Niranjan Bhattacharya
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Editors

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To all scientists – present, past, and future – whose sincere belief in God has made science the true source of religion (philosophy) in their lives.

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Preface

New areas of medical science are emerging every year, opening new vistas, stimulating new thoughts, and finding cures for age old diseases for which cures were not available earlier. Regenerative medicine is one of these areas, and interestingly, this field is focusing more and more on what is available from within the human body itself, particularly the fetal body, which can be used to regenerate and rejuvenate failing systems. While global attention is on the use of stem cells in regeneration, other fetal items like the fetal tissue, cord blood, placenta and placental tissue, and amniotic fluid, all of which contain stem cells of different types as well as things like growth factors that are important in the development of the fetus, have the potential to regenerate ailing organs. The implication is that stem cells do not have to be separated to be inserted in a host; the fetal tissue or cord blood or amniotic fluid can be transplanted in its totality or in a partial form in the patient, and the impact could even be more because of the additional growth elements in these items.

The issue, of course, has been the cause of a controversy in the West, where ethical issues are raised regarding the problem of acquisition of the fetal item. The fact is that approximately 205 million pregnancies occur each year (Cheng L Surgical versus medical methods for second-trimester induced abortion. The WHO Reproductive Health Library. World Health Organization. 2008. http://www.webcitation.org/5zVk3OSM4. Archived from the original on 17 June 2011. Retrieved 17 June 2011). Over a third of which are unintended, resulting in the abortion of around a fifth of all pregnancies annually. The numbers, therefore, amount to about 42 million abortions per annum, 20 million of which are done in unsafe circumstances (Shah I, Ahman E Unsafe abortion: global and regional incidence, trends, consequences, and challenges (PDF) J Obstet Gynaecol Can. 2009; 31(12):1149–58. PMID 20085681). The 22 million safely aborted fetuses are normally incinerated or buried underground (in societies that have such traditions or where incinerators are unavailable) or simply disposed of by throwing the fetus into a river (this too may be a custom, or it could be done to get rid of any sign of an unwanted pregnancy). These fetuses are unwanted (this is the cause of the abortion in the first place) and got rid of at the earliest in all societies; yet, they have the potential to serve the cause of medical science and human health, if they could be utilized for medical purposes.

Fetal tissue is an ensemble of varying stage and grade of differentiated and undifferentiated cells including fetal stem cells and stem cell-like progenitor cells, not necessarily identical, but from the same origin, that together carry out a specific function. Cell therapy is the process of introducing new cells into a tissue to treat a disease. These new cells can be obtained from fetal tissue, neo-natal tissue or any other growing tissue. Fetal tissue contains mostly immunologically naïve cells, depending on the stage of gestation. Along with their protective microenvironment, they constitute the niche for the fetal cell/tissue. Transplantation of fetal tissue can have unique results. Experiments have shown that fetal tissue transplanted to an accessible site in a human body, and then retrieved and examined after 10 years, showed the persistence of a group of primitive progenitor cells. This suggests that the transplanted tissue may have created its own microenvironment at the site of its transplantation in the adult host for its own survival. This also suggests the possibility that the primitive cells, which survive in the transplant site for long periods, may actually be migrating to the site of requirement/injury. Experiments in mice models have indicated that fetal cells similarly survive and sometimes differentiate to host cells. Thus, transmigration to the site of injury and differentiating to required cells would mean that this is an ideal form of cell therapy. Transmigration to the site of injury fulfills the requirements of an ideal cell therapy (Zeng XX, Tan KH, Yeo A, Sasajala P, Tan X, Xiao ZC, Dawe G, Udolph G. Pregnancy-associated progenitor cells differentiate and mature into neurons in the maternal brain. Stem Cells Dev. 2010;19(12):1819–30. Epub 2010 Sep 13.). This therapy has huge potential for treatment of intractable medical problems in human beings, particularly because it is noninvasive since it can be placed in a vascular site under the fold of the skin surface.

Every major discovery or invention in medical science has been fraught with controversy. For instance, vaccinations for different diseases, which are

so well accepted now and have led to the complete or partial eradication of dreaded scourges like small pox and polio, generated a lot of debate on various issues including ethical and safety concerns, during the early phases of experimentation. If the scientific community accepts the potential of fetal tissue transplant at a heterogenic site as an easy procedure for cell therapy, many currently intractable diseases can also be faced with their nemesis in the future as the matter is explored further and procedures are incrementally refined.

Since Western countries, which have the funds and facilities to carry out more research in this area, are bogged down by ethical concerns regarding the use of placental and fetal substances for cell therapy, it may be pertinent to say a few words here on medical ethics from ancient to modern times. In civilized societies, be it ancient India which produced the Ayurveda or ancient Greece where Hippocrates produced the Hippocratic system from which modern medicine is derived or be it the modern world which has produced the British Medical Association and the World Health Organization, which set the norms for medical practices today; the basic guideline for medical ethics is to benefit society and do good by it in general and the patient in particular.

The Oxford English Dictionary defines ethics as "the moral principle by which a person is guided," as well as "the rules of conduct recognized in certain associations or departments of human life" (Hawkins JM Oxford English dictionary, vol. III D-E. Oxford: Clarendon Press; 1970. p. 312 c2.). The Sanskrit term "dharma" has some equivalence with "ethics" in that dharma implies those principles that "support" or guide an individual in the passage through life (Monier Monier-Williams. A Sanskrit dictionary [Searchable digital edition] Mayapur: Bhaktivedanta Book Trust; 2002. p. 510 cited in Ran`es C Chakravorty. Some aspects of the ethics of stem cell research. In: Bhattacharya N, Stubblefield P, editors. Frontiers of cord blood science. London: Springer. 2009. p. 361–2, 10.1007/978-1-84800-167-1_18).

Medical ethics has to be seen in how far society can be served by curing the world of diseases, intractable or otherwise. Anything new is initially resisted, normally for safety concerns but sometimes for "ethical" reasons, until the benefits of the treatment are proven beyond question. Sometimes, they are still resisted on grounds of religion, but if it does not harm society, and on the contrary, cures particularly difficult diseases, the question is, should rational science be rejected by nonscientific irrationality?

Surgically aborted human fetal tissue is one of the most important sources of fetal stem cells, progenitor cells, and other stem cell-like cells with immense potential for clinical utilization in modern translational research, especially for end-stage degenerated human organs or tissues or systems. Stem cells may someday provide the means of treating and possibly curing diseases such as diabetes, Parkinsonism, Alzheimer's etc., which can only be symptomatically palliated currently. The promise of stem cell research products (medically and financially) is so great that in July 2005 the prestigious journal Scientific American devoted an entire section to stem cell research in conjunction with Financial Times.

We leave it to the judgment of the reader to decide whether the surgically aborted fetus should be thrown into a holy river like the Ganges for the eternal

bliss of the unborn, or just cremated or incinerated – as a waste that will be of no use to anyone – or should it be donated so that it and its contents can help some poor suffering patient overcome intractable diseases like Parkinsonism, myopathy, motor neurone disease, Alzheimer's, cardiomyopathy, and cirrhosis of the liver. More research needs to be done using human fetal tissue to perfect the mode of treatment, and donated fetal tissue can be of great help here. The fact is that fetal tissues have immense potential in rejuvenating degenerated cells because it is from the stem cells of the small human fetus that a fully grown baby with eyes, nose, limbs, and functioning organs takes shape, and it is magical how the transformation from a zygote to a baby takes place. When a healthy fetus is surgically discarded because the mother does not want the baby, instead of destroying it altogether, it can help in prolonging human life and give hope to many patients who have given up all hope of ever leading normal lives.

We would lastly like to salute one of the pioneers in reproductive medicine and in vitro fertilization, the legendary Sir Robert Geoffrey Edwards, CBE, FRS, who received the Nobel Prize for Medicine in 2011. In 1992, he published the first multiauthored book on human fetal tissue transplant. He understood the potential of this method of treatment in medicine. Science has moved forward since 1992, and sea changes have taken place in regenerative medicine in two decades since Prof. Edwards published his novel book. The present edited volume is an attempt to update the research work done in this field and integrate and bring together current findings in the field of research initiated by Sir Robert Edwards. We also salute Prof. B E Tuch (Australia) and Prof. JL Tourane (France), eminent scholars who participated in both the publications, Prof. Edwards' and the present one.

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Preamble

Hope for tissue and cell replacement should be based on rigorous pre-clinical testing and controlled clinical trials.

In a quest to replace or add specific cell types for clinical advantage to patients who are either missing such cells or have malfunctioning cells, investigators have focused on donor cell populations found in and isolated from embryonic, fetal, neonatal, or adult tissues. Such tissues contain very rare populations of early, non-differentiated cell populations termed stem cells. Stem cells have the capacity to self-renew (make more of themselves), a definition crucial to identification of a cell as a stem cell, and to differentiate down multiple cell lineages.

There are a number of different types of stem cells. Embryonic stem cells (ESCs) are found in the inner cell mass of the blastocyst and can be isolated and maintained in culture as an undifferentiated population with either a feeder layer of embryonic fibroblasts, certain growth factors or cytokines, or combinations of these cells and growth factors/cytokines. Upon removal from these cells/factors that allow ESCs to be maintained in an undifferentiated state, ESCs have the capacity to form cells of the three germ cell layers: endoderm, ectoderm, and mesoderm in vitro through formation of embryoid bodies and in vivo through formation of teratomas containing cells of the three germ cell layers. While both mouse and human ESC lines have been developed and their induced differentiation follows that seen in embryogenesis, allowing one to study the process of differentiation and to gain mechanistic insight into control of cell growth and differentiation [1] it is not yet clear if ESC lines are or will be of clinical utility for cell replacement and regenerative medicine [2]. Moreover, use of ESC lines as well as of fetal tissue, the latter tissue being the focus of this book, present ethical concerns to some.

Various types of stem cells can be found in fetal, neonatal, and adult tissues. These include, but are not necessarily limited to, hematopoietic stem cells (HSCs) and mesenchymal stem/stromal cells (MSCs) [3]. MSCs are much more limited in capacity than ESCs. While they have extensive renewal capability, they are quite restricted in their differentiation capacity compared to ESCs. Also, present in these tissues are a number of different progenitor cell types such as hematopoietic progenitor cells (HPCs) [4, 5] and high proliferative endothelial progenitor cells (EPCs; more precisely called endothelial colony-forming cells (ECFCs) [5, 6]). Progenitor cells are produced from stem cells (e.g., HSCs give rise to HPCs), have much more limited or no self-

renewal capacity compared to stem cells, and are much more limited in their cell-type differentiation capacity.

Translating bench laboratory research and preclinical animal model information to the clinic is a noteworthy goal that has a high significance and relevance impact for clinical medicine, hence the need to learn and understand as much as we can about stem/progenitor cells of all tissue sources. With the vast amount of new literature on the different stem and progenitor cell types, one can sometimes get caught up in the hype surrounding these cells with regard to application. At present, the most rigorously studied system is that of HSCs and HPCs. This hematopoietic system serves as a paradigm for other stem/progenitor cells. Also, these HSC/HPC types have clearly shown clinical efficacy in that they have been used to successfully treat a plethora of malignant and nonmalignant disorders, by replacing the defective and malignant cell types after intensive conditioning regimens and HSC/HPC transplantation [4]. *While HSC/HPC are found in fetal liver, blood, and bone marrow, in neonatal (*e.g.*, placental and umbilical cord blood (CB) blood), as well as in pediatric, and adult bone marrow, and in pediatric and adult blood in which the HSC/HPC have been* mobilized from bone marrow to the circulating peripheral blood, use of fetal tissue for HSC/HPC transplantation has not been successful. It is only CB, mobilized peripheral blood, and bone marrow from pediatric and adult patients that have been successfully used [4]. Some of the problems with use of fetal liver as a source of human HSC/HPC for transplantation may lie in difficulties in cryopreserving these cells since they would have to be collected, and typed for HLA antigens, and stored in a cryopreserved state prior to use. Old concepts that fetal liver cells will elicit no graft vs. host disease (GVHD), and could therefore be a useful source of these cells for transplantation, have not been proven. While neonatal blood (e.g., CB) has been shown to elicit less GVHD, allowing some latitude in using 1–2 HLA disparate donor samples [4], there is not an absence of GVHD, and previous thoughts that fetal liver HSC/HPC transplantation would not generate GVHD in recipients are highly unlikely. So if fetal liver is not an efficient source of transplantable HSC/HPC, there are other cell types one may find in fetal tissue that have potential clinical efficacy. This book reviews a number of different potential uses for fetal tissue.

A new technology that has garnered interest worldwide is that of induced pluripotent stem (iPS) cells [3, 7]. This new technology allows the dedifferentiation and reprogramming of many different mature and immature cell types found in neonatal, pediatric, or adult tissues, including those found in CB, to an ESC-like state by enforced, or other means of induced, expression of transcription factors known to be active in ESCs. Once these immature or mature cells are reprogrammed to this early ESC-like state, they can be differentiated, as can ESCs, to form cells associated with the three germ layers. This exciting new technology has opened up a window into enhanced insight into basic cell regulatory mechanisms and disease processes. As with any exciting new technology, there is the hope that it can be translated into clinical usefulness. However, there are likely many obstacles to overcome for ESCs and iPS cells before they can be used for cell replacement and regenerative medicine [2, 3, 8] and it is possible, although I hope not likely, that

ESCs and iPS cells may not soon, or ever, be ready for prime-time use as a source of transplantable cells. However, these technologies and primitive cell types may lead to breakthroughs and innovative thinking that will be of clinical benefit.

It is important when any new technologies and tissues are identified that suggest possible clinical utility that there is substantial and supportive basic science and preclinical animal model testing that rigorously evaluates the possible use of such treatments prior to moving too quickly into clinical trials in humans [3]. Hype regarding usefulness of treatments sometimes bypass reality, and such cannot and should not allow for human testing before the true facts are in. When the time is right, the clinical efforts must be well controlled to determine whether or not there is safety and, subsequently, true value to the procedure. Too often this is not done and will provide false hope to the patients and, at the worst, cause harm or death to the patient.

It is the review chapters in this book that will help us to learn about, and critically evaluate, potential new treatment modalities with regard to human fetal tissue transplantation.

Indianapolis, IN, USA Hal E. Broxmeyer

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Introduction

Over the past decade, there have been major advances in the field of regenerative medicine with the promise to bring to reality cures for debilitating diseases such as diabetes, heart failure, and Parkinson's disease. Cellular products from a variety of sources are being evaluated for their ability to replace damaged tissue. The isolation and characterization of human embryonic stem (HES) cells offers a unique cell source with the capacity to differentiate into all the cells types within the body. However, HES cells have the potential to form tumors and so limit the use of these cells in the clinical setting. In addition, HES would require matching with the recipient to avoid immunological rejection or other immune toxicities. Adult stem cells may provide a cell source in some limited settings; however, these stem cells have limited capacity for differentiation and finite numbers.

Cellular and organ transplantation has been delivered to patients for more than 50 years with many recipients alive, 20 years or more after transplantation. The success of these transplantations has increased the demand for tissue and organs resulting in increased numbers of patients on transplant waiting lists. The widening gap between supply and demand means many patients die each year while waiting on a donor organ. In the United States, the Organ Procurement and Transplantation Network currently has 110,169 people on their organ waiting list (www.unos.org.org) and a new patient is added every 10 min. In 2008, 27,963 transplants were performed while 23,955 transplants were performed in 2010 for the period January through October. These data suggest that despite the increased demand for organ transplantation, the number of transplants performed has been at the same level for many years.

Fetal tissues consist of stem cells and progenitor cells which have undergone initial commitment with varying states of differentiation. Stem cells from fetal tissues may also have a greater proliferative potential than their adult counterparts. In addition, fetal-derived stem and progenitor cells are immunologically naive, and some sources of fetal cells, e.g., cord blood, have been shown to be capable of crossing greater HLA mismatching resulting in less rejection and decreased immune-mediated toxicities. Given the increasing focus on HES and advances in our basic knowledge of regenerative medicine, it is an appropriate time to review the biology and use of fetal tissues.

There are a number of ethical and moral issues for some individuals around the use of fetal tissues; however, many organs and tissues from fetal sources are discarded on a daily basis. There is a need for dialogue to reach consensus

on how to ensure discard tissue can be made available for treatment of patients. As readers will see from the articles presented in this book, there is data that demonstrates the potential of fetal tissues as a source for regenerative medicine. In fact it may be unethical *not* to utilize this source of tissue and stem cells.

The book deals with aspects of basic biology of fetal tissues and organs, through animal models using fetal tissues and organs to clinical applications. Dr. Peter Hollands presents an overview of basic embryology and tissue development in the embryo and fetus. This chapter describes the development of embryonic stem (ES) cells and the generation of the three germ layers from ES cells (endoderm, mesoderm, and ectoderm) which give rise to all the cells of the body. The reader will learn how differentiation from ES cells leads to development of the organs and tissues of the fetus. Other chapters dealing with basic biology include topics of pregnancy and chimera development by Drs. Stubblefield and Niranjan Battacharya, fetal maternal cell trafficking also by Drs. Stubblefield and Bhattacharya, culture applications of hepatocytes by Dr. Sakai and colleagues, and the response of fetal cells to growth factors by Drs. Pratsinis, Armatas, and Kletsas. Several other chapters present details of specific fetal-derived cells including mesenchymal stromal cells from Wharton's jelly presented by Drs. Weiss, Lopez, and McIntosh; pluripotent stem cells from the early embryo by Drs. De Miguel, Schoorlemmer, and Garcia-Tunon; and placental-derived stem cells by Drs. Moodley and Manuelpillai. Finally in this section, Drs. Pleniceanu and Benjamin Dekel present their studies of fetal stem cells using the kidney as a model organ. The role of the stem cell niche and interactions of cells are highlighted. They also discuss methods for isolation of stem cells from fetal tissue and strategies for defining surface markers for stem cells.

The potential of fetal-derived organs and tissues has been studied in a number of in vitro models and in animal studies. Drs. Sumitran-Hlgersson, KJoshi, and Olausson present data on fetal liver transplantation focused on hepatocyte cells for drug testing, bioartificial livers, and transplantation. Differentiation of human embryonic stem cells has been used by Drs. Tuch, Gao, Wong, and Lees as a model for diabetes. Fetal hepatocytes have been grown in bioscaffolds by Drs. Sakai, Jiang, Hanada, Huang, Kojima, Teratani, and Ochiya, and their studies show promise for support of liver function. Drs Yutzis-Tchorsh and Reisner have identified the optimal time for harvest of fetal pig organs for transplantation into adult animals achieving successful organogenesis of functional organs. Tissue and progenitor cell transplant has been evaluated for the management of pituitary disorders by Drs. Pendleton and Quinones-Hinojosa.

Several chapters discuss the potential clinical applications of fetal tissues in a number of disease including:(1) diabetes (Drs. Joglekar and Hardikar),(2) transplantation of fetal thymus in DiGeorge syndrome (Dr. Touraine), (3) fetal neuron grafting for Parkinson's disease (Drs. Kondo, Okuno, Asari, and Muramatsu), and (4) fetal neural tissue for spinal cord injury repair (Dr. Venkatachalam). Some studies have been initiated in patients, and the results of clinical studies using fetal-derived tissues are presented in chapters by Dr. Bhattacharya.

Many studies have demonstrated the immature nature of the fetal immune system which offers the potential to achieve donor-specific tolerance. Drs. Silva, Koch, Lynch, and Platt discuss the mechanism of tolerance. In utero transplantation has been explored for treatment of congenital disorders, and several chapters discuss this application using hematopoietic stem cells (Drs. Nijagal and MacKenzie) and mesenchymal stem cell (Drs. Troeger, Perahud, Visca, and Holzgreve).

The final chapters of this book present discussion on critical aspects of the use of fetal tissue. Biobanking is a critical requirement for acquisition and availability of fetal tissue and organs, and the state of the art is presented by Drs. Zeisberger, Weber, and Hoerstrup. To round out the book, the final chapter by Dr. Bhattacharya discusses the ethics of fetal tissue transplant.

In summary, this is a timely publication that provides details of many aspects of the potential use of fetal tissues for therapeutic applications. As many tissues are wasted on a daily basis, it is appropriate to raise discussion on how to maximize access to discard tissue and at the same time engage in discussion of the ethics associated with fetal tissue procurement and clinical use.

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Part I

The Ideas Behind This Book

Alternatives of Human Organ/Tissue Transplantation

1

Niranjan Bhattacharya and Phillip Stubblefield

Introduction

The early years of the twentieth century brought a number of revolutionary advances in science and medicine. One of them is vascular anastomosis. This initiated the way for successful organ transplantation as well as avoiding unnecessary amputation of the injured extremities. Organ transplantation as a therapeutic strategy for patients with end-stage organ disease has been successfully attempted due to greater insight into the immunobiology of graft rejection and better measures for surgical and medical management.

It is now known that T cells play a central role in the specific immune response of acute allograft rejection. Strategies to prevent T cell activation or effectors' function are thus all potentially useful for immunosuppression. Standard immunosuppressive therapy in renal transplantation consists of baseline therapy to prevent rejection and short courses of high-dose corticosteroids or monoclonal or polyclonal antibodies as treatment of ongoing rejection episodes. Triple-drug therapy with the combination of cyclosporine, corticosteroids, and azathioprine is now the most frequently used immunosuppressive drug regimen in cadaveric kidney recipients. The continuing search for more selective immunosuppressant and less side effect has led to the discovery of new drugs like tacrolimus, mycophenolate mofetil and mizoribine (which selectively inhibit the enzyme inosine monophosphate dehydrogenase, the rate-limiting enzyme for de novo purine synthesis during cell division), and sirolimus (rapamycin) [which acts on and inhibits kinase homologues required for cell-cycle progression in response to growth factors, like interleukin-2 (IL-2)]. Other new pharmacological strategies and innovative approaches to organ transplantation are also under development.

According to United Network for Organ Sharing (UNOS) from the year 2000, there will be a severe donor organ shortage for those in the USA awaiting transplants, for example, for liver transplants in end-state liver disease. Previous attempts to address this shortage have included bioartificial livers and extracorporeal liver assist devices.

In the year 1999, UNOS reported 66,175 registered patients on their national organ transplant waiting list. Of these, 14,088 patients were registered for a liver transplant. The 1998 human donor liver pool was only 4,487 [1]; the permanent

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whole organ xenotransplants could be a potential alternative [2, 3], because there is a worldwide shortage of organs for clinical implantation and many patients awaiting replacement organs die while they are on the waiting list. What are the scientific options available?

1. Use and potentialities for organs taken from primates and from other species (xenotransplantation)
2. Using stem cell technologies for the end-state degeneration of an important organ
3. Use of clone technology for creation of specific cells/organ solely for the transplantation purpose
4. Tissue engineered three-dimensional organs
5. Artificial organ

Mary Shelley describes in a wonderful science fiction "Frankenstein" how a morally and physically superior creature is constructed taking parts collected from a graveyard. This creature turned violent when its creator rejected him. This is probably the first depiction of the positive and negative impact of the use of the dead body and its organs (1831).

Recent advances in understanding the mechanisms of transplant rejection have brought science to a stage where it is reasonable to consider that organs from other species, probably pigs, may soon be engineered to minimize the risk of serious rejection and used as an alternative to human tissues, possibly ending organ shortages to treat life-threatening and debilitating illnesses such as cancer, diabetes, liver failure, and Parkinson's disease. Other procedures, some of which are being investigated in early clinical trials, aim at using cells or tissues from other species.

Xenotransplantation

Xenotransplantation (xenos- from the Greek word meaning "foreign") is the transplantation of living cells, tissues, or organs from one species to another, such as from chimpanzee to man. Such cells, tissues, or organs are called xenografts or xenotransplants. In contrast, the term allotransplantation refers to a same-species transplant. Human xenotransplantation offers a potential treatment for end-stage organ failure; however, it also raises many novel medical, legal, and ethical issues.

Problem of Disease Transmission in Xenotransplantation

Zoonosis, also called *zoonotic disease*, refers to diseases that can be passed from animals, whether wild or domesticated, to humans. Evidence of the danger of zoonotic infections can be observed in the Ebola (from monkeys) and Nipah (from pigs) viruses, recent outbreaks of which have been observed in humans.

Researchers have postulated that retroviruses such as human immunodeficiency virus (HIV) entered the human population after cross-species infection. Xenotransplantation may increase the chance of disease transmission for three reasons: (a) loss of physical barrier, (b) immunosuppression of the host, and (c) human complement regulators (CD46, CD55, and CD59) expressed in transgenic pigs have been shown to serve as virus receptors and may also help to protect viruses from attack by the compliment system.

Of particular concern with respect to the xenotransplantation of pig organs is porcine endogenous retrovirus (PERV), which is permanently integrated into the pig genome. Other viruses which can affect pigs are herpes virus, rotavirus, parvovirus, circovirus, etc.

Although PERV has been shown to have the capacity to infect human cells in vitro, a recent study of 160 patients exposed to various living pig tissues did not demonstrate PERV infection [4]. Other potential carriers include the following: (a) Pigs are best known for carrying tapeworm but may also carry a large number of other infections including anthrax, influenza, and rabies. (b) Cattle may carry the organisms that cause anthrax, European tick-borne encephalitis, rabies, tapeworm, Salmonella infections, and many bacterial and viral diseases. (c) Dogs may carry plague, tapeworm, rabies, Rocky Mountain spotted fever, and Lyme disease. (d) Sheep and goats may carry rabies, European tick-borne encephalitis, Salmonella infections, and many bacterial and viral diseases. (e) Horses may carry anthrax, rabies, and Salmonella infections.

(f) Cats may carry anthrax, plague, cowpox, tapeworm diphtheria, etc. Unknown viruses, as well as those which are not harmful in the animal, may also pose risks [5].

Of particular concern are PERVS (porcine endogenous retroviruses), vertically transmitted microbes which are embedded in swine genomes. These retroviruses are remnants of ancient viral infections, found in the genomes of most, if not all, mammalian species. Integrated into the chromosomal DNA, they are vertically transferred through inheritance [6]. In addition, through complementation and genetic recombination, two defective PERV genomes could give rise to an infectious virus: there are three subgroups of infectious PERVs (PERV-A, PERV-B, and PERV-C). Experiments have shown that PERV-A and PERV-B can infect human cells in culture. Due to the many deletions and mutations, they accumulate over time; they usually are not infectious in the host species; however, the virus may become infectious in another species In 2005, the Australian National Health and Medical Research Council (NHMRC) declared a 18-year moratorium on all animal-to-human transplantation [7]. This was repealed in 2009 after an NHMRC review stated that "… the risks, if appropriately regulated, are minimal and acceptable given the potential benefits", citing international developments on the management and regulation of xenotransplantation by the World Health Organization and the European Medicines Agency [5].

The FDA requires retrovirus testing on xenotransplant recipients. In addition to the possible risk of animal-to-human infection, theoretically, not only could the transplant recipients be risking infection to their own selves, but they may also be risking infection to their immediate family and possibly even the society at large [8].

