it is still uncertain how to generate specific cell types of neurons or glia suitable for cellular grafts in great quantity, (ii) it is required to abate safety concern related to tumor formation following NSC transplantation, and (iii) it needs to be better understood by what mechanism transplantation of NSCs leads to an enhanced functional recovery. Steady and stepwise progress in stem cell research in both basic and pre-clinical settings should support the hope for development of stem cell-based therapies for neurodegenerative diseases. This review focuses on the utility of stem

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cells particularly NSCs as substrates for structural and functional repair of the diseased or injured brain.

Keywords Stem cell • Embryonic stem cell • Induced pluripotent stem cell • Neural stem cell • Mesenchymal stem cell • Cell therapy • Gene therapy • Neurological diseases • Transplantation • Parkinson's disease • Huntington's disease • Amyotrophic lateral sclerosis • Alzheimer's disease • Multiple sclerosis • Stroke

5.1 Introduction

In adult mammalian central nervous system (CNS), injured neurons exhibit low spontaneous capacity for regeneration (Ramon y Cajal 1928), although earlier studies have demonstrated regeneration in the injured adult CNS using fetal brain cells as transplants (cited by Kordower and Tuszinski 1999). It is important to adopt fetal CNS tissues as donors to promote repair in adult CNS since adult CNS cells do not survive in the grafted site. However, little progress has been seen for a long time in investigation related to the CNS regeneration until late 1970s. In 1979 two Swedish research groups reported independently that embryonic rat mesencephalic cells transplanted in the brain of parkinsonian rats survived and induced functional recovery (Perlow et al. 1979). This is the first time that fetal CNS cell transplants replace lost cells and restore functional deficits in animal models of neurological diseases. Since then, fetal CNS-derived cells were grafted into the brain and spinal cord of animal models of neurological disorders including Parkinson's disease (PD), Huntington's disease (HD), stroke and spinal cord injury. Starting late 1980s, transplantation of human fetal ventral mesencephalic tissues into the striatum of PD patients has been adopted as a successful therapy for patients with advanced disease (Lindvall et al. 1990; Olanow et al. 1996; Kordower et al. 1997a, b; Dunnett and Bjorklund 1999). However, this fetal brain tissue transplantation has grave problems associated with ethical and religious questions and limited supply of fetal tissues. To circumvent these difficulties, utilization of neurons with dopamine (DA) phenotype generated from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs) or neural stem cells (NSCs) could serve as a practical and effective alternative for the fetal brain tissues for brain transplantation.

Cell replacement therapy and gene transfer to the diseased or injured brain have provided the basis for the development of potentially powerful new therapeutic strategies for human neurological diseases. However, the paucity of suitable cell types for cell therapy in patients suffering from neurological disorders has hampered the development of this promising therapeutic approach. In recent years, neurons and glial cells have successfully been generated from stem cells such as ESCs, iPSCs, MSCs and NSCs, and extensive efforts by investigators to develop stem cell-based brain transplantation therapies have been carried out.

Stem cells are defined as cells that have the ability to renew themselves continuously and possess pluripotent ability to differentiate into many cell types. Two types of mammalian pluripotent stem cells, ESCs derived from the inner cell mass of blastocysts and embryonic germ cells (EGCs) obtained from post-implantation embryos, have been identified and these stem cells give rise to various organs and tissues (Thompson et al. 1998; Shamblott et al. 1998). Recently there has been an exciting development in generation of a new class of pluripotent stem cells, induced pluripotent stem cells (iPSCs), from adult somatic cells such as skin fibroblasts by introduction of embryogenesis-related genes (Takahashi et al. 2007; Yu et al. 2007; Park et al. 2008). In addition to ESCs and iPS cells, tissue specific stem cells could be isolated from various tissues of more advanced developmental stages such as hematopoietic stem cells (HSCs), bone marrow MSCs, adipose tissue-derived stem cells (ADSCs), amniotic fluid stem cells and NSCs. Among these, existence of multipotent NSCs has been known in developing or adult rodent brain with properties of indefinite growth and multipotent potential to differentiate into three major cell types of CNS, neurons, astrocytes and oligodendrocytes (McKay 1997; Flax et al. 1998; Gage 2000; Temple 2001; Gottlieb 2002; Kim 2004).

In human, existence of NSCs with multipotent differentiation capability has also been reported in embryonic and adult human brain (Flax et al. 1998; Kim 2004; Brustle and McKay 1996; Sah et al. 1997). In a group of cancer patients who had infusion of chemical bromodeoxyuridine (BrdU) for diagnostic purposes and later died, evidence that new neurons are continuously being generated in adult human CNS has been demonstrated (Eriksson et al. 1998). Why then there is only limited capacity to repair in adult CNS suffering from injury or diseases? It appears that endogenous brain environment that is responsible for induction of NSC proliferation and consequent NSC differentiation into neurons is not adequate in most of diseased or injured brain.

Recently continuously dividing immortalized cell lines of NSCs have been generated by introduction of oncogenes and these immortalized NSC lines have advantageous characteristics for basic studies on neural development and cell replacement therapy or gene therapy studies: (i) Stable immortalized NSC cells are homogeneous since they were generated from a single cell, i.e. a single clone; (ii) immortal NSC cells can be expanded readily in large numbers in short time; (iii) stable expression of therapeutic genes can be achieved readily (Flax et al. 1998; Kim 2004; Renfranz et al. 1991; Snyder et al. 1992; Lee et al. 2007a). Immortalized NSCs have emerged as highly effective source for genetic manipulation and gene transfer into the CNS *ex vivo*; immortalized NSCs were genetically manipulated *in vitro*, survive, integrate into host tissues and differentiate into both neurons and glial cells after transplantation to the intact or damaged brain. We have previously generated immortalized cell lines of human NSCs by infecting fetal human brain cells grown in primary culture with a retroviral vector carrying v-myc oncogene and selecting continuously dividing NSC clones. Both *in vivo* and *in vitro* these cells were able to differentiate into neurons and glial cells and populate the developing or degenerating CNS (Flax et al. 1998; Kim 2004; Lee et al. 2007a, Kim and deVellis 2009).

Stem cell-based cell and gene therapy could serve as potentially powerful new therapeutic strategies for a broad spectrum of human neurological diseases including PD, HD, AD, ALS, MS, stroke, spinal cord injury and brain tumors (Brustle and McKay 1996; Flax et al. 1998; Kim 2004; Lindvall et al. 2004; Goldman 2005, Kim and deVellis 2009). There are still many obstacles to be overcome before clinical application of cell therapy in neurological disease patients is adopted: (i) it is still uncertain how to generate specific cell types of neurons or glia suitable for cellular grafts in great quantity, (ii) it is required to abate safety concern related to tumor formation following NSC transplantation, and (iii) it needs to be better understood by what mechanism transplantation of NSCs leads to an enhanced functional recovery. Steady and stepwise progress in stem cell research in both basic and pre-clinical settings should support the hope for development of stem cell-based therapies for neurodegenerative diseases. This review focuses on the utility of stem cells particularly NSCs and MSCs as substrates for structural and functional repair of the diseased or injured CNS.

5.2 Parkinson Disease

Parkinson's disease (PD) is characterized by an extensive loss of dopamine neurons (DA) in the substantia nigra pars compacta and their terminals in the striatum (Kish et al. 1988; Agid 1991), and affects more than 500,000 people in the US. While the etiology of idiopathic PD is not known, several predisposing factors for the dopaminergic depletion associated with the disease have been suggested, including programmed cell death, viral infection, and environmental toxins. As an effective treatment for PD, patients have been given L-dihydroxyphenyl alanine (L-DOPA), a precursor of dopamine, but long-term administration of L-DOPA consequently produces grave side effects (Lang and Lozano 1998a, b). More recently surgical procedure of deep brain stimulation has been adopted as a successful treatment for PD patients (Lyons 2011).

Since late 1980s, transplantation of human fetal ventral mesencephalic tissues into the striatum of PD patients has been adopted as a successful therapy for patients with advanced disease (Lindvall et al. 1990; OLanow et al. 1996; Kordower et al. 1997a, b; Dunnett and Bjorklund 1999). However, this fetal tissue transplantation has grave problems associated with ethical and religious questions and logistics of acquiring fetal tissues. In addition, recent reports have indicated that the survival of transplanted fetal mesencephalic cells in the patients' brain was very low and it was difficult to obtain enough fetal tissues needed for transplantation (Hagell et al. 1999). To circumvent these difficulties, utilization of neurons with dopamine (DA) phenotype generated from ESCs, iPSCs, MSCs or NSCs could serve as a practical and effective alternative for the fetal brain tissues for transplantation. DA neurons were generated from mouse ESCs after treatment with fibroblast growth factor 8 (FGF8) and sonic hedgehog (Hagell and Brundin 2002), over-expression of Nurr1 (Wagner et al. 1999; Chung et al. 2002; Kim et al. 2003) or Bcl-XL (Shim et al.

2004), or co-culture with a mouse bone marrow stromal cell line (Kawasaki et al. 2000). Neurons with DA phenotype have been generated from monkey ESCs by co-culturing with mouse bone marrow stromal cells and behavioral improvement was seen in MPTP-lesioned monkeys following intra-striatal transplantaiton of these cells (Takagi et al. 2005). DA neurons were also generated from neural progenitor cells derived from fetal brain and induced functional recovery following brain transplantation in parkinsoinian monkeys (Takagi et al. 2005)

Transplantation of NSCs in the brain attenuates anatomic or functional deficits associated with injury or disease in the CNS via cell replacement, the release of specific neurotransmitters, and the production of neurotrophic factors that protect injured neurons and promote neuronal growth. Recently we have generated continuously dividing immortalized cell lines of human NSC from fetal human brain cell culture via a retroviral vector encoding v-*myc* (Kim 2004; Lee et al. 2007a; Kim et al. 2008a, b, c) and one of the immortalized NSC lines, HB1.F3, induced functional improvement in rat model of PD following transplantation into the striatum (Yasuhara et al. 2006).

Earlier studies have used gene transfer technology to develop treatment for PD by transferring tyrosine hydroxylase (TH) gene, a rate-limiting step enzyme in catecholamine biosynthesis process, into certain cell types and then implant these cells into the brain of PD animal models (Wolff et al. 1989; Fisher et al. 1991; Jiao et al. 1993; Anton et al. 1994; During et al. 1994). However, gene transfer of TH using genetically modified cells produced only partial restoration of behavioral and biochemical deficits in PD animal models, since the cells utilized did not carry sufficient amount of tetrahydrobiopterin (BH_4), a cofactor to support TH activity (Kang et al. 1993). Therefore, it is necessary to transfer additionally of GTP cyclohydrolase-1 (GTPCH-1) gene that is the first and rate-limiting enzyme in the BH_4 biosynthetic pathway (Bencsics et al. 1996). Immortalized CNS-derived mouse NSC line C17.2 was transduced to carry tyrosine hydroxylase (TH) gene and GTP cyclohydrorylase-1 (GTPCH-1) gene for production of L-DOPA and following intra-striatal implantation behavioral improvement was seen in 6-hydroxydopamine-lesioned rats (Ryu et al. 2005). We have similarly engineered HB1.F3 human NSC line to produce L-DOPA by double transduction with human TH and GTPCH-1 genes, and following transplantation of these cells in the brain of PD rat model led to enhanced L-DOPA production *in vivo* and induced functional recovery (Kim et al. 2006).

Previous studies have reported that mouse ES cell-derived DA neurons have shown efficacy in PD animal models, whereas DA neurons from human ES cells generally show poor performance. In addition, there are considerable safety concerns for ES cells related to risk of tumor formation and neural overgrowth. More recent studies have indicated that functional human DA neurons could be generated efficiently from human ES cells and upon transplantation in rat PD models ES cell-derived DA neurons induced behavior recovery in the animals (Cho et al. 2008; Kirks et al. 2011). These studies indicate that large scale generation of DA neurons is possible from human ES cells as cellular source for cell therapy in PD patients. Human DA neurons derived from iPS cells may provide an ideal cellular source for transplantation therapy for PD. However, developing effective cell therapy approach

Table 5.1 Stem cell-based cell therapy in experimental Parkinson's disease models

Reference (number)	Animal model	Transplanted cells	Additional treatment	Functional outcome
Kim et al. (2002)	Rat, 6-OHDA	NSC (rat)	None	Not tested
Hagell and Brundin (2002)	Rat, 6-OHDA	NPC (rat)	FGF8/SHH	Rotation↓
Takagi et al. (2005)	Monkey, MPTP	ESC (monkey)	Stromal cell (mouse) feeder	PFS-Parkinson factor score↓
Ryu et al. (2005)	Rat, 6-OHDA	Immortalized NSC (mouse, C17-2)	TH/GTPCH1 Gene transfer	Rotation↓
Kim et al. (2006)	Rat, 6-OHDA	Immortalized NSC (human, HB1.F3)	TH/GTPCH1 Gene transfer	Rotation↓
Yasuhara et al. (2006)	Rat, 6-OHDA	Immortalized NSC (human, HB1.F3)	NSC migration	Rotation↓
Redmond et al. (2007)	Monkey MPTP	NSC (human)	None	PFS-Parkinson factor score↓
Cho et al. (2008)	Rat, 6-OHDA	DA neurons from ES cells (human)	None	Rotation↓ Beam walking ↑
Werning et al. (2008)	Rat, 6-OHDA	DA neurons from ES cells (human)	WNT signal SHH	Rotation↓
Kriks et al. (2011)	Rat, 6-OHDA	DA neurons from iPS cells (human)	None	Rotation↓

ESC embryonic stem cell, *iPS cell* induced pluripotent stem cell, *NPC* neural precursor cell, *NSC* neural stem cell, *BMSC* bone marrow mesenchymal stem cell, 6-OHDA 6-hydroxydopamine, MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, TH tyrosine hydrpxylase, GTPCH-1 GTP cyclohydrOLyrase-1

for PD using iPS cells relies on optimizing in vitro production of iPS cell-derived DA neurons and preventing potential risk of teratoma formation in vivo. A recent study has reported generation of DA neurons from iPS cells derived from fibroblasts and improved behavior following transplantation of the DA neurons in PD model rats (Werning et al. 2008). Although further research is still required, cell therapy based on DA neurons derived from iPS cells will probably become a promising treatment technique in the coming days.

Summary of preclinical studies of stem cell transplantation in PD animal models in rat and monkey is shown in Table 5.1.

5.3 Huntington Disease

Huntington disease (HD) is an autosomal dominant neurodegenerative disorder characterized by involuntary choreiformic movements, cognitive impairment, and emotional disturbances (Greenamyre and Shoulson 1994; Harper 1996). Despite

identification of the HD gene and associated protein, the mechanisms involved in the pathogenesis of HD remain largely unknown and thus hamper effective therapeutic interventions. Transplantation of fetal human brain tissue may serve as a useful strategy in reducing neuronal damage in HD brain and a recent study has documented improvements in motor and cognition performance in HD patients following fetal cell transplantation (Bachoud-Lévi et al. 2000). This trial follows previous reports in experimental animals of HD that positive effects of fetal striatal cell transplantation to ameliorate neuronal dysfunction (Nakao and Itakura 2000) and that striatal graft tissue could integrate and survive within the progressively degenerated striatum in transgenic HD mouse model (Dunnett et al. 1998). The latter study is consistent with results obtained from HD patients indicating survival and differentiation of implanted human fetal tissue in the affected regions (Freeman et al. 2000). Cell replacement therapy using human fetal striatal grafts has shown clinical success in HD patients. However, a recent study has reported neural overgrowth of grafted tissue in a HD patient who survived 5 years post-transplantation (Keene et al. 2009). Overgrown grafts were composed of neurons and glia embedded in disorganized neutrophil. This report recalls safety concerns for fetal cell grafts related to potential risk of neural overgrowth following transplantation in HD patients.

Transplantation of NSCs to replace degenerated neurons or genetically modified NSCs producing neurotrophic factors have been used to protect striatal neurons against excitotoxic insults (Bjorklund and Lindvall 2000). At present, little is known regarding whether implantation of NSCs prior to neuropathological damage could alter the progressive degeneration of striatal neurons and motor deficits that occur in HD. This question is important since genetic study of Huntington disease gene mutation (Huntington's Disease Collaborative Research Group 1993) and neuroimaging can provide details on factors involved in the progression of HD (Harris et al. 1999; Thieben et al. 2002) suggesting early intervention using brain transplantation could be effective in "pre-clinical" HD patients carrying mutant HD gene. We have investigated the effectiveness of proactive transplantation of human NSCs into rat striatum of an HD rat prior to lesion formation and demonstrated significantly improved motor performance and increased resistance to striatal neuron damage compared with control sham injections (Ryu et al. 2004). The neuroprotection provided by the proactive transplantation of human NSCs in the rat model of HD appears to be contributed by brain-derived neurotrophic factor (BDNF) secreted by the transplanted human NSCs.

Rodents and primates with lesions of the striatum induced by excitotoxic kainic acid (KA), or quinolinic acid (QA) have been used to simulate HD in animals and to test efficacy of experimental therapeutics experiments on neural transplantation (DiFiglia 1990). Excitotoxic animal models induced by QA, which stimulates glutamate receptors, resemble the histopathologic characteristics of HD patients, were utilized for cell therapy with mouse embryonic stem cells, mouse neural stem cells, mouse bone marrow mesenchymal stem cells and primary human neural

precursor cells and resulted in varying degree of clinical improvement (Kordower et al. 1997a, b; Armstrong et al. 2000; McBride et al. 2004; Visnyei et al. 2006; Lee et al. 2005, 2006). We have recently injected human NSCs intravenously in QA-HD model rats and demonstrated functional recovery in HD animals (Lee et al. 2005, 2006). The systemic transplantation of NSCs via intravascular route is probably the least invasive method of cell administration (Lee et al. 2006). Neural cell transplantation into striatum requires an invasive surgical technique using a stereotaxic frame. Non-invasive transplantation via intravenous routes, if it may be effective in human, is much more attractive.

Systemic administration of 3-nitropropionic acid (3-NP) in rodents leads to metabolic impairment and gradual neurodegeneration of the basal ganglia with behavioral deficits similar to those associated with HD (Beal et al. 1993; Brouillet et al. 1995), and murine and human NSCs have been transplanted in the brain of 3-NP-HD animal models (Ryu et al. 2004). The compound 3-NP is a toxin which inhibits the mitochondrial enzyme succinate dehydrogenase (SDH) and tricarboxylic acid (TCA) cycle thereby interfering with the synthesis of ATP (Alston et al. 1977).

We have investigated the effectiveness of transplantation of human NSCs into adult rat striatum prior to striatal damage induced by 3-NP toxin (Ryu et al. 2004). Animals receiving intrastriatal implantation of human NSCs 1 week prior to 3-NP treatments exhibited significantly improved motor performance and increased resistance to striatal neuron damage compared with control sham injections. The neuroprotection provided by the proactive transplantation of human NSCs in the rat model of HD appears to be contributed by brain-derived neurotrophic factor (BDNF) secreted by the transplanted human NSCs. Previous studies have also demonstrated that BDNF could block neuronal injury under pathological conditions in animal models of HD (Bemelmans et al. 1999; Pérez-Navarro et al. 2000). These findings suggest that proactively transplanted human NSCs were well integrated in the striatum and supported the survival of host striatal neurons against neuronal injury.

Human NSCs derived from ESCs could provide a viable cellular source for cell and therapy in HD, since they can be expanded indefinitely and differentiate into any cell type desired. Three previous studies have shown that neurons expressing striatal markers could be induced from ESCs and brain transplantation of these ESC-derived neurons in QA-lesioned rats leads to behavioral recovery in the animals (Song et al. 2007; Aubry et al. 2008; Vasey et al. 2010).

We have recently written a review that focuses on the stem cell-based therapy for HD and investigators who wish to learn more about the subject are referred to the review article (Kim et al. 2008a, b, c). Summary of preclinical studies of stem cell transplantation in HD animal models is shown in Table 5.2.

Table 5.2 Stem cell-based cell therapy in experimental Huntington's disease models

Reference (number)	Animal model	Transplanted cells	Histology & lesion volume	Functional outcome
Kordower et al. (1997a, b)	Rat, Quinolinic acid/ QA	NSC (mouse)	Intact BBB Lesion vol↓	Not tested
Armstrong et al. (2000)	Rat, QA	BMSC (mouse)	GAD+cells 0.3 % No change	No improvement
McBride et al. (2004)	Rat, QA	NPC (fetal human)	NPC migration Lesion vol↓	Cylinder test↑
Visnyei et al. (2006)	Rat, QA	ESC (mouse)	NeuN+ cells↑ Lesion vol↓	No improvement
Lee et al. (2005)	Rat, QA	Immortalized NSC (human, HB1.F3)	NeuN+ cells↑ Lesion vol↓	Circling behavior↓
Ryu et al. (2004)	Rat, 3-NP	Immortalized NSC (human, HB1.F3)	NeuN+ cells↑ Lesion vol↓	Rotarod↓
Roberts et al. (2006)	Rat, 3-NP	NPC (rat)	NPC migration No change	Beam walking↑
Song et al. (2007)	Rat, QA	ESC-derived NSC (human)	NeuN+ cells↑	Circling behavior↓
Aubry et al. (2008)	Rat, QA	ESC-derived NSC (human)	DARPP+ neuron	Not done
Vasey et al. (2010)	Rat, QA	ESC-derived NSC (human)	Noggin-primed NSC migration	Not done

QA quinOLinic acid, 3-NP 3-nitropropionic acid, NSC neural stem cell, BMSC bone marrow stromal cells, NPC neural precursor cell, NSC neural stem cell, BBB blood-brain barrier, NeuN neuron-specific nuclear protein

5.4 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), known as Lou Gehric disease, is a relentlessly progressive, adult onset neurodegenerative disorder characterized by degeneration and loss of motor neurons in the cerebral cortex, brain stem and spinal cord, leading to muscle wasting and weakness, and eventually to death within 5 years after the onset of its clinical symptoms (Hudson 1990; Rowland and Shneider 2001). The proposed pathogenetic mechanisms of ALS, albeit not fully elucidated, include oxidative stress, protein aggregation, mitochondrial dysfunction, impaired axonal transport, glutamate-mediated excitotoxicity, and insufficient production of neurotrophic factors (Boillee et al. 2006). To date there is no effective treatment for patients suffering from ALS.

Recent studies have indicated that it is possible to generate motor neurons in culture from stem cells that include ESCs and NSCs (Wichterle et al. 2002; Harper et al. 2004; Miles et al. 2004; Li et al. 2005). Mouse ESC-derived motor neurons

transplanted into motor neuron-injured rat spinal cord survived and extended axons into ventral root (Miles et al. 2004), and human EGCs transplanted into cerebrospinal fluid of rats with motor neuron injury migrated into spinal cord and led to improved motor function (Kerr et al. 2003). Transplantation of NSCs isolated from fetal spinal cord (Xu et al. 2006) was also effective in delaying disease progression in mouse ALS model. These cell transplantation studies have shown functional improvement in animal models of ALS.

A recent study has reported that iPSCs isolated from an ALS patient were differentiated into motoneurons (Dimos et al. 2008) and these patient-derived neurons could be an ideal cellular source for transplantation. Neurons and glia induced from patient-derived iPSCs are ideal for cell therapy as the iPSC-derived neurons are autologous, easily accessible, without immune rejection and with no ethical problem although there is safety concern of tumor formation following the cell transplantation.

The systemic transplantation of NSCs via intravascular route is probably the least invasive method of cell administration in ALS. Non-invasive transplantation via intravenous routes is much more attractive than surgical technique. Recently rat NSCs labeled with green fluorescent protein were transplanted in rat ALS model via intravenous tail vein injection and 7 days later 13 % of injected cell were found in motor cortex, hippocampus and spinal cord. No improvement in clinical symptoms was reported (Miltrecic et al. 2010).

It is unrealistic to expect the transplantation of stem cells or stem cell-derived motor neurons in ALS patients in a clinical setting replaces lost neurons, integrates into existing neural circuitry and restores motor function. Rather preventing cell death in host motor neurons via provision of neurotrophic factors by transplanted stem cells or stem cell-derived motor neurons is more realistic and achievable approach (Lindvall and Kokaia 2006). Recent studies have shown that the application of an adenoviral vector encoding glial cell line-derived growth factor (GDNF) into injured rat facial motor nucleus rescued motor neurons from cell death (Watabe et al. 2000), and human cortical progenitor cells engineered to express GDNF and transplanted into the spinal cord of ALS rats survived and released the growth factor (Kerr et al. 2003). Several recent studies have also demonstrated that delivery of vascular endothelial cell growth factor (VEGF) significantly delayed disease onset and prolonged the survival of ALS animal models (Klein et al. 2005; Azzouz et al. 2004; Zheng et al. 2004; Storkebaum et al. 2005). VEGF is one of growth factors that can be used in combination with transplanted stem cells to improve therapeutic efficiency of cellular transplantation. VEGF is an angiogenetic growth factor acting as a potent mitogen and survival factor specific to endothelial cells, and also known for neurotrophic and neuroprotective effect against brain injury. Recently we have demonstrated that in transgenic SOD1/G93A mouse model of ALS (Gurney et al. 1994) intrathecal transplantation of human NSCs over-expressing VEGF (HB1.F3.VEGF) induced functional improvement, delayed disease onset for 7 days and extended the survival of animals for 15 days (Hwang et al. 2009a, b). Immunohistochemical investigation of SOD1/G93A mouse spinal cord demonstrated that the transplanted human NSCs migrated into spinal cord anterior horn and differentiated into motoneurons.

More recently, we generated motoneurons from human NSCs and transplanted these cells into spinal cord of SOD1G93A ALS mouse (Kim et al. 2011). Motoneurons were generated by treatment of human NSCs encoding Olig2 bHLH transcription factor gene (HB1.F3.Olig2) with sonic hedgehog (Shh) protein. HB1.F3.Olig2 NSCs treated with Shh for 4–7 days differentiated not motoneurons expressing motoneuron-specific markers HB-9, Isl-1 and choline acetyltransferase (ChAT) but did not express OLG markers such as O4, galctocerebroside or CNPase. Control HB1.F3.Olig2 NSCs grown in the absence of Shh did not express any of the motor neuron-specific cell type markers. Intrathecal transplantation of motoneuron-committed HB1.F3.Olig2+Shh human NSCs into L5 of spinal cord significantly delayed disease onset (9 days) and expanded the life span (22 days) of SOD1 G93A ALS mice, with two out of six mice living up to 35 days. Grafted NSCs were found within grey matter and anterior horn of the spinal cord. These results suggest that this treatment modality using genetically modified human NSCs might be of value in the treatment of ALS patients without significant adverse effects (Kim et al. 2011).

Summary of preclinical studies of stem cell transplantation in ALS animal models is shown in Table 5.3.

Table 5.3 Stem cell-based cell therapy in experimental ALS models

Reference	Injury model	Transplanted cells	Additional treatment	Functional outcome
Garbuzoba et al. (2002)	Mouse, SOD mutant	NT2 teratoma (human) –RA treatment	RA	Not done
Harper et al. (2004)	Rat, MN injury -sindivus virus	ESC (mouse)	RA+SHH agonist	Not done
Kerr et al. (2004)	Rat, MN injury -sindivus virus	EGC (human)	none	BBB-improvement Limb strength↑
Klein et al. (2002)	Rat, SOD mutant	NPC (human, primary)	GDNF Gene transfer	BBB-no impro-vement
Xu et al. (2006)	Rat, SOD mutant	NPC (human, primary)	None	BBB-improvement Extended survival
Hwang et al. (2008)	Mouse, SOD mutant	Immortalized NSC (human HB1.F3)	VEGF Gene transfer	Rotarod, limb placement↑ Extended survival
Miltrecic et al. (2010)	Rat, SOD mutant	NSC (rat)	GFP labeled	Not done
Kim et al. (2012)	Mouse, SOD mutant	Immortalized NSC (human HB1.F3)	Olig2 Gene transfer Shh treatment	Rotarod, limb placement↑ Extended survival

SOD superoxide dismutase, MN motor neuron, NPC neural precursor cell, ESC embryonic stem cell, EGC embryonic germinal stem cell, NSC neural stem cell, GDNF glial cell line-derived neurotrophic factor, RA retinoic acid, SHH sonic hedgehog, VEGF vascular endothelial growth factor, GFP green fluorescent protein

5.5 Alzheimer Disease

Alzheimer disease is characterized by degeneration and loss of neurons and synapses through out the brain particularly in basal fore brain, amygdala, hippocampus and cortical area. Memory and cognitive function of patients progressively decline, patients become demented and die prematurely (Whitehouse et al. 1981; Bartus et al. 1982; Coyle et al. 1983). No effective treatment is currently available except for acetylcholinesterase inhibitors which augment cholinergic function but is not curative and only a temporary measure.

As for the pathogenesis of AD, the amyloid cascade hypothesis postulates that memory deficits are caused by increased levels of both soluble and insoluble amyloid ß (Aß) peptides, which are derived from the larger amyloid precursor protein (APP) sequential proteolytic processing (Whitehouse et al. 1981; Bartus et al. 1982; Coyle et al. 1983). Recent study has reported that treatment of PDAPP mice, a transgenic mouse model of AD, with anti-Aß antibody completely restored hippocampal acetylcholine release and high-affinity choline uptake and improved habituation learning (Bales et al. 2006). Based on the study, a clinical trial in AD patients is underway in the US.

Chronically decreasing Aß levels in brain has been suggested as a possible therapeutic approach for AD, and several experimental evidence indicate that proteinases such as neprilysin (Iwata et al. 2001), insulin degrading enzyme (Farris et al. 2003; Miller et al. 2003), plasmin (Melchor et al. 2003) and cathepsin B (Mueller-Steiner et al. 2006) could be used as therapeutic agents to reduce Aß levels in AD brain. Recent studies have shown that intracerebral injection of a lentivirus vector expressing human neprilysin in transgenic mouse models of amyloidosis reduced Aß deposits in the brain and blocked neurodegeneration in the frontal cortex and hippocampus (Marr et al. 2003), and that intracerebrally injected fibroblasts overexpressing human neprilysin gene were found to significantly reduce amyloid plaque burden in the brain of Aß transgenic mice (Hemming et al. 2007). These studies support the use of Aß-degrading proteases as a tool to therapeutically lower Aß levels and encourage further investigation of ex vivo delivery of protease genes using human NSCs for the treatment of AD. We have recently generated a human NSC line encoding human neprylysin gene, transplanted these cells in lateral ventricle of AD transgenic mouse brain, and results are expected some time later.

Earlier studies have indicated that nerve growth factor (NGF) prevent neuronal death and improve memory in animal models of aging, excitotoxicity and amyloid toxicity (Hefti 1986; Fischer et al. 1987; Tuszynski et al. 1990; Emerich et al. 1994; Tuszynski 2002), and could be used for treating neuronal degeneration and cell death in AD brain. However, delivery of NGF into the brain is not possible via peripheral administration. Because of its size and polarity NGF does not cross the blood brain barrier. In order to overcome this difficulty, gene therapy approach could be adopted. Using *ex vivo* gene therapy approach via NGF encoding cells, NGF can be administered directly to the brain and diffuse for distance of 2–5 mm (Tuszynski et al. 1990). A phase 1 clinical trial of *ex vivo* NGF gene delivery was performed in 8 mild AD patients by implanting autologous fibroblasts genetically

modified to express human NGF into the forebrain. After mean follow-up of 22 months in 6 subjects, long-term adverse effects were not found. Evaluation by MMSE and AD A SCS suggested improvement in the rate of cognitive decline. Serial PET scans showed significant increases in cortical fluorodeoxyglucose after treatment (Tuszynski et al. 2005). Since fibroblasts are known for their immobility following brain transplantation (Kang et al. 1993), NSCs with high migratory capacity and pathology-tropic property (Flax et al. 1998; Kim 2004; Lee et al. 2007a; Kim and deVellis 2009) could be used in place of fibroblasts to deliver NGF into the AD brain. In learning deficit AD model rats induced by okadaic acid injection, transplantation of rat NSCs infected with adenovirus-NGF produced improvement in cognitive performance (Wu et al. 2008).

In a recent study, we used human NSCs in place of rodent NSCs or human fibroblasts to deliver NGF in ibotenic acid-induced learning deficit rats. Intrahippocampal injection of ibotenic acid caused severe neuronal loss, resulting in learning and memory deficit (Lee et al. 2012). NGF protein released by HB1.F3.NGF human NSCs in culture media is 10-fold over the control F3 naive NSCs at 1.2 μg/10^6 cells/day. Intra-hippocampal transplantation of HB1.F3.NGF cells was found to express NGF and fully improved the learning and memory function of ibotenic acid-challenged animals. Transplanted HB1.F3.NGF human NSCs were found all over the brain and differentiated into neurons and astrocytes (Lee et al. 2012). In another study, brain derived neurotrophic factor (BDNF), a member of neurotrophin family, secreted by transplanted mouse NSCs was responsible in enhancing cognitive function in triple transgenic mice that express pathogenic forms of myloid precursor protein, presenilin and tau. In these animals cognition was improved without altering Aβ or tau pathology (Blurton-Jones et al. 2009). In other studies in experimental rats with nucleus basalis of meynert (NBM) lesion induced by ibotenic acid, transplantation of mouse or rat neural precursor cells (NPCs) promoted behavioral recovery (Wang et al. 2006; Moghadam et al. 2009)

In AD patients, dysfunction of the presynaptic cholinergic system is one of the causes of cognitive disorders where decreased activity of choline acetyltransferase (ChAT), which is responsible for acetylcholine (ACh) synthesis, is observed (Terry and Buccafusco 2003). To date, AD therapy has largely been based on small molecules designed to increase ACh concentration by inhibiting acetylcholinesterase (Musiał et al. 2007). Since therapies with these drugs is only palliative without potential protection against progressive tissue destruction, there is a need for effective therapies for patients with AD, and stem cell-based therapeutic approaches targeting AD should fulfill this requirement. We have recently generated a human neural stem cell (NSC) line over-expressing human choline acetyltransferase (ChAT) gens and these HB1.F3.ChAT NSCs were transplanted into the brain of rat Alzheimer disease (AD) model which was generated by intra-hippocampal injection of kainic acid (KA) in CA3 region which results in severe neuronal loss and profound learning and memory deficit. Intraventricular transplantation of HB1.F3.ChAT human NSCs fully restored learning and memory (Park et al. 2012a). Similarly HB1.F3.ChAT human NSCs were transplanted in AD model rats generated by application of ethylcholine mustard aziridinium ion (AF64A) that specifically denatures cholinergic nerves and thereby leads to memory deficit as a salient

Table 5.4 Stem cell-based cell therapy in experimental Alzheimer's disease models

Reference (number)	Animal model	Transplanted cells	Special feature	Outcome
Wang et al. (2006)	Mouse NBM lesion Ibotenic acid -	ESC-derived neurosphere (mouse)	ChAT+ cells↑	Working memory↑
Wu et al. (2008)	Rat Forebrain Okadaic acid	NSC (rat)	NGF (human) Gene transfer	Memory↑
Moghadam et al. (2009)	Rat NBM lesion Ibotenic acid	ESC-derived NPC (mouse)	Shh-primed	Water maze↑ Spatial probe↑
Blurton-Jones et al. (2009)	Mouse 3X TG-AD	NSC (mouse)	BDNF-mediated effect	Working memory↑
Park et al. (2011)	Rat Hippocampus Kainic acid	Immortalized NSC (human, HB1.F3)	ChAT (human) Gene transfer	Water maze↑ Spatial probe↑
Park et al. (2012a, b)	Rat NBM lesion AF64A toxin	Immortalized NSC (human, HB1.F3)	ChAT (human) Gene transfer	Water maze↑ Spatial probe↑
Lee et al. (2012)	Mouse Hippocampus Ibotenic acid	Immortalized NSC (human, HB1. F3)	NGF (human) Gene transfer	Water maze↑ Spatial probe↑

NBM nucleus basalis of Meynert, NSC neural stem cell, ESC embryonic stem cell, NPC neural precursor cell, NGF nerve growth factor, BDNF brain derived growth factor, SHH sonic hedgehog protein, ChAT choline acetyltransferase, 3XTG triple transgenic/APP-presenilin-tau

feature of AD (Yamazaki et al. 1991). Transplantation of NB1.F3.ChAT human NSCs in AF64A-treated mice fully restored the learning and memory function of AF64A animals (Park et al. 2012b).

Summary of preclinical studies of stem cell-based cell therapy in AD animal models is shown in Table 5.4.

5.6 Multiple Sclerosis

In multiple sclerosis (MS), Oligodendrocytes (OLGs) and myelin are destroyed by inflammation-mediated mechanism (McFarlin and McFarland 1982; Ebers 1988). Although recent advance in treatment using immune moderators such as interferon-β has improved clinical outcome in some patients, functional recovery in most of MS patients is not achieved (Paty and Ebers 1998). Therefore, there is substantial need for effective therapies for MS patients.

Previous studies have reported that OLGs or OLG progenitor cells isolated from mouse or rat brain were transplanted in the brain of dysmyelination mutants or chemically induced demyelination lesions in rats and induced remyeliation in previously dysmyelinated or demyelinated lesion sites (Franklin and Blakemore 1997; Espinosa de los Monteros et al. 1997, 2001; Learish et al. 1999; Zhang et al. 1999; Ben-Hur et al. 2003). Experimental animal models for MS used in transplantation studies include shiverer dymyelination mutant mouse, demyelination lesions induced by ethidium bromide, experimental allergic encephalitis (EAE) or mouse hepatitis virus. Therapeutic approach with myelinating glia could be applied in MS patients by transplantation of human OLGs into demylination lesions. Intact embryonic human brain fragments or OLG progenitors isolated from fetal human brain have been placed in shiverer mouse brain, a mouse neurological mutant with defect in myelin basic protein gene, and remyelination was confirmed (Lachapelle et al. 1983; Gumpel et al. 1987; Seilhean et al. 1996; Windrem et al. 2004). Transplantation of human OLGs in MS patients to achieve remyelination of previously demyelinated axons, however, has not been undertaken to date. This therapeutic approach of transplantation of human OLGs or OLG progenitors derived from fetal brain is not widely acceptable because of moral, religious and logistic problems associated with tissue collection of human embryonic/fetal brain. In addition, the outcome of graft is not predictable since the implanted embryonic/fetal tissues contain mixed population of neurons, glial cells and CNS progenitor cells, and less than 10 % of cell population for graft expressed O4, a marker for young OLG (Gumpel et al. 1987). This difficulty can be circumvented by utilization of OLGs or OLG progenitor cells derived from human ES cells or NSCs. Recent studies have reported that OLGs could be generated from mouse and human ES cells (Brüstle et al. 1999; Liu et al. 2000; Glaser et al. 2005; Nistor et al. 2005), bone marrow mesenchymal stem cells (Akiyama et al. 2002) or immortalized mouse NSCs (Yandava et al. 1999). In a mouse EAE, systemically injected mouse neural precursor cells (NPCs) selectively enter the inflamed CNS in EAE model and induce apoptosis of blood-borne CNS-infiltrating encephalitogenic T cells, thus protecting against chronic neural tissue loss. NPCs display immune-like functions that promote neuroprotection in the CN (Pluchino et al. 2005). Similarly human ESC-derived NPCs transplanted into the brain ventricles of EAE mouse reduced clinical signs of EAE and transplanted NPCs were found in the white matter. These results indicate that NPCs act as immne-like cells in the CNS (Aharonowiz et al. 2008).

OLGs could also be generated from stable established cell lines of human NSC and used as cell source of transplantation. Previously we have produced immortalized cell lines of human NSC from human embryonic telencephalon using a retroviral vector encoding myc oncogene (Kim 2004; Lee et al. 2007a; Kim et al. 2008a, b, c). This human NSC line, HB1.F3 (F3), can be genetically engineered to express foreign transgenes, and following transplantation into brain of animal models of PD, HD and stroke, F3 NSCs survived, differentiate into neurons and astrocytes, and reversed functional deficits (Kim 2004; Lee et al. 2007a; Kim and deVellis 2009). Most recently we were successful in producing a new F3.Olig2 human NSC line by transduction of F3 with a retroviral vector encoding Olig2 bHLH transcription

factor gene. Olig2 is a member of Olig bHLH transcription factor family and plays a crucial role in generation of OLGs and ensueing myelination in the CNS, and also a key factor for generation of spinal motoneurons (Lu et al. 2000; Zhou et al. 2000; Takebayashi et al. 2000; Copray et al. 2006). F3.Olig2 human NSCs, we have generated, express cell type specific markers for OLG progenitors (PDGFRα and NG2), and also cell type specific markers for OLG (O4, galactocerebroside and CNP). F3.Olig2 NSCs were transplanted in contused rat spinal cord lesion site and at 7 weeks post-transplantation grafted NSCs were found in the white matter and differentiated into mature OLGs. Animals with F3.Olig2 grafts showed an improvement in hindlimbs locomotion (Hwang et al. 2009a, b).

Following transplantation into the shiverer mouse brain, a mouse neurological mutant with congenital dysmyelination, an extensive myelination was demonstrated (unpublished data). We expect this successful pre-clinical study could lead to cell-based therapy in MS patients, with provision of unlimited number of human OLG/OLG progenitor cells for transplantation from this human cell line.

No treatments are currently available that slow, stop, or reverse disease progression in established MS. Currently a phase II multicenter study of autologous MSC transplantation in secondary progressive MS is ongoing and the results of clinical outcome is expected 9 in a year or two (Connick et al. 2011).

Summary of preclinical studies of stem cell transplantation in MS animal models is shown in Table 5.5.

Table 5.5 Stem cell-based cell therapy in experimental multiple sclerosis models

Reference (number)	Animal model	Transplanted cells	Special feature	Outcome
Espinosa et al. (1997)	Rat, md mutant -	OPC (rat, CG4)	Beads label	Remyelination
Yandava (1999)	Mouse, shiverer mutant	Immortalized NSC (mouse, C17-2)	None	Remyelination
Liu et al. (2002) (121)	Rat, sp cord Ethidium bromide lesion	ESC (mouse)	FGF8/SHH	Remyelination
Akiyama et al. (2002)	Rat, sp cord contusion	BMSC (rat)	None	Remyelination
Glaser et al. (2004)	Rat, md mutant -	ESC (mouse)	None	Remyelination
Nistor et al. (2005)	Rat, sp cord contusion	ESC (human)	None	Remyelination
Copray et al. (2006)	Mouse, cuprizone	NPC (mouse) 20 % OLG	Olig2 bHLH Gene transfer (transient)	Remyelination
Hwang et al. (2009a, b)	Mouse, sp cord contusion	Immortalized NSC (human, HB1.F3)	Olig2 bHLH Gene transfer	Remyelination

OPC oligodendrocyte progenitor cell, NSC neural stem cell, ESC embryonic stem cell, BMSC bone marrow mesenchymal stem cell, NPC neural precursor cell, SHH sonic hedgehog protein, OLG oligodendrocyte, bHLH basic helix loop helix transcription factor

5.7 Stroke

Stroke represents the second highest among the causes of death in East Asia including China, Japan and Korea, and third highest in US. There are two major types pf stroke and they are ischemia and intracerebral hemorrhage (ICH). Ischemic stroke caused by abrupt and near-total interruption of cerebral blood flow, produces ischemic changes in the striatum and cortex, leading to a long-term sensorimotor deficit. The major cause of ICH is hypertension and less common causes include trauma, infections, tumors, blood clotting deficiencies, and abnormalities in blood vessels such as arteriovenous malformations. Once damage from a stroke occurred, little can be done to restore premorbid functions, and although numerous neuroprotective agents have been clinically tried, no specific agents replaced the lost neurons, improved the deteriorated functions, and reduced the long-term sequelae (Marshall and Thomas 1988, 130). There are numerous previous reports of stem cell transplantation in the stroke animal models (Savitz et al. 2002, 131), and various cellular sources such as rodent bone marrow MSCs (Sinden et al. 1997; Chen et al. 2001; Zhao et al. 2002; Modo et al. 2002; Chen et al. 2003), mouse neural precursor cells (Veizovic et al. 2001), human umbilical cord blood cells (Chen et al. 2001), human bone marrow MSCs (Kim et al. 2008a, b, c; Cho et al. 2010; Ding et al. 2011), human teratocarcinoma-derived neurons (Borlongan et al. 1998; Saporta et al. 1999) and ESC- or iPSC-derived NSCs (Daadi et al. 2010; Jin et al. 2010; Kawai et al. 2010) were grafted into the ischemic rodent brain, and reduced the neurological deficits. An earlier study has reported that in human with ischemic infarct, intracerebral implantation of human teratocarcinoma NT2-derived neurons has resulted in functional improvement (Kondziolka et al. 2000).

Neural stem cells (NSCs) could be isolated from embryonic, fetal or adult CNS tissues of mammals including human. NSCs and neurons could also be derived from ESCs or iPSCs and transplanted in animal models of stroke inducing functional recovery in the animals (Daadi et al. 2010; Jin et al. 2010; Seminatore et al. 2010; Kawai et al. 2010). However, risk of tumor formation is a major obstacle to cell therapy based on human ESC- or iPSC-derived cells. Human neural progenitor cells (NPCs) were isolated from four differentiation stage of ESCs and transplanted into rats with MCA lesions, and the tumorigenesis is linked to NPCs derived from later differentiation stages of ESCs (Seminatore et al. 2010). In another study, transplantation of iPS-derived cells into rat ischemic brain resulted in tumor formation at 4 weeks pos-transplantation (Kawai et al. 2010). ESC- and iPSC-derived cells have a promising potential to provide neurons and glia for the cell therapy in stroke. However proper and strict control of tumorigenesis has to be achieved before ESC- or iPSC-based cell therapy becomes a realistic clinical strategy.

We have previously investigated whether conditionally immortalized human NSCs could selectively migrate into lesioned brain sites, differentiate into new neurons and/or glia, and improve the functional deficits in rat stroke models of focal ischemia (Chu et al. 2003, 2005) and cerebral hemorrhage (Jeong et al. 2003; Lee et al. 2007a, b, 2009a, b, 2010a, b). NSCs can circumvent blood-brain barrier and

migrate to the specific pathologic areas of brain with tropism. We introduced immortalized human NSCs intravenously via tail veins or into lesion site and NSCs migrated into the adult rat/mouse brain with transient focal cerebral ischemia or with cerebral hemorrhage. Transplanted human NSCs migrated to the lesion site, differentiated into neurons and astrocytes, and a large number of the grafted human NSCs survived in the lesion sites for up to 12 weeks. Functional improvement was observed in the transplanted animals compared with non-grafted controls on rotarod and turning-in-an-alley tests. Transplantation of NSCs overexpressing neurotrophic factors such as vascular endothelial growth factor (VEGF), glial cell line-derived neurotrophic factor (GDNF) or brain derived growth factor (BDNF) induced good survival and neuroprotection of both host neurons and grafted NSCs in the lesion site and promoted functional improvement in the ICH model animals (Lee et al. 2007a, b, 2009a, b, 2010a, b).

Bone marrow-derived mesenchymal stem cells (MSCs) have been also engrafted in animal models of stroke and found to survive and ameliorate functional deficits in the animals (rodent MSCs – Zhao et al. 2002; Chen et al. 2003, 2009; Modo et al. 2002; Guzman et al. 2008; human MSCs – Kurozumi et al. 2004; Kim et al. 2008a, b, c; Cho et al. 2010; Pendharkar et al. 2010; Ding et al. 2011), raising the possibility of therapeutic potential of MSCs for repair of damaged brain following ischemic or hemorrhagic injury. It is worthy to note that transplantation of MSCs genetically modified to express neurotrophic molecules such as erythropoietin (Cho et al. 2010), or NGF and Noggin (Ding et al. 2011) in stroke model animals induced higher number of surviving cells and improved function. In another study, transplantation of human MSCs expressing Neurogenin1 (Ngn1), a proneural gene that directs neuronal differentiation of neural progenitor cells, in the rat ischemic stroke model improved motor functions as compared with control naive MSCs. This study indicates that the neurons induced from MSCs are far better cell source for cell therapy in stroke (Kim et al. 2008a, b, c).

We have previously generated a stably immortalized human MSC cell line derived from fetal bone marrow, and following brain transplantation in ICH mice, human MSCs were found to integrate into host brain, differentiate into neurons and astrocytes, and induce functional recovery in the animals (Nagai et al. 2007 147).

Only small number of clinical trials using MSCs were performed to date and one study on long-term safety and efficacy of intravenous MSC transplantation in a large population of stroke patients (52 patients) reported that the MSC therapy improved clinical outcome in 16 patients who received auologous MSCs. In another study, autologous MSCs derived from stroke patients were expanded in human serum and delivered intravenously in 12 patients. There were no CNS tumors, abnormal cell growths or neurological deterioration following MSC infusion. Mean lesion volume as assessed by MRI was reduced by >20 % at 1 week post-cell infusion. This study provides evidence indicating the feasibility and safety of delivery of a relatively large dose of autologous MSCs into stroke patients (Honmou et al. 2011).

Summary of preclinical studies of stem cell transplantation in stroke animal models is shown in Table 5.6.

Table 5.6 Stem cell-based cell therapy in experimental stroke models

Reference (number)	Animal model	Transplanted cells	Transplantation route	Functional outcome
Zhao et al. (2002)	Rat, MCA occlusion	BMSC (rat)		Limb placement↑
				Tactile stimulation
Chen et al. (2003)	Rat, MCA occlusion	BMSC (rat)	Intravenous	Rotarod↑
				Adhesive removal
Modo et al. (2001)	Rat MCA occlusion	BMSC (rat)	–	Rotarod↑
				Adhesive removal
Veizovic et al. (2001)	Rat, MCA occlusion	Immortalized NPC (rat)		Roarod↑
				Water maze↑
Borlongan et al. (1998)	Rat, MCA occlusion	NT2 Teratoma (human)	Intracerebral	Rotarod↑
		RA treatment		Neurol score↑
Chu et al. (2003)	Rat, MCA occlusion	Immortalized NSC (human, HB1.F3)	Intravenous	Roarod↑
				Neurol score↑
Jeong et al. (2003)	Rat, ICH collagenase	Immortalized NSC (human, HB1.F3)	Intravenous	Roarod↑
				Neurol score↑
Lee et al. (2005)	Rat, ICH collagenase	Immortalized NSC (human, HB1.F3)	Intravenous	Roarod↑
				Neurol score↑
Chu et al. (2005)	Rat, MCA occlusion	Immortalized NSC (human, HB1.F3)	Intravenous	Roarod↑
				Neurol score↑
Lee et al. (2007a)	Rat, ICH collagenase	Immortalized NSC (human, HB1.F3)	Intracerebral	Roarod↑
				Neurol score↑
Kuroizumi et al. (2004)	Rat, MCA occlusion	BMSC	Intracerebral	Limb placement↑
			BDNF gene	Treadmil↑
			Transfer (Adeno)	
Nagai et al. (2007)	Rat, ICH collagenase	Immortalized BMSC (human B10)	Intracerebral	Roarod↑
				Limb placement↑
Lee et al. (2007b)	Mouse, ICH collagenase	Immortalized NSC (human, HB1.F3)	Intracerebral	Roarod↑
			VEGF gene transfer	Neurol score↑
Lee et al. (2009a)	Mouse, ICH collagenase	Immortalized NSC (human, HB1.F3)	Intracerebral GDNF gene transfer	Roarod↑
				Limb placement↑
Lee et al. (2009b)	Mouse, MCA occlusion	Immortalized NSC (human, HB1.F3)	Intracerebral Akt1 gene Transfer	Roarod↑
				Limb placement↑
Lee et al. (2010a, b)	Mouse, ICH collagenase	Immortalized NSC (human, HB1.F3)	Intracerebral BDNF gene transfer	Roarod↑
				Limb placement↑

(continued)

Table 5.6 (continued)

Reference (number)	Animal model	Transplanted cells	Transplantation route	Functional outcome
Daadi et al. (2010)	Rat, MCA occulusion	ESC-derived NSC (human)	Intracerebral	Neurol score↑
Jin et al. (2010)	Rat, MCA occulusion	ESC-derived NSC (human)	Intracerebral	Neurol score↑
Kim et al. (2008a, b, c)	Rat, MCA occulusion	MSC (human)	Intracerebral	Roarod↑
			Ngn1 gene transfer	Limb placement↑
Ding et al. (2011)	Rat, MCA occulusion	MSC (human)	Intracerebral NGF/ Noggin gene (adeno)	Neurol score↑
Cho et al. (2010)	Rat, MCA occulusion	MSC (human)	Intracerebral EPO gene transfer	Neurol score↑
Lee et al. (2011)	Human stroke patients	MSC (human Autologous)	Intravascular	Neurol score↑ (16 out of 52 patients)
Honmou et al. (2011)	Human stroke patients	MSC (human Autologous)	Intravascular	Clinical outcome not available (12 patients)

MCA middle cerebral artery, *ICH* intracerebral hemorrhage, *BMSC* bone marrow mesenchymal stem cell, *NPC* neural precursor cell, *NSC* neural stem cell, *VEGF* vascular endothelial growth factor, *GDNF* glial cell line derived neurotrophic factor, *BDNF* brain derived neurotrophic factor, *NGF* nerve growth factor, *EPO* erythropoietin

5.8 Perspectives

There are a number of issues to be clarified before adoption of stem cells for cell replacement therapy and gene therapy is widely accepted in clinical medicine such as which type of stem cells are most suitable for cell replacement therapy in patients with neurological disorders or brain injury, and safety issues related to the risk of tumorigenesis by grafted stem cells. Since neurons could be derived not only from NSCs, but also from ESCs, EGCs, bone marrow MSCs, umbilical cord blood hematopoietic stem cells and even from iPS cells generated from adult somatic cells, the most pressing question is which cells are best suited for cell replacement therapy. Since the presence of NSCs in adult CNS is known, it is only a matter of time before neurons and glial cells are cultured from adult CNS tissue samples. There are ongoing debates as to why oocytes, embryonic or fetal materials should be used to generate stem cells when stem cells could be isolated from adult tissues. However, most of research up to now indicates that embryonic or fetal stem cells are significantly more versatile and plastic than adult counterparts.

Previous studies have demonstrated that ESC- or NSC-derived neurons or glial cells could be renewable cell source in cell based therapy for patients suffering from neurological diseases including PD, HD, ALS, AD, MS, stroke and spinal cord injury, however, there exist serious caveats that limit the use of stem cell-derived neurons or glial cells for the purpose. The considerations include (i) the long-term survival and phenotype stability of stem cell-derived neurons or glial cells in the

graft following transplantation are not favorable as earlier studies have demonstrated, (ii) highly purified populations of neuronal cell type derived from ESCs or NSCs may contain other neuronal or glial cell types that might produce unpredictable interactions among grafted cells or with host neurons, and (iii) a small number of ESCs or iPSCs that escaped differentiation and selection processes might expand and form tumor in the graft site following transplantation.

Continuously dividing immortalized cell lines of human NSCs as generated by introduction of oncogenes have advantageous features for cell replacement therapy and gene therapy and the features include that human NSCs are homogeneous since they were generated from a single clone, can be expanded to large numbers in vitro, and stable expression of therapeutic genes can be achieved readily. Immortalized human NSCs have emerged as highly effective source of cells for genetic manipulation and gene transfer into the CNS ex vivo and once transplanted into damaged brain they survive well, integrate into host tissues and differentiate into both neurons and glial cells. It is known that both extrinsic and heritable intrinsic signals play important roles in generating cellular diversity in the CNS. By introducing relevant signal molecules or regulatory genes into the human stem cell line, it is now possible to obtain a large number of selected populations of neurons or glial cells from continuously growing human NSCs. Further studies are needed in order to identify the signals for proliferation, differentiation and integration of NSCs and determine favorable conditions of host brain environment for implanted NSCs to survive, prosper and restore the damaged brain.

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Chapter 6
Regenerative Therapy for Central Nervous System Trauma

Kewal K. Jain

Abstract Functional regeneration and not merely structural restoration is important in the central nervous system (CNS) following loss of tissue due to trauma. Spontaneous regeneration in the CNS is poor due to a number of reasons, mainly the presence of inhibitory factors. This chapter reviews the mechanism of this inhibition on which some of the strategies to promote regeneration in the CNS are based. These strategies are considered for application in traumatic brain injury (TBI) and spinal cord injury (SCI) separately. Degradation of inhibitors such as chondroitin sulfate proteoglycans in the glial scar at the site of SCI by application of chondroitinase ABC promotes regeneration of corticospinal tract axons in experimental animals. Inhibitors of axonal regeneration in myelin include Nogo, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein. These can be blocked with antibodies or peptides to facilitate regeneration after SCI. Apart from acute TBI, chronic traumatic encephalopathy is being increasingly recognized as a cause of cognitive impairment and strategies for regeneration are similar to those for neurodegenerative disorders. Cell and gene therapies are under investigation for CNS regeneration. Developments in nanobiotechnology also show potential for CNS repair. However, experimental work in CNS regeneration has not yet been translated into clinical use. Combination of approaches, including stem cell transplantation with nanoscaffolds, supplemented with pharmacological enhancement of regeneration, hyperbaric oxygen, electrical fields, and physical therapies are promising for functional regeneration of the CNS following trauma.

Keywords Central nervous system (CNS) regeneration • CNS trauma • CNS repair • Neuroregeneration • Regenerative medicine • Spinal cord injury (SCI) • Traumatic brain injury (TBI)

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6.1 Introduction

The term "regeneration" is used to describe the sum total of activities leading to re-growth of cells and tissues of the body. It includes both anatomical and physiological structures; however, structural regeneration does not necessarily lead to restoration of function. The term "functional regeneration" implies recovery of the function that can occur without regeneration by compensatory mechanisms. Functional regeneration is important for recovery of the central nervous system (CNS) consisting of the brain and the spinal cord, following damage or loss of cells and tissues resulting from traumatic brain injury (TBI) and spinal cord injury (SCI).

6.2 Historical Background

Although regeneration was known to occurs to a variable degree in most body tissues, neural tissues (excepting peripheral nerves) were considered to be non-regenerative, an idea that was recognized as early as 1550 BC and well documented during the nineteenth century (Mitchell 1872). In the earlier part of the twentieth century, Ramon y Cajal reached the following conclusion in his monumental work on degeneration and regeneration of the nervous system:

Once the development has ended, the founts of growth and regeneration of the axons and dendrites dry up irrevocably. In adult centers the nerve paths are something fixed, ended, immutable (Ramon y Cajal 1959).

The view of axonal regeneration in the CNS as abortive or poor remained widely accepted for several decades. Evidence started to emerge during the last quarter of the twentieth century that, under certain circumstances, regeneration could occur successfully in the mammalian CNS. Discoveries in neurobiology have provided an insight into possible ways in which regeneration in the CNS may be encouraged.

Hughling Jackson, in his book "Principal of Compensation", explained the functional recovery that occurs following damage to the CNS, which was based on his theory of cerebral localization (York and Steinberg 1994). Functional recovery is related to plasticity of the CNS, i.e. its ability to adapt its structural organization, both anatomically and functionally, to new situations emerging during its maturation, in addition to those resulting from injuries. The concept of neuroplasticity was developed during the early part of the twentieth century (Goldstein 1931). Investigators in this field had already recognized the plasticity exhibited by brain microglia during development and under pathological conditions (del Rio-Hortega 1932). A further advance is the concept of reactive synaptogenesis, whereby the neighboring neurons make new synaptic contacts to replace those lost and play a major role in the restoration of function following brain damage (Cotman and Scheff 1979).

Some of the basic concepts of regeneration and repair taking place after CNS injury have led to strategies for treatment and rehabilitation of patients with brain

damage. Initial attempts to use neural grafts to repair the damage in experimental animals took place more than a century ago (Thompson 1890). During the last quarter of the twentieth century, neural grafting techniques were refined and investigated for TBI and SCI.

6.3 Basics of CNS Regeneration

Primary sensory neurons with cell bodies in the dorsal root ganglia have 2 branches: (1) a peripheral axon that regenerates itself if injured; and (2) a central axon that enters the CNS and does not regenerate after injury. The local environment of these branches explains the difference in regeneration: The peripheral axon contains Schwann cells, whereas the central axon contains oligodendrocytes and astrocytes. In the peripheral nervous system, myelin debris is cleared promptly, and Schwann cells dedifferentiate and down-regulate expression of the myelin protein, thus facilitating regeneration. The composition and organization of extracellular matrix in CNS lesions is a hindrance to regeneration. However, regeneration of CNS axons has been demonstrated in vivo after implantation of peripheral nervous tissue. Furthermore, regeneration of dorsal column fibers has been demonstrated beyond the lesion site in adult spinal cord injury by a preconditioning peripheral nerve lesion 1 to 2 weeks before the spinal cord lesion (Neumann and Woolf 2000).

6.3.1 Factors That Influence Regeneration in the Central Nervous System

Intrinsic factors that influence regeneration in the CNS. Various intrinsic factors that modulate regeneration in the CNS are listed in Table 6.1 and described in the following text. Neurotrophic factors are the most important of all the factors influencing regeneration.

6.3.2 Causes of Lack of Regeneration in the CNS

The CNS regenerative process is unsuccessful for three reasons: (1) neurons are highly susceptible to death after CNS injury; (2) multiple inhibitory factors in the CNS environments hinder regeneration; and (3) the intrinsic growth capacity of postmitotic neurons is constitutively reduced. Research is providing an insight into these areas and will form the basis of strategies to promote regeneration of the CNS.

Table 6.1 Factors that influence regeneration in the central nervous system

Factors	Role in regeneration and recovery
Neurotrophic factors	Cell survival, axon growth-cone stimulation, synapse regeneration.
Neuroprotective gene expression	Expression of genes such as Bcl-2 and c-fos/jun may occur within minutes of an acute brain injury and are a determinant of eventual recovery.
Neural stem cells	Neural stem cells can migrate to the site of injury in the brain and participate in regeneration.
Cadherins	These are involved in synaptogenesis in the CNS.
Intracellular levels of cyclic nucleotide in the neurons	These influence the capacity of mature CNS neurons to initiate and maintain a regrowth response.
Innate immune system, represented by activated macrophages	This can facilitate the processes of regeneration in the severed spinal cord.
Inducible nitric oxide synthase	This is not usually present in the brain but can be detected in the brain following injury and may be required for adequate repair.
Activin	Strong expression of activin is seen in repair processes of the brain and may have a role in neuroprotection. Although a transient overexpression of activin after tissue injury might be beneficial for the repair process, sustained expression of activin could be detrimental to regeneration.

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6.3.2.1 Factors Inhibiting Regeneration in the CNS

Glial scar. A glial scar containing extracellular matrix molecules including chondroitin sulfate proteoglycans develops at the site of injury and prevents regeneration.

Neurite outgrowth inhibitors. Various growth inhibitors are found in a glial scar. These include the following:

- Myelin-associated inhibitors of axonal regeneration.
- Astrocytes produce tenascin, brevican, and neurocan.
- Meningeal cells produce NG2 and other proteoglycans.
- Activated microglia produce free radicals, nitric oxide, and arachidonic acid derivatives.

Three inhibitors of axonal regeneration have been identified in myelin: Nogo, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein. Postmortem histopathological studies on SCI patients have shown that NG2 and phosphacan are both present in the evolving astroglial scar and, therefore, might have played an important role in the blockade of successful CNS regeneration (Buss et al. 2009). All of these proteins induce growth cone collapse and inhibit neurite outgrowth. Three of the four known myelin inhibitors, Nogo66, Myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp), although very different structurally, interact with the same receptor, NgR. They exert their

inhibitory effects by binding the NgR receptor that transduces the inhibitory signal to the cell interior via transmembrane co-receptors LINGO-1 and p75(NTR) or TROY. Although the receptor(s) for amino-Nogo-A are unknown, amino-Nogo-A and NgR ligands mutually activate the small GTPase RhoA (Walmsley and Mir 2007). However, the absence of NgR alone has no effect on inhibition of neurite outgrowth in culture, and a second receptor, PirB, was described for these myelin inhibitors (Filbin 2008).

In vitro neurite outgrowth studies have demonstrated that a significant part of myelin inhibition is mediated by Ephrin-B3, which remarkably equals to the inhibitory activity of Nogo, MAG and OMgp combined. Loss of EphA4, a receptor, leads to improved axon regeneration and functional recovery. Several other guidance cues are expressed by oligodendrocytes. Sema4D, a transmembrane class 4 Semaphorin, is transiently upregulated in oligodendrocytes that surround the lesion site after adult CNS injury and may inhibit CNS axonal regeneration. Another member of the Semaphorin family, Sema5A, has also been shown to be expressed by oligodendrocytesand induces growth cone collapse and inhibits neurite growth.

Humoral autoantibodies. Autoimmune responses directed against the CNS are generally considered pathogenic in nature, but autoreactive antibodies can also enhance endogenous myelin repair.

6.3.2.2 Role of Glial Cells in CNS Injury and Regeneration

Glial cells consist of microglia, which have a phagocytic function, and macroglia (astrocytes and oligodendrocytes). Astrocytes provide structural, trophic, and metabolic support to neurons and modulate synaptic activity. Therefore, impairment of astrocyte functions in TBI can compromise neuron survival. Functions of astrocytes that are known to influence neuronal survival include glutamate metabolism, free-radical scavenging, and the production of cytokines and nitric oxide. Neuron regeneration after TGI is influenced by the release of neurotrophic factors by astrocytes. Therapeutic approaches to TBI should be aimed at restoring the functions of both neurons and glial cells. Glial cells may also contribute to scar formation.