Success of Xenotransplantation

Xenotransplantation is not entirely novel, as pig heart valves have been used for many years without apparent ill effect, but they are essentially inert tissue and seldom elicit rejection. Transplantation of pig cells and tissues to treat diabetes and degenerative conditions such as Parkinson's disease and Huntington's chorea have also been done. Another example of xenotransplant is the attempted piscine–primate (fish to nonhuman primate) transplant of islet tissue. The latter research study was intended to pave the way for potential human use. The authors recommend an interesting review on cellular xenotransplantation which summarizes the current knowledge on immunological and functional aspects of xeno(allo)-cellular transplantation for cardiomyopathy, diabetes, liver failure, neural diseases, and bone regeneration just to name a few [9].

Xenotransplantation of ovarian tissue into immunodeficient nude mice has already been used in research to study the development of ovarian follicles. Mature follicles have developed, even after use of cryopreserved ovarian tissue. Both host and graft vessels contribute to the revascularization of xenografted human ovarian tissue in mice [10].

Similarly, human fetal testis tissue xenografts demonstrate normal structure, function, and development after xenografting, including normal germ cell differentiation. This provides an in vivo system to study normal human fetal testis development and its susceptibility to disruption by exogenous factors (e.g., environmental chemicals). This should provide mechanistic insight into the fetal origins of disorders of sex development (DSDs) and testicular dysgenesis syndrome (TDS) disorders. Human fetal testis xenografts are a comparable in vivo ex situ model of normal seminiferous cord formation, germ cell development, and testosterone production [11].

Potential Future Animal Organ Donors

Nonhuman primates were first considered as a potential organ source for xenotransplantation to humans. Since they are the closest relatives to humans, chimpanzees were originally considered to be the best option since their organs are of similar size and they have good blood type com-

patibility with humans. However, since chimpanzees are listed as an endangered species, other potential donors were sought out. Baboons are more readily available; however, they are also not practical as potential donors. Problems include their smaller body size, the infrequency of blood group O (the universal donor), and their long gestation period; moreover, they typically produce few offspring. In addition, a major problem with the use of nonhuman primates is the increased risk of disease transmission.

The biggest challenge in xenotransplantation is overcoming the aggressive response of the human body's immune system to the foreign tissue when antibodies attach to sugar molecules on the surface of the donor organ, for instance, from the pig. The gene that has been knocked out in cloned pigs, a-1, 3-galactosyltransferase, is responsible for making an enzyme that adds the sugar to the surface of the cells. However, two groups of scientists have produced genetically engineered, cloned pigs whose tissue may be suitable for transplanting into humans. Both teams have reported having bred pigs that lack the gene that causes rejection. According to Julia Greenstein, CEO, Immerge Biotherapeutics, Inc., whose company claimed to have knocked out the GATA1 gene, "Another advantage is that preliminary research shows that cells from this line of pigs, in contrast to most other cells tested don't have the capacity to spread porcine endogenous retrovirus to human cells in culture." [12] The potential market for pig organs is huge. PPL Therapeutics (Edinburgh) says that analysts put the value of the market for solid organs at $5bn (£3.6bn; €5.7bn), and cellular therapies for diabetes, Parkinson's disease, and Alzheimer's disease could be worth another $6bn [12]. Another leading concept is the transplant of whole organs from genetically modified pigs, which could make up the shortfall in human organs if immunological and physiological barriers can be overcome [13]. But transgenic pigs may heighten the risk that viruses with lipid envelopes, derived from the host cell membranes from which they bud, will be less likely to be inactivated by human complement [14].

Immunological Problems

To date, no xenotransplantation trials have been entirely successful due to the many obstacles arising from the response of the recipient's immune system. This response, which is generally more extreme than in allotransplantations, ultimately results in rejection of the xenograft and can in some cases result in the immediate death of the recipient. There are several types of rejection organ xenografts are faced with. These include:

a. *Hyperacute Rejection*. This rapid and violent type of rejection occurs within minutes to hours from the time of the transplant. It is mediated by the binding of xenoreactive natural antibodies to the donor endothelium, causing activation of the human complement system which results in endothelial damage, inflammation, thrombosis, and necrosis of the transplant [15]. Since hyperacute rejection presents such a barrier to the success of xenografts, several strategies to overcome it are under investigation like the use of cobra venom to deplete C3, anti-C5 antibodies, or C1 inhibitor (C1-INH) with very limited success. Other attempts include the use of transgenic organs (genetically engineered pigs), 1,3-galactosyltransferase gene knockout pigs [16]. These pigs do not contain the gene which codes for the enzyme responsible for expression of the immunogenic gala- 1,3Gal moiety (the a-Gal epitope). There has also been an attempt to use H-transferase (a-1,2-fucosyltransferase), an enzyme that competes with galactosyl transferase,. This may even reduce a-Gal expression by 70 % [17], and further prevent the expression of human complement regulators (CD55, CD46, and CD59) to inhibit the complement cascade [18].
b. *Acute Vascular Rejection*. This is due to graft endothelial cells and host antibodies, macrophages, and platelets. The response is characterized by an inflammatory infiltrate of mostly macrophages and natural killer cells with small numbers of T cells. The binding of XNAs ultimately leads to the development of a procoagulant state, the secretion

of inflammatory cytokines and chemokines, as well as expression of leukocyte adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) [19]. Overcoming acute vascular rejection includes strategy to prevent acute vascular rejection as well as the use of a synthetic thrombin inhibitor to modulate thrombogenesis; attempted depletion of antigalactose antibodies (XNAs) by techniques such as immunoadsorption, to prevent endothelial cell activation; and also inhibiting activation of macrophages (stimulated by CD4+ T cells) and NK cells (stimulated by the release of Il-2) [19].

Accommodation. If hyperacute and acute vascular rejection are avoided, accommodation is possible, which is the survival of the xenograft despite the presence of circulating XNAs. The graft is given a break from humoral rejection [20] when the complement cascade is interrupted, circulating antibodies are removed, or their function is changed, or there is a change in the expression of surface antigens on the graft. This allows the xenograft to upregulate and express protective genes, which aid in resistance to injury, such as heme oxygenase-1 (an enzyme that catalyzes the degradation of heme) [21].

c. *Cellular Rejection*. Cellular rejection is based on cellular immunity and is mediated by natural killer cells and T lymphocytes. In direct xenorecognition, antigen presenting cells from the xenograft present peptides to recipient CD4+ T cells via xenogeneic MHC class II molecules, resulting in the production of interleukin 2 (IL-2). Interesting strategy to avoid cellular recognition is creation of hematopoietic chimeras. These donor stem cells are introduced into the bone marrow of the recipient. Lymphoid progenitor cells are created by this process and move to the thymus where negative selection eliminates T cells found to be reactive to self. The existence of donor stem cells in the recipient's bone marrow causes donor-reactive T cells to be considered self and undergo apoptosis [22].

d. *Chronic Rejection*. Scientists are still unclear how chronic rejection exactly works, Fibrosis in the xenograft occurs as a result of immune reactions, cytokines, or the healing process. Perhaps the major cause of chronic rejection is arteriosclerosis. Lymphocytes, which were previously activated by antigens in the vessel wall of the graft, activate macrophages to secrete smooth muscle growth factors. It is also anticipated that chronic rejection will be more aggressive in xenotransplants as opposed to allotransplants.

Extensive research is required to solve many outstanding issues like the differences in organ size, longevity, life span, and temperature as well as environmental differences; that is, hydrostructural differences in the hormones and proteins of the donor and the recipient actually limit the range of malfunctions of important regulatory processes in the potential recipients of xenotransplants.

Ethicality of Xenotransplant Procedures

Xenografts have been a controversial procedure since they were first attempted. Many, including animal rights groups, strongly oppose killing animals in order to harvest their organs for human use [23]. Religious beliefs, such as the Jewish and Muslim prohibition against eating pork, have been sometimes thought to be a problem. Some ethical issues include informed consent complexities for research subjects as well as the selection of human subjects, rights of patients and medical staff, and public education (as many companies may go ahead with experiments without public awareness).

As medicine advances at what sometimes seems like lightening speed, it is important for society to make an ethical assessment of technology as it develops, instead of waiting for it to hit the marketplace. All said and done transgenic animal creation remains an answer to the problem of limited organ availability for transplantation purposes; however, the ethical concern will continue to focus on potential zoonosis and the probability of xenorejection.

Looking Further into the Future

Stem Cell Transplantation: To Combat End-State Degeneration of an Important Organ

Embryonal Stem Cell

Stem cells are the holy grail of modern biology. These root cells can, with proper stimulation, be used to produce virtually any type of cell in the body. Until now, the best source of stem cells has been human embryos. These have typically been obtained from fertility clinics. Considerable research is also underway to clone stem cells derived from nonembryonic tissue. The possibility of deriving stem cells from nonviable, asexually produced blastocysts may solve, at least for some, the ethical debate currently raging on the direction of therapeutic stem cell research.

One problem with present human embryonal stem cell technology is the critical problem of histocompatibility as the cells obtained from embryos derived during in vitro fertilization procedures, or from fetal sources, are essentially cells from another individual (allogeneic). This means that they, or any cells made from them, would be at risk of being rejected if transplanted into a human being. To solve this problem, the biotechnology industry is trying to manufacture embryonic cells identical to those of a human adult, this is to say, autologous embryonic cells. To do this, one of the following methods will eventually have to be employed and recent research shows that parthenogenesis might not only be possible but will be less ethically controversial.

1. Somatic Cell Nuclear Transfer: In this technique, commonly known as "human therapeutic cloning," a patient's body cell is combined with an egg cell that has had its DNA removed. As a result, the body cell's DNA is reprogrammed back to an embryonic state, and totipotent stem cells are produced identical to those the patient.
2. Ooplasmic Transfer: In contrast to nuclear transfer, ooplasmic transfer involves the removal of the cytoplasm of an oocyte and transferring it into the body cell of a patient, thereby transforming the patient's cell into a primitive stem cell.
3. Parthenogenesis: In this technique, a woman's oocyte is directly activated without the removal of its DNA to begin development on its own, forming a preimplantation embryo from which totipotent stem cells are isolated. The technique of parthenogenesis appears to both reduce transplantation problems and is perhaps less controversial.

Parthenogenesis is derived from the Greek word for "virgin birth." In modern biology, it refers to a form of reproduction in which an ovum develops into a new individual without having been fertilised. In many social insects, such as the honeybee and the ant, the unfertilised eggs give rise to male drones and the fertilized eggs to female workers and queens [24].

While the ancient Greeks may have been mystified by many elements of molecular biology, they would have easily been able to grasp parthenogenesis, a concept rooted in their oldest myths. Athena, daughter of Zeus, was among the most important of the Greek deities. The Parthenon is the name of the temple of Athena on the Acropolis. She was the patron goddess of Athens. The birth of Athena was most unusual. Classical scholars will probably recall that Zeus swallowed his first wife Thetis when she became pregnant, because he feared that she would bear him a son who would steal his throne. After a few months, he developed a severe headache and he went to his fellow god Hephaestus who history declares was good enough to split his head open with an axe. It is documented that Athena then emerged fully grown and wearing a suit of armor, from the head of Zeus [25].

The parthenogenetic creation of primate embryos with subsequent production of stem cells suggests a new, perhaps somewhat less ethically controversial, direction in research aimed at treating human diseases with stem cell-derived therapies. There is no doubt that the Catholic church would denounce it on the basis of two couples not being involved in the

creation of the initial embryo, but they would have certain difficulties explaining when exactly a life force actually entered the cell to make it a potential human being. This would become more difficult if human parthenogenesis was found to occur by some simple method such as heating or electrifying the cell, which is one of the reasons there are concepts that life is a continuum. Anyway, it is now known that a team of scientists from Mayo Clinic, Sloan Kettering Cancer Center, and Wake Forest University working with a Massachusetts biotechnology company recently managed to create primate embryos parthenogenetically [26].

Cord Blood Stem Cell Use

Hematopoietic stem cells (HSCs) are used in transplantation therapy to reconstitute the hematopoietic system or other failing systems. Human cord blood (hCB) transplantation has emerged as an attractive alternative treatment option when traditional HSC sources are unavailable; investigators have demonstrated that dimethyl–prostaglandin E2 (dmPGE2) increased HSCs in vertebrate models. Preclinical analyses have demonstrated the therapeutic potential of dmPGE2 treatment by using human and nonhuman primate HSCs. It has been shown that dmPGE2 significantly increased total human hematopoietic colony formation in vitro and enhanced engraftment of unfractionated and CD34(+) hCB after xenotransplantation, thus qualifying for FDA-approved phase 1 clinical trial [27].

In the future, we can envisage increasing use of "ex vivo" treatments by living cells, for which cultured human tissues may begin to compete with animal sources, for instance, through the routine preservation of blood stem cells from umbilical cords. In addition, cloning from mature cells, as was done with Dolly the sheep, might allow functional human tissues and, eventually, organs to be regenerated from somatic cells. With advances in reprogramming cellular differentiation, patients may themselves become donors for autografts, making xenografts superfluous for a disease condition which is essentially terminal because of irreversible damage to vital tissues or organs.

Use of Clone Technology for Creation of Specific Cells/Organs Solely for Transplantation Purpose

Human cloning is the creation of a genetically identical copy of a human being, human cell, or human tissue. The term is generally used to refer to artificial human cloning, although human clones in the form of identical twins are commonplace, with their cloning part being a natural process of reproduction, although genes influence behavior and cognition.

The Advantages of Cloning

The fundamental criterion of medical science must be the defense and promotion of the integral good of the human person. Do no harm and try to do good if feasible is the basic of ethics. Hence, every medical procedure performed on the human person is subject to limits determined by respect for human nature itself.

Human cloning (including the cloning of embryos) has stirred controversy in contemporary society since the success in the cloning of Dolly the sheep, in July 1997 at Roslin Institute, Ireland.

However, the potential advantage of cloning of selective cells are (a) saving an endangered species; (b) treatment for infertility – even gays and lesbians can have their own babies with the technique of cloning; (c) selective stem cell cloning; (d) therapeutic application in cancer and congenital, genetic, metabolic diseases, etc.; and (e) possibly the most important advantage is its potential to provide organs for transplantation. If human cloning succeeded, the comparability of the transplantation is 100 %. This can eliminate the various risks of transplantation and hence save more patients. But this concept has received more opposition than agreement. The opponents think that this will deprive the human value of life itself, because a child is made to be born for a predefined purpose, that is, to rescue another's life. The child will therefore become an equipment of therapy.

Clone and Transplant Solution

The most common technique used in human cloning is somatic cell nuclear transfer. Under this technique, the nucleus of an egg cell taken from

a donor is removed. Then the original cell gets fused with another cell of the same genetic material to be cloned. Another technique, which works only with the female, is parthenogenesis. This technique involves the inducement of an unfertilized egg to divide and grow as if it were fertilized. Disadvantages of cloning include uncertainty, inheriting disease or triggering an autoimmune phenomenon of the cloned product of life, as well as an enormous potential for abuse.

Clones would be the best organ donors. Imagine some bright young man suddenly detected to have a cancer of the lung, and he needs to have a transplant. Then after the operation, his body rejects the new, foreign organ. The solution to this problem and the answer to all prayers could be cloning. Cloning is the perfect solution for making many people incredibly happy and helping the human race remain healthy.

But many religious people do not accept this; for example, in Islam, cloning, either of animals or of human beings, is strictly forbidden, because its only Allah who creates all living creatures whether it is insects or birds or sea creatures or reptiles or land animals or trees or human beings. So any scientist or doctor who is doing experiment to create an animal by cloning can only be a devil worshipper or a follower of the devil.

Like everything in the universe, cloning has positive points as well as negative points. It will be considered positive if it is beneficial for mankind and can cause a revolution in medicine through the creation of better genes for a future breed, for instance; it will be considered negative if it causes any harm to society. According to the Organ Procurement and Transplantation Network (OPTN), 28,356 Americans received organ transplants in 2007 – around 78 % of those came from deceased people. Yet as of August 2008, more than 99,000 people in the United States were on the national waiting list for organs. The same waiting list for 18 April 2011 is 110,757 [28].

In a science–fiction movie, "The Island," Hollywood heartthrobs Scarlett Johansson and Ewan McGregor play dual roles portraying the rich and famous – and their genetically identical clones. In an appropriate Orwellian twist, doctors must murder the "spare" clones in order to harvest needed body parts.

3D Tissue Organ Culture

Biofabrication can be defined as the production of complex living and nonliving biological products from raw materials such as living cells, molecules, extracellular matrices, and biomaterials. An engineering approach translating into a developmental biology concept of embryonic tissue fluidity enables the creation of specific tissue and organ assembly. This tissue engineering technology promises to solve the organ transplantation crisis. However, assembly of vascularized 3D soft organs remains a big challenge [29]. Computer-assisted organ printing, along with 3D tissue engineering of living human organs, can offer hopes for the future. Organ printing depends on "blueprints" for organs or actual organ printing, organ conditioning, and accelerated organ maturation. A cell printer that can print gels, layer-by-layer sequentially placed and solidified thin layers of a thermoreversible gel, could serve as "printing paper" [30].

Artificial Organ Support for End-State Organ Failure Waiting for the Transplant

An artificial organ as transplant substitute is a man-made device that is implanted or integrated into a human to replace a natural organ for the purpose of restoring a specific function or a group of related functions so the patient may return to as normal a life as possible. There are practical devices to assist each and every system as noted below.

a. *Brain Pacemakers, Including Deep Brain Stimulators*: This instrument can send electrical impulses to the brain in order to relieve intractable epilepsy. The therapeutic effect of electrical stimulation on the brain has been studied for decades. Currently, the thalamus, subthalamic nucleus, hippocampus, cerebellar

nuclei, and cortical seizure foci are stimulated for treating epilepsy [31]; other indications include depression, tremors of Parkinson's disease, and other neurological conditions such as increased bladder secretions. Other neurostimulators that are therapeutically effective, include brain stimulations with vagus nerve stimulator (VNS), depth electrodes, subdural electrodes, external responsive neurostimulator, implantable brain stimulator, and transcranial magnetic stimulator [32]. These devices often serve by disrupting the output of existing malfunctioning nerve centers to eliminate symptoms.

b. *Cardia and Pylorus Valves:* The closure systems of the gastrointestinal canal are based on three major forms: constrictive sphincter, dilatory closure, and kinking closure. A constrictive sphincter is attached to the esophageal inlet and a dilatory closure to the cardiac orifice. Reflux esophagitis may result from failure of dilatory closure. Helical fibers in crosswise arrangement rather than isolated sphincters are recordable from the pyloric region. Their function is identical with that of dilatory closure. The principle of kinking closure applies to the Papilla Vateri and the ileocecal valve [33]. For therapeutic approach to problems of the closure systems, artificial cardia and pylorus can be used to fight esophageal cancer, achalasia, and gastroesophageal reflux disease. This pertains to gastric repairs, specifically of the valves at either end of the stomach.

c. *Corpora Cavernosa:* To treat erectile dysfunction, both corpora cavernosa can be irreversibly surgically replaced with manually inflatable penile implants in case of men suffering from complete impotence that has resisted all other treatment approaches. Two kinds of surgical procedures for augmenting penile corpora cavernosa were proved to be effective and reliable, with few complications. Both saphenous grafts and expanded polytetrafluoroethylene (ePTFE) artificial vessel patches are excellent materials for reconstructing the tunica albuginea. These augmenting phalloplasties can not only be used for patients with micropenis but also applied to satisfy the cosmetic and functional requests of patients with normal penile length and perimeter [34].

d. *Ear*: Cochlear implant is appreciated by most recipients. Thin-film array electrodes coupled with an insertion test device (ITD) have been successfully inserted into the human cochlea with limited trauma. With continued development and testing of this electrode design, the thin-film array may improve language perception achieved through cochlear implantation [35].

e. *Eye*: Visual prosthetic material is the most successful function-replacing artificial eye and so far is actually an external miniature digital camera with a remote unidirectional electronic interface implanted on the retina, optic nerve, or other related locations inside the brain. Semichronic implantation of a microelectrode–STS system has shown that it is safe and remains functional [36].

f. *Artificial hearts* are limited to patients awaiting transplants whose deaths are imminent; these can extend life up to a maximum of 18 months [37]. Artificial pacemakers are electronic devices, which can either intermittently augment (defibrillator mode), continuously augment, or completely bypass the natural living cardiac pacemaker as needed, and are so successful that they have become commonplace. Ventricular assist devices are mechanical circulatory devices that partially or completely replace the function of a failing heart, without the removal of the heart itself [38].

g. *Artificial Limb*: Artificial arms with semifunctional hands, some even fitted with working opposable "thumbs" plus 2 "fingers," and legs with shock absorbing feet capable of allowing a trained patient to even run have become available [39, 40].

h. *Artificial Liver*: Liver dialysis, liver dialysis devices, and hepatocyte: HepaLife is developing a bioartificial liver device intended for the treatment of liver failure using stem cells. It is only made possible by the fact that it uses real liver cells (hepatocytes), and even then, it is not a permanent substitute for a liver [41].

i. *Artificial lungs* promise to be a great success in the near future. An Ann Arbor company MC3 is currently working on this type of medical device. Actually, the artificial lung is a technical device for providing life support; it will be put in use when the natural lungs are failing and are not able to maintain sufficient oxygenation of the body's organ systems. From the viewpoint of long-term development, the artificial lung should be permanently implanted in the body, so that it will substitute for the human pulmonary function partially or completely [42].
j. *Artificial pancreas* is used for the treatment of diabetes; numerous promising techniques incorporate donated living tissue housed in special materials to prevent the patient's immune system from killing the foreign live components [43].
k. *Artificial bladders* represent a unique success in that these are autologous laboratory-grown living replacements [44].
l. *Artificial Ovaries*: An artificial human ovary can be created with self-assembled human theca and granulosa cell microtissues and used for IVM and future oocyte toxicology studies [45]. This will combat complications of early menopause with self-assembled microtissues created using novel 3D Petri dish technology, for the maturation of immature oocytes and the development of a system to study the effect of environmental toxins on folliculogenesis.

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2 Fetomaternal Cell Trafficking: A Window into the Long-Term Health Effects of Treating Disease with Fetal Cell/Tissue Transplants?

Niranjan Bhattacharya and Phillip Stubblefield

Introduction

During pregnancy, fetal hematopoietic cells carrying paternal human leukocyte antigens (HLA) migrate into maternal circulation, and vice versa; maternal nucleated cells can be detected in fetal organs and umbilical cord blood, indicating the presence of bidirectional cell traffic between mother and fetus. The result is that both mother and infant exhibit microchimerism (Mc); that is, both have small numbers of cells and cell-free DNA that come from two genetically distinct individuals. Evidence is mounting that this is not a rare phenomenon, but that it occurs commonly in humans and other placental species, and that the chimeric cells may persist indefinitely in many individuals with no evidence of an immune attack against those cells. In this chapter, we briefly review maternofetal immune tolerance and explore what is known about the effects of fetomaternal (FMc) and maternofetal microchimerism (MFc) on the biology of both individuals in health and in illness. Perhaps better knowledge of these effects of microchimerism will instruct as to how long-term allogeneic coexistence within an organism can impact chronic disease, cancer biology, regenerative medicine, and fetomaternal immunology.

Fetomaternal transfer was apparently first reported in 1893 when Schmori observed fetal in the tissues of women who died of eclampsia [1, 2]. Multiple investigators demonstrated fetal leukocytes in maternal circulation in the 1960s and 1970s [2]. The 1967 report by Taylor of cases where rhesus D-positive cells were found in genetically rhesus-negative fetuses, the so-called grandmother effect, is an early example of maternofetal trafficking [3]. Fetal cells can persist for decades in the mother. Bianchi and colleagues isolated male progenitor cells (CD34+CD38+) in six of eight women who had previously borne sons. In one case, the woman's last son was born 27 years prior [4]. Investigators have used polymerase chain reaction (PCR) and in situ hybridization analyses to detect the Y-chromosome and have demonstrated male DNA in maternal plasma or serum samples from 70 to 80 % of women tested and 17 % positivity for male cellular material [2, 5].

Studies of fetal cells and serum in the maternal circulation are now noninvasive prenatal diagnosis. The first use was to identify the Rh type of the fetus in Rh-negative women sensitized to the Rh factor [6]. These efforts have progressed

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to the point where current investigators are using new means for rapid sequencing of DNA to determine specific portions of the fetal genome by analysis of chromosome fragments in cell-free fetal DNA from maternal serum [7] and are close to being able to determine the entire fetal genome from a single fetal cell obtained from the maternal circulation (Van de Vyver I, 2011, personal communication).

Maternal Tolerance of Fetal Tissue

How the maternal organism tolerates the microchimera of fetal/maternal antigens is not fully understood. The mechanisms that allow this tolerance must be related to the other examples in nature where the homograft is tolerated by the host: how does the mother tolerate the entire fetus for 10 lunar months, and why are neoplasms tolerated?

Many local and systemic mechanisms have been identified that may contribute to the success of a pregnancy. Trophoblast expresses the nonclassical HLA molecules HLA-E, HLA-F, and HLA-G. While the function of HLA-F is unknown, protection of the fetus from allogeneic T-cell responses and NK cell-mediated damage has been attributed to HLA-G [8]. HLA-G can also induce apoptosis of lymphocytes which have been previously activated through the Fas/FasL pathway [9]. Apoptosis may be an important determinant in fetomaternal tolerance coming from studies which suggest that maternal tolerance of the fetus may be mediated by the Fas/FasL system, which plays a critical role in promoting apoptosis and was also identified some years ago as an important pathway for controlling maternal immune responses at the fetomaternal interface [10, 11]. Meanwhile, it has also been hypothesized that the effect of HLA-G on NK cell activity is not induced directly, but rather, that it requires the expression of HLA-E on trophoblastic cells.

It is also possible that fetomaternal tolerance is promoted through the active role played by indoleamine 2,3-dioxygenase (IDO). Contemporary evidences from a study by Munn et al. [12] suggest that synthesis of this tryptophan-catabolizing enzyme by placental cells can provide protection of the fetus from maternal T cells, with the observation that inhibition of this enzyme during murine pregnancy resulted in fetal allograft rejection. IDO is expressed by extravillous and villous trophoblast cells in humans, and its expression increases during the first week of pregnancy and diminishes during the second trimester [13]. A role for the complement system has also been hypothesized in the control of fetomaternal tolerance. This system is a component of natural immunity that can be activated by pathogens and also after transplantation of allogeneic or xenogeneic cells, resulting in induction of inflammatory cell chemotaxis, enhanced phagocytosis, and promotion of cell lysis by the membrane attack complex [14]. Recently, in the stromal layer of the amniotic membrane, two subpopulations of cells have been identified which differ in their expression of HLA-DR, CD45, CD14, CD86, and CD11b and which possess either T-cell suppressive or stimulatory properties [15]. Even though the roles of these two populations in the amniotic membrane are not yet known, it is tempting to speculate that they may both play a role in controlling fetomaternal tolerance. We have tried herein to briefly recapitulate the existing controversy on the mechanism of fetomaternal tolerance in placental pregnancy. Further clarification is provided by Dr Ornella Parolini et al., in our previous publication [16].

The survival of the pregnancy homograft also depends on a fine well-coordinated systemic and uterine support by the reproductive hormones progesterone (PR), estrogen (ER), human chorionic gonadotropin (HCG), prolactin (PRL), and others. These hormones and their cytokine hormonal nexus are essential for maintaining the pregnancy-specific immunomodulation, including its up and down regulations, for the entire pregnancy leading to the successful birth of a baby.

Recent studies in mice and humans have highlighted the significance of fetomaternal microchimerism in the induction and maintenance of CD4+CD25+ and CD8+ T regulatory cells that counterbalance the immune responses to fetal or maternal antigens mediated by T effector cells.

Consistent with these observations, T-cell-replete hematopoietic stem cell transplantation between mutually microchimeric mothers and their HLA-haploidentical offspring has been shown to be feasible, although the degree of microchimerism-associated tolerance appears to substantially differ among cases [17].

Neoplastic cells may be a special case, as they do not usually cross the placental barrier [18, 19]. Whereas placental metastases of the maternal malignant disease appear quite often, the risk of fetal involvement seems to be different between entities [20]. A possible defense mechanism has been recently brought up as a tendency of the malignant cells to be transformed back to benign cells in the fetal environment [21]. This mechanism might be insufficient to prevent the fetus from certain infections by cellular organisms, such as trypanosome or toxoplasma. In such cases, in the infected placentas, a destructive villitis and phagocytosis by Hofbauer cells support the transfer of infectious cells into the fetal circulation [22, 23]. For normal pregnancies, findings suggest that the maternofetal transplacental cell traffic does not appear in a dose-dependent manner, since an increased maternal white blood cell count does not correspond to the WBC count in the neonate [24]. Additionally, the "placental barrier" must be able to differentiate between normal and abnormal leucocytes, most probably through a receptor–ligand interaction, since it has been known for long that normal leukocytes traffic, whereas obviously neoplastic leukocytes only rarely cross into the fetal blood [25]. Maternal multipotent stromal cells, for instance, use a VEGF-A- and integrin-dependent pathway [26]. A further indication that the transfer is not dose- and size-dependent comes from the observation that the amount of maternofetal traffic is lower than the cell transfer from the fetus into the mother; the fetomaternal and maternofetal transfers do not correlate with each other [27, 28]. Both phenomena have implications on the health status of the chimeras in terms of immunization and tolerance [29]. It has been noted that occasional transfer of maternal lymphocytes into the fetus might lead to chimerism and possible graft-versus-host disease [30, 31]. It seems that microchimerism within the fetus might have different implications than similar cell traffic into the mother due to differences in the maturity of the immune system. Opposite outcomes of microchimerism in terms of tolerance or alloreactivity are also observed after solid organ transplants. Here, microchimerism in hosts with immature T cells have resulted in tolerance, whereas in immunologically mature hosts, immunity and graft rejection have occurred [32]. This may explain why maternal cells can persist in the offspring until adult life in individuals with a normal alloreactive immune system [33]. These cells may play an active role in the development of the fetal immune system.

Maternal activated T cells cross the placenta and can induce antigen-specific tolerance in developing fetuses. This may explain why certain individuals are to a certain degree tolerant to non-inherited maternal human leukocyte antigens (NIMA) [34, 35]. Whether microchimerism leads to beneficial regenerative effects or to autoimmunity might be related to MHC zygosity since it has been shown that low levels of microchimerism are associated with MHC heterozygosity, which tend to protect from autoimmunity [36, 37].

Microchimerism and Transplant Tolerance

Whether microchimerism influences transplantation tolerance is yet to be understood. Long-term acceptance of a recipient's cell in the donor may have a favorable or an unfavorable effect on the development of severe graft-versus-host disease (GVHD); or donor cell microchimerism in the recipient may facilitate graft acceptance [38].

The transfer and persistence of maternal leukocytes and stem cells in the fetal organs and blood may have implications for the use of umbilical cord blood for stem cell transplantation and for the vertical transmission of infectious agents [39, 40]. Increased GVHD has indeed been reported with syngeneic hematopoietic stem cell transplantation when either the donor or the recipient is parous compared with nulliparous women or men [41]. However, a large study that

analyzed living donor renal transplants to parents from their offspring found no difference in graft survival at 3 years in the mothers than in the fathers [42].

Microchimerism and Maternal Illness

Although massive transplacental transfer of red blood cells has probably the most serious consequences in regard to anemia and immunization, traffic of other blood particles is also of interest. The traffic of fetal cells into the maternal circulation and their persistence over decades may have implications on later maternal autoimmune disorders. This appears to be true for systemic sclerosis (scleroderma) [43]. Though one study revealed HLA compatibility as a risk factor for systemic sclerosis, another study did not [44, 45].

Increased levels of FMc were described in the peripheral blood of women with systemic sclerosis [46].

There is also some evidence that maternal microchimerism is associated with myositis, since two independent groups have described increased levels of maternal cells in the offspring's blood and tissue compared to a control group of unaffected siblings [47, 48]. Another possible example is neonatal lupus erythematosus, which is associated with maternal autoantibodies and maternal HLA genotype [49, 50]. A recent review has provided further detail [51].

Microchimerism may also be associated with beneficial effects for the mother in some autoimmune diseases. Though most autoimmune diseases do not improve in pregnancy, rheumatoid arthritis is ameliorated in pregnancy for most women [2]. FMc cells measured in blood samples throughout pregnancy were found to be higher when the arthritis was in remission and lower levels when the disease was active [52].

Even greater possibilities for maternal benefit are suggested in the works of Zeng and colleagues in the murine model [53]. They showed that pregnancy-associated progenitor cells (PAPCs) integrated and persisted in the maternal brain for up to 7 months after delivery. The PAPCs in the maternal brain expressed mature neuronal markers and displayed neuronal maturation that was not a consequence of cell fusion with maternal neural cells. In additional studies in mice with experimentally induced Parkinson's disease, the spatial distribution of PAPCs within the hippocampus was altered, suggesting that the disease context influenced initial attraction, long-term survival, and spatial distribution of PAPCs [53]. In summary, current knowledge of the biology of microchimerism and long-term allogeneic coexistence within the organism may alter existing paradigms of chronic disease.

Experience with Fetal Tissue Transplants

Since microchimerism with FMc cells is known to persist for decades in the host in some cases, what do we know about the fate of intentional grafts of much larger quantities of fetal cells in efforts to treat illness? Our group has previously reported on a series of patients suffering from severe Parkinson's disease. All were treated with grafts of human fetal neuronal tissue taken from consenting patients at the time of induced abortion [54]. The grafts were placed in a small pocket created in the anterior axillary fold as previously described [54]. In those 48 cases, the grafted tissue was surgically removed and studied at various intervals for as long as 10 years after grafting. None of the patients experienced any features of graft-versus-host response, and in all cases, the grafted fetal tissue was normal when examined after removal. There was no histologic evidence of graft rejection in any case. To this group, we add another case described below of the examination of grafted fetal tissue after a 12-year interval.

An Illustrative Case

Mr A.S., 27 years, Muslim, male, came to our outpatient department at Bijoygarh State Hospital, Calcutta, India, on 19 January 1999 on a stretcher with severe anemia (hemoglobin 5.6 g %) with pitting edema of the legs, COPD (chronic obstructive pulmonary disorder) aches

and pain all over the body, advanced rheumatoid arthritis (rheumatoid factor 370 mg), hypothyroidism (thyroid-stimulating hormone 11.2 Units), bed sores, and features of poor nutritional condition present. He was immediately admitted in the hospital. After investigations confirmed his diagnosis, antirheumatoid treatment was started with methotrexate 7.5 mg weekly schedule, Sazo 500 mg thrice daily, chloroquine 300 mg once daily, omeprazol 20 mg twice daily, salmeterol inhaler twice daily, eltroxyn 100 μg and other supportive hematinics, calcium and trace elements with nutritional supplements, and gradual physiotherapy for his fixed swollen knees. After obtaining Institutional Ethics Committee permission (which was prepared as per Indian Council of Medical Research guidelines), the patient was given a subcutaneous heterotopic fetal brain tissue transplant at the axillary site under local anesthesia on 26 January 1999. The patient was discharged after 15 days. He did not report to our outpatient department again in spite of repeated telephonic reminders and official letters.

On 24 February 2011, he appeared at Vidyasagore State Hospital OPD where he met the present author and offered profuse thanks. He stated he was practically free of disease and had not taken any antirheumatic drug for the last 12 years. Upon questioning his refusal to attend OPD, he said he was afraid that the fetal tissue would be retrieved and his crippling state would reappear. Now, after 12 years, he agreed to permit retrieval of the graft. Histologic study of the excised graft revealed the persistence of the fetal neuronal tissue as noted in the microphotographs attached below which was stained with hematoxylin and eosin and seen in different magnifications (Figs. 2.1, 2.2, and 2.3):

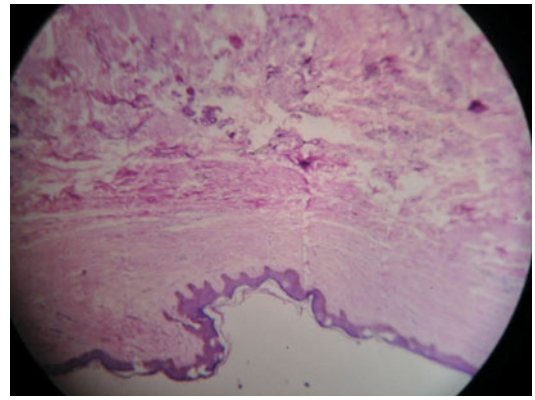

Fig. 2.1 Scan power (magnification 25 times) hematoxylin and eosin (H&E) stained 16 weeks fetal neuronal tissue seen after its retrieval on the twelfth year from the axillary site

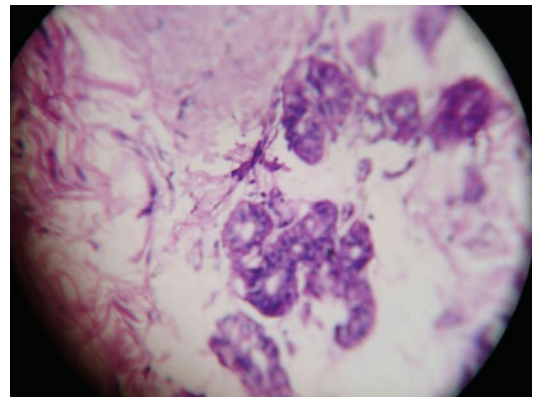

Fig. 2.2 Low-power (magnification 80 times) hematoxylin and eosin (H&E) stained 16 weeks fetal neuronal tissue seen after its retrieval on the twelfth year from the axillary site

Discussion

Persistence of fetal cells/tissue at the site of transplantation is seen in the retrieved tissue after more than a decade without provoking any clinical graft-versus-host reaction, autoimmunity, or any other specific problem. In this case as in the others already reported [54], the host was not pregnant, there was no selection based on HLA testing, no immunosuppressive treatment was used, and yet the grafted fetal tissue survived with apparent benefit to the patient. These are pilot studies. Controlled trials will be needed to confirm the benefit.

Why does this fetal tissue persist? Human fetal neuronal tissue from the cerebral cortex has survived in an extracortical site, which is not a privileged site such as the brain or the pregnant uterus. That there is no tumor formation or any other abnormal vascularity or any other acute chronic or subacute immunological or inflammatory condition is seen in the microscopic view and positively

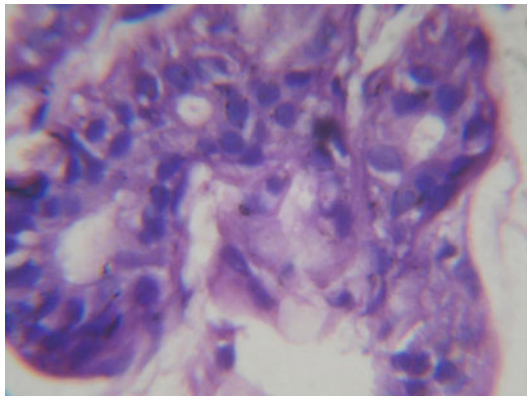

Fig. 2.3 High-power (oil immersion, magnification 375 times) hematoxylin and eosin (H&E) stained 16 weeks fetal neuronal tissue seen after its retrieval on the twelfth year from the axillary site

corroborated with clinical evidences from the patient. The patient also cited clinical remission of the disease despite discontinuation of anti-rheumatic drugs for 12 years.

The fetal cellular or tissue microenvironment is distinctly different from adult cellular/tissue neuroendocrine or metabolic microenvironment [55]. Hence, the most important answer should be that the fetal cells create their own niche that either prevents recognition by the host immune system, or alters the immune response, so that the graft is protected. In order to prevent recognition by the adult host system, the graft must downgrade its recognition antenna by altering its own cytokine network, thus behaving as stem cell or primitive progenitor cell, the molecular mechanism of which is presently under our intense scrutiny.

Here, we want to share our clinical experience with grafting of fetal tissue to treat illness in human volunteers for more than a decade:

(a) Sex-randomized fetal cells may survive in maternal/paternal or HLA and sex-unselected allogeneic hosts of any age group at least for more than a decade from its placement without support of any immunosuppression by drug or radiation.
(b) The fetal cells have not caused any detectable graft-versus-host reaction in hundreds of cases followed to date, many for more than a decade.
(c) The grafted cells that survive the maternal immune system either do not express any HLA or create their own niches of cytokine support which prevent immune rejection.
(d) This behavior mimics the mesenchymal stem cell, stem cell type, or progenitor cell behavior.
(e) Engrafted fetal tissue appears to be immunologically naïve; this behavior in the host provides it with a privileged status.
(f) The naivety depends on progression of gestation and immune stimulation, acquiring the phenomenon of immunocompetence.
(g) Gradual and selective transfer of fetal cells to the mother may have an immunomodulatory, rejuvenating impact on the maternal system.
(h) Compared to fetomaternal transfer, much less transfer of maternal cells to the fetal system may have an identical effect of immunomodulation. The suppression or stimulation of the mother cells in the fetal system will depend on the maturity of the fetal system. Under normal circumstances, the maternal cell load is mostly beneficial and rarely catastrophic.

Conclusion and Suggestions for the Future

Fetal tissue is an ensemble of varying stage and grade of differentiated and undifferentiated cells including fetal stem cells and stem cell-like progenitor cells, not necessarily identical, but from the same origin, that together carry out a specific function. The classical definition of cell therapy describes the process of introducing new cells into a tissue in order to treat a disease.

Fetal tissue contains mostly immunologically naïve cells, depending on the stage of gestation, with their protective microenvironment. Together, they constitute the niche for the fetal cell/tissue. Transplantation of fetal tissue has many unique properties. The retrieval of freshly collected fetal tissue transplanted into an accessible vascular site after over 10 years, showed the persistence of a group of primitive progenitor cells, thus suggesting the possibility that the graft

created its own microenvironment for its survival. The transplanted tissue gave no evidence of host response even after decades. Their persistence raises the question of whether fetal cells could migrate to the site of requirement or injury. Such migration has been described in studies in the mouse where fetal cells survive and sometimes differentiate similarly to host cells [53]. Transmigration to the site of injury fulfills the requirements of an ideal cell therapy. Thus, freshly collected fetal tissue may be redefined functionally as a cluster of fetal primitive cells with a varying degree of differentiation according to the microenvironmental niche, which may be used sometimes for effective therapy for a desperate or degenerative disease of the allogeneic host, if placed at a vascular site.

As with early experiments with vaccination to prevent infection, attempts at allogeneic fetal tissue transplant have been met with controversy since their inception, based on intrinsic suspicion and word of mouth, or unfounded information on the basis of animal experimentations or because of ethical, moral, political, scientific, medical, safety concerns for the future, religious, and other technical/social grounds. It is our hope that other medical scientists and clinicians will carry this work forward and further explore the efficacy of this simple means for regeneration of injury with fetal tissue implanted at peripheral vascular sites. We feel that this method of treatment has the potential to prevent the progression of many presently incurable degenerative diseases.

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Part II

Basic Science and the Unique Aspect of Fetal Growth and Maturation

Embryology of Fetal Tissue

3

Peter Hollands

Basic Preimplantation Embryology

When the first ever clinical embryologist, Bob Edwards, saw the fertilization of a human oocyte in vitro he was witnessing not only a momentous event in clinical medicine but also the creation of totipotent stem cells [249]. At the point of fertilization and up to the point of early compaction, the blastomeres of the human embryo are generally considered to be totipotent stem cells. These totipotent stem cells can differentiate into all cell types and therefore have the ability to create a complete new individual [60]. It is possible that blastomeres are totipotent to enable correction of early developmental errors in the embryo [87]. The data on totipotent stem cells come from experimental embryology using animal embryos as the legal and ethical restrictions on human embryo experimentation restrict such work [84]. From this animal experimentation, it is known that totipotent stem cells can develop into endoderm, mesoderm and ectoderm, germ cells, extraembryonic tissue, and trophoblast. In the mouse embryo, asymmetric divisions at the eight-cell stage result in two populations of cells [111]. The inner cell mass (ICM) of the blastocyst then develops from cells positioned inside the embryo, and those cells on the outside of the embryo develop into the trophectoderm which subsequently develops into the placenta [58, 72, 203]. Two ICM cell types then develop [76] which are:

1. The epiblast (EPI) which are pluripotent cells which form the future body and
2. Extra embryonic primitive endoderm (PE)

The overall mechanism of the cell differentiation at this stage is unknown but there is evidence to show that EPI and PE cells, carrying the markers *Gata6* and *Nanog*, are initially randomly distributed within the ICM and that they then move in an actin-dependent process to create the two distinctive cell lineages [38, 176, 207, 221]. Recent evidence shows that the origin of EPI and PE cells in the ICM is determined by the wave of cell division with those cells dividing late and on the inside forming PE [183]. This results in the presence of PE progenitors on the surface of the ICM and EPI progenitors deep inside the ICM. *Sox17* expression is also important for PE development in vitro suggesting that *Sox 17* is important in endoderm development [119, 211, 238].

The epiblast (EPI) is therefore the embryonic source of ectoderm (forming skin, central nervous system, and mammary/sweat glands), endoderm (forming gut, liver, and lung), and mesoderm (forming muscle and bone, connective tissue, bone marrow/blood cells, and allantois/yolk sac).

In 1981, Evans and Kaufman [67], and Martin [169] first described the creation of mouse embryonic stem cells (ES cells) from the ICM of mouse blastocysts. ES cells have been very useful in the study of mammalian development in vitro, but more recently evidence has been emerging to

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suggest that ES cells closely resemble the epiblast (primitive ectoderm). This is supported by observations that isolated EPI gives rise to ES cells better than those derived from isolated ICM and that isolated EPI can also give rise to ES cells in mouse species where previous attempts using isolated ICM failed [263, 296]. The field then extended to nonhuman primate ES cell derivation [267] and culminated in the derivation of human ES cells [268].

The Hemangioblast

It was in the early twentieth century when the concept of the hemangioblast was first proposed based on cells observed in the yolk sac giving rise to both blood cells and blood vessels [94, 223]. More recently, blast-colony-forming cells (BL-CFC), capable of forming both endothelial and hemopoietic cells, have been derived from ESC in media supplemented with vascular endothelial growth factor-A (VEGF-A) and bone morphogenetic protein-4 (BMP4) [40, 122, 165, 194]. Nevertheless, other workers have shown that hemopoietic and endothelial cells are independently derived from mesodermal cells [73, 128]. It is clear that the molecular identity and tissue plasticity of hemangioblasts are still poorly defined with a complex set of signals including BMP4, hedgehogs, fibroblast growth factor (FGF), and VEGF-A contributing to the differentiation of hemopoietic and endothelial cell lineages [30, 33, 47, 59, 147, 201, 284]. The use of genetically modified ESC has shown that BL-CFC formation in vitro is controlled by:

- Flk1 and SCL [41, 93]
- Scl [233]
- Transcription factor SCL/tal1 [218]
- Runx1 [138]
- Gata2 [164]
- Lysocardiolipin acyltransferase (Lycat) [280]
- Smad1 [291]

It is clear that the BL-CFC is a complex cell which interacts with many cells and proteins during its development and can be considered as the in vitro equivalent of the originally proposed hemangioblast. The hemangioblast has more recently been described as being present in the posterior portion of the primitive streak of the day 7 mouse embryo which then migrates to the extraembryonic mesoderm in the yolk sac [105]. These observations confirm earlier work on the identification of hemopoietic stem cells in day 7 mouse embryos capable of rescuing lethally irradiated and genetically anemic mice [96–99].

Experimental analysis of human ESC has shown that both hemopoietic and endothelial cells can be derived in vitro [120, 152]. This led to the identification of potential hemangioblasts in both mouse [40, 105] and human ESC [290]. Similar work has shown that there is a CD45-hemogenic endothelial cell expressing PECAM-1, Flk1, and VE-cadherin ($CD45^{neg}$PFV cell) which can be identified from day 10 human embryoid bodies [279]. As a natural progression from these observations, Kennedy et al. [122] have isolated a true BL-CFC from human embryoid bodies capable of producing endothelial and hemopoietic lineages, and Lu et al. [165] have shown that these BL-CFC can contribute to vascular regeneration of damaged blood vessels.

Growth and Maturation of Fetal Tissue

Hemopoietic Tissue

The developing fetus contains a complex and interacting array of tissues. The first and arguably the most critical tissue developing in the human fetus is the hemopoietic tissue which enables erythropoiesis and the oxygenation of the rapidly increasing fetal tissue mass. In humans, erythropoiesis begins in the yolk sac at 3–4 weeks of gestation. Large nucleated red blood cells develop (known as megaloblasts), and these cells contain predominantly embryonic hemoglobin [205]. The site of hemopoiesis switches to the liver by week 8, and by this time the cells are nonnucleated macrocytes. Finally, by week 11–12 of gestation hemopoiesis begins in the bone marrow, and liver hemopoiesis stops at birth [285].

The development of hemopoietic cells in the developing fetus has been found to be mediated

by time-dependent changes in the genetic programming of progenitor cells [247]. Subsequent in vitro studies using mouse ES cells have confirmed these concepts [230]. ES cell cultures have shown visible blood islands by day 8 of culture [53] and the cells are expressing *Epo* and *Epo receptor* (*EpoR*) genes [230].

While hemopoiesis appears to begin in the fetal yolk sac, a pre-yolk sac region in the fetus known as the dorsal aorta, genital ridge/gonads, and pro/mesonephros (AGM) has been identified as a very early source of multipotent hemopoietic progenitors [175]. These cells arising in the AGM are now generally accepted as being the origin of the definitive adult hemopoietic stem cells which colonize the fetal liver. Nevertheless, there is evidence to suggest that the precursors of primitive and definitive erythroid cells may be different types of cells [188]. The true identity of the precursor of the hemopoietic stem cell in the developing fetus is unknown, but the precursor cell may also have a dual role in endothelial cell formation as blood island formation, and vasculogenesis does not occur if the endothelial growth factor receptor *Flk-1* is absent in *Flk-1*-deficient mice [236]. It is also interesting to note that both endothelial cells and erythroid precursors have erythropoietin receptors and that erythropoietin has both a mitogenic and chemotactic effect on endothelial cells [5]. The principle Epo receptor on endothelial cells is the truncated receptor which is also that found on early erythroid progenitors [6]. The common embryological source of endothelial and hemopoietic precursors therefore seems highly likely.

The Adult Hemangioblast

Several workers have recently proposed the possible existence of hemangioblasts in adult tissue [16, 227]. Multipotent mesenchymal stem cells have been isolated both mouse and human bone marrow which are capable of producing mature endothelial cells both in vitro and in vivo [110, 216]. In addition, CD133+/CD34- cells isolated from mobilized human peripheral blood have been shown to be capable of producing either neutrophil or endothelial cells [162]. Such cells have been further analyzed in human and mouse using clonal analysis to reveal that they can differentiate into functional endothelial cells in vivo [17, 44, 80]. In the same context, vascular cells taken from the adult aorta or vena cava in the mouse can form hemopoietic cells in lethally irradiated recipient mice [182]. The hemangioblast clearly persists into adult life although its physiological role remains unclear.

Immune Privileges of Growing Fetal Tissue

Maternal-Fetal Tolerance

The establishment of human pregnancy requires a set of unique immunological processes which introduce the concept of "immunological privilege" and begins to explain how tolerance to tissue-specific antigen is attained [244]. The villous placental syncytiotrophoblast cells and the invading cytotrophoblast cells are in direct contact with maternal peripheral blood lymphocytes [21, 243]. The uterine leucocytes present at the fetal-maternal interface include natural killer cells, macrophages, dendritic cells, and T cells [86]. Potent immunomodulators are present on the trophoblast at the fetal-maternal interface including FasL, IDO, CD200, TRAIL, B7-H1, and galectin-1 [156, 206, 277]. The same immunomodulators have been proposed as being active in decidual cell-immune cell and immune cell-immune cell interactions [28, 166, 210]. In addition to these chemical immunomodulators, the class 1b HLA proteins HLA-G, HLA-E, and HLA-F, which are expressed on the extra-villous trophoblast, are known to modulate the actions of T and NK cells, dendritic cells, and macrophages [106, 108]. The extra-villous trophoblast cells also express the polymorphic class 1a protein HLA-C [129]. HLA-C can modulate decidual NK cells via paternally derived HLA-C and maternally derived killer inhibitory receptors (KIR) on decidual immune cells [91, 92].

It is generally thought that MHC class II molecules are absent from human trophoblast [186],

but expression of class II MHC is found on human trophoblast cells in cases of villitis, pemphigoid gestationis, and recurrent spontaneous abortion [15, 31, 137]. More recent work suggests that there is intracellular expression of HLA-DR and HLA-DO on trophoblast resulting in the requirement for further research to full establish the expression of class II MHC in the placenta [212].

The prevention of organ-specific antigens reaching the lymphatics and the blood has been proposed as the mechanism for immune privilege in some organs [35, 244]. The same concept has been used to explain maternal-fetal tolerance and the theory was supported by the observation that there are few lymphatics within the secretory phase endometrium [54, 134, 174]. Nevertheless, other workers have shown that the human decidua does contain lymphatic vessels and that cytotrophoblasts can stimulate lymphangiogenesis in vitro [213]. Two possible mechanisms for the way in which fetal antigens may reach uterus-draining lymph nodes have been proposed:

1. Soluble antigens from trophoblast cells are secreted into the decidual interstitial space and are carried directly to lymph nodes via afferent lymphatics. The antigen is then processed and presented to maternal T cells by maternal dendritic cells. This is achieved by cross-presentation of fetal antigen [65, 181, 234].
2. Decidual dendritic cells ingest fetal antigens from the interstitial space or phagocytose apoptotic extravillous trophoblast cells and transport the fetal antigen to draining lymph nodes via afferent lymphatics. This is known to occur in normal placentation [107, 115].