A study has shown how astroglia can be directly converted into the two main classes of cortical neurons, excitatory as well as inhibitory, by the selective transduction of transcription factors, specific proteins that regulate the transcription of DNA (Heinrich et al. 2010). This approach may provide new therapies for neurodegenerative diseases.

6.3.2.3 Role of Neurotrophic Factors in Neuronal Regeneration

The role of neurotrophic factors during neuronal regeneration differs little from their role during neuronal development in the expression of cytoskeletal genes or cellular protein synthesis, suggesting that regulatory events during regeneration recapitulate the patterns found during development.

Nerve growth factor. Within the central nervous system, the main neuronal system regulated by nerve growth factor is that of basal forebrain cholinergic neurons, which send topographically organized projections to the hippocampus and cerebral neocortex. Regeneration in the adult mammalian central nervous system has been viewed pessimistically in the past. Rapid progress of concepts and tools in developmental biology has been applied to approach the questions of regeneration. Important aims are cell survival, re-initiation of axon growth, target finding, and formation of functional connections. Significant recent developments include the availability of recombinant neurotrophic factors and stem cells for repair of the nervous system.

Neurotrophic factors and synapse regeneration. Synapses are the final common pathway for information exchange in the nervous system. They mediate a wide range of activities from a simple reflex arc to learning and memory. Synapse formation plays an important role in neuronal regeneration and survival. The presynaptic and postsynaptic parts of the synapse are separated by a synaptic cleft. At the neuromuscular junction, a specialized extracellular matrix known as the synaptic basal lamina occupies this cleft. Neurotransmitters such as acetylcholine or glutamate are released from the presynaptic vesicles, traverse the cleft, and bind to their receptors on the postsynaptic membrane. The signal is terminated by the reuptake or enzymatic destruction of the neurotransmitters. Synapses are formed by the expression of specific gene products such as synaptic vesicle proteins and neurotransmitter receptors. This process is regulated to some extent by a combination of neurotrophic factors and electrical activity.

6.3.2.4 Cadherins

These are found in the synaptic cleft near the transmitter release zone and may provide a molecular basis for the adhesive interactions between opposing synaptic membranes. Thus, they play a role in the formation and maintenance of synapses. Cadherins might directly regulate cell signaling to modulate synaptic connectivity.

Pan-cadherin is a good biomarker for neuronal recovery after cortical injury. Immunohistochemical staining of the injured cortex for pan-cadherin revealed a significant increase in staining in experimental animals treated with topical application of NEP 1-4, an inhibitory peptide that neutralizes Nogo-A, and preserves neuronal structures (Atalay et al. 2008).

6.3.2.5 Various Factors That Influence Plasticity in the CNS

The adult cortex undergoes plastic changes that are dependent on neuronal activity. Plasticity in the CNS following injury is influenced by several factors:

- The brain possesses a certain degree of biological plasticity that diminishes with age.

- Plasticity can be limited by a progressive neurodegenerative disease or severe damage to the brain.
- There is less room for plasticity in spinal cord lesions than in the cerebral hemispheres.
- Secondary damage that results from traumatic lesions of the CNS may reduce the role of plasticity in recovery.
- Neurotrophins and their receptors play a role in this plasticity.

Rapid and opposing effects of brain-derived neurotrophic factor and nerve growth factor on the functional organization of the adult cortex in the rat indicate that neurotrophins can modulate stimulus-dependent activity in the adult cortex. Such studies also suggest a role for neurotrophins in regulating adult cortical plasticity. Current knowledge enables some manipulation of plasticity and the induction of functional changes beneficial for vision.

GAP-43 plays an important role in axonal plasticity by guiding growth cones rather than supporting axonal elongation. The molecule GAP-43 is key to initiating axon growth, whereas other genes are necessary to develop a full regenerative program. Addition of GAP-43 gene can induce the formation of branched plexuses typical of sprouting growth.

6.4 Approaches to Regeneration of the CNS

Several technologies are being used to facilitate regeneration and repair of the CNS. A classification of these technologies is shown in Table 6.2.

Table 6.2 A classification of approaches to regeneration of the CNS

Inhibiting the factors that impede regeneration in the CNS
Reducing or eliminating scar formation
Providing cues to axons for regeneration
Repair of the injured nerve fibers
Cell therapy for promoting growth of neural tissues to replace the loss
Hyperbaric oxygen for neuroprotection and mobilization of intrinsic stem cells
Electrical fields (EF) for regeneration of the CNS
Pharmacological agents to promote growth of neural tissues
Restoration of neurotransmission
Struts for tissue engineering: self-degrading biomaterials, nanomaterials
Combinations of strategies

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6.4.1 *Inhibiting the Factors That Impede Regeneration in the CNS*

Various factors that inhibit regeneration following injury to the CNS have been identified in the earlier sections. In order to overcome the inhibitory environment of the glial scar, treatments should enhance the ability of neurons to elongate and manipulate the extrinsic inhibitors that block growth in the immediate environment of the glial scar. This combined approach may induce functional regeneration after CNS injury. Various strategies for blocking the inhibitory factors in SCI are described later in this chapter.

Humoral antibodies that promote remyelination bind to antigens on the surfaces of oligodendrocytes, suggesting that these antibodies might function through direct stimulation of the myelin-producing cells. An understanding of these mechanisms should open up significant new areas for the development of antibody-based therapeutics and perhaps also for small-molecule-based therapeutics and vaccines for induction of the reparative response.

6.4.2 *Guiding Axons in the CNS*

Most of the currently used methods of axonal regeneration approaches limit themselves to observe how axons elongate and migrate in response to signaling molecules presented on the substrate materials, or more recently, in response to different chemical and mechanical substrate properties. Many of these studies are encouraging in the hope of regenerating axons after disease or injury; however, numerous barriers remain. There is a need to optimize a permissive heterogeneous environment for axon elongation using tissue engineering approaches and a thorough understanding of the mechanical properties of the substrate, mechanotaxis, and both attractive and repulsive signaling mechanisms (Norman et al. 2009).

6.4.3 *Combination of Neuroprotection and Neuroregeneration*

It is generally believed that neuroprotection is required in the acute phase, and neuroregeneration is the long-term goal for restoring function following CNS injury, stroke, or neurodegenerative diseases. No clear-cut line of demarcation exists between neuroprotection and neuroregeneration. The measures for achieving these overlap in accordance with the generally accepted concept that rehabilitation should start in the acute phase of neurologic disease. No acute phase is identifiable in some chronic neurologic disorders. For example, chronic traumatic encephalopathy may be the cumulative result of repeated cerebral concussions in athletes or soldiers and

may have an insidious onset. Neuroregeneration efforts may not be successful without combination with neuroprotective strategies to counteract the progressive deterioration of neurologic function (Jain 2011). Cell transplants and gene therapy as methods for regeneration of the CNS may not be practical for application in the acute phase of injury, but both can have a long-term neuroprotective function.

6.4.4 Role of Enriched Environments

Considerable published evidence shows that living in an enriched environment alters dendrites and synapses in the brains of adult rodents. A study on adult primates shows that the brain remains highly sensitive to experiential complexity, and living in standard laboratory housing may induce reversible dendritic spine and synapse decreases in brain regions important for cognition (Kozorovitskiy et al. 2005). Currently, enriched environment is considered to be the single most efficient plasticity and regeneration promoting paradigm (Nilsson and Pekny 2007). This has important implications for neurorehabilitation

6.4.5 Role of Hyperbaric Oxygen in CNS Regeneration

Hyperbaric oxygen (HBO) therapy is therapeutic use of oxygen under greater than atmospheric pressure at sea level. Rationale for the neuroprotective effect of HBO in TBI is that it relieves hypoxia, improves the microcirculation, and relieves cerebral edema (Jain 2016). HBO also mobilizes instrinsic stem cells and can contribute to regeneration following TBI.

6.4.6 Role of Electrical Fields in CNS Regeneration

Electrical fields (EFs) have been generated over the brain and the spinal cord for diagnostic and therapeutic purposes. Cranial electrotherapy or transcranial direct current stimulation (tDCS) and repetitive transcranial magnetic stimulation (rTMS), i.e. noninvasive stimulation of the cerebral cortex using externally applied magnetic fields, are used for treatment of neurological disorders. Spinal cord and deep brain stimulation are recognized procedures for treatment of pain and movement disorders by using temporary or implanted electrodes.

Exogenous application of EFs to cultured neurons to supplement endogenous results in enhanced sprouting of neurites and directed growth along the fields. This is the basis for suggesting the use of EFs in a regenerative therapeutic setting (Haan and Song 2014). The mechanisms of effect of EF are not well understood. However,

the likely effect is the stimulation of release and/or production of various neurotrophic factors with AC stimulation, providing a nonspecific supportive environment for the regeneration of nervous cells. In contrast, DC is able to provide directional attractive cues for regeneration, altering gene expression, and accelerating reinnervation. Axonal regeneration and improved quality of life may be achieved in SCI by using EF stimulation. A phase I clinical trial of human oscillating field stimulator (OFS) in patients with SCI showed that it is safe, reliable, and easy to use (Shapiro et al. 2005). The stimulation provided significant improvement in sensation and improved motor scores in some cases compared to historical data for untreated patients. A physiological level of electric stimulation can be used to control directional recruitment of neural stem cells in a spinal cord slice culture model (Feng et al. 2012). This suggests potential for clinical use of EFs to optimize stem cell grafting in vivo for CNS injuries.

rTMS and tDCS show promise for repairing injured neural circuits. rTMS presents a unique opportunity to modulate brain excitability and plasticity in a precisely controlled manner, but there is need for determining how rTMS can be applied following neurotrauma to promote regeneration and rehabilitation of neural circuits (Rodger and Sherrard 2015).

6.4.7 Biomaterials for CNS Regeneration

Biomaterials can facilitate regeneration of the CNS as part of devices for targeted delivery of drugs or therapeutic proteins to the brain, as scaffolds for cell or tissue transplants, and to facilitate repair damaged neuronal pathways (Orive et al. 2009). Polyethylene glycol (PEG) and nanotechnology are providing the important methods for this regeneration and repair of the CNS.

6.4.7.1 Nanobiotechnology for Regeneration and Repair of the CNS

Nanotechnology is the creation and utilization of materials, devices, and systems through the control of matter on the nanometer (one billionth of a meter) scale. Nanobiotechnology is the application of nanotechnology in biotechnology leading to the development of nanomedicine (Jain 2012). Various nanomaterials have been designed to self-assemble into nanofibers and provide the framework for regeneration of nerve fibers in experimental studies on animal models of SCI. This enables greater control over material-cell interactions, which induce specific developmental processes and cellular responses, including differentiation, migration and outgrowth. In a nanofiber network, progenitor cells develop into neurons rather than astrocytes thus hindering the formation of scar tissue that hinders regeneration.

6.4.8 Biological Therapies for CNS Regeneration

Various biological therapies for CNS regeneration are listed in Table 6.3. These are described in detail in a special report on cell therapy (Jain 2015a). Some will be briefly described in the following text. Cell and gene therapies will be described separately along with regeneration of SCI and TBI.

6.4.8.1 Cells Therapy for Regenerating CNS

Cell therapy for CNS disorders involves the use of cells of neural or non-neural origin to replace, repair, or enhance the function of the damaged nervous system and is usually achieved by transplantation of the cells, which are isolated and may be modified, e.g. by genetic engineering, when it may be referred to as gene therapy.

The olfactory ensheathing cells play an important role in CNS regeneration. In clinical trials, olfactory ensheathing cells have produced some of the most promising results including a functional recovery in humans following CNS injury (King-Robson 2011).

Glial support cells play an important role in the CNS and attempts have been made to transplant astrocytes, the major support cells in the CNS system by generating them from embryonic human glial precursor cells. There are differences in effects depending on how the astrocytes are generated. One study has provided a specific population of human astrocytes that appears to be particularly suitable for further development towards clinical application in treating the traumatically injured or diseased human CNS (Davies et al. 2011).

Table 6.3 Biological therapies for CNS regeneration

Biological therapies
Cells therapy using non-neural somatic cells
Stem cell therapy
Embryonic stem cells (ESCs)
Adult stem cells: hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs)
Induced pluripotent stem cells (iPSCs)
Neural stem cells (NSCs)
Retinal progenitor cells (RPCs)
Gene therapy
Vector-mediated gene transfer for delivery of neurotrophic factors
Transplantation of genetically modified cells to release neurotrophic factors
Vaccines
Vaccine against inhibitors of neurite outgrowth after spinal cord injury
Immunotherapy to inhibit inflammatory response to spinal cord injury

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Neural stem cells (NSCs) migrate through the parenchyma along various routes in a precise, directed manner across great distances to injury sites in the CNS, where they might engage niches harboring local transiently-expressed reparative signals. Activation of endogenous NSCs is being considered along with stem cell transplantation for regeneration of the injured spinal cord.

Transplanted cells can be tracked in the CNS by using special labels and MRI. Labeling of human neural stem cells grown as neurospheres with magnetic nanoparticles was shown to not adversely affect survival, migration, and differentiation or alter neuronal electrophysiological characteristics (Guzman et al. 2007). Noninvasive cellular imaging has great potential for neurotransplantation as it enables real-time tracking of grafted cells as well as monitoring biodistribution and development (Walczak and Bulte 2007).

Cell therapy involving stem cell transplantation into the CNS to replace damaged tissue has been limited by low cell survival and integration upon transplantation. Although autologous stem cell transplants are well tolerated, rejection is the main adverse event with allogeneic cell transplants, particularly adult hematopoietic stem cells (HSCs). Implanted embryonic stem cells may be tumorigenic. Development of a donor stem cell-derived glioneural brain tumor has been reported in a patient affected by the ataxia telangiectasia 4 years following repeated transplantations of fetal neural stem cells (Amariglio et al. 2009). Molecular and cytogenetic studies showed that the tumor was of non-host origin, suggesting it was derived from the transplanted cells. There is difference in tolerance according to the type of stem cells used. Both adult HSCs and mesenchymal cells (MSCs) are used but MSCs are more relevant to regenerative therapy. Moreover, allogeneic MSCs are well tolerated. MSCs of bone marrow origin are considered as an alternative to HSC transplantation as they not only provide the supportive microenvironmental niche for HSCs but are capable of differentiating into various cell types. Ex vivo expansion of cells is necessary as large amounts are required, particularly for applications in regenerative medicine. Clinical use of expanded MSCs is accepted. MSCs can be used for delivery of drugs or nucleic acids to the brain across the BBB. Use of MSCs in some neurological disorders is currently in clinical trials. MSCs, unlike ESCs, do not form tumors. MSCs show pathotropism by migrating to sites of tissue insult. Due to the ability of MSCs to be transplanted across allogeneic barrier, drug-engineered MSCs can be available as off-the-shelf cells for rapid transplantation (Aleynik et al. 2014).

6.4.8.2 Gene Therapy Approaches for Repair of CNS Injuries

Gene therapy has the potential to overcome many of the difficulties associated with the delivery of anti-scarring and neurotrophic substances to the site of an injury. Suitable and safe vectors for the delivery of genes need to be developed. Although there are several obstacles to making gene therapy practical and effective in humans, it has the potential to provide a new approach to the treatment of TBI (Shen et al. 2007).

6.4.8.3 Vaccines for Neuroregeneration

Inability of neurons and axons to regenerate following injury to the nervous system is due mostly to the presence of myelin and oligodendrocyte-related inhibitors of neurite outgrowth. A vaccine-based approach can be used to circumvent this issue and promote axonal regeneration and repair following traumatic injury (Ang et al. 2006). A vaccine against Nogo-66 (NgR), the common receptor for three myelin-associated inhibitors (Nogo-A, myelin-associated and oligodendrocyte myelin glycoprotein), has been shown to significantly improve functional recovery in rats subjected to spinal cord hemisection (Yu et al. 2008). In a rat model of spinal cord injury, combination therapy of NgR vaccine and NSC transplantation exhibited significant advantages over single therapy with vaccine or stem cells (Xu et al. 2011).

Specific signaling molecules, such as some cytokines are involved in the inflammatory response following spinal cord injury. Therefore, antiinflammatory immunotherapy may be useful following acute spinal cord injury (Wang et al. 2015).

6.4.9 Pharmaceuticals to Facilitate Regeneration of the CNS

Several Drugs are being investigated or in development to enhance regeneration and repair of CNS injuries (Ibarra and Martiñón 2009). A classification of these is shown in Table 6.4.

6.4.9.1 Assessment of Potential of Drugs for Regenerating of CNS

Role of nitric oxide (NO)-cyclic guanosine-monophosphate (cGMP) transduction pathway in regulating axonal growth and neural migration has been demonstrated in an invertebrate locust embryo model, which lacks the growth-inhibiting factors found in the CNS of higher vertebrates (Stern and Bicker 2008). Application of exogenous NO or cGMP promotes axonal regeneration, whereas scavenging NO or inhibition of soluble guanylyl cyclase delays regeneration, an effect that can be rescued by application of external cGMP. This embryo-culture system is a useful tool for studying effect of various drugs on CNS regeneration.

A model system of mouse entorhino-hippocampal slice cultures has been used to assess the potential of pharmacological treatments with compounds targeting signal transduction pathways to promote growth of entorhinal fibers after mechanical lesions across the lesion site to their target region in the dentate gyrus (Bonnici and Kapfhammer 2009). This method can be used for selection of promising compounds for further development to promote regeneration of CNS.

Table 6.4 Pharmaceutical approaches to facilitate regeneration of the CNS

Agents that counteract the action of factors inhibiting regeneration following trauma
Anti-Nogo-A antibodies
Rho-ROCK inhibitors
Glial scar inhibitors
Local application of chondroitinase at the site of injury
Modulation of the proteoglycan receptor PTPσ
Axon guidance molecules
Pharmacological modulation of the signal transduction pathways
Cyclic AMP-enhancers
Inhibitors of the phosphoinositide 3-kinase pathway
Inhibitors of inositol triphosphate receptor
Nitric oxide-cyclic guanosine-monophosphate transduction pathway
Agents that promote regeneration
Bone morphogenetic protein 7
Immunophilin ligands
Neurotrophic factors
Retinoic acid
Agents that improve remyelination
Fampridine
Monoclonal antibodies
Drugs that mobilize intrinsic stem cells
Drug combinations with devices and biological therapies

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6.4.9.2 Delivery of Therapeutics to the CNS for Regeneration

Although several pharmaceutical regeneration strategies have shown promising results in experimental animal models, they have been difficult to translate into clinical use. Delivery of therapeutic molecules to the CNS using conventional methods, such as oral and intravenous administration, have been limited by diffusion across the blood-brain/spinal cord-barrier. Drug delivery to the central nervous system is challenging and is discussed in detail in other publications (Jain 2010; Jain 2015b). Some of the important issues relevant to therapeutic delivery for regeneration in CNS trauma include the following:

- Increased permeability of the blood-brain barrier (BBB) in TBI is not consistent or reliable and various strategies to enable systemically administered drugs to cross the BBB in a controlled manner need to be used.
- Various strategies are used, including nanobiotechnology, to improve the brain penetration of drugs for promoting regeneration after TBI.

- Cell and gene therapies may be introduced directly into the site of injury or by indirect minimally invasive introduction into the cerebrospinal fluid (CSF) pathways.
- Hydrogels, particularly injectable hydrogels, are important for controlled local delivery of cells and drugs for CNS regeneration (Tam et al. 2014).
- Devices used for delivery of therapeutics to the CNS should be safe and biocompatible if implanted.

6.4.9.3 Pharmaceutical Manipulation of Stem Cells

Pharmaceutical manipulation of stem cells has the following aims: (1) to increase the number of multipotential cells; (2) to enhance the survival of implanted cells; (3) to influence the fate of specific endogenous multipotential cell populations; and (4) to influence the differentiation of stem cells.. Several biological and small molecules can enhance the in vivo and ex vivo regenerative properties of stem cells. Mozobil™ (Genzyme), a small molecule drug, increases the number of stem cells in the circulating blood in human volunteers and enhances the effect of granulocyte colony stimulating factor. Lithium, a standard drug for manic depression with unknown mode of action, stimulates stem cells growing in culture to multiply faster, indicating that it could prompt stem cells in the brain to produce new cells to replace those that are damaged. The most important of the molecules for enhancing stem cells are neurotrophic factors.

6.4.9.4 Bone Morphogenetic Protein 7

Bone morphogenetic proteins (BMPs) are multifunctional growth factors that belong to the transforming growth factor-beta superfamily. BMPs regulate several crucial aspects of embryonic development and organogenesis. BMP-2/4 inhibits axonal regeneration and limits functional recovery following injury to the CNS, but BMP-2 is involved in the regeneration of peripheral nerves, and might function as a potential neurotrophic factor. BMP7, currently used in patients to treat non-neurological diseases, can induce neuroregeneration (Bani-Yaghoub et al. 2008). BMP may provide axon guidance cues, which may be used to force axons to grow in planned directions or patterns (Yaron and Zheng 2007).

6.4.9.5 Neurotrophic Factors

Neurotrophic factors (NTFs) are a class of naturally occurring protein growth factors that have multiple effects on the nervous system and potential therapeutic applications in neurological disorders. NTFs can be used for pharmacological control of endogenous neural stem cells (NSCs) to enhance brain repair and to improve integration of transplanted cells in the brain.

The most clinically advanced of NTFs, Glial Growth Factor 2 (GGF2), is a member of the neuregulin family of growth factors related to epidermal growth factor. The neuregulins bind to erbB receptors, which translate the growth factor signal to the cell and cause changes in cell growth, protein production and gene expression. The molecule was shown in published studies to stimulate remyelination in preclinical models of multiple sclerosis and to have a range of other effects in neural protection and repair. The neuroprotection and repair properties of neuregulins have led to promising results in a range of models of CNS injury. Research and development of neuregulin as a potential therapy in the treatment of TBI is in progress.

6.4.9.6 Retinoic Acid

Retinoic acid (RA) is involved in the induction of neural differentiation, motor axon outgrowth and neural patterning. Like other developmental molecules, RA continues to play a role after development has been completed. Elevated RA signaling in the adult triggers axon outgrowth with resulting nerve regeneration. RA is also involved in the maintenance of the differentiated state of adult neurons, and disruption of RA signaling in the adult leads to the degeneration of motor neurons. RA could be used as a therapeutic molecule for the induction of axon regeneration (Maden 2007).

6.4.9.7 PPAR Agonists

Injury to the nervous system activates immune cells, which infiltrate resulting in death of neurons and glia, mitochondrial dysfunction, and the secretion of substrates that inhibit axon regeneration. Some of these interrelated pathologic mechanisms can be managed by use of PPARs (peroxisome proliferator-activated receptors) agonists, a group of ligand-activated transcription factors (Mandrekar-Colucci et al. 2013).

6.4.9.8 Concluding Remarks on Pharmaceuticals for CNS Regeneration After Trauma

Clinical trials of most of the pharmaceutical agents so far have failed. Most of these aimed at showing neuroprotective effect in CNS injury. Drugs under early clinical trials include cyclosporin A, glibenclamide, minocycline, nerve growth factor, propranolol, statins, tranexamic acid, and valproic acid (Xiong et al. 2). Investigational drugs in preclinical development include thymosin β4 peptide, IL-1 receptor antagonist, and recombinant human tissue plasminogen activator.

6.5 Clinical Aspects of CNS Regeneration

6.5.1 Assessment of Regeneration and Plasticity

Various neurophysiological methods, clinical neuropsychological assessments, and brain imaging studies can be used for assessing regeneration, recovery, and plasticity in the CNS.

Neurophysiological techniques. These techniques are useful for evaluating spontaneous recovery from damage and the therapeutic benefits of training, as well as other therapies. Transcranial magnetic stimulation has been used for this purpose.

Brain imaging techniques. Positron emission tomography and functional magnetic resonance imaging can be used to monitor the recovery and plasticity of the brain following injury. PET has been used to demonstrate changes in the activation of cortical and subcortical brain areas in response to altered spinothalamic and spinocerebellar input in paraplegic patients. These techniques have also been used to map clinically relevant plasticity after a stroke.

6.5.2 Management of Spinal Cord Injury

Significant spontaneous functional recovery may occur over several years following incomplete SCI. Possible mechanisms involved are synaptic plasticity in pre-existing pathways and the formation of new circuits through collateral sprouting of lesioned and unlesioned fibers. Some evidence shows that plasticity can be facilitated by activity or experimental manipulations. These studies form a basis for the development of new rehabilitation approaches for SCI.

Several neuroregenerative approaches that are being pursued for acute SCI with complete functional transection are shown in Table 6.5.

6.5.2.1 Antagonism of Inhibitors of Regeneration Following SCI

Several inhibitors of regeneration of spinal cord following SCI have been identified and strategies to counteract most of these factors have been developed. Chondroitin sulfate proteoglycans (CSPGs) are a major class of axon growth inhibitors that are up-regulated after SCI and contribute to regenerative failure. Degradation of CSPGs after SCI by application of chondroitinase at the site of injury may promote regeneration of corticospinal tract axons. This approach has been effective in animal models of SCI and has potential for the treatment of human SCI. Therapeutic strategies aimed at inhibition of collagen matrix formation in brain and spinal cord lesions promote axonal regeneration and functional recovery (Klapka and Muller 2006).

Table 6.5 Strategies for neuroregenerative in acute SCI

Strategy	Rationale
Neuroprotection	To counteract the progression of damage following initial injury in order to facilitate regeneration.
Careful modulation of the inflammatory response following trauma	Usually beneficial to recovery of function but may be detrimental in some situations.
Antagonists of inhibitors of regeneration	To facilitate regeneration (see text for details)
Restoration of neurotransmission	Based on the concept that the dysfunctions that occur after SCI are primarily due to damage to neurotransmission (Xu and Onifer 2009).
Cell transplantation	Replacement of damaged tissue.
Neurotrophic factors	Enhance axonal plasticity and regeneration after SCI (Hollis and Tuszynski 2011).
Peripheral nerve transplantation	Bridging the disrupted segment of spinal cord to provide a pathway for regenerating nerve fibers.
Nanofiber struts with stem cells	Scaffolds to promote regeneration.
Synthetic/biodegradable gel/polymer implants	To serve as substrates for neurite outgrowth and synapse formation.
Transfer of acidic fibroblast growth factor (aFGF) gene with adeno-associated virus	aFGF was shown to improve functional recovery in spinal cord-contused rats (Huang et al 2011)
Vaccines	Act against inhibitors of neurite outgrowth
Anti-Nogo-A antibodies for neutralizing the inhibitory effect of Nogo-A	These antibodies have been shown to enhance fiber growth, regeneration, and functional recovery in primate models of SCI (Buchli et al 2007).
Peptide mimic of protein tyrosine phosphatase σ (PTPσ) that binds to PTPσ receptor	Relieves chondroitin sulfate proteoglycans (CSPGs)-mediated inhibition of regeneration within the glial scar (Lang et al 2015).
Forced upregulation of mTOR (mammalian target of rapamycin) activity by deletion of PTEN (phosphatase and tensin homolog), a negative regulator of mTOR to enable successful regeneration of corticospinal axons past a spinal cord lesion.	An experimental study showed that the regrowth potential of corticospinal tract axons was lost in the fully grown mouse and this was accompanied by a downregulation of mTOR activity in corticospinal neurons, which further diminished following axonal injury (Liu et al. 2010).

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Chondroitin sulfate proteoglycans (CSPGs) are a major class of axon growth inhibitors that are up-regulated after spinal cord injury (SCI) and contribute to regenerative failure. Chondroitinase ABC (chABC) digests glycosaminoglycan chains on CSPGs and can counteract CSPG-mediated inhibition, but it loses its enzymatic activity rapidly at 37 °C, requiring the use of repeated injections or local infusions for prolonged periods. A thermostabilized chABC and a system for its sustained local delivery have been developed to overcome this problem (Lee et al. 2010).

Animals treated with sustained delivery of thermostabilized chABC in combination with neurotrophin-3 showed enhanced growth sensory axons and sprouting of serotonergic fibers.

Knowledge of axon regeneration inhibitors provides new opportunities for therapeutic development of counteracting these inhibitors for spinal SCI (Xie and Zheng 2008). Neurite outgrowth inhibitors and their receptors can be blocked with antibodies or peptides to facilitate regeneration. Some of the specific antagonists are commercially available for experimental investigations. Administration of an inhibitor of Sema3A, a guidance molecule that is expressed by the fibroblast component of the scar tissue, and leads to multiple beneficial effects including enhanced regenerative response from axons (Kaneko et al. 2006).

Rho-kinase (ROCK) is a serine/threonine kinase and one of the major downstream effectors of the small GTPase Rho. The Rho-ROCK pathway is involved in many aspects of neuronal functions including neurite outgrowth and retraction. The Rho-ROCK pathway is an attractive target for the development of drugs for treating CNS disorders, since it has been recently revealed that this pathway is closely related to the pathogenesis of several CNS disorders such as SCI. The effects of regeneration inhibitors are reversed by blockade of the Rho-ROCK pathway in vitro, and the inhibition of this pathway promotes axonal regeneration and functional recovery in the injured CNS in vivo (Kubo et al. 2008).

Eph receptor tyrosine kinase family and their ligands inhibit axonal regeneration following CNS injury. Antagonism of one of these, EphA4 is a potential therapy to promote recovery from SCI based on the demonstration that axonal regeneration following SCI is promoted in an EphA4 knockout animal (Goldshmit et al. 2011). Further studies by these authors in animal models of SCI provide definite evidence that soluble inhibitors of EphA4 function offer considerable therapeutic potential for the treatment of SCI.

Cortical gene expression profiling studies with microarrays in the rat have shown that anti-scarring treatment attenuates SCI-triggered transcriptional changes of genes related to inhibition of axon growth and impairment of cell survival, while upregulating the expression of genes associated with axon outgrowth, cell protection, and neural development (Kruse et al. 2011). This treatment not only modifies the local environment impeding spinal cord regeneration by reduction of fibrous scarring in the injured spinal cord, but, in addition, strikingly changes the activity of cortical neurons that is favorable for axonal regeneration.

6.5.2.2 Cell Transplantation for SCI

Considerable advances have been made during the past decade in devising and evaluating axon regeneration strategies based on cell transplants for SCI patients. Table 6.6 lists various types of cells used for this purpose.

Grafted cultured keratinocytes secrete growth factor scan induce growth of cell that have survived as well as neural differentiation of stem cells surrounding the injured spinal cord, leading to functional recovery (Inoue et al. 2011). A chitosan

Table 6.6 Types of cells used for transplantation in SCI

Cell type	Rationale for use
Autoimmune T cells against CNS myelin-associated peptide.	Neuroprotective effect in experimental models by reducing the spread of damage and promotion of recovery in injured rat spinal cord.
Glial cells: progenitor-derived astrocytes or cultured	Glia-depleted areas of the CNS can be reconstituted by introducing glial cells
Olfactory ensheathing glial cells	Facilitate regeneration
Keratinocytes	Secrete growth factors
Embryonic stem cells (ESCs)	To replace the lost neurons and supporting cells
Autologous bone marrow stem cells (BMSCs)	A clinical trial in patients with chronic complete SCI produced slight neurologic improvement (Deda et al 2008)
Neural stem cells (NSCs)	Generated from ESCs and can be developed into motor neurons
Induced pluripotent stem cells (iPSCs)	iPSC clone-derived NSCs may be a promising cell source for transplantation therapy for SCI.
Mesenchymal stem cells (MSCs)	Allogeneic MSCs are equally effective but better tolerated than BMSCs.

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conduit loaded with bone marrow stem cells (BMSCs) was shown to significantly reduce the spinal cord cavity volume at the injured site in adult rats and the results suggest that it may become a promising approach to the repair of SCI in humans (Chen et al. 2011). In another study, Rats with thoracic SCI could walk with weight bearing and showed recovered motor evoked potentials following transplanted of neurospheres (NS) derived from BMSCs (Suzuki et al. 2011). Histological analysis of spinal cords showed neuronal or axonal sproutings, which were replaced by host cells. Also, transplanted BMSCs-NS expressed neuronal lineage biomarkers.

A study has directed neural differentiation of murine iPSCs and examined their therapeutic potential in a mouse SCI model (Tsuji et al. 2010). Safe iPSC-derived neurospheres, which had been pre-evaluated as nontumorigenic, were transplanted into the spinal cord after contusive injury. The neurospheres produced remyelination and induced axonal regrowth promoting locomotor function recovery. These results show that iPSC clone-derived NSCs cells may be a promising cell source for transplantation therapy for SCI.

Progress in stem cell biology has made it feasible to induce the regeneration of injured axons after SCI in experimental animals by transplanting neural stem cells (NSCs) generated from the ESCs, which can be developed into motor neurons by using special techniques and culture media with growth factors. In an open trial, intravenous injection of autologous bone marrow cells in conjunction with the administration of granulocyte macrophage-colony stimulating factor led to improvement in patients with complete spinal cord injury (Park et al. 2005). Cell transplantation alone may not suffice for regeneration of the spinal cord and may need to be combined with other methods such as neurotrophic factors, blocking of

inhibitors of neural regeneration, and modulation of inflammatory response following injury (Ronsyn et al. 2008).

Transplantation of autologous bone marrow-derived mesenchymal stem cells (MSC), expanded ex vivo, has been tested in a clinical trial on SCI patients (Pal et al. 2009). After quality control and characterization for cell surface markers, MSCs were administered to the patients via lumbar puncture. Safety of the procedure was demonstrated by 1–3 years of follow-up. Therapeutic use of stem cells in SCI with unverified claims of recovery of paraplegia is being reported from countries without strict regulatory controls.

Several studies have shown that transplantation of ESCs to replace the lost neurons and other supporting cells into adult rats that were partially paralyzed through spinal cord damage led to some recovery of spinal cord function. Introduction of stem cells into the cerebrospinal fluid (CSF) via lumbar puncture is as effective as direct injection into the spinal cord. A clinical trial showed that subarachnoid placement of stem cells is safe with no long-term adverse effects (Mehta et al. 2008).

A spinal contusion injury model in the rat has been used to assess the efficacy of hESC-derived oligodendrocyte progenitor cells (OPCs) for cervical SCI (Sharp et al. 2010). hESC-derived OPC transplants attenuated lesion pathogenesis and improved recovery of forelimb function. Histological effects of transplantation included robust white and gray matter sparing at the injury epicenter, and in particular, preservation of motor neurons that correlated with movement recovery. These findings further an understanding of the histopathology and functional outcomes of cervical SCI, define potential therapeutic targets, and support the use of these cells as a treatment for cervical SCI.

Several clinical trials of stem cells in SCI are ongoing. These include NSCs, MSCs and HSCs.

6.5.2.3 Polyethylene Glycol for Repair of Nerve Fibers in SCI

Polyethylene glycol (PEG) can reunite and fuse transected cell processes and seal anatomical disruptions in cell membranes produced by mechanical injury. Topical application of PEG has been used for repair of spinal axons after severe, standardized SCI in experimental animals. PEG reduces both necrosis and apoptosis through two distinct yet synergistic pathways: repair of disrupted plasma membranes and protection of mitochondria through direct interaction (Luo and Shi 2007). An intravenous preparation of PEG has been used safely in a trial on dogs with SCI with some recovery of paraplegia (Laverty et al. 2004). Safety of PEG for human use is already established and it is feasible to translate this technique into application for human SCI. However, further studies have shown that although PEG protects key axonal cytoskeletal proteins after SCI along with axonal preservation, the modest extent of locomotor recovery after treatment with PEG may not be sufficient for clinical use as a single treatment (Baptiste et al. 2009).

6.5.2.4 Fampridine for SCI

Fampridine-SR is a sustained-release tablet form of the K^+ channel-blocking compound 4-aminopyridine that has been shown to restore conduction in focally demyelinated axons, to enhance synaptic transmission in many types of neurons and to potentiate muscle contraction. Most of the clinical development is focused on multiple sclerosis but it also includes trials on SCI. Results of two phase III clinical trials of sustained-release fampridine (25 mg twice daily) showed that it was well tolerated but had no effect on spasticity of chronic SCI (Cardenas et al. 2014). Fampridine is approved for multiple sclerosis but not for SCI.

6.5.2.5 Artemin for Regeneration in SCI

Artemin is a naturally occurring growth factor. GDNF family receptor GFRα3, a known receptor for artemin, is expressed on both myelinated and unmyelinated sensory neurons, consistent with artemin's ability to promote regeneration of large and small sensory neurons. In experimental studies, systemic treatment with artemin was shown to promote regeneration of sensory axons away from their site of interruption in dorsal roots to the brainstem, where they reestablish functional connections (Wong et al. 2015). Artemin is a potential therapy for restoring sensory function after SCI.

6.5.2.6 Monoclonal Antibodies for Repair in SCI

Natural autoreactive Ig monoclonal antibodies (MAbs) bind surface antigens on specific CNS cells, activating intracellular repair-promoting signals. Ig MAbs that bind to neurons stimulate neurite outgrowth and prevent death of neurons and have potential application for repair in neuron-damaging diseases, such as SCI (Wright et al. 2009). Neural cell adhesion molecule L1 constitutes is a viable MAb target to promote regeneration after SCI. Infusion of αL1 Fab (fragment, antigen-binding) into the lesioned spinal cord of mice was shown to enhance functional recovery after thoracic spinal cord compression injury (Loers et al. 2014). αL1 Fab treatment resulted in reduced scar volume, enhanced number of tyrosine hydroxylase-positive axons and increased linear density of vesicular glutamate transporter 1 on motoneurons. In addition, the number and size of choline acetyltransferase-positive motoneurons were increased. Stimulation of endogenous L1 by application of the αL1 Fab provides new opportunities for therapeutic applications of recombinant antibody technology for CNS injuries.

Recombinant remyelination-promoting Ig MAbs have been produced and undergone a phase 1 clinical trial after toxicology studies. Rather than pharmacologically compensating for myelin loss in some axons as in the case of fampridine, MAbs may actually replace the lost myelin, which may be more beneficial. Currently no product in this category is in advanced clinical development.

6.5.2.7 Role of RNAi and Other Knockdown Technologies in Regeneration Following SCI

Following SCI, there are numerous changes in gene expression that appear to contribute to either neurodegeneration or reparative processes. RNAi, ribozymes, and antisense technologies are used to suppress these. In animal experimental studies, several genes including the small GTPase Rab13 and actin-binding protein Coronin 1b, show significantly increased mRNA expression after SCI and this enhances neurite outgrowth. RNAi gene silencing for Coronin 1b or Rab13 in NGF-treated PC-12 cells markedly reduces neurite outgrowth (Di Giovanni et al. 2005). Modulation of these proteins may provide novel targets for facilitating restorative processes after SCI.

The expression of Nogo-A and the receptor NgR1 limits the recovery of adult mammals from CNS injury and several studies have demonstrated efficacy from targeting this pathway for functional recovery and neural repair after SCI (Schwab and Strittmatter 2014). RNAi therapeutics that inhibits this pathway could potentially reduce or prevent paralysis caused by such injuries.

There are changes in expression levels of microRNAs (miRNAs), a class of small non-coding RNAs, during SCI, which suggest that miRNAs play an important role in inflammation, oxidative stress, apoptosis, glial scar formation and axonal regeneration (Yan et al. 2012). Because of small size as well as specificity of action of miRNAs, and availability of techniques for delivering them, they are potential therapeutics for treating SCI.

Another knockdown technology uses deoxyribozymes, which are catalytic DNA molecules. Deoxyribozyme against xylosyltransferase (XT-1) mRNA not only prevents glycosylation of proteoglycans but also avoids the assembly of their core protein into the extracellular matrix. Thus it alters the inhibitory nature of the scar and promotes axonal growth in the injured spinal cord (Grimpe 2011). The immunological status of deoxyribozyme is not known and it does not penetrate the blood-brain or blood-spinal cord barrier. If used cautiously with an appropriate delivery technology, deoxyribozyme technology has the potential to become a major in CNS regeneration.

6.5.2.8 Physical Methods for Promoting Regeneration in SCI Patients

Several physical methods have been applied to promote regeneration in SCI. One of these is functional electrical stimulation (FES). An EF oriented in the direction of a damaged neural fiber can enhance axonal regrowth. The technique of oscillating field stimulation applied to SCI has been shown to promote bidirectional regeneration in the injured nerve fibers (Hamid and Hayek 2008).

Neuroprostheses are machines designed to artificially restore lost neurologic function. Recent emphasis is on development of neuroprosthetic devices that utilize the information recorded directly from the CNS. Development of neurorobots for simple walking movements may require elaborate systems for timed interaction

between sensory input and rhythmic motor output programs. There is some evidence that continuous CNS machine interaction and repeated activation facilities regeneration of the injured spinal cord and development of plasticity.

6.5.2.9 Combined Approaches to SCI

Following complete spinal cord transection that removes all supraspinal inputs in adult rats, spinal locomotion was shown to emerge from a combination of functional electrical stimulation and pharmacological approaches (Courtine et al. 2009). The development of central pattern-generating capability and the ability of these spinal circuits to use sensory afferent input to control stepping provide a strategy by which individuals with SCI could regain substantial levels of motor control.

6.5.3 Management of Traumatic Brain Injury

TBI or neurosurgical procedures may cause extensive loss of cerebral parenchyma. Reconstruction and regeneration is desirable, not only to replace the lost brain substance, but also to restore lost function and prevent formation of scar tissue. However, no clinically effective method is available as yet.

6.5.3.1 Cell Therapy for TBI

Cell therapy is expected to play an important role in the repair of TBI (Jain 2009). It is important that cells are transplanted into an environment that is favorable for extended survival and integration within the host tissue (Tate et al. 2009). Extracellular matrix proteins such as fibronectin and laminin are involved in neural development and may mediate subsequent cell signaling events. Enhanced cell survival was demonstrated following transplantation of a NSC construct containing laminin-based scaffold into the traumatically injured mouse brain.

Stem cell-based cellular replacement strategies have a potential therapeutic role following TBI, but the mechanism by which stem cells produce their effect (e.g. via integration into surviving neuronal circuits, local neurotrophic support, or modification of the local microenvironment to enhance endogenous regeneration and neuroprotection) remains to be assessed further (Maegele and Schaefer 2008). One of the functions of stem cells is to decrease inflammation whereby regeneration can be facilitated.

NSCs transplanted directly into the injured brain were shown to survive, differentiate into neurons and promote functional recovery in a rat model of TBI (Ma et al. 2011). There was an increase in the expression of SYP and GAP43 in the injured brain of NSC-transplanted rats, suggesting it as one of the mechanisms underlying the improved functional recovery.

A clinical trial to gauge the safety and potential of treating children suffering from TBI using HSCs derived from their own bone marrow started in 2008 (Harting et al. 2008). The clinical trial is based on laboratory and animal research indicating that HSCs can migrate to an injured area of the brain, differentiate into new neurons and support cells, and induce brain repair. A few other clinical trials of cell therapy for TBI have not produced any significant results so far.

6.5.3.2 Gene Therapy for TBI

Currently available information from preclinical studies reveals that there are several gene targets with therapeutic potentials in TBI and vectors that can be used to deliver the candidate genes. However, there are difficulties in translating these techniques into effective gene therapy in humans. Examples of some of these studies are given in the following paragraphs.

NSCs genetically modified to encode BDNF gene (BDNF/NSCs) have been shown to significantly improve neurological motor function on selected behavioral tests following transplantation into brains of rats with TBI (Ma et al. 2012). The number of surviving engrafted cells and the proportion of engrafted cells with a neuronal phenotype were significantly greater in BDNF/NSCs than in naive NSCs-transplanted rats that served as controls.

Neuronal regeneration can be induced by transgenic integrin expression. Integrins are nerve cell receptors that have been linked to the growth of nerve cells. Nerve cells taken from developing animals typically have high levels of integrins compared with those taken from adult animals. In experimental studies, the regenerative performance of adult neurons can be restored to that of young neurons by the gene transfer-mediated expression of a single alpha-integrin, which has the potential to be developed into a gene therapy approach to regeneration. The therapy would be based on the modification of integrin genes with a type of "switch" controlled by a drug. Physicians would then inject the modified genes into damaged area of the brain. A recombinant adenovirus vector expressing Bcl-2 fusion protein can suppress apoptosis and promote cell survival in experimental TBI in rats (Yang et al. 2006).

6.5.3.3 Nanomaterial Scaffolds for Repair of TBI

The peptide nanofiber scaffold is an effective technology for tissue repair and restoration and is a promising treatment for TBI. This peptide nanofiber scaffold has several advantages over currently available polymer biomaterials. The network of nanofibers is similar in scale to the native extracellular matrix and, thus, provides an environment for cell growth, migration, and differentiation. This peptide disintegrates and is immunologically inert. Self-assembling peptide nanofiber scaffold may help to reconstruct the acutely injured brain and reduce the glial reaction and inflammation in the surrounding brain tissue (Guo et al. 2009; Webber et al. 2010).

This creates a permissive environment for axons, not only to regenerate through the site of an acute injury, but also to knit the brain tissue together.

Challenges of using a tissue engineering approach for regeneration in TBI include a complex environment and variables that are difficult to assess. For optimal benefit, the brain should be in a condition that minimizes immune response, inflammation and rejection of the grafted material. Tissue engineering, using a bioactive scaffold counters some of the hostile factors and facilitates integration of donor cells into the brain, but transplantation of a combination biologic construct to the brain has not yet been successfully translated into clinical use (Stabenfeldt et al. 2011).

The next generation of tissue engineering scaffolds for TBI may incorporate nanoscale surface feature dimensions, which mimic natural neural tissue. Nanomaterials can enhance desirable neural cell activity while minimizing unwanted astrocyte reactivity. Composite materials with zinc oxide nanoparticles embedded into a polymer matrix can provide an electrical stimulus when mechanically deformed through ultrasound, which can act as a cue for neural tissue regeneration (Seil and Webster 2010).

6.5.3.4 Neuroregeneration in Chronic Traumatic Encephalopathy

Chronic traumatic encephalopathy (CTE) is the term used for neuropathological changes consistent with long-term repetitive concussive brain injury traditionally seen in football players and boxers, but now in US soldiers returning from Iraq and Afghanistan wars. Clinical manifestations include cerebral dysfunction with cognitive impairments and neurobehavioral disturbances, which may progress to dementia as tearing of neuronal connections (axonal shearing) disconnects or impairs cortical and thalamic circuits. Neuropathological changes in the brain are those of a tauopathy with neurofibrillary deposits. The cumulative effect of repeated concussions is different from acute TBI and resembles more the course of a neurodegenerative disease. Apart from avoidance of trauma, neuroprotective and neuroregenerative measures may be considered for CTE, which may be similar to those for neurodegenerative disorders. HBO has been found to be useful for treatment of CTE due to blast injury (Harch et al. 2009). Pharmacological strategies include development of anti-tau drugs to clear tau deposits.

6.6 Concluding Remarks and Future Prospects

Considerable knowledge has been gained about regeneration in the CNS in experimental animals by studying factors inhibiting neurite outgrowth and using substances to inhibit these. Worldwide, research on regeneration in SCI has historically preceded that in TBI and there has been more activity in the former. Currently TBI has gained priority in research in the US due to a large number of injured soldiers from various wars and greater recognition of CTE in soldiers and athletes. However, translation of these into application in human CNS injury has only limited success.

Although many strategies are being pursued currently, several challenges still remain. Areas that are promising for future research in regeneration following CNS trauma are:

- Stimulation of neurovascular remodeling by enhancing angiogenesis, neurogenesis, oligodendrogenesis, and axonal sprouting; acting in concert, these may improve functional recovery after TBI (Xiong et al. 2015).
- Cell therapies, particularly by use of mesenchymal stem cells.
- Use of biodegradable materials such as polymer nanofibers to provide support and a favorable environment for CNS regeneration.
- Strategies for inhibiting signaling mechanisms that hinder regeneration.
- Efforts continue to develop more effective treatment to inhibit glial scarring and to promote regeneration in SCI.
- There is a focus on functional recovery rather than mere structural restoration.
- Combination of multiple methods such as use of biological therapies and pharmaceuticals.

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Chapter 7
Peripheral Nervous System: Regenerative Therapies

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Abstract There is a general belief that regeneration in the Peripheral Nervous System (PNS) is a successful event, however complete functional regeneration is seldom achieved in patients that have suffered a nerve traumatic injury. In fact, what is clinically observed is that these patients live with permanent disabilities that interfere

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negatively in their daily routine activities. In injuries where there is tissue loss a direct neurorraphy is not possible without causing nerve tension and, therefore, another repair technique is needed. Clinically, these lesions are repaired by nerve autograft, a technique that requires a second surgery to harvest a segment of a donor nerve, a disadvantage of the method. Also, the area covered by the donor nerve becomes denervated and its function is lost. Other techniques that are used by surgeons when the proximal stump is not available are end-to-side coaptation and nerve transfer. Experimental studies aiming at developing alternative strategies that can improve nerve regeneration have increased over the last decades. Particularly, the search for nerve guiding conduits that can be used to bridge the nerve defect has received much attention by researchers all over the world. These conduits can be made by either synthetic or biological materials, but ideally, they should be biodegradable and biocompatible, have adequate permeability so as to allow the entrance of nutrients into the tube lumen and yet avoid the passage of cells that can interfere negatively in the regeneration processes, such as fibroblasts and inflammatory cells. Other therapeutic strategies such as gene, cell and molecular therapies as well as physical therapies (exercise, electrical and LASER therapy) have also been tested in experimental studies with positive results. In this chapter we review the literature covering all these strategies in terms of experimental studies and existing clinical trials.

Keywords Peripheral nerve regeneration • Cell therapy • Gene therapy • Microsurgical techniques • Physical therapies

7.1 Introduction

7.1.1 Nerve Trauma and Diseases

Peripheral nerve injuries result in total or partial loss of motor, sensory and autonomic functions of the denervated segment of the body. Despite the well documented capacity of peripheral axons to regenerate, full functional recovery is almost never achieved. Each year in the United States there are about 200,000 new cases of patients with peripheral nerve injury, and of this total, only 10 % can achieve complete functional recovery (Scholz et al. 2009). Apart from traumatic injury, the other peripheral nerve disorders can result from dysfunction of the cell body, myelin sheath, axons or neuromuscular junction. There are several kinds of nerve disorders that can disrupt the normal function of the nerve. Peripheral neuropathies may affect one nerve (mononeuropathy), several discrete nerves (multiple mononeuropathy), or multiple nerves diffusely (polyneuropathy). Some may be caused by diseases, such as diabetes, also by syndromes like in the case of Guillain Barre and be a result of nerve compression, as for instance, in the tunel carpal syndrome. In most cases, however, the problem begins after an injury (Kimura 2006).

Regardless of the etiology, nerve disorders decrease the quality of life of the affected patients who also suffer from secondary problems, such as neuropathic pain and psychosocial difficulties (Rosberg et al. 2005).

7.1.2 Mechanisms of PNS Degeneration and Regeneration

Following nerve injury, the axon is divided into two segments: one proximal segment, which remains in contact with the cell body, and a distal segment, which is disconnected from it. The interruption of axon continuity causes disruption of axonal conduction, degradation of the myelin sheath and degeneration of the axons distal to the lesion site (Gaudet et al. 2011). This degenerative process, called Wallerian degeneration, is necessary to create a microenvironment that is favorable to the regeneration of the surviving neurons (Rotshenker 2011).

In the PNS, Wallerian degeneration involves mainly two types of cells, the Schwann cells and macrophages. Immediately after a nerve injury, Schwann cells dedifferentiate to a progenitor-like state and, together with the infiltrating macrophages, clear the degenerated myelin and axon. First, around 24 h after injury, denervated Schwann cells begin to phagocyte myelin debris. However, from 2 to 3 days, there is an important recruitment of hematogenous macrophages which become the main phagocytic cells. They are attracted by cytokines secreted by reactive Schwann cells, such as monocyte chemoattractant protein1 and interleukin (IL-1) (Tofaris et al. 2002). The removal of myelin sheaths enables the clearance of regeneration-inhibitory factors associated to myelin, especially myelin-associated glycoprotein (MAG).

At 48 h after the lesion, Schwann cells acquire a non-myelinating phenotype, begin to express regeneration-related genes such as the growth-associated protein 43 (GAP-43), and assume an intense proliferative activity (Murinson et al. 2005), which reaches its peak about 4 days after injury. Then, Schwann cells line up to form the bands of Büngner, which will provide support for regenerating axons. Once Schwann cells contact with their axons, re-myelination process is started (Griffin and Thompson 2008).

Parallel to Wallerian degeneration, the neuron cell body undergoes a series of phenotypic changes known as neuronal reaction or chromatolysis, which represents the metabolic changes necessary for regeneration and axonal elongation (Navarro et al. 2007; Wood et al. 2011). The regeneration process requires the transformation of a stable axonal segment into a mobile segment called growth cone. The growth cone is formed at the distal portion of the proximal segment of the nerve, is guided by local factors until it reaches its target and restores function (Kato and Ide 1994).

The movement of growth cone is guided by gradients of molecular cues present on the site of injury and at the distal nerve segment. Some of these cues are neurotrophic factors such as nerve growth factor (NGF), glial derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BNDF) and neurotrophin-3 and 4/5 (NT-3 and NT-4/5). Some extracellular matrix molecules such as laminin, fibronectin and collagen can also guide the growing axon (Allodi et al. 2012). The gowth cone undergoes three main steps: protrusion, enlargement and consolidation (Mortimer et al. 2008). These mechanisms enable the advancement of the central area towards the target organ and the consolidation of a new axon (Dent and Gertler 2003).

The elongation process is slow and depends on the presence of growing-promoting molecules, however the ability of supporting cells to produce these molecules diminishes over time. That is the reason why, in humans, the functional recovery is often incomplete. Hence, strategies to improve axon regeneration through long distances are necessary. In this sense, some experimental strategies have been recently used, such as: the use of tubular prosthesis, also called nerve conduits; gene therapy associated with prostheses (Lopes et al. 2013); cell therapy associated with prostheses (Lopes et al. 2006; Oliveira et al. 2010, 2014a); physical therapy and exercise (Goulart et al. 2014). The ideal treatment should take into account the nerve degeneration and regeneration process, and should be initiated as early as possible. Therefore, there is a continuing search for new techniques to optimize the regeneration and functional recovery after peripheral nerve injury.

7.1.3 Central Nervous System Mechanisms Associated to Peripheral Nerve Lesion

The central nervous system is capable of modeling neural circuits in response to deafferentation and tissue injury (Mendonca et al. 2010; Espirito-Santo et al. 2012). Despite the morpho-functional subdivision of nervous system into the categories of peripheral or central, it behaves as an unit. Thereby, long-lasting central reorganization occurs in response to peripheral nerve injury, leading to functional adaptative and/or maladaptative changes, such as neuropathic pain, disesthesia, hyperreflexia and dystonia (Navarro 2009; Oliveira et al. 2014a).

Following axotomy of a peripheral nerve, a variable number of dorsal root ganglion (DRG) and spinal cord dorsal horn neurons undergo apoptosis, decreasing the possibility of proper re-innervation of dorsal column neurons in the spinal cord and restoration of receptive fields (Groves et al. 1997; Azkue et al. 1998; Valero-Cabre et al. 2001). Besides, primary sensory neurons down-regulate neurotransmission-associated genes (Caldero et al. 1992; Hökfelt et al. 2006), which might affect the input to the dorsal column, leading to maladaptive neuroplasticity.

Regenerating peripheral axons up-regulate Nav (voltage-gated sodium channel) 1.3 and 2, while other subtypes are decreased (Wood et al. 2004). Moreover Kv (voltage-gated potassium channel) 1.1, 1.2, 1.3 and 2.1 are down-regulated in DRG neurons after injury (Ishikawa et al. 1999). These alterations lead to reduction in the excitation threshold, increase of spontaneous activity and prolonged *after-discharges*, leading to hyperexcitability of regenerating axons (Abdulla and Smith 2001; Hökfelt et al. 2006). Spontaneous activity is also increased in the dorsal horn of the spinal cord, spinal trigeminal nucleus and thalamus after peripheral injury and dorsal rhizotomy (Dalal et al. 1999; Basbaum and Wall 1976; Macon 1979; Albe-Fessard and Lombard 1982). Besides, sciatic nerve experimental lesion, such as ligation, induces an increase in glutamate release by DRG central axons and promotes up-regulation of GluN2B subunit of the NMDA receptor in DRG axon terminals, positively modulating excitatory pos-synaptic potentials within the dorsal horn

(Yan et al. 2013). Additionally, GluN2A subunit is also up-regulated in dorsal lamina II of the dorsal horn after lesion (Chen et al. 2014). These changes might increase neurotransmission and compensate the lower sensitive drive due to loss of DRG neurons and poor re-innervation.

However, central projections of DRG regenerated neurons are not well-matched with their peripheral related receptors. Instead, their receptive fields become larger and scrambled, reflecting the distorted regeneration pattern (Koerber et al. 2006). This diffuse somatotopy is reproduced in all levels along the neuro-axis. Immediately after nerve transection, there is a period of denervation-induced loss of evoked activity into the somatic neuro-axis. Within a few days, the representation of neighbor regions of the body surface expand into areas previously involved in the processing of the damaged nerve (Hökfelt et al. 2006; Oliveira et al. 2014a). This effect is probably reflected by dis-inhibition of pre-existent excitatory synapses, since both GABA and its receptors are reduced after lesion (Castro-Lopes et al. 1993; Moore et al. 2002). Indeed, Nicolelis and co-workers (Nicolelis et al. 1993) have shown immediate expansion of neighbor regions' representation in the dorsal column nuclei and thalamus after a peripheral anesthetic block in the face. In addition to disinhibition, sprouting of the preserved pathway underlies central somatotopy enlargement. Somatosensory(?) A fibers were found to sprout from dorsal column laminae III and V to the nociceptive laminae I and II, activating them (Woolf et al. 1995; Kohama et al. 2000). These connectivity alterations are related to neurophatic pain. However, during specific physiological conditions, central plasticity impairs pain development. In this context, during puerperium there is an increase in descending noradrenergic mediated inhibition and a decrease in the expression of the excitatory regulators dynophin-A and neuregulin-1, preventing the development of damage-induced neuropathic pain (Gutierrez et al. 2013). On contrary, if neuroplasticity is inhibited by metalloproteinase blockade, neuropathic pain can be reduced (Liou et al. 2013). Not only pain-related plasticity is modulated after extracellular matrix degradation. Indeed, chondroitin sulfate breakdown enhances spinal cord reflexes after peripheral lesion (Bosch et al. 2012).

During early regenerative stages, expansions of subcortical receptive fields are more pronounced than that of neocortex. The expansion of somatosensory representation in the neocortex depends on subcortical plasticity. Following dorsal rhizotomy in rats, Sengelaub and collaborators (Sengelaub et al. 1997) found that gracile nucleus neurons sprouts to the cuneate nucleus, bringing hind limb sensory information to be relayed within the forelimb pathway. As concluded by Navarro (Navarro 2009): *"Within a subcortical nucleus, a few millimeters shortcut may be sufficient to induce the rapid shift of cortical representation areas"*. Although stimuli to the body surface of neighbor region of the denervated area evokes activity in the cortical area originally related to the lesioned nerve, direct cortical stimulation still evokes sensations below the spinal cord transection on paraplegic subjects (Cohen et al. 1991). This phenomoenon suggests that there is a change in the input pattern to these cells, but their output pattern remains the same. This conserved perception that follows neocortical stimulation reflects partial/incomplete plasticity and may lead to phantom limb sensations in amputees.

After a peripheral lesion, motoneurons are usually spared. However, they switch into a regenerative state, increasing growth associated protein expression and decreasing translation of neurotransmission related genes (Hökfelt et al. 2006; Navarro 2009). Moreover, somatic and vegetative spinal motoneurons loose their distal dendritic segments and associated excitatory synapses, becoming partially disconnected from the neuronal network during regeneration (Purves 1975; Sumner and Sutherland 1973; Navarro 2009). In addition, cortical motor representation of the denervated muscles becomes initially silent, and its stimulation does not evoke any movement. Later on, the stimulation of the same region elicits movement of muscles mapped adjacent to the paralyzed ones (Navarro 2009). This expansion of intact nerve-related cortical representation is partially due to motoneuron sprouting (Chen et al. 2002; Makwana et al. 2010).

After re-innervation is accomplished, somatosensory and motor properties normalize in the case of nerve crush, recovering the cortical representation of the lesioned and regenerated nerve, but they are still scrambled after peripheral nerve transection (Koerber et al. 2006; Oliveira et al. 2014a). This phenomena is probably related to factors implicating in the neuroplastic capacity of the nervous system, such as age, as Chemnitz showed that when nerve transection and repair occur in subjects before 13 years of age, patients present normal cortical representation at 28 years of age. In contrast, when patients were lesioned after 13 years old, cortical representation is not restored until 28 years (Chemnitz et al. 2013). The recovery of motoneuron activity is dependent on astrocyte activation. Briefly, astrocytes express STAT3, which regulates perineuronal astrocytic process formation and re-expression of the synaptogenic molecule thrombospondin-1 and re-expression of the adhesion molecule SynCAM-1 (Zelano et al. 2009; Tyzack et al. 2014).

Thus, several central plastic mechanisms work in concert to reestablish function after peripheral lesions. Very often, however, these events are not properly orchestrated, leading to maladaptive changes. Research in strategies to boost or fine tune central plasticity is welcomed and will probably bring great contributions to improve functional recovery after peripheral lesions.

7.1.4 Peripheral Nerve Injuries Classification

Peripheral nerve injuries may arise from different sources, including traumatic, metabolic and immune origins. Independent of the source, there will be consequences on the normal function of the nerve that will depend on the magnitude of the lesion, the presence of other comorbidities, and a series of biological aspects such as age and sex.

Seddon in 1943 developed the first classifications of peripheral nerve injuries. The degree of nerve injuries in this classification is related to the ability of the injured nerve to conduct action potentials, and connective layers integrity (*epi*, *peri* and *endoneurium*).

Seddon's lowest degree of injury is called Neuropraxia (Class I). In this type of lesion, axons and connective tissue layers remain intact, but there is local conduction block in the axon. As axonal conduction remains intact both, proximal and distal to the injury site, neuropraxia is generally reversible over a period of hours to months. Wallerian degeneration is not seen in this type of lesion. Sensory and motor dysfunction may develop in various degrees, and may include tingling, pain, numbness and other paresthetic sensations. The lesion may also be associated with muscle flaccid paresis or paralysis, but sensory fibers are generally more susceptible (Hofmeijer et al. 2013). Electrophysiologic testing will show conduction block and increased sensory and/or motor latencies. Needle electromyography may have a prognostic value in some lesions (Sittel and Stennert 2001; Grosheva and Guntinas-Lichius 2007).

The second degree of nerve injury according to Seddon is Axonotmesis (Class II). In this type of injury, there is axonal interruption, but the connective layers are preserved. Consequent to axonal lesion, there is Wallerian degeneration distal to the injury site, which leads to loss of sensory and motor functions. Retrograde neuronal loss will happen in some axons, with changes in brain sensory and motor representation of the affected regions. If there is no physical barrier, axons will regenerate in the direction of target organs. As connective tissue layers remain intact, regenerating axons will be driven to reinnervate their original organs, in a rate of approximately 1 mm/day in humans.

The worst degree of injury of Seddon's classification is Neurotmesis (Class III), where there is rupture of the nerve, compromising both axons and connective tissue layers. The anatomical interruption leads to total loss of function. Wallerian and retrograde degeneration are present. Spinal cord and brain reorganization may be severe, and even associated with phantom phenomena. Nerve reconstruction is mandatory by means of suture, grafts or tubulization. Nerve transfers (neurotization) have been used to overcome the impossibility of other surgical procedures.

In order to detail Seddon's Class II classification, Sunderland added some categories. Classes I and II from Seddon and First and Second-degree classifications of Seddon represent the same lesions. Sunderland further added two degrees on the classification, taking into account two possible lesions of the connective layers. Third-degree Sunderland classification includes disruption of the endoneurium, but preservation of the epi and perineurium, and in Fourth-degree there is also perineurium damage.

Finally, two weak types of peripheral nerve lesions include physiological conduction blocks type a and b, that are not referred in Seddon's or Sunderland's classifications. Local conduction block type a is associated with intraneural circulatory arrest and reversible metabolic dysfunction, but without axon pathology. Type b lesion presents intraneural edema, metabolic dysfunction with or without discrete nerve fiber pathology, and increased endoneurial pressure.

7.2 PNS Regenerative Therapies

7.2.1 Microsurgical Techniques and Biomaterials

Nerve trauma is a common condition in the clinical setting, and despite great advances in the microsurgical techniques for nerve repair, it still represents one of the most challenging reconstructive problems in the field of restorative medicine. The treatment success of peripheral nerve injuries will depend on some limiting factors, such as: age, the wound itself, injury site, time between injury and surgical intervention and the nerve repair microsurgical technique. The nerve repair microsurgical technique will, in turn, depend on the etiology of nerve damage, once it determines the site (proximal or distal to the cell body) and extent (no, minimal or significant loss of nerve segment) of injury. There are several microsurgical techniques available for nerve repair: direct nerve repair, which comprises end-to-end and end-to-side coaptation; nerve transfer; and the techniques of bridging nerve defects, which comprise nerve grafting (autograft, allograft and xenograft) and nerve conduits (synthetic and biological conduits) (Fig. 7.1).