A combination of these mechanisms along with other possible currently unknown mechanisms may be involved in the processing of fetal antigen. It is also known that decidual dendritic cells in human pregnancy have an immature surface phenotype and physiological function which correlates with tolerogenic properties [29]. In the secretory phase of the menstrual cycle, dendritic cells express CD83 which is characteristic of mature antigen-presenting cells, and in the majority of first trimester, decidua the dendritic cells express CD209 which is typical of immature dendritic cells [77, 118, 217]. Interestingly, pathological pregnancies are associated with an excess of decidual cells of which a significant proportion expresses the mature phenotype [14, 103]. Decidual dendritic cells also secrete lower levels of IL-12 than those found in peripheral blood which results in decidual dendritic cells being more able to preferentially drive the differentiation of T cells to the Th2 phenotype [180]. The evidence above supports the concept that in pregnancy there are dendritic cells of the immature phenotype which may promote immune tolerance of the fetal allograft.

In addition to these phenotypic changes in dendritic cells in pregnancy, the local microenvironment may also play an important role in dendritic cell function and phenotype. High levels of estrogen or transforming growth factor beta (TGFβ) in the dendritic cell microenvironment may promote immune tolerance to fetal antigens [219, 234]. Elevated levels of IL-10 and prostaglandins may also enhance the tolerogenic properties of dendritic cells during pregnancy [100, 248], and IL-10 along with suppressor CD8+ T cells may also enhance tolerance in dendritic cells [37, 168, 237]. It is also interesting to note that when dendritic cells are induced by IL-10 and CD8+ T cells, they express the HLA-G receptor ILT4. It is therefore possible that the HLA-G may further modulate dendritic cells by stimulating Th2 cytokines thus reducing T cell stimulation [8].

Stem Cells and Their Niche in Fetal Tissue

The understanding of the fetal stem cell niche requires the amalgamation of experimental embryology, cell biology, and in vitro models of fetal and adult microenvironments [50]. The following examples are given to provide an overview of this developing field.

Fetal Liver

The fetal liver arises from an endodermal stem cell population in the embryonic foregut [149, 293]. Mature hepatocytes and cholangio-

cytes are then formed under the guidance of paracrine cells in the portal vasculature [70, 239, 240]. This paracrine activity has been identified as originating from angioblasts which are producing a range of fibroblast growth factors [114, 170]. The transcription factors HNF1 beta and HNF6b have been shown to regulate the developmental process [42, 43]. The ductal plate cells, which are found in the portal triad region in fetal and neonatal liver [27, 222], form the niche for hepatic stem cells which are distinguished from hepatoblasts by in vitro and in vivo studies in which they form mature human liver [171, 172, 228, 229, 242, 264, 273]. The ductal plates develop during hepatic organogenesis and involve hedgehog protein signaling [170, 189, 222, 242]. These represent the niche for the fetal hepatic stem cells.

Fetal Lung

Normal lung development in the fetus relies on the growth and differentiation factor modulation of epithelial and mesenchymal cell interactions notably by members of the fibroblast growth factor (FGF) and the transforming growth factor beta (TGFβ) family [177, 281]. FGF9 and FGF10 appear to play an important role in lung branching morphogenesis and may be involved in the development of the fetal lung stem cell niche [45, 179, 235]. The transcriptional activator Cited2 [CBP/p300-interacting transactivators with glutamic acid (E) and aspartic acid (D)-rich tail 2] may play a role in the fetal lung stem cell niche and is certainly involved in maturation of fetal lung. Hypoxia, lipopolysaccharides, cytokines, and shear stress have all been reported as being able to induce expression of Cited2 [22, 257, 289]. Cited2 is expressed in embryonic and extraembryonic tissues and is essential for normal development [57, 282]. It is also interesting to note that Cited2-null embryos develop adrenal agenesis [18, 275] which in turn results in defects in fetal lung septal thinning and air-space development [185]. The interaction of all of these factors may be involved in the development of the fetal lung stem cell niche.

Fetal Aorta

Normal adult arterial wall has been shown to contain a "side population" of progenitor cells [224]. These cells must have their own niche which to date is uncharacterized. Angiogenesis in the fetus begins by differentiation of endothelial cells from the splanchnic mesoderm notably Flk1+ mesoderm cells [286]. Endothelial progenitors in the peripheral blood and bone marrow appear to be involved in *postnatal* neovascularization [12, 13, 226]. In addition, it has been shown that embryonic vascular endothelial cells can delaminate, migrate into the subendothelium, and differentiate into smooth muscle cells [49]. There are clearly stem cells present in the fetal aorta which have an important role not only in embryogenesis but also potentially in adult repair.

Cytokines in Fetal Tissue

Erythropoietin in the Fetus

The developing fetus contains an unknown number of cytokines, some of which are characterized and some are not, and these cytokines undergo complex interactions to guide differentiating tissue in the fetus. Erythropoietin was thought to be synthesized predominantly by fetal liver [81, 292]. Nevertheless, more recent studies have shown that the fetal kidney is the site of erythropoietin synthesis from early gestation in the sheep [155]. This has been confirmed in the human with erythropoietin synthesis detected in the area of the fetal proximal tubules [154]. Erythropoietin mRNA is detectable in the mid-trimester human kidney [197], and fetal plasma erythropoietin levels increase throughout gestation independent of maternal erythropoietin [150, 265].

Experimental evidence suggests that erythropoietin may have an important role in the development of the fetal brain. This follows the observation that there is a 50 % increase in murine fetal septal region choline actyl-transferase activity 48 h after treatment with recombinant erythropoietin [133]. Erythropoietin binding sites

have been demonstrated in murine corpus callosum, zona incerta, fimbria hippocampus, and mammillothalamic tract [51]. At 13–17 weeks gestation, when neurogenesis is at a maximum, the ependymal cells enclosing the central canal of the spinal cord in the human fetus express erythropoietin receptor mRNA [52, 153]. Similar work has shown that there are higher levels of erythropoietin protein in the cerebrospinal fluid (CSF) of preterm and term neonates when compared to infants or adults [116] and that erythropoietin and erythropoietin receptor mRNA are detectable in human fetal brain aged 5–24 weeks gestation [117]. Erythropoietin clearly plays an important role in fetal development as well as being the basis of normal erythropoiesis in the adult.

Umbilical Cord Serum Cytokines

Cytokines have been shown to be important in the regulation of uterine function in both pregnancy and parturition [198]. Maternal infection will result in the production of a range of cytokines such as tumor necrosis factor α (TNFα) and interferon γ (IFNγ) resulting in the production of prostaglandins which can trigger premature labor [193]. There appears to be a clear link between elevated levels of pro-inflammatory cytokines such as TNFα, IFNγ, and interleukins (IL) 1β, 6, and 8 and preterm birth [61, 79, 83, 104, 121, 202, 269]. The relationship between cytokine concentration and a small-for-gestational-age (SGA) fetus is less clear [85, 88, 245]. Nevertheless, three studies have found that increasing levels of TNFα and IL-8 in association with decreasing levels of transforming growth factor beta (TGFβ) may result in intrauterine growth retardation [143, 144, 200]. Maternal polymorphisms resulting in increased production of TNFα, IL-1, and IL-6 have been associated with preterm delivery [63, 64]. Interestingly in subsequent work, similar polymorphisms for TNFα, IL-6, and IL-10 were found to be unassociated with abnormal pregnancy [253–255]. Overall, it appears that higher levels of TNFα are associated with preterm delivery [64, 79, 121]. The overall role of IL-6 in fetal development is controversial, but one group did find that an increasing level of IL-6 is related to a lower risk of preeclampsia-associated fetal growth restriction [195].

There are, as would be expected, complex interactions between cytokines in the developing fetus. It is possible that where increased levels of Th1 pro-inflammatory cytokines are seen there is a strong immune response from a healthy fetus [78]. It is also reported that low levels of IFNγ, which is a Th1 cytokine produced in villous trophoblast cells, are an indicator of placental insufficiency and trophoblast malfunction [187]. The study of cytokine actions and interactions in vivo is complex and often contradictory. Future research using in vitro models may help toward a deeper understanding of the critical role which cytokines play in fetal development.

Fetal and Adult Tissue Interaction in Health and Disease

Fetal Tissue Transplantation

This subject will be dealt with in detail elsewhere in this book, but it is useful to state the basic concepts here to place the subject in context with fetal microchimerism.

The interaction of fetal and adult cells on transplantation has been described many times with the first transplant using fetal bone marrow to treat Rhesus disease [48]. Perhaps the most innovative approach was the use of fetal liver cells to treat another fetus suffering from bare lymphocyte syndrome in utero [271, 272]. Subsequent attempts at fetal cell transplantation into adults or vice versa have been less successful except in the treatment of severe combined immunodeficiency [71, 141, 283]. More recently, fetal stem cells have been proposed as potentially useful in a range of regenerative medicine procedures [82, 220, 256, 262]. Ethical objections to the use of fetal cells for transplantation remain a stumbling block for such technology.

Fetal Microchimerism in Health

Fetal genetic material was first identified in the maternal peripheral circulation by Herzenberg et al., in 1979 [90]. Subsequent work confirmed these observations by using molecular biology technology to detect single-copy fetal DNA sequences in the maternal blood, and such technology even enabled prenatal sex determination by fetal DNA amplification from maternal peripheral blood [157, 158]. Male fetal cells have been detected in a mother 27 years *postpartum* [23], and fetal microchimerism has now been described many times in maternal peripheral blood mononuclear cells [24, 68, 139, 295]. The incidence of fetal microchimerism in maternal peripheral blood has been reported as 30–58 % T cells, 45–75 % B cells, 44–62 % natural killer cells, and 26–58 % of antigen-presenting cells [163]. Aclear and well-documented exchange of cells between the fetus and mother has therefore been reported, and these cells may contribute to tissue repair in both the mother and the fetus [26, 260]. Fetal cells which cross to the mother during pregnancy and maternal cells which cross into the fetus can clearly persist throughout life resulting in microchimerism [125].

Fetal microchimerism, in the form of fetal cells and fetal DNA, has been found to be a relatively common phenomenon with 70–80 % of maternal plasma positive for male DNA and up to 17 % positive for fetal cells [159]. Further studies have shown that male DNA represents 3–6 % of the total DNA in maternal plasma [160] raising possibilities for noninvasive prenatal diagnosis. It is interesting to note that there seems to be a defined timescale for the development of fetal microchimerism with the earliest detection in maternal blood at 4–5 weeks of gestation [266]. There is a then steady increase in fetal microchimerism after 24 weeks of gestation which peaks at parturition and then declines *postpartum* [9].

Some studies have shown that free fetal DNA microchimerism is rapidly cleared from the maternal plasma with a half-life of 16 min and 100 % clearance by 24 h *postpartum* [161], and similar observations have been made for the clearance of fetal cells from the maternal circulation [132]. Other workers have shown the persistence of fetal microchimeric cells over many years *postpartum* [109], and these differences may be explained by technical differences in the maternal plasma processing which yield different results [39]. The presence of apoptotic fetal cellular material in the maternal plasma may also influence fetal microchimerism data [276] as does the variation of free fetal DNA in maternal plasma and serum [148].

Fetal microchimerism has also been described in a range of maternal organs as well as the maternal hemopoietic system [124, 125]. Other workers have shown a male fetal hepatic cell lineage present in maternal liver [251]. In addition to this, fetal microchimerism has been found in mobilized maternal hemopoietic stem cells and within the maternal bone marrow mesenchymal stem cell population [2, 196].

Fetal Microchimerism in Abnormal Pregnancy

It was in 1893 when Georg Schmorl first described the presence of multinucleated cells in maternal lung and other tissues at the *postmortem* of 17 women who had died as a result of eclampsia [231]. Schmorl deduced that these cells were of placental origin and he also described the concept of fetomaternal trafficking in normal pregnancy with the hypothesis that there would be quantitative differences in fetomaternal trafficking in eclampsia [142]. More recent work has confirmed the findings of Schmorl showing that there are increased numbers of fetal erythroblasts and increased amounts of free fetal DNA in the plasma of preeclampsia patients [101, 294]. Similar fetal microchimerism has been shown in the severe version of preeclampsia known as HELLP syndrome (hemolysis, elevated liver enzymes, low platelets) which may be attributed to either increased fetomaternal transfer or decreased maternal clearance capacity [146, 259]. Fetal growth restriction and preterm labor has also been associated with increased fetomaternal trafficking [4, 151].

Obstetric interventions such as changing the orientation of the fetus in the uterus by the use of external cephalic version (ECP) has been shown to increase the free fetal DNA in the *maternal* circulation [145] with similar results seen as a result of termination of pregnancy [25]. There are also data to suggest that the rapidly differentiating cells in early pregnancy have a greater potential to create fetal microchimerism than later fetal cells [123, 173]. This hypothesis is based on the observation that women with no history of childbirth but a history of spontaneous and elective abortion have high levels of fetal microchimerism [287].

Fetal Microchimerism in Malignant Disease

Since fetal-maternal microchimerism is now an accepted phenomenon, attention has recently turned to the possible role of microchimerism in disease. There are currently three proposed mechanisms for the role of microchimerism in disease, these are:

1. Microchimerism acting as an effector of alloimmune reactions. This theory is supported by the observation that women suffering from scleroderma have T cells which are alloreactive to maternal antigens in peripheral blood and skin [225].
2. Microchimeric fetal antigen presenting cells may display maternal antigens, or allogeneic tissue microchimerism could be attacked by the maternal immune system. These concepts are supported by the fact that differentiated tissue fetal microchimerism has been found in thyroiditis and goiter, hepatitis, lupus, and other autoimmune diseases [19, 66].
3. Fetal microchimerism may provide a source of endogenous progenitor cells capable of repairing damaged or inflamed tissue as described in the murine model of liver repair by fetal microchimeric cells [127].

Recent studies have investigated the incidence of breast cancer in relation to fetal microchimerism and discovered that women in which fetal microchimerism has been identified are less likely to develop breast cancer [74, 75]. Nevertheless, a further study assessed the presence of fetal microchimeric cells in pregnant women presenting with breast cancer and found that fetal-derived cells are found in the tumor stroma. This of course raises the possibility of targeting fetal cells in tumor therapy [55, 56]. Other cancers have also been associated with fetal microchimerism such as cervical cancer [36], thyroid carcinoma, and adenoma [215, 246]. The significance of fetal microchimerism in maternal malignancy is still unclear, but this is clearly an interesting phenomenon deserving further investigation.

The Role of Fetal Microchimerism in Autoimmune Disease

It has long been known that pregnancy can result in a reduction in symptoms in those women who suffer from rheumatoid arthritis [89]. It is equally well reported that the symptoms of rheumatoid arthritis resume within 3 months after delivery [199]. Hormonal changes (especially cortisol) have been uncertainly proposed as the cause of these changes, but more recently it has been shown that fetal-maternal HLA class II disparity correlates with pregnancy-related improvement of arthritis [190]. The greater the HLA class II difference between mother and fetus, the greater the reduction of symptoms, whereas when the HLA class II difference is small, the symptoms of rheumatoid arthritis stay the same and can even worsen during pregnancy. More recent analysis of such patients, both during pregnancy and *postpartum*, for the presence of fetal microchimerism has shown that higher levels are fetal microchimerism are present when rheumatoid arthritis is quiescent [288]. This observation promoted the development of the "changing maternal self hypothesis" [3] in which the need for tolerance of fetal antigens in pregnancy results in a decrease in autoimmune symptoms. A similar mechanism may be present in other autoimmune diseases, but this is an area where much more research is required to fully understand the relationship between autoimmunity and fetal microchimerism.

The possible role of fetal microchimerism in systemic sclerosis has also been assessed. It was found that women suffering from systemic sclerosis had previously given birth to a baby who had a close similarity to the mother for the HLA class II gene HLA-DRB1 [192]. Other workers have indentified that the male offspring HLA class II genotype DQA1*0501 is associated with fetal microchimerism in the maternal T (CD3+) cells in the mother suffering from systemic sclerosis [11]. It appears that the specific HLA alleles fetal microchimerism and the specific HLA alleles of the mother have the capacity to contribute to the risk of developing systemic sclerosis [191]. Such an understanding may lead to the development of new therapies for systemic sclerosis and other autoimmune diseases [274].

In Sjögren's syndrome, there is a possible correlation between fetal microchimerism in salivary glands and the occurrence of the disease [34, 62, 136, 178, 270], and in systemic lupus erythematosus, the link to fetal microchimerism is also controversial [1, 126] although lupus nephritis may be associated with increased levels of fetal microchimerism in the circulation and in kidney tissue [102, 184]. *Postmortem* examination of systemic lupus erythematosus patients has shown fetal microchimerism in abnormal tissue but not in normal tissue [112].

The role of fetal microchimerism in autoimmune thyroiditis is a possibility as first time and flare episodes often occur *postpartum* although the current literature does not support this hypothesis [204, 278]. Nevertheless, fetal microchimerism has been detected in the thyroid tissue of autoimmune thyroid disease patients [7, 130, 215, 246].

The role of fetal microchimerism in primary biliary cirrhosis has also been investigated by several workers, but the data are relatively inconsistent with fetal microchimerism also identified in the liver of patients suffering from other hepatic pathologies [46, 232, 261]. One study has described higher levels of fetal microchimerism in primary biliary cirrhosis compared to the controls [69]. Fetal microchimerism clearly has a role to play in autoimmune disease although its true significance and relationship to other pathological factors is yet to be understood.

Maternal Microchimerism in the Fetus

The bulk of the study on microchimerism focuses on fetal microchimerism of the mother, but there are a few workers interested in maternal microchimerism in the fetus with evidence that such cells can persist into adult life [167]. Such maternal cells may be responsible for the paternal transmission of cancer [113]. The historical observation of transplacental metastasis from mother to fetus of various malignancies also supports the concept of maternal microchimerism [32, 95, 208, 209]. More recently, metastasis of melanoma from mother to placenta has been described [241] along with the similar placental metastasis of breast cancer [20].

Maternal microchimerism has been linked to dermatological and systemic autoimmune symptoms in a child who was shown to have significant maternal microchimerism in his CD34+ cell population [135]. In addition, maternal microchimerism has been shown to be present in the lung, heart, and bone marrow of a systemic sclerosis patient who died following an autologous bone marrow transplant [140] and also in the hearts of neonatal lupus syndrome patients where the maternal cells express α-actin [250]. The peripheral blood of twins and triplets discordant for neonatal lupus syndrome has been found to have maternal microchimerism which increases with time in a child suffering from progressive congenital heart block [252]. Juvenile idiopathic inflammatory myopathy and dermatomyositis have also been associated with maternal microchimerism in the peripheral blood and muscle tissue of these patients [10, 214], and maternal microchimerism has also been identified in the liver of biliary atresia patients [131, 258]

Maternal microchimerism is clearly a complex and multifunctional phenomenon in a range of childhood diseases. A thorough understanding of microchimerism in the future may enable a clearer understanding of both adult and pediatric pathology.

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Three-Dimensional Culture of Fetal Mouse, Rat, and Porcine Hepatocytes

4

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Introduction

Fetal liver cells have a much higher capacity to proliferate and reorganize themselves even in vitro than adult hepatocytes, but the expression of liver-specific functions is significantly lower than that of adult hepatocytes. Therefore, establishments and integrations of methodologies for both in vitro culture and in vivo implantation of fetal hepatocytes would be of great significance because such methodologies can be applied to the use of immature hepatocytes induced from iPS/ES cells whose induction to mature hepatocytes has not yet achieved [1].

As can be seen in the results on adult hepatocytes, 3D culture of fetal hepatocyte is expected to be a method suitable for obtaining high cell density and is even more advantageous not only

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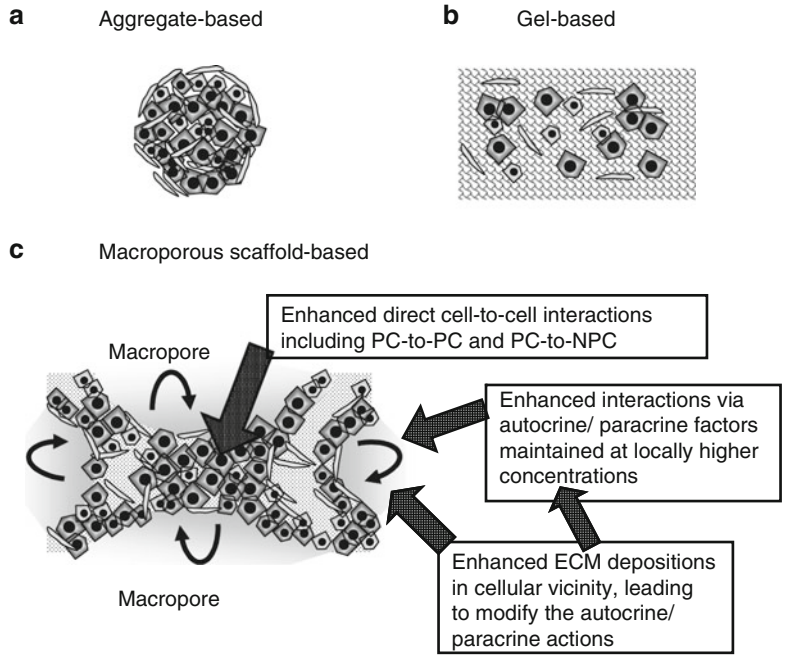

Fig. 4.1 Typical 3D cultures (**a**–**c**) and expected favorable effects in the case of macroporous scaffold-based 3D culture (**c**). *PC* parenchymal cell, *NPC* non-parenchymal cell

in obtaining enhanced hepatic functions but also in accommodating higher total cell number per unit volume of scaffolds, on the condition that oxygen and nutrients are satisfactory supplied. The term "3D culture" is ambiguous, but the most essential issue is that cells are forming direct connections in 3D manners. Aggregates culture simply meets this most essential condition (Fig. 4.1a). Gel-based culture is generally gel-entrapment culture, but when cells migrate and organize into 3D aggregate structure, we may say as 3D culture (Fig. 4.1b).

More general 3D culture is based on macroporous or fibrous scaffolds where cells are attached onto the inner surfaces of the scaffolds and subsequently grow there to form various cell-to-cell interactions in a 3D manner (Fig. 4.1c). In this 3D culture system, possible mechanisms for the 3D effects from the biological point of view are categorized into three as follows (Fig. 4.1c) [2]: enhanced cell-to-cell contact that makes them recognize they are in in vivo mimicking cellular community via various adhesion molecules such as cadherin or integrins (both between hepatocytes and hepatocyte-to-other non-parenchymal cells), secretion and enhanced disposition of liver-specific ECM in 3D architecture, and enhanced concentrations of various autocrine/paracrine growth factors in the localized areas, which is partly modulated by the deposited ECMs. However, 3D culture is not widely used in scientific research, presumably because conducting morphological observation is more difficult in 3D culture than in conventional 2D culture.

One earliest study on 3D culture is concerning hepatocyte aggregate (spheroid) formation of fetal rat mouse liver cells on non-cell-adherent hydrophobic surfaces [3]. After that, 3D cultures of hepatocyte progenitors from fetal livers have been tried mainly from the standpoint of basic biology. Namely, these studies used gel-based ECM materials, which are not advantageous in terms of mass transfer. Tanimizu et al. tried in vitro culture of fetal mouse liver cells in Matrigel where they formed a cyst-like structures found in the in vivo liver development and examined influences of various growth factors [4]. Turner et al. reported enhanced morphological development of human hepatoblast in hyaluronan hydrogels [5], and Xiong et al. reported active growth of human fetal liver progenitors cells in coculture with endothelial cells in fibrin gel [6].

To ensure better flexibility in scaling up for implantation, macroporous scaffold-based

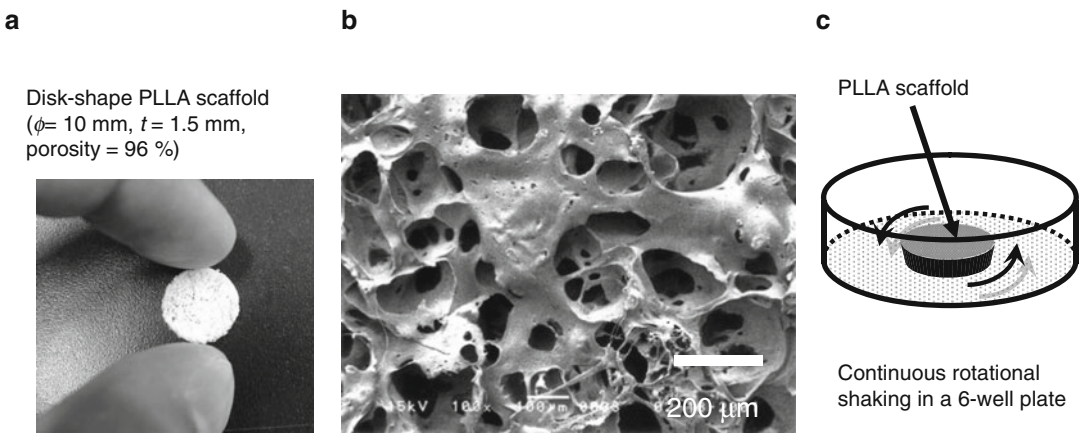

Fig. 4.2 Appearance of the disk-shape PLLA scaffolds (**a**), their inner macroporous structure by SEM (**b**), and illustration of gentle rotational culture of the scaffold in a 6-well plate (**c**)

3D culture system should be more advantageous. Dvir-Ginzberg et al. reported enhanced maturation of newborn rat hepatocytes in macroporous alginate-based 3D scaffolds [7]. The cells formed spheroids and spontaneously developed to organoid having an external mature hepatocyte layers encasing ECMs- and nonparenchymal cell-rich cores. Ehashi et al. used macroporous 3D scaffolds made from nonbiodegradable polyvinyl formal for the culture of fetal mouse [8] and porcine [9] hepatocytes for future uses in bioartificial lives. As an evidence of in vivo efficacy of 3D-cultured fetal hepatocytes, Cusick et al. [10] report that freshly isolated fetal rat hepatocytes heterotopically transplanted onto mesenteric leaves have a significant survival advantage over adult hepatocytes in biodegradable PLLA sponges. All of them demonstrate the enhanced functionality and duration in 3D culture over conventional 2D culture. However, growth and per cell-based functions, which are necessary to address the functional maturation and expected per volume-based functions for liver tissue equivalents, are not always distinguished.

In this chapter, we introduce and summarize the results from our groups on typical macroporous and biodegradable scaffolds-based 3D culture of fetal hepatocyte populations isolated from mice, rats, and pigs (Fig. 4.2). To fully utilize various synergistic effects occurring in 3D culture, we supplemented various cocktails of growth factors or other soluble factors. To better avoid the possible problems in mass transfer between culture medium and inner space of the scaffolds, we fabricated a thin disk-shape poly-L-lactic acid (PLLA) scaffold and applied continuous rotational shaking during the entire culture periods (Fig. 4.4). In addition to the fundamental growth factors or soluble factors such as insulin, dexamethasone, EGF, and ascorbic acid 2-phosphate, we examined the effects of nicotinamide (NA), dimethyl sulfoxide (DMSO), OSM, HGF, FGF-1, FGF-4, and sodium butyrate (SB). In some 3D culture such as mice and rat, we performed preliminary implantation studies to the mesentery leave where we can expect better angiogenesis upon implantation. In the case of rat implantation, we used hyaluronic acid sponge having a 3D macroporous structure.

General Experimental Protocols

3D PLLA scaffolds (1.5 mm thick, Φ10 mm, about 0.1 cm^3) having a well-developed open-pore structure were prepared by a gas formation method with ammonium chloride particles (250–500 m) as a porogen and a gas-forming reagent as reported by Nam et al. (Fig. 4.2) [11]. This was washed, sterilized, and precoated with 0.03 % collagen acid solution and equilibrated with culture medium before use.

Fetal mouse liver cells were isolated from the embryonic liver of C57Bl/6CrALc mice (Sankyo

Lab, Tokyo, Japan) at E14.5 by the same procedure as previously described [12]. Briefly, minced embryonic livers were first dissociated with the preperfusion buffer containing 0.5 mM EGTA (ethyleneglycoltetraacetic acid) for 10 min and then enzymatically digested for 15 min at 37 °C with a 0.05 % collagenase-containing buffer. This protocol was similarly used in the isolation of fetal rat hepatocytes at E17 from the fetuses from pregnant Wistar rats. This protocol enabled to have a good cell population of over 85 % viability for both spices. The final cellular yields were $2.0–3.0 \times 10^7$ cells from one pregnant mouse and $1.0–1.4 \times 10^7$ cells from one pregnant rat. Adult mouse and rat hepatocytes were also isolated by the conventional two-step collagenase perfusion digestion method by Seglen [13].

Porcine fetus was obtained from a local slaughterhouse with a gestation length of 96 ± 5 days weighting 700–1,200 g (full term is about 115 days), stored in phosphate buffered saline (PBS) on ice, and transported to the laboratory within 4 h of their death. The fetal porcine hepatocytes were also isolated by the collagenase digestion method [13] after several time scaling up of that for the rats. Typically, $2.9–4.3 \times 10^8$ cells were isolated from a single isolation, and the viability was 87.3 ± 2.1 %.

The basal culture medium (BM) was Williams' medium E supplemented with 2 mM L-glutamine, 10^{-6} M hydrocortisone or dexamethasone, 10 ng/mL mouse EGF, 10^{-6} or 10^{-7} M insulin, 0.5 mM ascorbic acid phosphate, 10 mM nicotinamide (NA), antibiotic/antimycotic solution, and 10 % FBS. Various soluble factors including growth factors as indicated in each experiment were added at the first medium replenishment performed on Day 1. Thereafter, it was changed every 2 days.

For 2D monolayer culture, isolated fetal hepatocytes were usually inoculated at $1.6–2.0 \times 10^5$ viable cells per one well of a 0.03 % Type-I collagen-precoated 12 well plates with a 1 mL culture medium ($4.0–5.0 \times 10^4$ viable cells/cm^2-culture surface, $1.6–2.0 \times 10^5$ cells/mL). For PLLA scaffold-based 3D culture, 4.0×10^5 viable cells were inoculated to one scaffold in one well of a 6 well bacterial grade plate with a 2 mL culture medium (4.0×10^6 cells/cm^3-scaffold, 2.0×10^5 cells/mL).

Albumin secreted into the culture medium was measured with the sandwich-type enzyme-linked immunosorbent assay (ELISA). Cytochrome P450 IA1/2 capacity is evaluated from ethoxyresorufin O-deethylase (EROD) capacity [14] in terms of the produced resorufin from 10 μM ethoxyresorufin in 1 h. In addition, fetal porcine hepatocytes were exposed to culture medium containing 1 mM NH_4Cl, and its decrease in 2 h was measured by the indophenol method, and simultaneous urea production was measured by the diaceylmonoxium method followed by trichloroacetic acid protein precipitation as previously reported [15]. Cellular DNA content was measured by 4′,6-diamidino-2-phenylindole (DAPI) fluorometry and sometimes used to calculate per cell-based functionality [16].

For implantation studies in mice using cell-loaded PLLA scaffolds, they were implanted into the mesentery leaves of 70 % hepatectomized C57Bl/6CrALc mice. After 2 weeks, the animals were sacrificed, and the frozen thin sections of the tissues were prepared by a cryostat. Albumin immunostaining was performed using the same antibody set used in the albumin ELISA. The percent area covered by albumin-positive cells was digitized and analyzed in terms of the color by PhotoShop (version 7, Adobe Systems Ind., Mountain View, CA, USA) and NIH Image (version 1.63) developed by Rasbund in the National Cancer Institute (NIH), USA.

For implantation study in rats, we used LEC (Long Evans Cinnamon) rats as an animal model of Wilson's disease (WD) [17]. Instead of the use of conventional PLLA scaffold, we used a 1.0-mm-thick photo-crosslinked HA sponge (the dimension is $12.5 \times 2.5 \times 1.3$ mm after swollen in culture medium; mean pore size is 60 m). Fetal hepatocytes were isolated from normal LEA (Long Evans Agouti) rats, inoculated to the HA sponge at $2–4 \times 10^6$ cells/piece, and cultured for 4 days prior to transplantation. The cell-loaded HA sponges were implanted into the mesentery of LEC rats, and the animals were fed a conditioned diet containing ten times more concentrated copper (100 ppm) than a general diet. The animals were sacrificed

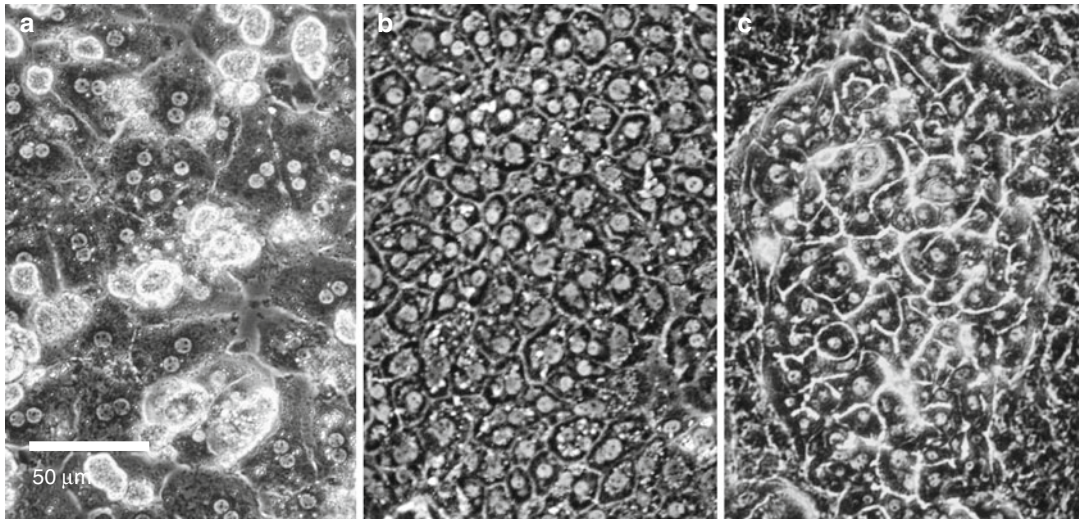

Fig. 4.3 Morphologies of adult mouse hepatocytes on Day 3 in the basal culture medium (**a**) and fetal mouse hepatocytes at Week 5 in the complete culture medium (**b**, **c**)

at Week 3; various blood serum indices including cupper concentrations were measured, and the implanted tissues were analyzed by the HE staining.

Fetal Mouse Hepatocytes: In Vitro and In Vivo Studies

3D Culture Using PLLA Scaffolds Versus 2D Monolayer Culture

We started the series of 3D culture studies with fetal mouse hepatocytes with addition of NA, DMSO, and OSM. This was because NA and OSM, in the presence of EGF, were the established soluble factors inducing the emergence of small hepatocytes from the non-parenchymal cell fraction of adult livers [18]. In addition, oncostatin M (OSM), an interleukin-6-related cytokine produced by hematopoietic cells, has been shown to stimulate liver cell maturation in vitro [12].

In 2D monolayer culture, up to week 5 growth of non-parenchymal cells was enhanced by OSM alone, whereas the emergence of small hepatocytes, whose diameter was almost 1/3 to 1/2 of adult hepatocytes (Fig. 4.3a and b), was elicited in the presence of both NA and DMSO. In the presence of the three factors (complete medium), both growth and emergence of such small hepatocytes were remarkable compared with other conditions so that almost 60–70 % of the culture surface was covered with such small hepatocytes (Fig. 4.3b). Some of the small hepatocyte developed a distinctive appearance, that is, piling up over the existing monolayer, better development of gap junctions, and possible bile canaliculus structures (Fig. 4.3c). In addition, a small number of binucleated cells began to be observed in those areas (Fig. 4.3c) [19].

In 3D culture using PLLA scaffolds, enhanced growth and hepatic maturation in the complete medium were further augmented as evidenced from the increase in albumin production (Fig. 4.4) [20]. Basal culture medium, which contained fundamental growth factors authorized in culture of adult rat hepatocytes, did not support any functional maturation. OSM has some synergistic effects on the albumin production with 3D culture (data not shown), although this just enhanced nonspecific growth of entire cell populations including non-parenchymal cells in 2D culture.

DNA measurement at the end of culture showed the threefold and fourfold growths in 2D and 3D cultures, respectively. This indicates that 3D culture does not support extensive growth in vitro in spite of the fact that there is much

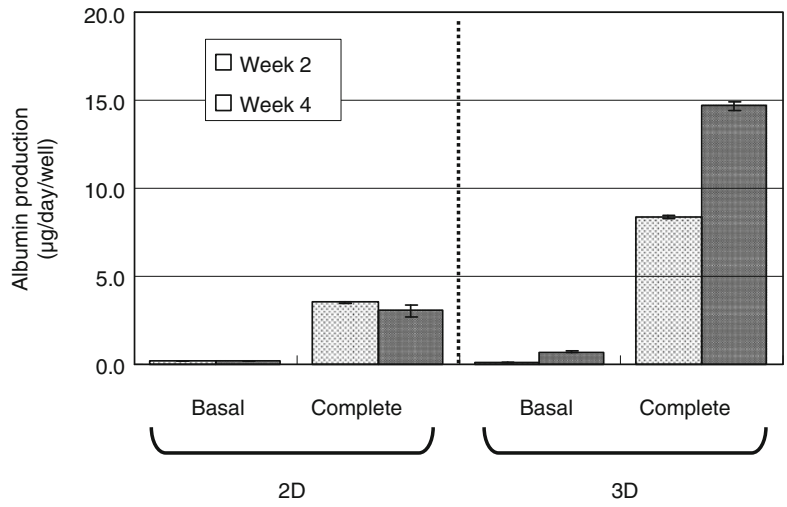

Fig. 4.4 Albumin production of fetal mouse hepatocytes culture in 2D monolayers and 3D PLLA scaffolds at Week 2 and 4. Mean ± SD ($N = 6$)

larger surfaces available for growth. Per cell-based functional comparison of the fetal hepatocytes in the complete medium in 2D and 3D culture with those in adult hepatocytes showed that albumin productivity was almost the same in the 3D culture, but the EROD was still in very low levels (Fig. 4.5). This suggests that whole maturation comparable to that found in adult hepatocytes has not yet been achieved even under 3D culture in the presence of the three factors and that further improvement is needed to obtain the best functionality comparable to that of adult hepatocytes [21].

Implantation of Cell-Loaded 3D Scaffolds into the Mesentery

We further conducted a simple in vivo study using the same series of adult mice as recipients [21]. The mesentery was selected as the site of implantation of the cell-loaded PLLA scaffolds. This is because microvascular system is well developed there, and therefore, we can expect quick angiogenesis of the implanted tissue. In this experiment, we confirmed the advantages of fetal hepatocytes in 3D culture, particularly of their preculture in vitro before implantation. HE staining clearly showed that PLLA with fetal cells cultured for 2 weeks in the complete medium accommodated larger number of hepatic cells (Fig. 4.6d), compared with PLLA with fetal cells without such preculture (Fig. 4.6c) or with other control cultures, that is, PLLA with adult cells (Fig. 4.6b) and PLLA alone (Fig. 4.6a). The cells observed in Fig. 4.6d were mostly albumin-positive ones. The percent area covered by such albumin-positive cells was larger in PLLA with fetal cells cultured in the complete medium than other conditions such as fetal cells without preculture or adult cells (Fig. 4.7). These results strongly show that the basic concept of combining 3D culture and in vitro selective propagation/maturation of fetal hepatocytes in a suitable culture medium before implantation shows promise for engineering liver tissues effective after implantation.

Fetal Rat Hepatocytes: In Vitro and In Vivo Studies

3D Culture Using PLLA Scaffolds Versus 2D Monolayer Culture

Rats are the most suitable animal model prior to preclinical studies with larger animals such as pigs, because it is technically far easier to perform surgical operations, including accessing blood supplies, on rats than on mice [22]. We started first with the combination of NA, DMSO, and OSM as established in fetal mouse hepatocyte culture, but it did not work at all in rats. In particular, addition

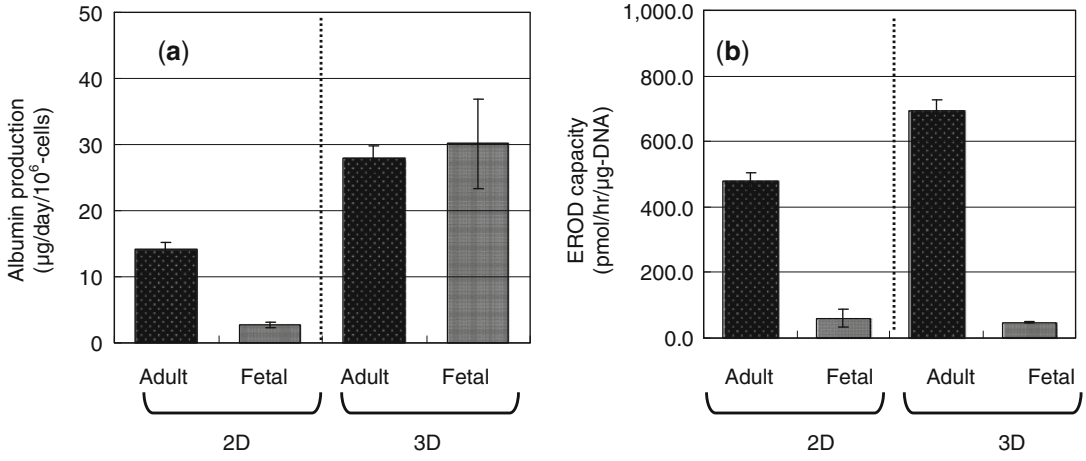

Fig. 4.5 Per cell number-based comparison of albumin production (**a**) and EROD capacity (**b**) between adult mouse hepatocytes on Day 3 and fetal mouse hepatocytes culture for 4 weeks in the complete medium. Mean ± SD ($N = 6$)

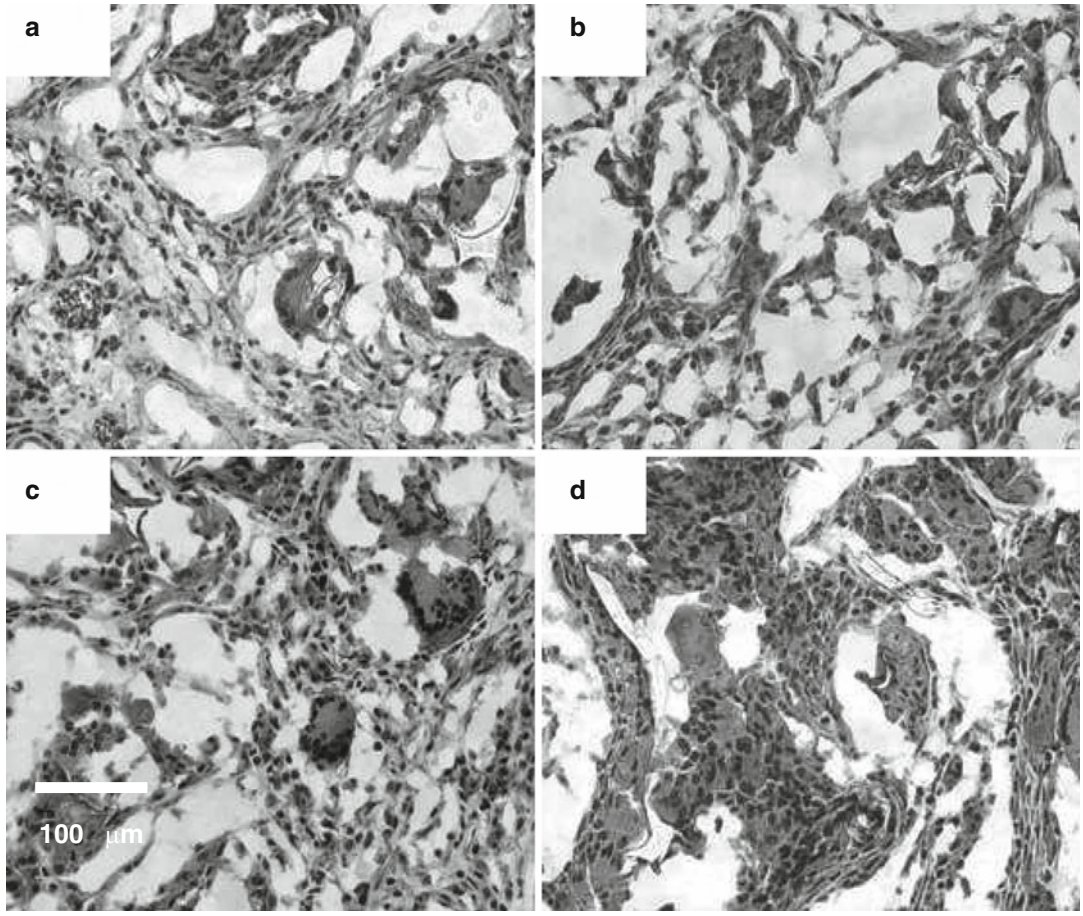

Fig. 4.6 HE staining of the implanted tissues recovered at Week 2. (**a**) PLLA without cells; (**b**) PLLA with adult mouse hepatocytes, (**c**) PLLA with fetal mouse hepatocytes without any in vitro culture, (**d**) PLLA with fetal mouse hepatocytes cultured in the complete medium for 2 weeks before the implantation

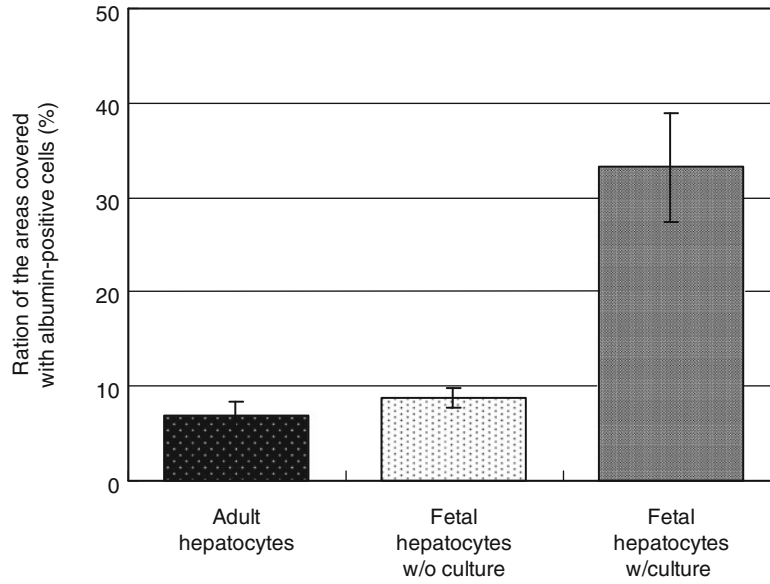

Fig. 4.7 Ratio of the area of covered with albumin-positive cells in the immunostaining performed on thin section of the PLLA-based implanted tissues formed from adult mouse hepatocytes, fetal mouse hepatocytes without any in vitro culture and fetal mouse hepatocytes with 2 weeks of culture in the complete medium. Mean±SD in ten different microscopic areas

of DMSO blocked the growth of fetal rat cells isolated at E14.5. Therefore, in addition to NA and OSM, we added other strong growth factors such as HGF, FGF-1, FGF-4, and butyrate on fetal rat hepatocytes in the same 3D culture using the PLLA scaffolds [23]. Combination of HGF, FGF-1, and FGF-4 was employed in one representative protocol for the induction of hepatic differentiation from ES cells [24].

In 2D culture, addition of all the factors (complete medium) strongly enhanced the emergence and growth of immature hepatocytes on Day 6 (Fig. 4.8). However, at week 2, such cells were dramatically reduced in all culture conditions. Independent effects of each factor were evaluated by complete mediums without relevant factor. Lack of the three growth factors, HGF, FGF-1, and FGF-4 (3GFs), diminished the morphologies of hepatocyte-like cells and enhanced the growth of non-parenchymal cells. Lack of butyrate decreased the diameter of the hepatocytes, and the resulting cell morphology was dominated by small hepatocyte-like cells in the similar manner as in mice culture (Fig. 4.3b). Lack of OSM slightly enhanced the growth of non-parenchymal cells.

In terms of albumin production, there was synergistic effect of 3D culture and the established complete medium (Fig. 4.9). Although the complete medium succeeded in enhancing albumin production at the initial stage in 2D culture, the long-term functional stability was inferior to those of 3D culture; 3D culture in the basal medium partly supported long-term functional stability. Concerning the effects of individual factors on the functions in 3D culture, 3GFs were fundamentally important, but OSM in addition to the 3GFs was necessary in its prolonged expression. Addition of sodium butyrate, which is a product of intestinal flora and is shown to promote maturation of various progenitor cells, was very effective for EROD expression. Brill et al. reported that butyrate tended to increase the overall gene expression because of the inhibition of a histone deacetylase [25]. The most striking difference in EROD capacity between the 2D and 3D cultures was that it was maintained in the 3D culture until week 2, whereas it deteriorated dramatically in the 2D (data not shown). From the per cell-based functional comparisons based on the remaining DNA measurements at Week 2, the complete medium significantly enhanced the hepatic maturation in terms of both albumin secretion and EROD capacity in 3D culture; the former reached about the same level as that of the adult hepatocyte (Fig. 4.10a), and the latter reached about half that of the adult hepatocyte (Fig. 4.10b).

From the DNA measurement at the end of culture, cellular amplification was limited to below

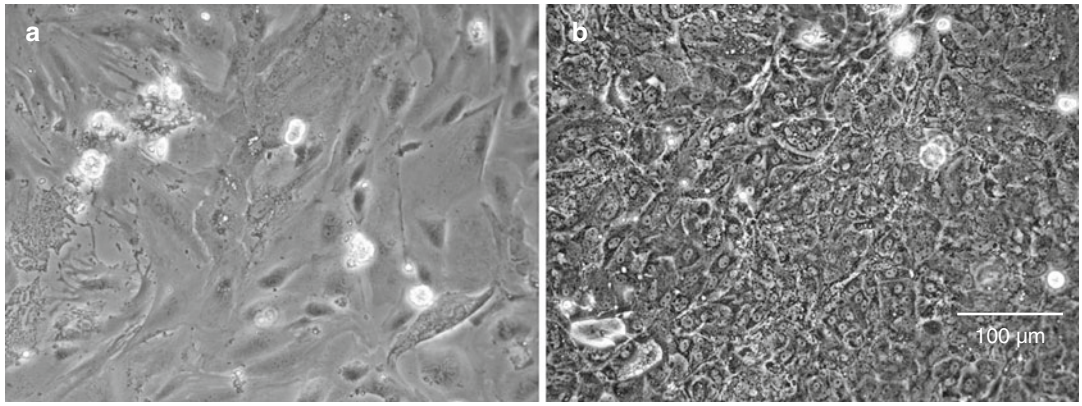

Fig. 4.8 Morphologies of fetal rat hepatocytes on Day 6 in the basal medium (*BM*) (**a**) and the complete medium (*CM*) (**b**)

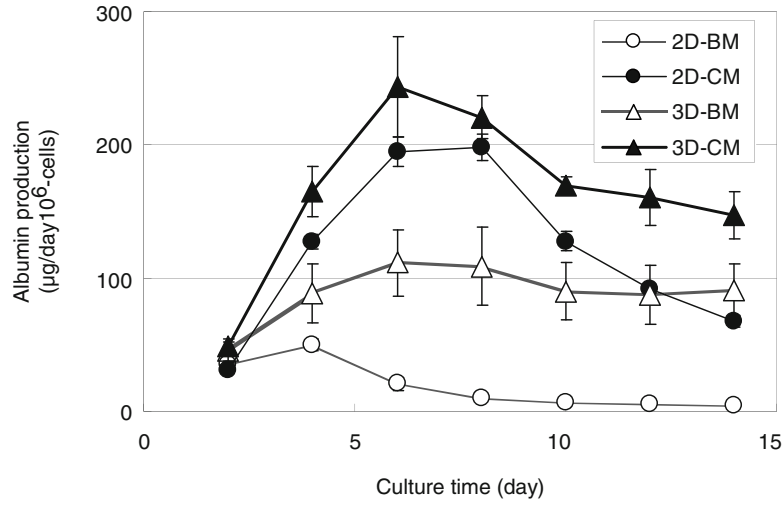

Fig. 4.9 Time-course changes in albumin production of fetal rat hepatocytes cultured in 2D and 3D with the basal and complete culture mediums (*BM* and *CM*)

three- to fourfold in both the monolayer and 3D cultures (data not shown). From the cross section and SEM pictures, we found that the cells attached sparsely in the 3D PLLA scaffold and were aligned mostly in layers around the inner surfaces of the scaffold's large pores (Fig. 4.11a). In such layers, hepatocyte aligned at high density or formed small clusters around the pore, and non-parenchymal cells surrounded such hepatocytes (Fig. 4.11b). SEM images showed the same situation and showed extensive deposition of ECMs with cells (Fig. 4.11c, d). In 3D cultures, the final populations of parenchymal and non-parenchymal cells seemed to be well balanced and well organized with each other with enriched ECMs in their vicinity, leading to not only high functional maturation but also long-term stability in vitro.