Following a complete nerve injury with a small gap (<2.5 cm), the primary repair, also called neurorrhaphy, direct suture or end-to-end coaptation, is the preferred method of repair because nerve ends can be approximated with no or minimal tension. The tension-free microsurgical method is crucial for allowing the microvascular flow (Driscoll et al. 2002) thus preventing ischemia and fibrosis, and, ultimately, impaired nerve regeneration and poor functional outcomes. In addition, minimal number of epineurial or fascicular sutures and the correct topographic nerve ends alignment are desired when performing the meticulous primary repair technique. The identification of longitudinal intraneural blood vessels may be helpful for correct alignment and, in specific cases, it is possible to identify individual fascicular groups, allowing group fascicular nerve repair (Dahlin 2008). Despite the fact that group fascicular repair may provide a better alignment, preventing nerve misdirection (Brushart et al. 1983) it requires higher number of sutures compared with epineurial repair. Alternatively to the nylon sutures that can lead to undesired increase of intraneural scar formation and impaired blood flow, nerve coaptation can be accomplished by means of an atraumatic coaptation using fibrin glue. The outcomes of both methods of tissue fixation, the suturing and the fibrin glue, are similar but the use of fibrin glue alone in the clinical setting is still limited, since human nerves are usually of larger caliber and therefore more difficult to repair (Félix et al. 2013). Thus, surgeons combine both methods for nervous tissue fixation.

The indicated microsurgical technique when the proximal nerve stump is unavailable or a significant nerve gap exists is the end-to-side nerve repair. In this method, the distal nerve stump is coapted to the side of an uninjured donor nerve. The putative mechanisms of nerve regeneration by using this microsurgical technique are the invasion of the transected proximal stump of the injured nerve; the regeneration from donor nerve axons that were damaged during nerve preparation; or the

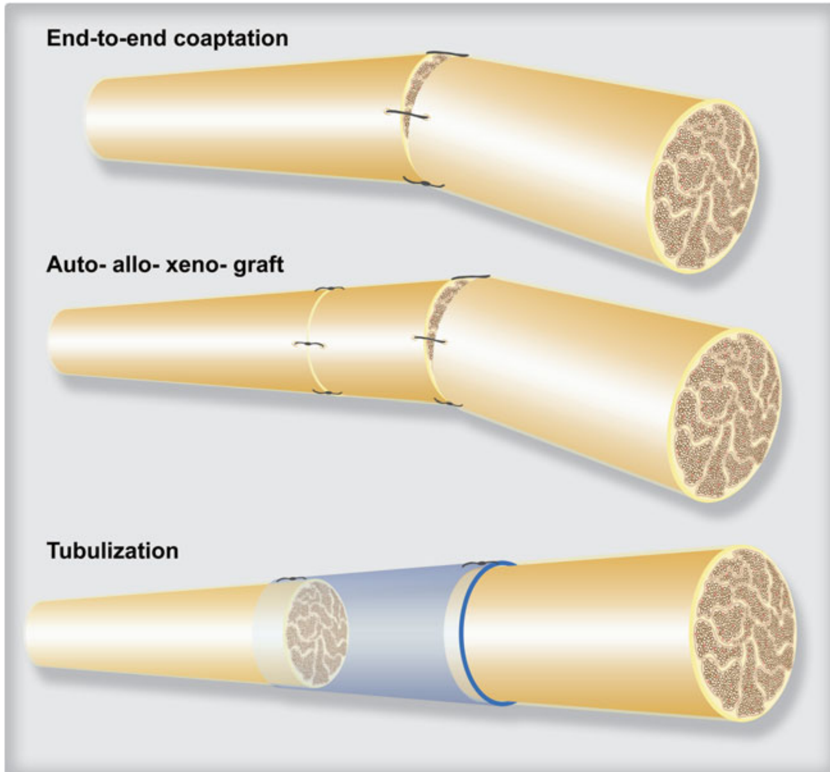

Fig. 7.1 The most currently used microsurgical techniques in experimental and clinical trials. End-to-end coaptation is a tension-free microsurgical method indicated when there is not loss of tissue, while grafts and tubulization microsurgical methods are indicated when there is significant loss of tissue and the coaptation of nerve ends are not possible without tension. All procedures require epineurial sutures for the fixation of nerve ends to each other, to the graft or to biodegradable nerve conduits.

regeneration from collateral sprouting from the closest Ranvier nodes on the side of the anastomosis, which is the most accepted by researchers (Dvali and Myckatyn 2008). There is evidence that epineurial window and neurectomy of the donor nerve may enhance regeneration of the repaired nerve with end-to-side coaptation, likely because of a larger area of contact between nerves and the creation of a suitable microenvironment for growth and neurothophic factors (Liu et al. 2014). Although end-to-side coaptation is an alternative to end-to-end coaptation in specific cases, the last standard method provides better outcomes when compared to the first (de Almeida et al. 2015). In addition, the end-to-side coaptation is controversial in the literature, leading to discussion on the axonal growth and the degree of motor and sensory recovery. Thus, at present this method is not encouraging in the clinical practice.

Nerve transfer, also called end-to-side neurotization, has become a major method for reconstructing the brachial plexus after avulsion injury. This microsurgical technique, which is a "reverse end-to-side" nerve repair, involves the transference of nerves or its fascicles that have less important roles onto distal ends of crucial nerves. Several donor nerves for the repair of brachial plexus injuries have been investigated in pre-clinical and clinical trials, for example phrenic to the musculocutaneous nerve transfer (Liu et al. 2015), radial to ulnar nerve transfer (Phillips et al. 2014), among others. Despite the fact that there is a lack of studies comparing end-to-side-nerve repair with nerve transfer, in particular, for treating nerve root avulsions injuries, the "reverse" method, in general, shows more satisfactory results (Wolfe et al. 2014).

In severe injuries with segmental nerve loss due to debridement or the trauma itself leaving therefore a gap between nerve ends which can not be approximated without tension, the current gold standard for peripheral nerve repair is autologous nerve grafting (autograft). Autograft provides Schwann cells, surface adhesion molecules and extracellular matrix components, such as the basal lamina that recreate the native nerve microenvironment. Sensory donor nerves are most often used, in particular the sural nerve. However, there is evidence that sensory and motor neurons have different Schwann cell phenotypes suggesting that if the cells are placed in the incorrect microenvironment it may limit their regenerative ability (Höke et al. 2006). Also, the architecture of sensory and motor nerves in terms of size of Schwann cell basal lamina tubes is different, which may play an important role in nerve regeneration in mixed nerve gap model (Moradzadeh et al. 2008). Some authors proposed the harvesting of sensory donor nerves from the same surgical area in attempt to avoid a second surgical intervention (Poppler et al. 2015). However, autograft still have some limitations, such as morbidity, donor site mismatch, limited supply and possible painful neuromas.

When the gap exceeds the available nerve autograft length, a nerve allograft harvested from a cadaver donor represents an alternative for transplant. Nerve allograft does not have the aforementioned drawbacks associated with the gold standard nerve autograft. However, a considerable concerning on the use of nerve allograft is the immunogenicity, which elicits tissue rejection. There are two main ways of avoiding immunogenicity, which are by administrating immunosuppressive agents or by denaturating nerve allograft. Among the immunosuppressive agents available the FK506 (Tacrolimus) is often used in nerve allografting. Although there is evidence that FK506 has neuroprotective and neurotrophic actions in experimental models, increasing neurite elongation and accelerating the rate of nerve regeneration (Konofaos and Terzis 2013), it has potentially severe adverse effects, such as infections, neoplasia, drug toxicity and metabolic derangement (Tung 2010). Antibody-based therapies that induce immune unresponsiveness represent an alternative to nonspecific immunosuppression. Although administration of antibodies prevents rejection and allows regeneration of peripheral nerve allografts the effect is transient with restoration of immunocompetence shortly after withdrawal of therapy (Brenner et al. 2004).

The denaturating methods often used in nerve allograft are irradiation, freezing-thawing, lyophilization, cryopreservation, decellularization with detergents or a combination of them. Although the cellular components are removed by denaturating methods, nerve architecture and guidance cues, such as laminin, are preserved. The acellular nerve allograft is effective on regenerating small to moderate nerve gaps and one of the mechanisms provided by this method is the upregulation of protein and mRNA expression of BDNF and calcitonin gene-related peptide CGRP in spinal cord (Zhang et al. 2012).

Besides the administration of immunosuppressive agents and the denaturating methods, adipose-derived stem cells have gained considerable attention in the field of regenerative medicine due to the paracrine immunomodulatory capacity as well as the reparative function (Davis et al. 2014). Thus, the combination of both, adipose-derived stem cells and nerve allografs, may facilitate allograft long-term survival and improve nerve regeneration. Indeed, the adipose-derived stem cells loaded in acellular nerve xenografts resulted in transient depression of CD4+ lymphocytes proliferation (Huang et al. 2012). Despite nerve xenografts represent an available and unlimited source of material for transplant, they are unlikely to be of clinical value for nerve repair. Nerve allografts show superior nerve regeneration compared with nerve xenografts (Wood et al. 2014). In addition, the risk of cross-species disease transmission should be considered for the use of xenografts.

Biological and synthetic nerve conduits have emerged as alternatives to nerve grafts. However, in clinic, conduits are only effective and, thus, indicated for repairing small gaps (<3 cm) in small-caliber nerves (Pfister et al. 2011).

Biological conduits for nerve repair include epimysium conduit filled with skeletal muscle fibers (Yang et al. 2013), vein (Chiu et al. 1982), artery (Gulati 1989), skeletal muscle (Jiming et al. 1986), epineurial sheath (Karacaoğlu et al. 2001) or muscle-vein combined conduit (Geuna et al. 2004). Inside-out vein and artery grafts provide the growing axons to be in contact with the adventitia, which is rich in collagen, and the normal orientation of the grafts provides the growing axons to be in contact with the endothelial basal membrane which is rich in laminin, a component of Schwann cell basal membrane. In this way, both methods, natural and inverted orientations, provide adhesion and surface molecules that may improve nerve regeneration. Epineurial sheath has the advantage of its naturally occurring benefits of neurotrophism and skleteal muscle contains directionally arranged basement membrane and extracellular matrix.

Epineurial sheath showed improved functional and morphological parameters compared with vein conduit, but both biological conduits were not superior to nerve grafts (Karacaoğlu et al. 2001). Epimysium conduit showed repair effectiveness superior to the synthetic polyurethane conduit but inferior to that achieved in the autologus nerve graft (Yang et al. 2013). Experimental studies comparing each biological available conduit with the nerve allograft are needed to evidence their efficacy over the standard of care for bridging nerve gaps.

Progress in tissue engineering has enabled the introduction of several synthetic conduits for peripheral nerve repair. The ideal conduit should be biocompatible, avoiding elicitation of immunologic response; be biodegradable, providing a

substrate for growing axons while regeneration takes place; provide a suitable substrate for adhesion, migration, proliferation and differentiation of Schwann cells; be obtained from a feasible source; have low costs; have adequate flexibility and stiffness to be able to bear the stresses of the surgical procedure (handling and suturing) and to prevent collapse provided by adjacent tissue; and have adequate porosity and permeability to allow diffusion of nutrients while preventing cellular and molecular additives to escape from the lumen. The great advantages of synthetic conduits are that they do not lead to donor-site morbidity; there is no necessity of a second surgical intervention for harvesting material for transplant; and they can be designed to present structural, mechanical and biochemical features that can potentially enhance nerve regeneration. For example, besides the hollow conduit, there are other conduit design strategies such as intraluminal guidance structures (Schmidt and Leach 2003), micro/nano-grooved luminal design (Huang et al. 2015), electrospun micro/nanofibrous conduit (Huang et al. 2015), multi-channel conduit (Dinis et al. 2015), electrically condutive conduit (Kim et al. 2010) as well as conduit surface functionalization. These varied features may influence Schwann cell behaviors and provide alignment of growing axons. In addition, cellular and molecular-based therapies can be associated to the conduit repair for the creation of a more conductive nerve microenvironment.

Biopolymers such as polyesters, proteins, polysaccharides and composite have been used in peripheral nerve regeneration studies. Collagen is the major component of the extracellular matrix and has been widely used in nerve regeneration repair as a channel, as filaments or as additive (Sahakyants et al. 2013). In spite of being a biological material that aid in peripheral nerve regeneration process, collagen conduits are not superior to the gold standard autologous nerve repair (Lee et al. 2012; Sahakyants et al. 2013). In addition to attempts of enhancing nerve regeneration by variations on the conduit architecture, another appealing strategy is the use of composite conduits. Chitosan/silk (Gu et al. 2014), poly(lactic acid-caprolactone) (Liu et al. 2011), poly(lactic-co-glycolic acid) (Wang et al. 2014b), collagen-polyvinyl alcohol (Marinescu et al. 2013), among others have being used in experimental trials for peripheral nerve repair. Keratin (Pace et al. 2013), collagen (Sahakyants et al. 2013), among other proteins in form of hydrogels, cross-linking or coating the conduit lumen have been used as additives. Interestingly, due to the inherent electrical excitability of neurons and the beneficial effects of electrical stimulation on nerve regeneration, biomaterial scaffolds that are electrically conductive, such as polypyrrole and polyaniline, have also been combined with other polymers for improving nerve regeneration (Kim et al. 2010).

In spite of great advances in nerve tissue bioengineering, to date most of the experimental studies demonstrate that outcomes of using synthetic conduits for nerve repair are equivalent or inferior to outcomes provided from autologous nerve graft. In addition to the progress in biotechnology, allowing variations in the design of nerve conduits, celullar and molecular additives may be associated with nerve conduits in an attempt of enhancing nerve regeneration and providing better functional outcomes after injury.

7.2.2 Cell Therapy

Cell therapy has emerged as a promising strategy in the field of regenerative medicine. In attempts to aid the regenerative cellular response to injury, cell-based therapies are being considered as potential additives to nerve repair techniques. Following a peripheral nerve injury, the Schwann cells play the main intrinsic supportive role, acting in pivotal stages of nerve regeneration by the phagocytosis of myelin and axons debris, by secreting growth promoting and adhesion molecules and by axon remyelination. Thus, autologous transplantation of Scwhann cells has been used in experimental studies of peripheral nerve injury, confirming that this cell source is able to improve nerve regeneration (Keilhoff et al. 2006). This evidence in addition to the inherent properties of Schwann cells would make these cells the ideal source for transplants if there were not technical limitations hindering their clinical implementation. Schwann cells have restricted mitotic activity, expanding poorly in culture conditions, and its use leads to donor site morbidity because another nerve has to be sacrificed for harvesting material for transplant. All these technical limitations have motivated efforts to seek for alternatives to Schwann cells for cell-based therapy and stem cells have been considered a potential candidate in their place.

Several sources of stem cells for improving peripheral nerve regeneration have been investigated, such as mesenchymal stem cells, adipose-derived stem cells, olfactory ensheathing stem cells, skeletal muscle-derived stem cells, embryonic stem cells, hair follicule pluripotent stem cells and skin-derived stem cells.

The embryonic stem cells are pluripotent stem cells from the inner cell mass of the blastocyst. These pluripotent stem cells can differentiate into virtually any cell lineage, including neural progenitor cells (Bain et al. 1995), and have the capacity of self-renew, thus, proliferating efficiently *in vitro* (Itskovitz-Eldor et al. 2000). Under defined culture induction conditions, the pluripotent embryonic stem cells can be limited to progenitors/multipotent stem cells, and, ultimately to specific cell types (Cui et al. 2008). Neurally-induced embryonic stem cells transplanted after a rat sciatic nerve severe injury were able to express the Schwann cell markers, such as S100, P75, GFAP and MBP, likely differentiating into myelin-forming cells, thus promoting nerve regeneration (Cui et al. 2008; Ziegler et al. 2011). If embryonic stem cells present the effective proliferation capacity unlike the Schwann cells, on the other hand this capacity makes the risk of teratoma formation *in vivo* higher (Johnson et al. 2010). Additionally, there are immunological rejection and ethical concerns surrounding the use of the embryonic stem cells in cell-based therapy.

Mesenchymal stem cells are adult multipotent cells that, under adequate culture conditions, are capable of transdifferentiating into endodermal (Di Bonzo et al. 2008) and ectodermal (Bossolasco et al. 2005) lineages. A number of experimental studies have shown the potential of mesenchymal stem cells to improve peripheral nerve regeneration (Oliveira et al. 2010; Frattini et al. 2012). The mechanisms by which these cells may act on this process are by paracrine, neuro/axonoprotective or immunomodulatory effects; by transdifferentiation into Scwhann cells; by fusion mechanisms; by modulating cellular behaviors; or even a combination of the above

mechanisms (Oliveira et al. 2013). However, most of the beneficial effects exerted by the mesenchymal stem cells are strongly correlated with the production of neurotrophic factors (Oliveira et al. 2013). Schwann cells can foster mesenchymal stem cells phenotype using a cocktail of cytokine stimulation *in vitro* and these cells may be considered a viable alternative to authentic Schwann cells (Shimizu et al. 2007). Autologous bone marrow mesenchymal stem cells-derived Schwann cell demonstrated to be an effective and safe cell system for peripheral nerve regeneration for up to 1 year (Wakao et al. 2010). Thus, regardless of phenotype maintenance *in vivo*, these cells are able to improve nerve regeneration in a long term observation. Further studies should be done to shed light on the prolonged or maintained beneficial effects of these cells, either by stabilization of the Schwann cell phenotype in the nerve microenvironment or by significantly acting in the first stages of nerve regeneration.

Adipose-derived stem cells are multipotent adult stem cells that have many similarities to mesenchymal stem cells in terms of embryonic origin and cell surface markers. However, adipose-derived stem cells are more abundant and easier to isolate than msenchymal stem cells, which make them an attractive cell source for improving nerve regeneration. Experimental studies have shown that both differentiated and undifferentiated adipose-derived stem cells exert beneficial effects on nerve regeneration. Schwann cell-like adipose stem cells improved sciatic nerve regeneration in similar manner of bone marrow mesenchymal stem cells-derived Schwann cells (di Summa et al. 2010). Regardless of the induced or natural phenotype used, the adipose-derived stem cells have remarkable paracrine effects, secreting growth factors and neurotrophic mediators that enhance nerve regeneration (Widgerow et al. 2013). However, the adipose-derived stem cells comprise heterogenous cell populations, including mesenchymal stem cells, and identification of the optimal cell subpopulation as well as the adipose tissue source that has strong nerve regeneration properties is of great importance for the regenerative medicine field.

Skeletal muscle-derived stem cells have been studied as a novel alternative cell source for peripheral nerve regeneration (Tamaki et al. 2014). These stem cells can be obtained with safety and several works have demonstrated their multipotency. These stem cells have the capacity to differentiate into mesodermal lineage, such as pericytes, endothelial cells and vascular smooth cells, as well as ectodermal lineage, such as Schwann cell and perineurial cells (Tamaki et al. 2005, 2014). The skeletal muscle-derived cells presented greater engraftment and higher number of blood vessels when compared with Schwann cells and bone marrow mesenchymal stem cells in the sciatic nerve crush lesion model. However, there was no difference between the studied cell sources regarding number of myelinated fibers. Further studies using skeletal muscle-derived stem cells in the sciatic nerve transection lesion model should be done to confirm the superior positive effects of these cells over Schwann cells transplant and the autologous nerve graft treatment.

Another novel source of adult multipotent stem cells for cell-based therapy is the hair-follicle stem cells. Interestingly, it was demonstrated that stem cells isolated from the dermis reside in a niche of hair follicle (Li et al. 2003), thus these cell are also called skin-derived stem cells. There is evidence that these cells are capable of

differentiating into several cell types, including glial cells, neurons, smooth muscle cells and melanocytes *in vitro* (Amoh et al. 2005). The follicle bulge region contains cells that are considered, by several criteria, true stem cells. The hair-follicle stem cells were capable of differentiating into Schwann cells and support nerve regeneration in the model of mouse sciatic nerve crush lesion (Amoh et al. 2005). Although hair-follicle stem cells provide an accessible source for autologous transplant, it is a relatively novel cell-based approach in the regenerative medicine field and further studies are needed to clarify their mechanisms of action in the nerve regeneration microenvironment. In addition, comparison of efficacy of hair-follicle stem cells with other well described sources of cell for improving nerve regeneration should be considered in further experimental studies.

Recently, skin mesenchymal precursors have been differentiated into functional Schwann cells under adequate culture conditions (Krause et al. 2014). However these cells were not capable of differentiating into Schwann cells *in vivo*, suggesting that the *in vivo* environment restricts these dermal mesenchymal precursors so that they do not cross lineage boundaries.

With the advent of cellular reprogramming biotechnology, pluripotent stem cells can be induced from adult cells. The great advantage on the use of these cells is the possibility of transplanting patient-specific cell while circumventing the limitations surrounding the embryonic stem cells. Induced pluripotent stem cells are capable of accelerating nerve regeneration and improve functional recovery following a sciatic nerve transection. However, they are not superior to the gold standard of care, autologous nerve repair (Ikeda et al. 2014). Additionally, the mechanisms that shed light on the peripheral nerve regeneration promotion potential of these cells are not clarified yet, and problems related to low production efficiency and safety are not completed solved. So far, there is no protocol available of reprogramming induced pluripotent stem cells or of direct reprogramming of adult cells towards Schwann cells.

7.2.3 Molecules Delivery

Bridging nerve defects with an empty conduit may be insufficient to obtain satisfactory functional recovery. In order to potentiate nerve regeneration, substantial attention has been directed to structural components and growth-promoting factors delivery. The molecules delivery can be mainly accomplished through their incorporation in the conduit wall, allowing a diffusion-based release; through their incorporation in the inner surface of the conduit by affinity; in suspension; or by the loading in hydrogel microspheres. The incorporation in the conduit wall and hydrogel microspheres systems have the advantage of controlled delivery of growth factors over time.

The extracellular matrix not only provide structural support to cells but also plays an important role in successful nerve regeneration, as its constituents interact with Schwann cells, macrophages, endothelial cells and fibroblasts, acting on

cellular behaviors and axonal growth and myelination. The adhesion protein laminin is the major constituent of the basal lamina, which is present in the Schwann and endothelial cells, and may interact mainly with integrin receptors mediating axonal growth. The surface functionalization of polycaprolactone and chitosan scaffolds by cross–linking of laminin favored Schwann cell attachment and proliferation (Junka et al. 2013). Collagen is another important constituent of the extracellular matrix and basal lamina. In form of hydrogels this constituent may improve nerve regeneration. Schwann cells in cell-hydrogel constructs of collagen, laminin and hyaluronic acid did not have their viability altered but increased the production of NGF and BNDF (Suri and Schmidt 2010). Recently, it was demonstrated that the use of thermosensitive collagen hydrogel had an effect on cell viability by avoiding cell loss in the initial adhesion stage *in vitro* (Huang et al. 2014). Another role of collagen is on the improvement of nerve regeneration by promoting machophage recruitment and antiinflammatory phenotype polarization, which represent critical steps on nerve degeneration and regeneration (Chen et al. 2015).

Besides extracellular matrix constitutes, neurotrophic factors are often used as additives for improving nerve regeneration. The neurotrophic factors comprise a family of proteins that plays crucial and complex roles in modulating neural and non-neural cellular behaviors in the regenerating nerve microenvironment. However, the supportive role of these factors released by endogenous cells at the distal nerve stump is not sustained until complete reinnervation takes place due to the decline over time of growth factors synthesis. In this way, delivery of exogenous growth factors is an attempt to provide not only nerve microenvironment with these growth-promoting molecules over a longer period but also at larger amounts. The most used neurotrophic factors are NGF, BDNF and NT-3/4. Other growth factors, such as ciliary neurotrophic factor (CNTF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) have been also used for improving nerve regeneration.

Sustained release of NGF by microspheres improved rat sciatic nerve regeneration after nerve transection lesion followed by conduit repair (Wang et al. 2014c). The growth promoting action of NGF has been studied in adult sensory neurons and it is determined by gp130 signaling via STAT3 activation (Quarta et al. 2014). Since angiogenesis has been long recognized as an important and crucial step during tissue repair, the potent angiogenic fator VEGF-A/B has also been investigated on peripheral nerve regeneration studies (Gnavi et al. 2014). Interestingly, VEGF-B also promotes nerve regeneration independent of vascular effect and it requires PI3K and Notch signaling (Guaiquil et al. 2014).

7.2.4 Gene Therapy

The delivery of growth factors may interfere at different stages of the nerve regeneration process as these molecules can exert additional beneficial actions on the refined molecular microenvironment. However, the delivery system must be sustained and

should overcome the short half-life of the bioactive molecules. The use of gene therapy alone or associated with cell therapy has gained the attention of neuroscientists worldwide because it lies mainly in providing prolonged extra bioactive trophic factors support, within the site of injury, seeking the improvement of nerve regeneration.

Viral vectors have emerged as the most efficient way to the forced expression of therapeutic genes into several tissues. Currently, vectors based on Adeno-Associated and Lenti Virus represent the most commonly used systems for gene transfer in the nervous system (Mason et al. 2011). Non-viral vectors methods encoding therapeutic genes, are also alternatives as gene transfer vehicles in the peripheral nervous system as these vectors systems overcome the citotoxicity elicited by some virus vectors systems.

The three main targets for gene transfer in the peripheral nervous system are Schwann cells, stem cells, injured neurons and skeletal muscle fibers (Oliveira et al. 2014b). After a peripheral nerve injury, Schwann cells play the main supportive role, both by secreting growth-promoting molecules and by axonal remyelination. However, under conditions of chronic injury, Schwann cells lose the capacity of expressing growth factors, which make these glial cells ideally suited as cellular platforms for driving prolonged forced expression of neurotrophic factors.

The use of this therapeutic strategy holds great hope in the nerve repair field because it lies mainly in providing prolonged extra bioactive trophic factors support, within the site of injury, resulting in the improvement of survival of sensory and motor neurons and Schwann cells; increased Schwann cell motility; axon regrowth and remyelination; reinnervation; and, ultimately, functional recovery.

7.2.5 Physical Therapies (LASER, Electrotherapy and Exercises)

Physical Therapy has a key role in the diagnosis and treatment of individuals with peripheral nerve injuries, as they are frequently associated with pain, and sensory and motor dysfunction. A detailed diagnosis of these dysfunctions and its relation to movement and daily activities will help patients to deal with the problems secondary to the peripheral nerve lesion. The main treatment goals are addressed to maintain and restore joint mobility, muscle activity and sensory function, as well as to decrease secondary nerve fiber death after the primary lesion, and to increase regeneration. To achieve these goals, the use of electrical fields, light and ultrasound therapies and exercises have been investigated.

As the literature arising from laboratory animals studies is diversified, and there is very few studies in humans, this review will approach some basic studies, but aims to present the results of clinical trials using physical resources to improve peripheral nerve regeneration.

7.2.5.1 Electrotherapy

The use of weak electrical fields to prevent neuronal loss after a peripheral nerve injury and to promote nerve regeneration has been investigated predominantly in rats and mice. Electric fields applications aim to reestablish an adequate electric environment, increase blood circulation and cell metabolism, and to stimulate the expression of trophic factors. Direct (DC) and Alternate (AC) electrical currents are generally used. Electrical currents are applied by means of needle, surface or special implanted electrodes.

As axonal regeneration is generally directed to the target-organs, monophasic currents like DC may increase the rate of neurite outgrowth through galvanotropism (Rajnicek et al. 1998). Neurites are generally directed to the cathode, as membrane proteins will tend to deposit over the side of the cell membrane oriented to this pole (Yao et al. 2009, 2011; Haan and Song 2014). DC may also increase angiogenesis (Bai et al. 2011), which is generally associated to neurogenesis through VEGF mechanisms.

Although this type of electrical stimulation was frequently used in the first works to investigate its influence on peripheral nerve regeneration, its disadvantages apparently overwhelmed the benefits. DC may provoke tissue lesions through electrophoresis. As in peripheral nerve lesions there is usually loss of sensibility, translating this procedure to humans would increase the risk of burns bellow or around the electrodes.

Alternating electrical currents, conversely, do not lead to electrophoretic phenomenon, but have no directionally properties such as DC. However, this characteristic does not seem to have an important role, as AC has been shown to accelerate peripheral nerve regeneration through the increase in the expression of regeneration-associated genes and BDNF (Al-Majed et al. 2000a, 2004). TGF-Beta may be also upregulated by electric fields. In the initial phase of peripheral nerve regeneration this factor promotes growth cone sprouting, but at the late phase it may maintain regenerated nerve fibers in an unmyelinated condition (Guenard et al. 1995).

In the first study to translate laboratory animals studies into humans, alternating 20Hz electrical currents were used to treat grade III Carpal Tunnel Syndrome patients after surgery (Gordon et al. 2010). They were submitted to 20 Hz, 2 h stimulation just after surgical release of the median nerve, and then assessed periodically for motor and sensory gains. Stimulation promoted axonal regeneration, and accelerated sensory function restoration, without influencing other variables.

Although there is evidence to support the use of DC and AC to promote peripheral nerve regeneration, care should be taken to avoid potential detrimental effects arising from chronic use. One study has shown the use of high- and low-frequency Transcutaneous Electrical Nerve Stimulation (TENS) 5 days a week, over a period of 5 weeks, led to inhibition of crushed sciatic nerves in mice (Baptista et al. 2008). Four or more days of TENS may provoke endogenous opioid pharmacological tolerance (Chandran and Sluka 2003), which is associated with neuronal apoptosis (Mao et al. 2002). Conversely, 2Hz TENS applied for 2 h just after the same crush lesion lead to signs of increased regeneration (Assis et al. 2014). These results

confirm previous findings (Al-Majed et al. 2000a, b; Gordon et al. 2003) that this timeframe is adequate to promote peripheral nerve regeneration regardless of the electrode type. As TENS is a low risk and low cost therapy, it may be regarded as a promising option to stimulate peripheral nerve regeneration in the future.

7.2.5.2 LASER Therapy

Low-level LASER therapy has been investigated since the 1980 decade as an option to promote peripheral nerve regeneration. Its advantages include no sensation during stimulation, no need of contacting electrodes over the skin and locality. However LASER will only act superficially, limiting its use to irradiate deep nerve structures. LASER influences peripheral nerve regeneration through ATP synthesis, cell proliferation and signaling, growth proteins expression (Wang et al. 2014a; Farivar et al. 2014; Agrawal et al. 2014), neurite sprouting and outgrowth (Anders et al. 2014; Mohammed and Kaka 2007).

Wavelength, type of radiation and dose must be selected according to the clinical / pathological condition. The most frequent wavelengths investigated to promote peripheral nerve regeneration are 540 nm, 632.8 nm, 780 nm, although 820/830 nm and 910 nm are also used (Gigo-Benato et al. 2005). The nerve may be irradiated both indirectly through the skin, and directly, over the surgical wound. Doses around 10 J/cm^2 to 150 J/cm^2 are reported, with applications varying from 1 to 90 min (Gigo-Benato et al. 2005).

7.2.5.3 Exercise

Physical exercise is another promising approach for the enhancement of axonal regeneration. The use of exercise training has been advocated by researches and physiotherapists due to its potential to improve motor function after clinical and experimental peripheral nervous lesion (Ilha et al. 2008; Goulart et al. 2014). Recent studies have shown that the use of different exercise protocols can produce the same effects of the electrical stimulation on nerve regeneration (Sabatier et al. 2008; Navarro 2009).

After a nerve injury it is necessary to maintain the muscle activity during the time of reinnervation, and it is well known that passive and active exercise preserves the structure of the end-plate as well as enhances reinnervation of the target organ (Marqueste et al. 2004). Physical activity can increase the expression of many genes associated with synaptic function, such as NF68, synapsin and synaptophysin (Ferreira et al. 2010).

Low intense aerobic exercise is described to increase motor and nerve function especially when performed combining prophylactic and therapeutic approaches (Bobinski et al. 2011). Exercise can also induce pain relief after a nerve trauma, reducing the incidence of a chronic pain condition, called neuropathic pain (Cobianchi et al. 2010).

Despite the numerous reports about the benefits of physical activity, only recently the researchers started to investigate the cellular mechanisms underlying the effectiveness of these activity-associated therapies. Among the many molecular systems that could potentially participate in the benefits of exercise, neurotrophic factors are the most studied.

One of the most important trophic factors is the BDNF. Therapies such as electrical stimulation or exercise usually promote axon regeneration in the PNS by a BDNF signaling mechanism. BDNF has neurotrophic and neuroprotective properties and can affect functions that underlie brain plasticity. Voluntary wheel-running increased the levels of BDNF, NGF and FGF-2 not only in the motor-sensory systems of the brain, but also in hippocampus, a structure normally associated with higher cognitive functions rather than motor activity (Neeper et al. 1996). BDNF is secreted by growth cones of regenerating axons and binds to TrkB receptor.

Peripheral nerve injuries are a common clinical problem, and there are few effective therapies to minimize, or reverse, the associated damage. The approach of combining therapies has proven to be promising, as it allows the sum of the beneficial effects provided by each treatment itself. Thus, the approach of combining two or more therapeutic strategies has been chosen by several research groups as a treatment in order to achieve full functional recovery.

7.3 Translational Studies in PNS Regenerative Therapies

7.3.1 Clinical Relevance

Peripheral nervous system can recover from injuries, however the regeneration can be very slow and incomplete. Many experimental studies have shed light on this field and some of these works have already been tested in clinical trials. All studies attempt to augment the regeneration and accelerate motor and sensory recovery.

7.3.2 Regenerative Therapies in Clinic

7.3.2.1 Autologous Graft

The common procedure after a complete lesion to a nerve is to suture both ends reconnecting them, a procedure called neurorrhaphy (Wang et al. 1996). It is associated with high levels of functional recovery. Nevertheless, there are limitations associated with the use of this technique, such as the loss of nerve substance, delay in the operative repair or severe concomitant injury. When using end-to-end repair, the surgeons have to be careful about putting tension in the coaptation, since stretches greater than 15 % comprises tissue perfusions and results in ischemic damages (Clark et al. 1992; Wood et al. 2011). In cases where there is loss of tissue the autogenous graft has been the procedure of choice, but the functional recovery

is, not as good as direct suture (Wilgis and Maxwell 1979; Millesi et al. 1976; Millesi 1984). The disadvantage of autogenous graft is that it can increase the time of surgery, it can cause morbidity such as local site donor neuroma, sensory loss and hypertrophic scarring (Staniforth and Fisher 1978; Rappaport et al. 1993). These are the reasons that move scientists and clinicians to search for alternative ways of reconnecting the nervous tissue in order to get improvement on functional recovery after severe injuries. In this rationale there are many experimental and clinical studies on bridging the nerve gap left by traumatic lesion.

7.3.2.2 Nerve Guides and Allografts

Those bridges are made of biopolymers and synthetic polymers as primary scaffolds with tailored physical and mechanical properties. They are known as nerve growth conduits. The first generation of nerve conduits used in the clinic was a silicone tube which did not succeed, instead presented many complications, such as compression syndrome and often required a second surgery for removal (Lundborg et al. 1982). Different biomaterials have been approved for clinical use, such as type I collagen, polyglycolic acid (PGA), poly-DL-lactide-co-caprolactone (PLCL), and polyvinyl alcohol (PVA). Currently, there are five types of conduits approved by the U S food and drug administration (FDA). Four of them have a reasonable rate of degradation – 3 months to 4 years – they are the Neurotube (PGA), Neurolac (PLCL), NeuraGen (type I collagen), and NeuroMatrixNeuroflex (type I collagen). However, there is one with an unreasonable FDA-approvement, since it is not biodegradable – SaluBridge (Schlosshauer et al. 2006). Another effective technique that has been adopted by clinical studies of peripheral nerve regeneration is the use of allografts. They have the advantage of keeping the extracellular matrix of the nerve, serving as a mechanical guidance to the axons. The only decellularized allograft commercially available at the moment for clinical studies is one from AxoGen Avance. Nerve allografts can be decellularized by many different protocols and there are not much clinical studies comparing the regeneration performance according to the decellularized protocol. The nerve allograft from AxoGen Avance has been used in facial and hand nerve defect with successful results (Gunn et al. 2010; Karabekmez et al. 2009). Both conduits and allografts are useful tools for dealing with nerve gaps, however, the critical length in nerve gap size for both are not known (Isaacs and Browne 2014). The next generation of nerve conduits will have to be able to associate trophic factors, extracellular matrix proteins and cells into it; experimental studies have had great advance in this field, as discussed here in other section.

7.3.2.3 Electrical Stimulation

Electrotherapy may be used both, to promote peripheral nerve regeneration, and to treat denervated muscles. These two different targets are associated with distinct methods and electrica current patterns. As the scope of this text is to cover peripheral nerve regeneration, we will not include the treatment of the denervated muscle.

Experimental studies show the benefits from electrical stimulation on axon regeneration. Direct electrical stimulation on a transected femoral nerve, immediately after the repair increases the target organ reinnervation and this is associated with accelerated and enhancement overexpression of BDNF and its receptor, tyrosine kinase B receptor (Al-Majed et al. 2000a, b). There is only one study that investigated the effects of ES in humans; it included 21 patients diagnosed with carpal tunnel syndrome ranging from moderate to severe lesion (Gordon et al. 2010). Patients were subjected to 1 h treatment of electrical stimulation or sham stimulation after surgical treatment for carpal tunnel decompression. The group treated with electrical stimulation presented evidences of accelerated axon regeneration and target innervations. More multicenter studies need to be performed in order to evaluate different parameters and types of peripheral nerve injuries to standard clinical practices.

7.3.2.4 LASER Therapy

Low-level LASER therapy to improve peripheral nerve regeneration in humans has been investigated in two studies (de Albornoz et al. 2011). The first (Hazrati 1997) included participants with sensory abnormalities secondary to inferior alveolar nerve lesion. They were irradiated with 880 nm LASER, 48 J/cm^2 in many spots over the lips and internally into the mouth, during 20 sessions. Assessment of nerve damage showed that participants in the active group improved mechanosensitivity and sensory function in general.

The second study included (Rochkind 2009) participants with peripheral nerve and brachial plexus lesions. They were treated with 780 nm LASER, 5 h a day, for 21 consecutive days. Three regions from the damaged portion of the nerve were irradiated for 3 h (45 J/cm^2 a day for each one), and two regions of the corresponding spinal cord for more 2 h (30 J/cm^2 a day for each one). Placebo treatment was accomplished with the same probe, but distant 40 cm from the skin. The results showed that LASER irradiation improved motor, but not sensory function in the active treatment group, but not in the placebo group. Although there are some methodological flaws in this study, especially when reporting data, it has the importance to show that a very intense regimen of treatment with LASER may influence chronic peripheral nerve lesions.

The basic literature about the use of physical resources to improve peripheral nerve regeneration covers a broad spectrum of studies, from cell cultures to laboratory animal experiments. However, few of the knowledge coming from those data have been translated into the recovery of human peripheral nerve injuries. Clinical trials assessing Electrotherapy and LASER therapy to promote nerve healing show promising results, but study design should improve to generate consistent data.

7.3.2.5 Cell Therapy and Gene Therapy

Cell therapy and gene therapy are very exciting therapies to be used in the future in patients with peripheral nerve injuries. Up to date, there are no published studies on humans. This is because many issues still need to be clarified before translation to humans, for instance, appropriate number of cells that need to be injected to cause effect on axon regeneration, the rate of survival of those cells after being injected, type of cell used in the therapy that shows the better outcome on regeneration. There is one clinical trial ongoing that aims to investigate the role of mesenchymal stem cell transfusion on patients with diabetic neuropathy. Hyperglycemia blocks the production of angiogenic and neurotrophic growth factors, which are required for normal function of neurons and glial cells and this leads to diabetic neuropathy a disabling disorder and the sensory symptoms are more prevalent than motor symptoms. The rationale of the study is to take advantage on the paracrine effect of mesenchymal stem cell which secretes growth factors such as bFGF and VEGF (University 2015). Gene therapy has reached a state of progress that is now more suitable to clinical studies, lately the viral vectors are produced completely free of viral gene. There is also one clinical trial using gene therapy, it aims to investigate the safety and efficacy of pl-vegf165 (Neurovasculogen). This is an angiogenic medication that induces growth of new vessels and also nerve regeneration and muscle reinnervation in animals. The investigators want to learn whether this plasmid gene therapy is safe and has efficacy on severe lesion to peripheral nerves (Russia 2015).

7.4 Conclusions and Future Perspectives on PNS Regenerative Therapies

Peripheral nerve injuries with either long-distance defects or occurring far from the target tissue are particularly challenging and, in general, the regeneration process is as difficult as those occurring in central tracts, where regeneration does not occur spontaneously. In these cases of severe nerve injuries a direct neurorraphy is not possible and surgeons make the use of nerve autograft/allograft or nerve transfer to repair the lesioned nerve. Unfortunately, all these techniques present disadvantages and the results obtained are far from being satisfactory. More recently, nerve conduits have been used in the clinical set but only for short defects or very thin nerves such as the digital ones. In the experimental set there is a continuous search for ideal conduits that can overcome the limitations of the commercially available nerve conduits. Since these tubes can be associated with other pro-regenerative strategies such as cell therapy, gene therapy, growth-factors, among others, it is possible that in the near future more complex tubes will be available for clinical use. In addition, the clinical use of other strategies such as electrical stimulation, LASER therapy, exercise and others can bring better outcomes in terms of full reestablishment of the lost function.

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Chapter 8
Regenerative Therapies for the Ocular Surface

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Abstract Integrity of ocular surface depends on adequate tear film and stability of the surface epithelium consisting of two specialized phenotypically different epithelial cells, the central transparent corneal epithelium and the peripheral conjunctival cells, separated by a more specialized transition zone, called the limbus. Similar to the epithelial regeneration in other parts of the body, the corneal epithelium is regenerated from the stem cells located in limbus. Severe chemical burns and other diseases can cause damage to the limbus, resulting in a condition called Limbal Stem Cell Deficiency (LSCD). Effective therapeutic modalities for this vision-threatening condition include use of human amniotic membrane, replenishing the stores of limbal stem cells by limbal transplantation. However last decade has witnessed the use of ex-vivo expanded sheet of limbal epithelial cells for ocular surface reconstruction in such cases. Our group has established a simple, feeder-cell free, cost-effective way of culturing the corneal epithelium from limbal tissues within 2 weeks, using human amniotic membrane as a vehicle. The interim results of a clinical trial involving 700 patients with severe unilateral and bilateral LSCD revealed

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70 % and 50 % success at the end of 3 and 5 years respectively. For patients affected by bilateral disease, options include use of allogenic tissues with immunosuppressive therapy or use of autologous alternative sources of epithelium like oral mucosal epithelium, both of which show limited success. The pre-requisites for cell therapy are that the desired cells should be grown in sufficient amounts, should survive, integrate and network with the host tissues and cause no harm to the recipient. All these criteria are fulfilled when Limbal Epithelial cell therapy is used for ocular surface reconstruction thus making it a successful model in the emerging field of regenerative medicine.

Keywords Limbal stem cells • Limbal stem cell deficiency • Cultivated limbal stem cell transplantation • Tissue engineering • Scaffolds • Microarrays • Proteomics • Cryopreservation • Clinical outcome

8.1 Introduction

The integrity of ocular surface is a cumulative function achieved by the co-ordination and interdependence of the ocular surface elements (epithelia and adnexa of the eye) and the tear film. The cornea, the central transparent part of the eye that contributes significantly to normal vision, is made up of five layers; the epithelium, Bowman's membrane, stroma, Descemet's membrane, and the endothelium. The homeostasis of epithelium is maintained by stem cells located in the region know as the limbus (Thoft and Friend 1983). In the normal uninjured state, LSC are mitotically quiescent and maintained in a specialized limbal stromal microenvironment or "niche". However, on corneal epithelial wounding, stem cells located in the limbus (Hanna 1966; Davanger and Evensen 1971) proliferate to generate more stem cells and transient amplifying cells and migrate centripetally so as to replace the damaged corneal epithelium. Any damage to this functional and physical interdependent structures leads to vision threatening conditions ranging from decreased to complete loss of vision posing a challenge to the clinicians to reconstruct the ocular surface. The most important of such conditions is the Limbal Stem Cell Deficiency (LSCD) caused by a number of individual or environmental or incident specific factors (Dua and Azuara-Blanco 2000; Chen and Tseng 1991; Kruse et al. 1990). Limbal stem cell deficiency is a challenging clinical problem, the current treatment of which involves replenishing the depleted limbal stem cell (LSC) pool by either limbal tissue transplantation or use of cultivated limbal epithelial cells (LEC).

8.2 Limbal Stem Cells: Development, Stem Cell Function

Cornea is the transparent window on the ocular surface that allows light rays to pass through the anterior chamber and contributes to 60 % of the total refractive power of an eye. Corneal surface consists of a 5–6 cell thickness stratified epithelial layer.

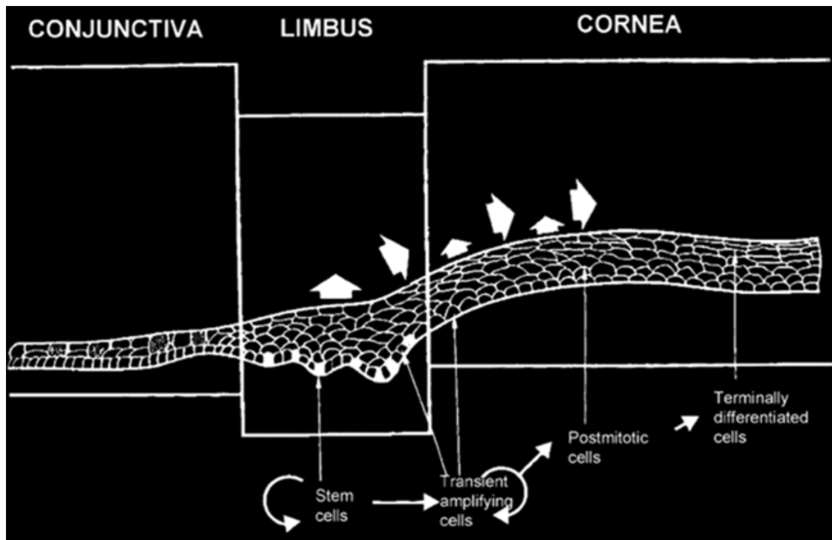

Fig. 8.1 Schematic diagram showing location of cornea, limbus and corneal epithelia. During homoestasis as well as during wound healign, the limbal stem cells from limbal region, proliferate, form transient amplying cells that move centripetally into the cornea, mature and differentiate into the corneal epithelial cells

The narrow zone of about 2 mm thickness between the cornea and the bulbar conjunctiva is known as the limbus and is widely accepted as the niche for the corneal epithelial stem cells (Fig. 8.1) (Hanna 1966; Srinivasan et al. 1979; Cortsarelis et al. 1989). The limbal epithelium is 10–12 cell layers thick, and contains melanocytes, Langerhan cells and an underlying network of blood vessels in the limbal stroma. The limbal stroma with its overlying epithelium is arranged in radial fibrovascular elevations called the Palisades of Vogt, which alternate with epithelial rete ridges (Van Buskirk 1989). These palisades are present 360° all around the corneo-scleral rim but are most well defined in the inferior and the superior zones.

The stem cells located at the limbal region play an important role in the normal maintenance of the corneal surface. The corneal epithelium is a highly dynamic structure and is constantly renewed all through the adulthood of an eye. The activated stem cells from the limbus migrate centripetally to the central cornea and helps in tissue homeostasis. As explained by XYZ theory (Thoft and Friend 1983) of corneal maintenance, the combined rate of cell proliferation and centripetal migration of the activated limbal stem cells (XY component) is equal to the rate of cell loss by desquamation (Z component).

Limbal epithelial stem cells (LESC) share common features with other adult somatic stem cells including small size (Bickenbach 1981; Holbrook and Odland 1975) and high nuclear to cytoplasmic ratio (Epstein et al. 2005). They also lack expression of differentiation markers such as cytokeratins 3 and 12 (Schermer et al. 1986; Fatima et al. 2006). LESCs are slow cycling during homeostasis and therefore retain DNA labels for long time periods, however in the event of corneal injury they can become highly proliferative (Park et al. 2006; Cotsarelis et al. 1989; Haskjold

et al. 1989; Thompson et al. 1991) and contribute actively to healing of the wound. The basal epithelial stem cells located at the limbal niche express some of the markers such as, ΔNp63α (Pellegrini et al. 2001; Di Iorio et al. 2005), ABCG2 (Watanabe et al. 2004; Chen et al. 2004), C/EBPδ, Bmi1 (Barbaro et al. 2007), TCF4 (Lu et al. 2012), Frizzled 7 (Mei et al. 2014), ABCB5 (Ksander et al. 2014), SSEA4 (Mariappan et al 2014) and few others. These stem cells divide asymmetrically to maintain the reserve stem cell pool within the limbal niche. The limbal location of corneal epithelial stem cells provides a number of functional advantages. The basal cells of the cornea are devoid of any pigments and are highly susceptible to radiation induced damages. But the limbal region is pigmented and therefore the basal stem cells do not face this constraint. Also, the limbal epithelium has a highly undulating epithelial- stromal junctions and therefore are resistant to the shearing forces and provides maximum protection to the corneal stem cells (Van Buskirk 1989). It is important tot note that while the cornea is avascular, the limbal epithelium has a vascular bed which provides for the nourishment and maintenance of the limbal stem cells.

During wound healing process, the stem cells get activated and the transiently amplifying cells migrate in a centripetal manner which is a unique characteristic of the limbal epithelium. They undergo cell division and migrate centripetally towards the central cornea and also show a basal to upward movement towards the corneal surface. Once the epithelial cells leave the limbal basal layer or the limbal niche, they gradually activate the differentiation programme and differentiate into transiently amplifying cells (TAC) with lesser proliferative capacity and reduced stemness. Prior to or during the course of migration, these TAC become terminally differentiated and contribute to corneal wound healing by establishing cell-cell contacts through the formation of desmosomes, hemidesmosomes and tight junctions (Chen and Tseng 1990). Several reports in the past two decades have confirmed the limbal location of corneal epithelial stem cells and the role they play in regenerating the corneal epithelium (Sangwan and Tseng 2001; Zieske et al. 1992; Cotsarelis 1989). Recent reports have reconfirmed this fact using multi colored genetic tracers and *in vivo* imaging of proliferating cells within and between clonally propagating corneal epithelial stripes by fluorescence spectral imaging (Di Girolamo et al. 2015; Amitai-Lange et al. 2015).

8.3 Ocular Surface Diseases- Regenerative Principles

The mechanism by which ocular surface health is ensured is built into the intimate relationship between ocular surface epithelium and the periocular tear film. Damage to ocular surface caused by chemical, thermal, mechanical injuries, or immune-mediated diseases results in limbal stem cell deficiency. This is manifested as vascularization and chronic inflammation of the cornea, ingrowth of conjunctiva on to the cornea (conjunctivalization), corneal opacification, and persistent or recurrent corneal epithelial defects (Chen and Tseng 1990, 1991; Kruse et al. 1990). This

leads to the decrease or loss of vision in the affected eye with pain, redness, watering, and light sensitivity. There are a variety of causes for the development of limbal stem cell deficiency (LSCD) Table 8.1. LSCD is typically characterized by the invasion of conjunctival epithelium onto the corneal surface leading to conjunctivalization, neo-vascularization, subepithelial scarring and symblepharon formation

Table 8.1 Etiology of limbal stem cell deficiency (LSCD)

(A) Primary LSCD
1. Hereditary aniridia
2. Ectodermal dysplasia or other congenital connective tissue disorders
3. Neurotrophic keratopathy
4. Sclerocornea
(B) Secondary LSCD
1. Trauma
(a) Chemical injuries
(i) Acid injuries
(ii) Alkali injuries
(b) Thermal injuries
(c) Radiation injury
(i) Ultraviolet radiation
(ii) Ionizing radiation
2. Systemic conditions
(a) Steven Johnson Syndrome (SJS)
(b) Ocular cicatricial pemphigoid (OCP)\Pseudopemphigoid
(c) Multiple endocrine disorders
(d) Vitamin A deficiency
3. Iatrogenic:
(a) Multiple ocular surgeries, eg:-excision of pterygia, pseudoptherygia, limbal neoplasm
(b) Cyclocryotherapy
(c) Antimetabolites (topical mitomycin C (MMC))
(d) Systemic chemotherapy
(e) Contact lens wear
4. Severe ocular surface diseases:
(a) Keratoconjunctivitis sicca
(b) Post-infectious keratitis
(c) Neurotrophic keratitis
(d) Vernal/atopic keratoconjunctivitis
(e) Rosacea blepharoconjunctivitis
(f) Phlyctenular disease
(g)Tumors
(h) Pterygium

resulting in corneal opacity and visual impairment apart from varying degrees of discomfort which includes redness, irritation and watering in the affected eye (Vemuganti et al. 2009). The definitive treatment for LSCD is limbal transplantation using auto or allografts of limbal tissues, each of which are associated with different risks and benefits. The success of limbal stem cell transplantation is determined by a variety of factors and may be adversely affected by concomitant lid pathology, dry eye and uncontrolled systemic disorders. Hence, the management of associated adenexal conditions such as eyelid/eyelash disposition and management of dry eye is a pre-requisite for a successful ocular surface reconstruction and needs to precede stem cell transplantation. Increasing knowledge in the biology of limbal stem cells have opened up new avenues of ocular surface regeneration using the principles of stem cells, regenerative medicine and tissue engineering (Sangwan and Tseng 2001; Tsubota et al.1999; Sangwan et al. 2003).

8.4 Clinical Principles, Diagnostics, Indications of Ocular Surface Regenerative Therapies

The clinical principle in treating severe Limbal Stem Cell Deficiency is the replacement of depleted limbal stem cell stores by surgical intervention. Various surgical techniques of limbal transplantation using cadaveric (Tsubota et al.1999) or live-related donor tissues have evolved for the treatment of bilateral limbal stem cell deficiency (LSCD) (Rao SK et al. 1999a, b; Daya and Ilari 2001). However, these methods have met with limited success and allografts require immunosuppression for indefinite period to avoid rejection (Solomon et al. 2002; Ilari and Daya 2002). Long-term systemic immunosuppression involves the risk of serious eye and systemic complications, apart from being a significant economic burden. For unilateral total or partial limbal stem cell deficiency (LSCD) direct or cultivated autologus limbal transplantation works very well. With direct limbal stem cell transplantation there is a need for transplanting 3–6 o' clock hours of healthy limbus from the contralateral eye. This technique could potentially lead to limbal stem cell deficiency at the donor site. To overcome this, a novel way of expanding the stem cell population of limbal tissue has been adopted and has revolutioned the field of ocular surface reconstruction in last decade.

In case of unilateral LSCD, the treatment option includes transplantation of (i) human amniotic membrane (hAM) (Tseng et al. 1998) (ii) healthy limbal tissues or (ii) in vitro cultured limbal epithelial stem cells. Over the past decade, cultured limbal epithelial stem cell transplantation (CLET) has been widely accepted as a standard approach for the ocular surface reconstruction of patients with LSCD. (Pellegrini et al. 1997; Schwab et al. 2000; Tsai et al. 2000a, b; Sangwan et al. 2005; Baradaran-Rafii et al. 2010; Kolli et al. 2010; Mariappan et al. 2010). Cultured limbal epithelial transplantation (CLET) followed by penetrating keratoplasty (PKP) has resulted in long term graft survival and improved visual outcome (Sangwan et al. 2005) (Fig. 8.2). Another technique described for focal LSCD is

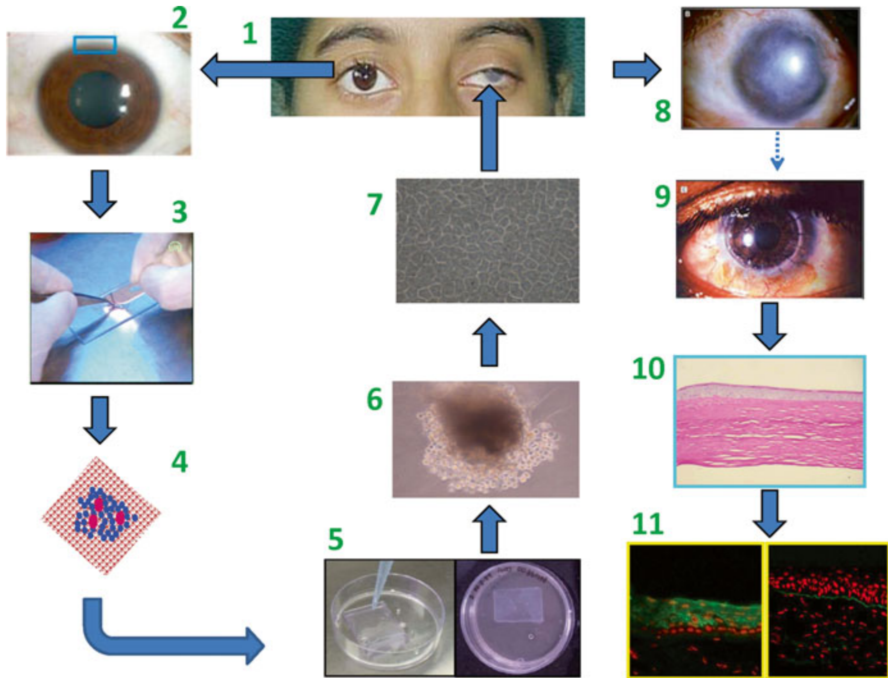

Fig. 8.2 The figure shows the chronological events for cultivated limbal epithelial transplantation. *1* Identifying patients with clinical features of unilateral severe limbal stem cell deficiency. *2* Harvest limbal biopsy from the healthy contralateral eye for ex-vivo expansion. *3* The harvested biopsy is fragmented into tiny explants for cultivation. *4* Ex-vivo Expansion of cells: By a feeder cell free method of explant culture on denuded human amniotic membrane. *5* The cultures are incubated at 37 °C × 2 weeks using human corneal epithelial cell medium. *6* The cultures are observed for growth of cells. Note the presence of cells arising from the edge of the explant by day 2–3. *7* Formation of a monolayer of epithelial cells by day 10–12. *8* Reconstruction of the epithelium of the diseased eye – By transplanting the epithelial sheet derived from autologus limbal tissue on HAM. *9* After stablization of the ocular surface by the transplanted cells,some of the patients may undergo penetrating keratoplasty for visual rehabilitation. *10* The study of the corneal button removed from the patient, after cultivated limbal epithelial transplantation provides the objective proof of survival , maturation and integration of transplanted cells. *11* The corneal epithelim is intact, stratified and adherent to the corneal stroma (Note the formation of its basement membrane as seen by immunofluorescence studies for Collagen IV- a basement membrane marker)

ipsilateral translocation of healthy limbal tissue to an area of partial LSCD. In a small series of patients, this has been shown to provide good outcomes (Nishiwaki-Dantas et al. 2001). However this approach is not well studied and understood for the management of partial LSCD. The visual acuity of a patient with ocular surface disease who has undergone limbal stem cell transplantation may improve without any further surgical intervention. However, in cases where the corneal stromal opacification hampers visual recovery, a penetrating keratoplasty (PK) may be necessary. While some authors suggest PK and limbal stem cell transplantation to be done at one sitting (Rao et al. 1999a, b; Theng and Tan 1997); Croasdale et al.

(1999) recommends an interval of 3 months between the two and some others (Ilari and Daya 2002) recommend a deep lamellar keratoplasty one year post limbal stem cell transplantation, if the endothelium is healthy. In our experience, the latter approach of staged surface reconstruction followed by subsequent corneal transplantation (lamellar in any situation where the endothelium is deemed healthy and penetrating in eyes with endothelial injury) is the preferred approach to visual rehabilitation of such eyes.

The technique of ex vivo expansion of limbal stem cells and their subsequent transplantation offers certain advantages. A small amount of tissue needs to be harvested – either from the healthy fellow eye of a patient with unilateral LSCD (contra lateral) or from healthy limbus of the partially affected same eye (ipsilateral) or from the phthisical other eye with healthy limbus or from the healthy eye of a living related donor of a patient with bilateral LSCD or from cadaveric limbal rings for cultivated limbal allograts. This reduces the risk of an iatrogenic LSCD in the donor eye and allows the possibility of a second biopsy being taken, if required. Owing to the absence of the antigen-presenting Langerhans' cells, these bioengineered sheets are less immunogenic. In vitro culture also ensures that cells are viable and proliferating as opposed to the cadaveric limbal transplantation. We have shown in our laboratory that (Vemuganti et al. 2004) chances of cell growth from live limbal tissues is 100 % while those from the cadaveric tisues showed only 42 % cell growth.

LSCD may occur due to primary or secondary causes (Puangsricharen and Tseng 1995) Primary LSCD is characterized by absence of identifiable external factors. In these cases, the dysfunction of stromal microenvironment of limbal epithelial stem cells results in gradual loss of stem cell population or generation and amplification of transient amplifying cells (Vemuganti et al. 2009). Secondary LSCD occurs due to the destruction of LSCs by external factors as seen in trauma, systemic conditions, iatrogenic causes and severe ocular disease (Vemuganti et al. 2009).

We have earlier reported our surgical techniques for cultivated limbal epithelium transplantation (Sangwan et al. 2005). After obtaining informed consent from the patients or guardians, limbal biopsy is performed on the healthy contralateral eye or a healthy area of the same eye for autologus procedure and for allograft biopsy is harvested of live-related donor or cadaveris limbal rings obtained from the eye bank. The procedure involves careful dissection of a 2×2 mm piece of limbal epithelium with 0.5 mm into clear corneal stromal tissue at the limbus under strict aseptic conditions under local or general anesthesia, depending on patient's preference and age. The limbal tissue that contains limbal epithelial cells at the pigmented line (palisades of Vogt) and a part of the corneal stroma is excised. If limbal stem cell deficiency is bilateral, then tissue can be taken from live-related donor or cadaveric source. Earlier reports from our group (Vemuganti et al. 2004) have shown that fresh limbal tissue with 100 % viability is preferable to a preserved cadaveric limbal tissue with 51.6 % viability for culture.

However the technique of culturing these cells has been varying with different investigators. The variations in the culture technique involves the preference for the type of biopsy (cadaveric or fresh), choice of substrate (human amniotic membrane, Fibrin, Collagen), presence or absence of feeder cells, type of medium

(HEC, DMEM), type of culture inserts used, use of autologous human serum or fetal calf serum, submerged or air-lift technique (Vemuganti et al. 2009). Studies on *in vitro* expanded limbal cells have shown that explants cultures retained the native niche and allowed expansion of both the niche cells and stem cells in culture (Polisetty et al. 2008; Mariappan et al. 2014) and could contributes to better clinical outcomes. Based on our experience from treating more than 800 cases, we believe that a feeder-cell free method of submerged explant culture technique on human amniotic membrane using the autologus serum is a cost effective and efficient method of generating corneal epithelial monolayer for clinical transplantation.

8.5 Standardized Treatment, Technologies

An ideal substrate for cultivation of limbal epithelial cells, should have high optical clarity, appropriate refractive index, dimensions as that of cornea, toughness to withstand surgical procedure i.e., adequately robust for implantation, nontoxic, non-immunogenic, non-inflammatory, and most importantly promote regeneration of corneal cells and nerves. Human amniotic membrane has been extensively used for ocular surface reconstruction and has properties, which facilitates the growth of epithelial cells, suppresses inflammation, and has antibacterial and anti-apoptotic properties, thus making it an ideal scaffold for clinical application.

We used the standard protocol proposed by Kim et al. (1995) to prepare the human amniotic membrane. In brief, the placenta (which has two layers called amnion and chorion) obtained from the caesarian section delivery was used to prepare human amniotic membrane (hAM). After screening the donor for HIV, HBs Ag and VDRL, the placenta is placed in a sterile pan and washed repeatedly with antibiotic containing ringer lactate/normal saline until clear water is obtained. The placenta is then transferred aseptically to another sterile pan and carried to the laminar flow hood, which is pre-cleaned, and UV sterilized. AM is peeled, separating the amnion and chorion. The stretched membrane is cleaned using a cotton swab and intermittent wetting with ringer lactate/normal saline using wash bottle. Once a clear membrane surface is available, a sterile nitrocellulose paper of dimension 7.5 × 7.5 cm is attached to the chorion side, keeping the epithelial side exposed to the top surface. The AM was cut around the paper while rolling the edges on the other side of the paper. (AM should be sticked to the nitrocellulose paper perfectly without gaps or air-bubbles). The nitrocellulose paper was then cut to get small pieces of membrane as required. The AM pieces (2.5 × 2.5, 2.5 × 5, 5 × 5 cm) were then inserted in vials containing Dulbecco's Modified Eagles Medium (DMEM) and stored at −80 °C. Just before use, the AM was thawed at 37 °C for 30 min. Apart from hAM, several other substrates have been tested and successfully used for the culture, expansion and transfer of limbal epithelial cells. Some of them includes the mitotically inactivated mouse NIH 3T3 feeder cells (Pellegrini et al. 1997), fibrin gels (Rama et al. 2001), temperature-responsive culture inserts (Hayashida et al. 2006), Myogel (Francis et al. 2009), biodegradable matrices made

Table 8.2 Composition of Human Corneal Epithelium Medium (HCEM) for culture of limbal epithelial cells

S.No.	Ingredients	Quantity per liter	Company
1.	Minimal essential medium	5.05 g	Sigma, Cat. No. M0644
2.	Nutrient mixture Ham's F-12	5.30 g	Sigma, Cat. No. N6760
3.	Sodium bicarbonate	1.688 g	Sigma, Cat. No. S5761
4.	Penicillin G sodium salt (100 U/ml)	0.1 MU	(Sigma, Cat No. P3032)
5.	Stereptomycin sulphate (100 µg/ml)	100 mg	(Sigma, Cat No. S9137)
6.	Gentamicin sulfate (5 µg/ml)	5 mg	(Sigma, Cat No. G1264)
7.	Amphoterecin B (2.5 µg/ml)	10 ml of 100X stock	Sigma, Cat No. A2942
8.	Epidermal growth factor (10 ng/ml)	100 µl of 100 µg/ml stock	Sigma, Cat No. E9644
9.	Insulin (5 µg/ml)	100 µl of 50 mg/ml stock	Sigma, Cat No. I0259

Method of Preparation: Add the first two ingredients in 500 ml of Milli Q water, in a sterile 1 l flask/beaker, add sodium bicarbonate, add the antibiotics and stir to dissolve. Adjust the pH to 7.2 with 1 N HCl or 1 N NaOH while stirring and make up the volume to 900 ml. Add the growth factors, mix well and filter sterilize the medium by vacuum filtration through a 0.22 µm membrane filter. Perform sterility check by streaking on to chocolate agar and blood agar plates and by inoculating into thioglycollate broth before use. The medium is then stored at 4 °C. The shelf life of the medium is about 15 days as after that the pH of the medium increases as indicated by change in color (phenol red) towards more pinkish

Separation of autologous serum: About 10-ml blood is drawn from the patient in non-heparin, 15 ml graduated falcon tubes. The blood is kept at room temperature undisturbed for a couple of hours to allow the settlement of cellular components. It is then centrifuged at 2000 rpm for 10 min. The serum supernatant is pipetted out in a fresh falcon and filter sterilized using 0.22 µm millipore syringe filters, and used in the culture medium at 10 % concentration

of human recombinant collagen (Merrett et al. 2008), synthetic PLGA-based scaffolds (Deshpande et al. 2013) and plasma polymer–coated surfaces (Deshpande et al. 2010; Brown et al. 2014). The composition of Human Corneal Epithelial (HCE) medium used to culture limbal cells is described in Table 8.2.

A corneal tissue replacement or a substrate for cultivation of limbal epithelial cells, should have high optical clarity, appropriate refractive index, dimensions as that of cornea, toughness to withstand surgical procedure i.e., adequately robust for implantation, nontoxic, non-immunogenic, non-inflammatory, and most importantly promote regeneration of corneal cells and nerves.

A careful clinical examination is required for all the potential patients of cultivated limbal epithelial transplantation (CLET). Ocular adnexa should be examined for any defects, if found to have lid notch or defects, then it should be repaired prior to CLET. The patients should not have surface anesthesia as found in patients with herpes zoster ophthalmicus or due to seventh nerve paralysis. If there is underlying immune-mediated inflammation as seen in ocular cicatricial pemphigoid (OCP) or Stevens-Johnson syndrome (SJS), then it should be treated with appropriate therapy before doing CLET. If the limbal stem cell deficiency (LSCD) is due to chemical injury then enough time should be allowed before attempting ocular surface recon-

struction so that inflammation subsides and chances of survival of transplanted epithelial cells improves. The visual potential of the eye should be carefully assessed before doing the surgery especially in children where there is a possibility of amblyopia.

The surgery is performed under local or general anesthesia, based on patient's choice as well as patient's age. In younger children general anesthesia is preferred. A drop of epinephrine (1: 1000) is instilled in the conjunctival cul-de-sac prior to pannus excision to decrease bleeding during the procedure. Dissection of ocular surface pannus is started 2–3 mm behind the limbus if this landmark is still visible. Blunt tip spring scissors or conjunctival scissors are used to initiate the dissection by lifting the pannus and dissection is continued all around the limbus with a # 15 blade on Bard Parker handle. A combination of sharp and blunt dissection from the periphery towards the central area carried out to clean the cornea. It is important to pay attention to the thickness of the corneal tissue, which may be variable due to the effects of chemical or disease process, and it is vital not to perforate the cornea. Any accidental perforation, which can be identified by softening of the globe, should be repaired either by suturing or by application of fibrin tissue sealant. If the perforation is too large to be repaired then lamellar or penetrating keratoplasty must be performed along with cultivated limbal stem cell transplantation. Perforation is uncommon if the tissue planes are respected by the surgeon. The ocular surface is thus cleared of the fibrovascular pannus and hemostasis is achieved by judicious use of bipolar cautery. The adhesions between the eyeball and lids (symblephara) are released taking due care not to injure the extraocular muscles and other tissues. Fornix is reconstructed using amniotic membrane, and fornix-forming suture if indicated. At this stage the corneal thickness is assessed clinically and by pachymetry. If the recipient corneal thickness is less than 300 μm, then a lamellar corneal grafting is done to restore the thickness of the cornea; this will help in supporting the epithelization of ocular surface following cultivated epithelial transplantation. At this stage, the cultivated limbal epithelium on human amniotic membrane is brought on to the ocular surface, cell side up, aseptically, and the membrane containing the cultured cells is gently spread over the cornea as well as limbus without damaging or dislodging the cells. The area of maximum number of visible explants with surrounding cells is kept at the center of cornea and the peripheral part of membrane is trimmed using blunt tipped scissors. There are two ways of anchoring the composite of membrane and cells on to the ocular surface. If sutures are used then, 10-0 monofilament nylon material is preferred. The tissue should be anchored using circumferential, interrupted sutures at the limbus. Peripheral edge of the membrane is sutured to conjunctiva with 8-0 polyglactin (Vicryl™) sutures. Usually 6-8 interrupted sutures are enough to secure the membrane and care should be taken to avoid any folds or wrinkles of the membrane. If they are present, then the tissue should be stretched and unfolded, holding the peripheral part of the membrane. The central part of the membrane, which has maximum cellular growth, is never touched because it is critical for re-epithelization. If there is inadvertent needle perforation, recognized by softening of the eye, then the suture should be taken at another location. Needle perforations usually do not require repair. If sutureless surgery is

planned , the fibrin tissue sealant (Tissel™ Kit; Baxter AG, Vienna, Austria) is put on the ocular surface using a tuberculin syringe with 27 gauge needle. Both components are put one after another and the membrane with grown cells is brought on the ocular surface and spread as explained above. Excess membrane is trimmed and peripheral part of the membrane is ironed out in order to avoid wrinkles or folds of the membrane. The membrane is touched with a surgical sponge in order to test if the adhesion is sufficient to hold the tissue in place. Sometime there may fluid accumulation under the membrane during postoperative period. To avoid this complication two small holes are made in the membrane at limbus at 5 o' clock and 7 o' clock so the fluid is drained out.

At the conclusion of the surgery, one drop of 2.5 % povidone iodine is instilled; use of a bandage contact lens is optional. Some surgeons recommend using a bandage contact lens for the first few weeks, to avoid loss of cells due to blinking. For the same reason some surgeons prefer to induce ptosis by injecting botulinum toxin in orbicularis oculi. We do not use any of these techniques because we believe that there are firm adhesions between the membrane and growing cells. The technique described above is for total limbal stem cell deficiency where fibrovascular pannus covers the entire ocular surface. If there is focal or sectoral deficiency the surgical technique is tailored to cover the defect created by removal of abnormal tissue. Either sutures or fibrin glue can be used to anchor the membrane with cultivated cells to the ocular surface. Briefly, the procedure for the transplantation of cultivated limbal epithelial cells is represented in Fig. 8.2.

Following the cultivated limbal epithelium transplantation, all the patients are treated with prednisolone acetate 1 % eye drops or any other equivalent topical steroids drops, 8 times a day, tapered to once a day in 5–6 weeks, and ciprofloxacin hydrochloride 0.3 % eye drops, 4 times a day, for 1 week. Ciprofloxacin hydrochloride 0.3 % eye drops are continued if an epithelial defect is present or as long as the bandage contact lens is used. If a limbal allograft is done then the patient should be given systemic immunosuppression, as has been reported earlier (Sangwan et al. 2005). The patients are examined on postoperative day one, one week, two weeks, five weeks and monthly thereafter for 6 months. Thereafter, follow up is customized to success or failure of the procedure. If successful and does not require further interventions then follow up is every 6 month for 3 years and thereafter annually. For failed surgeries process could be repeated or appropriate alternative therapy offered. Each examination includes a complete history, noting of new ocular or systemic symptoms, and complete evaluation of recipient as well as donor sites and any signs of neovascularization or surface instability. Sutures are removed when indicated (loose or vascularized). Epithelialized sutures are left indefinitely. Some of the patients with CLET may require penetrating keratoplasty or deep anterior lamellar keratoplasty if the vision does not improve with CLEt alone.

Ocular surface reconstruction in limbal stem cell deficiency is a multi-staged procedure, with the patient often requiring several surgical interventions for visual rehabilitation. Some of these patients may require cataract extraction after successful CLET which can be done in a standard way. If there is stromal scarring and

CLET have been successful then penetrating keratoplasty (PKP) or deep anterior lamellar keratoplasty (DALK) can be done to improve the vision.

Sangwan et al. (2005) described the early results of penetrating keratoplasty (PKP) in patients who had previously undergone cultivated limbal epithelium transplantation. Demographics, primary etiology, type of limbal transplantation, ocular surface stability, visual acuity, graft clarity, and complications were reviewed. Histopathologic features of the recipient corneal buttons were studied with special attention to epithelial status (Fig. 8.2). PKP was performed at a mean interval of 7 months (range, 2–12 months) following cultivated limbal epithelium transplantation (autologous, n = 11; allogenic, n = 4). Fourteen (93 %) of the 15 eyes had a successful corneal graft with a stable corneal epithelium. Preoperative best-corrected visual acuity was less than 20/200 in 14 of the 15 eyes. At a mean follow- up of 8.3 months after PKP, the best-corrected visual acuity was more than 20/60 in 8 eyes, 20/200 to 20/60 in 5 eyes, and less than 20/200 in 2 eyes. Three of the 15 eyes experienced corneal allograft rejection, which was managed successfully. None of the limbal epithelial allografts showed signs of rejection.

These cases did not show a high rejection rate (overall rejection rate, 20 %) despite the young age of the recipients and stromal vascularization nor a high non–rejection-related failure as expected in cases of chemical burns. This could be attributed attributed to the cultivated limbal epithelium transplantation procedure preceding the PKP, which continued to supply healthy epithelium after PKP. Similarly, the fewer corneal graft rejection episodes could be due to the stepwise, versus the simultaneous, approach, which included ocular surface reconstruction by cultivated limbal epithelium transplantation in the first step and PKP in the second.

The proponents of simultaneous limbal stem cell transplantation and penetrating keratoplasty highlight the potential of this approach to minimize recipient antigenic load by using the same donor tissue for both the limbal stem cells and the keratoplasty procedure. However, ex vivo expanded limbal epithelium transplantation requires a minimum period of 2 weeks for ex vivo expansion of limbal stem cells. A staged approach allows achievement of a stable ocular surface prior to the penetrating keratoplasty and allows sufficient time for inflammation to subside, thereby increasing the chances of a successful penetrating keratoplasty. The low rejection rate may also be ascribed to the fact that the cultivated limbal epithelium is devoid of Langerhans cells (Holland et al. 1987). The anti-inflammatory role of the amniotic membrane carrier should also be kept in mind.

As such patients have already undergone pannus resection with or without superficial keratectomy, the recipient corneal stromal bed is usually thin and irregular, which could result in postoperative astigmatism. In addition, associated conditions, such as eyelid abnormalities, glaucoma, and dry eye syndrome, may affect the outcome and hence must be treated before PKP.

8.6 Clinical Studies, Experience, Outcome/Side Effects of "Ocular Surface " Regenerative Therapies

While the literature has consistently seen reports on the short and intermediate term efficacy of stem cell transplantation for ocular surface reconstruction, it must be mentioned that all of these studies are limited in that they are retrospective, non-randomized, the number of eyes in the cohorts being small and relatively short durations of follow-up. It is clear from the literature that autograft transplantation is efficacious for both transplanted limbal tissue (Kenyon and Tseng 1989; Rao et al. 1999a, b; Shimazaki 2006) as well as for cultivated epithelial transplants (Tsai et al. 2000a, b; Rama et al. 2001; Sangwan 2005), but is limited by the relatively short follow-up .

Ocular surface reconstruction by cell therapy provides a unique opportunity to document the survival, networking, and integration of transplanted cells, through various techniques. A thorough clinical evaluation for presence of epithelial integrity, lack of staining by fluorescein dyes, absence of vascularization, and corneal clarity is indicative of successful transplantation. A novel form of non-invasive method of documenting the survival and stratification of the transplanted cells is by clinical confocal microscopy which documents the multilayering of corneal epithelial cells, and remnants of the degraded HAM (if any). A minimally invasive method of documenting the proof of survival of transplanted cells is by impression cytology of the ocular surface which provides information on the phenotype of the cells lining epithelium the central corneal region. Presence of corneal phenotype (K3+, K19-) and the absence of goblet cells indicate suggest successful transplantation.

A recent study by Pauklin et al. 2009 on pannus tissue excised from LSCD patients showed that epithelial lineage marker expression (Keratins (K)3, K12, K19 and MUC5AC) was close to conjunctiva than cornea in conditions of LSCD, but were reverted to a corneal phenotype with significant reduction in inflammatory markers (IL-1alpha, IL-1beta, ICAM-1, VCAM-1 and VEGEF) post cultivated LSCT.

It is important to note that transplantation of limbal tissue or cells is to restore the surface epithelium and corneal clarity and visual acuity needs to be assessed and evaluated in due course of time. Patients who show surface stabilization but lack corneal clarity may require corneal transplantation for visual rehabilitation. The corneal specimen received from patients undergoing PK following CLET for visual rehabilitation provide us the unique opportunity to objectively document the proof of surviving cells through histological studies.

Earlier results of PKP in these eyes was not very encouraging probably because of the limited life span and limited proliferative potential of the TACs from PKP specimen, was insufficient to restore the ocular surface epithelium on a long-term basis. Our data (unpublished) showed the presence of stratified corneal epithelium and the establishment of basement membrane (collagen IV staining) in about 75 % of cases. (Fig. 8.2) This proves the concept that the transplanted limbal stem cells, repopulate the damaged host corneal tissue and becomes functional as evident by

the formation of its own basement membrane and by the expression of cornea specific markers.

If resources and facilities are available, the corneal epithelial cells from the PK tissues can be harvested to provide a molecular proof of cell survival through DNA finger printing in allogenic CLET treated patients. Studies have shown that the surviving donor limbal epithelial cells in allogenic transplantations are responsible for the long-term graft survival. The clinical efficacy of limbal transplantation does not necessarily correlate with the survival of donor cells on the ocular surface. Some reports have shown the presence of a chimera, wherein the surviving corneal epithelium constitutes both the donor and the recipient cells, probably indicating that the surviving donor cells have participated in tissue repair. The use of laser capture microdissection and corneal impression cytology (Nelson 1988; Tseng 1985; Williams et al. 1995) allows for a clean examination without involving the stroma or other contaminants.

Williams et al. (1995) have investigated the survival of donor-derived epithelial cells based on impression cytology after limbal stem cell transplantation (allo), using short tandem-repeat DNA polymorphisms (microsatellites) to distinguish the donor and recipient cells and showed that cells of donor genotype were present over the grafted areas at the time of surgery but were not detected in the central cornea until 12 weeks postoperatively, indicating that repopulation of the epithelial surface from the transplanted limbal stem cells took considerable time while only the recipient-type cells were detected in the grafted eye by 20th week. Henderson et al. (1997) attempted to investigate donor cell survival following corneal Limbal stem cell grafting using amelogenin gene probe (a Y-specific DNA probe) with PCR. The same group later (2001) demonstrated that a combination of impression cytology and single cell DNA fingerprinting is suitable for detecting transplanted cells after corneal limbal allografting.

Although there are no known cases of limbal dysfunction after removal of donor tissue from a healthy eye, the effect of initial trauma of healthy eye cannot be ruled out. Irreversible damage caused by the removal of limbal tissue from a partially stem cell–deficient eye may be reduced by cultivated epithelial sheets limiting the amount of excised tissue.

8.7 Outcome of Cultivated Limbal Epithelium Transplantation

Clinical outcome is determined by noting the improvement in ocular surface stability, as judged by non-recurrence of conjunctivalization and the absence of epithelial defects. There are several case reports and small case series reported in the literature using this technique. Table 8.3 summarizes the clinical outcome of CLET. We have reported the largest series of autologous cultivated limbal epithelial transplantation (CLET) in 2006 (Sangwan et al. 2006). Eighty-eight eyes of 86 patients underwent

Table 8.3 Clinical outcome of CLET

Author/year	Type of LSCD	Intervention	No of eyes	Mean follow up (Months)	Overall success rate	Improved BCVA from baseline No. /total (%)	Comments
1. Pellegrini et al. (1997)	Total-2	Autologus LSCT	2	24	Stable ocular surface	Improvement in BCVA	
2. Schwab et al. (2000)	Total-7 Partial-7	Autologus LSCT-10 Allograft LSCT-4	14	13	Stable ocular surface in 6/10 of autografts and all 4 of allografts	All patients improved in BCVA >20/200 in 12/14 patients And > 20/30 in 7/14 patients	
3. Tsai et al. (2000a, b)	Total	Autografts	6	15	All eyes had stable ocular surface	83 % BCVA improved from 20/112 to 20/45	
4. Rama et al. (2001)	Unilateral severe	Autologous LSCT	18	18.6		7/18 (38.9 %)	
5. Koizumi et al. (2001)	Total	Allografts-13	13	11.2	All eyes had stable ocular surface	BCVA improved by two lines or more in 10/13 eyes	Subconjunctival tissue treatment with 0.04 % mitomycin C
6. Shimazaki et al. (2002)	Total-13	Allograft LSCT-13	13	NA	46.2 % 3/8 eyes developed partial conjunctival invasion two eyes later developed epithelial defects		Corneal perforation-4 eyes Infectious keratitis-2 eyes
7. Sangwan et al. (2005)	Total-14, partial-1	Autologous LSCT -11 Living related allograft LSCT-3 nonrelated allograft LSCT-1	15	15.3		10/15 (67 %)	In 15 cases PKP was done later
8. Sangwan et al. (2006)	Bilateral-4 Unilateral-84	Autologus LSCT	86	18.3			

9. Daya et al. 2000	Total-10	Allografts-10	10	28	Improvement in ocular surface –70 %	BCVA improvement –40 %	
10. Nakamura et al. (2006)	Total-9	Allograft LSCT-7	9	14.6	Stable ocular surface at last follow up	Improvement in more than two lines of preoperative BCVA	
		Autologus LSCT-2					
11. Kawashima et al. (2007)	Total-6	Autologus-2	6	25	Stable ocular surface at last follow up	Improvement in BCVA in 4 out if 6 eyes	All eyes subsequently underwent keratoplasty for visual rehabilitation
		Allografts non related-3					
		Allograft related -1					
12. Shortt et al. (2008)		Autologus-3	10	13	60 % (autografts 33 %, allografts 71 %)	Improvement in more than two lines of preoperative BCVA	
		Allografts-7					
13. Baradaran-Rafii et al. (2010)	Total unilateral -8	Autologus – 8	8	34.0	7 eyes – stable corneal epithelium with decrease in opacification and vascularization	Progressive sectorial conjunctivalization in all cases with subsequent PKP	
		Subsequently PKP -4					
14. Kolli et al. (2010)	Unilateral total -8	Autologus		19 months	100 % stable ocular surface epithelium	BCVA improved in 5 eyes, remained same in 3	
15. Rama et al. (2010)	Unilateral	Autologus	113 eyes	Mean follow-up: 2.91 years (Range: 1–9.5 years)	76.6 %	Permanent recovery of vision (visual acuity: 0.6–1.0) in 21 patients	78 % success for cultures with >3 % p63 (+) cells. 11 % success for cultures with ≤3 % p63 (+) cells
		CLET			Permanent restoration of avascular corneal epithelium and amelioration of symptoms	Partial recovery of vision (visual acuity up to 0.5) in 25 patients	
16. Sangwan et al. (2011)	Unilateral	Autologus	200 eyes	Mean follow-up: 3 ± 1.6	71 %	Two line improvement in visual acuity without further surgical intervention was seen in 60.5 % of eyes	CLET using limbal grafts on hAM, prepared under xeno-free culture conditions
		CLET		(Range 1–7.6) years	Completely epithelised, avascular and clinically stable corneal surface		

autologous CLET between 2001 and 2003, alkali burn was the most common cause of LSCD (n = 56), and 61 of these 88 eyes had total LSCD. Success was achieved in 73.1 % (95 % CI 63.3–82.9) and BCVA on Snellen's chart improved from 17/78 (21.8 %) eyes to 41/78 (52.6 %) having functional ambulatory vision (>20/200) postoperatively. We have reported early results of penetrating keratoplasty (PKP) following CLET. 15 of 125 patients underwent PKP following CLET at a mean interval of 7 months. Fourteen of the 15 eyes had a successful corneal graft with stable corneal epithelium (Sangwan et al. 2005).

Koizumi et al. (2001)) reported transplantation of allogenic cultivated corneal limbal epithelium from donor corneas. The cells were cultivated on denuded amniotic membrane with 3 T3 fibroblast feeder cell layer. Air-lifting technique was used to achieve stratification of the epithelium. Thirteen eyes with total LSCD from 11 patients were selected for the procedure. The cultivated epithelium was transplanted to the diseased eye. Simultaneous lamellar keratoplasty was performed in 5 patients with associated stromal scarring. All the recipients were started on an immunosuppression regime. After 48 h all of the 13 eyes showed regained epithelial integrity. On follow-up for 6 months, 10 of the 13 eyes (77 %) showed improved BCVA, a gain of at least 2 lines in the Snellen's chart. However, 3 eyes experienced epithelial rejection. This finding underscores the importance of proper immunosuppression for the allograft technique.

8.8 Alternate Stem Cell Sources and Treatment Modalities for Ocular Surface Reconstruction

While the need for a cGMP compliant cell culture facility is mandatory for executing CLET procedures, a recently reported innovative method circumvents this requirement and enables direct transplantation of limbal explants on the recipient corneal surface. This procedure is termed as Simple Limbal Epithelial Transplantation (SLET), wherein the surgical procedure for collecting autologous limbal biopsy from the donor eye, preparing the corneal surface of the recipient eye and limbal graft transplantation are all completed in a single sitting. The donor tissue pieces are directly transplanted onto the hAM covered corneal surface and held in place by fibrin glue and a bandage contact lens. The stem cells in the limbal grafts proliferate *in vivo* and enables successful reconstruction of the corneal surface. One-year success rate of SLET was reported to be better than autologous CLET in both adults (76 % vs 71 %) and children (74 % vs 37 %) (Sangwan et al. 2012; Amescua et al. 2014). However, in case of bilateral LSCD, allografting is done using limbal tissue from a live related donor or from cadaveric sources. This involves the associated risk of graft rejection and therefore requires long term administration of immune suppressive drugs which becomes unaffordable for low income groups resulting in non-compliance, graft rejection and are predisposed to opportunistic microbial infections as well. Therefore there exists a need for an alternative autologous tissue source that can functionally replace the corneal epithelium. In order to address these

issues, a few groups have attempted to use autologous conjunctival or oral mucosal epithelial cells as an alternative to limbal epithelium for corneal surface reconstruction. Cultivated oral mucosal epithelial transplantation (COMET) has been successfully used to treat patients with severe bilateral corneal defects with good clinical outcome in terms of ocular surface stabilization and marginal improvement in terms of visual acuity (Ang et al. 2006; Hayashida et al. 2005; Nishida et al. 2004b; Nakamura et al. 2011; Satake et al. 2011; Gaddipati et al. 2014; Sangwan et al. 2014). Optical PK following the COMET treatment has been reported by a group to achieve good improvement in the visual outcome with a stable corneal graft (Ma et al. 2009). Recent studies have also shown that mesenchymal stem cells derived from various sources such as bone marrow, dental pulp, adipose tissues and limbal stroma are effective in controlling inflammation and regeneration of corneal surface (Gu et al. 2009; Gomes et al. 2010; Nieto-Miguel et al. 2013; Rohaina et al. 2014; Katikireddy et al. 2014; Acar et al. 2015; Syed-Picard et al. 2015). Another group has reported the presence of stromal stem cells at the limbal niche and demonstrated the usefulness of these autologous, mesenchymal-like stem cells in the treatment of corneal stromal scars (Li et al. 2012; Basu et al. 2014).

Apart from various adult stem cell sources, pluripotent stem cell (PSCs) sources such as the human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been tested for their ability of to generate corneal epithelial, endothelial and stromal cells (Ahmad et al. 2007; Yoshida et al. 2011; Hayashi et al. 2012; Chan et al. 2013; Sareen et al. 2014; Mikhailova et al. 2014; Zhang et al. 2014). Spontaneous differentiation of PSCs results in the generation of heterogenous cell types representing all three germ layers of the body. Therefore it becomes a major challenge to establish enriched populations of any particular tissue-specific cell type from PSCs. However a thorough understanding of various signaling events that regulate organogenesis and tissue-specific micro environments have enabled few groups to device efficient, step-wise differentiation protocols *in vitro* to mimic the native *in vivo* conditions.

Corneal epithelium originates from the ocular surface ectoderm during embryogenesis and the ectoderm lineage specification is regulated by noggin-mediated inhibition of BMP signals. Also TGFβ, Wnt and bFGF signaling are known to regulate corneal epithelial cell proliferation and terminal differentiation. Mikhailova *et al* have demonstrated that hiPSCs could be efficiently differentiated to corneal epithelial cells by culturing them in the presence of Noggin, bFGF, SB-505124 (TGF-β inhibitor) and IWP-2 (Wnt inhibitor). The basement membrane of the corneal epithelium is composed of Fibronectin, Type IV collagen and Laminin. These ECM components, together with other cell types such as melanocytes, peripheral neurons, vasculartures and limbal stromal keratocytes constitutes the limbal stem cell niche. In an attempt to mimic this micro environment *in vitro*, Ahmad et al have cultured hESCs on Fibronectin, Collagen, and Laminin (FCL) matrix in the presence on stromal fibroblast conditioned medium to induce corneal epithelial differentiation. Also, the epigenetic memory of parental somatic cells is known to influence lineage differentiation propensity of iPSCs. To address this problem, a couple of recent studies have attempted to generate iPSCs from limbal fibroblasts

and epithelial cells and shown that these iPSCs could efficiently differentiate into corneal cells (Hayashi et al. 2012; Sareen et al. 2014). These stem cell sources are amenable for large scale expansion and are suitable for genetic manipulations *in vitro*. Therefore, PSC-derived corneal cells are now considered as useful donor cell sources in the treatment of corneal endothelial and stromal dysfunction, bilateral LSCD and in the treatment of various genetic disorders affecting corneal functions.

8.8.1 Bilateral LSCD

Compared to unilateral LSCD, bilateral LSCD presents an entirely different set of challenges with regard to visual rehabilitation. To begin with, the underlying causes of unilateral and bilateral LSCD are quite often different. Whereas ocular surface burns are the leading cause of unilateral LSCD, bilateral LSCD is often caused by autoimmune conditions such as cicatrizing conjunctivitis following SJS and OCP. These represent the severest form of ocular surface disease. In these conditions, in addition to LSCD, there is damage to the adnexa including eyelids, cilia, meibomian glands and conjunctiva. Combined with dry eye, this creates an environment that is extremely hostile for most conventional surgical procedures for surface reconstruction. Another pertinent difference is that, unlike cases of unilateral LSCD, there is no autologous source of donor limbal tissue available in bilateral LSCD. Autologous LSCT, which is by and large the procedure of choice for ocular surface reconstruction in unilateral LSCD, is therefore unavailable as an option in bilateral LSCD. Thus the surgical management in bilateral LSCD is based on two broad approaches – cell-based therapies or keratoprostheses.

8.9 Conclusions and Future Perspectives on "Ocular Surface" Regenerative Therapies

Though severe limbal stem cell deficiency is a rare disease, it has a unique role in unraveling the mysteries of stem cell concept in the limbus, and has set precedence in the field of cell therapy. This also has the unique distinction of bringing together the clinicians, scientists and cell biologists together in taking the science from bench to bedside ,and from protocol to practice. Thus ocular surface reconstruction has served as a model system which fulfilled the principles of successful cell therapy wherein the desirable cell source is available in adequate quantities, well characterized and the surviving transplanted cells were documented to survive, network, integrate and restore the function of the target tissue. Though the clinical outcome is definitely rewarding in most experienced hands, a few aspects that require further attention include objective quantification of transplanted cells, development of tissue and cell banking facilities, and a non-invasive method of in-vivo cell tracking so as to understand the homing and survival of transplanted cells into the limbal niche.

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Chapter 9
Regenerative Therapies for Retinopathy

Ramesh Periasamy and Rajashekhar Gangaraju

Abstract Studies conducted in animal models and human tissues have suggested that retinopathies occur through loss of cells resulting in vascular leakage, excessive immature retinal angiogenesis, and neuronal degeneration eventually leading to loss of vision. Regenerative therapy offers a great promise for such terminally differentiated organs with a stem cell-based therapy. A variety of stem cells including tissue specific endogenous stem cells, hematopoietic stem cells, embryonic stem cells, endothelial progenitor cells, induced pluripotent stem cells and adult mesenchymal stem cells have been considered. Although we made great progress in regenerative therapies in the last two decades, much of the stem cell work on retinopathies came from animal models that do not mimic human retinopathies. In addition, the key molecular and cellular signaling mechanisms in these stem cells in relation to the hostile disease environment have not been thoroughly investigated. Last but not least, the unwanted, unintended differentiated cell types from stem cells likely affect the function, efficacy, and safety of a stem cell product and therefore long-term studies relevant to human conditions must be addressed. As we attempt to translate these cell therapies from preclinical studies into the clinic, challenges remain to be solved center on reproducible manufacturing ability and testing in clinically validated end points relevant to human retinopathies. This chapter describes the current aspects of stem cell therapy in retinopathy, specifically for Diabetic Retinopathy and Retinopathy of Prematurity.

Keywords Diabetes • Retina • Stem cells • Angiogenesis • Inflammation • Pericyte • p38 MAPK • Hypoxia

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9.1 Introduction

In normal-physiological condition retina plays a vital role in vision and visual-perception of the external environment. Retina is a light-sensitive organ that lines the back of the eye. It is responsible for photo transduction, where light energy is translated into chemical and electrical energy (Sia et al. 2014). These signals are further transmitted via optic nerve from the retina to the brain. Any impairment affecting the function of this vital organ could lead to a condition known as retinopathy. It can develop gradually (chronic) or abruptly (acute) depending upon the underlying pathology mostly leading to an irreversible damage. This impairment can cause partial or complete loss of vision.

A number of underlying systemic diseases leading to a variety of retinopathies have been described in literature such as Diabetic Retinopathy due to Diabetes (Nentwich and Ulbig 2015); Retinopathy of Prematurity due to premature birth (Hartnett 2015); Hypertensive Retinopathy due to arterial hypertension (Katsi et al. 2012); Radiation Retinopathy due to radiation exposure (Reichstein 2015); Solar Retinopathy due to direct sunlight exposure (Roh and Weiter 1994); vascular disease such as retinal vein or artery occlusion; trauma, especially to the head, and several diseases may cause Purtscher's retinopathy (Agrawal and McKibbin 2006); and autoimmune retinopathy due to an underlying rare autoimmune diseases (Braithwaite et al. 2012). Among these retinopathies, Diabetic Retinopathy and Retinopathy of Prematurity are the most common vascular complications in patients with long-standing diabetes and premature births respectively, and are the leading cause of blindness in working-age adults and pediatric population. This chapter describes these retinopathies in greater detail.

9.2 Development of Retinal Vascular System

The retinal layers inside the human eye are nourished with retinal and choroidal vascular system. Oxygenated blood enriched with nutrients is delivered to the inner retina by the retinal vascular system and outer retina by the choroidal vascular system (Sapieha et al. 2010). To better understand retinopathy, it is imperative to describe normal vascular development within the retina.

The retinal vascular system undergoes two developmental phases namely vasculogenesis and angiogenesis. During vasculogenesis, the *de novo* formation of blood vessels occurs from the endothelial progenitors-angioblasts (McLeod et al. 2006) and begins at the optic disk at about 16 weeks of gestation. During the next phase of angiogenesis, new blood vessels are formed from the pre-existing vessels (Hughes et al. 2000) and this phenomenon is important for increasing the vascularization to the periphery of the inner retina and is completed by 36–40 weeks of gestation. Astrocytes emerge from the optic nerve and migrate just ahead of the developing vascular network and act as a guide for the endothelial cells to form vascular

network (Dorrell et al. 2010; Weidemann et al. 2010). Importantly, astrocytes, a sub-type of glial cells of central nervous system are capable of sensing low oxygen levels or hypoxic condition and release one of the most important vascular mitogen factors known as vascular endothelial growth factor (VEGF). Consequently, VEGF becomes a crucial factor that aids in the formation of adequate angiogenesis during hypoxic condition. On the other hand, excess oxygen or hyperoxia inhibits new blood vessel formation by down-regulating VEGF expression by astrocytes, limiting the hypoxic stimulus (Pierce et al. 1996; Stone et al. 1995). In an *in vivo* environment for instance, the fetus growing inside the intrauterine is in a relative hypoxic condition that promotes VEGF production, which in turns stimulates the development of retinal vascular system. Indeed, the developing retina is highly sensitive to oxidative stress but not efficient to deal with such stress in premature infants leading to increased reactive oxygen species (ROS). However, the retinal vascular system in most premature infants has an inefficient blood flow system that lead to further retinal complications.

Premature birth results in lack of normal maturation of retinal vascular system. Exposure of newborn premature infants to excessive oxygen downregulates VEGF resulting in constricted or obliterated immature vessels. Often, this hyperoxia-vasocessation is known as stage I of retinopathy of prematurity. Over time, when the retina matures, the increase in demand for oxygen from immature vessels leads to retinal hypoxia stimulating angiogenesis. This is often referred to as stage II of retinopathy of prematurity or hypoxia driven vasoproliferation stage.

Much similar to Retinopathy of Prematurity, angiogenesis plays a vital role in Diabetic Retinopathy. High blood glucose apparently induces hypoxia in retinal tissues, leading to the production of VEGF and subsequent uncontrolled angiogenesis leading to proliferative retinopathy. It is noteworthy to mention that this uncontrolled angiogenesis is a result of an imbalance between VEGF and angiogenic inhibitors that drive the retina into a vasoproliefrative phase of Diabetic Retinopathy (Crawford et al. 2009).

9.3 Diabetic Retinopathy

Diabetic Retinopathy is a leading cause of blindness in adults (age 20–65) suffering from long-term diabetes. In the USA, around 7.7 million adults are estimated to be suffering from Diabetic Retinopathy and it is expected to increase further due to the surge in the obese and /or diabetic population (Kempen et al. 2004).

Diabetic Retinopathy, a heterogeneous metabolic disease that is characterized by hyperglycemia develops due to the defective insulin secretion (type-1) or resistance to insulin action (type-2), or both. In 2014, an approximate figure of 387 million people worldwide suffered from diabetes with a rapid projected increase in this figure (Shaw et al. 2010; Wild et al. 2004; Diabetes-atlas 2014). It is not only the people suffering from diabetes are at risk but also the people diabetic for an extended time period are at a greater risk of developing Diabetic Retinopathy. Simply put, all

diabetics over 20 years develop some form of retinopathy. Despite all medical advancement, there is a significant increase in Diabetic Retinopathy amounting to approximately 5 % of the US adults suffering from diabetes (Gong and Rubin 2015).

9.3.1 Classification of Diabetic Retinopathy

The changes in vascular and neuronal bed within the fundus of Diabetic Retinopathy patient follow a progressive course over the years. The classification of Diabetic Retinopathy into two major forms, non-proliferative Diabetic Retinopathy (NPDR) and Proliferative Diabetic Retinopathy historically were deduced from angiographies performed with sodium fluorescein as well as from autopsy specimens from diabetic eyes (Ashton 1950) and later trypsin digests of neural tissues (Cogan et al. 1961).

In the early stages of NPDR, one notices intra-retinal changes presumably develop concomitant with pericyte loss with residual acellular occluded ghost vessels with thickened basement membrane. These changes lead to endothelial dysfunction involving loss of its barrier integrity. The occlusion of capillary bed can easily be seen with fluorescein angiography with non-perfusion areas in the fundus (Kohner et al. 1967). This relative lack of perfusion results in hypoxia, dilation of retinal vessels to compensate blood flow. This results in capillary obliteration, occlusion and microaneurysm formation (Stefansson et al. 1983). In mild-to-moderate NPDR, microaneurysms compounded with intra-retinal hemorrhages, hard exudates and clinically significant macular edema are common. Finally, moderate to severe NPDR features include cotton wool spots, dot and blot hemorrhages, venous beading and intra-retinal microvascular abnormalities.

In proliferative Diabetic Retinopathy, closure of retinal capillaries results in retinal ischemia and aberrant growth factor secretion eventually leading to neovascular formation (Yang et al. 2010; Davis 1992). These new blood vessels arise at optic disc and proliferate along the retinal surface or into vitreous with or without fibrous component leading to retinal detachment.

Our understanding of the pathophysiological mechanisms underlying the development of Diabetic Retinopathy is constantly evolving with new research. It is becoming increasingly evident that Diabetic Retinopathy is now more accurately defined as a neurovascular rather than a microvascular disease as neurodegenerative disease precedes and coexists with microvascular changes (Barber et al. 2011).

9.3.2 Role of Inflammation and Angiogenesis in Diabetic Retinopathy

New evidence indicates that Diabetic Retinopathy may be an inflammatory disease (Tang and Kern 2011). In diabetic mice induced by Streptozotocin, it is shown that chronic, low-grade subclinical inflammation is responsible for many of the

signature vascular lesions of Diabetic Retinopathy. Tumor necrosis factor (TNF-α), a well-known pro-inflammatory cytokine, has been shown to be a detrimental player in proliferative Diabetic Retinopathy (Spranger et al. 1995; Armstrong et al. 1998; Limb et al. 1996; Demircan et al. 2006; Behl et al. 2008). TNF-α induces changes in endothelial cells, including the expression of intercellular adhesion molecule ICAM-1, an essential player in leukocyte recruitment. Importantly, diabetic retinal vascular leakage, capillary non-perfusion, and endothelial cell damage are temporally and spatially associated with retinal leukocyte stasis in early experimental diabetes (Kern 2007). Previously, Joussen et al. have demonstrated that angiogenesis in Diabetic Retinopathy is closely associated with the appearance of pro-inflammatory macrophages and endothelial activation markers including the vascular adhesion molecule VCAM-1 (Joussen et al. 2004), implicating that either monocyte's as a source of growth factors or intrinsic endothelial activation comprise a mechanism for angiogenesis in Diabetic Retinopathy. This claim is also supported by the evidence that pathological but not physiological retinal neovascularization is altered in TNF-Rp55-receptor-deficient mice (Kociok et al. 2006). These data highlight the central and causal role of TNF-α in the pathogenesis of Diabetic Retinopathy and underscore the potential utility of anti-inflammatory treatment in Diabetic Retinopathy (Joussen et al. 2004). Downstream of TNF signaling it was observed that TNF-α can switch the endothelial cells to become (p38 MAP kinase)-dependent pro-angiogenic showing enhanced cell migration facilitated by (MMP-9)-induced proteolytic activity (Rajashekhar et al. 2007) (Fig. 9.1). Towards this end, p38 MAPK has been shown to play an important role in diabetes-induced inflammation in the retina, and inhibition of p38 MAPK was suggested to offer a novel therapeutic approach to inhibiting the development of early stages of Diabetic Retinopathy and other complications of diabetes (Du et al. 2010). Although inhibition of p38 MAPK prevented capillary degeneration they had minimal or no effects on diabetes-induced degeneration or dysfunction of retinal neurons suggesting future therapies need to consider neurodegeneration as well.

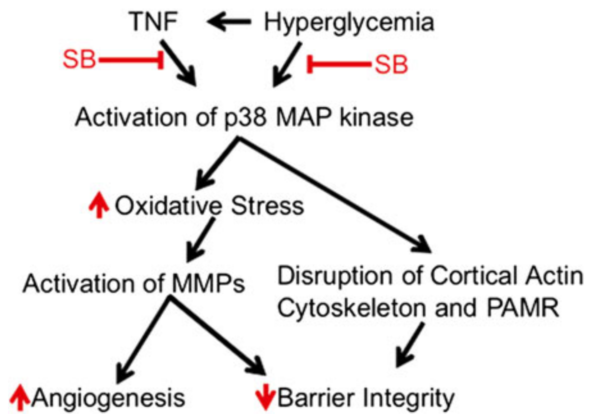

Fig. 9.1 Hypothesis concerning potential mechanisms mobilized by hyperglycemia and TNF-α leading to endothelial dysfunction resulting in increased angiogenesis and decreased barrier integrity. PAMR = Peri junctional Actomyosin Ring. SB = SB203580, p38 MAP kinase inhibitor

9.3.3 Role of Hyperglycemia and Oxidative Stress in Diabetic Retinopathy

Although insulin treatment can delay the onset and progression of diabetic complications (1993) good glycemic control after hyperglycemia does not decrease the rate of progression of retinopathy (Sustained effect of intensive treatment of type 1 diabetes mellitus on development and progression of diabetic nephropathy: the Epidemiology of Diabetes Interventions and Complications (EDIC) study 2003), suggesting that hyperglycemia-induced chronic cellular changes are long-lasting in nature. Hyperglycemia causes tissue damage through five major mechanisms: Increased flux of glucose and other sugars through the polyol pathway, increased intracellular formation of advanced glycation end-products (AGEs), increased expression of the receptor for advanced glycation end products and its activating ligands, activation of protein kinase C (PKC) isoforms and over activity of the hexosamine pathway (Giacco and Brownlee 2010). All five mechanisms are activated by a single upstream event: mitochondrial overproduction of ROS (Yang et al. 2010; Giacco and Brownlee 2010).

The vascular endothelium is the main target of hyperglycemic damage and the common denominator of diabetic complications. Biochemically, endothelial cells are susceptible to hyperglycemic damage as they do not downregulate glucose transport in response to hyperglycemia. This results in intracellular accumulation of glucose and increased flux through glycolysis. As a consequence, mitochondria generate increased reactive oxygen species (ROS) (Busik et al. 2008), which influences intracellular signaling and gene expression, including TNF-α expression in human endothelial cells (Valen et al. 1999). In addition to endothelial cells, oxidative stress in retina leads to the damage of other vascular and retinal ganglion cells (Kern and Barber 2008). This triggers a cascade of events such as the release of inflammatory factors (angiotensin-II, TNF-α, ICAM-1 and IL-1β), microglial cell activation, and apoptosis of capillary endothelial/retinal ganglion cells. Finally, this leads to hypoxic condition, breakdown of the Blood-Retinal Barrier (BRB), and retinal neovascularization (Gong and Rubin 2015).

Overproduction of superoxide, hydroxyl radicals, and peroxynitrite are the products of oxidative stress and improper electron transport chain (Yang et al. 2010). In Diabetic Retinopathy, ROS affects the functionality of the retinal capillary cells, cause damage to the mitochondrial DNA, destroys the mitochondrial membrane potential, and initiates apoptosis of mitochondria by activating caspase-3 and caspase-9 signaling pathways (Kowluru 2005). Other pathways in Diabetic Retinopathy such as NF-κB have major role in cell death, JNK and p38 MAPK pathways are involved in the ROS production (Miranda et al. 2012; El-Remessy et al. 2011).

DNA breakage due to ROS leads to activation of enzyme-PARP that inhibits the GAPDH activity finally leading to endothelial abnormality in Diabetic Retinopathy (Pacher and Szabo 2005) . Studies conducted in retinal endothelial cells with inhibition of PARP under hyperglycemia condition suggested a decrease in NF-κB signaling and protection against cell death (Pacher and Szabo 2005). Other signaling mecha-

nisms involving downregulation of Notch1, phosphorylation of Akt, higher activation of PARP and cleaved caspase-3 domain have been linked to retinal apoptosis under hyperglycemia.

9.3.4 Role of Retinal Barrier Integrity in Diabetic Retinopathy

Loss of BRB is one of the key events in Diabetic Retinopathy. Vascular basement membrane containing tight and gap junctions between endothelium play a significant role in limiting the paracellular flux thus maintaining normal physiology in the retina (Roy et al. 2015). Elegant studies in particular implicated hyperglycemia induced downregulation of Connexin-43 (Cx43), a prominent gap junction protein expression may contribute to the breakdown of barrier tight junctions by compromising occludin and zonula occludens-1 (ZO-1) levels in endothelial cells and Muller cells ultimately leading to glial and vascular cell loss associated with Diabetic Retinopathy (Tien et al. 2013; Muto et al. 2014). In other studies involving vascular endothelium, TNFα is known to induce barrier dysfunction concomitant with disruption of actin cytoskeleton (Goldblum et al. 1993; McKenzie and Ridley 2007; Petrache et al. 2001; Wojciak-Stothard et al. 1998) and microtubule disassembly (Petrache et al. 2003a) through mechanisms involving activation of ROS, (Gertzberg et al. 2004) RhoA GTPase, (Petrache et al. 2003b) MAPKs, (Birukova et al. 2005; Petrache et al. 2003a) transcriptional activation of myosin light chain kinase (MLCK) (McKenzie and Ridley 2007; Petrache et al. 2001) and/or heat shock protein 27 (Hsp27) (Wang et al. 2005). However, a preponderance of the evidence collected to date seems to indicate a major role for p38 MAP kinase as it is also implicated in the activation of ROS production through activation of components of NADPH complex (Hashimoto et al. 2001), Hsp27 (Wang et al. 2005) and microtubule disruption (Birukova et al. 2005; Petrache et al. 2003a) (Fig. 9.1). Other important potential mechanisms of vascular permeability include PKC activation, VEGF-induced caveolar transcytosis, modulation of aquaporins and loss of any cells including endothelial, pericyte and glial cells that govern the retinal barrier integrity (Klaassen et al. 2013).

9.3.5 Role of Lipid Mediators in Diabetic Retinopathy

Much similar to diabetic hyperglycemia, dyslipidemia results in metabolic insults that affect retinal degeneration in diabetes. Although the role of hyperglycemia in inducing Diabetic Retinopathy has been studied in detail, much less attention has been paid to dyslipidemia (Busik et al. 2012). Several clinical studies such as DCCT/EDIC and ETDRS have demonstrated a strong association between increased serum lipids and development of Diabetic Retinopathy (Lyons et al. 2004; Ferris et al. 1996), highlighting the importance of understanding the exact changes in

retinal lipid metabolism in diabetes. More recently several elegant studies in animal and cell culture models suggested various bioactive sphingolipids (Opreanu et al. 2011), lipoxygenase (Al-Shabrawey et al. 2011), polyunsaturated fatty acids (Chen et al. 2003) within the diabetic retina are differentially expressed suggesting future therapies should target diabetes induced perturbations of known lipid classes in the retina may yield beneficial effects.

9.4 Retinopathy of Prematurity

Incomplete vascularization in the retina of preterm infants results in severe hypoxic environment which in turn leads to excessive immature retinal angiogenesis, a serious complication known as Retinopathy of Prematurity commonly seen in pediatric ophthalmology. In USA, nearly 50 % of low-birth weight infants develop Retinopathy of Prematurity. It is primarily characterized by the degeneration of micro-vessels and retinal ischemia. This condition can further lead to secondary abnormal-neovascularization, hemorrhages and even blindness (Campochiaro 2000). Such abnormal phenomenon can be controlled by either inhibition of the pathological neovascularization or by decreasing the loss of the retinal vessels, which will suppress the hypoxic condition that drives the retinal damage. In an Retinopathy of Prematurity condition, the following sequence are observed (Hartnett 2010b):

– Delay in the development of retinal vascular system in preterm infants
– Hyperoxia leading to the reduction of growth factors intake
– Hypoxia leading to surge in metabolic demand

In a hypoxic condition, lack of oxygen in retina stimulates the oxygen regulated pro-angiogenic factors. This leads to retinal neo vascularization with sprouting of irregular blood vessels occurring from the retinal layer to the vitreous body of the eye (Hellstrom et al. 2013). Based on studies in animal models of oxygen-induced retinopathy (OIR), exogenous factors such as oxygen levels, oxidative stress, inflammation, and nutritional capacity have been linked to severe Retinopathy of Prematurity through dysregulated signaling pathways involving hypoxia-inducible factors and angiogenic factors like VEGF, oxidative species, and neuroprotective growth factors to cause phases of Retinopathy of Prematurity (Hartnett 2015).

9.4.1 Role of Oxidative Stress in Retinopathy of Prematurity

The retina is highly sensitive to oxidative stress caused by ROS. During the ischemic damage of retina, the normal balance of ROS production and capacity of endogenous antioxidant to scavenge the ROS is aggravated. Excess ROS then stimulate many signaling pathways that trigger the damage of DNA/lipids leading

to further oxidative stress and ultimately cell death. Studies have shown, in animal models of OIR, oxidative damage can be controlled/inhibited by antioxidants that suppress the degeneration of the vascular system (Niesman et al. 1997). Antioxidant 'Lutein' is under clinical trial and its effects are studied in the neonatal eye of both preterm and term infants (Perrone et al. 2014). Among the xanthophylls (carotenoid pigment), lutein and zeaxanthin account to 80–90 % of the total carotenoids in the human retina. A metabolite of lutein known as Meso-zeaxanthin is localized specifically in the retinal layer of the human eye. These xanthophylls are densely packed at the center of the fovea region known as macula lutea or macular pigment area. Indeed, these pigments are cell protective having antioxidant, anti-inflammatory, and light-protective properties (Bone et al. 1997). Low levels of lutein and zeaxanthin in the retina are associated with Retinopathy of Prematurity, including Age–related Macular Disease (AMD) and Diabetic Retinopathy (Musch 2014; Abdel-Aal el et al. 2013). In addition, preterm birth is also linked with the reduction of enzymatic/non-enzymatic antioxidants (such as lutein, zeaxanthin, catalase, superoxide dismutase (SOD), vitamin C and E) that are produced and/or accumulated later in pregnancy (Qanungo and Mukherjea 2000). An inhibitor known as apocynin inhibits nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that results in the inhibition of ROS and prevention of apoptosis of the avascular retina (Hartnett 2010a).

9.4.2 Role of Growth Factors in Retinopathy of Prematurity

The severity of the retinal disease is based on the level of immaturity at birth making the retina more vulnerable with the exposure of detrimental factors and deficiency of protective factors in utero. It has been shown that preterm birth and hyperoxia can inhibit the production of insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF) and erythropoietin (EPO), leading to hypoxic and oxidative retinal damage. Consequently, a number of signaling mechanisms driven by hypoxia-inducible transcription factors that bind DNA at the hypoxia-responsive element and enable transcription of a number of downstream genes that are angiogenic, including VEGF, angiopoietins, and EPO as well as other signaling pathways including Janus kinase signaling transducer and activator of transcription-3 (JAK/STAT3) have been implicated in the promotion of retinal neovascularization (Byfield et al. 2009; Hartnett 2015; Gong and Rubin 2015).

9.4.3 Role of Inflammation in Retinopathy of Prematurity

Inflammation has long been implicated in Retinopathy of Prematurity etiology (Dammann 2010). Specifically from animals model studies we know that inflammatory processes interfere with normal retinal vascularization in the most vulnerable

retinas. For example, TNFα knockout mice appear to be relatively protected from OIR injury (Gardiner et al. 2005). In another study omega-3-polyunsaturated fatty acids decreased the size of the avascular area and this protective effect appeared to be mediated, at least in part, via suppression of TNFα (Connor et al. 2007). Recent studies conducted in human subjects with Retinopathy of Prematurity correlated with differential expression of chemokines and cytokines between early and later time periods suggesting that perinatal inflammation may be involved in the pathogenesis of Retinopathy of Prematurity (Sood et al. 2010). Such systemic inflammation early in the postnatal life using animal models has been implicated in abnormal retinal vascular development, increased vessel anastomosis and, ultimately, a permanent compromise in retinal function predisposing to Retinopathy of Prematurity (Tremblay et al. 2013).

9.5 Current Therapies for Retinal Diseases

Different therapeutic strategies are currently employed to prevent retinopathies related to ischemic injury. The approach is to stop or reduce the ischemic condition by inhibition of abnormal neovascularization, promoting vascularization or reducing the loss of the vasculature (Hartnett 2010a; Kermorvant-Duchemin et al. 2010; Sapieha et al. 2010). Developing such strategies could lead to increase of retinal re-vascularization or/and reduction of retinal vascular degeneration. Although early markers of neovascularization identified as VEGF, due to redundancy of signaling systems and the interrelationship with other pathophysiological mechanisms lead us to think new ways to treat these blinding disorders. Furthermore, unlike tumor vasculature, the mechanisms by which vasculature can be stabilized would be beneficial against the already existing ischemia in Retinopathy of Prematurity rather than blocking the angiogenesis. Thus, the success of future treatments of retinal neovascular disease such as Retinopathy of Prematurity will not depend on simply blocking angiogenesis *per se*, but rather on our ability to shift pathological neovascularization towards healthy revascularization (Scott and Fruttiger 2009).

Neonatal intensive care has been improved drastically over the time leading to greater survival of preterm (≤31 gestation weeks) infants (Costeloe et al. 2012). Despite such medical improvements, there is no significant reduction in Retinopathy of Prematurity cases (Hellstrom et al. 2013). Most of the treatment that are currently used for retinal neovascularization are invasive and has low efficacy in preventing the disease. Pan-retinal laser photocoagulation is used to treat vitreous hemorrhage and neovessels located near optic disc. Laser photocoagulation and early treatment of retinopathy of prematurity (ETROP) are used for treating severe Retinopathy of Prematurity (Good 2004). Anti-VEGF treatment for Retinopathy of Prematurity is widely performed but they have inadequate follow-up time (Klufas and Chan 2015). The most common drugs used as an anti-VEGF in Retinopathy of Prematurity condition are Bevacizumab and Ranibizumab. Despite the beneficial property of the Ranibizumab, reactivation of Retinopathy of Prematurity is always a threat and there is no observation for an extended time to study further (Wong et al. 2015).

Laser photocoagulation combined with intravitreal bevacizumab injection was used in Retinopathy of Prematurity with some favorable results (Kim and Kim 2014). A combination therapy of anti-VEGF drugs such as photodynamic therapy with verteporfin (PDT-V) has been developed to treat the ocular neovascularization (Bradley et al. 2007). Aminophylline, a broncho dilator at low dosage has been shown to decrease the risk of post-operative apnea in premature infants (Mohajerani and Roodneshin 2014). Despite all the development, there is a risk of reoccurrence of the disease, contracting infection while administration and limited success in these therapeutics. More recently, novel technology platforms like intravitreal administration of nanoparticle releasing essential drugs are being explored that aid in the treatment with reduced risk of infection, increased bio-availability and sustained release (Hennig and Goepferich 2015; Kompella et al. 2013).

Current strategies for the therapeutic management of Diabetic Retinopathy include laser photocoagulation, intravitreal triamcinolone, and intravitreal injection of VEGF neutralizing agents (e.g., Bevacizumab). Patients undergoing Laser treatment continue to remain at risk for new bleeding episode requiring multiple laser treatments. Triamcinolone, which is relatively more successful in suppressing macular edema, requires intravitreal administration. Usage of intravitreal injections of Bevacizumab or ranibizumab in macular edema appeared to be promising but may cause systemic or ocular complications such as subcapsular cataract, onset of steroid-induced glaucoma, and potential for endophthalmitis (Shima et al. 2008; Nguyen et al. 2012). Additional therapies targeting retinal oxidative stress, for example, Catalase therapy (Giordano et al. 2015); specific inhibition of PKC pathways (Aiello et al. 1997; Ishii et al. 1996); aldose reductase inhibitor pathways (Tang et al. 2013); tyrosine kinase pathways (Thakur et al. 2011); Wnt signaling pathways (Lee et al. 2012) have shown improvement in the initial stages of Diabetic Retinopathy.

9.6 Regenerative Cell Therapies as a New Paradigm in Treating Retinopathy

The stem cell field in the recent time has shown promising strategies and approaches regarding cell-based therapy. The stem cells that are used for developing therapies for retina can be of two origin: (1) Exogenous stem cells that are of not origin in retina. For instance: neural stem cells, and embryonic/induced pluripotent stem cells, hematopoietic stem cells and mesenchymal stem cells. (2) Endogenous stem cells that are origin of retinal stem cells. For instance: Müller glia (Ooto et al. 2004; Reichenbach and Bringmann 2013), retinal pigment epithelial (RPE) stem cells and ciliary epithelia-derived stem cells (Tropepe et al. 2000; Ahmed et al. 2011). It has been well documented that there is limited retinal regeneration in adult mammals due to both intrinsic inability of retinal neurons to reinitiate robust regeneration and lack of a permissive environment for such growth (Yu et al. 2014). As such, most of the literature pertinent to regeneration in retinopathy predominantly comes from exogenous stem cells (Fig. 9.2).

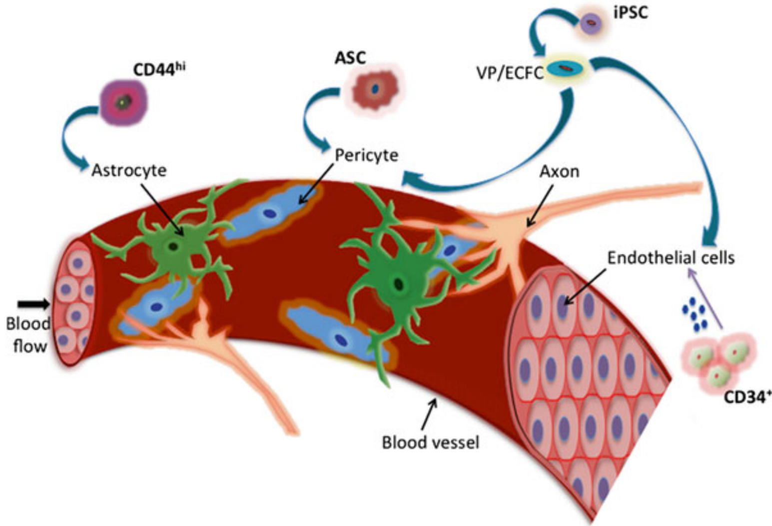

Fig. 9.2 Current regenerative cell therapy efforts in retinopathy. A variety of stem cells including hematopoietic stem cells, embryonic stem cells, endothelial progenitor cells, induced pluripotent stem cells and adult mesenchymal stem cells have been considered in retinopathies. Arrows point to the stem cells that either differentiates into vascular or neural cells to replace lost cells or provide a trophic support (Please refer to the text for more details)

Among cell-based approaches intended to address Diabetic Retinopathy or ischemic retinopathies suggested that Lin^- hematopoietic stem cells from bone marrow injected directly into the mouse eye targeted activated astrocytes, and participate in normal developmental angiogenesis in neonatal mice or injury-induced neovascularization in the adult (Otani et al. 2002; Ritter et al. 2006). Particularly, $CD44^{hi}$ hematopoietic stem cells were more effective in an OIR model in providing neurotropic support to form neovascularization (Friedlander et al. 2007; Ritter et al. 2006). Unlike these, bone marrow-derived myeloid cells other than endothelial progenitor cells via SDF-1 mediated mechanism promoted vessel growth after assuming direct perivascular localization in a VEGF driven model (Grunewald et al. 2006). Similarly, hematopoietic stem cells were also differentiated into pericytes and astrocytes thereby increasing vascular ensheathment of pericytes and decreasing apoptosis of pericytes and retinal neurons in the OIR model influenced by insulin-like growth factor binding protein-3 (IGFBP-3) (Kielczewski et al. 2011).

Since pericytes and endothelial cells play an important role in pathogenesis of Diabetic Retinopathy and in combination with glial cells form a tight BRB integrity (Cai et al. 2008), majority of the studies aimed at rescue or repair of these cell types. Some of the early studies include, intravitreal injection of CD34+ endothelial cells that have been very successful to prevent vascular regression and protect neurons in genetic mouse models of retinal degeneration (Caballero et al. 2007). It is noteworthy to mention that only healthy CD34+ endothelial precursor cells but not diabetic cells were able to integrate into damaged vasculature. These studies demonstrate

that healthy endothelial progenitor cells can effectively repair injured retina and that there is defective repair of vasculature in patients with diabetes (Caballero et al. 2007; Li Calzi et al. 2010). More recently, vascular progenitors derived from either embryonic stem cells or cord blood derived induced pluripotent stem cells (iPSC) possessed augmented capacity to home, integrate into, and repair damaged retinal vasculature in an retinal ischemia reperfusion injury model (Park et al. 2014). Interestingly, these cells engrafted into both luminal endothelial and abluminal pericytic locations. Human endothelial progenitor cells or endothelial colony forming cells (ECFC) derived from cord blood or bone marrow have shown a remarkable angiogenic potential in several disease models (Yoder 2012; Jarajapu and Grant 2010). Likewise hiPSC-ECFCs prepared from cord blood demonstrated a significant reduction of the post-injury avascular area as well as reduced pre-retinal neovascular tufts in OIR model (Prasain et al. 2014) suggesting highly efficient production of patient-derived ECFCs for the treatment of retinal disease. While iPSC need extensive cell manipulation prior to potential utility, other more readily available adult stem cells such as Adipose Stromal Cells (ASC) were shown as a promising therapeutic. In a Streptozotocin (STZ) induced chronic hyperglycemia Diabetic Retinopathy model intravitreal injection of ASC pair with host vasculature in a perivascular location, possibly suggesting pericyte replacement (Rajashekhar et al. 2014). However, one must note that not all mesenchymal cells are pericytes and more careful defined sub populations in future studies may yield more favorable results (Blocki et al. 2013).

Several adult stem cells that are of non-retinal origin are considered for developing treatments for traumatic and degenerative eye disease such as glaucoma and AMD, which will also shed light on how these stem cells may be useful in treating retinopathies. Bone marrow derived stem cell (BMSC) transplanted intravitreally but not systemic route was found neuroprotective in a rat glaucoma model (Johnson et al. 2010). Similarly, intravitreal transplants of dental plulp stem cells (DPSCs) promoted significant neurotrophin-mediated retinal ganglion cell (RGC) survival and axon regeneration after optic nerve injury (Mead et al. 2013). There are only few studies that have used neural stem cells (NSC) in the eye for retinal diseases with their limited capacity to produce neurons and glia (McGill et al. 2012), but have not yet been shown to generate neural retina or retinal pigment epithelium (Aboody et al. 2011). Secreted (neuro) trophic factors from mesenchymal stem cells provide axonal development and neuroprotection to cells in the retina and/or inducing the endogenous retinal cell population to activate the repair mechanism by cell replacement via differentiation pathway and/or further by paracrine support (Lamba et al. 2009). For example, ASC are known to produce a variety of paracrine neurotropic factors (Wei et al. 2009a, b; Rehman et al. 2004). Among these progranulin, a cysteine-rich protein produced by ASC has been shown to be neuroprotective in the light-induced retinal-damage model (Tsuruma et al. 2014). The latter study establishes a mechanistic basis supporting the therapeutic application of ASC for neurological disorders, specifically through paracrine support provided by trophic factor secretion, which may be of paramount importance in regeneration of photoreceptors/astrocytes in retinopathies (Rajashekhar 2014).

Umbilical cord blood and tissue contains unique and powerful stem cells that are being investigated for their ability to help repair and heal the body in different diseases. Intravitreal transplantation of human umbilical cord blood stem cells (UCBSCs) results in increase in the RGC survival in the retina, following traumatic optic neuropathy. It was shown that this beneficial effect could be associated with the release of Brain-derived neurotrophic factor (BDNF) and Glial cell-derived neurotrophic factor (GDNF) by the hUCBSCs (Zhao et al. 2011); up regulation of immunoglobulin heavy chain-binding protein, GRP78 and down regulation of endoplasmic reticulum (ER) stress-C/EBP homologues protein (CHOP) expression in retinal cells (Jiang et al. 2013). In addition to supporting the neuronal cells, UCBSCs are also known to differentiate into functional endothelial progenitor cells (Aoki et al. 2004) and have the potential to exhibit pericyte-like phenotype and help in network formation of endothelial progenitor cells *in vitro* (Peters et al. 2015). Stem cells derived from the Warton's jelly of fetal umbilical cord have the potential to differentiate into retinal progenitor cells (Hu et al. 2013). Umbilical Cord Blood Mesenchymal stromal cells (UCBMSC) secrete several immunomodulatory and neurotropic factors such as BDNF, neurotrophin-3 (NT-3), Ciliary neurotrophic factor (CNTF), and Transforming growth factor- β1 (TGFβ1). These cells upon grafting can contribute toward neural repair in rat optic tract model (Zwart et al. 2009). Despite the MSC's beneficial secretome properties, human UCBMSC do not differentiate into neural cell types or integrate into retina after intravitreal grafting in neonatal rats (Hill et al. 2009).

Recently, peripheral blood stem cells, a small proportion of pluripotent stem cells, have been reported to mainly exist in the peripheral blood mononuclear cells (PBMNCs). Freshly isolated adult human PBMNCs pre-induced with the conditioned medium of rat retinas for 4 days and transplanted into the sub-retinal space of the rd1 mice migrated into the degenerative region expressing human photoreceptor-specific marker, Rhodopsin (Zhang et al. 2013; Peng et al. 2014). In another study PBMNCs trans differentiated into neural precursor cells and retinal progenitor cells *in vitro* and migrated and integrated into retina *in vivo* (Liu et al. 2011). However, their differentiation into retina like cells is controversial.