Implantation of Cell-Loaded 3D Hyaluronic Acid Scaffolds into the Mesentery

In rat implantation study, we used another 3D scaffold, hyaluronic acid (HA)-based one [26], and LEC rats as an animal model of Wilson's disease (WD) [27]. Different from PLLA, HA is one of the natural ECMs, and we may expect some biological influence on hepatic maturations [5]. In this

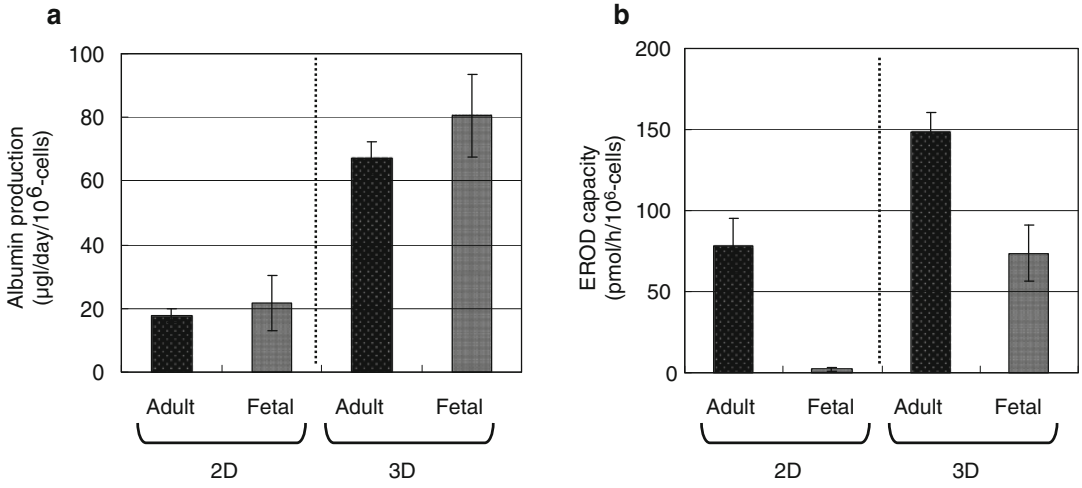

Fig. 4.10 Per cell number-based comparison of albumin production (**a**) and EROD capacity (**b**) between adult rat hepatocytes on Day 3 and fetal mouse hepatocytes cultured for 2 weeks in the complete medium. Mean ± SD ($N = 6$)

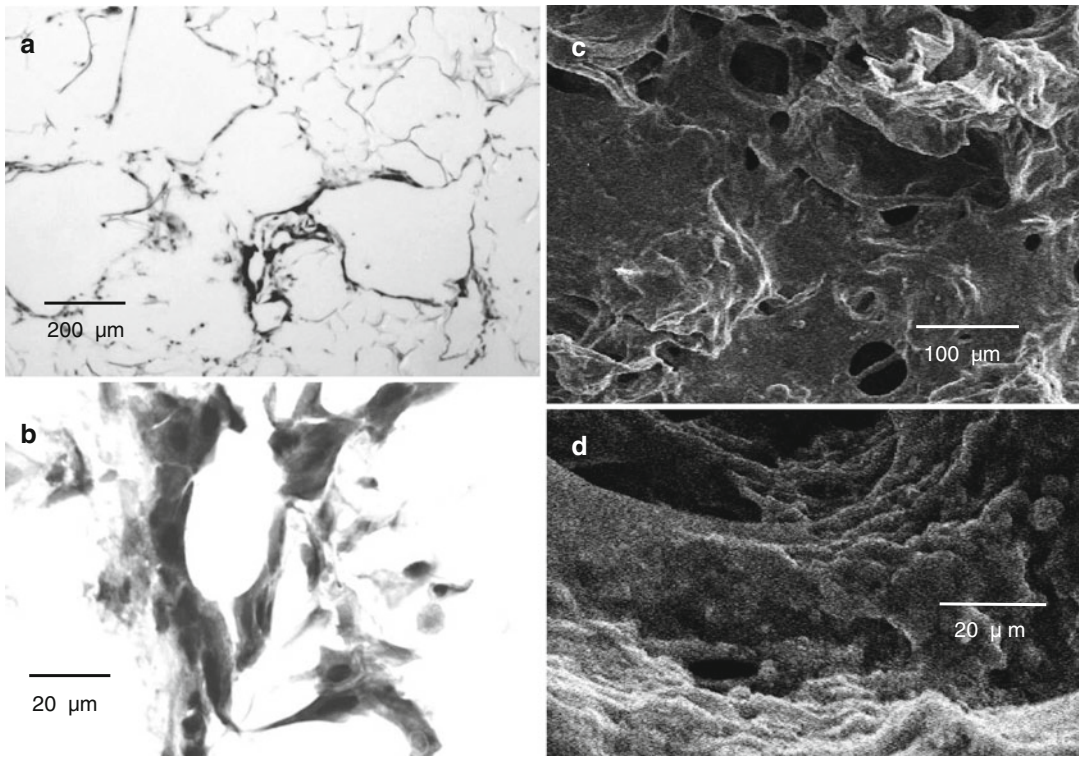

Fig. 4.11 HE staining (**a**, **b**) and scanning electron microscopy (**c**, **d**) of fetal rat hepatocytes cultured in 3D PLLA scaffolds for 2 weeks with the complete culture medium

animal model, the excessive deposition of copper leads to hepatic, neuropsychiatric, and other clinical manifestations. A wide variety of mutations in the P-type copper transporting ATPase (ATP7B) gene are responsible for defective hepatic copper excretion in WD [28]. LEC rats develop many clinical and biochemical features of WD, including liver copper accumulation [29].

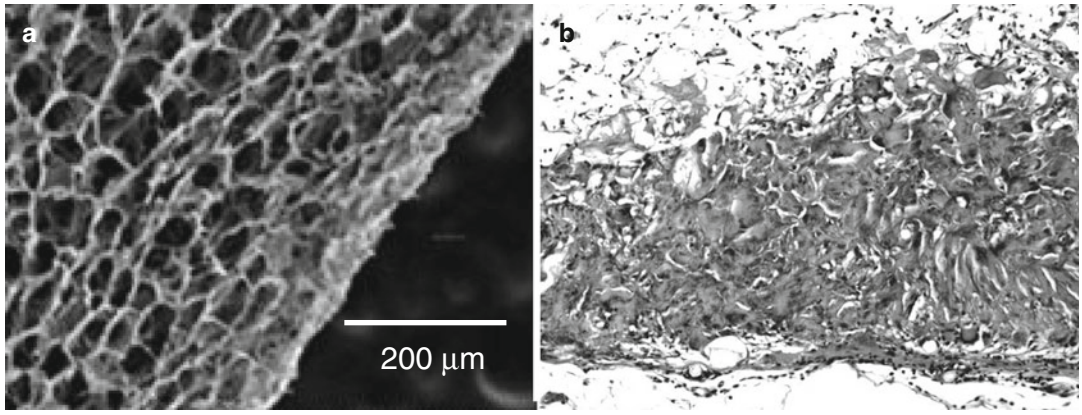

Fig. 4.12 Scanning electron microscopy (**a**) of a hyaluronic acid-based 3D macroporous scaffold (**a**) and HE staining (**b**) of fetal rat hepatocytes recovered at Week 2 after implantation

Isolated fetal rat hepatocytes from wild-type Long Evans Agouti (LEA) rats were inoculated into hyaluronic acid-based 3D macroporous scaffolds (Fig. 4.12a) and cultured in the complete medium for 4 days. Different from the results on PLLA scaffolds, hepatocytes were mainly immobilized on the outer surface, and no growth was observed in the deeper spaces. This was due to the differences in pore sizes between the PLLA scaffolds (250–500 m) and HA ones (60 m). However, the graft implanted to the mesentery and excised at Week 3 showed significant proliferation of cells of hepatocyte-like morphology inside the scaffolds (Fig. 4.12b). This was very much similar to the result of the implantation of cell-loaded PLLA scaffolds to mice, where active cell growth occurred, resulting almost complete packing of the macroporous structures, although in vitro growth was limited to the scaffold surface or surfaces of large pores even in the best culture medium. Two weeks after being fed with a conditioned diet, the control LEC rats showed severe jaundice, typically on their tails, ears, and limbs. In contrast, the rats transplanted with FLC-loaded HA sponges showed no jaundice within 3 weeks after transplantation. Accordingly, the treated LEC rats showed a significant reduction in blood Cu concentration compared to the control LEC rats (Fig. 4.13).

In the liver, HA is present in the matrix of embryonic and fetal tissues and near the presumptive stem cell compartment, the Canals of Hering, located in zone 1 of adult livers [5], but is not in association with the mature parenchymal

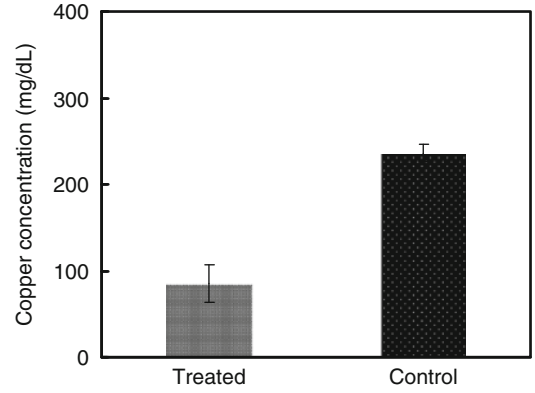

Fig. 4.13 Copper concentrations with SD in the blood sera of the treated and control animals. Mean ± SD ($N = 3$)

cells. Therefore, HA is a candidate matrix component for 3D scaffolds for liver progenitor cells. In fact, several studies besides ours [24] have demonstrated the suitability of HA for cultures of liver progenitor cells including FLCs [30].

Fetal Porcine Hepatocytes: In Vitro Study

When we think about the processes to human clinical trials of engineered liver tissue, particularly when the tissue is aimed at severe liver failure that necessitate implantation of much larger masses, large animal experimentations are necessary, and pigs have been regarded as promising candidates. This is because of their tissue availability as well as for their physiological

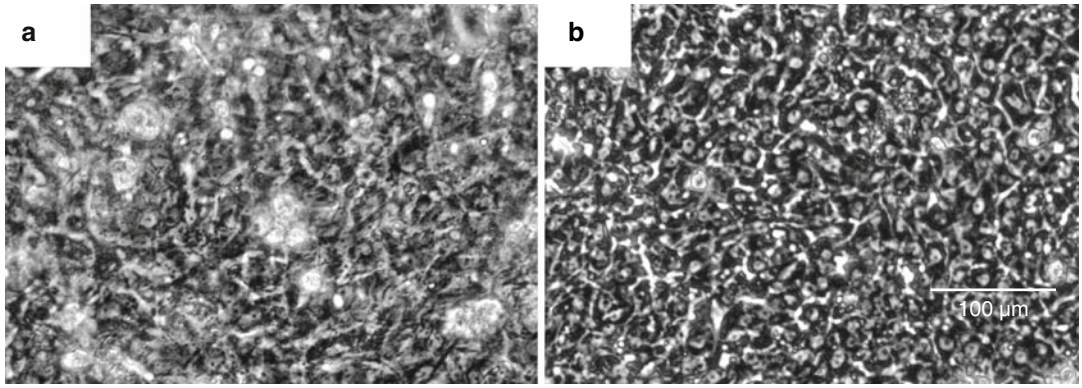

Fig. 4.14 Morphologies of fetal rat hepatocytes on Day 6 in the basal (**a**) and the complete culture medium (**b**)

similarities to humans [31]. However, there have been only two articles that reported the culture of fetal porcine hepatocytes, including the study by Ehashi et al. [9]. Elcin et al. showed good attachment and functional maintenance of the cells on 2D chitosan membranes [32]. They also tried xenotransplantation of the cells attached onto chitosan–albumin microcarriers in rats [33]. However, their approaches were based on 2D culture systems, and they did not observe any functional maturation that may be affected by various soluble factors. Therefore, we applied our PLLA scaffold-based simple 3D culture to fetal porcine hepatocytes, which can easily be obtained from a local slaughter house and be isolated with conventional collagenase digestion method [34].

In 2D monolayer culture, combination of HGF and sodium butyrate (SB) in the presence or absence of NA, DMSO, and OSM (NDO) gave the best cellular morphology on Day 15. In these conditions, nearly 100 % cells showed typical hepatocyte morphology such as polygonal shapes or distinct junctions with adjacent cells (Fig. 4.14). They had a smaller size than mature hepatocytes and had only a single nucleus; addition of NDO slightly decreased the diameter of the cells. Such appearance indicated that they can be referred to as small hepatocyte. However, NDO cocktail alone did not support well the emergence of such small hepatocytes.

In terms of various functions such as albumin production, EROD, ammonium removal, or urea synthesis showed that combination of HGF and Sb without NDO overall gave the best results both in 2D and 3D cultures; thus, this prescription was used as the complete culture medium. However, dependency on soluble factors of hepatocyte functions was lower than the culture of mice or rat fetal hepatocytes. Instead, 3D culture itself had controlling effects on the functions as clearly evidenced in albumin production (Fig. 4.15). In addition, the functional expression was more stable in 3D culture than in 2D culture. On a per cell-based functional comparison, finally attained functional levels in 3D-PLLA culture were almost completely comparable to those reported in adult porcine hepatocytes cultured in 3D spheroids [15] (Fig. 4.16). Thus, 3D culture itself had very significant effects on functions and their duration in culture, with less effects of supplementation of growth factor cocktails. This is presumably because the fetus became better matured compared with those in mice or rat.

Concerning cellular growth in 3D culture, porcine hepatocytes grew relatively well up to about fivefold during the 15 days of culture, but the finally attained cell density in the PLLA scaffold in the complete medium was about 1.8×10^7 cells/cm^3, which was over one order of magnitude lower than that of the in vivo liver tissue. In histological observations, cells grew only along the surfaces of large pores of the PLLA scaffolds, and they locally formed aggregate (Fig. 4.17). This agreed well with the measured cellular density in the scaffolds.

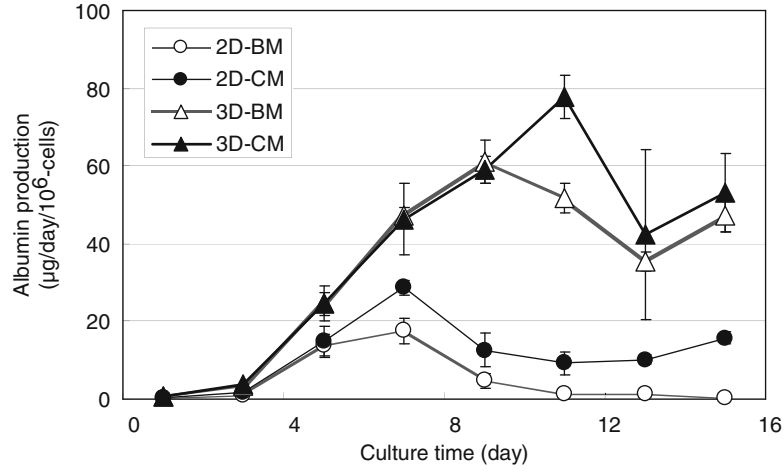

Fig. 4.15 Time-course changes in albumin production of fetal porcine hepatocytes cultured in 2D and 3D with the basal and complete culture mediums (*BM* and *CM*). Mean ± SD ($N = 6$)

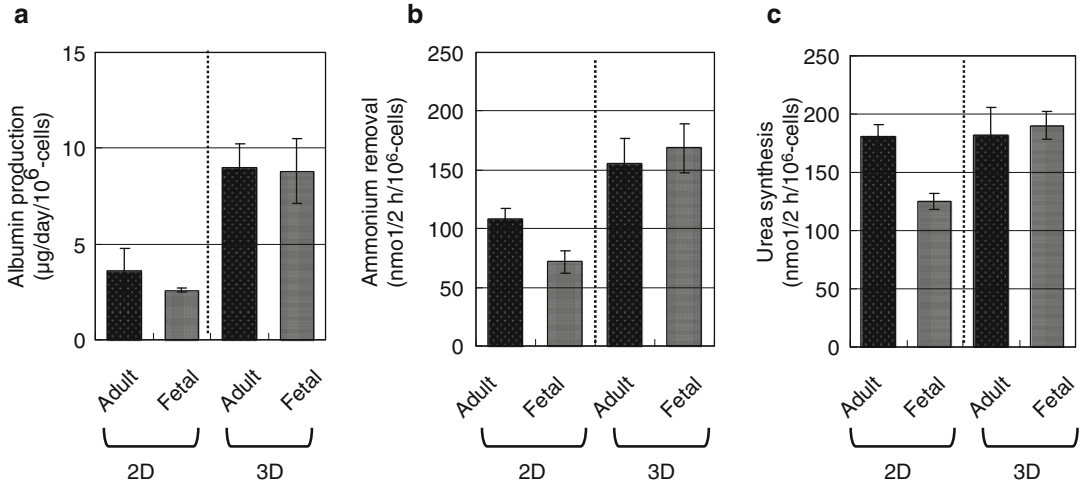

Fig. 4.16 Per cell number-based comparison of albumin production (**a**) ammonium removal (**b**) and urea synthesis (**c**) between adult porcine hepatocytes and fetal porcine hepatocytes cultured for 15 days in the complete medium. Mean ± SD ($N = 6$) (Adult porcine data are reploted from [14])

General Discussion and Future Perspectives

In this chapter, we introduced our results on small-scale 3D culture of fetal mouse, rat, and porcine hepatocytes using macroporous and biodegradable PLLA scaffolds-based 3D shaking culture and on some preliminary implantation of the cell-loaded scaffolds to the mesentery leaves of mice and rats. Synergistic effects of such 3D environments and soluble factor cocktails were observed in all the species, although some species differences existed in terms of the sensibility to these factors and 3D cultures. This synergy enabled better functional maturation and its maintenance in vitro over conventional 2D monolayer cultures. Although the cellular growth was limited on the surfaces of large pores in vitro, upon implantation to mesentery leaves of animals, almost all the remaining spaces in the scaffolds were finally filled with proliferated hepatocytes as evidenced from the in vivo studies on mice and rats. These results show promise of auxiliary liver tissues based on 3D-cultured fetal hepatocytes with appropriate soluble factor cocktails. This strategy can also be applied when hepatic

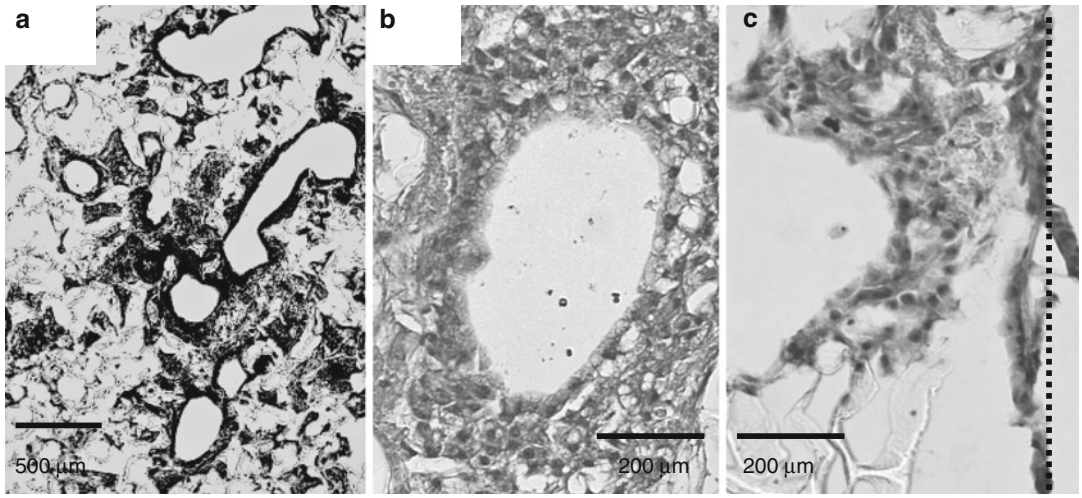

Fig. 4.17 HE staining of fetal porcine hepatocytes cultured in 3D PLLA scaffolds for 15 days with the complete culture medium. (**a**) and (**b**) thin section horizontal to the surface of the disk; (**c**) thin section vertical to the surface of the disk. *Dotted line* shows the surface of the disk (**c**)

induction from iPS/ES cells becomes practically feasible in the near future.

Although the combination of NA and DMSO in the presence of OSM was very effective in mice in inducing small hepatocyte among the inoculated fetal liver cell population, it did not support the growth and maturation of rat and porcine fetal hepatocytes. In the mice experiments, spontaneous organization toward 3D structure was even observed in 2D monolayers (Fig. 4.3c), but similar phenomena were not observed in rat or porcine fetal hepatocytes induced with other growth factor cocktails. As recently reviewed by Mitaka [35], who is one of the pioneers of small hepatocyte research using adult livers, small hepatocyte populations from adult liver are overall different from that of fetal hepatocytes. Therefore, combination of NA and DMSO itself is not likely to be effective in growth and maturation of overall population of fetal hepatocytes. Rather, NA (without DMSO), HGF, FGF-1, FGF-4, and OSM, which strongly support the rat fetal cell growth and maturation (Figs. 4.9, 4.10, and 4.11), seem to be generally effective for fetal hepatocyte culture. Less sensitivity of fetal porcine hepatocytes to growth factors in 3D culture (Fig. 4.15) compared with that of rats (Fig. 4.9) can be explained by the fact that the cells obtained from fetus are far better maturated than fetal rat hepatocytes; such better matured fetal hepatocytes should have stronger capability to spontaneously grow and mature in 3D microenvironment where various synergistic effects exist (Fig. 4.1c), even without exogenous supply of growth factors.

Three-dimensional culture system simultaneously realizes a microenvironment where both direct and indirect interactions among the cells, involving secretion/accumulation of autocrine and paracrine factors with the help of deposited ECMs (Fig. 4.1c), occur. Effect of exogenous growth factors or soluble factors should be maximized in such favorable microenvironments in manners mimicking actual in vivo liver environments. However, the functional maturation was still lower than those of fully matured adult cells, particularly in detoxification capability such as EROD. Further improvement in growth factor cocktails is thus necessary. To achieve this, we are thinking that we need to study again the biological/physiological situations of in vivo liver development after birth and to try to realize them in vitro. As partly evidenced from the remarkable effect of sodium butyrate, which is a metabolite of intestinal flora, on EROD capacity, in vivo liver is exposed to various exogenous stimulations, and there is high possibility that such metabolites flowing into the in vivo liver along the development may have another key to further optimize

the culture conditions as well as optimized inclusion of growth factors.

Sparse cellular growth, specifically along the surfaces of large inner pores, and the overall cell density less than one-tenth that of the in vivo liver should be another problem of current 3D culture results. Although this situation is completely reversed upon implantation, that is, cells began to grow again so that all the inner spaces are filled with cells, the lower total cell number after in vitro cultivation should result in lower efficacy in disease treatments particularly immediately after implantation. Most plausible cause for this low growth in vitro 3D culture is likely to be the limitation of oxygen supply in the scaffolds. Oxygen supply is the controlling factor in hepatocyte because they consume almost ten times higher amount than fibroblast [36]. Recently, we showed that direct oxygenation of cells through oxygen-permeable membranes enables advanced organization of hepatoma cells [37] or fetal rat liver cells [38] in static culture. In this culture system, cells spontaneously grow and organize into multilayers composed of 5–6 layers, which has never been observed in conventional oxygen impermeable culture surfaces. In the case of fetal rat hepatocytes, they formed heterogenic thick sheet-like tissues (up to 100 μm in thickness) composed of hepatocyte on the top of the layer and ECM-enriched stromal cell layer when we first used 5 % oxygen and then increased to 21 %. Such a thickness seems to be the limitation without arranging vascular systems, and therefore, we need to include functional vascular-like system in further scaling up [2].

Realization of tissue polarity in terms of bile acid secretion is a remaining big challenge. In usual heterotopic liver tissues, bile acids are likely to be leaked back to the blood flow from the tissue and finally removed from the blood by the remaining host liver [39]. One clue to the solution of this issue is the experimental results by Sudo et al. [40] concerning the formation of functional bile canaliculi networks formed in small hepatocyte colonies, where formed bile acids are actively transported to small bile pools by synchronized mechanical constriction of the entire colony. Next trial is to guide the formation of bile canaliculi network over some larger regions of the engineered tissues with the help of various microtechnologies so that we can recover secreted bile acid from the heterotopic liver tissues and connect them to the intestine as in vivo.

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Response of Fetal and Adult Cells to Growth Factors

5

Harris Pratsinis, Andreas A. Armatas†, and Dimitris Kletsas

Introduction

Cell replacement therapies represent a major tool in regenerative medicine, aiming at the restoration of the architecture and function of damaged or nonfunctional tissues. In this context, cells derived from fetuses could provide an additional advantage as they have not been previously exposed to environmental noxes and possess a more extended lifespan from cells derived from adult organisms. Accordingly, fetal tissues are more vigorous than adult ones and with a superior ability for tissue repair. In this vein, it is well known that fetuses and adults follow different strategies in tissue repair. In contrast to the adult-like healing, characterized in several tissues, such as skin, by a scar formation, fetuses from various species are able for a scar-free tissue regeneration. Consequently, an in-depth investigation of the mechanisms of fetal repair is needed, aiming at improving the healing process in the adults [73].

†The present chapter is devoted to the memory of our late colleague Andreas Armatas.

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Differences in Wound Repair Between Fetuses and Adults: Environmental or Intrinsic?

Tissue injury activates a sequence of processes leading to repair. These overlapping events can be temporally categorized in the following phases: inflammation, tissue formation, and remodeling [23]. Immediately after injury and vascular rupture, blood coagulation process is activated. In parallel, a provisional matrix, necessary for the migration of cells into the wound area, is created. In this phase, the role of platelets is of utmost importance as they aggregate and release several mediators of the healing process, among them being a series of growth factors. These factors attract in a chemotactic manner immune cells, such as monocytes/macrophages, that are able to produce and secrete growth factors and cytokines in the wound area. Among the cells that migrate in the provisional matrix are fibroblasts, major players in the healing process, as they populate this area and produce the components of the extracellular matrix (ECM), such as collagen, hyaluronic acid, and proteoglycans. Thus, provisional matrix is replaced by a more permanent granulation tissue. Fibroblasts play an additional role in this phase as they can be converted to myofibroblasts, leading to wound contraction. In parallel, other important parameters are reepithelialization (the formation of a new epithelium covering the injured tissue) and

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neovascularization (an increased angiogenesis in the newly formed tissue). Several weeks later, tissue remodeling is activated aiming at the restoration of the initial tissue. In this phase, crucial is the balance between matrix metalloproteases (MMPs) and their inhibitors (tissue inhibitors of metalloproteases – TIMPs) [30]. In extended wounds or in pathological cases (as in keloids), an imperfect healing is observed, characterized by non-remodeled scar. Another case of imperfect healing is chronic wounds featuring an intense and perpetuating inflammation.

In contrast to the above, as already mentioned, fetuses are able to heal their wounds by a perfect tissue regeneration, marked by the absence of scar formation. This is determined by the size of the wound and the age of the fetus [18], as this scar-free healing occurs in the first two trimesters of gestation. Then, a transition from scarless repair to adult-like scar formation is observed. In humans, this transition is after approx. 24 weeks of gestation [72]. Tissue repair in the fetus is achieved in a sterile fluid environment with a relative lack of inflammation [78]. Accordingly, it has been hypothesized that these alterations are responsible for the differential repair process in fetuses and adults. In this direction, it has been shown that incorporation of bacteria in fetal wounds can lead to neovascularization and fibroplasia, typical responses of adult-like healing. However, a series of experimental data support the importance of intrinsic differences between the fetal and the adult tissue. Specifically, it has been shown that grafts of human fetal skin when placed subcutaneously in adult athymic nude mice healed their experimental wounds in the absence of scar, while cutaneous grafts healed with scar [72]. In addition, the wounds in full-thickness adult sheep skin transplanted onto the backs of early fetal lambs healed with scar formation [71]. Finally, important information stems from observations on the opossum *Monodelphis domesticus*. Its embryos are born at a very early developmental stage and with a poorly developed immune system and can still heal in a fetal mode, characterized by rapid reepithelialization, minimal inflammatory and angiogenic responses, and lack of fibrosis [78]. All the above collectively indicate that although the environment can affect scar formation, fetal skin healing properties are primarily intrinsic to the fetal tissue.