Retinal stem cells of endogenous origin have multipotential and self-renewal properties. The Müller glia retinal stem cell is capable of differentiation into cell types including photoreceptors (Liu et al. 2013; Del Debbio et al. 2010), Ciliary epithelial- derived stem cell in the development of photoreceptor (rhodopsin), and RPE cells in differentiating into neuronal phenotype (Del Debbio et al. 2013). Despite the promising differentiation and transplantation properties of the endogenous retinal stem cells on developing therapy for retinal degeneration functional RGC replacement is still elusive.

9.7 Concluding Remarks and Future Prospects

We have made great progress in understanding retinopathies in the last few decades. Anti-VEGF therapies and emerging stem cell therapies are in the forefront of novel therapeutics. However, approximately 40–50 % of patients with diabetic macular

edema do not respond completely to anti-VEGF therapy suggesting that rationally designed therapies targeted to VEGF-independent pathways may offer the promise of expanded treatment efficacy. Regenerative medicine has seen a remarkable progress in the last few years promising the use of stem cells in the treatment of retinal disorders. Some of the challenges we have include but not limited to are (1) there are major anatomical differences between animal models and humans making preclinical studies difficult to translate into humans (2) the clinical end points in humans and animal studies differ posing another issue (3) it remains unknown how exogenous stem cells integrate within retinal circuits and contribute to vision restoration and (4) there may be some unknown genetic factors that may play a role. Future studies should concentrate on new stem cell approaches that provide novel insights into both pathogenesis and treatment of retinopathies. Specifically, proper correlations of histopathology, molecular abnormalities with proper and clinically validated endpoints relevant to human are necessary. We need therapies that are non-destructive to the sensitive retina, perhaps non-invasive and infrequent dosage form. Before advancing to clinical therapy, it is necessary to perform extensive investigations to improve the processes of obtaining, differentiation and implantation of these cells to overcome the challenges, which are still present to date (Alonso-Alonso and Srivastava 2015). Last but not least, the unwanted, unintended differentiated cell types from stem cells likely affect the function, efficacy, and safety of a stem cell product and therefore reproducibility of manufacturing conditions should be addressed.

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Chapter 10
Lacrimal Gland Regeneration: Progress and Promise

Geeta K. Vemuganti and Shubha Tiwari

Abstract The lacrimal gland is a tubuloacinar gland located in the groove of the frontal bone, which is involved in synthesis and secretion of major tear proteins and other aqueous components of the trilayered tear film. Any injury to the lacrimal gland, which may be age related, drug or radiation therapy induced leads to dysfunction of the gland with a resultant hyperosmolarity of the tear film and its subsequent instability. This tear film instability leads to destabilization of the ocular surface homeostasis and to a number of morbid complications like the dry eye syndrome.

The current treatment modality for chronic dry eye remains palliative, which provides only temporary symptomatic relief. One of the modalities of providing long-term benefit to these patients would be cell therapy to restore or replenish the damaged gland. This review describes the progress and promise of cell therapy for lacrimal gland regeneration for potential clinical application.

Keywords Lacrimal gland development • Secretion • Stem cells • Lacrimal functional unit (LFU) • Tear film • Secretory acinar cells • Ductal cells • Dry eye syndrome • Etiopathogenesis • Androgens • Treatment • Animal models • Keratoconjunctivitis sicca (KCS) • Lacrimal gland dysfunction • Evaporative dry eye • Meibomian gland dysfunction • Tear film stability • Tear hyperosmolarity • Radiation therapy • Lacrimal gland acinar cells • *In-vitro* culturing

Method of Literature Search
A search of Pubmed database (1979–till date) was conducted. Medline, Elsevier-EMBASE and Ophthalmic literature databases was also searched. The following key words were used: *Lacrimal gland/lacrimal in-vitro studies/ dry eye syndrome.*
Additional sources include review of publications cited in other articles. Google search was also used to find publication that may be missed in the above databases.

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10.1 Introduction

The human lacrimal gland is a tubuloacinar, almond shaped gland located superior and lateral to the eye in the shallow depression of the frontal bone. The lacrimal gland can be divided into the main lacrimal gland (orbital and palpebral portions) and the accessory lacrimal glands (Gland of Wolfring and Krauss). The lacrimal gland forms an important entity of the lacrimal functional unit (LFU), which comprises of the lacrimal gland, the ocular surface (cornea, conjunctiva and the meibomian gland) and the sensory and motor nerves that connect them. The LFU controls the secretion of the major components of the tear film and is overall responsible for maintaining the stability of the tear film, transparency of the cornea and the quality of the image projected onto the retina (2007a).

Tear film is composed of the secretions of the lacrimal gland, meibomian gland and the conjunctival goblet cells. It has three basic layers: aqueous, which is 3–4 microns thick and is composed of the secretions of the lacrimal gland; lipid, which is 0.2 microns thick and is secreted by the meibomian gland; and the mucin layer, 1 micron thick, secreted mainly by the conjunctival goblet cells (Fig. 10.1). The important constituents of human tear are electrolytes like sodium, potassium, calcium, magnesium, bicarbonate and chloride; and major proteins like lysozyme, lipocalin, lactoferrin, scIgA, albumin and IgG (Table 10.1). Other components of tear film include lipids like phosphatidylcholine and phosphatidylethanolamine; mucins like MUC4, MUC5AC, MUC1. Minor components like defensins, catalase cytokines also form a part of the tear film composition (Tiffany 2008) (Table 10.2).

The tear film, as already mentioned, maintains the transparency of the cornea and determines the quality of image projected on the retina for cortical sensing. The periocular tear film is also responsible for providing nutrition to the cornea by acting as a coupling medium for the environmental oxygen; protecting the ocular surface due to the antimicrobial properties of lysozyme, lactoferrin and lipocalin present; and also providing physical protection to the ocular surface against the shearing force of blinking due to the mucins present in it (Tiffany 2008).

10.2 Embryology and Development

The development of the human lacrimal gland has been the subject of numerous studies since the early 1900s. Most of these studies, other than the one published by Tripathi and Tripathi, 1990 (Tripathi & Tripathi 1990), report that the gland develops from the ectoderm of the superior conjunctival fornix in human embryos with a crown to rump length of 22–24 mm (de la Cuadra-Blanco et al. 2003).

The two main lacrimal gland lobes- the orbital and the palpebral lobes- originate not simultaneously but one after the other. The orbital lobe originates from the proliferation of conjunctival fornix epithelial cells in the form of five or six epithelial buds and its formation concludes by the end of the second month. This is followed

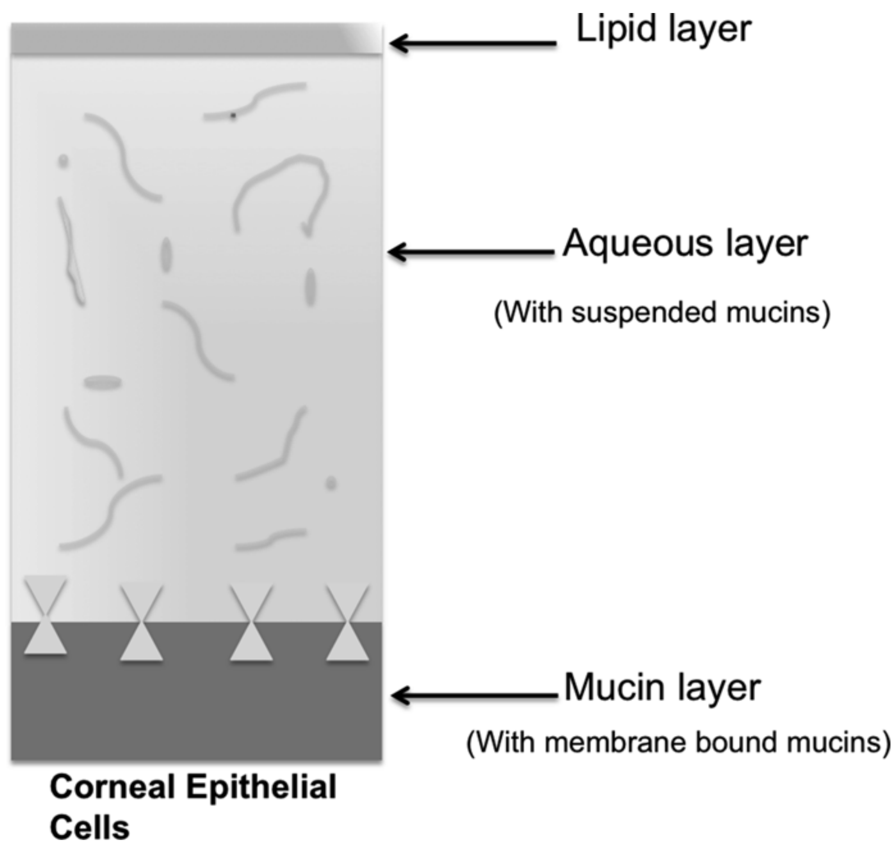

Fig. 10.1 Layers of tear film

Table 10.1 Composition of normal human tears

Composition	Concentration
Electrolytes	**mmol/litre**
Sodium	128.7
Potassium	17
Calcium	0.32
Magnesium	0.35
Bicarbonate	12.4
Chloride	141.3
Major proteins	**mg/litre**
Lysozyme	2.07
Lactoferrin	1.65
scIgA	1.93
Lipocalin	1.55
Albumin	0.04
IgG	0.004

Table 10.2 *In-vitro* lacrimal gland research: Information matrix

Year	Species	*In-vitro* research	Reference
1987	Rat	Established culture	Oliver et al. (1987)
1991	Rat	Importance of media & growth factors for in-vitro cultures	Hann et al. (1991)
1994	Rabbit	Physiologically responsive *in-vitro* cultures	Menerey et al. (1994)
			Rismondo et al. (1994)
2000	Rabbit	Purified acinar cell preparation	Guo et al. (2000)
2000	Rabbit	Use of EGF, DHT, Matrigel and HepatoSTIM for culture	Schonthal et al. (2000)
2000	Human	*In-vitro* culture from cadaveric tissue	Yoshino (2000)
1984	Rat	Effect of androgen on synthesis and secretion by lacrimal gland	Sullivan et al. (1984; 1990)
1990			Hann et al. (1991)
1991			Kelleher et al. (1991)
2006	Rat	*In-vitro* culture in polyethersulfone dead end tube	Long et al. (2006)
2007	Rabbit	*In-vitro* culture on amniotic membrane	Schrader et al. (2007)
2009	Rabbit	Rotary cell culture system	Schrader et al. (2009)
2011	Mouse	Report of mesenchymal stem cells in lacrimal gland post injury	You et al. (2011)
2012	Human	Established functionally viable cultures from fresh tissue	Tiwari et al. (2012)
		Preliminary report on presence of stem cells in native human lacrimal gland	
2012	Rat	Progenitor cells in uninjured rat lacrimal gland	Shatos et al. (2012)

by initiation of the palpebral lobe formation. The orbital and the palpebral lobes are separated by the levator muscle tendon, which forms during the third week of development.

Epithelial-mesenchymal interaction has been considered by a number of authors to be responsible for morphogenesis, organogenesis, cell differentiation and growth (Makarenkova et al. 2000; Martin 1998; Sanders 1988). Lacrimal gland development is an example of such an interaction in which the bud-like invagination of conjunctival epithelium at the fornix is the process, which initiates lacrimal gland development (Kammandel et al. 1999). Factors like pax6, FGF10, Runx 1 to 3 and Barx2 have been shown to be involved in lacrimal gland morphogenesis and development (Makarenkova et al. 2000; Tsau et al. 2011; Voronov et al. 2013). The mesenchymal cells surrounding the point of epithelial budding are the periocular cells of neural crest origin (Johnston et al. 1979). The tubular invaginations of the lacrimal gland extends and branches multiple times to form the lobular structure of the mature lacrimal gland.

10.3 Histology, Anatomy and Physiology

The lacrimal gland is a tubulo-acinar gland that consists of secretory epithelium arranged in a lobular pattern. These secretory acinar cells empty their secretions into ducts that anastomose into a larger nasolacrimal (NLD) duct, which drains onto the ocular surface. Enveloping the secretory acinar cells are myoepithelial cells that contract and squeeze them facilitating the draining of the secretory components into the ducts. Between the lacrimal lobes are fibroblasts, which produce the collagen and matrix of interstitial regions, and mast cells, which secrete histamine and heparin (Fig. 10.2). In addition to this basic tissue architecture, the lacrimal gland is highly inundated with trafficking B and T lymphocytes as well as plasma cells (Walcott 1998).

The secretory acinar cells of the gland are columnar epithelium with basally located nucleus and a large perinuclear Golgi body. The ductal cells are more cuboidal in shape. The apical portion of the acinar and ductal cells has a number of vesicles and the cell base has an associated basement membrane that imparts the cells their polarity. Large junctional complex is found near the luminal pole that couples these cells electrically and chemically as well as mechanically attaches them with each other. Gap junctions like connexin 26 and 32 are also found here. The presence of a large number of junctional complexes between the epithelial cells indicate that these cells are very closely associated with each other (Walcott 1998).

The lacrimal gland is innervated by the sympathetic as well as the parasympathetic arms of the autonomic nervous system (Matsumoto et al. 1992). These nerves have a large number of cholinergic fibers and fewer adrenergic fibers. The parasympathetic postganglionic neural cell bodies are found in the pterygopalatine (sphenopalatine) ganglion as well as the ciliary ganglion. Sympathetic fibers arise in the superior cervical ganglion. There is also some amount of sensory innervation of the gland from the trigeminal ganglia (van der Werf et al. 1996). Even though the inner-

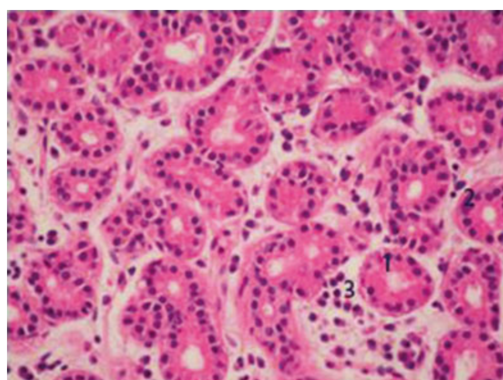

Fig. 10.2 Histology of normal human lacrimal gland. *1*: Secretory epithelial/acinar cells that synthesize and secrete major tear proteins. *2*: Myoepithelial cells that envelope the acinar cells. *3*: Interstitium that has fibroblasts that secretes collagen and other extracellular matrix

vation is similar across different species yet the nature and pattern of innervations as well as the pathway from these ganglia to the gland vary significantly from species to species.

The lacrimal gland secretes a number of proteins like lysozyme, lactoferrin, lipocalin, scIgA (Tiffany 2008). The secretion of these proteins is regulated by the nerves that innervate the gland and their associated nurotransmitters/ neuropeptides (Walcott 1998). The important receptors present on the lacrimal gland are acetylcholine receptors like muscarinc M3 (Mauduit et al. 1993), vasoactive intestinal peptide type I and II, norepinephrine like alpha 1 and beta. Other receptors present are for neuropeptide Y, adenocorticotrophic hormone (ACTH) and alpha- melanocyte stimulating hormone. Since the epithelial cells of the gland are extensively coupled by junctional complexes, secondary messengers like inositol triphophate can easily diffuse between cells and activate the unstimulated cells too (Walcott 1998).

The muscarinic receptors in the gland are linked to G proteins, which are in turn linked to phospholipase C. This, on activation, releases inositol phosphate 3 (IP3) and diacyl glycerol (DAG) (Dartt 1989). IP3 induces the release of intracellular stores of calcium and opens calcium channels. DAG, on the other hand, activates protein kinase C isoenzymes, which further stimulates secretion. VIP receptors activate protein kinase A, which in turn causes cAMP release stimulating protein secretion (Hodges et al. 1997). Alpha adrenergic compounds cause protein secretion by activating protein kinase C (Walcott 1998).

Protein secretion in the acinar cells involves the fusion of vesicles with the apical membrane. There is also a basolateral membrane trafficking that is seen in these cells. This is responsible for the entry of molecules like prolactin into the cells. In addition, this basolateral membrane trafficking has also been implicated in antigen presentation and secretion of autoantigens (Mircheff et al. 1994) which leads to immune mediated apoptosis of acinar cells and loss of physiological function as seen in conditions like Sjogren's syndrome.

10.4 The Dry Eye Syndrome

The lacrimal gland forms an integral part of the lacrimal functional unit (LFU) due to its physiological function of secreting the aqueous component of the tear film. Any perturbation in the functioning of this gland, which may be age related, drug induced, autoimmune or due to orbital radiotherapy leads to destabilization of the tear film which in turn leads to a chronic debilating condition called the dry eye syndrome or keratoconjunctivitis sicca (KCS) (Fig. 10.3).

The International Dry Eye Workshop, 2007 (2007a) defined dry eye as:

> *Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbances and tears film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of tear film and inflammation of the ocular surface.*

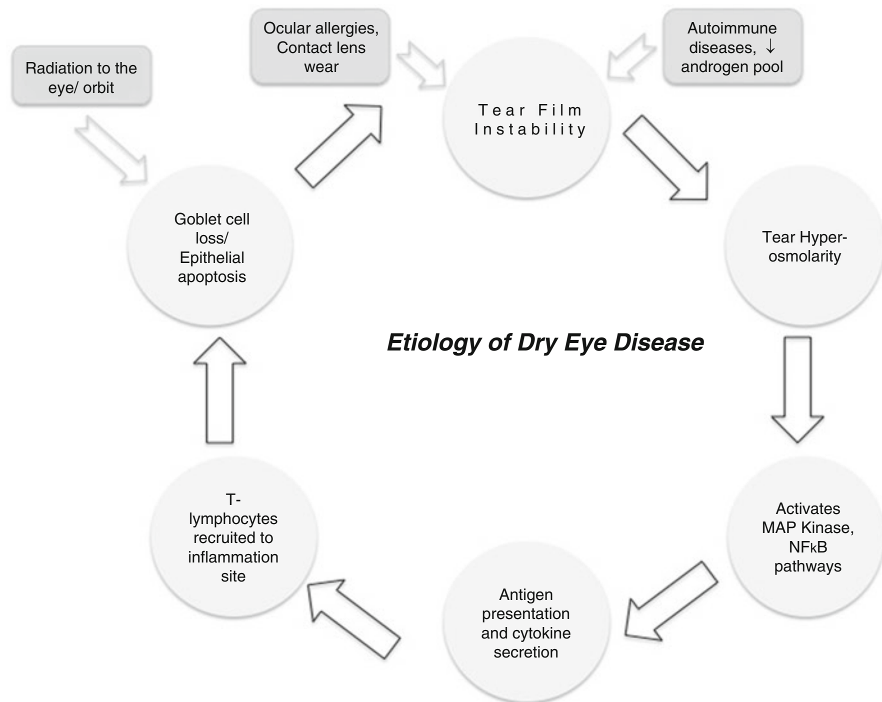

Fig. 10.3 Etiology of dry eye disease

The most important causative/ contributing factors for dry eye are (2007a):

- Primary lacrimal gland dysfunction due to reduction in circulating androgens
- Secondary lacrimal gland dysfunction due to sarcoidosis, lymphoma etc.
- Autoimmune diseases like Sjogren's syndrome
- Reflex hyposecretion as in contact lens wear, diabetes, exposure to systemic drugs like antihistamines, beta blockers etc.
- Orbital radiotherapy for ocular malignancies
- Meibomian gland dysfunction

10.5 Etiology of Dry Eye Syndrome

Dry eye may be classified as aqueous deficient dry eye, caused due to lacrimal gland dysfunction; or evaporative dry eye, caused due to meibomian gland dysfunction. In the former, there is a deficiency in tear production itself due to perturbations in lacrimal gland function; and In evaporative dry eye, the rate of evaporation of tear

from the ocular surface increases due to an unstable lipid film secreted by the damaged meibomian gland (2007a).

There has been a considerable increase in knowledge about the etiopathogenesis of dry eye syndrome in the past few years. The pathologic features of this condition include increased epithelial proliferation,stratification and abnormal differentiation with maintenance of a basal phenotype (Jones et al. 1998). This is accompanied by reduced expression of secretory and membrane-bound mucins by the ocular surface conjunctival epithelial cells (Danjo et al. 1998) compounding the effects of existing lacrimal dysfunction.

The two most important factors that contribute to the initiation and progression of dry eye are *tear hyperosmolarity* and *tear film instability* that adversely affects the ocular surface epithelial function and differentiation (Gilbard et al. 1989a). Trauma to a poorly lubricated and unprotected ocular surface due to blinking or environmental factors becomes a confounding factor, which worsens the condition (Danjo et al. 1998; Lemp 1995). Tear film stability, which is important in maintaining clear and sharp vision, is threatened when the interactions between stabilizing tear film constituents are compromised either by decreased tear secretion, delayed clearance, or altered tear composition as is seen in xerophthalmia and allergic eye diseases. Ocular surface inflammation is secondary consequence. Reflex tear secretion in response to ocular irritation is seen as the initial compensatory mechanism, but, with time, due to severe inflammation and chronic secretory dysfunction a decrease in corneal sensation occurs which compromises the reflex response and results in even greater tear film instability. Dysfunction of the LFU is considered to play an important role in the evolution of different forms of dry eye (2007a).

Even though tear fluid is secreted as a hypotonic fluid, yet due to excessive evaporation from the exposed ocular surface or low rate of aqueous tear flow (or a combination of the two) tear hyperosmolarity may arise. This hyperosmolarity stimulates various inflammatory pathways involving the MAP kinases, inflammatory cytokines (interleukins, tumor necrosis factor alpha) and matrix metalloproteinases (MMP9). This cascade of events attract the circulating T cells within the lacrimal glands. Under physiological conditions, the trafficking lymphocytes, finding no inflammation, would undergo apoptosis. However, in the presence of inflammatory signals, these lymphocytes become activated and secrete pro-inflammatory cytokines, which cause homing of additional T cells to the tissue and an increase in the level of inflammation thereby aggravating the existing condition and forming a vicious loop of inflammation (Gao et al. 1998). Any condition that results in hyper activity of the functional unit can also initiate inflammatory response within the lacrimal glands resulting in antigen presentation and cytokine secretion by the epithelial cells of the gland (Meggs 1993; Mircheff et al. 1998). These pro-inflammatory mediators cause epithelial cell death by apoptosis and also loss of conjunctival goblet cells – a combinatorial assault that leads to worsening of the existing condition.

10.6 Aqueous Deficient Dry Eye

In addition to release of inflammatory mediators, the etiology of dry eyealsoinvolves the loss of anti-inflammatory environment within the lacrimal glands, which may occur due to a drop in the levels of circulating androgens (Azzarolo et al. 1997; Sullivan et al. 1984). At the time of menopause in women, or due to various pathologic conditions, the level of circulating androgens may drop below a threshold level, thus making the tissues vulnerable for the initiation and progression of immune-based inflammation. A number of studies have shown a significant correlation between the levels of these inflammatory cytokines and the severity of ocular surface irritation symptoms, corneal fluorescein staining and the severity of conjunctival squamous metaplasia in patients (Pflugfelder et al. 1999).

Severe dry eye is also seen in patients of Sjogren's syndrome,which is an autoimmune disorder. In Sjogren's, autoantigens are expressed at the surface of the epithelial cells which causes homing and retention of tissue specific CD4 and CD8 cells. These lymphocytes cause loss of lacrimal acinar and ductal cells due to an immune mediated attack leading to tear hyposecretion and destruction of the gland. The precise trigger factors for Sjogrens is not known but risk factors include genetic profile, low androgen pool and certain viruses.

Radiation therapy, which represents a commonly used modality in the treatment of ocular and oculoadenexal disorders including benign and malignant tumors, also contributes to the development of dry eye syndrome in patients. Despite a rapid evolution in the field of radiotherapy over the past years, a significant number of patients are still seen with acute and chronic ophthalmic complications including severe dry eye (Alberti 1997; Durkin et al. 2007). Preliminary data from our institute indicates that chronic dry eye develops in over 49 % of the patients who undergo external beam radiation therapy for ocular malignancies (unpublished data).

The causes of dry eye post- radiotherapy are multifactorial: (a) decrease in the lacrimal secretion leading to loss of aqueous layer; (b) ocular surface damage with goblet cell loss leading to loss of mucin secretion; (c) meibomian gland atrophy with loss of lipid layer secretion. In the early post-radiotherapy phase, lacrimal gland loss has been shown to be due to inflammation mediated apoptotic loss of acinar cells. In contrast, stem cells becoming sterile is proposed to be the main cause in late phase resulting in insufficient replacement of acinar cells with resultant decrease in tear secretion. This combined with the radiation induced fibrosis leads to loss of lacrimal function with progressive decrease in tear volume and finally dry eye (Barabino et al. 2005; Konings et al. 2005; Parsons et al. 1996; Stephens et al. 1991).

Contact lens wear is yet another condition that may lead to the development of sever dry eye in long -term users. The mechanism underlying the development of dry eye in these patients is probably the reduction in corneal sensitivity and an increase in tear film osmolarity due to chronic usage. Similar arguments have been proposed for the development of dry eye post-LASIK therapy.

10.7 Current Therapies for KCS

Even though there has been significant advancement in the knowledge about lacrimal gland dysfunction and development of dry eye syndrome, yet the same cannot be said for the management of the condition. Dry eye is still a chronic debilating condition, the treatment and management of which aims at palliation and improving the quality of life of the patient. The current treatment modalities available are lubricating agents like hydroxy methylcellulose, solutions containing bicarbonates and potassium,hyposmotic artificial tears (HypoTears, Novartis Ophthalmics) and artificial serum. In cases of severe dry eye, therapies such as anti-inflammatory medications (cyclosporins A, corticosteroids), pharmacological tear stimulants like diquafosol, rebamipide, ecabet sodium, pilocarpine etc. are employed. In certain instances, where the patient does not get any relief in symptoms by these, surgical interventions like punctal occlusion and salivary gland autotransplantation are done to slow down the progress of the condition (2007b).

On recommendation of the committee on the therapy and management of dry eye (DEWS 2007a, b), the treatment/ management protocol for this condition is now shifting towards employing strategies that would increase the natural production of tears, maintain ocular surface integrity and reduce/eliminate the levels of existing inflammation. With these objectives in mind various therapeutic avenues are being explored with the inclusion of cell therapy for restoring the damaged lacrimal gland.

10.8 Research on Lacrimal Gland

Given the sparsity of data on the etiopathogenesis and treatment of dry eye, it is still not clear how alteration in tear film composition can cause such a vicious cycle of tear film instability and chronic ocular inflammation. Even though a lot of research is being directed towards profiling the proteins, lipids and other constituents present in human tear yet there is a glaring lack of comparative data between normal individuals and dry eye patients.

10.9 Animal Studies

10.9.1 In-Vitro Cultures

An important area of investigation in this field is to find a common link between tear film osmolarity, tear film break up response and the resultant inflammatory stress. In order to facilitate these studies, not just *in-vivo* models but also *in-vitro* models are being developed that would greatly assist the investigation into the secretory repertoire of lacrimal gland epithelia, regulation of secretion and etiopathogenesis of lacrimal gland conditions like Sjogren’s syndrome.

Procedure for *in-vitro* culturing of lacrimal gland acinar cells has been evolving for nearly two decades now (Table 10.2). Oliver et al. published one of the first reports on *in-vitro* culture of rat lacrimal gland acinar cells in 1987 wherein they described a method for culturing a dividing population of morphologically differentiated rat lacrimal acinar cells on a three-dimensional, reconstituted basement membrane gel. The cultured acinar cells proliferated on the basement matrix and showed the presence of cytoplasmic secretory granules (Oliver et al. 1987). However, their culture system could only maintain the epithelial cells for 6–7 days after which fibroblast overgrowth was observed. Successful *in-vitro* culture of lacrimal acinar cells was first achieved and published by Meneray and Rismondo in two separate reports in 1994 (Meneray et al. 1994; Rismondo et al. 1994). The importance of media formulation, supplement profile and extracellular matrix composition for optimal growth and functionality of these cells was first reported by Hann et al. (1991) and these findings were supported by a number of subsequent reports.

A major problem faced by all these investigators was that the lacrimal acinar cells could not be induced to proliferate significantly *in-vitro*. This issue was resolved by Schonthal et al. who reported in 2000 that the i*n-vitro* proliferation of lacrimal acinar cells could be improved significantlyby the use of EGF, dihydrotestosterone (DHT), Matrigel® and HepatoStim culture medium (Schonthal et al. 2000). A number of recent publications also report the use of polyethersulfone dead-end tube (Long et al. 2006), denuded amniotic membrane (Schrader et al. 2007) as scaffolds and rotary cell culture system (Schrader et al. 2009) for successful *in-vitro* culture of rat or rabbit lacrimal glands.

The effect of androgen on the control of secretory component output by the lacrimal gland has been well established. The effect of androgens and androgen analogues on *in-vitro* culture of lacrimal acinar cells has helped elucidate the control that the androgens exert on the synthesis and secretion of secretory component (Sullivan et al. 1984; Hann et al. 1991; Kelleher et al. 1991; Sullivan et al. 1990) as well as other biochemical parameters related to the lacrimal secretion including the basal tear flow rate (Azzarolo et al. 1997).

The culture systems developed for the lacrimal acinar cells have also been optimized to assess the functionality of these cells. The currently employed conditions for the *in-vitro* culture of these secretory cells support the *in-viv*o mimicry of their secretion pattern as elucidated by the detection of scIgA, lactoferrin, lysozyme, lacritin and a number of other tear proteins in the culture supernatant.

10.9.2 Animal Models

In order to better understand this condition, animal models have been developed which mimic the features of human dry eye syndrome. The important animal models that have increased our knowledge of this condition are:

Mouse models created using scopolamine and environmental dessicating stress show that osmolarity of tear as well as secretion of inflammatory cytokines is increased under such condition (Gilbard et al. 1989b; Stewart et al. 2005).

Mouse model of Sjogren syndrome dry eye have also been developed and it has been shown in these models that androgens have the potential to reduce the inflammatory response due to autoantigen presentation.

Neurturin deficient mice that develop dry eye and show elevated levels of inflammatory mediators in their tears (Song et al. 2003).

Rabbit model of KCS created using the technique of ablation, which shows that steroids like dexamethasone can be used to reverse the ocular surface damage and also to increase the low tear film break up time (Nagelhout et al. 2005).

Even though it is undeniable that these animal models have indeed increased our basic understanding behind the etiopathology of dry eye, yet the fact remains that we are not completely sure of how well these animal models mimic the human condition. In order to bridge this gap in understanding the similarity/ differences between the animal form and the human form of the dry eye condition, it is imperative that studies be conducted on human tissue. Since it is unethical to undertake such studies without sufficient background information, *in-vitro* models of human lacrimal gland become very important tool for research.

10.10 Human Lacrimal Gland In-Vitro Studies

In-vitro work on human lacrimal gland cultures is scarce, possibly due to the difficulty in obtaining human tissue for research. To the best of our knowledge and literature search, there is just one report, prior to ours, published by Yoshino in 2000 (Yoshino 2000), which dealt with establishing human lacrimal cultures from cadaveric tissue. However, the study reported no detectable proliferative potential of the cells when cultured on Matrigel™, even though the cells had a central lumen containing the secretory protein lactoferrin.

Our group has been working with human lacrimal gland cultures since 2008 and we were the first to report the establishment of functionally viable human lacrimal gland *in-vitro* culture system from fresh exenteration specimens (Tiwari et al. 2012). Our results deal with the establishment of functionally viable human lacrimal gland cultures on three matrices: Matrigel®, collagen and denuded human amniotic membrane; and more recently as 3D functional lacrispheres (unpublished data). We have extensively characterized our culture system using markers for epithelial, myoepithelial and mesenchymal origin by immunohistochemistry, immunocytochemistry and flow cytometry. Our established cultures show the capacity to synthesize and secrete quantifiable levels of major tear proteins like scIgA, lactoferrin and lysozyme into the culture supernatant (Fig. 10.4). We have successfully shown that these lacrimal epithelial cultures show a polymorphous population of cells (similar to the native gland) and can be maintained *in-vitro*, with intact secretory function, for a minimum period of 21 days. In addition, we also report that by day 16–18, these *in-vitro* cultures show the appearance of spherules and structures that look like ductal connections between them. We believe that this indicates their potential for *in-vitro* gland formation.

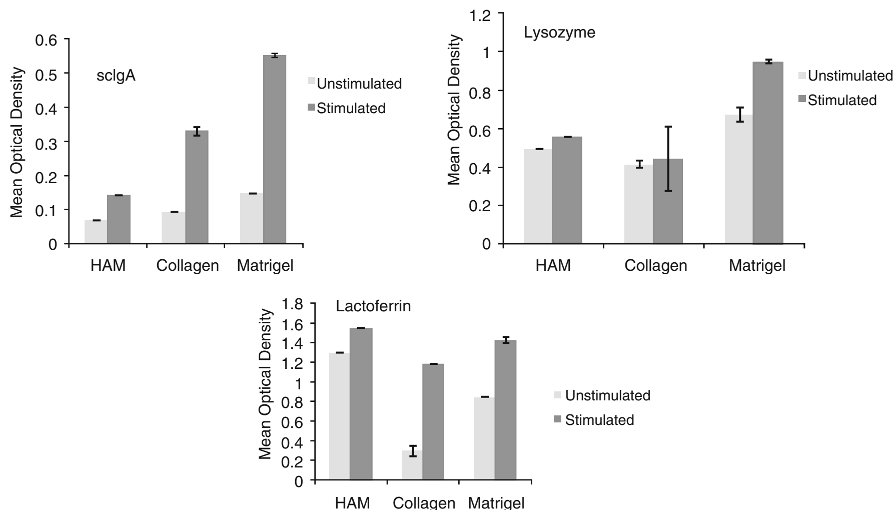

Fig. 10.4 Tear protein secretion by in-vitro human lacrimal gland cultures pre and post carbachol stimulation (Mean optical values can be correlated to protein levels)

Towards the long-term goal of cell therapy in chronic dry eye condition, our group evaluated the presence of stem-like cells in the native human lacrimal gland as well as in the established cultures. Our preliminary results indicate the presence of stem-like cells (ABCG2$^{+,}$ CD117^{+}, ALDH high) in the native human lacrimal gland as well as in our *in-vitro* cultures.

10.11 Regeneration of Lacrimal Gland and Future Directions

Dry eye can be an extremely debilitating condition with high incidence of associated morbidity. The current prevalence of dry eye in the world is estimated at around 11–22 %(Abelson et al. 2009). In the Indian context, these numbers are estimated to be around 18.4–20 % (Gupta et al. 2008; Sahai & Malik 2005). These epidemiological numbers are a good indicator of the need for research on dry eye syndrome.

While there has been tremendous improvement in management of dry eye; clinical and biochemical prognostic markers to predict the severity of the disease and its progression are yet to be defined. The current management regime is far from adequate in terms of patient compliance and outcome.

To look at long term management of the condition an important area that needs to be explored carefully is the potential to use cell therapy in chronic cases of dry eye. The last couple of years have seen an increase in the knowledge about the presence of stem-like cells in the lacrimal gland of mice (You et al. 2011), rat

(Shatos et al. 2012) and humans (Tiwari et al. 2012). These studies indicate the inherent potential of the gland to heal itself following an insult. The study published by You et al. (2011) showed that post injection of interleukin into the mouse lacrimal gland which destroys areas in the gland, stem-like cells migrate towards the site of injury and heal the wound. These cells can be harvested and grown under *in-vitro* conditions too. However, the authors report minimum *in-vitro* growth from uninjured gland. In contrast, the recent study by Shatos et al.(2012) on rat lacrimal gland and our own experience with human lacrimal gland (Tiwari et al. 2012) showed that stem-like cells are present in the native, uninjured gland too which can be maintained under appropriate *in-vitro* conditions. The study published by Mishim aet al. has shown that transplantation of lacrimal gland derived clusterin^{+}side –population (SP) cells could restore function in mice with radiation induced lacrimal dysfunction, possibly by inhibiting oxidative stress induced hypofunction of the gland (Mishima et al. 2012). Another study worth highlighting is by Hirayama et al. which has demonstrated functional recovery of lacrimal function in mice with extra-orbital lacrimal gland defect, post orthotopic transplantation of bioengineered lacrimal gland (Hirayama et al. 2013).

The presence of stem cells in the lacrimal gland is an important finding that leads us to believe that these cells can be recruited to salvage the damaged gland. However, before we take a leap of faith the viability, homing and functionality of these cells need to be established by more extensive *in-vitro* studies and independent animal experimentation.

10.12 Conclusion

Progress in the research in the dry eye disease and lacrimal gland regeneration appears to be very promising and is likely to pave way for identification of biomarkers to prognosticate the disease as well as in long-term clinical cell therapy for chronic dry eye syndrome.

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Chapter 11
The Development of a Stem Cell Therapy for Deafness

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Abstract Medicine is at the doorstep of a phenomenal revolution, brought about by the advances in the stem cell field and the development of new technologies to engineer cells and tissues into more complex organs. The promise of a true regenerative approach to organ damage and loss of function is closer than ever to becoming a reality. The auditory field is participating in these developments with high expectations. Since the cochlea is an organ of difficult access and with very limited regenerative capacity, conventional therapeutic approaches have failed and, currently, the only treatments available are in the form of hearing aids and cochlear implants. The potential restoration of hearing by the use of exogenous stem cells will offer a solution to a condition that has very limited options. In this chapter, we review the increasing volume of research on this emerging field and discuss the key elements that need to be developed further, in order to translate the basic science into a clinical reality.

Keywords Hearing • Hearing impairment • Cochlea • Sensorineural hearing loss (SNHL) • Deafness • Cochlear implant • Cellular replacement • Supporting cells (Scs) • Vestibular organ • Hair cells • Notch signalling • $p27^{Kip1}$ • Cochlear epithelium • Neurotrophic factors (NTFs) • Neurotrophin-3 (NT-3) • Brain derived neurotrophic factor (BDNF) • Evoked auditory brainstem response (eABRs) • Auditory brainstem implant (ABI) • Stem cell transplantation • Deafness • Embryonic stem cells (ESCs) • ESC-derived neuroprogenitor cells • Neural stem/progenitor cells • Auditory progenitor/stem cells • Mesenchymal stem cells (MSCs) • Induced pluripotent stem cells (iPSCs) • MESC-derived embryoid bodies • Sensory cells • Sensory neurons • Neurospheres • Human fetal auditory stem cells • Cell isolation •

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Auditory stem cells • Oligonucleotide arrays • Small compound libraries • Target-based approach • Phenotype-based screening • Perilymphatic transplantation • Scala tympani • Scala media • Transplantation • Modiolar nerve trunk • Cochlear nerve trunk • Rosenthal's canal • Inner ear cell transplantation • Distortion product otoacoustic emissions (DPOAEs)

11.1 Introduction

Hearing, one of our primordial senses, is crucial for communication, pleasure and awareness. The impact of a hearing deficit, especially during childhood, is huge. It can lead to problems with the development of speech and language having implications for social integration and affecting quality of life as a whole. According to 2015 estimates by the World Health Organization (WHO), 360 million people worldwide have moderate to profound hearing loss in both ears www.who.int/mediacentre/factsheets/fs300/en/index.html

Hearing impairment is most commonly caused by both inherited and acquired factors such as noise, prescription of ototoxic drugs or age (Holley 2005). The pathological changes underpinning hearing loss are concentrated mainly in the inner ear, which is the primary centre for hearing. The process of sound perception begins at the *cochlea*, a small organ situated within the temporal bone, which converts the incoming mechanical sound waves into an electrical, nerve-transmitted signal. The cochlea contains two major types of sensory receptor cells, the inner hair cells (IHCs) and the outer hair cells (OHCs). The IHCs are the primary transducers, translating the displacement of their apical hair bundles, induced by the sound waves, into a depolarizing signal. The OHCs, on the other hand, receive efferent stimuli from higher control centres to modulate the auditory signal and contribute to magnifying the sensitivity of the system (Webster 1992). The IHC signals are conveyed to higher auditory nuclei in the brainstem via the spiral ganglion neurons (SGNs), the first order neuron of the auditory pathway (Fig. 11.1). There are several nuclei in the brainstem responsible for adjusting and tuning the signal from the cochlea before sending fibres for the final interpretation of sound to the auditory cortex. The loss of sensory cells in the cochlea accounts for the majority of hearing deficits (90 %), and is classified as sensorineural hearing loss (SNHL). Such deafness can be caused by primary degeneration of the spiral ganglion neurons, in what is known as auditory neuropathy or by the primary loss of hair cells (Hardie and Shepherd 1999; Webster and Webster 1981). Secondary degeneration of neurons commonly follows HC loss and cell death occurs due to lack of trophic support (Fritzsch et al. 1997). However, in humans, this process is highly variable and depends on several factors, such as the nature of the original ototoxic insult (Nadol 1997; Nadol et al. 1989).

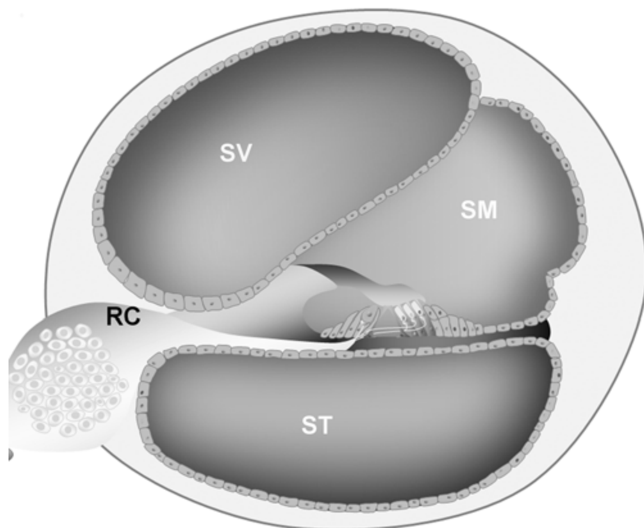

Fig. 11.1 Schematic illustration showing the anatomy of the cochlear duct. The cochlea comprises three fluid-filled chambers, separated in part by a bony structure, the osseous spiral lamina. The cochlear duct can be divided according to the type of fluid. The perilymph, contained in the Scala Vestibuli (SV) and Scala Tympani (ST) has an ionic composition similar to the extracellular medium, with high sodium concentration and low potassium. The Scala Media (SM) contains the endolymph, which in contrast to the perilymph, is high in potassium and low in sodium. The SM compartment harbours the organ of Corti, where the sensory hair cells play a crucial role in converting the mechanical sound wave into an electrical neural signal that is then sent to the auditory neurons. These auditory neurons have a bipolar morphology, with a dendrite that receives information from hair cells in the organ of Corti and the axon, which resides in the auditory nerve. The cell bodies of these neurons form the spiral ganglion, located in a discrete compartment, the Rosenthal's canal (RC)

11.2 The Adult Mammalian Cochlea Does Not Regenerate Its Hair Cells or Neurons

While non-mammalian species can repair and heal their damaged sensory epithelia, the mammalian cochlea does not have the potential to regenerate either the hair cells or the sensory neurons (Raphael 2002). In avians and lower vertebrates, support cells can be triggered into proliferative or transdifferentiation states by signals from dying hair cells, leading to a limited degree of hair cell regeneration (Morest and Cotanche 2004). As in birds, the mammalian supporting cells share a common progenitor with hair cells during development (Fekete et al. 1998). However SCs of the mammalian organ of Corti fail to show any regenerative response to HC loss, via either direct transdifferentiation or mitosis (Forge et al. 1998). The vestibular organ

(including the utricle, the saccule and the cristae ampullaris) is also a mechanosensory structure located in the inner ear that conveys information on spatial position and gravitational acceleration. A minor regeneration of hair cells has been observed in the vestibular sensory epithelia in guinea pigs following ototoxic drug treatment (Forge et al. 1993). Moreover, a study on the murine utricle has confirmed evidence that vestibular hair cells can spontaneously regenerate after exposure to the ototoxic drug gentamycin, with large numbers of immature hair cells being seen as early as 2 weeks after the lesion. However, neither the numbers of regenerated cells nor their appearance were normal (Kawamoto et al. 2009). A population of stem cells can be isolated from the adult mouse utricle (Li et al. 2003a), but these cells virtually disappear from the mouse cochlea after the third week of age (Oshima et al. 2007). The proliferation and differentiation of mouse inner ear stem cells in vitro seems to be regulated by Notch signalling, similar to the progenitors in vivo. Inhibition of Notch signalling by γ-secretase enhances hair differentiation by upregulating the expression of Atoh1. On the other hand, activation of Notch signalling by its intracellular domain (NICD) increases proliferation but also the differentiation of neural phenotypes by upregulating Sox2 and the neuronal transcription factor Ngn1 (Jeon et al. 2011).

The mature vestibular and auditory organs differ greatly, not only in their ability to contain stem cells but also in their potential for regeneration. What is the underlying explanation for the differences of these two related, neighbouring sense organs? Part of the answer may lie in the fact that cochlear supporting cells become terminally postmitotic during embryogenesis whereas supporting cells in the vestibular organ show a lingering proliferative capacity (Rubel et al. 1995). However, it is not clear whether the lack of mammalian hair cell regeneration could be due to an intrinsic loss of competence for SCs to divide and differentiate, to the absence of appropriate mitogenic signals, or to the presence of instructive signals that actively block any regenerative capacity. The fact that proliferative stem cells cannot be isolated from the murine cochlea after the third week of age, despite of the presence of mitogens in the media and the removal from any potential inhibition present in the tissue, would suggest that changes are more likely intrinsic and fixed. Cell cycle regulators are believed to be key factors in maintaining the post-mitotic state of SCs. Upregulation of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor (CDKI) is found in the cochlear epithelium at the same time that terminal mitosis begins during embryogenesis, and it remains in the SCs till adulthood (Chen and Segil 1999). Evidence has been presented that a perinatal population of supporting cells can downregulate $p27^{Kip1}$ *in vitro* and re-enter the cell cycle. This ability is severely reduced by the time they reach P14. However, even at this stage, a small proportion of cochlear supporting cells can transdifferentiate in vitro into hair cell-like cells (White et al. 2006). Supporting further the idea that the ability of these cells to proliferate is $p27^{Kip1}$-dependent, cells taken from $p27^{Kip1}$-null animals had an enhanced proliferative capacity in culture when compared with wild type-ones (White et al. 2006). The level of expression of $p27^{Kip1}$ protein remains robust in differentiated cochlear SCs *in vivo*, suggesting that $p27^{Kip1}$ imposes strong inhibition on cell mitosis in differentiated SCs and may prevent them from dividing after HC loss. The inhibition of SC

proliferation seems to be a major factor that blocks the possibility of HC regeneration in the mammalian cochlea. Likewise, the SCs may lose their ability to convert directly into HCs during development. Experiments have shown that new HCs are generated when existing HCs are ablated in the mouse organ of Corti prior to E16, but this ability is lost after E16 (Kelley et al. 1995). Early postnatal rats treated with the ototoxic drug amikacin generated cells that had mixed features between hair and supporting cells. These cells have been interpreted as having attempted direct transdifferentiation but failing to achieve complete HC morphological characteristics (Daudet et al. 1998). Together, these findings suggest that the ability of auditory SCs to undergo direct transdifferentiation is limited during the course of cochlear development. Mature SCs may have differentiated too far for functional, direct transdifferentiation to occur.

In contrast to cochlear HCs, there is some evidence to suggest the presence of neural progenitor cells in the adult auditory nerve. Although Oshima et al failed to isolate stem cells/progenitors from the spiral ganglion of adult mice (Oshima et al. 2007), Rask-Anderson et al isolated nestin-positive neural progenitors that also expressed TrkB and TrkC from adult human and guinea pig spiral ganglion tissues (Rask-Andersen et al. 2005). However, regeneration of SGNs cannot be observed after degeneration; therefore damage to neurons can lead to permanent deafness (Sekiya et al. 2003). Even when the cell body and central axon survives, deafness can still occur due to degeneration of peripheral processes (Nadol 1997). SGN degeneration has been described in a variety of pathologies. Exposure to sound pressure levels that do not harm HCs or cause HC loss can still insult the SGN and trigger neuronal degeneration (Kujawa and Liberman 2006). In humans, the regrowth of SGNs does not appear to be clinically significant (Nadol 1997). In summary, although there are indications that some reparative potential is still present in the adult spiral ganglion, recovery does not seem to take place at any substantial rate after damage to HCs or neurons in the adult mammalian cochlea.

11.3 A Therapeutic Solution for Deafness: The Cochlear Implant

Giving the lack of a regenerative response in the mammalian ear, the development of a surgically implanted prosthesis was seen as the only medical effective therapy for hearing loss. The first attempts at cochlear implantation were performed in the 1950s by the collaboration between Djourno and Eryiès, a French surgeon and an engineer, to place a coil of wire in the inner ear of two deaf patients. Although these trials failed after a short time, they kick-started an area of research that was going to deliver substantial advances (Eisen 2003). A modern cochlear implant is an electronic device that can be divided into two major parts: the external head piece that includes a microphone and speech processor and is placed on the skin close to the temporal bone area, and the internal cochlear electrode. The external component functions as a transmitter to process sound signals and is connected to the receiving

coil which, secured on the temporal bone beneath the skin, is responsible for converting the signals into electric impulses and delivering them through an internal cable to the implanted electrode in the cochlea. An array of up to 22 electrodes winding up through the scala tympani of the cochlea stimulates the SGNs, which in turn send the information to the brain via the auditory nuclei. The cochlear implant can give a quality of sound discrimination fine enough to understand speech but post-implantation rehabilitation is critical for ensuring the effectiveness of treatment. Modern cochlear implants allow the typical patient to understand more than 90 % of words in unfamiliar sentences when presented in quiet listening conditions (Spahr and Dorman 2004). In certain parts of the world, the high costs of the operation and prosthesis have a heavy influence in their accessibility. Because many patients were unable to benefit from cochlear implantation due to an auditory nerve dysfunction, this led to the development of the auditory brainstem implant (ABI). The principle of ABI is similar to cochlear implant but bypasses the function of SGNs by stimulating the cochlear nucleus (CN) directly via surface mounted 'button' electrodes (Moore and Shannon 2009). This requires an electrode implanted directly into the brainstem, making this device more risky and still not widely used. The best performance of ABI is still poor when compared with cochlear implantation (Moore and Shannon 2009).

11.4 Cellular Replacement: A Promising Therapeutic Strategy

Given the lack of endogenous regeneration and the limited range of therapeutic options available, the potential to develop a treatment based on the delivery of exogenous cells offers new hopes. Cell-based approaches have been proposed directed at the replacement or restoration of damaged HCs and/or SGNs. Stem cells are excellent candidates for biological implantation as they have the potential to proliferate and differentiate, both required features for a regenerative strategy option. The optimisation of a cell transplantation strategy is a phenomenal task, since there are huge variables to consider in each experimental paradigm. The range of different stem cells and cell lines of potential use, the state of the host tissue and the routes for delivery are all issues that could affect the efficiency of transplantation. Stem cell transplantation experiments could also be excellent models in the context of drug discovery and development. They may be combined with supplementary treatments such as neurotrophic factors (NTFs) or exogenous compounds that could provide the niche for transplanted cell survival or, alternatively support differentiation into the target cell type. In the following sections, we will discuss the current evidence on the achievements and challenges lying ahead for stem cell-based therapeutic strategies for hair cell and spiral ganglion cell loss, with emphasis on the different cell types and delivery routes used in each experimental condition.

11.5 Cell Candidates for Transplantation

Several stem cell types and lines have been used in transplantation experiments, ranging from directly transplanting undifferentiated embryonic (ESCs) and mesenchymal stem cells (MSCs) to various kinds of chemically differentiated lineages, including some genetically modified cells.

11.5.1 Embryonic Stem Cells

Most of the stem cells used so far in transplantation studies have been of murine origin. Many ESCs have also been genetically modified by tagging them with green fluorescent protein (GFP), a reporter used for tracing after transplantation. In a study performed in deafened guinea pigs, undifferentiated and partially differentiated mouse embryonic stem (ES) cells were delivered into the cochlea (Hildebrand et al. 2005). Cells survived in the site of transplantation and were also observed in the cochlear chambers, stria vascularis, endolymph fluid of the scala media and spiral ligament for a postoperative period of at least 9 weeks. However, the transplanted cells failed to differentiate. There was no evidence of significant immunological rejection of the transplanted cells, this being quite relevant since no apparent immunosuppressant treatment was provided. Sekiya et al described extensive migration of mESCs in the cochlear modiolus after transplantation (Sekiya et al. 2006). Cells were observed not only in the cochlear nerve but were also present in the Rosenthal's canal and scala media. Although they appeared to migrate more extensively into the damaged auditory nerve than on undamaged ones, cells on intact nerves showed more signs of neuronal differentiation. In a different study, ESCs co-implanted with embryonic dorsal root ganglion neurons migrated to the area close to the ventral cochlear nucleus (Hu et al. 2004). mESCs genetically modified to conditionally express neurog1 have also been implanted into deafened animals. After a short induction in vivo with doxycycline to induce transient expression of the neurog1 transgene, a continuous supplement of glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) was provided for over 3 weeks to provide trophic support for the transplanted cells (Reyes et al. 2008). The results showed the transplanted cells in several areas of the cochlea- the modiolus, Rosenthal's canal and the site of transplantation, the scala tympani. Surviving cells were found mainly in the scala tympani, they had more of a neuronal-like appearance and had increased expression of the neuronal maker TUJ1 than cells in the modiolus. Moreover, the majority of TUJ1 positive cells were co-labelled with the vesicular glutamatergic neuron marker VGLUT (Altschuler et al. 2008).

11.5.2 Neural Stem Cells (NSCs)

The use of undifferentiated ES cells as an initial substrate population for transplantation may have the benefits of an increased proliferative capacity, but the dark side to this potential is the risk of tumorigenicity. Consequently, much work has focussed on the use of committed progenitors, those in which the pathway to terminal differentiation has already been embarked upon. Neural stem/progenitor cells have also been used for otic transplantation. Neurospheres, obtained from the adult mouse lateral ventricle, were introduced into either normal or deafened inner ears in the guinea pig. These neural stem cells (NSCs) were transduced with Neurogenin2 by retroviral transfection prior to transplantation. The survival rate of transplanted cells was relatively poor, even in the neurogenin-transduced group with there being no significant difference in survival rate between deafened and normal animals. The transplanted cells distributed themselves in the perilymphatic chamber and in the Rosenthal's canal (Hu et al. 2005b). Interesting results were obtained from Parker et al. using the c17.2 cell line, a NSC line derived from immortalized male murine foetal cerebellar cells and implanted into a sound-damaged model (Parker et al. 2007). The transplanted cells were traced by Y-chromosome fluorescence in situ hybridization (Y-FISH) and NSCs were later found with characteristic of both neural tissues (satellite, spiral ganglion and Schwann cells) and cells of the organ of Corti (hair cells and supporting cells). Although still a single report that may need further confirmation, this study shows that potentially, several lineages could be obtained from neural stem cells, besides the expected neurons and glia. A caveat to this approach, requiring fetal tissues, is that there are considerable ethical implications of using material derived from pregnancy terminations which may preclude their widespread use clinically.

Recent studies on transplantation using NSCs were done in undamaged animal model to observe their distribution and potential effect on auditory function (Fu et al. 2009). NSCs were dissociated from the hippocampal tissue of rat embryos and transferred to neurosphere culture media with the combination of bFGF and epidermal growth factor (EGF). Transplanted NSCs were able to survive in the perilymphatic space 2 weeks after transplantation, some of them were also observed in the endolymphatic space and Rosenthal's canal. Unfortunately, there was no report of differentiation from this experiment. Hu et al. carried out a study to look at the effects of combining cell therapy with chronic electrical stimulation (CES) and exogenous neurotrophic growth factor (NGF) (Hu et al. 2009). Embryonic dorsal root ganglion neurons (DRGs) were implanted into a deafened animal model and these were found close to Rosenthal's canal in the adult host cochlea for up to 4 weeks after transplantation. They also showed extensive neurite projections penetrating into the bony modiolus and reached the spiral ganglion region in animals supplied with CES and/or NGF, suggesting that a 'cells alone' approach may need to be supplemented with exogenous factors to assist grafting and functional recovery

11.5.3 Auditory Progenitor/Stem Cells

Tissue specific stem/progenitor cells are probably the cell type that will more accurately recapitulate the process of normal differentiation that takes place in vivo. Although they may lack an extensive proliferative capacity, which could make difficult the scaling up of their production for therapeutic applications, their cochlear origin validates them as important systems for development and analysis. Several papers have reported the isolation of progenitor cells from either developing or early postnatal mouse cochleae. However, the lineage potential for the majority of these systems seems to be restricted to one given phenotype. Many of these cells have been shown to produce *in vitro*, either HCs (Malgrange et al. 2002; Oshima et al. 2007; Savary et al. 2007; Zhai et al. 2005) or SGNs (Rask-Andersen et al. 2005), but rarely both. Interesting exceptions are the human foetal auditory stem cells (hFASCs), a population isolated from 9 to 11 week old human foetal cochleae and described below in detail (Chen et al. 2007, 2009).

11.5.4 Mesenchymal Stem Cells

The potential use of MSCs as a source for cells for transplantation is an attractive option therapeutically, since they are relatively easy to obtain and amplify from the patient, allowing for the possibility of autografts. Only a few studies have looked at mesenchymal stem cells (MSCs) transplanted into the cochlea. Mouse MSCs labelled with GFP were xenografted into the perilymphatic space of a gerbil host. Seven days post-operation, most transplanted MSCs were found in the scala tympani and scala vestibuli, and only a small number located in the scala media. No GFP-positive MSCs were found in the cochlear modiolus (Matsuoka et al. 2006). In a later experiment, the same group compared a deafened model with a normal host. This experiment also studied the effect of different routes of transplantation, comparing between intraperilymphatic and cochlear modiolar injection. Transplantation into the perilymphatic duct was unable to deliver cells into Rosenthal's canal. However, with the modiolar injection, undifferentiated MSCs were able to survive both in the control and deafened cochleae, with the average number of transplanted cells found in the modiolus being greater in the deafened ear than in the control (Matsuoka et al. 2007). Unfortunately, there was no indication of differentiation of transplanted cells from either experiment. In a study carried out by Cho and co-workers (Cho et al. 2011), MSCs derived from human bone marrow were generically neuralised and transplanted into a guinea pig neuropathy model, where not only did the cells graft into Rosenthal's canal, an improvement in hearing thresholds was also recorded. In an intriguing set of results, human olfactory mucosa-derived stem cells, a subtype of MSC, were found to protect against hearing loss in a mouse model of early-onset SNHL (Pandit et al. 2011). However, although cells survived in the perilymphatic compartments, no evidence of integration in to the sensorineural

apparatus was found, leading the authors to speculate that the cells may be exerting some manner of paracrine protective effect. Although the replacement of sensory cells by MSCs remains to be unequivocally demonstrated and their potential in this regard is very limited, they could be used to target different types of SNHL. In some cases, the original problem lies not with the sensory cells but in the stria vascularis, a tissue in the lateral part of the cochlear duct responsible for the control of potassium homeostasis and for the generation of the endocochlear potential. A model for SNHL has been developed that generates dysfunction of the fibrocytes critical for the normal flow of potassium ions in the endolymphatic compartment. The application of the mitochondrial toxin 3-nitropropionic acid (3NP) induces acute SNHL by selective degeneration of the fibrocytes of the cochlear lateral wall, without inducing any significant damage to HCs or SGNs (Kada et al. 2009; Kamiya et al. 2007). Using this model, bone marrow MSCs were allogeneically transplanted via the lateral semicircular canal. MSCs were observed to localize at the ampullary area close to the transplanted site and in the perilymphatic ducts. Transplanted MSCs cells were found to replace the fibrocytes of the cochlear lateral wall, confirmed by co-localization of BrdU and connexin 26 markers. Moreover, a recovery of hearing was detected by using ABR measurements. There was no report of the distribution in any other areas of the cochlea and also no evidence to show differentiation of MSCs into another cell lineage (Kamiya et al. 2007).

11.5.5 Induced Pluripotent Stem Cells

The generation of induced pluripotent stem cells (iPSCs) appears to hail a new era in stem cell research. The potential to produce patient-specific stem cells with properties resembling those of hESCs should have huge impact in the development of cell-based therapies. Cells could be applied as autologous transplants, bypassing the need for immunosupression. Moreover, iPSCs are not surrounded by the ethical concerns associated with the use of hESCs. By using a relatively ‘simple’ protocol, that is the forced expression of four critical pluripotency factors (*Oct3/4*, *Sox2*, *c-Myc*, and *Klf4)* under ESC culture conditions, differentiated cells such as fibroblasts can be reprogrammed and turned into undifferentiated, ESC-like cells (Takahashi and Yamanaka 2006).

Although still a novel technique, its potential for repair of the auditory system is being explored. Mouse iPSCs were neuralized by exposing them to stromal cell-derived inducing activity (SDIA), showing evidence of neuronal differentiation *in vitro*. Neuralized iPSCs were then transplanted into the cochlea, where differentiation was observed 1 week after transplantation. The iPS-derived neural progenitor cells survived and were able to project their neurites toward cochlear hair cells. A marker for glutamatergic neurons, Vglut1, was expressed in some of transplanted cells indicating the possibility of differentiation in the host tissue (Nishimura et al. 2009). But more interestingly, using a combination of growth factors, cell signalling inhibitors and growing them over chicken stromal fibroblasts, Heller’s group showed

that mouse iPSCs can generate hair cell-like cells with the ability to mechanotransduce (Oshima et al. 2010). One must approach the use of iPS cells with caution, however – Nishimura et al (Nishimura et al. 2012) describe the formation of a teratoma in the cochlea in one of five animals transplanted with fibroblast-derived iPS cells, which will certainly be a red flag for any future regulatory procedures.

A key factor regarding the different types of cells for transplantation is their stage of differentiation. The more undifferentiated ESCs seem to have greater potential than progenitor cells in terms of migration and distribution in the cochlea. Neuroprogenitor cells are more committed to undergo differentiation and show a higher capacity to generate neuronal-like cells in the host tissues. The transplantation of neuroprogenitor cells into the cochlear nerve trunk has so far, provided the most convincing evidence that transplanted cells can integrate into the host tissue and project processes toward the organ of Corti. However, the transplanted cells are generally established as an ectopic ganglion, remaining at the site of transplantation rather than migrating into the Rosenthal's canal. MSCs, on the other hand, show a very strong propensity to differentiate into mesodermal cell types suggesting them to be a promising therapy for patients with SNHL attributed to the degeneration of cochlear fibrocytes. Nevertheless, migration and differentiation are not just dependent on the characteristics of transplanted cell types. Of critical importance is the technique for delivering them to the right place within the cochlea (see Sect. 11.8 for a more in-depth discussion).

11.5.6 Neuroprogenitors Induced from Pluripotent Stem Cells

Mouse ESCs were differentiated, before transplantation, into neuroectoderm-containing embryoid bodies (EBs) by a combination of basic fibroblast growth factor (bFGF) and insulin-transferrin-sodium selenite (ITSS). Although low number of cells survived in both the site of transplantation and the target area, the remaining cells showed the potential to undergo differentiation in the xenografted host by expressing both neuronal and glial phenotypes (Coleman et al. 2006). In an independent study, EYFP-labelled neuroprogenitors were derived from mouse ESCs by culturing in the presence of bFGF and were subsequently used in the ouabain-induced model of SGN damage. Transplanted cells at the cochlear nerve trunk showed strong evidence of neuronal differentiation by extending their perpheral processes toward the organ of Corti. Cells survived for more than 3 months and formed abundant processes projecting through the Rosenthal's canal (Corrales et al. 2006). In a different approach, mESC-derived embryoid bodies were co-cultured with SGN or HC explants, isolated from P5 (postnatal day 5) rats. Embryoid bodies co-cultured with hair cell explants showed a significantly greater number of neurofilament-positive and neural-like cells (Coleman et al. 2007). mESCs induced into neural differentiation by retinoic acid (RA) were used to compare the effects of different periods of time between the onset of injury and transplantation (Lang et al. 2008). This experiment showed a significant difference in the survival of

transplanted cells, with greater survival rates obtained when transplantation occurred soon after injury (i.e, 1 to 3 days after induction of damage). The ability of RA to produce sensory neuron-like differentiation from mESCs can be enhanced when combined with BMP4. However, although these neurons are sensory in nature (as implied by the expression of peripherin and Brn3a) there is no evidence that they express otic–specifc markers (Nayagam and Minter 2011). Most examples using mESC-derived cells aim to replace neurons. The replacement of HCs is still very difficult due to the limitations in the surgical techniques for cell delivery. Nevertheless, mESCs have been explored in *vitro* with the aim of producing hair cell-like cells. Sensory cells were derived from mESCs by culturing them in a chemical defined media with several growth factors. The mESC-derived phenotypes expressed the HC markers, Atoh1, Brn3c and Myosin VIIa and a small proportion of them also showed the characteristic HC stereociliary morphology, co-labelling with espin and phalloidin (Li et al. 2003b). The same group has now published an improved protocol that produces otic progenitors and then hair cell-like cells from mESCs. By culturing the progenitors with chicken-derived stroma cells, a robust differentation of the apical bundle could be obtained, and more importantly, the cells expressed mechano-transduction currents (Oshima et al. 2010).

Efforts to produce sensory neurons from human embryonic stem cells have also produced encouraging and ground-breaking results. In initial studies, human embryonic stem cells (hESCs) were induced to form embryoid bodies and latterly transferred to differentiation media in the presence of NT-3, BDNF, FGF or bone morphogenetic protein 4 (BMP4). The hES-derived neuroprogenitor cells showed fibres projecting to denervated *ex vivo* sensory epithelia and expressed synaptic markers. Moreover, when the neuroprogenitor cells were transplanted into the cochlear nerve trunk of deafened animals, the transplanted cells engrafted in the auditory nerve trunk and sent out processes which grew toward the auditory sensory epithelium (Shi et al. 2007). Furthermore, functional and specific auditory sensory neurons have been produced from hESCs using a step-wise protocol that generates otic progenitors (Chen et al. 2012). These cells survive, differentiate and grow neurite projections from their graft site in the modiolus to both the organ of Corti and the cochlear nucleus when transplanted into deafened cochleae. Not only did the host cells survive, but the animals showed a substantial restoration of hearing function, the first time that this had ever been shown and providing an excellent 'proof of principle' paradigm (Jongkamonwiwat et al. 2009), (Chen et al. 2012).

11.6 Challenges for the Large Scale Production of Clinical Grade Cells

11.6.1 Cell Generation and Isolation

The translation of any experimental stem cell approach into a real clinical therapy requires the development of standards and quality control (QC) processes that are subjected to very strict scrutiny by the regulatory bodies. Clinical production of

cells will need to adhere to good manufacturing practices (GMP) to insure the delivery of a "cell drug" that is safe, reproducible and efficient. For this, reliable and efficient protocols will have to be developed and all parts of the process would have to be optimized and well defined. To achieve this final goal, it is necessary to develop the tools that will facilitate the scaling up of a controlled production process.

11.6.1.1 Human Fetal Auditory Stem Cells: A Model for Cochlear Stem Cell Biology in Humans

Despite the advances obtained in rodents, until recently hearing research has suffered from the lack of a suitable model to study stem cell biology of the auditory organ in humans. This started to change a few years ago when a population of stem cells was identified in the human fetal cochlea (Chen et al. 2007) and later, a protocol was developed that allowed their isolation and expansion in vitro (Chen et al. 2009). By culturing dissociated cells from sensory epithelia from 9 to 11 weeks-old foetuses in a serum-free media supplemented with EGF, IGF1 and bFGF (and referred as OSCFM, *O tic S tem C ell F ull M edia*), an homogenous population that expressed stem cell markers such as NESTIN, SOX2, OCT4 and REX1, among others, was selectively expanded.

Several stem cell lines were established that retained expression of these stem cell markers and remained proliferative for several months. When cells were grown under defined culture conditions and passaged non-enzymatically, the cells remained undifferentiated, growing as an adherent monolayer and displaying an epithelial-like morphology. However, when they were passaged using trypsin, neuronal differentiation was readily induced. Cells grew processes and elongated. After 24–36 h they displayed the characteristic bipolar morphology of spiral ganglion neurons. The differentiation process was further supported by exogenous factors such as Shh, NT3 and BDNF, as measured by the expression of the neuronal markers *NEUROGEN1*, *BRN3A*, b-TUBULIN III and NEUROFILAMENT 200. Moreover, 5–7 days after inducing differentiation bipolar cells displayed potassium delayed rectifiers and voltage-gated sodium currents. These findings are important as some neurons obtained from mouse embryonic stem cells have failed to express sodium channels (Balasubramaniyan et al. 2004). On the other hand, culture in the presence of RA and EGF favoured the differentiation into hair cell-like phenotypes by inducing the expression of *ATOH1* and *BRN3C* as well as MYOSIN VIIA and PARVALBUMIN. Furthermore, these cells showed a rearrangement of the actin cytoskeleton, resembling the cuticular plate, and expressed the inward rectifier K^+ current (I_{K1}), whose slow decay and voltage activation range closely resembled those recorded in pre-hearing mouse cochlear hair cells (Marcotti et al. 1999). Besides I_{K1}, hair cell-like cells also showed a small outward I_K and a sustained inward Ca^{2+} current. This correlates with the expression of the Cav1.3 subunit by cells under 'hair cell conditions', confirmed by RT-PCR. In summary, these cells are an excellent system to study human ear differentiation and, as a biotechnological tool, allowed to define the conditions needed to induce differentiation into neurons and hair cells. However, as these human auditory stem cells undergo replicative

senescence after prolonged passaging in culture, they are unsuitable for use in a clinical transplantation setting. Therefore it is crucial to identify and isolate a source of renewable cells, suitable for both clinical transplantation and large scale production.

11.6.1.2 Generation of Otic Progenitors from Human Pluripotent Stem Cells

Since their initial derivation from human blastocysts in 1998 (Thomson et al. 1998), human pluripotent stem cells have rapidly altered the scope and potential of disease modelling in vitro and have introduced a significant possibility of producing differentiated progeny for clinical transplantation therapeutics. Pluripotent stem cells have the innate ability to self-renew indefinitely in vitro, and given a specific set of molecular cues, can differentiate into any of the cell types of the three primary germ layers (endoderm, mesoderm and ectoderm). These pluripotent stem cells can be characterised by the expression of known "pluripotency genes" such as OCT4, NANOG and SOX2, and also cell surface markers, including SSEA3, TRA-1-60 and TRA-1-81. As mentioned above, it has now been established that terminally differentiated cell types (for example, skin fibroblasts) could be reprogrammed back to a stem cell-like state using viral vector delivery of a small cocktail of four transcription factors; OCT4, SOX2, KLF4 and c-MYC (Takahashi et al. 2007). Referred to as induced pluripotent stem cells, these cells have opened up the potential for deriving patient-specific therapies, while circumventing immune rejection and the ethical issues associated with the use of embryonic stem cells.

For the possibility of a stem cell based therapy for deafness becoming a future reality it will be necessary for appropriate protocols to be derived in order to generate progenitors of the otic lineage in an efficient and reliable manner. In recent years a small number of such protocols have been described that take advantage of known signalling events crucial for the initiation and further development of the otic structures of the embryo. Fibroblast growth factor (FGF) signalling, in particular FGF3 and FGF10, has previously been shown to be both necessary and sufficient to promote the development of the otic placode, the primordium of the inner ear, in vivo (Wright and Mansour 2003). Chen et al. (2012) utilised this knowledge of FGF signalling to direct the differentiation of human embryonic stem cells into otic progenitors. Upon dissociation of the embryonic stem cells with trypsin, the developing progenitors were cultured in serum-free medium supplemented with FGF3 and FGF10 in a monolayer format on laminin-coated culture vessels for approximately 12 days. Assessed by immunolabelling and quantitative RT-PCR the resultant progenitors treated with the FGF ligands expressed the characteristic otic markers PAX2, PAX8, FOXG1 and SOX2 when compared to the control medium condition. A subset of the cells co-expressed high levels of various combinations of the markers, and these were believed to represent the true otic progenitor population.

Two cellular morphologies of particular interest are produced during this protocol; a neural-like progenitor phenotype and an epithelial-like phenotype. Using differentiation methods derived from experiments with the human fetal auditory stem cells, the neural-like progenitors were able to efficiently further differentiate into a more neural phenotype expressing BRN3A, β-tubulin III and NEUROFILAMENT 200, whereas the epithelial-like progenitors gave rise to hair cell-like cells expressing BRN3C, ATOH1 and MYOSINVIIA. More importantly the neural-like progenitors, when grafted into the modiolus of the cochlea in a gerbil model of neuropathic deafness, formed an ectopic spiral ganglion and restored functional ABR thresholds by up to 46 %.

Although the protocol developed by Chen et al. (2012) has been shown to produce otic progenitors capable of restoring functional hearing in animal models, the efficiency of the protocol is somewhat limited in the number of otic progenitors produced and therefore could be detrimental to large scale clinical production. An explanation of this limited efficiency could be due to the relatively simple protocol requiring the supplementation of just two growth factor ligands to the differentiating pluripotent stem cells. Other recently published protocols have attempted to improve the efficiency of the directed differentiation of human pluripotent stem cells to mature phenotypes using developmentally informed step-wise events, synchronising multiple signalling pathways either sequentially or simultaneously. This in vitro mimicking of in vivo events may help to increase the proportion of progenitors generated. Differentiation into progenitors of the various neural lineages has been a rich area of human pluripotent stem cell research for a number of years. Research conducted by Chambers et al. (Chambers et al. 2009) highlighted the importance of manipulating multiple signalling pathways in order to improve on progenitor differentiation in vitro. Inhibiting the TGF-β and BMP signalling pathways during differentiation simultaneously with SB413452 and Noggin respectively resulted in the induction of approximately 80 % PAX6-expressing neural progenitors, compared to only 10 % when the differentiating cells were treated with SB413542 or Noggin alone. The timing of manipulation of signalling pathways has also been reported to be important in the efficient induction of a progenitor cell type. Dincer et al. (2013) modified the protocol derived by Chambers et al. (Chambers et al. 2009) to determine if the enhanced neural differentiation protocol driven by the dual inhibition of TGF-β and BMP signalling could be further modified to generate cranial placodal precursors. It was found that the removal of BMP inhibition after 3 days was sufficient to induce approximately 70 % of SIX1-expressing cranial placode progenitors by day 11 of the protocol. These placodal precursors were capable of being coerced into developing into other placodal-derived phenotypes, for example lens by inhibition of FGF signalling with SU5402, or anterior pituitary by treatment with Shh.

Not all in vitro differentiation protocols adopt a monolayer culture approach. A landmark study carried out by Eiraku et al. (2011) differentiated mouse embryonic stem cells into self-directed optic cup structures via an embryoid body formation step.

In a similar fashion, protracted differentiation of aggregated human pluripotent stem cells has been used within the hearing research field to induce otic progenitor differentiation and improve the generation of hair cell-like cells (Ronaghi et al. 2014). Embryoid bodies were formed, and by inhibiting the formation of endoderm and mesoderm lineage identity using Wnt and TGF-β signalling antagonists with concomitant IGF supplementation to support cranial ectoderm formation, the differentiating cells were pushed towards adopting a pre-placodal identity after 15 days in culture, marked by the expression of SIX1, EYA1, DLX5 and GATA3 transcription factors. Following this, a 6 day period of FGF signalling activation with bFGF and FGF19 was carried out to enhance otic induction. At this stage, some progenitors co-expressed PAX2 and PAX8, and PAX2 and DLX5. When these embryoid bodies were then subjected to self-guided differentiation with decreasing concentrations of knockout serum replacement in the culture medium, a 20 % fraction of the cells expressed the hair cell marker Atoh1 (using a nuclear GFP Atoh1 reporter system), with approximately 9 % of this fraction also expressing MYO7A and MYO15A. A similar study has also been carried out using mouse pluripotent stem cells (Koehler et al. 2013), subsequently leading to a large number of hair cell-like cells being produced within the three-dimensional embryoid bodies (up to 1500 hair cell-like cells per aggregate) after 30 days of in vitro culture. This has yet to be reported with the use of human pluripotent stem cells.

Despite the three-dimensional approach seemingly being more conductive to otic progenitor and hair cell-like cell differentiation in vitro, it presents a number of caveats for clinical translation and large scale production in comparison to the simple monolayer system. An increased level of complexity and expenditure is added compared to a monolayer approach as the embryoid body protocols are protracted and may take up to 30 days to produce the desired cell phenotypes. Embryoid bodies also remove a large element of control. Cells within an aggregate may spontaneously differentiate, or those at the centre of the aggregate may not receive the same amount of exogenous signalling as those cells closer to the outside of the aggregate, forcing a concentration gradient which may affect the efficacy of differentiation; a phenomena unlikely to be present when culturing differentiating cells in monolayer. In addition, purification and isolation of a cell type of interest from an embryoid body aggregate has a tendency to require a harsher dissociation method to produce single cells and this may also have a detrimental effect on cell yield and quality. Essential components for embryoid culture are usually of animal origin and this could hinder the translation of research into a clinical setting. Monolayer culture systems however are more amenable to transition onto xeno-free, GMP compliant substrates.

As techniques in human pluripotent stem cell differentiation rapidly evolve over the coming years, more sophisticated protocols will be developed to give rise to an enriched population of otic progenitors which will be readily isolated and purified to aid in the large scale expansion and production of clinical grade cells for therapeutic transplantation.

11.6.2 *Cell Purification and the Need of Reliable Markers*

11.6.2.1 Isolation of Relevant Cell Types from Tissue and Characterisation of in vivo Protein Expression

An early, important element to address is how to purify the relevant cells in an efficient and non-invasive manner. To prospectively isolate cells from complex tissues or mixtures, being these the cochlea or a mixed population induced from pluripotent cells, we need suitable markers. An ideal approach would be to use cell surface markers to allow their purification by Fluorescent Automated Cell Sorting (FACS).

The use of cell-specific regulatory elements driving reporter proteins such as GFP has been applied to the successful isolation of supporting cells from the postnatal mouse cochlea (White et al. 2006). Although highly useful for research in animal models, this approach requires genetic modifications of the target cell (or the generation of transgenic mutants in the case of animal models) that makes it either undesirable or totally unfeasible for clinical applications in humans. The elucidation of the transcriptome of the human auditory stem cells should yield strong candidates of surface molecules to screen with antibodies, as is has been done with glial precursors (Campanelli et al. 2008). This targeted, informed approach should be more advantageous than the random screening of available antibodies. Identification and isolation of Lgr5 positive cells within supporting cells of the cochlea in mice has highlighted a potential 'stem cell' source within the organ of Corti (Chai et al. 2012) (Shi et al. 2012). This potential was inferred from shared Lgr5 expression in stem cells of the adult intestinal epithelium, and highlights how a more comprehensive understanding of protein expression within unique cell populations of the inner ear may prove invaluable in opening up new avenues for therapeutic targeting.

In an effort to address the lack of known cell surface markers, Hertzano et al. (Hertzano et al. 2010) identified a cohort of 107 'cluster of differentiation (CD) antigens' expressed in the postnatal mouse inner ear. From these, CD44 was detected as a reliable marker for the outer pillar cells. In a follow-up study, they describe that CD326 stains sensory and non-sensory epithelial cells at day P0, while CD49f is specific to sensory epithelial cells (Hertzano et al. 2011).

A potentially useful strategy for the purification of key cell populations within tissues could be the isolation, by flow cytometry, of 'side populations' based on the ability of certain stem cell-like cells to exclude Hoechst dye. This method was successfully used by Savary et al. (2007) to isolate a population of supporting cells from the mouse cochlea that retained progenitor properties. It is important to note that hFASCs express the ABCG2 transporter, which is believed to be the molecule responsible for the 'side population' phenotype. However, because this is a functional assay, it is difficult to standardize and different laboratories have obtained very dissimilar results while working in other systems (Sales-Pardo et al. 2006).

It is clear that much more work is necessary in identifying proteins expressed on the surfaces of the different populations of inner ear cells, both to enable their purification from tissues and to improve understanding of inner ear development. A greater understanding of protein expression in vivo will also aid the development of differentiation protocols *in vitro*, though as the environment in which cells are being directed to develop in the lab is very different to their natural developmental niche it is important to also consider the two systems separately.

11.6.2.2 Purification of Potential Therapeutic Cell Populations

Regenerative medicine involving the transplantation of cells derived from multipotent lines suffers from safety concerns regarding carryover of self-renewing cell populations, and their potential to give rise to tumours. For this reason, identification of robust extracellular markers that can be used to sort the therapeutic cell population from other contaminating cell types is essential for stem cell-derived treatments to progress from animal models into humans. In this regard, inner ear research lags behind other fields, such as haematology, where haematopoetic stem cells advanced quickly into clinical application because of the availability of well-defined surface markers. These markers have made possible the specific isolation and purification of progenitors for different lineages using FACS and the monitoring of their downstream differentiation (Wognum et al. 2003). This highlights a greater need for cell sub-population identification and sorting experiments to be conducted within an auditory research context, as it would considerably aid our understanding of the biological processes supporting sensory neuronal and cochlear hair cell differentiation *in vitro*. Current selection methods for progenitor populations are crude and it is likely that groups of cells unified by morphology or expression of single transcription factors are heterogeneous beyond these simple criteria. Further dissection of the progenitor populations may yield subsets that respond more efficiently to differentiation regimes and improve therapeutic outcomes. Alternatively, it may transpire that a balance of different progenitor sub-types is necessary for cells to progress down neuronal and/or hair cell lineages. Whatever the outcome, it is clear that further characterisation of the progenitor sub-populations is critical for both therapeutic and in vitro research to progress.

Selection of reliable markers requires a suitable method for accurately probing the proteome of individual cells within differentiating populations and analysing large resultant datasets. There are a variety of methods available to screen protein expression, each with limitations. Panning with phage display libraries and hybridoma technologies are well established methods used to detect protein expression, but are large undertakings requiring much downstream validation, hence, are being replaced by other technologies. Microarrays and PCR arrays infer protein expression indirectly through comparing mRNA (after reverse transcription) between sample populations. Protein arrays, consisting of antibodies attached onto glass/membranes,

can be used to investigate peptide expression directly from purified protein samples, as can mass spectrometry methods. Whilst capable of generating insightful data, these methods lose morphological information and represent averages from cell populations, and RNA-based inferences suffer from inadequacy in reliably predicting relative protein expression levels (Carter et al. 2004). As the differentiation protocols of pluripotent cells produce highly heterogeneous populations, screening methods capable of maintaining expression data associated with single cells are preferable in this context.

As an important aim in therapeutic terms is to find markers suitable for sorting out relevant progenitors prior to surgical implantation in the inner ear, FACS antibody screening methods have obvious attraction. In FACS and mass cytometry screens, cell populations are dissociated into single cell suspensions then exposed to panels of antibodies. This is an efficient way of probing the extracellular proteome of cells in a population as it simultaneously verifies antibodies that could then be used to sort the cells. However, this method inevitably loses useful morphological data as the cells are dissociated prior to antibody exposure. Furthermore, pre-purification of the desired cell population combined with huge numbers of cells are required to complete such screens, make them impractical in many circumstances. Bioimaging of cells exposed to antibody panels offers a way to maintain cell morphology data whilst characterising individual cells, though hits require further validation via FACS.

If a stem cell based treatment for hearing impairment is to be realised, it is essential that the cell surface proteome of potential biological therapeutics is well characterised and sub-categories of cells identified for rigorous potency and safety testing. The wealth of information a cell surface marker screen will yield may not only provide precise, reliable methods for separating neural and hair cell progenitor populations from heterogeneous populations, but also offer further insights into the mechanisms by which cells are directed along otic lineages. Both aspects will ultimately lead to improved cellular differentiation and better outcomes for patients.

11.7 Cell Delivery

The routes for surgical delivery are another major factor for consideration when transplanting cells in the very small and delicate cochlear tissue. The main objective for transplantation is to deliver the cells into the damaged target area. This will obviously depend on which type of degeneration has occurred and whether the primary aim is to replace HCs and/or SGNs (Fig. 11.2). Another challenge is to distribute the transplanted cells throughout the length of cochlea while minimizing further damage from the transplantation. A few articles have been published showing different cell delivery techniques into mammalian cochleae, which are summarized in the following section.

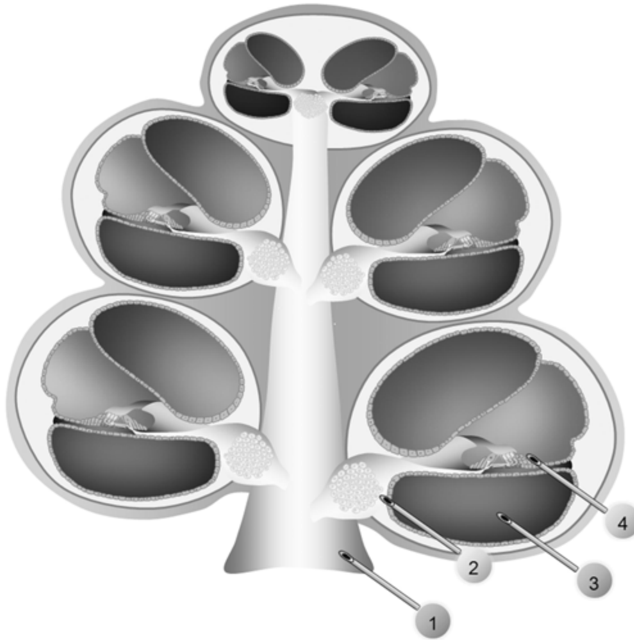

Fig. 11.2 Schematic illustration showing the routes for cell delivery into the cochlea. For neuronal replacement, cells can be delivered into the cochlear nerve trunk (*1*) or directly into the Rosenthal's canal in the modiolus (*2*). Alternatively, cells can be injected into the scala tympani (*3*) or, in order to target the organ of Corti, directly into the scala media (*4*)

11.7.1 Intra-Perilymphatic Transplantation

Perilymphatic transplantation via the scala tympani is probably the most used delivery technique so far. Positive features are the relative bigger volume of the space when compared to the other cochlear compartments together with the fact that its fluid runs along the entire cochlear length, making it the best vehicle to distribute transplanted cells throughout the cochlea. Moreover, surgical access to the perilymphatic duct is believed to cause less trauma to the cochlea, and it can be done either through a cochleostomy in close proximity to the round window or through the round window itself. Access to the perilymphatic compartment is not just limited to the scala tympani in the basal turn. Iguchi et al. (2004) have shown that it is possible to reach it via the lateral semicircular canal (LSCC). Transplanted neurospheres derived from mESCs were found in every cochlear turn in all experimental animals, with a larger number of cells found in the scala vestibuli than in the scala tympani. A few cells were also found in the scala media (around 0.4 %). This experiment suggested that this surgical technique would cause only a minimal ABR threshold shift (~10–15 dB SPL) in the high frequency response region (40 kHz) (Iguchi et al. 2004). There was no evidence of cells being able to reach the modiolus by this approach. Even though the number of cells in the scala media was modest, it would

suggest that they could migrate into the endolymphatic compartment from the perilymphatic region. How cells could manage to cross into this tightly sealed compartment and survive in an environment with high potassium concentration, remains to be established. However, several other transplantation experiments using mESCs and MSCs delivered into the scala tympani have found cells localized only to the perilymphatic space (Coleman et al. 2006; Matsuoka et al. 2006, 2007). In a different study, using neural stem cells transplanted into the scala tympani, cells were able to migrate to an area very close to Rosenthal's canal (Hu et al. 2005b). However, the number of surviving cells was very low, and differentiating cells were only obtained when transduced with neurogenin2. Supplementation with GDNF has also been found to promote survival and migration of transplanted cells into the cochlear modiolus (Altschuler et al. 2008). Dorsal root ganglion neurons co-grafted with mESCs would appear to promote migration and survival of the undifferentiated cells. In this experiment, mESCs were found to migrate into the SGN area via intrascalar access, suggesting that DRG co-grafts not only release growth factors to support the survival but also provide a structural matrix for cell growth in the fluid-filled compartment (Hu et al. 2005a). Cells transplanted in the scala tympani are thought to migrate to the modiolus via holes located in the internal bony wall, known as '*canaliculae perforantes*'. This area is highly porous, and may provide windows big enough for transplanted cells to migrate into Rosenthal's canal. However, the size of transplanted cells would be a major factor limiting cell migration through these bony pores (Sekiya et al. 2007). A report by Parker et al. (2007) described general distribution of cells throughout different cochlear compartments when using a perfusion system (Parker et al. 2007). Since their initial scala tympani delivery produced a localized lump of cells, they explored perfusion by using a syringe pump. As described above, this group used the Y-FISH technique to trace the transplanted cells and showed migration to different areas in the cochlea including the organ of Corti, Rosenthal's canal and even to the spiral ligament. Transplanted cells would appeared to have differentiated into both hair cells, supporting cells (pillar, Deiter's, phalangeal), SGNs, satellite celsl and cells in the spiral ligament, although cell fusion could not be categorically ruled out.

11.7.2 Transplantation Aimed at the Organ of Corti Via the Scala Media

There have been very few reports aiming to introduce stem cells directly into the scala media, and it remains the most technically difficult. The main reason for this approach would be to target the replacement of HCs, however several biological barriers would need to be overcome. The endolymph, the fluid located in this compartment, contains high levels of potassium (~150 mM). These concentrations are toxic to many cell types, and it could lead to a very limited viability of transplanted cells. Moreover, the complex cytoarchitecture of the organ of Corti represents another challenge. The epithelium is a tightly sealed barrier and it will be difficult

for the transplanted cells to break through the adherent and tight junctions between hair cells and supporting cells in order to home and graft – indeed, in the situation where there is long-term loss of the hair cells, the organ of Corti often regresses into a non-specialised, flattened epithelium which may lack any relevant positional cues for hair cell grafting (Taylor et al. 2012). An initial attempt at scala media transplantation was described in mice, by delivering cells through the cochlear lateral wall (CLW) of the second cochlear turn. This approach revealed the distribution of transplanted cells in all three cochlear chambers, that is scala media, scala vestibuli and scala tympani, with a relative distribution of cells of 62.1 %, 20.8 % and 17.1 % respectively. There were no reports of differentiation and integration into the host tissue, nor confirmation of survival of transplanted cells in the endolymph. To compound the problem, the surgical intervention produced a significant elevation of ABR thresholds over all frequency ranges 3 days post- operation and there was no significant recovery of threshold observed at any frequency (Iguchi et al. 2004). The access via the CLW obviously damaged cochlear function, specifically the structures needed to maintain homeostasis of K^+ and/or endocochlear potential. It has been shown that by this lateral approach to the scala media, it is possible to damage the stria vascularis and cochlear blood supply (Izumikawa et al. 2005). A different surgical approach to the scala media was then developed by piercing the basilar membrane with a cannula via the cochlear round window. This technique was established by Hildebrand et al. (Hildebrand et al. 2005) to deliver neuroectoderm-like embryoid bodies to deafened guinea pigs. There was no evidence of damage to the organ of Corti or Reissner's membrane attributable to the surgical procedure in any animal after surgical delivery. This evidence was confirmed by retention of the ABR threshold level after surgery when compared to the control group. The transplanted cells were found in all three cochlear chambers, scala media, scala tympani and scala vestibuli. The overall survival rate of transplanted cells was around 19.1 %, 9 weeks after transplantation. Around 14 % of these surviving cells were found in the scala media, and some were localized close to the damaged organ of Corti; however, there was no evidence of extensive cell differentiation and integration into the host tissue (Hildebrand et al. 2005). This would indicate that some cells are able to survive, probably after partial differentiation, in a potassium-rich environment. However, conclusive evidence for the integration of transplanted cells into the damaged organ of Corti is still missing.

11.7.3 Transplantation Via the Modiolus and into the Cochlear Nerve Trunk

The modiolar and cochlear nerve trunk routes for transplantation are mostly aimed at replacing degenerated SGNs. Because of the difficulties described above, hair cell replacement is still a long way away. However, targeting sensory neurons appears as a far more realistic application in the short term. Moreover, a cell

based-therapy to reconstitute the nerve cells could be implemented in combination with the currently available cochlear implants. As mentioned before, these devices can substitute for hair cell function but still require the existence of SGNs to function, as the conduit for signals to the central nervous system. Secondary degeneration of SGNs after hair cell loss is hugely problematic and this severely affects the chances of deaf patients in their candidacy to receive cochlear implantation. For these reasons, some research groups have turned their interest to study the regeneration of SGNs and transplantation via the cochlear nerve seems to be the most reasonable approach to deliver stem cells to the target location of Rosenthal's canal. Interesting results from mESC transplantation via cochlear nerve trunk access has shown both of peripheral and central migration along the cochlear nerve from the injection site. Transplanted cells were found close to the ventral cochlear nucleus (VCN) but the number of cells that migrated to Rosenthal's canal was still limited (Hu et al. 2004). Similar results were obtained when rat E13 embryonic SGN progenitors were used. The transplanted cells grafted in the nerve trunk between the brain stem and the internal auditory meatus (IAM), but cells could not migrate further than the CNS/PNS transition boundary and failed to reach the cochlea (Palmgren et al. 2011). There is evidence of cells undergoing differentiation to SGN-like morphology after transplantion via the IAM of the intact cochlear nerve (Sekiya et al. 2007). Mouse conditionally immortalised neuroblasts (VOT-N33) were used in this study and were found to be distributed in the cochlear modiolus at different levels. Surprisingly, the transplanted cells via the IAM approach differentiated into a bipolar morphology and showed a very strong staining for the neuronal marker β-tubulin III just 7 days after transplantation. Exploring a similar route, mESC-derived neuroprogenitor cells were implanted into the cochlear nerve trunk of deafened animal models. The surgical approach was made at the bony area that separates the cochlear nerve from the floor of round window niche. This study has shown very nice engraftment of transplanted cells into the host tissue – however, only a very small number of cells showed migration to Rosenthal's canal but instead, cells formed ectopic ganglia at the transplantation site with projections both into Rosenthal's canal and the organ of Corti (Corrales et al. 2006). Successful use of this transplant approach was also employed in the gerbil model using hESC-derived otic neuroprogenitors (hONPs), in which access to the cochlear nerve was made via the round window, allowing for the infusion of hONPs, which were then found to settle in Rosenthal's canal and send out bipolar projections, synapsing both in the organ of Corti and in the brainstem, leading to functional recovery (Chen et al. 2012). Functional hearing measurements imply that the level of mechanical damage from the surgical techniques does not further deteriorate the environment in residual auditory nerve.

Two studies have specifically compared these different delivery routes using the same cell type. The first one, produced by Lang et al., explored the perilymphatic approach via the scala tympani, the scala media and the Rosenthal's canal approach through the round window niche using neuralized mESCs (Lang et al. 2008). Two weeks after transplantation, cell survival in the endolymphatic compartment was very low when compared to cells delivered into the perilymphatic space. Most of the transplanted cells were dead or dying, showing evidence of apoptosis. The Rosenthal's

canal approach performed the best, with large numbers of surviving transplanted cells labelling with neuron and glial markers. The microenvironment in Rosenthal's canal might be vital to provide the appropriate niche for the transplanted cells. The results of this study are highly encouraging for the delivery into Rosenthal's canal, but there was no evidence about migration beyond the transplantation site. The other study aimed to compare between a cochleostomy into the scala tympani, the auditory nerve via a translabyrinthine approach and direct access to Rosenthal's canal through the osseous spiral lamina wall of the scala tympani (Backhouse et al. 2008). The transplantation was not performed with cells, but with biocompatible microspheres delivered with or without hydrogel, a matrix to minimize dispersal. Endogenous SGN survival was measured, and the generation of areolar fibrous tissue and bone formation was assessed to indicate the level of inflammatory tissue response. The translabyrinthine approach produced the largest inflammatory responses and also damaged the SGNs.

Cell encapsulation within a hydrogel matrix may well prove to have a great therapeutic benefit in terms of minimising cell dispersal and increasing cell targeting to Rosenthal's canal – embedding mouse ESC-derived neurospheres in such a biodegradable matrix prior to transplantation into a deafened guinea pig model restricted the degree of cell dispersal from the injection site in Rosenthal's canal to the scala tympani of the basal turn (Nayagam et al. 2012). However, these experimental observations did note a degree of inflammatory response at the injection site, which is not generally found when naked cells are infused – there may be scope for the infusion of glucocorticoids within the hydrogel though, to dampen this response. Several groups are working on the lessening on of the foreign body response in the cochlea by the employment of such methods (see (Honeder et al. 2015) for examples of this in the context of cochlear implantation).

11.8 Models to Study Cell Transplantation in the Inner ear

Several factors are critical for the success of a transplantation project. These include, for instance, the survival and homing of cells into the host tissues, the migration of transplanted cells to the target site, the differentiation of cells into appropriate phenotypes and their ability to regenerate the functional connections. All these variables are the result of a balanced interaction between the intrinsic potential of the donor cells and the properties provided by the microenvironment of the host tissue. Therefore, the models used for transplantation experiments should be considered carefully. In the different models, deafness can be induced in a targeted manner, hitting primarily the hair cells, the spiral ganglion neurons or the fibrocytes of the lateral wall. Various kinds of chemical substances have been used, showing different cellular targets. Aminoglycoside antibiotics have been widely used to damage primarily hair cells, subsequently causing SNHL. A secondary degeneration of SGNs may or may not take place later, depending on the in vivo model – for example, there is a progressive loss of SGNs in the guinea pig (Coleman et al. 2006),

whereas there is almost complete neural preservation for several months post hair cell loss in the gerbil (Abbas and Rivolta 2015). The glycoside and Na + K+ ATPase inhibitor, ouabain, has been broadly employed to specifically damage SGNs. Outer hair cells and the stria vascularis are not affected, as shown by histology and the preservation of distorted product otoacoustic emissions (DPOAE) and endocochlear potential (EP), respectively (Schmiedt et al. 2002). Ouabain application via the cochlear round window niche can increase the cochlear compound action potential (CAP) threshold in just only 3 h after application and SGNs begin to undergo apoptosis after 12 h. This drug specifically targets type I spiral ganglion neurons (Lang et al. 2005). Because of this specificity, ouabain application has been used as a model of neuropathic deafness in several transplantation studies (Corrales et al. 2006; Lang et al. 2008; Shi et al. 2007; (Chen et al. 2012). A comparative study between transplantation in the intact cochlea versus a model of hair cell injury by neomycin, revealed that the survival rate of transplanted cells was better in the neomycin-treated animals. Moreover, the differentiation potential of transplanted cells was also greater in the deafened model (Hu et al. 2005a). This result suggests that the microenvironment provided by the damaged host tissue is a significant factor for viability and differentiation of transplanted cells. The length of time after injury may have a major impact in the survival of implanted cells. In an early post-injury transplantation, made 1–3 days after the deafening protocol, cell survival was substantially better than when transplantation was done 7 days after induction of deafness (Lang et al. 2008), although Chen et al (2012) did not detect a significant difference between transplants after 3–5 days and 2 weeks. Finally, noise-induced deafness has also been employed as a model for transplantation. Exposure to loud noise (112 dB level) for 72 h can induce deafness in guinea pigs and generate damage to different cell types including cells in the stria vascularis, hair cells and supporting cells of the organ of Corti. The area of damage corresponded with frequency of sound stimulus (Parker et al. 2007).

11.9 Replacing the Spiral Ganglion Neurons: The Role of the Glia

The encouraging results supporting the replacement of spiral ganglion neurons with exogenous stem cells has renewed interest into trying to understand the relationship between auditory neurons and glial cells within the peripheral auditory system, and how they respond to various types of pathology. All peripheral neurons are supported by glial cells, which are known to play an important role in maintaining neuronal function (Hanani 2005). The glial cell types of peripheral nerve fibres include Schwann cells, which support neuronal axons and neurites, and satellite cells, which surround and myelinate ganglionic neuronal cell bodies. As with other nerves in the human body, the cochlear nerve is also composed of both neurons and glia. These two cell types have differing embryological origins; glial cells are derived from neural crest cell progenitors (NCC) (D'Amico-Martel and Noden 1983),

whilst the neurons almost exclusively derive from the otic placode (Breuskin et al. 2010). The exclusive placodal origin of auditory neurons is somewhat unusual, as most cranial sensory nerves contain neurons derived from NCCs and sensory placodes.

11.9.1 The Potential Importance of the Glial Transitionary Zone

In vertebrates, the peripheral and central nervous system connect at specific zones and the boundary between the two can be distinguished by a change in glial cells associated with neurons. Typically, the principle glial components of the central nervous system are astrocytes and oligodendrocytes (derived from the neural tube), whereas Schwann cells are the principle glial cell type of the peripheral nervous system (Berthold and Carlstedt 1977). The boundary between the CNS and PNS is usually demarcated by a junction, which is known as the glial transitionary zone (Fraher 2000). The glial cells that are close to this transitionary zone usually respond to neural damage. Schwann cells can cross this boundary in response to demyelinating conditions in both rodents and humans (Itoyama et al. 1983), and also in spinal cord lesions in humans (Guest et al. 2005). Astrocytes may also respond to neural injury known as the 'astroglial reaction' which prevents further neuronal damage but also creates an inhibitory microenvironment for neuronal repair (Fraher 2000). The glial transitionary zone of the auditory nerve is located at the internal auditory meatus and has an unusually long segment of central nervous tissue extending distally (Toesca 1996). Understanding the changes that take place at this transitionary zone are extremely important in the context of neuronal repair; in particular, if any changes in the glial environment are to be permissive for regenerated auditory neurons to allow the rewiring of the organ of corti to the cochlear nucleus in the brainstem.

As our knowledge of glial responses to auditory nerve injury furthers, studying the interaction between transplanted ONPs and glial cells in the pathological auditory system is particularly relevant. Fundamentally, it is vital that the experimental models sensorineural hearing loss employed in such studies share similarities with those seen in humans, to facilitate the process of validating stem cells as a viable therapy.

11.9.2 Responses of Auditory Glial Cell to Cochlear Injury

The most common mechanism of cochlear injury in sensorineural hearing loss occurs as a result of injury to the inner hair cells of the cochlea, which prevents to cochlea from converting the mechanical energy from sound waves into an action potential. Following hair cell loss, a secondary neuronal degeneration occurs, which suggests that hair cells provide trophic support to peripheral auditory neurons.

Interestingly there is increasing evidence to suggest that the supporting cells within the organ of Corti are also an important trophic source for auditory neurons. In fact, supporting cells seem to share a similar symbiotic relationship with auditory neurons as peripheral glial cells do with their associated neurons, for example, ErbB receptor signaling (Coppens et al. 2001) and trophic support through neurotrophin-3 (Sugawara et al. 2005) have both been implicated between cochlear supporting cells and auditory neurons. Given that cochlear supporting cells are known to express glial markers such as S100, PLP and GFAP (Gómez-Casati et al. 2010; Rio et al. 2002; Stankovic et al. 2004), have been proposed as belonging to the glial family. Given that in some situations, secondary neuronal degeneration can occur following loss of support cells whilst the hair cells remain intact, the role of support cells in the pathogenesis of sensorineural hearing loss ought to be probed further.

Aminoglycoside injury is perhaps one of the most common experimental paradigms used to study sensorineural hearing loss, and is also a frequently observed as an iatrogenic form of deafness. In response to the co-administration administration of gentamycin and the loop-diuretic furosemide, Schwann cells in the peripheral auditory system have been shown to change phenotype, changing from a myelinating to a non-myelinating cell type, raising the possibility that such a phenotypic change might contribute to the demyelination and SGN loss that is frequently seen in the cochlea (Hurley et al. 2007). Another common chemically induced model of sensorineural hearing loss is through the local delivery of Ouabain to the cochlear round window, which provides a useful model for auditory neuropathy in which type I SGNs are damaged whilst the hair cells are preserved. When oabain is administered in mice, activation and proliferation of glial-like cells has been observed, expressing classic glial markers such as SOX10 and S100 (Lang et al. 2011). These cells also began expressing SOX2 and increased expression of BrdU, implying that these cells have reverted from a quiescent state to a more active one, and raises the exciting possibility that if peripheral glial cells in the cochlea are somehow de-differentiating, they may be recruited to facilitate the repair of SGNs in early deafness.

Astrocyte proliferation has also been observed in a number of experimental models of auditory nerve pathology. Sekiya and co-workers (Sekiya et al. 2011) damaged the auditory nerve by applying mechanical pressure, and observed the response of astrocytes at the glial transitionary zone. They illustrated a profound gliosis in the auditory nerve in response to nerve compression at the internal auditory meatus by a steel wire, whereby GFAP labeled central glia crossed the glial boundary well into the peripheral auditory system. More recently, astroglial reactions have also been documented to occur following aminoglycoside injury, with GFAP labeled astrocyted crossing the transitionary zone and migrating peripherally along with auditory nerve over a period of 6 weeks (Hu et al. 2014).

Although much research has been conducted in understanding how hair cells and SGNs are damaged in response to an insult to the peripheral auditory system, it is clear that the glial cells of the auditory system warrant further investigation, and their study will be of paramount importance in the quest to translate cell based therapies for deafness from the bench to the bedside. In particular comparing central

and peripheral glial responses must be evaluated carefully, so that the resulting microenvironment can be harnessed in order to re-connect the peripheral auditory system with the CNS.

11.10 Functional Tests to Explore Recovery of Hearing After Transplantation

To complete the analysis of a transplantation study, it is necessary to monitor for functional recovery. Many types of hearing measurements have been conducted to evaluate the level of deafness, establishing the severity of injury and detecting recovery after transplantation. Otoacoustic emissions are sounds produced as a consequence of electromechanical feedback from the outer hair cells (OHC). Distortion product otoacoustic emissions (DPOAEs) are a measure of the sensitivity of the cochlea. Distortion products are generated when two tones with frequencies F1 and F2 are mixed in a nonlinear amplifier. In the cochlea, the amplifier is the OHCs and a principle distortion product is generated at 2F1 –F2. The magnitude of the distortion products generated by the cochlea is determined by the level of the primary tones and also by their ratio, which for the mouse cochlea is about 1.24. By varying the frequency and levels of the primary tones it is possible to derive an audiogram and also to derive input-output functions for each frequency region of the cochlea. DPOAEs can therefore be used to check the functional status of OHCs in a given region of the cochlea (Kemp 2002). Some experiments had obtained DPOAE measurements to evaluate the degree of deafness after drug application and/or the level of cochlear perturbation after transplantation (Corrales et al. 2006; Lang et al. 2008). Compound action potential recording is the measurement that relates to both HC and SGN function. This technique can give a sensitive read-out of inferred cochlear function since the recording electrode is placed at the cochlear round window and it can detect the evoked responses from sound stimulation at different frequencies. Evoked auditory brainstem responses (ABRs) work in a similar way, but the recording is done from distant, surface electrodes. This technique measures the discharge of auditory neurons in response to a given tone and the subsequent series of potentials generated by the nuclei of the central auditory pathway. The ABR is recorded as a pattern of a series of waves that represent different aspects of the pathway. Because of this, it is an ideal tool to study the integration of grafted cells into host tissues and the reconnection of the cochlear nerve to auditory brainstem nuclei. ABRs have been used in transplantation studies to verify the level of injury after surgery (Bogaerts et al. 2008; Iguchi et al. 2004), to determine deafness after drug application (Coleman et al. 2006) and to ascertain recovery after transplantation (Ito et al. 2001; Sekiya et al. 2007). There is a correlation between the three main parameters measured in ABRs (amplitude of the signal, threshold and latency of the response) and their anatomical significance. The amplitude of the first wave reflects primarily the sum of neural firings of a large population of SGNs, whereas threshold level represents the function of only the most sensitive auditory nerve fibres (Agterberg et al. 2009). A rise in latency after induction of deafness correlates well

with the degeneration of dendrites and a reduction of the number of myelin layers in the fibers. In summary, the use of a single test or a combination of them should be determined depending which part of the auditory pathway the cell transplantation experiment is aiming to explore.

11.11 Conclusions and Future Perspectives

Although a substantial volume of information is starting to mount about this potentially revolutionary technique, a lot more research is still needed to establish the ideal conditions for the system to work. A correct balance between the right intrinsic factors (such as the donor cell type) and extrinsic factors (such as the host environment and means of delivery), still needs to be achieved. Undifferentiated ESCs have shown a good migratory capacity but less ability to differentiate into the target cell types. The neuroprogenitor cells appear to differentiate into SGNs but seem to stay mostly at the transplanted site. Systems that allow the control of directed differentiation, such as the neurog1 inducible one (Reyes et al. 2008), are elegant solutions to establish proof of principle but, since they involve substantial genetic modifications of the donor cell, are unlikely to be of clinical application. Studies with model cell systems of murine origin are highly valuable. However, given the important differences encountered between species (primarily in the stem cell biology field) more studies with human cell types are required to establish conditions of clinical relevance. Since the use of factors such as NTF supplements seems to promote survival and differentiation, the combination of cell transplantation together with NTF supplementation should be worthwhile to explore. Along similar lines, studies combining the potential of stem cells and cochlear implants are critically needed. Regarding the transplantation route, the cochlear modiolar approach to gain direct access to Rosenthal's canal appears to be the best system for SGN replacement. However, for hair cell replacement, the delivery techniques into the scala media are still rudimentary and produce a substantial amount of damage. Further development and refinement is clearly needed.

There is also an issue of presumptive immunogenicity of these cells – would a patient be willing to subscribe to a lifetime of immunosuppressive drugs in exchange for hearing? Or will the suggested immunoprivilege of the cochlear environment protect a xenograft from a host-mediated attack? Perhaps the increase in stem cell banks will lead to the existence of a 'haplotype for all', meaning that neural progenitors can be tissue-typed as one would expect for a transplanted liver or a blood stem cell graft. These questions remain to be answered with information from animal studies.

Finally, promoting transdifferentiation of supporting cells into hair cells can be considered cell replacement, albeit from an endogenous source. Alteration of the supporting cell phenotype, by removing the cell cycle inhibition imposed by $p27^{Kip1}$ or by promoting the expression of the hair cell transcription factor *Math1/Atoh1* (Kawamoto et al. 2003; Izumikawa et al. 2005) could have great potential for hair cell generation *in vivo*.

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Chapter 12
Oral and Maxillo-Facial

Kristina Arvidson, Michele Cottler-Fox, Sølve Hellem, and Kamal Mustafa

Abstract Regenerative medicine and dentistry are two rapidly growing fields of research with important clinical implications. Recent advances in cell biology, biotechnology, material science and tissue transplantation have been translated into new approaches to clinical repair and replacement of tissues and organs. In dentistry, a number of regenerative therapies and materials have been in clinical use for many years, to repair small and large defects involving multiple tissue types. Currently, various strategies are applied to stimulate healing of bone defects and to restore lost maxillofacial bone and periodontal support following traumatic insult, tumor ablation, disease or congenital deformities.

Bone tissue engineering is an emerging field using bone-forming cells seeded onto synthetic scaffolds to form hybrid constructs that can be used to regenerate tissues. There are numerous published case reports of the application of bone tissue engineering for oral and maxillofacial surgical reconstruction, periodontal tissue regeneration and sinus floor augmentation.

Mesenchymal stem cells (MSC) are currently the cells of choice for bone tissue engineering and can be isolated from many different tissues such as bone marrow, periosteum, and trabecular bone as well as from muscle, adipose tissue and synovial membrane. MSC have also been found among the cells derived from human umbilical cord: *in vivo*, these cells have demonstrated that they are capable of osteogenic differentiation, leading to bone formation and *in vitro* have shown adipogenic, chondrogenic, and osteogenic differentiation. Further, MSC have been identified in periodontal ligament, deciduous and permanent molar teeth. Recent research has shown that these cells have promising regenerative potential. Thus stem cell-based bone tissue engineering is a promising concept for reconstruction/ regeneration of craniofacial defects but much work remains before this approach becomes a routine part of clinical practice.

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12.1 Background

Modern dentistry is not limited to maintenance of dentition but has many subspecialties encompassing diagnosis and treatment of conditions affecting the oral and maxillofacial structures. In this relatively small area of the body, many different cells and tissue types occur in morphologically complex structures. Thus defects often involve multiple tissue types, including teeth and craniofacial bones, nerves and blood vessels, soft tissues such as mucosa, skin and muscles, salivary glands and specialized sensory organs.

The oral cavity plays an important role in daily living, including selection of nutritional intake through the complicated neural interactions of taste and smell. It is well documented in the scientific literature that teeth are important to both general health and quality of life through masticatory function, as well as to esthetics and speech. The oral cavity is important to general health and the quality of life because it is the initial organ of digestion: the first stage of the digestive process or mastication, the mechanical breaking up of solid food particles into smaller pieces by chewing and mixing them with saliva and its enzymes, occurs here. Natural dentition or a properly functioning substitute (fixed or removable prostheses) is of major importance to this function. The oral cavity is important to esthetics and speech because the physical appearance of the mouth, *i.e.* the teeth and lips, are essential to these functions and help in defining social and sexual attractiveness.

Over the past 50–60 years there have been major overall improvements in oral health, reflecting advances in dental research during this period. One of the most exciting developments is a change in traditional concepts of disease and its sequelae; from mechanical repair of damage to teeth and surrounding tissues caused by dental disease, to a more biologically-based approach to treatment options and the etiology of dental diseases. Advances in basic science using techniques from cellular and molecular biology have been translated into clinical practice. At the same time, clinical and epidemiological studies have improved methods of diagnosis, treatment and prevention of a wide range of oral health problems.

A striking development is the decrease in the number of edentulous people over the past 40 years. The elderly are retaining their natural dentition and the mean number of standing teeth is higher than a generation ago. Improvements in periodontal health and oral health care are obvious. Many children are caries free or without active caries and the caries rate in adults has decreased. Important contributing factors to caries prevention are water fluoridation and the widespread use of fluoride toothpaste, but it has also been shown that social, economic and geographic factors play important roles.

The focus of restorative care has shifted from ‘black to white’, as new tooth-colored resin-based materials have been widely adopted as alternatives to amalgam. The longevity and stability of the resin materials have also been improved. With increasing patient awareness of oral esthetics, not only are posterior composite restorations preferred to amalgam, but bleaching materials have also been introduced for a whiter, brighter smile.

12.2 State of the Art

12.2.1 Loss of Permanent Teeth

One of the most common challenges for the dental clinician today, however, is rehabilitation following loss of the permanent teeth and the surrounding structures. Maintenance of good oral function is significant for general wellbeing, nutritional status and general health (Buhlin et al. 2002, 2003; Sheiham and Steele 2001; Nowjack-Raymer and Sheiham 2003). Loss of all the teeth or even of one tooth is a dramatic life event. For many people replacing missing teeth with complete dentures is unsatisfactory: not only are oral factors such as pain, taste perception and chewing capacity adversely affected, but the patient may also undergo marked psychological changes such as reduced self-image and loss of confidence in social situations (Trulsson et al. 2002).

Bone resorption is a common sequela to tooth extraction, but both the rate and the total amount of resorption may vary between individuals. While the causes of this variation are still unclear, it is recognised that resorption of residual ridges after loss of all the teeth is a complex biophysical process. Successful replacement of the dentition with complete removable dentures that merely rest on the mucosa presents a challenge, not only for dentists but for the wearer: in order to eat, drink, or talk whilst wearing dentures, patients must master amazing adaptations of the oral musculature (Fig. 12.1).

The concept of treating edentulism by osseointegration of dental implants was first proposed in the 1960’s by two independent groups: Professor Schroeder at the University of Berne, Switzerland and Professor Brånemark at the University of Gothenburg, Sweden. Their data were based on treatment protocols using endosseous, root analogue, titanium implants. These investigators were the first to document

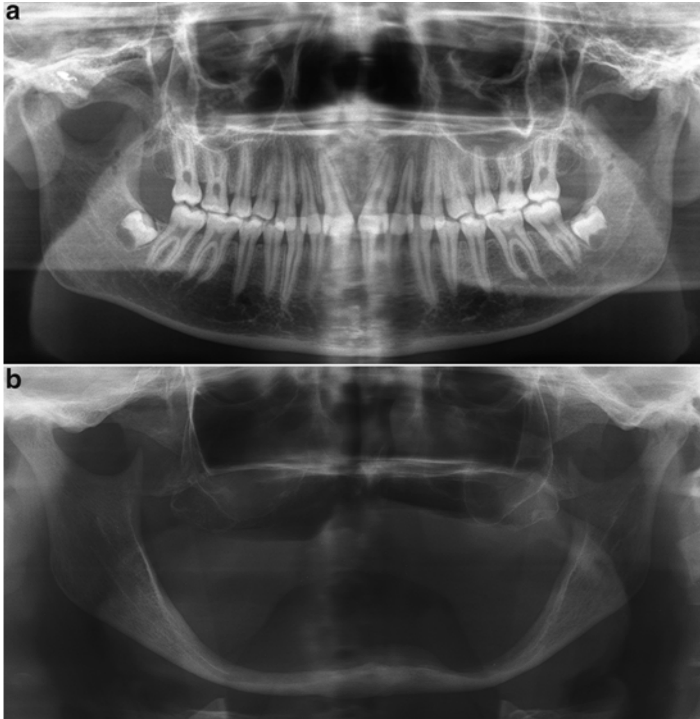

Fig. 12.1 Radiograph of fully dentate jaws with no signs of bone loss or other defects (**a**). Advanced resorption of the mandible in an edentulous patient (**b**)

the fundamental requirements for osseointegration and the interaction between the titanium surface and bone (Brånemark et al. 1969, 1977; Schroeder et al. 1981). They also addressed the primary biomechanical requirements for dental implant design. Both research teams obtained excellent results through the integration of basic biological and biomechanical knowledge and the initiation and application of clinical research projects.

Most of the endosseous cylindrical implant systems subsequently developed, both for submerged and non-submerged implant procedures, followed the guidelines for successful osseointegration by Adell et al. (1981), *i.e.* a 3- to 6-month unloaded healing period. It was argued that implants required an undisturbed healing time for successful tissue integration and that premature loading might prevent direct bone apposition and lead to fibrous tissue encapsulation. Improved understanding of the osseointegration process, bone resorption and re-modelling and the interaction between bone and metal surfaces has resulted in recent departures from the traditional conservative approach established some 40 years ago. The importance of the surface characteristics and choice of the implant material in determining the quality of bone anchorage was recognized early (Albrektsson et al. 1981; Buser et al. 1991; Johansson 1991). Various surface treatments have been successfully used to achieve more rapid and more stable bone integration *i.e.* bone-metal anchorage

(Buser et al. 1999; Albrektsson et al. 2000; Arvidson 1998; Arvidson et al. 1998, 2008; Fischer et al. 2008, for recent reviews see, Esposito et al. 2004, 2007a, b; Wennerberg and Albrektsson 2009).

Successful endosseous implantation in the alveolar ridge requires sufficient quality and quantity of bone at the recipient site. Several surgical techniques have been described to augment bone before or in combination with dental implant installation (for a review see Hammerle and Jung 2003). More recently, in a relatively limited RCT study, Jung et al. (2009) demonstrated that implants installed in defective bone sites grafted with demineralised bovine mineral with or without a growth factor (rhBMP-2) had excellent clinical and radiological outcomes after 5 years.

12.2.2 Loss of Periodontal Tissues

The main function of the periodontium is to attach the tooth to the alveolar bone and to maintain the surface integrity of the masticatory mucosa. Epidemiological studies have shown that infections are the main cause of destruction of bone as a supporting tissue of the teeth. The etiology has, however, been shown to be multi-causal. Periodontal disease, especially the most severe forms, is no longer regarded as a simple infection, but rather as the result of a complicated interaction with systemic factors or disorders. In the most severe cases the outcome can be the loss of most or all teeth (Fig. 12.2).

An important goal of periodontal therapy is to achieve a reduction in the depth of the periodontal pocket in order to prevent further disease progression. In patients with moderate periodontitis, *i.e.* pocket depths $\leq$6 mm, this goal can be accomplished by non-surgical therapy, whereas in severe cases, particularly in the presence of intrabony defects and furcations (Fig. 12.3), the treatment must be supplemented with periodontal surgery. There is increasing use of regenerative procedures to restore lost periodontal support.

Periodontal regeneration has been defined as the process by which the architecture and function of the periodontal tissues are completely renewed (The American Academy of Periodontology 1992) and includes the formation of a new connective tissue attachment, cementum and supporting bone (Ellegaard et al. 1973, 1974; Karring et al. 1993). Regenerative periodontal therapy comprises procedures which are specially designed to restore, by reattachment or new attachment, those parts of the supporting apparatus which have been lost due to periodontitis, *i.e.* gingiva, periodontal ligament, root cementum and alveolar bone. For true regeneration, the root surface must therefore be repopulated by epithelial cells and cells derived from the gingival connective tissue, bone and periodontal ligament. Guided tissue regeneration (GTR) is a treatment modality intended to promote regeneration of periodontal tissue lost through periodontitis. Animal studies have confirmed that in intra-bony defects, this treatment results in true regeneration, albeit with some limitations (Laurell et al. 2006). GTR has also been used in implant rehabilitation, using different techniques and membrane materials (for a review see Hammerle and Jung 2003).

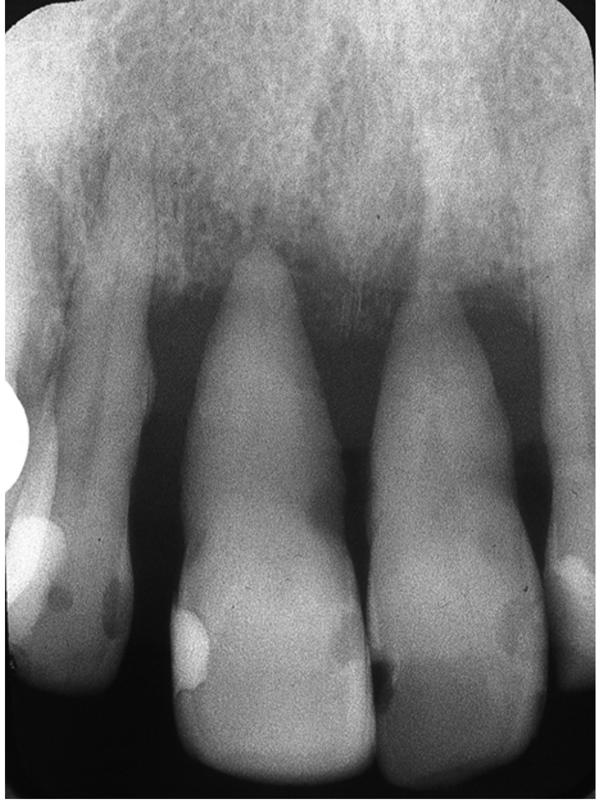

Fig. 12.2 Radiograph of very severe bone loss around the maxillary anterior teeth

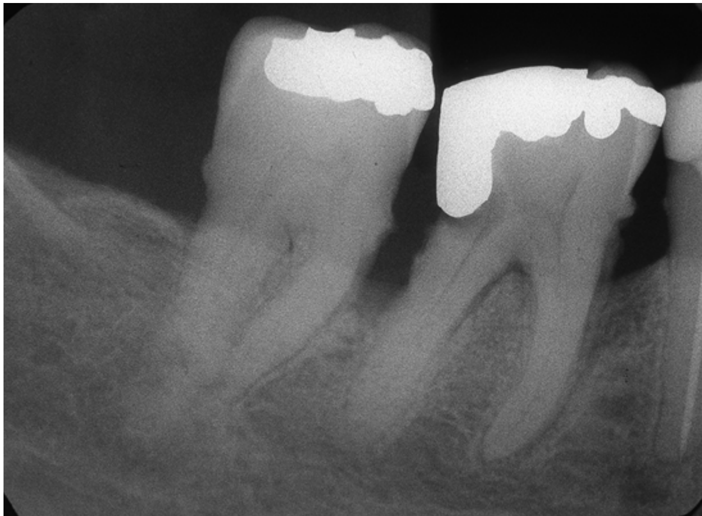

Fig. 12.3 Radiograph of vertical bone loss around a mandibular molar

The most commonly used clinical methods for regeneration of the periodontal attachment apparatus are GTR (Sculean et al. 2008) and a derivative of enamel matrix proteins (EMD). GTR, using bioabsorbable barriers made of e.g. polylactide acetyltributyl citrate or polydioxanon, has shown stable clinical results in both short and long term studies (Eickholz et al. 2004). EMD are acidic extracts of extracellular enamel matrix, and include a heterogeneous mixture of polypeptides encoded by several genes (Bosshardt 2008). It is unclear which of the enamel matrix proteins induces the regeneration, and the underlying molecular mechanisms have yet to be determined.

The use of bioactive molecules to induce local bone formation is an active field of research. Bioactive agents are used alone or together with grafting or GTR for treatment of intra-osseous and furcation defects (Trombelli and Farina 2008). A variety of growth factors have also been tested for local bone regeneration (for a recent systematic review see Jung et al. 2008).

At present, regimes that encounter bone formation and attachment formation and hold the promise of significantly increasing bone density and volume and leads to the formation of a new functional periodontal attachment, have yet to come available. In this context, the concept of tissue engineering has emerged as a valid approach to the current therapies for periodontal tissue regeneration and attracting considerable attention. It has been shown that the use of MSCs in Platelet-rich plasma gel could be useful for periodontal tissue regeneration (Yamada et al. 2006). However, not much has been reported on the application of tissue engineering for regeneration of periodontal tissues.

12.2.3 Loss of Bone

Bone defects in the oral and maxillo-facial region may arise following surgical treatment of tumors, cysts and other pathological conditions as well as traumatic insults to the facial and dento-alveolar structures. As such defects often involve structures of different origins, the reconstructive procedures are very demanding. Maxillofacial tumors and cysts may arise from both soft and hard tissues and may be of odontogenic or nonodontogenic origin. Lesions located within the jaws thus include odontogenic cysts and tumors, nonodontogenic cysts and benign tumors and malignant, nonodontogenic neoplasms. Benign cysts and tumors occur frequently, are clinically and radiologically well-delineated and treated by curettage or enucleation, whereas highly proliferative lesions are treated by resection. Malignant primary neoplasms of the jaws are rare, the most common being osteosarcoma. Much less common are chondrosarcoma, plasmocytoma and Ewing's sarcoma. Some of these tumors may require extensive surgical treatment and reconstruction. Further examples of pathological conditions of the jaws requiring treatment by extensive bone resection are osteoradionecrosis or extensive, proliferative benign lesions which have proved resistant to other therapies.

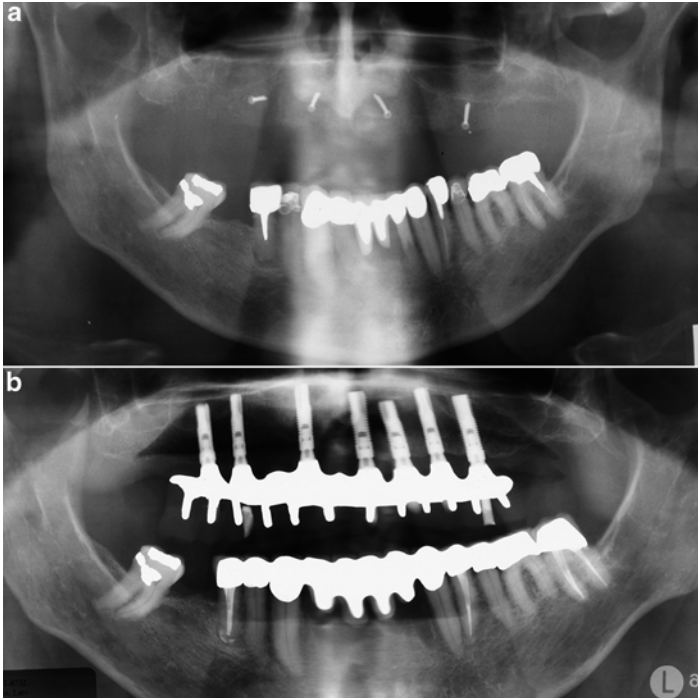

Fig. 12.4 Edentulous maxilla with extensive resorption of the alveolar crest. Reconstructed with free cortical onlay blocks from the iliac crest, fixed with miniscrews (**a**). After a healing period, a full arch maxillary bridge was retained on seven osseointegrated implants (**b**)

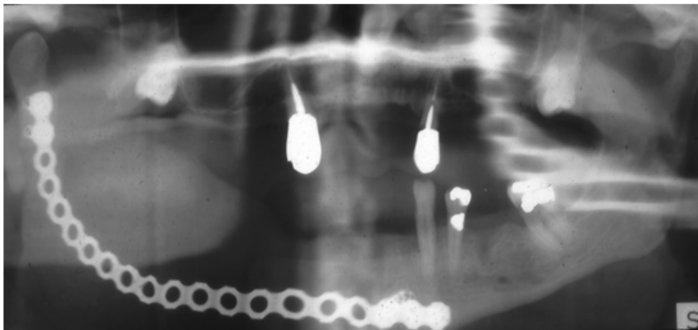

Fig. 12.5 Mandibular defect after tumor resection. Treated with a free revascularized forearm soft tissue graft and a bridging reconstruction plate

Despite progress in the field of reconstruction as a result of new surgical techniques, improved biomaterials and advances in cell biology, autologous bone grafting remains the “gold standard”, especially for the reconstruction of large bone defects (Chiapasco et al. 2008; Raveh et al. 1987) (Figs. 12.4 and 12.5).