The intrinsic ability of the fetal tissue for complete tissue regeneration is most probably the reflection of the unique features of its cells. Accordingly, the present chapter will focus on the characteristics of fetal cells, and especially fibroblasts, and in particular on their response to growth factors that represent an important determinant of tissue homeostasis and repair.

Growth Factors

As mentioned above, the initial sources of a battery of growth factors triggering the wound healing process are the serum of injured blood vessels containing the products of the degranulating platelets; hence, the first growth factors present in the wound are PDGF, TGF-β, and EGF [7]. Furthermore, during the next phases of wound healing, many other growth factors and cytokines are produced locally from various cell types, most important being FGFs, IGFs, and VEGFs. A brief description of these growth factor families follows.

PDGFs

Platelet-derived growth factor (PDGF) appears as a family of dimeric disulfide-bound isoforms with a molecular weight of approximately 30 kDa, originally isolated from human platelets [3]. Traditionally, the homodimers PDGF-AA and PDGF-BB and the heterodimer PDGF-AB – the most common isoform in human platelets – were the only known PDGF ligands [53]; more recently, however, two new members of the PDGF family were identified: PDGF-CC and PDGF-DD [52]. The A and B polypeptide chains are synthesized as precursor molecules that undergo proteolytic processing before their cell secretion; the mature A and B chains contain approximately 100 amino acid residues, and they have approximately 60 % amino acid sequence identity [41]. The PDGF-C and PDGF-D chains, on the other hand, are

activated after their secretion by cleavage of their amino-terminal domains [10, 64, 69]. PDGFs act on target cells through activation of two structurally related protein tyrosine kinase receptors, α and β, with molecular weights of approximately 170 and 180 kDa, respectively [53]. Binding of PDGFs to their receptors causes homo- or heterodimerization and cross-phosphorylation on specific tyrosine residues, thus triggering signaling cascades including among others the PLCγ, the PI 3-K/Akt, and the Ras/Raf/MEK/ERK pathways [68].

These pathways are transducing a variety of biological effects of PDGFs, cell proliferation being the most prominent of them, as PDGF is a most potent mitogen for cells of mesenchymal origin [13, 107]. Other effects encompass cell survival, actin cytoskeleton rearrangements, chemotaxis, stimulation of ECM molecules' production, as well as secretion of ECM-degrading enzymes, and induction of collagen matrix contraction [53]. Hence, PDGFs are implicated in the early phases of wound healing by attracting various cell types in the wound area and promoting their proliferation, during ECM deposition and remodeling, as well as during the phase of contraction [7].

TGF-βs

The transforming growth factor-β (TGF-β) superfamily of cytokines are encoded by 42 open reading frames in human, 9 in fly, and 6 in worm, and can be classified to two subfamilies, the TGF-β/Activin/Nodal subfamily and the BMP (bone morphogenetic protein)/GDF (growth and differentiation factor)/MIS (Mullerian-inhibiting substance) subfamily, based on sequence similarities and the specific signaling pathways activated [105]. Most of them encode dimeric, secreted polypeptides regulating cell proliferation, differentiation, adhesion, migration and death, in a developmental context-dependent and cell type-specific manner [82]. Among these members the ones participating in mammalian wound closure are TGF-β1, -β2, and –β3, activin, and BMP-2, -4, -6, and –7 [110]. All TGF-β ligands act on cells through binding to type I and type II receptors that form heterotetrameric complexes in the presence of the dimeric ligand [82] and are featuring a cytoplasmic kinase domain that has strong serine/threonine kinase activity and weaker tyrosine kinase activity, which classifies them as being dual specificity kinases. Downstream signaling of all TGF-β superfamily members is mainly effected by members of the Smad family: the receptor-activated Smads or R-Smads (SMAD2 and SMAD3 for the TGF-β subfamily and SMAD1, SMAD5, and SMAD8 for the BMP subfamily, see above) are phosphorylated by type I receptor and associate with the common mediator Smad or Co-Smad, i.e., SMAD4; this complex is transferred to the nucleus to regulate gene transcription, while the inhibitory Smads or I-Smads (SMAD6 and SMAD7) negatively regulate signaling strength and duration [82]. Besides this so-called canonical pathway of Smad proteins [59], TGF-β superfamily members' signaling can also involve one or more of the mitogen-activated protein kinases (MAPKs) ERK, Jun N-terminal kinase (JNK), and p38, as well as, PI 3-K kinases, PP2A phosphatases, and Rho family members [32].

TGF-β is the prototype of the multifunctional growth factor [77]. It inhibits the proliferation of epithelial and endothelial cells, while for fibroblastic cells, it can be either inhibitory or stimulatory [75]. The members of the TGF-β-superfamily are considered as the main regulators of ECM synthesis and degradation through the coordinated regulation of complex gene sets [55, 99]. TGF-β, furthermore, regulates migration, and collagen gel contraction, and exhibits immunosuppressant activities, thus playing central role in all phases of wound repair, from inflammation and granulation tissue formation to reepithelialization and matrix formation and remodeling [7, 51, 75, 110].

One of the target genes of TGF-β is connective tissue growth factor (CTGF), a cysteine-rich heparin-binding mitogenic peptide, which is selectively induced by TGF-β in cells of mesenchymal origin [40, 65], and it further regulates fibroblast growth and ECM production [56]. On the other hand, in epithelial cells CTGF seems to

be induced by TGF-β through the Ras/MEK/ERK pathway [104]. Hence, in the wound repair process CTGF is involved in granulation tissue formation, reepithelialization, and matrix formation and remodeling [7].

EGF Family

The ever-growing epidermal growth factor (EGF) family of mitogens comprises several members, including EGF, transforming growth factor-α (TGF-α), heparin-binding EGF (HB-EGF), amphiregulin, epiregulin, betacellulin, epigen, as well as proteins encoded by Vaccinia virus and other poxviruses [83, 110]. In addition, more distantly related proteins known as neuregulins (heregulins, neu differentiation factors, NDF 1–4) can also bind to some EGF receptor family members [110]. All these growth factors exert their functions by binding to four different high-affinity receptors with tyrosine kinase activity, EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4, which upon ligand binding form homo- or hetero-dimers and undergo autophosphorylation. Substrates involved in the downstream transduction of the signal include the Ras/Raf/MEK/ERK axis, G-proteins, and the Jak/Stat pathway, among others [83].

EGF family members promote the proliferation of most cell types in the wound area, such as keratinocytes, fibroblasts, macrophages, and vascular endothelial cells [103]. Furthermore, they regulate keratinocyte differentiation, as well as the migration of fibroblasts and endothelial cells. Hence, EGF family members play an important role during the wound healing process, mainly through promotion of the reepithelialization and neovascularization processes [7, 51, 103].

FGFs

The fibroblast growth factor (FGF) family comprises 23 members up to now characterized by their strong affinity for heparin and heparan-like glycosaminoglycans, as well as a central core of 140 amino acids exhibiting high homology throughout all family members [91]. Among them, the three most important molecules involved in the wound repair process are FGF-2 or basic FGF (bFGF), FGF-7 or keratinocyte growth factor-1 (KGF-1), and FGF-10 keratinocyte growth factor-2 (KGF-2) [7]. FGFs mediate their cellular responses by binding to and activating a family of four receptor tyrosine kinases (RTKs) called high-affinity FGF receptors (FGFR1 to FGFR4) – the low-affinity ones being heparin and heparan sulfate proteoglycans, which do not transmit a signal but play accessory and regulatory roles [39]. The diversity of FGFRs is further expanded due to the expression of numerous splice variants of each gene [91]. The main downstream effectors of FGFR signaling include the Ras/Raf/MEK/ERK pathway, the PLCγ/DAG/PKC pathway, as well as the PI 3-K/Akt axis [39].

bFGF is mitogenic for fibroblastic cells [87], and it regulates the growth and function of vascular cells such as endothelial and smooth muscle cells [86]. Furthermore, bFGF regulates the turnover of various ECM molecules [9], while FGF-7 and −10 are both mitogenic for keratinocytes [91]. Hence, FGFs during the wound repair process are implicated in granulation tissue formation, in reepithelialization, as well as in matrix formation and remodeling, and in angiogenesis [7].

IGFs

The insulin-like growth factor (IGF) family comprises IGF-I and –II, two polypeptides of 70 and 67 kDa, respectively, that share 62 % homology with proinsulin [8]. However, the IGF family in the broad sense includes three ligands (IGF-I, -II, and insulin), six IGF binding proteins (IGFBPs), and three receptors: the type I insulin-like growth factor receptor (IGF-IR), the insulin receptor (IR), and the IGF-II/mannose 6-phosphate receptor (IGF-IIR); hybrid IGF-IR and IR have also been reported to interact with IGF [98]. Responsible for most of IGFs' actions is mainly IGF-IR, which upon ligand binding on its extracellular α subunits undergoes conformational changes leading to activation of the intracellular

tyrosine kinase domains of the β subunits and autophosphorylation [58]. Downstream signaling proceeds through the direct phosphorylation of the insulin receptor substrates IRS-1 to –4 and Shc and subsequently the PI 3-K/Akt/mTOR and the Ras/Raf/MEK/ERK axes [98].

IGFs exert proliferative and survival effects on an astonishing variety of target cell types, and they furthermore can affect differentiation, ECM-regulation, chemotaxis, secretion of hormones, neurotransmitters, and other growth factors, as well as the uptake of amino acids and glucose [58]. They often operate as local mediators of the actions of hormones or other growth factors [58]. IGFs can act in autocrine or paracrine manner [6]; hence, they affect the reepithelialization of the wound; generally, lower levels of IGFs are observed in various cases of impaired wound healing [110], while, on the other hand, their overexpression seems to lead to hypertrophic scars due to increased collagen deposition [44].

VEGFs

The vascular endothelial growth factor (VEGF) family currently includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PLGF) [110]. These dimeric molecules are distant relatives of PDGF, since their monomers share a cysteine knot motif comprising an 8-residue ring formed by 3 disulphide bridges [53, 113]. The variety of VEGF family increases further due to alternative splicing of the above genes. They bind to heparin, and, with high affinity, to three different transmembrane tyrosine kinase receptors, designated VEGFR-1 (or Flt-1), VEGFR-2 (or KDR), and VEGFR-3 [110].

VEGF receptors, like the ones of PDGF or EGF, upon ligand binding undergo dimerization and autophosphorylation and then transmit their signal through activation of the MAPK cascade, PLCγ, PKC, as well as FAK and paxillin [113].

The biological functions of VEGFs are mainly focusing on the regulation of vasculogenesis and angiogenesis during development [43], while they play an important role in wound angiogenesis [38] and granulation tissue formation [7, 110].

Fibroblasts' Responses to Growth Factors

Normally, fibroblasts – central players of the repair process – express the respective receptors and are capable of responding to members of the PDGF, the TGF-β, the EGF, the FGF, and the IGF families [13, 16, 58, 87]. Furthermore, fibroblasts have been shown to express and produce PDGF [94], TGF-β [93, 114], EGF [63], FGF [4, 70], IGF [2, 6], and VEGF [27]. Consequently, various attempts to delineate the mechanisms underlying the intrinsic ability of the fetal tissue for complete tissue regeneration are focusing on the differential expression of these growth factors and/or their receptors between fetal and adult cells. Through a series of studies examining spatiotemporal aspects of growth factor expression in the wound, it appears that bFGF and especially TGF-β levels are reduced in fetal wounds; perhaps more importantly, TGF-β1 mRNA and protein are only transiently expressed in mouse embryo wounds, and these differences could be attributed to the various cell types present, such as to the macrophages, but also mesenchymal cells [74, 85, 111]. In a preliminary report, TGF-β1,2,3, a/bFGF, KGF, PDGF-AA,-AB,-BB mRNA expression in fetal and adult human skin fibroblasts was tested; the main finding was that fetal and adult fibroblasts express acidic and basic FGF, and TGF-β1; however, adult fibroblasts show twice the relative expression of these growth factors compared with fetal fibroblasts, which is thought to contribute to suboptimal wound healing in adult wounds compared with the scarless healing of fetal wounds [14]. On the other hand, the same team reported reverse results at the level of the protein, i.e., higher levels of TGF-β1, aFGF, and bFGF expression in fetal fibroblasts [66], attributing this contradiction mainly to technical issues and/or differences in mRNA translation and degradation. The latter observations were reinforced by the finding that TGF-β1 protein levels in serum-free conditioned medium from human fetal fibroblast cultures were higher than in those from adult cells as assessed by an ELISA [49]. Another study, using fetal rat skin fibroblast cultures derived from various gestational ages – i.e.,

before and after the transition from the scarless repair to the scar-forming healing – found no differences in the EGF- and the PDGF-B-gene expression, although they observed a decrease in the expression of both genes when they used mRNA extracted from whole skin at the same gestational stages [89]. On the other hand, in a similar approach using primary mouse skin fibroblast cultures, it was observed that fetal cells express lower basal CTGF mRNA levels than adult ones, although the induction of this gene by TGF-β was much more intense in fetal than in adult fibroblasts [25]. Furthermore, the same team has shown that murine fetal skin fibroblasts express higher levels of the TGF-β3-isoform than adult ones, while no differences were observed in the expression of TGF-β1 and -β2 and of both TGF-β-receptor types I and II [26], a very important observation given the anti-scarring properties of TGF-β3 (see below). In contrast, regarding human skin fibroblasts, no differences in the gene expression levels of all three TGF-β-isoforms were observed between fetal and adult cells by using quantitative real-time PCR [96].

Fibroblast Proliferation

PDGF is considered as a potent mitogen for cells of mesenchymal origin, and from relatively early reports, it has been shown to stimulate the proliferation of both fetal and adult human skin fibroblasts [11]. Moreover, it has been shown that the intensity of PDGF-induced proliferative response is comparable in both fetal and adult cells [92]. Similar observations have been obtained with fetal and adult fibroblasts from different tissues, such as lung [21]. EGF is also mitogenic for human skin fibroblasts from both developmental stages [11], with comparable potency, which is, however, less than that of PDGF (our unpublished data). Results obtained from cultures of fetal and adult prostatic fibroblasts suggest that this response to EGF could be independent of the tissue of origin [67]. Similarly, FGF-2 is mitogenic for human skin fibroblasts originating from both fetal and adult donors, with potency higher than that of EGF but lower than that of PDGF (our unpublished observations). On the other hand, regarding IGFs, it was reported that human fetal skin fibroblasts exhibit a weaker mitogenic response to both IGF-I and -II than postnatal ones, although they express comparable levels of IGF-IR, and that fetal cells – in contrast to postnatal ones – failed to phosphorylate ERK I (p44) and Shc (p46) in response to IGF-I and to IGF-II, respectively [95].

As mentioned above, TGF-β is the prototype of the multifunctional growth factor [108], a fact apparent also at the level of cell proliferation regulation, as exemplified by the early observations that this factor can either inhibit or stimulate proliferation depending on the cellular context [54]. With regard to human skin fibroblast proliferation, it has been reported initially that TGF-β1 does not stimulate DNA synthesis in neonatal fibroblasts, and it inhibits the mitogenic activity of PDGF in a density-dependent fashion [88]. In contrast, several other studies reported a mitogenic action of TGF-β in confluent cultures of newborn and adult skin fibroblasts [57, 106, 112], while in a more recent study, a weak mitogenic response to this growth factor was described for both fetal and postnatal cells [97]. However, through a direct systematic comparison of different cell strains cultured under identical conditions, a clear-cut difference between fetal and adult human skin fibroblasts in their proliferative response to TGF-β was observed, i.e., this growth factor is inhibitory for fetal cells, while it is stimulatory for adult ones [92]. This was observed for all three isoforms TGF-β1, -β2, and -β3, and since neonatal fibroblasts were found to respond similarly to adult ones [92], it seems that this change is coinciding with the transition from scarless repair to scar-forming healing. Regarding the mechanisms underlying these responses, it was shown that the inhibition of human fetal skin fibroblast-proliferation by TGF-β is mediated through the activation of PKA and the subsequent induction of the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p15^{INK4B}$ [45]. In human adult skin fibroblasts, TGF-β does not activate PKA, but induces an autocrine loop involving the upregulation of extracellular

bFGF, and through FGFR1 activation of the MEK/ERK pathway, thus leading to cell proliferation [45].

Fibroblast Migration

Migration of various cell types in the wound area is an important parameter in the early healing process, as they support many functions leading to the formation of the new tissue. Especially fibroblasts play a central role, as their migration leads to the repopulation of the tissue. Furthermore, these cells are the main producers of the extracellular matrix components that are necessary for the formation of the granulation tissue. Considerable differences exist between fetal and adult fibroblasts, as the former display an increased migratory activity. The observed changes are both due to intrinsic features, as well as due to a different response to exogenous growth factors.

It has been reported that fetal fibroblasts migrate to a greater extent into 3-D collagen gel matrices, compared to adult fibroblasts [102]. In addition, it has been observed that fetal cells can migrate irrespective of cell density, while adult fibroblasts migrate easier when are plated on collagen matrix at low density, compared to confluent cultures [102]. The differences mentioned above may be directly linked to the abundance of hyaluronic acid, as high levels of this extracellular matrix component enhance the migratory activity of cells, while treatment with hyaluronidase blocks completely cell migration [100, 101]. Fetal fibroblasts seem to have more receptors for hyaluronic acid than adult cells [1]. Furthermore, they also secrete a soluble factor (migration stimulation factor – MSF), which stimulates cell migration through novel hyaluronic acid synthesis [101]. Consequently, it seems that the differences in migratory activity between fetal and adult fibroblasts are partly due to the differential secretion and response to hyaluronic acid. Moreover, fetal skin fibroblasts can migrate in the presence of serum-depleted medium, while adult fibroblasts require the presence of serum for migration [62]. It has been shown that this autonomous migration on plastic surfaces of fetal cells may be linked to the secretion of bFGF, as in the presence of an anti-bFGF antibody the migratory activity was inhibited [61].

Exogenous growth factors regulate the migration of fetal and adult fibroblasts in a differential manner. Interestingly, their action depends largely on the presence of extracellular matrix components, as well as on culture density. Specifically, PDGF stimulates the migration of both fetal and adult human fibroblast on plastic surfaces, while when plated on three-dimensional collagen gels, it stimulates only adult fibroblasts and has no effect on fetal cells [35]. Similarly, EGF stimulates the migration of adult, but not fetal, fibroblasts on 3-D collagen gels [35]. On the other hand, as mentioned above, bFGF is important for the autonomous migration of fetal fibroblasts, as an anti-bFGF antibody inhibits their migratory activity [61]. However, exogenously added bFGF cannot reinforce their migration [61], while stimulating the migration of adult fibroblasts [35]. As these cells also secrete this growth factor, it seems that probably a different threshold of bFGF concentration is needed for cell migration in these two developmental stages. Finally, the action of TGF-β is substratum, density, and isoform dependent. On plastic surfaces, TGF-β1 stimulates the migration of both fetal and adult fibroblasts, most probably via the secretion of hyaluronic acid [36]. On collagen gels, TGF-β1 inhibits fetal cell migration when cultures are confluent, while having no effect on subconfluent ones; in contrast, in adult fibroblasts, it is inhibitory in subconfluent cultures but not in confluent ones. Interestingly, TGF-β2 exerts the same action with the TGF-β1 isoform [37]. On the other hand, TGF-β3 inhibits its migration in subconfluent cultures in cells from both developmental stages. However, in confluency, it is inhibitory for fetal fibroblasts and stimulatory for adult ones [37]. All the above strongly indicate that the migration of fetal and adult cells depends largely on intrinsic features, as well as on a complex network of exogenous growth factors, extracellular matrix components, and cell-cell interactions.

Extracellular Matrix Synthesis and Remodeling

As mentioned above, the most obvious difference between fetal and adult skin wounds is the healing with scar formation in the latter. As scar is a tissue rich in collagen, especially type I, but with a less organized pattern, synthesis and remodeling of collagen is important for the maintenance of homeostasis and the proper tissue repair in these two developmental stages. Fetal skin has an increased type III/type I collagen ratio, compared to the adult. Interestingly, this change is reflected at the cellular level, as fetal fibroblasts produce higher levels of type III collagen [47, 79]. In addition, fetal fibroblasts express increased activity of prolyl hydroxylase, the enzyme for a rate-limiting step in collagen production [34]. Finally, collagen synthesis by fibroblasts in vivo follows different kinetics between fetuses and adults. While in the former it starts immediately after wound formation, in the adults, a delayed collagen synthesis is observed [50].

In accordance with the above, fetal fibroblasts secrete increased quantities of latent TGF-β, a major stimulant of collagen synthesis and accumulation [47, 84]. Consequently, adult cells seem to be more responsive to TGF-β (our unpublished data). In line with the increased expression of type I collagen in the adult skin, it has been shown in mouse fibroblasts that mid-gestational cells showed decreased expression of procollagen 1α1 in response to TGF-β1. In cells from late-gestational stages, procollagen 1α1 production is increased, while that of procollagen type III is decreased [17]. Other growth factors also display differential effects on fetal versus adult fibroblast. In particular, it has been shown that adult cells are stimulated for collagen production by IGF-I, in contrast to fetal fibroblasts. On the other hand, IGF-II increases significantly collagen synthesis by fetal cells but had no effect on collagen synthesis by adult ones [95].

One major difference between fetal and adult skin is that the former contains more hyaluronic acid (HA), a negatively charged, nonsulfated glycosaminoglycan [15, 76]. This change probably gives unique structural and functional features in the fetal tissue, such as increased fibroblast migration in the wounded area, as a relation between high HA synthesis and increased migratory activity in fetal fibroblasts has been proposed [20]. In addition, fetal fibroblasts express more HA receptors than adult fibroblasts [1]. Concerning wound formation, an increased deposition of HA in fetal tissues compared to adult ones was observed 6 days after wounding [31].

Differences between fetal and adult fibroblasts in hyaluronic acid production are also directed by cell density. It has been shown that fetal fibroblasts produced high level of HA in subconfluent and confluent cultures, while the production by adult fibroblasts is decreased significantly when cells reached confluence [20]. Fetal and adult fibroblasts respond also differently to exogenous factors toward HA synthesis. TGF-β1 induces HA synthesis for both cell types in confluent cultures, while in subconfluent ones, it inhibits adult but not fetal cells [36]. In addition, TNF-α regulates differentially the three enzymes synthesizing HA; in fetal cells, it increases HAS-1 mRNA levels, while in adult fibroblasts, only HAS-3 gene expression is upregulated [60].

Extracellular matrix-degrading enzymes play a significant role in all phases of tissue repair and especially in tissue remodeling. Prominent among them are matrix metalloproteases, a large family of zinc-dependent endopeptidases, which are secreted in latent forms and activated by limited proteolysis [12, 46]. Their action is also regulated by their specific inhibitors TIMPs (tissue inhibitors of metalloproteases) [5, 46]. In addition, MMP production is influenced by the environment; for example, fibroblasts grown in 3-D collagen gels secrete much higher levels of MMPs [115]. In non-wounded human and mouse tissues, it was found that the expression of several MMPs and TIMPs increases from early to late-gestational stages and even more in adult tissues [19, 29]. However, it was hypothesized that the lower TIMP expression may be crucial for scarless repair, facilitating the turnover of extracellular matrix components, as well as other important functions of tissue formation, such as cell migration [19, 29]. In human fibroblasts, it has been shown that fetal cells secrete much higher levels of activated MMP-9 than adult ones, while

these of MMP-2 remain essentially similar [28]. In the same study, a differential effect of two of the major growth factors released in the wound area (i.e., TGF-β and PDGF) on the expression of these MMPs was reported. TGF-β induces the secretion of MMP-2 and MMP-9 only in fetal and not in adult cells. On the other hand, PDGF reduces MMP-2 secretion in fetal cells, while it stimulates MMP-9 secretion in both cell types [28].

Contraction

Wound contraction is a major process in adult wound healing, and it is mediated by fibroblasts that transdifferentiate into myofibroblasts, characterized by an increased expression of α-smooth muscle actin, a contractile cytoskeletal protein [42]. On the other hand, one of the unique features of fetal skin healing is the absence of contracture. In order to understand if these differences are intrinsic to the fetal tissue, in vitro studies have been performed by several laboratories by using as a model the ability of cells from both developmental stages to contract cell-populated 3-D gels of polymerized collagen, the latter being the major extracellular matrix component in dermis [48]. It has been shown that indeed human fetal fibroblasts have a much lower contractile capacity, compared to adult cells [80]. The data have also been supported by studies on cells from other species, such as mice or lamb. In both species, it has been found that skin fibroblasts from early gestational stages display a lower ability to contract collagen gels, compared to cells from late-gestational stages or from adult animals [24, 90].

TGF-β is considered an important regulator of contraction, as it provokes the differentiation of fibroblasts to myofibroblasts by enhancing α-smooth muscle actin [33]. In murine skin fibroblasts, the differences in the contractile ability between early fetal cells and late fetal and adult cells are linked to the secretion of total and active TGF-β [24]. However, it has been reported that while TGF-β stimulates the contraction in adult human skin fibroblasts, it is inhibitory in fetal cells, being unable to increase α-smooth muscle actin expression [81]. On the other hand, other investigators have shown that TGF-β is able to induce collagen gel contraction by fetal cells ([90]; our unpublished observations). Similarly to TGF-β, PDGF is also secreted in the wound area by degranulated platelets and somatic and immune cells, and it is able to potently stimulate the contraction in adult skin fibroblasts [22, 109]. We have found that PDGF, when used in high concentrations, can also enhance the contractile capacity of both fetal and adult cells (our unpublished observations), indicating that it is not only the intrinsic features of fibroblasts but also the local concentrations of circulating growth factors that regulate the overall phenomenon of tissue repair in these two developmental stages.

Collectively, it seems that fetal fibroblasts possess unique features that may affect significantly the speed and quality of tissue repair, in agreement with the results from previous xenographic studies. They also show quantitatively and/or qualitatively different responses to several growth factors present in the wound area. So, the effect of a complex network of autocrine and exogenous growth factors on fetal cells and in a dynamic interaction with the continuously changing extracellular matrix direct scarless fetal regeneration. More detailed understanding of the fine mechanisms regulating these interactions may lead to future therapeutic interventions toward the improvement of the quality of wound repair in the adults.