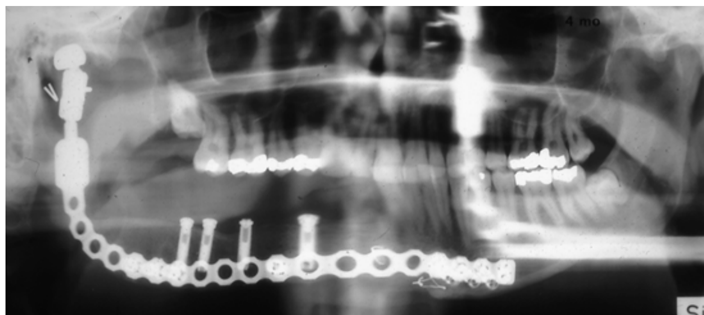

Fig. 12.6 Hemi-mandibular defect after tumor resection. Treated with a temporo-mandibular joint prosthesis, a bridging reconstruction plate with free iliac crest graft and osseointegrated dental implants

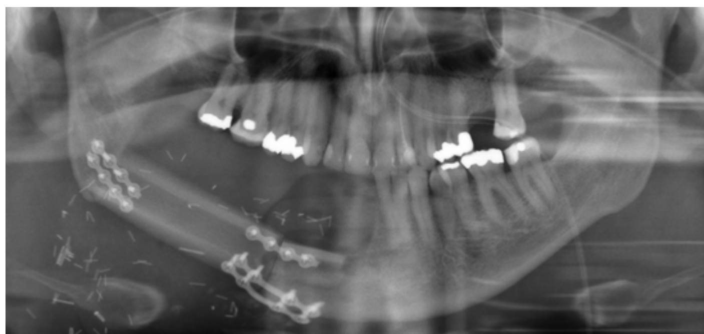

Fig. 12.7 Lateral mandibular defect after tumor resection. Treated with a free revascularized compound fibular graft

Free, nonvascularized autologous transplants are function for bridging of defects and as volume fillers by inducing bone growth. In some cases, however, the prognosis may be guarded, due to the risk of inadequate vascular regeneration and impaired tissue repair following hypoxia. Of vital importance to success are adequate microvascularity of the recipient tissues and optimal fixation of the grafts, in order to prevent infection and loss of osteogenic cells. Segmental osteodistraction may have potential as a treatment solution. In cases of compromised tissue healing or composite tissue defects, the treatment of choice is the use of revascularized hard and soft tissue free flaps (Torroni et al. 2007; Smolka and Iizuka 2005; Emerick and Teknos 2007; Chepeha et al. 2008; Chiapasco et al. 2006). Despite the above risk factors, good functional and esthetic outcomes have been reported (Chiapasco et al. 2008; Louis et al. 2008) (Figs. 12.6 and 12.7).

For reconstruction of minor and single tissue defects, a wide range of autografts, allografts, xenografts and synthetic substitutes has been extensively used in recent years, in some instances showing outcomes comparable with autologous grafts (Hallman and Thor 2008; Hellem et al. 2003). In a review article Kretlow et al. (2009)

presented an excellent summary of newer materials and methods in bone and soft tissue regeneration. Compared with autologous transplants, the disadvantages of allografts, xenografts and synthetic biomaterials include lack of osteoinductive properties and relatively varying osteoconduction. Varying, and to some degree uncontrolled resorption rates may represent a challenge in a clinical situation when assessing the amount and progression of tissue regeneration. The risks of bacterial, viral or prion transmission from allo- and xenografts as well as immunologic reactions are minimal and dependent on the method used for tissue preservation (Kretlow et al. 2009).

Bone and soft tissue defects due to traumatic high velocity insults may be extensive, and involve several areas of tissue loss and progressive necrosis, demanding extensive surgery. These defects often have to be reconstructed by two stage surgery, using revascularized free or pedicled compound flaps or osteodistraction (Bertele et al. 2005; Pereira et al. 2007). Defects due to non-optimal repositioning of fractures in the periorbital and naso-ethmoidal regions remain a challenge for the surgeon. The midface and mandibular regions, however, may be reconstructed using osteodistraction devices.

Severe dento-alveolar trauma occurring in isolation or in combination with facial trauma, is often associated with loss of teeth and defects in the alveolar crest. Cases involving primary or secondary loss of teeth and bone tissue have to be reconstructed as a prerequisite for treatment with dental implants. In some cases replacement of lost mucosal/gingival soft tissue must also be addressed. Where functional and esthetic outcomes are priorities, the treatment of choice would be reconstruction of bone defects in the maxillary anterior alveolar crest, bone grafting and local osteodistraction (Lundgren and Sennerby 2008) or even non resorbable bone substitutes (Hallman et al. 2009; Hellem et al. 2003).

12.3 Future Directions

12.3.1 Oral Stem Cells in Regenerative Dentistry

Physiological bone tissue regeneration is a remarkable process that results in healing without scarring. It is a multi-faceted process, beginning with angiogenesis, followed by callus formation and eventually bone remodelling. Key contributing factors in this process are growth factors [VEGF, PDGF-BB, plGF, BMPs, basic Fibroblast Growth Factor (bFGF)] osteocytes and angiocytes of the surrounding bone tissue, adult mesenchymal and hematopoietic stem cells. However, the prognosis is uncertain in the presence of large defects (>1 cm) or conditions associated with healing impairment such as old age, diabetes or radiation therapy. Under such suboptimal conditions, the gold standard of autologous bone transplantation is however associated with disadvantages, such as the limited amount of bone which can be harvested, unpredictable donor bone turnover, donor site morbidity, and the added cost incurred by surgical procedures to harvest the bone as well as pain at the harvest site.

Currently, various strategies are applied to stimulate healing of bone defects and to restore lost maxillofacial bone and periodontal support following traumatic insult, tumor ablation, diseases or congenital deformities. Despite the fact that materials science and technology has markedly improved the field of bone regeneration, none of the currently available treatment regimes stimulates bone and attachment formation. They therefore lack the potential to increase bone density and volume significantly and to form a new, functional periodontal attachment. For this reason large defects/injuries still represent a major challenge for dentists and oral maxillofacial surgeons. The clinical challenges have stimulated interest in developing new therapies that involve regeneration of bone and periodontal ligament.

Bone marrow has been shown to contain a population of rare cells capable of differentiating into the cells that form various tissues. These cells, referred to as mesenchymal stem cells (MSC), are located within the bone marrow and, depending on the culture conditions chosen, have the potential to differentiate into fibroblastic, osteogenic, adipogenic or reticular cells (Friedenstein 1976; Bianco et al. 2001). The lack of immunogenicity of MSC heightens the potential of these cells for bone repair. Human bone marrow osteoprogenitors can be isolated and enriched from the CD34+ fraction using selective markers such as STRO-1 (Stewart et al. 1999). In recent years there has been increasing interest in the possibility of using adult MSC for regeneration of oral tissues, not only to enhance attachment around periodontally compromised teeth, but also to augment alveolar bone before and/or after placement of oral implants. Adult stem cells, previously thought to be limited in potential, have increasingly been shown to be able to differentiate into tissues of an entirely different germ layer, with potential clinical application in the treatment of a number of diseases. Cell based therapies are promising new therapeutic tools in regenerative medicine. By using mesenchymal stem cells (MSCs), good results have been reported for bone engineering in a number of clinical studies (Gomez-Barrena et al. (2011). Thus, stem cell-based bone tissue engineering is a promising concept for reconstruction/ regeneration of craniofacial defects, but much work remains before this approach becomes a routine part in clinical practice. The reconstruction of bone defects using stem cells seeded onto biodegradable carrier materials or scaffolds requires timely formation of functional blood vessels. After implantation, complex tissues are dependent on a functional vasculature, not only for cell survival, but also for tissue organization. Recently, the ability of MSC to support development of blood vessels as perivascular cells was reported (Pedersen et al. 2013, 2014). The data showed that generation of endothelial microvascular networks *in vitro* affected the angiogenic and osteogenic potential of tissue-engineered constructs.

One of the most extensively studied populations of multipotent stem cells has been mesenchymal stem cells (MSC) from bone marrow. It has been demonstrated that their precursors are typically associated with the blood vessels, and found in most of the human tissues (Crisan et al. 2009), thus making it theoretically possible to obtain MSC from an unlimited number of organs and tissues. It has been reported that from a small volume (0.1–3 ml) of marrow aspirate, alveolar bone mesenchymal cells (BMSC) can be expanded successfully 70 % of the time (Matsubara et al. 2005). Alveolar BMSC might be useful for regenerative medicine, because small marrow

aspirates from alveolar bone can be made with minimal pain. Furthermore, Matsubara et al. (2005) demonstrated a high osteogenic potential from alveolar BMSC. Although this raises few ethical issues, harvesting of cells from bone marrow is still an invasive procedure, and stem cell numbers decrease significantly with the age. The search for more readily accessible sources of pluripotent stem cells has led to investigation of other tissues, including mobilized peripheral blood, umbilical cord blood and more recently, fat (adipose) tissues, periodontal ligament (PDL), deciduous and permanent teeth.

Although potential of BMSC for future clinical use in bone tissue engineering (BTE) is undoubted, recently, adipose tissue stem cells (ASC) have shown a great promise as an alternative to BMSC in BTE for several reasons (Sándor et al. 2013). Firstly, the stem cell yield and proliferation rate is much higher than that of BMSC. Secondly, harvesting procedure of ASC is less complicated and is associated with less morbidity and complications. Several recent studies indicate potential clinical use of ASC in BTE (Zuk et al. 2001; Sándor et al. 2013; 2014; Gotoh et al. 2014). Despite the above advantages, both *in vitro* and *in vivo* osteogenic ability of ASC seem to be inferior as compared to that of BMSC (Liao and Chen 2014).

The PDL is one of the tissues that has attracted interest as a source of stem cells and its potential for regeneration. It contains a heterogeneous cell population that can differentiate into cementoblasts or osteoblasts. Recent findings suggest that PDL cells have osteoblast-like properties. They have the capacity to form mineralized nodules *in vitro*, express bone-associated markers such as alkaline phosphatase and sialoprotein, and also respond to bone inductive factors such as parathyroid hormone, insulin -like growth factor 1, bone morphogenetic protein 2, and transforming growth factor β_1. Seo et al. (2004) showed that human PDL cells participate in periodontal tissue repair in immunocompromised rats, indicating that the PDL contains stem cells.

Dental pulp tissue is also a readily accessible source of pulp-derived mesenchymal stem cells (PDSC). PDSC express the endothelial and smooth muscle marker STRO-1 (Shi and Gronthos 2003) and display a pericyte phenotype, with expression of the pericyte-associated antigen 3G5 (Shi and Gronthos 2003). It is therefore assumed, but not yet confirmed, that the perivascular region in the pulp is the niche for PDSC and that pericytes give rise to dental pulp stem cells. Isolated dental pulp stem cells have been shown to be plastic-adherent and express the MSC markers STRO-1, CD90, CD29, CD44, CD166, CD105, CD106, CD146, CD13 and are also negative for CD14 and CD34 (Shi et al. 2005; Ikeda et al. 2006). *In vitro*, PDSC are capable of self-renewal, display plasticity and mutilineage potential (adipocytes, chondrocytes, osteoblasts, neural cell progenitors and myotubes) and can therefore be considered as stem cells (Gronthos et al. 2002).

For tissue engineering purposes, PDSC have shown potential for both dentin and bone production. From the pool of human dental pulp cells, odontoblasts capable of forming dentin-like structures can be differentiated when cultured under mineralization-enhancing conditions (About et al. 2000). Moreover, in immunocompromised mice, subcutaneously implanted cells derived from human dental pulp generate a dentin-pulp-like complex without lamellar bone (Shi et al. 2005).

Using a similar model, another research group has also shown that PDSC are able to generate vascularized bone tissue that *in vivo* was remodelled into a lamellar bone (Laino et al. 2005, 2006a, b; d'Aquino et al. 2007). Further, when implanted into immunocompromised rats, a distinguishable STRO-1 positive subpopulation of cells was found to produce woven bone efficiently and to remodel lamellar tissue (d'Aquino et al. 2007; Laino et al. 2006b). After implantation, PDSC expressed bone markers including osteocalcin, Runx-2, collagen I and alkaline phosphatase (d'Aquino et al. 2007). Furthermore, it might be possible for PDSC to contribute to the formation of new bone containing Haversian channels with appropriate vascularization *in vivo* (Huang et al. 2008; Pierdomenico et al. 2005; Shi et al. 2005; Young et al. 2002; d'Aquino et al. 2007; Laino et al. 2006b; Ikeda et al. 2006; Gronthos et al. 2000; About et al. 2000; Batouli et al. 2003; Cheng et al. 2008). Even when removed from their native location, dental pulp cells maintain the potential to contribute to the formation of both dentin and alveolar bone (Diep et al. 2009).

The transition from deciduous (baby) teeth to permanent (adult) teeth is a unique, dynamic process in which the development and eruption of the permanent teeth is co-ordinated with the resorption of the roots of deciduous teeth. In humans, it may take >7 years to complete the orderly replacement of 20 deciduous teeth. It was found that a naturally exfoliated human deciduous tooth contains a population of stem cells (SHED) and are thus available without surgical intervention (Laino et al. 2006b). These cells have been shown to be plastic-adherent, have great proliferative capacity and positive for MSCs markers STRO-1, CD29, CD106, CD146, while negative for CD14, CD34 (Shi et al. 2005). Further, they exhibited a high degree of plasticity with the capacity to differentiate into neurons, adipocytes, osteoblasts and odontoblasts (Miura et al. 2003; Huang et al. 2008). SHED are not only derived from a very accessible tissue resource but are also capable of providing enough cells for potential clinical application. Thus, exfoliated teeth may be an unexpected, unique resource for stem cell therapies including autologous stem cell transplantation and tissue engineering. These cells could aid the repair of damaged teeth and perhaps even treat neural injuries or degenerative diseases. Stem cells isolated from deciduous teeth (SHED) have several advantages. Although unlikely to have the differentiation and proliferative potential of ESC, deciduous tooth stem cells require no invasive harvesting procedure. Furthermore, there are no ethical issues, as in the normal course of events deciduous teeth exfoliate and are discarded.

12.3.2 Artificial Scaffolds in Regenerative Dentistry

In contrast to the conventional biomaterials approach, tissue engineering is based on an understanding of tissue formation and regeneration, and aims at inducing new functional tissues, rather than just implanting replacement parts. There are numerous published case reports of the application of bone tissue engineering for oral and maxillofacial surgical reconstruction, periodontal tissue regeneration and sinus

floor augmentation. Tissue engineering is the application of scientific principles to the design, construction, modification and growth of living tissues, using biomaterials, cells, and factors alone or in combination. Skeletal tissue engineering requires a scaffold conducive to cell attachment and maintenance of cell function, in combination with a rich source of osteoprogenitor cells and osteoinductive growth factors. Crucial to success is an understanding of how cells function and form a matrix, and the development of appropriate materials for fabrication of scaffolding designed to promote cell attachment and maintain cell function.

Recently, much effort has been devoted to synthesis methods and fabrication techniques used to design and select a scaffold with properties that most closely match those required for bone regeneration. Highly porous and degradable aliphatic polyester scaffolds with varying pore size and interconnected pores were fabricated by bulk copolymerization of poly(L-lactide) (PLLA), 1,5-dioxepan-2-one (DXO-co-LLA) and ϵ-caprolactone (CL-co-LLA) (Dånmark et al. 2010). The degradation rates of polyester scaffolds and loss of mechanical integrity were greatly increased in porous scaffolds made with hydrophilic co-monomers (Dånmark et al. 2011). By incorporating hydrophobic co-monomers with limited ability to crystalize instead of hydrophilic co-monomers, the mechanical stability was retained longer during degradation. It has been shown that these scaffolds are biocompatible and stimulate bone regeneration both *in vitro* and *in vivo* (Arvidson et al. 2011; Dånmark 2011; Idris 2010; Xue 2011; Xing 2012). These polyester scaffolding materials show great potential as bone tissue constructs (Suliman et al. 2015; Yassin et al. 2015). However, the scaffolds need to be optimized to control cell differentiation and growth as well as to achieve angiogenesis before they are ready for human use.

12.3.3 Paracrine Effects of Stem Cell-Derived Growth Factors

Tooth regeneration by cell transplantation is a meritorious approach. However, there are hurdles in the translation of cell-delivery-based tooth regeneration into therapeutics. The inaccessibility of autologous embryonic tooth germ cells for human applications, the limited availability of autologous postnatal tooth germ cells (e.g. third molars) and the low survival rates of the implanted cells may undermine the efficacy of the cell-based treatment. Furthermore, other factors such as the availability of autologous stem cells, the excessive costs of cell isolation, handling, storage, shipping and *ex vivo* manipulation, liability issues if contamination occurs, and potential for transmission of infectious disease are all potential drawbacks to cell transplantation (Inanc and Elcin 2011; Yildirim et al. 2011).

It has been reported that stem cells secrete multiple metabolites, growth factors, signaling molecules, and extracellular matrix proteins during *in vitro* culture that affect cellular behavior (Kinnaird et al. 2004; Barcelos et al. 2009; Cai et al. 2009; Perin and Silva 2009; Osugi et al. 2012). Stem cell-conditioned medium (CM) can be used, transplanted or injected with or without scaffolds to induce cell homing, migration, proliferation and differentiation (Ueda and Nishino 2010; Kim et al. 2009;

Yang et al. 2009). Therefore, the use of stem cell-conditioned medium as an alternative to transplanting stem cells might be a feasible approach for tissue engineering. The paracrine effects of the growth factors in CM on recruiting circulating progenitor/stem cells and/or endogenous adjacent cells to the treatment site is attracting considerable research attention at present. Although the molecular mechanisms that direct mobilization and homing of cells in response to the paracrine factors secreted by stem cells are not fully understood, cell homing represents a novel concept for regenerative dentistry and may offer a clinically useful approach (Kim et al. 2010).

The therapeutic effects of CM derived from stem cells derived from different sources have been demonstrated in experimental animal models (Cho et al. 2012; Osugi et al. 2012). It has been shown that conditioned medium derived from mesenchymal stem cells as well as SHED-conditioned medium is able to accelerate wound healing as well as that seen with stem cell transplantation, and thus may become a new therapeutic method for wound healing in the future (Tamari et al. 2011; Ueda and Nishino 2010). Thus conditioned medium might be used to create a highly inductive microenvironment, with many possible uses in regenerative dentistry. However, further studies are required to address the underlying mechanisms involved in organogenesis mediated by conditioned medium.

12.4 Conclusion

ASC, PDL, PDSC, SHED and BMSC stem cells appear to be appropriate candidates for tissue engineering involving restoration of dental and periodontal tissues, as well as bone, suggesting a potential future therapeutic role of these cells for craniofacial regeneration. Artificial scaffolds are currently underdevelopment and may, together with cells from these different sources, lead to improvements in tissue engineering of bone defects in the oral cavity. The use of paracrine factors to improve tissue regeneration is a very promising new concept. However, much work remains before this approach will be ready for routine clinical use.

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Chapter 13
Regenerative Therapies – Trachea

Silvia Baiguera and Paolo Macchiarini

Abstract No preferred treatment, which could offer a functional solution, has been so far developed for patients affected by extensive airway damages. As the field of tissue engineering attempts to develop tracheal replacements, multiple types and combinations of cells, scaffold materials, and/or culture conditions have been used. Interest has been evoked by decellularized natural matrices, which affecting cell proliferation, migration and differentiation, could play an active part in tissue regeneration and remodeling. Using the detergent-enzymatic method, we were able to obtain decellularized human tracheal matrices lacking MHC antigens (bypassing rejection), having structural, mechanical and *in vivo* pro-angiogenic properties similar to that of native airways and supporting *in vivo* recellularization. Starting from these results, we have developed an *in vivo* tissue engineered strategy, based on airway bioengineered grafts combined with autologous stem cells and pharmacological intervention (to boost progenitor cell recruitment commitment), which resulted to be a clinically successful alternative for patients with serious airway disorders.

Keywords Tracheal damage • Regenerative medicine • Trachea • Tracheomalacia • Stenosis • Primary tracheal tumors • Palliative treatment • Tracheal allotransplantation • Airway replacement • Tracheal substitute • Decellularized tracheal matrix • Chondrocytes • Chondrogenesis • TGF-β3 • In vitro cultures • In situ tissue engineering • In vivo tissue engineering • Granulocyte colony-stimulating factor (G-CSF) • Erythropoietin (EPO) • In vivo tracheal strategy • Artificial tracheal scaffold • Bioengineering airway transplants

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Abbreviations

DEM	detergent-enzymatic method
EPO	erythropoietin
G-CSF	granulocyte colony-stimulating factor
MSCs	marrow stromal cells
TGF-β	transforming growth factor-β
TNF-α	tumour necrosis factor-α
β-FGF	basic fibroblast growth factor

13.1 Introduction

Any tracheal damage can compromise a wide range of important functions for survival, such as speech, deglutition (swallowing), respiration, mucociliary clearance, and immune protection from inhaled or ingested antigens. In the world, approximately 0.1 every 100,000 persons per year are affected by primary tracheal cancer (Honings et al. 2010), and a smaller, but severely impaired, group of patients are affected by benign disease and trauma linked to non-functioning airways (Nouraei et al. 2007). To date, no conventional solution to treat these patients has been developed.

Regenerative medicine is an interdisciplinary field that "replace or regenerate human cells, tissues or organs, to restore or establish normal function" (Mason and Dunnill 2008) and it has been recently accepted as a useful clinical discipline that ensures and enhances the quality of life in patients undergoing organ reconstructions. Among the different regenerative strategies, tissue engineering is a promising technology that has already provided functional human organ replacements in various clinical settings (Atala et al. 2006). Combining living cells with biocompatible and biodegradable scaffolds, tissue engineering can be applied to the obtainment of an anatomically, physiologically and biomechanically airway replacement which could transform functional outcomes for patients with advanced structural tracheal disorders.

13.2 Tracheal Anatomy and Pathology

The trachea is a fibro-cartilagineous, tubular structure, extending from the cricoids cartilage to the bronchial bifurcation. Structurally, it consists of 18–24 C-shaped cartilaginous rings joined by fibroelastic tissue and closed posteriorly by a membranous muscular structure, named *pars membranacea* (Fig. 13.1a). The trachea functions as an air conduit: the cartilaginous rings prevent collapse during inspiration and widen during expiration, the fibro-elastic tissue prevents over distention, while

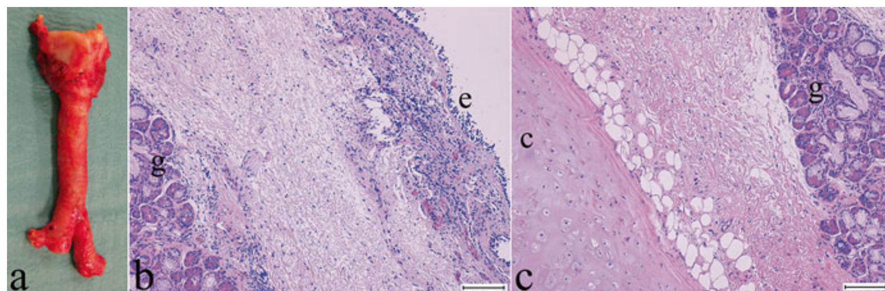

Fig. 13.1 (**a**): Human airway consisting of larynx, trachea and carina. *b* and *c*: H&E stain of human trachea showing tracheal luminal part covered with a mucosal surface (**b**) and the supporting cartilagineous connective tissue layer beneath the epithelium (**c**). '*c*' cartilaginous part, '*g*' seromucinous glands, '*e*' epithelium (Scale bar = 100 μm)

muscle contraction reduces lumen size facilitating airway clearance. The lateral flexibility of the trachea allows cervical rotation, flexion and extension, while maintaining a constant patency for air passage. The tracheal luminal part is covered with a mucosal surface, which serves to protect against infection and aids in mucous clearance, lined with a pseudostratified columnar respiratory epithelium (Fig. 13.1b), containing basal (classical stem cells, which can play a role in airway epithelium homeostasis and regeneration after injury), ciliated, secretory (goblet, serous and Clara cells), neuroendocrine and less well categorized 'indeterminate' or 'intermediate' cells (Mercer et al. 1994; Rock et al. 2010). The supporting connective tissue layer beneath the epithelium, mostly cartilaginous, forms a scaffold hosting blood vessels, nerves and undifferentiated adult stem cells of mesenchymal derivation (which could played a pivotal role in cell repair, regeneration and functional restoration) (Fig. 13.1c) (Okubo et al. 2005; Cardoso and Lü 2006).

A wide spectrum of benign and malignant pathology, most leading to central airway obstruction with subsequent respiratory insufficiency, may afflict the trachea. The exact epidemiology of the tracheal diseases is not perfectly known, being included together with the pathologies affecting the total airway system.

Between benign lesions, tracheomalacia and stenosis (congenital or acquired) are the two most common tracheal anomalies. Tracheomalacia is described as a decreased rigidity of the trachea, due to a structural abnormalities of its wall. In response to variations in intrathoracic pressure or to compression by adjacent intrathoracic structures (esophagus, ascending aorta or aortic arch), tracheomalacia may result in functionally significant interference with air flow and impaired clearance of tracheobronchial secretions. Acquired tracheomalacia may occur following any injury that results in loss of cartilage (such as postintubation injury), chronic external compression or relapsing polychondritis. Stenosis is a fibrotic narrowing of the airway, which can result in severe dyspnea. Although tracheal stenosis is a rare disease, the mortality rates for patients with long-segment tracheal stenosis and atresia (abnormally closed or absent trachea) are nearly 11 % and 100 %, respectively (Fuchs et al. 2002). There are many causes of stenosis: postintubation injury

and tracheostomy are the most common causes of acquired stenosis. Congenital tracheal stenosis, which may involve all or a portion of the tracheal length, is almost always secondary to a development defect in which the *pars membranacea* is deficient and the wall consists of complete or almost complete cartilaginous rings.

Primary tracheal tumors are the least common neoplastic lesions of the airways, representing the 2 % of upper airway tumors (Macchiarini 2006). A majority of primary tumors (more than 90 %) in adults are malignant (Macchiarini 2006). By far the most common are adenoid cystic (50 %) and squamous cell (20–40 %) carcinomas. The adenoid cystic carcinoma is considered to be a slowly growing neoplasma; while squamous cell carcinomas are frequently locally advanced and associated with high incidence of lymph node metastasis (Macchiarini 2006).

In almost all patients with tracheal diseases, resection followed by end-to-end anastomotic reconstruction (surgical joining of two trachea ends to allow air flow from one segment to the other) yields better results and successful outcome than any other treatment. High success rates of over 70 % have been reported (Grillo et al. 1995; Omori et al. 2005). However, if a tracheal segment >6 cm long needs to be resected, direct anastomosis is impossible because of the high mechanical tension at the anastomosic site, leading to severe and fatal postoperative complications (Mulliken and Grillo 1968; Grillo 2002). In these cases palliative treatment, such as irradiation, stents and T tubes, are the only solutions (Grillo 2002). Related to primary malignant tracheal tumors, epidemiological studies demonstrated that, due to the difficulties in the definitive diagnosis, most patients present with already advanced local disease and the only possible treatment is the palliative management with stents or neoadjuvant radiotherapy (Gelder and Hetzel 1993; Yang et al. 1997; Licht et al. 2001; Bhattacharyya 2004). For patients non-surgically treated, the 5-year survival pass from 39 to 7 % for squamous-cell tumors and from 52 to 33 % for adenoid cystic tumors (Licht et al. 2001) and a median survival of less than 12 months has been consistently reported (Chao et al. 1998; Choi 2004).

For these reason, researchers have paid high attention to exploring a solution for tracheal function reconstruction; however, to date, there is no well established good conventional solution. An effective tracheal replacement, displaying anatomical, physiological and biomechanical properties equivalent to normal human airway, could provide an alternative for patients for which standard surgical procedures are not an option.

13.3 Clinical Application of Tracheal Replacement Strategy

In the last century, numerous studies have been made to identify the ideal airway substitute. The various techniques adopted ranges from synthetic stents and prosthetic materials, to autologous/allogenic tissue flaps and patches (Osada 2006; Baiguera and Macchiarini 2011). However, none has proven satisfactory for clinical use mainly because of the lack of adequate vascularisation and respiratory epithelium along the lumen (Grillo 2002; Doss et al. 2007). Moreover, it has to be

underlined that the trachea is not located in a mesenchymal environment but it is in direct contact with the breathing air, making infection and contamination more likely to occur. More recently, the enormous efforts in the field of airway replacement allowed the development of strategies which could have relevant clinical applications.

Clinical tracheal allotransplantation have been tried in humans obtaining different results. Fresh or cryopreserved aortic allografts were used, without aid of immunosuppressive therapy, for tracheal, carinal or bronchial replacements (Wurtz et al. 2006; Wurtz et al. 2010; Wurtz 2010; Martinod et al. 2011): presence of respiratory epithelium and no graft rejection phenomenon were observed. However, fistula development, sparse calcification, progressive graft contraction, stent necessity and/or no evidence of cartilage regeneration were also reported (Wurtz et al. 2006; Wurtz et al. 2010; Wurtz 2010; Martinod et al. 2011). Non-vital allogenic tracheal grafts resulted suitable only for tracheal patch replacement in the pediatric population, having however the big limitation not to grow with the recipients and resulting in long-term stenosis (Bujia et al. 1991; Elliott et al. 1996; Jacobs et al. 1996; Jacobs et al. 1999). Recently, the Leuven Tracheal Transplant Group have reported successful tracheal allotransplantation, after withdrawal of immunosuppressive therapy (Delaere et al. 2010). Indirect revascularization was achieved by placing the graft, for 4 months, in the recipient's forearm fascia. During this period, immunosuppressive therapy was necessary, the cartilaginous viability was maintained, while the membranous posterior wall of the allograft underwent avascular necrosis. At the time of transplantation, the tracheal graft (3.5 cm) had viable cartilage fully lined with squamous epithelium, and 1 year after tracheal reconstruction, the patient was satisfied with the outcome and lung functions resulted to be normal (Delaere et al. 2010). Even if clinically successful, the number of operation needed (two major plus eight minor) and the requirement of an 8-month immunosuppression (preventing the use of this strategy in a tumor context) limited the therapeutic potential of this approach.

A regenerative solution providing anatomical restoration of the airway, consisting of a graft with pliability, elasticity, and mucosal lining similar to that of native tissues, which resists stenosis and tolerate implantation, with minimal risk of infection, extrusion, migration or failure, may provide functional airway replacement in a manner superior to that provided by present techniques.

13.4 Regenerative Tracheal Strategy

The tissue engineered approach is normally based on three fundamental components: (i) cells, acting as "seeds" for tissue regeneration, (ii) scaffold, where cells can proliferate and grow, and (iii) regulatory/growth/boosting factors, mediating cell behaviors (Table 13.1). Tissue engineering resulted to be the only technique that seems to offer any real promise, avoiding immunosuppression, for airway replacement and regeneration (Walles et al. 2004a; Walles 2004; Mertsching et al. 2005).

Table 13.1 Main components on which the tissue engineered technique is based.

	Requirements	Type	Problems
Cells	Non immunogenic	Autologous	Adult primary cell yields and proliferation rates tend to be low
	Highly proliferative	Allogenic	
		Differentiated	
	Easy to harvest	Progenitor cells	Embryonic stem cells linked to ethical dilemmas and to risks of immunological rejection and tumor formation.
		Embryonic/adult stem cells	
	Able to differentiate into a variety of cell types with specialized functions	Inducible pluripotent stem cells	
Scaffold	Biocompatible	Natural	Structures have to be tailored to the size and shape required for a particular patient
	Non-immunogenic		
	Suitable three dimensional template for tissue growth	Synthetic	Material properties (strength, degradation time, porosity and microstructure) have to be similar to that of native tissue
	Sustain/promote cellular growth		
	Allow cell adhesion, proliferation and differentiation	Biodegradable	Natural matrices related to a limited donor pool.
	Facilitate the delivery of vital cell nutrients and waste products		
	Exert mechanical properties similar to that of native tissue	Permanent	
Factors	Local and/or systemic pharmaceutical intervention to promote autologous progenitor cell mobilization and improve graft integrity	Boosting	Clinical grade
		Recruitment	Possible pro-coagulant effect and hemodynamic changes
			Possible thrombembolic risk
		Commitment	Pro-angiogenesis and indirect tumor promotion

A variety of regenerative approaches have been proposed for airway replacement, ranging from collagen scaffolds supported by silicones stents, cartilaginous tubes created by *in vitro* culture methods or Marlex mesh tube covered by collagen sponge (Teramachi et al. 1997; Kojima et al. 2002; Omori et al. 2005; Kanzaki et al. 2006; Yamashita et al. 2007; Omori et al. 2008); however, none of these strategies resulted adequate for clinical tracheal replacement due to incomplete epithelialisation, with associated stricture, and to a lack of mechanical integrity with consequent tracheomalacic development (Grillo 2002). To provide a biocompatible tracheal substitute with sufficient biological stability, the trachea itself has been hypothesize as the most suitable airway bioprosthesis. This approach relies on the fact that, for tracheal cartilage reconstruction, complex anatomically shaped scaffolds demonstrated to support tissue development better than simple highly modelled designs,

the imperfections in the anatomical construct create local niches for increased cell-cell contact and the thinner (nanometric) fibers allow better chondrocyte attachment (Moroni et al. 2007). A tracheal scaffold, conditioned with basic fibroblast growth factor (β-FGF), has been recently implanted in patients affected by stenosis and, 6 months postoperatively, all patients were able to breathe easily (Kanemaru et al. 2010). Even if this new regenerative therapy showed great potential for the treatment of airway diseases, the procedure involved two-staged operations (to enlarge the stenotic region and to implant tracheal scaffold) and β-FGF could not be applied to oncological patients because of tumor recurrence.

Starting from the success of biological scaffolds, derived from decellularized tissues and organs (Ott et al. 2008; Petersen et al. 2010; Uygun et al. 2010; Song et al. 2011), attention has been driven to the possible use of decellularized tracheal matrix to realize functional tracheal replacement. Using a simple and effective procedure, based on osmotic lysis, detergent cell-extraction and DNA digestion (the detergent-enzymatic method) (Meezan et al. 1975), we were able to obtain bioengineered decellularized human tracheal matrices characterized by preserved tissue matrix integrity and biomechanical properties, of sufficient length for clinical application, containing pro-angiogenic factors and supporting *in vivo* recellularization (Fig. 13.2) (Baiguera et al. 2010; Go et al. 2010). This scaffold (seeded with autologous epithelial respiratory cells and mesenchymal stromal cell-derived chondrocytes *via* a bioreactor) allowed to perform the world's first successful transplant of a bioengineered airway (Macchiarini et al. 2008; Baiguera and Macchiarini 2011). Starting from this clinical promising result, we have improved our tissue engineered tracheal approach focusing on the idea that the use of *in vivo* seeded bone marrow stromal cells (MSCs) and of an adequate stimulation (to directly differentiate stromal cells, to mobilize progenitor cells out of the bone marrow and to recruit them at the desired site of transplantation), could allow to obtain a faster tissue repair and remodeling (Bader and Macchiarini 2010).

13.4.1 **In Vitro** ***Cell Culture or*** **In Vivo** ***Cell Seeding?***

Chondrocytes result essential for the tracheal mechanical properties and the development of a functional cartilage provides the needed support to prevent tracheal collapse. Chondrocyte culture is a well-established procedure, however, de-differentiation of chondrocytes during culture is a limitation (von der Mark et al. 1977), and *in vitro* engineering of cartilage with valuable quality and functional characteristics is still very challenging. Chondrogenesis is promoted by several growth factors, such as transforming growth factor-β (TGF-β), insulin-like growth factors, fibroblast growth factors, platelet derived growth factor-BB, parathyroid hormone related protein, bone morphogenic proteins and Wnt/β-catenin signalling pathway, and a positive effect on chondrogenic differentiation has been demonstrated by combining various growth factors (Tsutsumi et al. 2001; Mastrogiacomo et al. 2001; Bianchi et al. 2003; Barbero et al. 2003; Solchaga et al. 2005; Kafienah

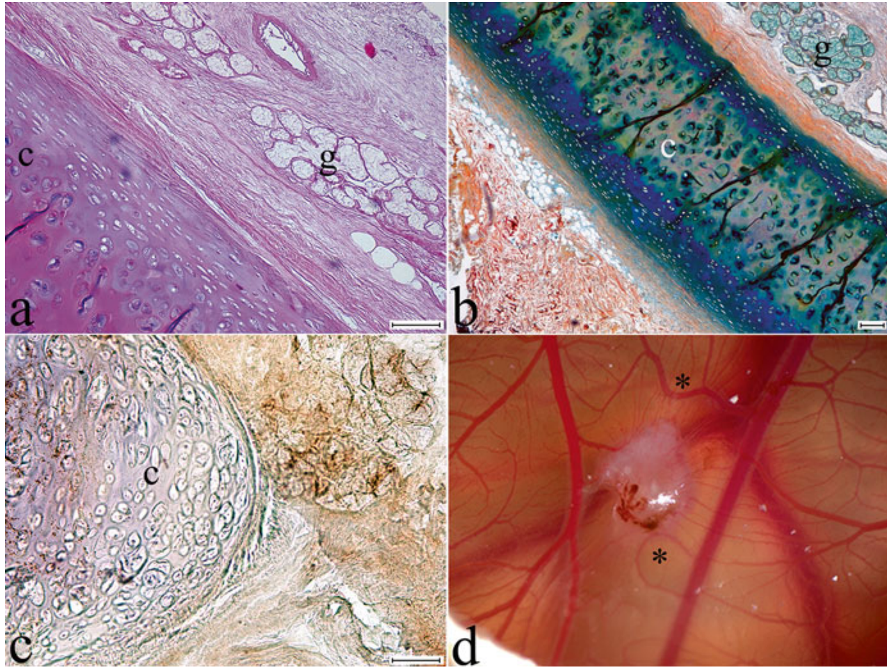

Fig. 13.2 (**a**): H&E stain of decellularized trachea showing the presence of a well preserved and intact extracellular matrix and the absence of cellular elements after 25 detergent-enzymatic cycles. (**b**): Movat pentachromic staining (*connective tissue staining*) of decellularized matrices. *Yellow* indicates collagen and reticulum fibers; *green* to *blue green*, ground substance; and *red*, muscle. (**c**): Immunostaining of decellularized tracheal matrix thin sections showing strong immunoreactivity (*brown staining*) against anti-bFGF, a well-known angiogenic factor. (**d**): Representative example of chicken chorioallantoic membrane (CAM) implanted with fragments of decellularized tracheal matrices. Sample was placed on CAM surface of 8-day-old embryos and photographed 4 days later. The sample resulted totally enveloped by CAM vessels and induced a "*spoke-wheel*" patterns of the new vessels, exerting an influence on vessel network development, as suggested by the looping of vessels toward the matrices (*). '*c*' cartilaginous part, '*g*' seromucinous glands, '*e*' epithelium (Scale bar = 100 μm)

et al. 2006; Kafienah et al. 2007; Augello and De Bari 2010). However, the optimal combination has not been elucidated so far. TGF-β has been used in different experimental studies, it revealed to be one of the most promising factor for chondrogenic formation (Johnstone et al. 1998; Ronzière et al. 2010) and the different members of the TGF-β family produce a wide range of effects in different cells and tissues in the body (Cals et al. 2012). Among them, TGF-β3 is emerging as one of the relatively newer isoforms to be discovered and studied. *In vitro* and *in vivo* researches, evaluating the effect of TGF-β3 on chondrogenic differentiation of stem cells, suggested that the implantation of TGF-β3 with chondrocytes in a suitable scaffold may offer a potential biological therapy for cartilaginous regeneration (Tang et al. 2009).

The *in vitro* use of factors poses great concerns because transplanted cells should be clinical grade and animal by-products free for clinical application. Another concern for *in vitro* cultures is that static and mono-dimensional condition cannot mimic the physiological environment. It has been demonstrated that maintaining differentiated chondrocytes in monolayer culture induces a shift of their biosynthetic profile to a fibroblast-like phenotype (Nehrer et al. 1999), with a consequent *in vivo* formation of fibrocartilage instead of hyaline cartilage (Hedbom et al. 1992). On the contrary, chondrocytes cultured in three-dimensional conditions or in macroaggregates remained vital, functional and with a stable phenotype (Walles et al. 2004b; Wu et al. 2007). The use of an *in vitro* bioreactor or of an *in vivo* subcutaneous pre-conditioning has been also evaluated. However, all these approaches requires time and money, introduces regulatory challenges and are related to bacterial contamination risks.

Recently, it has been suggested that a suitable regenerative strategy could be to avoid any *in vitro* cellular or construct manipulation and to directly implant the construct together with a pharmacological mix of specific growth factors to induce *in vivo* physiological regeneration (Bader and Macchiarini 2010). In this approach, the replacement is designed to take place in the body after implantation and thus is referred to as *in situ* or *in vivo* tissue engineering (Bader and Macchiarini 2010). This concept has been recently used by Nakamura and colleagues (2009), which, seeding intraoperatively a synthetic polypropylene scaffold with autologous bone marrow MSCs, were able to *in vivo* rebuild a trachea-like organ, with a good epithelial lining. The positive results obtained suggested that starting from i*n vivo* seeded bone marrow stromal cells and using the body of the recipient patient as a biological, natural bioreactor, the *in vivo* tissue engineering approach may facilitate tracheal reconstruction.

Based on these idea, we improved our strategy by seeding the decellularized human tracheal graft with a mixture of mononuclear cells, MSCs and patient own blood directly after isolation in the surgery theatre, avoiding therefore any *in vitro* culture step.

13.4.2 Pharmacological Therapy

Direct intraoperative application of cells is an interesting and promising approach, however we suppose that graft survival could be supported by the activation of the endogenous repair system. A proper pharmaceutical intervention, by which mobilize endogenous stem and progenitor cells, could result then essential to promote a successful long-term graft regeneration.

It has been demonstrated that growth factors and cytokines, produced by injured and inflammatory cells, are able to activate, mobilize and attract different types of progenitor cells (Singer and Caplan 2011; Marquez-Curtis et al. 2011), and cytokines, in particular, have been accepted as “standard” agents to mobilize progenitor stromal cells (like hematopoietic) from bone marrow to peripheral blood. To date,

the granulocyte colony-stimulating factor (G-CSF) has become a standard agent in current clinical practice because it has been shown to mobilize significantly more peripheral blood stromal cells and resulted less toxic than other cytokines (Takeyama and Ohto 2004). Moreover, addition of erythropoietin (EPO), a cytokine mainly active on erythropoiesis, to G-CSF has been demonstrated to increase peripheral blood stromal cell mobilization and resulted in significantly higher yields of $CD34^+$ cells (Takeyama and Ohto 2004). The EPO influences in the peripheral circulation is well described and it has been observed in different tissues (Brines and Cerami 2008; Jungebluth et al. 2012). It has been determined that, in the presence of an inflammatory region, local EPO production is repressed by high tumour necrosis factor-α (TNF-α) levels, while the EPO receptor becomes up-regulated by pro-inflammatory cytokines, resulting a potential target region for pharmaceutical intervention (Brines and Cerami 2008; Brines 2010). Moreover, it has been demonstrated that EPO, by binding to β–common receptor (which does not have any contribution to the erythropoiesis but significant impact on tissue protection), induces a tissue protective effect in different tissue types and diseased conditions, particularly in ischemic settings of the lung, liver, heart, chronic skin ulcera or burn, trauma, cytokine-related acute injuries (Brines and Cerami 2008). Based on these data and to counteract apoptotic events within the trachea graft, which occur due to surgery induced injury and to a lack in vascularization within the newly transplanted scaffold, we decided to submit transplanted patients to a regenerative therapy, by giving post-operative injections of G-CSF and EPO in order to increase construct regeneration.

G-CSF is almost an acceptable agent for peripheral blood cell mobilization, but it has obvious toxicities, such as bone pain, fever, reversible elevations of alkaline phosphatase, lactate dehydrogenase, asparate aminotransferase and uric acid and rare case of thrombosis, myocardial infarction, adult respiratory distress syndrome, and splenic rupture (Takeyama and Ohto 2004). Also EPO treatment is related with negative side effects, such as hemodynamic changes and thrombembolic risks. Close careful monitoring of the patient's condition, especially hematocrit, during regenerative therapy administration is then necessary for detecting early signs of complications.

13.5 *In Vivo* Regenerative Tracheal Strategy

The new developed *in vivo* tracheal strategy is based on decellularized human tracheas intraoperatively seeded with autologous bone marrow MSCs, for the external, and respiratory epithelial cells, for the internal, tracheal surface (completely avoiding *in vitro* cell culturing and using the patient's own body as a "living natural" bioreactor), and intraoperatively conditioned with differentiative (TGF-β3, to allow stem cell's differentiation into cartilage-tissue), growing (G-CSF, to promote stem cell mobilization and recruitment to the injured sites), and 'boosting' (EPO, to activate and stimulate stem cell recruitment and wound healing) factors (Table 13.2)

Table 13.2 *In vitro* and *in vivo* bioengineering tracheal procedures

		Procedure		Timing	
		In vitro	***In vivo***	***In vitro***	***In vivo***
Scaffold		Human donor trachea decellularized by DEM	Human donor trachea decellularized by improved DEM	2 months	3 weeks
Cells	External surface	Autologous bone marrow MSCs *in vitro* differentiated into chondrocytes	Autologous bone marrow MSCs	3 months	2 h
	Internal surface	*In vitro* cultured autologous respiratory epithelial cells	Autologous tracheal (or nasal) respiratory epithelial cells	3 months	15 min
Bioreactor		Double-chamber bioreactor	Patient body Growthing and boosting factors		
Construct obtainment		Cells seeded onto the matrix and *in vitro* dynamically cultured	Cellular conditioning, boosting and differentiative factor injection performed immediately before transplant.	96 h	intraoperative

DEM detergent-enzymatic method, *MSCs* mesenchymal stem cells

(Kalatur et al. 2010). After implantation, the therapy is intensified by giving post-operative systemic injections of G-CSF and EPO at 'regenerative' dose levels every other day for 14 days.

This *in vivo* regenerative approach has been so far adopted in 5 patients with benign tracheal diseases and in 2 patients with primary tracheal cancers, involving the entire trachea (abcnews). The cytofluorimetric analysis of peripheral blood cell mobilization showed a steady increase in the number of the hematopoietic progenitor cells ($CD34^{+}$) during the peri-transplantation period, suggesting that peripheral blood (hematopoietic) stromal cell recruitment may play a vital role in the overall success of the replacement airway. To date, the new *in vivo* engineered transplanted tracheas are able to support themselves, have proved to possess a good epithelial coating, immediate vascularization and, upon all, a constantly wide open lumen for air passage.

These early successful results demonstrate that our strategy, based on optimally bioengineered materials combined with autologous cells and pharmacological intervention, to boost progenitor cell recruitment and thereby promoting tissue formation and regeneration *in situ*, could provide a therapeutic option and eventually a better alternative solution for patients with serious clinical tracheal disorders.

Besides these clinical successful outcomes, this approach has some limitations, like a relatively long period for the decellularization process (15–20 days), the risks for altering long-term natural matrix mechanical properties and for bacterial con-

tamination during the *in vitro* natural graft manipulation and, most importantly, the absolute requirement of obtaining a donor organ and the reliance on donor tissues. A suitable approach to obtain a long-term preservation is one of the methods to help solve the limitation related to donor tissue shortage. We have reported that the immunological and mechanical characteristics of the acellular pig matrices remained unaffected by a 2-month storage in phosphate-buffered saline solution (containing 1 % antibiotic and antimycotic, at 4 °C) (Jungebluth et al. 2009), however longer preservation periods have not been evaluated. Very recently, we have demonstrated that after 1-year storage, human decellularized tracheas were characterized by a general damage of the extracellular matrix histoarchitecture, particularly collagenous and elastic fiber structure, resulting in decreased mechanical and angiogenic properties. Considering that the quality of the structural matrix at implantation may predetermine durability or failure of preserved graft, human decellularized tracheas stored for 1 year in phosphate-buffered saline solution at 4 °C, would not meet the demands for a tissue engineering matrix and likely would not yield a suitable graft for lifelong implantation. Moreover, the degradation phenomenon observed *in vitro* may be further enhanced *in vivo*, having clinical relevance for tissues that will be transplanted long-term and this should be carefully evaluated in pre-clinical settings. (Baiguera et al. 2012).

Another solution could be represented by the development of a synthetic construct, that mimics the structural and mechanical tracheal properties. Very recently, based on our experience on human tracheal matrix characteristics (Baiguera et al. 2010) and using a nanocomposite polymeric material, we were able to obtain a tailored-made artificial tracheal scaffold with physical and mechanical properties similar to native tissue. The artificial scaffold, *ex-vivo* seeded with autologous progenitor cells (*via* bioreactor) and conditioned with pharmacology therapy, has been successfully implanted into a patient affected by primary recurrent tracheobronchial tumour (Jungebluth et al. 2011). After 3 postoperative months, the nanocomposite was lined with a well-developed healthy mucosa and no distal ischemic necrosis was observable. Moreover, cellular biochemistry analyses have provided new insight into the mechanisms by which the utilized regenerative therapy contribute to cell mobilization, differentiation and ultrastructural organization of the fully engrafted tracheobronchial construct (Jungebluth et al. 2011). The successful overall clinical outcome of this first-in-man bioengineered artificial tracheobronchial transplantation provides ongoing proof of the viability of this approach, where a cell-seeded synthetic graft is fabricated to patient-specific anatomical requirements and incubated to maturity within the environment of a bioreactor.

13.6 Conclusions

Successful bioengineering airway transplants have been obtained, within the past 3 years, thanks to a thorough interdisciplinary work. Patients transplanted can now breathe normally without any immunosuppression, and the application of the *in vivo*

tissue engineering technique in cancer patients, although requiring further follow-up, is very promising and may be curative. These early successful results demonstrate that the *in vivo* tissue engineered strategy, based on optimally bioengineered materials combined with autologous cells and pharmacological intervention, to boost progenitor cells, could provide a therapeutic option and eventually a cure for patients with serious clinical airway disorders. However, important questions, such as the evaluation of the long-term biomechanical properties of the bioengineered grafts, the optimization of tracheal synthetic graft and of bioreactor design, function and fate of the seeded cells in tissue regeneration and mechanisms of angiogenesis, remain to be answered before a full clinical trial accreditation may be obtained.

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Chapter 14
Stem Cell Therapy for Neonatal Lung Diseases

Maria Pierro, E. Ciarmoli, and B. Thébaud

Abstract Despite advances in perinatal care, neonatal lung diseases characterized by disrupted alveolar and vascular development, such as bronchopulmonary dysplasia and congenital diaphragmatic hernia, remain a therapeutic challenge and economic burden with long-term consequences that may affect a lifetime. Stem cell therapies appear promising for yet untreatable diseases. Several studies have already proven stem cell efficacy in a variety of experimental settings, including neonatal lung diseases. Among stem cells, mesenchymal stem cells and endothelial progenitors cells may offer new hope to the neonatal population. These two cell types share a similar mechanism of action, including recruitment to site of injury and subsequent paracrine secretion of bioactive molecule and microvesicles. The evidence of a paracrine mechanism as the crucial effector of stem cells is directing research towards cell-free products, that may further change the face of regenerative medicine. Current clinical translation of regenerative medicine is mainly involving mesenchymal stem cells, thanks to their unique properties and extensive preclinical evidence. Given the essential role of vasculogenesis in lung development, further insight into endothelial progenitor cells may prove them as an important therapeutic option in neonatal lung diseases. Eventually co-transplantation of mesenchymal stem cells and endothelial progenitors cells may increase their therapeutic potential.

Keywords Bronchopulmonary dysplasia • Congenital diaphragmatic hernia • Mesenchymal stem cells • Newborn • Lung injury

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The revolutionary progress of perinatal care during the past few decades has led to a remarkable reduction of neonatal mortality (Gregory et al. 2014; MacDorman et al. 2012). However, this progress has created new challenges. Bronchopulmonary dysplasia (BPD), the chronic lung disease of prematurity, now occurs in extreme premature infants and consequently disrupts normal lung growth at an early developmental stage. Likewise, infants with congenital diaphragmatic hernia (CDH), a defect of the diaphragm resulting in lung hypoplasia, develop severe pulmonary hypertension as a consequence of severe underdevelopment of the pulmonary vascular bed. Given their peculiar pathogenesis, these diseases may benefit from regenerative medicine.

14.1 Stages of Lung Development

Lung development is typically divided into five stages: embryonic, pseudoglandular, canalicular, saccular, and alveolar (Fig. 14.1). During the *embryonic phase* (third-seventh week of gestation), the human lung originates from the primitive foregut as a ventral endodermal bud, which will divide into the two lung buds (primary bronchi) and then into the secondary bronchi, further branching into the major airways. Histologically, these structures are lined with undifferentiated columnar epithelium. The *pseudoglandular stage* begins at the end of the fifth week of fetal life, with the branching of all conductive airways down to the terminal bronchi. The epithelium of the conducting airways starts to differentiate into non-ciliated columnar Clara cells, pulmonary neuroendocrine cells, and ciliated cells. The distal cuboidal epithelium differentiate into type II epithelial cells. Between 16 and 26 weeks the *canalicular phase* takes place, leading to the formation of the respiratory bronchioles and the pulmonary acinus. During this time, the capillary bed of the distal lung remarkably increases. Histologically, the epithelium of the distal lung begins to differentiate from cuboid type II cells into squamous type I cells. During the *saccular* stage (26–36 weeks), the distal epithelium further mature: type 2 cells start producing surfactant, while type 1 cells form the thin layer, needed for future gas exchange. The interstitial mesenchyme becomes thinner. Saccules and ducts, the characteristic elements of this stage, consist of thick primary septae, containing a double pulmonary capillary layer. In the proximal epithelium ciliated, non-ciliated Clara, basal and neuroendocrine cells increase in number. The larger vessels of the pulmonary vasculature start to muscularize.

Starting from 36 weeks up to the first few years of life, the process of *alveolarization* and secondary septation ensure the formation of mature and well-organized alveoli. Equally important, the microvascular maturation takes place and the capillary bilayer merge into the single-layer vascular network, creating the efficient air-blood gas-exchange unit (reviewed in Wert 2011). Much more needs to be learned about normal lung development and how these mechanisms are impaired during disease in order to discover novel treatment options. Nonetheless, current knowledge suggest various cell-based therapies a promising interventions for neonatal lung disease such as bronchopulmonary dysplasia and congenital diaphragmatic hernia.

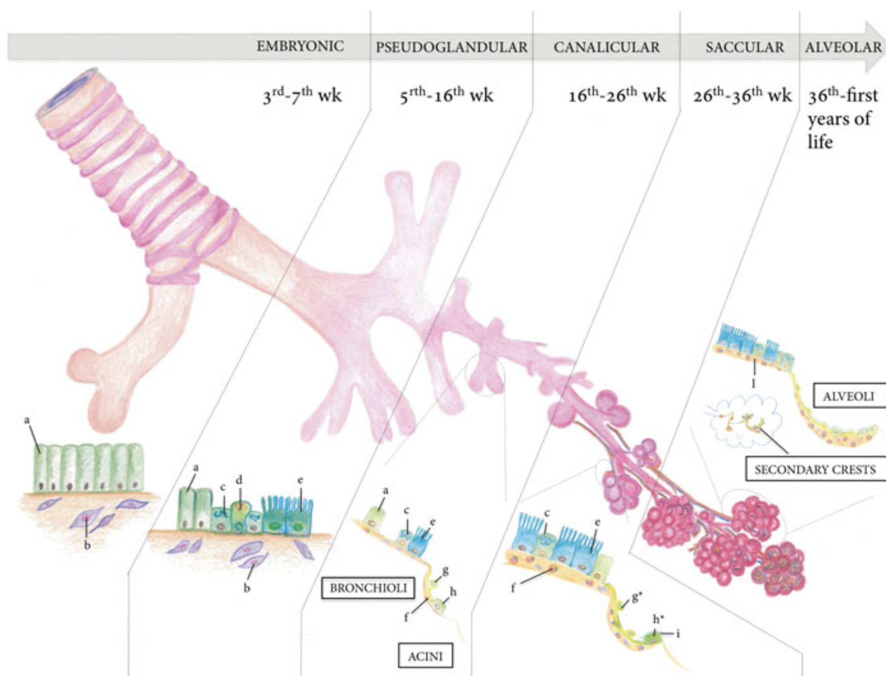

Fig. 14.1 Stages of lung development. *Embryonic phase (3rd–7th weeks of gestation)*: formation of the two lung buds (primary bronchi) further branching into the secondary bronchi and into the major airways. Histologically, these structures are lined with undifferentiated columnar epithelium (*a*). Mesenchymal cells (*b*) are crucial for epithelial-mesenchymal "cross-talk" cell interactions that regulate branching morphogenesis and cellular differentiation. *Pseudoglandular stage* (*5–16th weeks of gestation)*: formation of the conductive airways. The epithelium starts to differentiate into non-ciliated columnar Clara cells (*c*), pulmonary neuroendocrine cells (*d*), and ciliated cells (*e*). *Canalicular phase (16–26th weeks of gestation)*: formation of the respiratory bronchioles and the pulmonary acinus. Histologically, the epithelium of the distal lung begins to differentiate from cuboid type II cells (**g**) into squamous type I cells (*h*). *Saccular stage* (*26–36th weeks of gestation*): Maturation of the distal epithelium. Mature type 2 cells (*g**) start producing surfactant (*i*), while type 1 cells form the thin layer. In the proximal epithelium ciliated (*e*), non-ciliated Clara (*c*), and neuroendocrine cells (*d*) increase in number. *Alveolar stage (36 weeks of gestation-first few years of life)*: *alveolarization* and formation of the secondary crests. The microvascular maturation takes place and the capillary bilayer (*f*) merge into the single-layer vascular network (*f**)

14.2 Neonatal Lung Diseases

14.2.1 Bronchopulmonary Dysplasia (BPD)

14.2.1.1 Incidence and Definition

BPD is a form of chronic lung disease, peculiar to the premature infants born before the lungs are fully mature. The first report of BPD dates back to the pre-surfactant era, when Northway described a form of chronic lung disease (Northway et al. 1967),

defined as oxygen need for at least 28 days in conjunction with specific radiographic changes, in late preterm infants surviving from respiratory distress syndrome at birth (Report of Workshop on Bronchopulmonary Dysplasia 1979). Subsequently, oxygen dependency at 36 weeks post-menstrual age (PMA) was shown to better predict long-term respiratory outcomes (Shennan et al. 1988). Roughly 10 years later, in the NICHD Workshop Summary, infants below 32 weeks requiring supplemental oxygen for at least 28 days were stratified into three severity groups (mild, moderate and severe BPD), depending on the presence and the amount of supplemental oxygen and mode of respiratory support at 36 weeks PMA (Jobe and Bancalari 2001). The 'physiologic' definition of BPD was proposed in the attempt to compensate for the significant inter-center variability in oxygen administration (Vermont Oxford Network 2012). At 36 weeks PMA infants receiving less than 30 % of supplemental oxygen and no respiratory support are challenged by reducing the fraction of administered oxygen during a standardized test. Infants who are unable to maintain saturations above 90 % during the test are diagnosed with BPD (Walsh et al. 2003).

Although, by definition, BPD cannot be diagnosed before 28 days of life, the respiratory disease characterized by oxygen and/or ventilator-dependency from 7 to 28 days of life, represents the initial phase of the chronic process leading to BPD, thus classified as "evolving BPD" (Walsh et al. 2006).

While some data suggest a trend towards a reduction of BPD rates, most studies report a slight increase over the past two to three decades. This is likely linked to the higher survival of more immature infants at higher risk for BPD (Stoll et al. 2010). Incidence of BPD varies depending on the definition. The NICHD Neonatal Research Network has compared the BPD rates in extremely preterm infants (22–28 weeks gestation) according to the three definitions currently in use. The NICHD classification is associated with the highest rates (68 %) due to the inclusion of infants requiring oxygen for 28 days, although breathing room air at 36 weeks PMA. Oxygen need at 36 weeks PMA and the physiological definition obtained similar results (42 and 40 % respectively) (Stoll et al. 2010). It is still controversial which definition better predicts the long-term outcomes and if grading system actually improves its positive predictive value (Ehrenkranz et al. 2005; Landry et al. 2011; Lefkowitz and Rosenberg 2008).

14.2.1.2 Pathogenesis of BPD

Arrest of Alveolar and Vascular Development

The form of chronic lung disease described by Northway, now referred to as "old BPD", was mainly a consequence of the aggressive ventilatory approach on a relatively mature lung, although deficient in surfactant. Histologically, "old BPD" was characterized by a diffuse injury, with significant inflammation and parenchymal fibrosis, despite numerically and structurally normal, mature and complex alveoli. Thanks to the improvement of neonatal care, in particular to the introduction of antenatal steroids in order to induce lung maturation, the discovery of exogenous surfactant and the

use of "gentle" ventilatory techniques, nowadays infants born after the canalicular stage of lung development exceptionally suffer from chronic lung disease. At the same time, the increased survival of extremely premature infants has led to the appearance of a new form of chronic lung disease ("new BPD"), typical of the infants born at the early stages of lung development (22–28 weeks of gestation) (Jobe and Bancalari 2001). The "new BPD" is the expression of the lung immaturity, rather than the iatrogenic damage (Fig. 14.2). Histologically, the BPD lungs are characterized by less numerous, enlarged, and simplified alveoli, typical of the early canalicular stage, while fibrosis and inflammation are less represented. (Coalson 2003). BPD is also characterized by an abnormal distribution of pulmonary vessels and a significant reduction in the number of small arteries, which are functionally hyperreactive and hypertonic, culminating in pulmonary arterial hypertension and right ventricular hypertrophy (Rossor and Greenough 2015). Stem cells, thanks to their unique possibility of restoring and regenerating damaged tissue, may structurally revert the alveolar and vascular architectural changes that make traditional therapies unsuccessful in treating BPD.

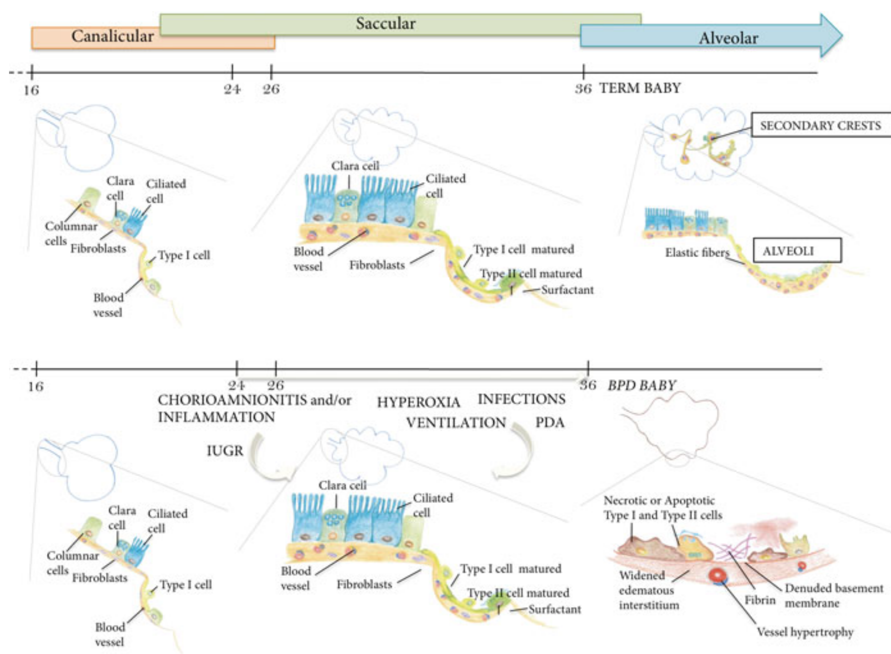

Fig. 14.2 Events leading to the development of BPD. The "new BPD", typical of the infants born at the early stages of lung development (22–28 weeks of gestation), is the expression of the lung immaturity. Prenatal and postnatal inflammatory stimuli, such as corioamnionitis, ventilator induced lung injury, oxygen toxicity and neonatal sepsis contribute to the development of BPD. Histologically, the BPD lungs are characterized by less numerous, enlarged, and simplified alveoli, with no secondary crests, typical of the early canalicular stage. BPD is also characterized by an abnormal distribution of pulmonary vessels and a significant reduction in the number of small arteries

Contributory Factors

Prenatal and postnatal inflammatory stimuli, such as corioamnionitis, ventilator induced lung injury, oxygen toxicity and neonatal sepsis, can trigger an inflammatory cascade leading to cell necrosis and repair (Speer 2006) (Fig. 14.2). In premature infants that will develop BPD, inflammation is documented by higher levels of pro-inflammatory cytokines and lower levels of anti-inflammatory cytokines (Thompson and Bhandari 2008). Moreover, the imbalance between anti-oxidant defense mechanisms and increased exposure to oxygen reactive species (Jankov et al. 2001) exacerbates cell apoptosis and disruption of the extracellular matrix, leading to tissue remodeling (Saugstad 2003). These events contribute to the development of BPD, by further perturbing the developing lung and intensifying the alveolar and vascular dysregulation, primarily caused by the premature birth (Jobe and Bancalari 2001). The well-documented anti-inflammatory, anti-oxidant and anti-fibrotic potential of stem cells would further target the pathological events contributing to the development of BPD.

14.2.1.3 Outcomes of BPD

Premature infants, with or without BPD, continue to show worse respiratory performances compared to their peers up to late childhood (Hennessy et al. 2008). Among premature infants, BPD increases the risk for re-hospitalization during the first 2 years of life, due to acute respiratory distress and respiratory tract illness (Ralser et al. 2012). Later in childhood, ex-preterm infants affected by BPD need more often respiratory medications and present more frequently with respiratory symptoms (Hennessy et al. 2008). Respiratory symptoms have been objectified by signs of airway obstruction at lung function tests (Landry et al. 2011). Airway obstruction in these patients seems to be caused by structural changes rather than airway hyperreactivity (Baraldi et al. 2005). Response to asthma medication is less pronounced and levels of exhaled nitric oxide are lower in symptomatic BPD patients as compared to asthmatic children born at term with similar grade of airway obstruction. (Baraldi et al. 2005). In agreement with these results, structural BPD findings have been documented at the autopsy of a child diagnosed with BDP, died from asthma attack at 12 years of life (Cutz and Chiasson 2008).

BPD is also associated with worse long term developmental outcomes, including higher rates of cerebral palsy at 18–22 months' corrected age and psychomotor delay, attentional impairments, speech and language disorders, executive deficits and behavioural problems at school age (Doyle and Anderson 2009)

14.2.1.4 Current Treatment of BPD

The irreversible arrest of lung development make traditional therapies ineffective in treating this disease. “Gentle” ventilatory techniques starting from the delivery room and optimal timing for surfactant administration seem to be partially effective in preventing BPD. However, in terms of medications, most of the therapies are either ineffective, inconsistently evaluated or unsafe. A recent meta-analysis (Beam et al. 2014) analyzed all the therapies available to prevent or treat BPD. Among the 21 drugs, 16 showed no efficacy. Out of the 5 effective drugs, only three (vitamin A, caffeine and dexamethasone) have been assessed in large or multi-centric randomized controlled trials (RCTs). However, none of them is currently recommended for prevention or treatment of BPD. The need for a long course of intramuscular injections of vitamin A may not be balanced by the modest reduction of BPD rates (Darlow and Graham 2011). Treatment with caffeine was associated with a significant reduction in the incidence of BPD in one large RCT that enrolled more than 2000 patients (Schmidt et al. 2006). However, BPD was only a secondary measure and the results need to be evaluated in further trials. Early dexamethasone is the only drug that has been repeatedly shown to significantly reduce the incidence of BPD. However, the higher risk of cerebral palsy at 18 months of life after this treatment (especially if administered during the first week of life) have greatly limited its use. On the other hand, patients at higher risk for BPD (ie ventilator dependent after 2 weeks of life) seem to benefit from a short course of low-dose dexamethasone (Doyle et al. 2014). In case they survive, these patients will be almost certainly diagnosed with BPD. Dexamethasone, by reducing the incidence of BPD, reduces its associated neurological complications as well, improving the overall outcome (Watterberg 2012). However, dexamethasone use should be cautious and restricted to severe cases, with ventilator dependency after the first 2–3 weeks of life.

In summary, no effective and safe treatment has yet been developed for BPD.

14.2.2 Congenital Diaphragmatic Hernia (CDH)

14.2.2.1 Incidence and Definition

Congenital diaphragmatic hernia (CDH) is a defect of the diaphragm allowing the abdominal content to ascend into the thorax. CDH has an overall prevalence rate of 3.5 per 10.000, of which 69 % result in live births (Wright et al. 2011). CDH can be classified based on the anatomical position of the defect as posterolateral (Bochdalek hernia), anterior (Morgagni hernia) and central. Bochdalek hernia occurs in 70–75 % of the cases, usually (85 % of the times) on the left side, Morgagni hernia in 23–28 % and central defects in 2–7 % of the cases. (Veenma et al. 2012). CDH patients present with associated congenital abnormalities (mostly pulmonary and cardiovascular) and/or genetic syndromes in approximately 40 % of the cases. However, no specific gene defect has yet been identified (Pober 2008).

Survival of isolated CDH at 1 year can be as high as 77 % of affected live births. However, when considering all the prenatal diagnosis of CDH (including pregnancy termination, miscarriages and patients born with associated anomalies) survival to 1 year decreases to 42 % (Wright et al. 2011).

14.2.2.2 Pathogenesis of CDH

Lung Hypoplasia and Arrested Pulmonary Vascular Development

The failure of the diaphragm closure and the disruption of lung and vascular development are the major features of CDH. Both are supposed to happen independently at the beginning of the pseudoglandular stage of lung development, when the chest and abdomen are not yet separated (Guilbert et al. 2000). The events altering the normal branching of the airways and the formation of the vascular networks, normally happening during this stage, lead to the characteristic findings of CDH (Kotecha 2000). Lung hypoplasia and disrupted diaphragm formation have been linked to a perturbation of the retinoid signaling pathway, which is known to play an important role in normal lung development (Thébaud et al. 1999). In a later phase, the mechanical compression of the abdominal organs on the ispilateral lung to the diaphragm defect worsen the picture. (dual hit hypothesis) (Keijzer et al. 2000). Histologically, the lungs are characterized by fewer alveoli and reduced gas-exchange surface area, markedly thickened alveolar walls and increased interstitial tissue. These findings are bilateral, although more pronounced on the side of the defect (George et al. 1987).

At the same time disruption of the pulmonary vascular bed occurs. Structural changes of the lung vasculature, with reduced size of the pulmonary vascular bed have been documented in CDH patients. The pulmonary arteries are hypertrophied (O'Toole et al. 1996) and composed of more abundant contractile vascular smooth muscle cells, disposed more distally than normal; the media and adventitia are thickened (Sluiter et al. 2013). These findings of underdevelopment and hypertrophy of the vascular bed determine the "irreversible" component of pulmonary hypertension, highlighting the importance of promoting pulmonary vascular growth in CDH, a possibility that may be provided by cell-based therapies.

14.2.2.3 Pathophysiology: Altered Vasoreactivity

A "reversible" hyperresponsiveness of the pulmonary vasculature worsen the "fixed" pulmonary hypertension (Shinkai et al. 2005). The contribution of this increased "reversible" vasoreactivity is variable. Dysregulation of vascular reactivity mediators, such as Endothelin-1, a potent vasoconstrictor synthesized by endothelial cells or nitric oxide, an effective endogenous vasodilator, seem to be involved in the pathogenesis of the reversible component of pulmonary hypertension in CDH (Pierro and Thébaud 2014). In clinical practice, depending on the degree of vascular

underdevelopment, different and sometimes absent responses to vasodilator therapies are seen, highlighting the need for a structural rather than functional reversal of the pulmonary hypertension of CDH.

14.2.3 Outcomes of CDH

At follow-up, recurrent respiratory infection, often needing hospital readmission, are reported as the most common complication during the first 5 years of life (Rocha et al. 2012). Long-term pulmonary morbidity in CDH persist into childhood and sometimes into adulthood. (Kamata et al. 2005; Spoel et al. 2013). Persistent pulmonary hypertension complicate the course of the disease and greatly determines pre and post-discharge mortality (Wynn et al. 2013; Zussman et al. 2012). Moreover, CDH survivors, independently from the treatment with extracorporeal membrane oxygenation (ECMO), needed by the most severe cases, have been found to have a higher incidence of motor and language problems compared to their pares (Bouman et al. 2000). At school age learning disabilities, attention deficit hyperactivity disorder, or developmental disability are also more frequent (Frisk et al. 2011).

14.2.4 Current Therapies for CDH

No postnatal therapies to treat pulmonary hypoplasia and vascular disruption of CDH are available. Techniques of gentle ventilation and stabilization of the hemodynamics to minimize the right to left shunting due to pulmonary hypertension have improved the outcomes (Guidry et al. 2012; Acker et al. 2014). In particular, permissive hypercapnia has decreased ECMO need and mortality (Guidry et al. 2012), although, with the improved survival of most severe cases, a new pattern of chronic and refractory pulmonary hypertension has emerged. Most of the pharmacological therapies aim to correct the reversible component pulmonary hypertension. However, results are inconsistent, due to the variable degree of structural pulmonary hypertension. In case oxygenation is not achieved with pharmacological therapies, ECMO may represent an important tool in the treatment of intractable cardiorespiratory failure (Harrington and Goldman 2005). However, in the most compromised cases, survival is unlikely unless pulmonary hypoplasia and underdevelopment of pulmonary vascular bed are structurally reversed. In this regard, the prenatal fetoscopic endoluminal tracheal occlusion (FETO) has sparked progressively more interest in the last few years. The technique consists of a fetal tracheal occlusion through the positioning of a balloon during fetoscopy (Deprest and De Coppi 2012). As it happens during physiological periods of fetal apnea, fetal lung liquid, produced by the alveolar cells, accumulate within the airways, increasing the transpulmonary pressure gradient, which is the most potent stimulus for fetal lung growth and lung cell proliferation (Deprest and De Coppi 2012). Limitation of this

technique include the invasive procedure to the mother and the fetus, the operator learning curve to perform the procedure associated with higher rates of complications (i.e. premature delivery, fetal and maternal infections with longer procedure time), the variable lung growth following the procedure, the difficulties in finding the optimal prenatal severity marker and the higher risk for a complicated delivery (ie increased need for ex-utero intrapartum technique) (Jani et al. 2009). An easily reproducible, safe and effective therapy, to permanently revert lung and vascular disruption is urgently needed.

14.3 Regenerative Medicine for Neonatal Lung Diseases

14.3.1 Mesenchymal Stem/Stromal Cells (MSCS)

Among stem cells, mesenchymal stromal cells (MSCs) have been extensively investigated, thanks to their peculiar properties that make them particularly interesting for clinical practice. MSCs are easy to isolate and display multilineage potential and compelling immunomodulatory properties (Dominici et al. 2006). MSCs represent a broad and heterogeneous cell population, defined by three minimum criteria: (i) adhesion to plastic when cultured in a tissue culture flask under standard culturing conditions (ii) expression of specific surface markers (CD73, CD90, CD105) and lack of expression of hematopoietic markers [CD45 (leukocytes), CD34 (hematopoietic progenitors), CD14 or CD11b (monocytes/macrophages), CD19 or CD79a (B-cells), or HLA-DR (human leukocyte antigen DR major histocompatibility complex type II], (iii) ability to differentiate into mesodermic (osteogenic, chondrogenic, and adipogenic) lineages upon in vitro stimulation (Dominici et al. 2006).

14.3.1.1 Origin of MSCs

The prevailing hypothesis, is that MSCs originate from perivascular precursors, named pericytes (Crisan et al. 2008). Long-term cultured perivascular cells from a variety of tissues start expressing typical markers of MSCs and each individual long-term cultured pericyte becomes able to differentiate into osteocytes, chondrocytes, and adipocytes (Crisan et al. 2008). Pericytes surround the blood vessels throughout the body and protrude into the endothelial lumen to sense chemotactic signals and allow prompt and efficient MSC recruitment and response to injury (Murphy et al. 2013).

14.3.1.2 Source of MSCs

Bone marrow is the first described and best known source of MSCs. However, bone marrow as a source of MSCs demonstrate some limitation due to the aging of the cells with the donor aging (Mueller and Glowacki 2001), the paucity of the MSCs

among the other cells of the bone marrow (Castro-Malaspina et al. 1980) and the painful and invasive procedure needed to obtain the cells (iliac crest bone marrow aspirate).

Adipose tissue is an emerging source of MSCs. It offers a greater number of cells and it is more accessible than the bone marrow, although aging of the cells with donor aging remains an issue (Dmitrieva et al. 2012)

Recently, perinatal tissues have emerged as a promising source of MSCs. The use of perinatal stem cells would be particularly suitable and practical in treating neonatal diseases. A significant advantage of perinatal tissues is their availability without invasive and painful harvesting procedures (Batsali et al. 2013). Being discarded at birth, most of these tissues and fluids are entirely free from ethical concerns. Furthermore, MSCs from perinatal tissues, as compared to adult MSCs, display superior cell biological properties, such as stronger immunomodulatory and immunosuppressive potential (Li et al. 2014), improved proliferative capacity, life span (Jin et al. 2013) and stemness (Hsieh et al. 2010), higher trophic (Hsieh et al. 2013) and anti-inflammatory (Jin et al. 2013) activity as well as improved cardiac function after myocardial infarct (Yannarelli et al. 2013). Perinatal tissues are divided into extra-embryonic tissues (chorion, amniotic membranes, and umbilical cord) and placental fluids (amniotic fluid and umbilical cord blood); MSCs have been isolated from any of them, with some differences between one another. Amniotic membrane-derived MSCs cells show a pronounced inter-donor variability (Wegmeyer et al. 2013). Chorion-derived MSCs may be contaminated with maternal cells, which present lower proliferative potential as compared to cord MSCs from the same donor (Zhu et al. 2013). Amniotic fluid is an interesting source of MSCs, although isolation and characterization protocols need further investigation (Zhou et al. 2014). MSCs from the umbilical cord blood and umbilical tissue display high regenerative and immunosuppresive potential. However, cell presence in the cord blood is extremely rare (Batsali et al. 2013). To date, among the different perinatal sources, the most practical and effective one is the cord tissue (in particular the Wharton's Jelly), disposing of robust and reproducible techniques for harvesting and expansion (Batsali et al. 2013).

14.3.1.3 Lung Resident MSCs (L-MSCs) are Involved in Lung Development and Lung Homeostasis

MSCs can be isolated virtually from any tissue (da Silva et al. 2006). Although most of harvesting sites are not clinically relevant due the difficult access, the understanding of the tissue-specific MSC role in organ homeostasis and disease pathogenesis, may optimize the impact of novel therapies. MSCs from any tissue share the three minimum criteria needed to be defined MSCs. In addition, tissue-specific MSCs, including lung resident MSCs, also display distinct functional characteristics to support their specific microenvironment (in 't Anker et al. 2003). In particular, L-MSCs display higher gene expression of lung-specific extracellular matrix proteins, growth

factors, and chemokines (Hoffman et al. 2011), promote proliferation of epithelial progenitor cells (Ingenito et al. 2012) and differentiate into epithelial cells *in vitro* (Gong et al. 2014). Moreover, lung mesenchyme actively guide branching of the airways and alveolar maturation during lung development, thanks to the "cross-talk" interactions with the epithelial cells (McCulley et al. 2015). Interstitial myofibroblasts during the pseudoglandular stage and secondary crests myofibroblasts during the alveolar stage, both derived from mesenchymal precursors, have a key, yet elusive, role in lung development (Boström et al. 1996; Li et al. 2015). Interestingly, MSCs from the tracheal aspirate of preterm infants undergo myofibroblastic differentiation upon TGF-beta1 stimulation, as opposed to bone marrow derived MSCs (Popova et al. 2010a). L-MSCs can be isolated from fetal and adult animal (Summer et al. 2007; Ingenito et al. 2012), and human lung (Lama et al. 2007; Hua et al. 2009). The presence of donor MSCs years after a sex-mismatched lung transplant in the recipients further confirms the lung origin of L-MSCs that reside and self-renew in the adult lung (Lama et al. 2007). The existence of lung specific MSCs and the evidence of their contribution to normal lung development have provided rational support to the documented MSC therapeutic efficacy in experimental diseases, corroborating the great potential of exogenous MSC administration and/or endogenous MSC restoration in the treatment of neonatal lung disorders.

14.3.1.4 Mechanism of Action of MSCs

Homing to Site of Injury and Regeneration

Chemotactic cytokines and signaling proteins, released by different types of injury, recruit resident and remote host MSCs in a process called "homing" (Ren et al. 2012). MSCs migrate into the local or systemic circulation through the endothelium upon interaction of their membrane receptor with chemokines (Ma et al. 2014). Once recruited into the damaged area, MSCs exert their therapeutic function (Fig. 14.3). Although MSCs have been proven to differentiate *in vitro* along different lineages, including lung epithelial cells and alveolar type 2 cells (Krause et al. 2001; Sueblinvong et al. 2008), this property has not been convincingly confirmed *in vivo*. Moreover, independently from the ability to generate various differentiated cell lineages, it has been repeatedly shown that only few exogenously-administered cells engraft and differentiate into the damaged organs in neonatal (van Haaften et al. 2009; Pierro et al. 2013) or adult lung injury models (Chang et al. 2005), despite a significant beneficial effect, suggesting that MSC therapeutic benefits cannot be ascribed to cell replacement.

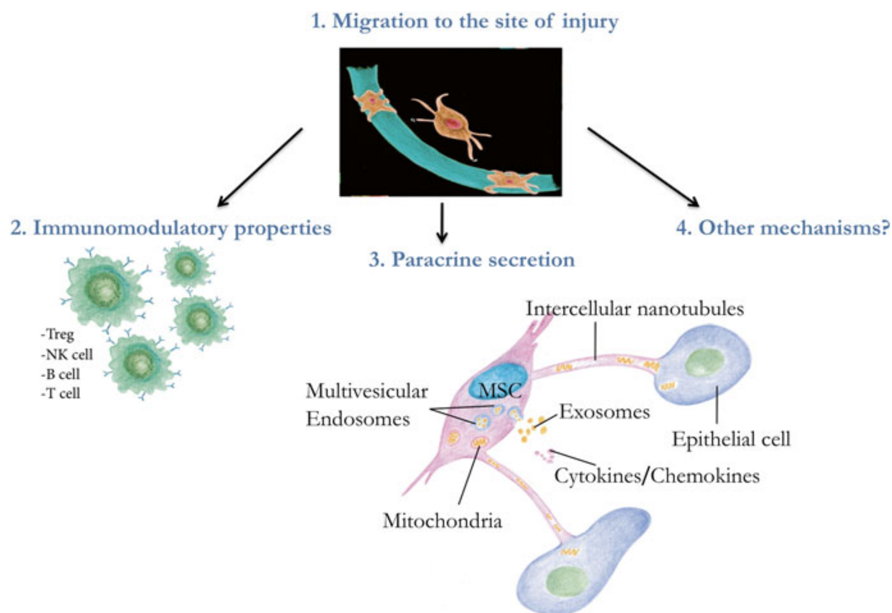

Fig. 14.3 MSC mechanism of action. Activated MSCs, derived from pericytes, which surround the blood vessels, are recruited to the site of injury. Once homed to the damaged organ, MSCs can exert their therapeutic benefit through the interaction with immune cells, including T cells, natural killer cells, B cells, monocytes, macrophages and dendritic cells by cell-to-cell contact (*2*) and the paracrine secretion of anti-inflammatory, antimicrobial, anti-apoptotic and anti-scarring molecules and growth factors and other chemokines to induce cell proliferation and angiogenesis. MSC may "reprogram" the injured tissue by delivering nanoparticles in the form of extracellular vesicles (exosomes), subsequently incorporated by other cells and also through mitochondrial transfer from MSCs to resident lung cells via nanotubules (*3*). Other uninvestigated mechanisms may be involved in MSC mechanism of action (*4*)

Paracrine Effect

The discrepancy between the impressive preclinical results and the low rate of engraftment has introduced the hypothesis that the few engrafted cells may secrete healing factors in a paracrine fashion to boost the local response to injury (Murphy et al. 2013; Fung and Thébaud 2014) (Fig. 14.3). Growth factors and cytokines to induce cell proliferation and angiogenesis, as well as anti-apoptotic, anti-oxidant, anti-fibrotic, anti-inflammatory and anti-microbial factors have been detected in the MSC media (Lee et al. 2011) and likely explain the pleiotropic effect of these cells, making them particularly appealing for the treatment of a multifactorial disease such as BPD. Recently, a new intriguing theory has been suggested. MSC may

“reprogram” the injured tissue by delivering nanoparticles in the form of extracellular vesicles, subsequently incorporated by other cells (Fig. 14.3). These nanoparticles, also named exosomes, are membrane vesicles, formed through the fusion of the endosomes with the plasma membrane, and can be distinguished by their size (range in size from 30 to 100 nM) (Chaput and Thery 2011). Exosomes are secreted by several cell types, including stem cells and are involved in cell-to-cell communication. They can be considered as nano-packages of bioactive molecules and non-coding microRNA (non-coding RNA involved in transcriptional regulation of gene expression) that can been transferred from one cell to another and could mediate tissue repair and remodelling (Kourembanas 2015). Exosomes can be isolated from cultured cells and delivered in vivo (Muller et al. 2014).

Mitochondrial transfer from MSCs to resident lung cells via nanotubes seems to be another mechanism of action of MSCs (Islam et al. 2012) (Fig. 14.3).

Immunomodulatory Properties

The interaction with the immune system is a striking component of MSC function and contributes to their clinical outstanding appeal (Fig. 14.3). Undifferentiated MSCs express low levels of human leukocyte antigen (HLA) Class I and low levels of HLA Class II, enabling MSCs to avoid recognition by the immune system (Le Blanc et al. 2003). MSCs also exert immunomodulatory effects by direct cell-to-cell contact, preventing proliferation and function of many inflammatory immune cells, including T cells, natural killer cells, B cells, monocytes, macrophages and dendritic cells (Gebler et al. 2012). Although, immune rejection cannot be entirely ruled out (Huang et al. 2010b), MSC are the good candidates for allogeneic therapies, thanks to their immunomodulatory potential.

14.3.2 Endothelial Progenitor Cells (EPCs)

EPCs are the vascular progenitors designated to generate blood vessels *de novo* during embryogenesis, in the process of *vasculogenesis*. The formation of new vessels for tissue maintenance and repair after fetal life was believed to happen exclusively as an extension from existing capillaries (*angiogenesis*), driven by mature endothelial cells. However, the possible existence of *vasculogenesis* throughout postnatal life was proven nearly 20 year ago, when circulating EPCs were identified in adult peripheral blood for the first time (Asahara et al. 1997). Since then, EPCs have been under intensive investigation. However, their definition and characterization remains controversial. Flow cytometry fails to select EPCs, since no single marker or combination of markers is specific to EPCs, being shared by hematopoietic cells and mature endothelial cells (Case et al. 2007). Selection of adherent cells after culturing peripheral blood mononuclear cells with endothelial growth factors for 4 to 7 days (Vasa et al. 2001) collects a mixed population, composed of activated

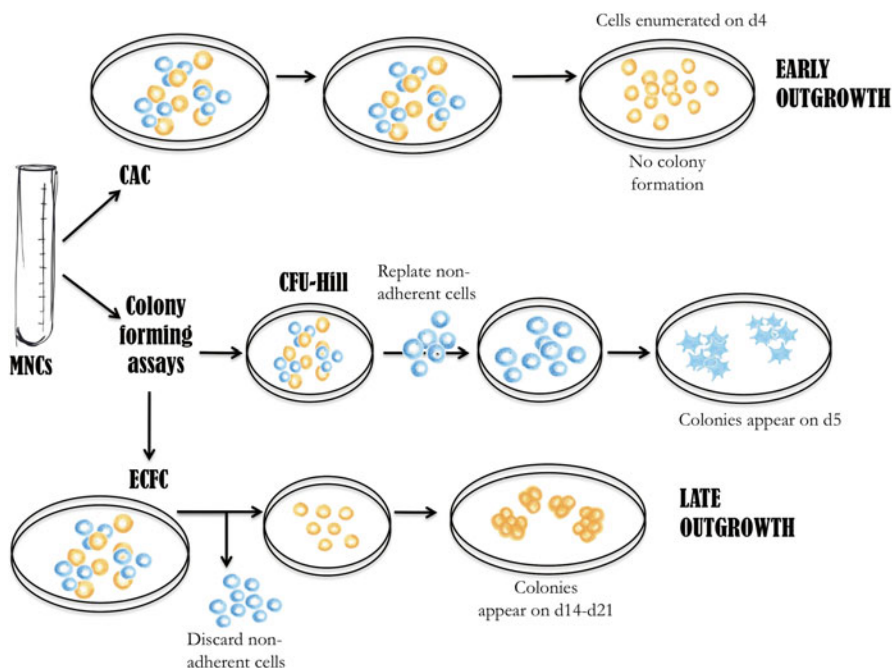

Fig. 14.4 Methods to obtain EPCs. Enumeration of adherent cells after culturing peripheral blood mononuclear cells (MNCs) with endothelial growth factors for 4 to 7 days obtains circulating angiogenic cells (CACs). After replating non-adherent MNCs, early-outgrowth EPCs can be obtained within 7 days of culture based on *in vitro* colony forming cell assays (colony forming unit-Hill, CFU-Hill). CACs are categorized within early-outgrowth EPCs, because of their similar mechanism of action. Endothelial colony forming cells (ECFCs), known as late-outgrowth colonies, are obtained through colony forming cell assay between 14 and 21 days of culture

macrophages with enhanced wound healing and angiogenic activities, named circulating angiogenic cell (CACs) (Fig. 14.4). CACs, although phenotypically indistinguishable from EPCs, are unable to generate blood vessels in vivo (Zhang et al. 2006; Schmeisser et al. 2001). Given the inaccuracy of flow cytometry and standard cell culture in obtaining true EPCs, *in vitro* colony forming cell assays have been developed. The colony forming assays recognize two types of EPCs, based on the time of the appearance in culture: early-outgrowth EPCs and late outgrowth EPCs (Fig. 14.4). Early-outgrowth EPCs (colonies obtained within 7 days of culture) have myeloid/hematopoietic characteristics, share features with monocytes/macrophages, and do not incorporate in the vasculature or differentiate into endothelial cells *in vivo* (Hill et al. 2003). Due to their similar behavior, CACs are now categorized within early-outgrowth EPCs. Early-outgrowth EPCs seem to participate to vessel formation mainly through the release of pro-angiogenic molecules (Richardson and Yoder 2010). On the other hand, endothelial colony forming cells (ECFCs), known as late-outgrowth colonies because they are obtained between 14 and 21 days of culture (Yoder et al. 2007), express cell surface antigens like primary

endothelium, lack hematopoietic and myeloid markers, and are able to form capillary-like structures *in vitro*, and human blood vessels *in vivo* (Urbich et al. 2003). These cells can be considered genuine EPCs, responsible for the process of *vasculogenesis* during embryogenesis and throughout post-natal life. A hierarchy has been demonstrated within the ECFC populations with regards to their proliferative potential. Using a single-cell clonogenic assay, ECFCs give rise to high proliferative potential (HPP-ECFCs) and low proliferative potential (LPP-ECFCs) populations. Both display self-renewal activity and capacity to differentiate into non-dividing endothelium. In addition, HPP-ECFCs are capable to generate new clusters with same and lower levels of proliferative potential (Ingram et al. 2004).

14.3.2.1 Source of ECFCs

EPCs can circulate in the bloodstream. Most of the circulating endothelial cells are vessel derived. However, the presence of bone marrow-derived circulating EPCs has been documented in the recipients of sex mismatched bone marrow transplantation (Lin et al. 2000). Circulating ECFCs have been cultured either from the adult peripheral blood or the umbilical cord blood. Cord blood-derived ECFCs seem to have higher proliferative potential, as documented by more numerous and more effective HPP-ECFCs in cord blood as compared to adult peripheral blood. Moreover, cord blood-derived ECFCs do not show signs of senescence in long-term cultures as opposed to adult ECFCs (Ingram et al. 2004). In normal conditions, there is a low number of circulating EPCs in the peripheral blood. EPCs are mobilized from the bone marrow in response to tissue ischemia or inflammation (Takahashi et al. 1999). However, increasing evidence suggests that resident EPCs, rather than circulating EPCs, orchestrate vascular maintenance and repair (Hagensen et al. 2012). ECFCs have been isolated from the endothelium of several human and animal tissues. Vessel wall-derived ECFCs, isolated from the endothelium of human umbilical vein and adult aorta (Ingram et al. 2005), present the same hierarchy with non-uniform differentiation potential, as circulating ECFCs. ECFCs can be cultured from bone marrow, white adipose tissue (Lin et al. 2013), corneal endothelium (Huang et al. 2010a) and hepatic sinusoidal vessels (Wang et al. 2012). Moreover, it has been reported that mature endothelial cells from different organs, and likely their progenitors, exhibit different and tissue-specific genetic expression patterns of growth factors, cytokines, chemokines and transcription factors for endothelial proliferation (Nolan et al. 2013).

14.3.2.2 Resident Lung ECFCs

Endothelial progenitors cells have been detected in the murine pulmonary microvasculature and the pulmonary artery (Alvarez et al. 2008; Clark et al. 2008), with a higher number of HPP-ECFCs in the microvasculature rather than in the

pulmonary artery (Solodushko and Fouty 2007). Resident ECFCs capable of self-renewal and de novo vessel formation *in vivo* have been detected in the developing human lung as well (Alphonse et al. 2014). However, their role is still elusive.

14.3.2.3 Mechanism of Action of ECFCs

Homing at the Site of Pre-existing Vasculature and Vasculogenesis

Resident and remote ECFCs, home at sites of pre-existing damaged vasculature and form neovessels under the influence of growth factors and chemokines (Heissig et al. 2002). Once activate, EPCs migrate through the endothelium into the interstitial extracellular matrix (ECM) to exert their functions (Chavakis et al. 2005) (Fig. 14.5).

EPCs have been shown to create new vessels *in vitro* and to incorporate into sites of active neovascularization inducing the formation of neovessels and the differentiation into mature endothelial cells *in vivo* (Gehling et al. 2000; Gunsilius et al. 2001).

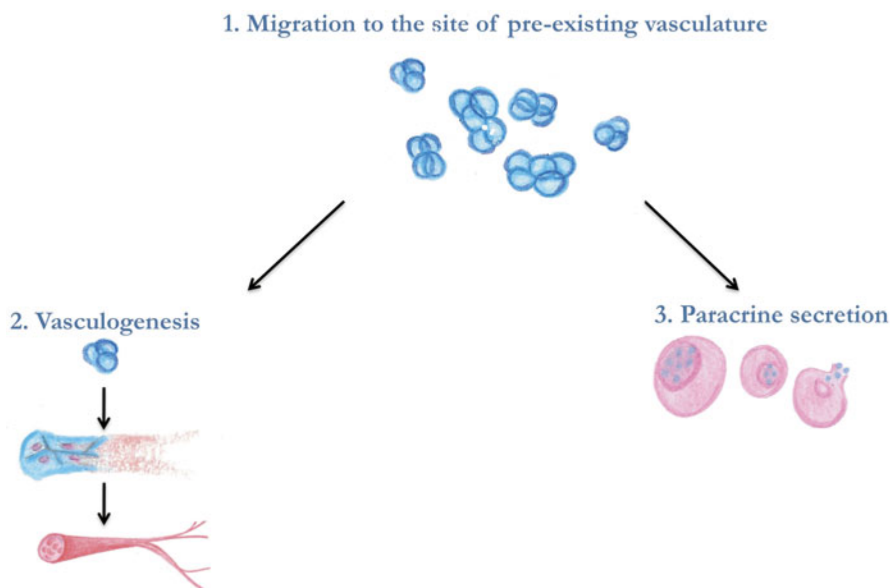

Fig. 14.5 ECFC mechanism of action. ECFCs known as late-outgrowth EPCs, home at sites of pre-existing damaged vasculature, where they incorporate into sites of active neovascularization inducing the formation of neovessels (*2*). ECFC also release paracrine factors to contribute to vascular repair (*3*)

Paracrine Mechanism

Quantification of human ECFCs after endotracheal administration in nude rats showed a low rate of engraftment in the recipient lungs, despite a significant therapeutic effect (Alphonse et al. 2014). These data suggest that the primary ECFC contribution to vascular repair happens through the release of paracrine factors (Fig. 14.5) not only for early-outgrowth EPCs, but also for ECFCs . Moreover EPC-derived microparticles (extracellular vesicles in the micron range, potentially containing a broader array of cell surface proteins in addition to exosomes) have been identified in ECFC cultures. These microparticles are incorporated into endothelial cells, activating the angiogenic program (Deregibus et al. 2007) and inducing vessel regeneration by promotion of mature endothelial cells proliferation (Dignat-George and Boulanger 2011).

14.4 Disruption of Cell Homeostasis in Neonatal Lung Diseases

14.4.1 Disruption of MSC Homeostasis

14.4.1.1 Disruption of MSC Homeostasis in BPD

A disruption of L-MSCs seems involved in the development of BPD. Circulating and lung resident MSCs are reduced in number in experimental BPD (van Haaften et al. 2009). On the contrary, the presence of MSCs in the tracheal aspirates of premature infants undergoing mechanical ventilation during the first days of life is associated with higher incidence of BPD (Hennrick et al. 2007; Popova et al. 2010b). Unstimulated MSCs from preterm lung, as opposed to bone marrow-derived MSCs, can become myofibroblasts under TGF-beta1 stimulation with a similar pattern as lung fibroblasts (Popova et al. 2010a) and show activated pathway of myofibroblast differentiation (Popova et al. 2010a). Furthermore, these MSCs can drive their own myofibroblastic differentiation by producing TGF-beta1 and they may represent myofibroblasts progenitors. (Popova et al. 2010a). Although myofibroblasts are required for secondary septation (Boström et al. 1996; Li et al. 2015), excessive proliferation of myofibroblasts may impair alveolarization in premature infants (Toti et al. 1997). These data suggest that lung MSCs, acquiring a profibrotic phenotype, could eventually contribute to the disease process. Moreover, MSCs from infants who will develop BPD show persistent alterations of the platelet-derived growth factor gene expression that favor hypoalveolarization (Popova et al. 2014) It is possible that a perturbed function rather than a reduced pool of L-MSCs is involved in the development of BPD.

14.4.1.2 Perturbation of MSC Homeostasis in CDH

Little is known about the role of MSCs in CDH. The mesenchyme-derived fibroblast layer is primarily defective and guide the epithelial disruption according to the *in vitro* cell recombinant model from nitrofen exposed animals. The malfunctioning fibroblasts exhibit decreased apoptosis and an arrest in proliferation, as compared to control (van Loenhout et al. 2012), possibly explaining the reduced branching morphogenesis seen in CDH. Recently, amniotic fluid-derived MSCs from healthy fetuses were shown to accelerate branching morphogenesis of experimental CDH lungs *ex vivo*; on the contrary amniotic fluid-derived MSCs from CDH had no effects. The authors suggested that nitrofen-induced lung hypoplasia could be exacerbated by dysfunctional amniotic fluid-derived MSCs surrounding the fetus (Di Bernardo et al. 2014). Possibly, the investigation of L-MSC function in CDH hypoplastic lungs may generate better insight into the pathogenesis of the disease.

14.4.2 Disruption of ECFC Homeostasis

14.4.2.1 Disruption of ECFC Homeostasis in BPD

Perturbation of *vasculogenesis* in the developing lung may directly alter normal lung growth. Given the pivotal role of EPCs in this process, their disruption may be responsible for the defects seen in BPD. Confirming this assumption, circulating ECFCs are markedly decreased in the cord blood of extremely premature infants who will develop moderate or severe BPD (Borghesi et al. 2009; Baker et al. 2012). Moreover, growth rate of preterm ECFCs is significantly reduced by hyperoxia exposure, as opposed to term ECFCs (Baker et al. 2009; Fujinaga et al. 2009). In agreement with these results, human fetal lung resident ECFCs, when exposed to 40 % hyperoxia for 24 h, formed fewer capillary-like structures in Matrigel and generated fewer HPP-ECFC colonies in comparison with fetal lung ECFCs cultured in room air (Alphonse et al. 2014). Chronic hyperoxia in neonatal rats (a model used to mimic BPD) disrupts rat lung ECFC self-renewal and angiogenic capacity (Alphonse et al. 2014).

14.4.2.2 Perturbation of ECFC Homeostasis in CDH

ECFCs are more numerous in the cord blood of CDH infants and can proliferate significantly more rapidly, compared to ECFCs from healthy infants (Baker et al. 2013b). It is possible that ECFC overproduction is one of the response mechanisms to impaired lung development in CDH. Interestingly, highly proliferative lung resident endothelial cells (HP-PAECs) obtained from pulmonary arteries of

sheep with surgical-induced CDH, were decreased in number and impaired in function (defective growth and tube formation in vitro) compared to HP-PAECs from control animals. HP-PAECs from fetal sheep with CDH also had increased levels of VEGF and decreased eNOS protein and NO production, compared to HP-PAECs from control animals. Exogenous VEGF and NO, were able to restore PAEC function (Acker et al. 2013).

14.5 Preclinical Evidence of MSC Effects on Neonatal Lung Diseases

14.5.1 Models of Neonatal Lung Diseases

In order to test the efficacy and safety of a novel therapy, it is paramount to dispose of reliable animal models. This section briefly reviews the experimental settings adopted to investigate stem cell administration for neonatal lung diseases.

14.5.1.1 Experimental BPD

Few models in different animal species have been able to mimic BPD or some of its aspects. *The hyperoxia-induced lung injury* (Fig. 14.6). is the most adopted one and displays several BPD features, such as alveolar and vascular disruption, inflammation, fibrosis and oxidative stress (Berger and Bhandari 2014; O'Reilly and Thébaud 2014). Rats and mice are born at the canalicular stage of lung development, similarly to premature infants at risk for BPD.

Hyperoxia-induced lung remodeling persists histologically and functionally at least up to 6 to10 months (equivalent to 25–30 years in humans) (Pierro et al. 2013; Alphonse et al. 2014). Animals exposed to hyperoxia in the neonatal period showed functional impairment later in life, as documented by significantly reduced distance running on the treadmill (Pierro et al. 2013; Alphonse et al. 2014). Neonatal exposure of mouse pups to hyperoxia, similar to clinical findings, worsens the severity of subsequent viral infections, increasing mortality and inflammatory response following adult influenza A inoculation (O'Reilly et al. 2008).

Few other models, although much less employed, may approximate BPD. *Postnatal injection of intraperitoneal bleomycin* in newborn rats from day 4 to 14 induces significant pulmonary hypertension and alveolar simplifications, with no fibrosis (Tourneux et al. 2009), as opposed to adult rats that suffer mostly from fibrotic lung lesions after bleomycin administration (Chua et al. 2005).

Larger animals (lamb, sheep and baboon) can be delivered prematurely and undergo mechanical ventilation, reproducing the chain of events that leads to BPD. These animals show acute lung inflammation, abnormal pulmonary function, altered lung architecture, alveolar simplification, and mild fibrotic changes

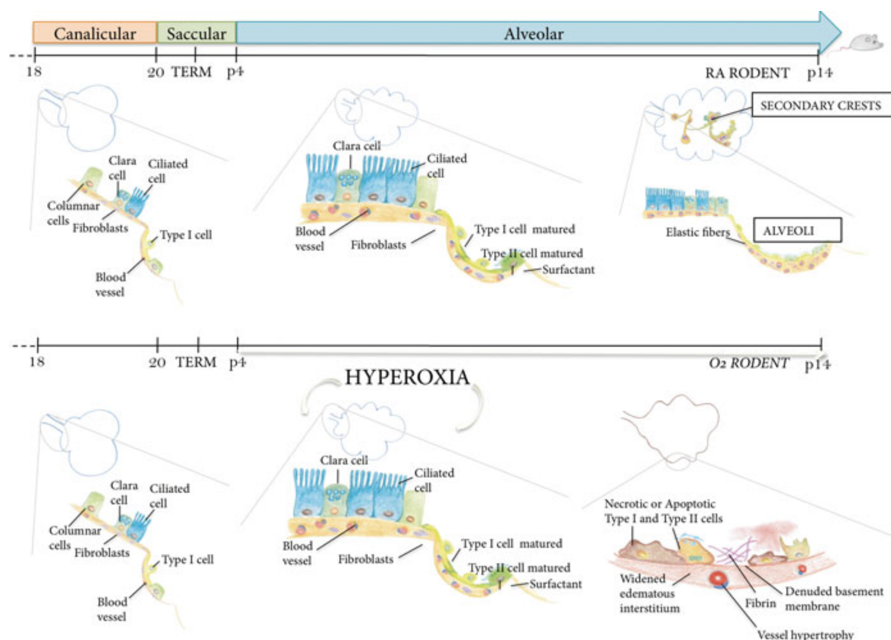

Fig. 14.6 Experimental BPD: the hyperoxia-induced lung injury. Rat pups are naturally born during the saccular stage of lung development. Exposure of the developing rat lung to various concentration of hyperoxic gas (O_2 rodent), during the alveolar stage (day 4 to day 14 of life) impairs alveolarization, resulting in fewer and enlarged alveolar air spaces, resembling BPD lungs, as opposed to controls in room air (RA rodent)

(D'Angio and Ryan 2014; Albertine 2015; Yoder and Coalson 2014). However only few facilities are equipped to work with large animals and most of the data on stem cell therapy are obtained with the murine models, particularly the hyperoxia-induced lung injury.

14.5.1.2 Experimental CDH

The herbicide nitrofen (2,4-dichloro-phenylp-nitrophenyl ether), when administered during pregnancy in rats, causes developmental anomalies in the lung, heart, diaphragm and skeletal tissue of the offspring, with no obvious harm to adult rodents (Kluth et al. 1990). Diaphragmatic defects resulting from a single oral dose of nitrofen are remarkably similar to those documented in human Bochdalek CDH, including lung hypoplasia with reduction in the number of terminal bronchioles and alveolar volume, hypoplastic pulmonary vascular bed, and increased thickness of the pulmonary arterial smooth muscle coat (Tenbrinck et al. 1990). Animal studies showed increased contractility and impairment in endothelium-dependent relaxation of pulmonary arteries in nitrofen-exposed animals (Luong et al. 2011).

Moreover, nitrofen generates a perturbation in the retoinid pathway (Thébaud et al. 1999), that may be, at in least in part, responsible for typical CDH phenotype in humans (Beurskens et al. 2010, 2013) .

The surgical model of CDH (rabbit or lambs) requires a diaphragmatic defect to be created through fetal surgery. This model presents some limitations. First, it is more expensive and time-consuming and requires facilities equipped for bigger animals. More importantly, it cannot be used to study the earliest origins of the CDH diaphragmatic defect and lung hypoplasia that occur before the diaphragm closure and is better reproduced by the rodent models (Chiu 2014). However, the surgical model may be better suited to test postnatal therapies for CDH, since nitrofen-exposed animals do not survive in the neonatal period.

14.5.2 MSCs for Neonatal Lung Diseases

14.5.2.1 MSCs for BPD

Prophylactic Treatment for BPD

Systemic and endotracheal prophylactic administration of MSCs (before exposure to hyperoxia) improves survival, prevents lung injury, reduces inflammation and attenuates pulmonary hypertension in experimental BPD (van Haaften et al. 2009; Aslam et al. 2009; Chang et al. 2009, 2011, 2013; Pierro et al. 2013) (Fig. 14.7). Moreover, MSC administration promotes proliferation of bronchio-alveolar stem cells (BASCs), a class of putative epithelial lung progenitor cells, capable of self-renewal and differentiation in culture (Tropea et al. 2012). Lung compliance and exercise tolerance are also significantly improved by MSCs administration (van Haaften et al. 2009; Pierro et al. 2013). Results seem to be most beneficial by endotracheal, as compared to intravenous or intraperitoneal administration (Chang et al. 2009, 2013) in a dose dependent manner (Chang et al. 2011).

The long-term effects of MSC administration have not been extensively investigated yet. Neonatal prophylactic endotracheal MSC treatment significantly improves exercise capacity and almost normalizes alveolar architecture at 6 months of age in animals exposed to hyperoxia in the neonatal period (Pierro et al. 2013). Intrapulmonary delivery of MSCs on day 4 of life was safe up to 6 months, as assessed by lung morphometry and exercise capacity (Pierro et al. 2013). Total body computerized tomography (CT scans) showed no signs of tumor formation. These data need to be confirmed by systematic sampling of tissue histology.

Rescue Treatment for BPD

Rescue administration of MSCs (after exposure to hyperoxia), have led to inconsistent results, with no (Aslam et al. 2009) or negligible (Chang et al. 2013) improvement. However, in both of these rescue studies, the same absolute dose as the

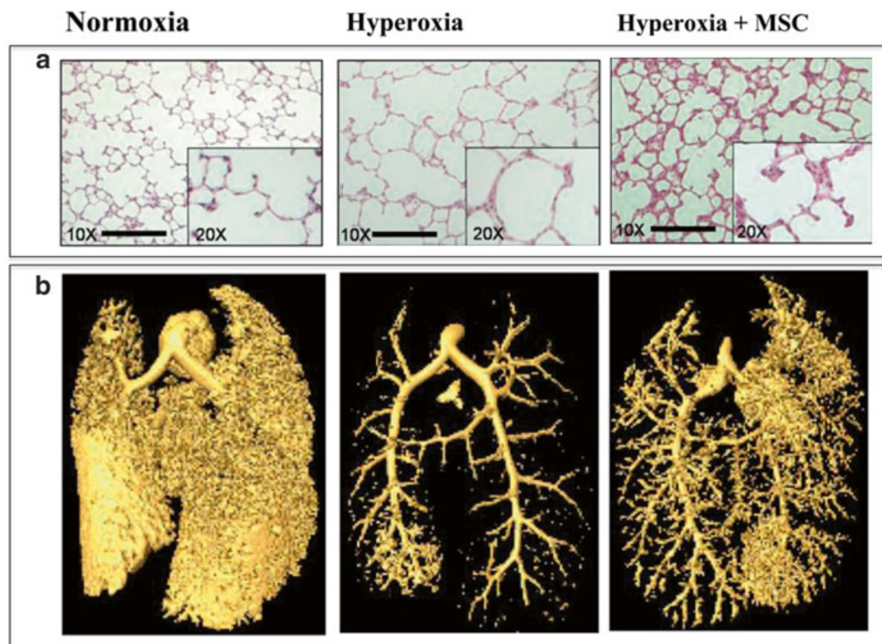

Fig. 14.7 MSC administration improves the hyperoxia-induced lung injury. (**a**) *Hematoxylin and eosin–stained lung sections:* Oxygen-exposed lungs (Hyperoxia) display the characteristic features of alveolar simplification, as compared to control lungs (Normoxia). Animals treated with intratracheal bone marrow–derived mesenchymal stem cells (Hyperoxia + MSCs) on day 4 of life, before O2 exposure, have smaller and more numerous alveoli. (**b**) *Microangiograph computed tomography (CT) scan:* intratracheal MSC administration promote s lung angiogenesis and restore a denser capillary network after hyperoxia induced lung vascular disruption (Adapted from van Haaften et al. 2009, Official Journal of the American Thoracic Society. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society)

prophylactic experiments was used, without adjusting for the increase in body weight of the older animals. When the dose was adjusted per body weight, although the prophylactic treatment still elicited a superior response, the rescue administration of MSCs was able to ameliorate lung injury (Pierro et al. 2013). No studies report on the effects of rescue MSC administration on the vascular component of the lung injury. Rescue administration of MSCs shows a milder improvement in terms of inflammation and oxidation as compared to early administration (Chang et al. 2013). Taken together these data suggest a possible beneficial effect of a rescue MSC treatment, but early timing seems to be more efficient.

14.5.2.2 MSCS for CDH

Prenatal administration of lung MSCs from adult rat lung into the uterine vein through the bilateral horn of the uterus of the dams improves fetal lung alveolarization and vascular development. MSC administration reduces the nitrofen-induced

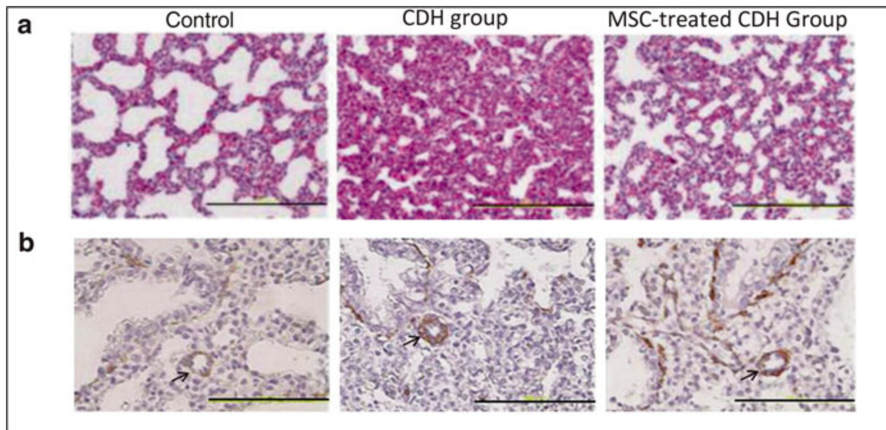

Fig. 14.8 MSC administration improves the nitrofen-induced lung hypoplasia. (**a**) *Hematoxylin and eosin–stained lung sections:* The alveolar walls are thicker and the alveolar air space area is smaller in CDH animals as compared to controls. Prenatal lung-derived MSC transplantation, injected via the uterine vein in the bilateral horn of the uterus of the dams, significantly improves alveolar wall thickness and alveolar air space area. (**b**) *Immunohistochemical staining for alpha smooth muscle actin:* the wall medial thickness of the pulmonary arteries in CDH lungs, significantly higher than controls, is restored by prenatal MSC administration (Adapted from Yuniartha et al. 2014; with permission)

alveolar walls thickness and saccules underdevelopment, increases the endogenous pool of surfactant, and normalizes medial wall thickness of the pulmonary artery (Yuniartha et al. 2014) (Fig. 14.8). Lung surface and number of terminal buds were significantly increased by prenatal amniotic administration of amniotic fluid MSCs from healthy rats (Pederiva et al. 2013). These proof of concept experiments are promising but clinically challenging. Alternate strategies exploring safe and efficient cell administration should also be validated in larger (rabbit, sheep) animal models.

Engineered tendons coated with MSCs have been suggested for repair of large diaphragmatic defects. In animal models, prosthetic patches seem to give better outcomes when engineered with amniotic MSCs as compared to an equivalent acellular bioprosthesis, with no local or systemic adverse effects (Turner et al. 2011).

14.5.3 ECFCS for Neonatal Lung Diseases

14.5.3.1 ECFCs for BPD

Systemic (intrajugular) delivery of cord-derived ECFCs at P14 restores established hyperoxia-induced pulmonary hypertension in immunocompromised mice and rats as documented by echocardiography and right ventricle hypertrophy. Similarly, ECFC therapy restored normal alveolar architecture, lung function and lung vascular growth (Alphonse et al. 2014) (Fig. 14.9).

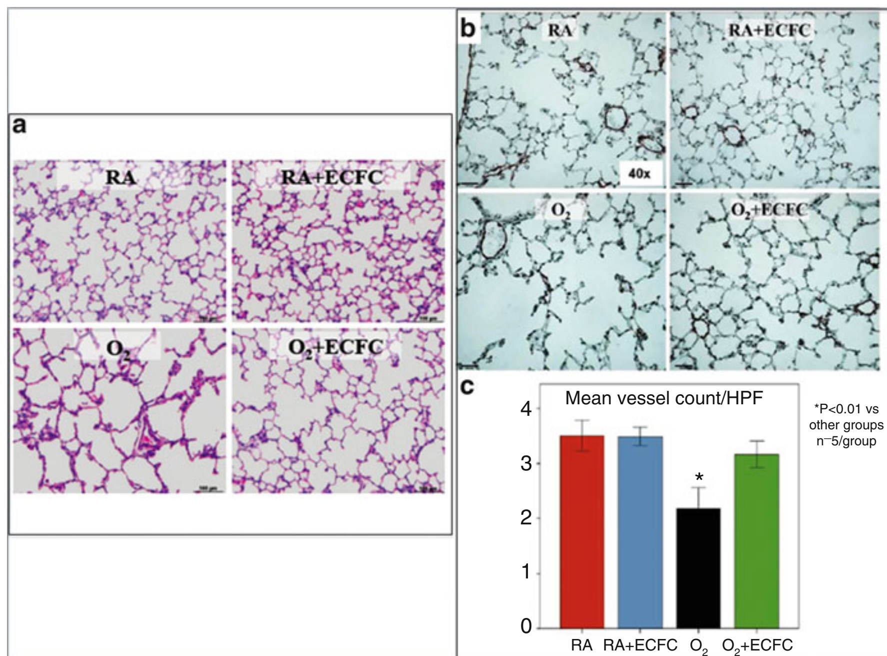

Fig. 14.9 ECFCs revert Oxygen-induced lung injury. _A. Hematoxylin and eosin–stained lung sections:_ untreated hyperoxia-exposed (O2) lungs show larger and fewer alveoli in comparison with control mice housed in room air (RA) at P28. Intrajugular administration of ECFCs at P14 after oxygen exposure restored alveolar growth. (**b**, **c**) _Effects of ECFC treatment on pulmonary vessel density assessed on lung slides stained with von Willebrand Factor._ The pulmonary vessel density per 10 high-power fields was significantly decreased in the lungs of O_2-exposed animals in comparison with RA. Intrajugular injection of ECFCs significantly improved pulmonary vessel density (Adapted from Alphonse et al. 2014)

This rescue therapy appeared to be safe and effective up to 10 months of life: hyperoxic-exposed neonatal mice treated in the neonatal period had preserved alveolar architecture, significantly improved exercise capacity and reduced pulmonary hypertension in these long-term experiments (Alphonse et al. 2014). In immunocompetent rats, ECFCs from term cord were able to reduce bleomycin-induced pulmonary hypertensionas determined by right ventricular hypertrophy, although the treatment did not improve lung histology (Baker et al. 2013a) ECFCs from preterm cord did not elicit any response. The discrepancy between these two studies may be due to choice of a different model and the different status of the animal immune system.

14.5.3.2 ECFCs for CDH

No studies have yet investigated the efficacy of ECFCs in CDH

14.5.4 Cell-Free Therapy for Neonatal Lung Diseases

The evidence of low engraftment of both, MSCs and ECFCs, despite exciting therapeutic results, strongly confirms the stem cell paracrine activity as their major mechanism of action. This notion has paved the way to test novel cell-free derived products.

14.5.4.1 Conditioned Media (CdM)

CdM is the serum free media, where confluent cells have released their functional effectors in different forms. After 24 h of culture, the media is collected, centrifuged, concentrated and stored at −80°.

MSC CdM

Several studies have documented the efficacy of MSC CdM in experimental models of different diseases. In particular, MSC CdM could effectively prevent and treat the hyperoxia-induced lung injury. *In vitro*, cell-free bone marrow-derived MSC CdM prevents hyperoxia-induced alveolar epithelial cell apoptosis, accelerates wound healing of alveolar epithelial cells and preserves endothelial cord formation under hyperoxic conditions (Van Haaften et al. 2009). *In vivo*, daily intraperitoneal administration of MSC CdM prevents oxygen-induced alveolar simplification, improves lung function and reduces pulmonary hypertension (Pierro et al. 2013) (Fig. 14.10). The beneficial effects in terms of lung architecture and exercise capacity of the 2 week course of CdM administration in the neonatal period were documented up to 6 months of life, with no evident side effects (Pierro et al. 2013). Although the therapeutic benefit of CdM is remarkable, the down side of this strategy is the lack of cell homing and cell adaptation to the local injurious environment. Waszak et al. exposed bone marrow derived MSCs *ex vivo to* hyperoxia for 24 h. This preconditioning strategy enhanced the production of the anti-oxidant stanniocalcin-1 in the CdM. The beneficial effects of the CdM collected from preconditioned cells and injected into hyperoxic rats were more pronounced as compared to non-preconditioned media (Waszak et al. 2012).

In the setting of hyperoxic injury, a single prophylactic injection of cell-free bone marrow-derived MSC CdM seems to be even more effective on alveolar and lung vascular growth than cell therapy itself (Aslam et al. 2009), which has been documented up to 3 months (Sutsko et al. 2013). A single rescue dose of CdM, after exposure to hyperoxia, is also able to reverse hyperoxia-induced parenchymal fibrosis, partially reverse alveolar injury, fully reverse pulmonary hypertension and normalize lung function (Hansmann et al. 2012). In addition to the pleiotropic effects on lung structure, inflammation and oxidative stress, MSC CdM may also boost local repair activity such as the proliferation of resident lung progenitor cells (Tropea et al. 2012).

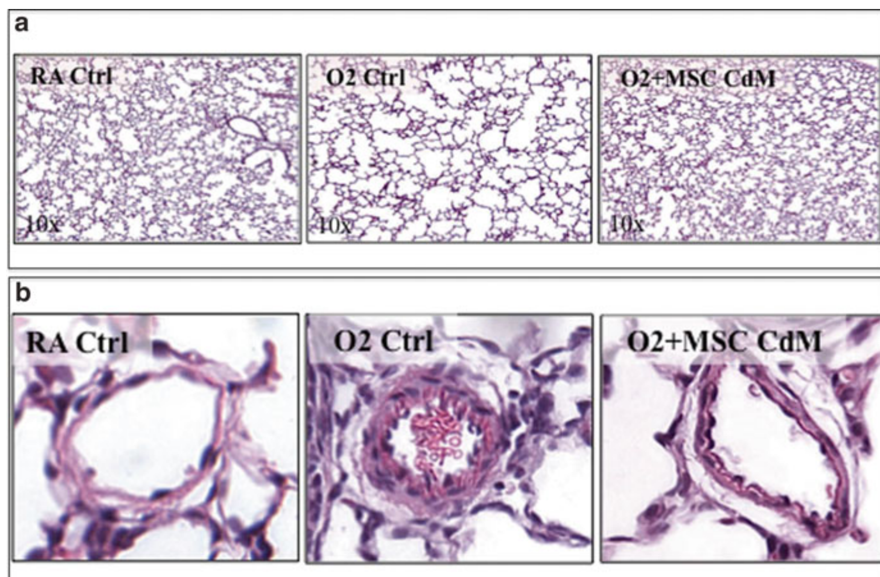

Fig. 14.10 MSC CdM administration improves the hyperoxia-induced lung injury. (**a**) _Hematoxylin and eosin–stained lung sections:_ larger and fewer alveoli are present in hyperoxia-exposed lungs compared with lungs from rats housed in room air (RA) and RA animals treated with MSC conditioned media (CdM). Daily intraperitoneal administration of MSC CdM in oxygen-exposed animals improved alveolar growth. Representative H&E sections of pulmonary arteries from the six experimental groups. (**b**) _Hematoxylin and eosin–stained lung sections:_ Hyperoxic-exposed rats had a significant increase in media wall thickness compared with RA housed rat pups. MSC CdM significantly reduced media wall thickness (Adapted from Pierro et al. 2013)

The compelling and persistent results of a single dose of CdM may be explained by the presence of microparticles and exosomes in the CdM. These particles act as therapeutic packages carrying combinations of bioactive molecules and miRNA, that have the ability to incorporate in resident cells and transfer functional RNA (Kourembanas 2015). In variety of animal models, the use of MSC-derived exosomes, purified by ultracentrifugation and size exclusion (Muller et al. 2014), is strongly associated with improved organ function following experimental injury (Akyurekli et al. 2015). Lee et al. demonstrated that exosomes from bone marrow-derived MSCs, in the setting of hypoxia-induced pulmonary hypertension, prevent pulmonary hypertension, right ventricular hypertrophy and hypoxia-activated inflammation, whereas exosome-depleted CdM had no effect (Lee et al. 2012). Exosomes from bone marrow-derived MSCs significantly reduce lung edema, lung protein permeability and lung inflammation in LPS-induced acute lung injury (Zhu et al. 2014).

ECFC CdM

CdM from ECFCs *in vitro* promotes proliferation and angiogenesis of fetal pulmonary artery endothelial cells (PAEC) and alveolar type 2 alveolar cells (Baker et al. 2013a). *In vitro* cell-free bone marrow-derived ECFC CdM, accelerates wound healing of alveolar type 2 cell and preserves endothelial cord formation under hyperoxic conditions (Alphonse et al. 2014). When administered *in vivo* either as a single iv injection or as a 2 week course, ECFC CdM, prevents bleomycin-induced right ventricular hypertrophy. Neither single nor repeated administrations were able to improve lung architecture. ECFC CdM from preterm cord blood did not elicit any response independently from the duration of the treatment. On the contrary, Alphonse et al. showed that, a 2 week course of daily intraperitoneal injections of ECFC-CdM to neonatal rats exposed to hyperoxia preserved alveolar growth, lung vascular growth, and attenuated pulmonary hypertension (Fig. 14.11). Co-transplantation of ECFCs and MSCs significantly enhances MSC engraftment by reducing early apoptosis and preserving MSC stemness (Lin et al. 2014).

Endothelial cells have been demonstrated to secrete exosomes and incorporate exosomes from various cell types to directly induce angiogenesis (van Balkom et al. 2013; Hood et al. 2009). ECFC exosomes have not yet been investigated for neonatal lung diseases.

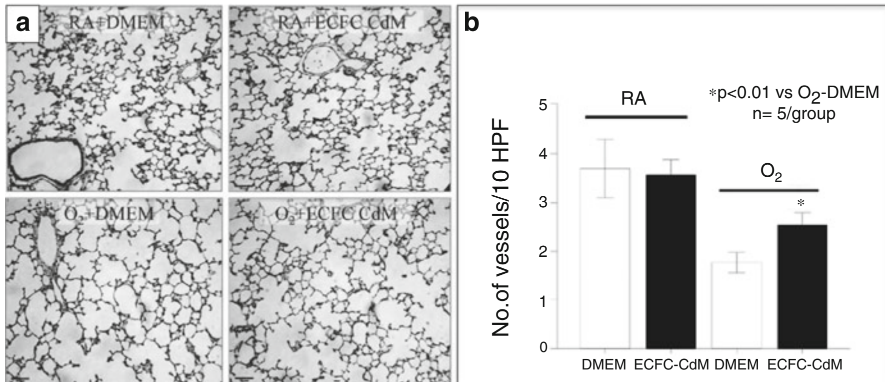

Fig. 14.11 ECFC CdM reverts Oxygen-induced lung injury. (**a**) *Hematoxylin and eosin stained lung sections:* Oxygen-exposed newborn rats (O_2 + DMEM) show arrested alveolar growth in comparison with room air controls (RA + DMEM). ECFC conditioned media (CdM) significantly preserved alveolar growth. (**b**) *Vessel count:* Mean data showing decreased pulmonary vessel density in O_2-exposed newborn rats in comparison with RA controls as assessed by the number of barium-filled pulmonary vessels. ECFC CdM significantly attenuated the loss of pulmonary vessels in hyperoxia (Adapted from Alphonse et al. 2014)

14.6 Clinical Translation of Stem Cell Therapy for Neonatal Lung Diseases

Although the option of a cell free product is appealing, the administration of the entire cocktail entails a risk of unanticipated side effects, due to presence of yet undetermined active molecules. Technological advances are still required for the isolation, characterization, quality control, and large-scale manufacturing of exosomes. At present, regenerative medicine seems to favor cell therapy. While ECFCs have not been yet tested in premature infants, preliminary trials investigating the potential of MSCs to treat BPD have already been started. Chang et al. showed that the endotracheal transplantation of two different doses of human cord-derived MSCs in preterm infants at high risk for BPD is feasible and apparently safe (Chang et al. 2014). The treatment was well tolerated, without dose-limiting toxicity or immediate (up to 6 h) complications after transplantation. Six severe adverse events were recorded. However they were typical complications of extreme prematurity and not attributed to the MSC therapy. This trial was designed to test safety and was not randomized and powered to investigate efficacy measures. The long-term follow-up study is underway (NCT01632475). Six studies are currently investigating the effects of MSC administration in patients at risk for BPD (https://clinicaltrials.gov/ct2/results?term=mesenchymal+stem+cell+and+BPD&Search=Search). Although safety of MSC administration in the adult (Lalu et al. 2012) and pediatric (Zheng et al. 2013) population has been widely documented, efficacy can be hampered by several product and patient related factors.

14.6.1 Cell-Related Variables

Several factors, including isolation and characterization techniques, production to scale-up, cryopreservation and banking have been shown to affect the efficacy of MSC therapy and have been reviewed elsewhere (Thirumala et al. 2013). Number of passages can affect senescence and thus the efficacy of ECFC and MSC treatment (Sethe et al. 2006; Ingram et al. 2004). A passage is the process of removing cells from a culture flask and plating them into more culture flasks. Passaging is necessary to obtain a sufficient number of cells for transplantation. The number of passages is inversely proportional to the efficiency of MSC homing, with freshly isolated cell performing superior results than cultivated cells (Rombouts and Ploemacher 2003). Moreover MSC and ECFC tend to show genotypic and phenotypic variation, when cultured for extended periods of time (Sethe et al. 2006; Ingram et al. 2004), although cord-derived cells seem to be less sensitive to aging in culture (Kern et al. 2006; Ingram et al. 2004). The type of transplant is another paramount variable. Considering the low immunogenicity of MSCs, although immune rejection is still possible (Le Blanc et al. 2003), allogeneic transplantation (from a donor) may be the most suitable option for preterm infants at risk for BPD (Alagesan and Griffin 2014),

providing a readily available, quality controlled, off-the-shelf product. On the contrary, ECFC transplantation, may need the autologous source (from the cord of the patient), due the lack of ECFC immunomodulatory properties and the difficulties in pharmacological immunosuppression of the fragile preterm infant. Unfortunately, autologous transplantation, has some important shortcomings: (i) impracticality of the prophylactic/early administration due to lead time, (ii) need for on site GMP (Good Manufacturing Practice) facilities to handle clinical grade cell products, (iii) questionable therapeutic properties of preterm cord blood-derived ECFCs compared to term ECFCs.

14.6.2 Patient-Related Variables

With regards to BPD, several factors need to be considered when designing a trial. The timing of the treatment is crucial. Waiting until BPD is officially diagnosed (at least 28 days of life), may hamper the efficacy of the treatment. The early phases of evolving BPD may represent the optimal target for MSCs. Eventually, prophylactic administration may be an interesting option for MSCs, once safety is well documented.

Route of administration greatly impacts efficacy, bioavailability, and functionality of any pharmacological drug. With regards to lung diseases, the two most relevant routes of administration are the intravenous (iv) and the endotracheal (ET) ones. The iv route favors stem cells migration towards multiple sites of injury, even though most of the cells will pass through the lung and is less invasive than the ET route, especially in spontaneously breathing patients. On the other hand, ET administration provides superior therapeutic benefit at least in the experimental BPD settings for MSCs. In addition, co-administration with surfactant may facilitate the distribution of stem cells.

14.7 Conclusions

Neonatal lung diseases such as BPD and CDH remain a major therapeutic challenge as they still lack safe and effective treatments. Regenerative medicine, either as cell administration or, in the future, in the form of cell free products, may offer promising therapeutic options to these infants. While much more needs to be learned about their mechanism of action and optimal manufacturing procedures, MSCs have been widely investigated in pre-clinical studies and are now entering early phase clinical trials to assess the feasibility, safety and efficacy of this intervention in preterm infants at risk of developing BPD. Other cell therapies, such as ECFCs to promote vasculogenesis are still at the preclinical stage of investigation, but may enter the clinical arena in years to come as a stand alone product or in combination with MSCs.

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