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Accommodation and the Fetus

6

Ines Silva, Cody A. Koch, Raymond J. Lynch, and Jeffrey L. Platt

Introduction

The term "accommodation" refers to acquired resistance of a graft to injury by the immune system of the recipient of that graft. Accommodation was "discovered" in the 1980s as an explanation for the unanticipated survival and well-being of organs transplanted across ABO blood group barriers (blood group A or B organs expressing blood group A or B transplanted into recipients producing antibodies against one or both of those blood groups) [7, 15]. Organs transplanted across blood group barriers usually undergo early and severe rejection; approximately 75 % fail within 3 months [52]. However, if anti-blood group antibodies are removed from recipients or the binding of these antibodies is blocked, the prospects for long-term survival and function of the graft approach that of grafts matched for ABO blood groups [1]. Although several explanations including spontaneous tolerance [24] and loss of the antigenic target [2] might explain this phenomenon, the organs were found to persist in expression of antigen, and the recipients were found to continue to produce anti-blood group antibodies [7, 15]. These findings led to the suggestion that the organ might have changed in some way that would allow it to "accommodate" to what would otherwise be a toxic assault by antibodies of the recipient and activation of complement [42]. In this communication, we discuss the possibility, yet theoretical, that accommodation may explain the survival of the fetus as an allograft.

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Mechanisms Underlying Accommodation

Accommodation of organ transplants may involve one or more of three mechanisms of resistance. Accommodation may reflect heightened control of complement activation such that sub-toxic rather than toxic amounts of complement are activated upon binding of anti-graft antibodies. Dalmasso et al. [21] showed that activation of complement on endothelial cells heightens expression of CD59, which inhibits killing of cells by terminal complement complexes. Williams et al. [53] showed that for a given amount of antibody binding, accommodated organs activate less complement than rejecting organs, and this decrease reflects control of complement at

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the level of C3 or C4. Koch et al. [28] showed that hepatocytes, which, like accommodated organs, resist complement-mediated killing, also exhibit intrinsic inhibition at the level of C3 or C4.

Accommodation may result from changes in the way complement and other toxic factors signal cells. For example, NF-kappaB, activated by complement and cytokines, can induce transcription of IkappaB [43, 47], which inhibits NF-kappaB, preventing apoptosis and injury by a range of potentially toxic cytokines and other substances. Similarly, IL-1α, transcribed in response to insertion of terminal complement complexes in endothelial cells [48], represses utilization of the IL-1α promoter.

Accommodation might also result from expression of "protective genes, substances, and pathways" [3, 6, 28, 39]. As only a few examples, complement and other noxious factors induce heme oxygenase-1 [39], anti-apoptotic genes such as Bcl-2 and A20 [4] and the PI 3-kinase – AKT pathway [28] which prevents cell activation and death. Regardless of which of these mechanisms, or mechanisms yet to be found, operate in accommodation, the point to be taken is that organs need not be viewed as passive targets of immunity but rather they exert powerful and variously specific defenses that protect against "autotoxicity."

Viewed in this way, accommodation shifts the canonical theories of immunity and tolerance [35]. To the extent that accommodation operates, immunity need not be so specific as to avoid recognition of "self" nor must tolerance function perfectly to avert such recognition. Indeed, both accommodation and tolerance can be invoked to explain how immunity avoids autotoxicity, and both might be integral to Ehrlich's concept of *horror autotoxicus* [49]. Put in another way, accommodation "relieves" the immune system of having to perfectly control autoreactivity because autoreactivity need not damage tissues and incite disease. And, if fetal tissues were accommodated, they might be inured to toxic cells and substances generated by the maternal autoimmune response.

Might the Fetus "Need" Accommodation?

If immunity against the fetus is generated and if the fetus is accessible to the cytotoxic cells, antibodies, and cytokines produced in the mother, the fetus might "need" accommodation. Both reasons for "need" appear established.

Immunity to the Fetus

Although fetal cells and tissues are sometimes found to be less immunogenic than mature cells and tissues [23], the fetus elicits immunity in the mother. Fetal antigens, particularly paternal histocompatibility antigens, elicit humoral and cell-mediated immunity in the pregnant female (see [46] for review). Indeed, the sera of previously pregnant women provide a useful source of antibodies specific for major histocompatibility (MHC) antigens [22, 46].

More pertinent may be investigations of the impact of repeated pregnancy on survival of the fetus. Those previously pregnant are usually found to be more likely to have future successful pregnancies than those never pregnant, suggesting that sensitization during or after pregnancy does not necessarily harm the fetus by immunological means [8, 46]. This subject has been discussed in detail [36]. Prior sensitization may impact on the size of the fetus and placenta [17], both sometimes found to be larger and, on the number of fetuses in mice, sometimes found to be less [8, 9, 36]; but a female can be repeatedly sensitized to paternal antigens without compromising the fate of a fetus carrying the sensitizing antigens [16]. While sensitization has been associated with spontaneous abortion and preeclampsia, these events are relatively infrequent, and fully manifest immunological memory does not preclude successful pregnancy. Therefore, either the fetus is protected from the immune response, perhaps by the placenta and/or by circulating blocking factors [44], or the fetus and/or placenta resist injury inflicted by antibodies and cells that penetrate the protective barrier, that is, the fetus

exhibits accommodation to maternal immunity or both.

Impact of Immunity on Transplants

Before evidence suggesting accommodation may operate in the fetus is reviewed, let us consider how the immune response impacts on the "ordinary" transplant. Most transplants, like pregnancy, induce both cellular and humoral immune responses. The impact of those responses, however, depends profoundly on how the transplant receives a vascular supply. Transplants consisting mainly of cells and free tissues are vascularized by blood vessels originating with the transplant recipient, and these are mainly susceptible to cellular rejection [12, 40]. Although antibodies specific for cell and tissue transplants may be produced in large amounts, those antibodies penetrate poorly through the recipient vasculature and have access to only small amounts of complement and effector cells residing in extracellular spaces. Even cell and tissue xenografts which are highly susceptible in vitro to killing by xenoreactive antibodies and complement survive and function in recipients with high levels of xenoreactive antibodies [38]. T lymphocytes, however, migrate actively and efficiently through recipient blood vessels to reach cell and tissue allografts and in so doing can initiate and mediate cellular rejection.

On the other hand, organ transplants are fed by blood vessels of donor origin, and these vessels can be readily targeted by donor-specific antibodies in the circulation and by complement. As a result, organ transplants are highly susceptible to various types of antibody-mediated rejection [12, 40]. If the circulation of the recipient contains antibodies against the transplant at the time of reperfusion, those antibodies can bind and activate complement and cause immediate destruction of the graft [14]. This process is called hyperacute rejection. Antibodies produced after the transplant is perfused by the blood of the recipient also cause devastating if somewhat less dramatic injury [13]. This type of rejection, variously called antibody-mediated humoral or acute vascular rejection causes destruction of the graft in days to weeks unless intensive therapies, such as plasmapheresis, are instituted.

Whether a tissue or organ transplant would better model the fetus is not entirely clear. The circulatory system of the fetus is, for the most part, distinct from the circulatory system of the mother, although some cells of the fetus migrate to the mother and vice versa. Some antibodies, especially IgG, produced by the mother pass through the placenta to the fetus; some antibodies, particularly IgM, do not. Since IgG can, and often does, cause hyperacute and antibody-mediated rejection, the fetus would seem in this respect more like an organ than a tissue transplant.

The fetus also might in principle be targeted by alloreactive T lymphocytes of the mother as these cells appear to reach the fetus, albeit in small numbers. Because small numbers of alloreactive lymphocytes transferred to an immunodeficient recipient of an organ allograft can cause the rejection of the graft, the fetus might in principle be subject to injury by maternal cells. Given these considerations, there is no reason to exclude a priori the possibility that alloreactive T cells and antibodies would reach the fetus especially from mothers previously sensitized by paternal antigens.

Local Regulation or Suppression of Immunity

If the fetus is demonstrably immunogenic, the mother mounts both cellular and humoral immune responses against paternal antigens, and both antibodies and alloreactive lymphocytes of the mother reach the fetus, then the absence of injury to the fetus must be explained either by barrier or suppressive properties of placenta and fetus or by resistance of the fetus to injury by the mother's immune system. Evidence from several quarters suggests that even though some maternal cells penetrate to the fetus, the trophoblast locally suppresses and/or blocks some of the effector responses of maternal T cells. We have reviewed various mechanisms of suppression that might be exerted [30, 31]. These mechanisms are local in

the sense that lymphocytes that reach the placenta may be active, but within the placenta, they do not exert cytotoxic or other effector properties, and migration is decreased. Although some T cells may be deleted, perhaps in some cases via CD95, for the most part, deletional mechanisms do not apply since the mother does not become tolerant to fetal paternal antigens and indeed in experimental systems remains able to reject transplants of paternal tissues, as mentioned above.

Our own work using embryonic stem cells as a model system has pointed to the possibility that TGF-beta, locally produced, might suppress immunity [32]. This action of TGF-beta, and perhaps other factors, allows semi-allogeneic and even fully allogeneic grafts of embryonic stem cells to survive and grow in a dose-dependent way (smaller doses of cells are immunogenic, larger doses are less so and more likely to survive). However, survival is independent of immunogenicity. Still, whether the ability of embryonic stem cells to evade immunity represents the mechanism by which the fetus evades the maternal immune system is unknown.

Susceptibility of the Fetus to Antibody-Mediated Injury

Maternal antibodies do enter the fetal circulation and are an important source of passive immunity until the fetus is able to generate its own humoral immune response after birth. In some cases, antibodies transferred from the mother to fetus lead to cellular injury and death. Pregnant rats immunized with nerve growth factor mount an antibody response to the antigen that is transferred to the fetus leading to destruction of peripheral sympathetic neurons [25]. Maternal antibodies directed against antigens on the surface of fetal erythrocytes do generate lysis of erythrocytes. Antibodies directed against Rh antigen can cause complement-mediated lysis of fetal erythrocytes leading in extreme cases to congestive heart failure and death of the human fetus between 18 and 34 weeks gestation [10]. Thus, complement is available in the fetus as early as the second trimester (complement does not appear to cross the placenta but rather originates from fetal cells [18, 19]) and can potentially destroy fetal cells.

If maternal antibodies can reach the fetus and complement is available in at least some quantities, then humoral injury to the fetus ought to be possible. This possibility might be amplified by maternal antibodies specific for paternal MHC-encoded antigens, yet anti-HLA antibodies of maternal origin have been detected in fetuses but have not been shown to be incompatible with fetal health [22, 46].

Blocking Immunity Versus Accommodation

If the immune system of the mother mounts an immune response to paternal antigens and if the products of that response enter the fetus, then either the interaction of maternal T cells and antibodies with the fetus must be blocked or the fetus and trophoblast are inured to whatever interaction with maternal immunity occurs. Both possibilities merit consideration.

Blockade by Soluble Antigen

Soluble forms of otherwise cell-associated antigen can block binding of alloreactive antibodies [44]. In vitro and in vivo experiments suggest that such blocking can occur since the antibodies are detected in the circulation and because plasma can inhibit antibody binding [46]. However, as a practical matter, the amounts of antigen that would be needed to block binding are quite substantial. However, for several reasons blockade by soluble antigen seems unlikely to explain all absence of antibody-mediated damage. First, the amount of soluble antigen needed to block high-affinity antibodies is large, and failure of even one antigen to be produced in sufficient quantity would doom a fetus to humoral injury and death. Second, genetic modifications of MHC or regulatory genes that change the level of MHC expression and/or the expressed sequences have not been reported to have a profound effect on fetal survival. Third, this mechanism would not explain how antibodies against other

histocompatibility antigens fail to induce discernable tissue damage.

Enhancement

Antibodies directed toward histocompatibility antigens can sometimes "enhance" rather than retard the growth of tumors [26, 27, 50]. This striking phenomenon is called enhancement. Enhancing antibodies can also protect grafts of normal tissue, sometimes indefinitely [11, 51]. Most evidence suggests enhancement results from the "blocking" of immunological recognition [37], although the antibodies might also suppress T cell responses [20]. Such blocking of recognition or suppression of response might be exerted when immunity is initially elicited (afferent inhibition) or when immunity attacks the graft (efferent inhibition) [20].

The importance of enhancement in pregnancy was suggested by the observations that mice previously mated with males of allogeneic strains better retain tumors and skin grafts from the allogeneic strain. This observation led to the idea that blocking of paternal antigens by alloreactive antibodies might explain the failure of maternal immunity to injure the fetus. Consistent with this concept, the low level of expression of histocompatibility antigens would favor both enhancement and blocking [33, 41].

Accommodation

Another explanation for the failure of alloreactive antibodies to injure or destroy a fetus is that the fetus may develop accommodation, that is resistance to injury by antibodies and complement and possibly T cells [29, 34]. Accommodation might involve control of complement activation and protection of cells from activated products of complement or other noxious substances. Both forms of protection would seem needed by the fetus.

One facet of accommodation that might protect the fetus is heightened control of complement activation. Successful pregnancy depends on expression of complement regulatory proteins in the fetus; fetal mice deficient of Crry, which controls complement at the level of C3 and C4, invariably die in utero, while fetuses deficient in both Crry and C3 survive [54]. These observations suggest that complement activation occurs in the fetus and survival depends on complement control. However, the mice in which these experiments were performed were presumably inbred to the extent that one would not expect production of alloreactive antibodies. Hence, activation of complement in the fetus must proceed by the alternative or lectin pathways.

The fetus also appears to need "cytoprotection" conferred by various cellular pathways and protective agonists. Both heme oxygenase-1 and the Akt/PI3-kinase pathways implicated in accommodation of transplants [3, 28] have been associated with successful fetal survival and development [5, 45]. Although fetuses deficient in heme oxygenase-1 can survive [55], the function of that enzyme might be replaced by other heme oxygenases. However, PI3-kinase and Akt appear to be required for multiple stages of development. Whether this requirement is vitiated to any extent by immunodeficiency or complement deficiency is unknown.

Concluding Remarks

Whether accommodation, as resistance to immune-mediated injury, promotes survival or development of the fetus is unknown. The potential involvement of accommodation is suggested by the capacity of both cellular immunity and humoral immunity of the mother to target fetal cells. Survival of the fetus cannot be explained by tolerance in a classical sense but may be explained in part by blocking of cell-mediated immunity and possibly of antibodies at the level of the placenta or in the fetal circulation. Still, maternal T cells and pathogenic antibodies do reach the fetus and could potentially trigger injury and fetal demise. In this setting, acquired resistance of the fetal cells and organs to injury, mediated by maternal cells and antibodies and fetal complement, would seem a reasonable adaptation. It would seem reasonable to postulate that the fetal cells and organs would be able to amount some form of resistance to maternal cells and antibodies and to fetal complement. Whether this resistance, such as it may be, represents accommodation of organ

transplants is also unknown. If it does, we may hope to acquire new insights into mechanisms of *horror autotoxicus* and reproductive immunology in the same arena.

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Wharton's Jelly-Derived Mesenchymal Stromal Cells as Immunoregulatory Cells

7

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Mesenchymal Stromal Cell Populations

Mesenchymal stromal cells (MSCs) have generated interest because of their therapeutic potential in cellular therapy, tissue engineering, and drug delivery (e.g., [1]). Since MSCs can be isolated from many different tissues and from tissues at different time throughout the life of the individual, and since MSCs can be derived from discarded tissues such as umbilical cord or placenta, or from adipose tissue following liposuction, the question arises: What source of MSCs will serve best for these different applications?

Many groups report physiological differences between the properties of MSCs derived from different time points from the same tissues (such as bone marrow) or from different tissue sources. These results may be confounded, however, since MSCs are affected by health/disease status, medium components, passage, freeze/thaw, cytokines, plating density, oxygen conditions, and perhaps other factors such as isolation technique. MSCs, at first glance, appear to be a homogeneous population that can readily be collected, expanded, and used clinically. Closer inspection reveals that MSCs are heterogeneous, in terms of colony-forming unit-fibroblast (CFU-F) incidence, proliferation rate, number of passages to senescence, ability to avoid immune surveillance, etc. The reasons for the heterogeneity may be the tissue source, the expansion conditions, or other factors. It is not clear currently.

MSCs can be expanded in a variety of conditions. There is no universal standard operating procedure (SOP) for MSC isolation and expansion, and MSCs derived and expanded in one lab most likely have different physiological properties. Furthermore, the definition of MSCs provided by the International Society for Cellular Therapy casts a "broad net" that "catches" many cells. The defining properties of MSCs per the ISCT definition are as follows: fibroblastic morphology, cells that adhere and grown on tissue culture plastic, cells that self-renew and have the ability to be differentiated into bone, fat, and cartilage in vitro, with cells with characteristic mesenchymal (and not hematopoietic cell) surface markers [2]. As stated above, one can obtain cells from virtually every tissue compartment that fit this definition. This definition is adequate. Since tools are lacking to differentiate the more primitive and more stemmy mesenchymal cells from the unipotent or multipotent stromal progenitor populations, so this definition holds for the present. In the future, there may be a culture medium that maintains or expands the mesenchymal stem cell population preferentially, or a unique surface marker to allow prospective selection for stemmy

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or for progenitor cells (such as CD271). Right now, scientists do not know whether they are all working on the same MSC population and most likely they are not.

Freshly isolated bone marrow-derived MSCs from healthy young individuals likely contain a small, quiescent, multipotential stem cell population and a larger population of multipotential and unipotential progenitor cells and null-potential (differentiated) cells. While one would presume that mesenchymal stem cells (vs. the stromal cells) are in each isolate, in the current state of the art, one cannot be sure. It is likely that culture expansion conditions do not maintain the stem cell population, but we cannot be sure of this either. We presume that this is so because this is observed in other stem cells such as the CD34 hematopoietic stem cells and because the percentage of senescent cells in MSC cultures increases with passage. Many scientists claim to work with "mesenchymal stem cells" without the data to support their claims conclusively. We note that mouse embryonic stem cell lines may possess many qualities of pluripotent cells, but not all mouse embryonic stem cell lines contribute to the germline with equal efficiency. In contrast to MSCs, the defining qualities of the embryonic stem cells are much better known than MSCs.

Is it worth taking issue over mesenchymal stromal versus mesenchymal stem cells nomenclature? Yes, because, first, claims have been made that having more stem cells in a cell population increases the potency of those cells therapeutically. This is a thin claim. Some support can be gained by correlating outcomes of transplantation of MSCs with higher colony-forming unit-fibroblast (CFU-F) incidence (discussed below). CFU-F is an indicator of proliferation, however, and does not prove "stemness." Second, it makes the scientific literature confusing, and it confuses the general public. Like the boy who cried "Wolf" until the public no longer heard him, we have scientists who cry "stem cell." This mesenchymal stem versus stromal cell issue has not damaged the field, apparently. It has blurred the line between real "mesenchymal stem cells" and stromal cells and polarized the field into "stromal" and "stem cell" camps. Things will become more pitched once pure populations of true mesenchymal stem cells are available for scientific work.

MSCs have received the most attention as a potential cellular therapy since they can be collected and used in an autologous fashion, avoiding immune rejection, and they are in clinical trials for various maladies (274 MSC clinical trials were found on clinicaltrials.gov on 26 November 2012). An explosion of experimental and clinical investigations followed the discovery that cellular therapy provides functional improvements following myocardial infarction (MI); see reviews [3–5]. Cellular therapy following MI using mesenchymal stromal cells (MSCs) derived from bone marrow (BM-MSCs) demonstrated long-term cardiac rescue/repair in completed clinical trials [5–9] and ongoing trials (see clinicaltrials.gov for the latest). A meta-analysis concluded that MSC transplantation produces a significant, albeit modest, long-term improvement in cardiac function with no additional risks following MI [8]. This begs the question whether optimized populations of MSCs can be derived that will produce a more robust and long-term functional improvement post MI. Since shortcomings of autologous BM-MSCs have been identified, one presumes that allogeneic cell products might be manufactured for a particular malady. For example, the capacity of BM-MSCs to proliferate and differentiate decreases with age, since the marrow cavity fills with yellow fat and CFU-F frequency (an indicator of MSC clonality in culture) decreases with age, too [10]. The decrease in CFU-F frequency is significant because higher CFU-F in MSC transplants correlated with better functional outcomes in MI patients [11]. In addition, cardiovascular disease compounds the effects of aging on BM-MSC number and function [12, 13]. Heeschen et al. noted, "…significantly reduced migratory and colony-forming activity *in vitro* and a reduced neovascularization capacity *in vivo*," in BM-MSCs isolated from patients with chronic ischemic cardiomyopathy (ICMP). The authors stated that "This functional impairment of BM-MSCs from patients with ICMP may limit their therapeutic potential for clinical cell therapy" [13]. Finally, BM-MSC collection involves an invasive, painful

surgical procedure. Thus, autologous BM-MSCs may not be available in clinically relevant numbers, for every MI patient and aged BM-MSCs may be less capable of effective rescue/repair. This suggests that alternative sources of MSCs should be considered for MI cell therapy.

Scientists in the MSC field are looking for the means to identify and maintain the mesenchymal stem cell population, since bone marrow-derived MSCs appear to lose therapeutic potency through the normal aging processes, or due to disease processes [14–16]. Therefore, alternative sources of therapeutic MSCs have been sought that may be relatively enriched for therapeutic cells (be they stemlike or progenitor cells) and that cells that can be subjected to extended passage as needed to derive clinically relevant numbers of cells. Our group and other groups have examined components of the umbilical cord as a source of therapeutic MSCs.

Umbilical Cord as a Source of MSCs

The umbilical cord is a fetal structure that contains two umbilical arteries and one umbilical vein surrounded by a loose connective tissue and covered by amnion along part of its length. The connective tissue that supports and cushions these vessels is called Wharton's jelly. Wharton's jelly contains an extracellular matrix rich in collagen and hyaluronic acid and contains a population of MSCs distributed from the perivascular space to the subamnion. Some people have suggested that the more primitive MSCs are located just beneath the amnion and the more differentiated cells in the perivascular region [17, 18]; others believe that the perivascular cells adjacent to the blood vessels are the more primitive MSCs [19, 20]. It is unclear if either is correct.

MSCs derived from the umbilical cord Wharton's jelly (WJCs) merit evaluation as an alternative source of allogeneic MSCs for therapy because WJCs are collected painlessly from discarded umbilical cord and have MSC-like properties [18, 21–26]. Important for their use as an allogeneic therapeutic MSC, WJCs have higher proliferation rate in vitro and greater CFU-F frequency than adult BM-MSCs and can be collected and banked for use as an off-the-shelf allogeneic cell therapy product [18, 21–24, 27]. That WJCs have utility in MI is suggested by WJCs' capacity to differentiate along the cardiac lineage [25, 28] and by their ability to improve circulation in a hind limb ischemia model [29]. Still more relevant, human WJCs given by myocardial injection into rats that had MI 2 weeks previously showed significant improvement in cardiac function 2 and 4 weeks following transplantation [30]. It is important to note that these results are confounded since the transplanted rats received cyclosporine treatment and the control group did not receive immune suppression. WJCs, like BM-MSCs, release a plethora of cytokines and growth factors [27, 31–33]. The paracrine release of cytokines and growth factors is one mechanism that MSCs use to produce their benefits in MI [34]. WJCs release significantly different levels of cytokines than BM-MSCs: For example, when conditioned medium was measured by ELISA, WJCs secreted more human growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage-colony-stimulating factor, leukemia inhibitory factor, and interleukin-1 (IL-1), IL-6, and IL-8 and secreted less vascular endothelial growth factor (VEGF) and stromal cell derived factor 1 (SDF-1) into the medium compared to BM-MSCs [31]. In BM-MSCs, VEGF and SDF-1 have a role in repair/rescue via pro-angiogenic effects and anti-apoptosis of cardiomyocytes [35–38]. Based upon their cytokine production, WJCs and BM-MSCs may have different mechanisms for cardiac rescue/repair, and the differences in functional recovery may not be apparent without direct head-to-head comparison.

MSCs from sources other than BM-MSCs have been evaluated in the experimental MI; see review [4]. However, to date, few head-to-head comparisons between different sources have been made [15, 39–41]. In contrast, the effect of BM-MSCs derived from young animals versus adult animals on repair/rescue following MI has been examine in rodent MI model [42, 43], with diametrically different conclusions. Markel et al. determined that neonatal BM-MSCs were less

effective than adult BM-MSC in protecting from acute effects of ischemia in a Langendorff-isolated heart preparation [42]. In follow-up work, they concluded that neonatal BM-MSCs were less able to release of VEGF and IL-6 compared to adult BM-MSCs [44]. Note: This finding does not fit with other work that indicates that the WJCs release more IL-6 and less VEGF than BM-MSCs [31]. In contrast, Wang et al. transplanted BM-MSCs derived from young or adult rats into rats with MI and observed a greater improvement in recovery when animals received the younger BM-MSCs versus older BM-MSCs [43]. This finding fits with observations that rescue/repair responses are enhanced in younger individuals and may be lost with aging [15, 45]. We speculate that much of the variation observed following clinical MSC therapy is due to variation in donor MSCs (age, health history, in vitro culture conditions, etc.) and that significant clinical improvement of MI treatment would be seen by optimizing cell source and cell preparation. We speculate that MSCs from different sources may differ in their ex vivo expansion properties and their ability to home to pathology and to improve function following transplantation. The best way to address this hypothesis is by direct comparison.

One property that WJCs share with other MSCs and MSC-like cells is their immune properties [46–48]. For example, WJCs poorly stimulate allogeneic or xenogeneic immune cell proliferation in lymphocyte proliferation assays or other expansion experiments, and WJCs suppress proliferation in mitogen-stimulated splenocyte proliferation and mixed lymphocyte proliferation assays [33, 49]. What is known of the physiological mechanisms for these properties is discussed in the next section.

Comparison of the Immunologic Properties of MSCs from Different Sources

MSCs derived from adult tissues are non-immunogenic when cultured with allogeneic T cells, and they suppress a variety of immune functions through inhibition of T cell proliferation, prevention of cell maturation, induction of regulatory T cell proliferation, and modification of induced cytokine production. Adult MSCs have been derived from bone marrow BM-MSCs and adipose tissue (ASCs). Although it is beyond the scope of this review to summarize the entire field of immune suppression by MSCs, we have summarized what we believe are the most important findings of adult MSC suppression and have compared them to fetal-derived MSCs. This latter group of cells includes MSCs derived from Wharton's jelly (WJCs) as well as MSCs derived from other fetal tissues including blood, placenta, and amnion.

Immune regulation by MSCs is summarized in Fig. 7.1:

- The behavior of MSCs is regulated by the sum total of input signaling. MSCs are exposed to a mixture of signals provided by stimulation of cells of the immune system. In various assay systems, T cells were activated by mitogens, soluble protein recall antigens, alloantigens, and superantigens. Antigen-presenting cells (monocytes, dendritic cells) were activated by lipopolysaccharide (LPS).
- The interaction of MSCs with cells and cytokines in each circumstance may be expected to provide different results unique to the induction stimulus. The resulting output from MSCs dictates whether immune deviation takes place, Tregs are induced, or a certain pattern of immunosuppressive molecules is produced. The high variability of results from studies of MSC-mediated immune modulation is likely due to this point; for example, MSCs may enhance or suppress immune inflammatory responses.
- The pattern of immune suppression elicited by MSCs may affect outcomes in MSC transplantation studies. For example, a short burst of immunosuppressive molecules would be expected to provide transient suppression of immune responses in vivo, immune deviation from a Th1 to a Th2 response may preferentially affect inflammatory responses, and induction of Tregs may maintain immunologic tolerance.

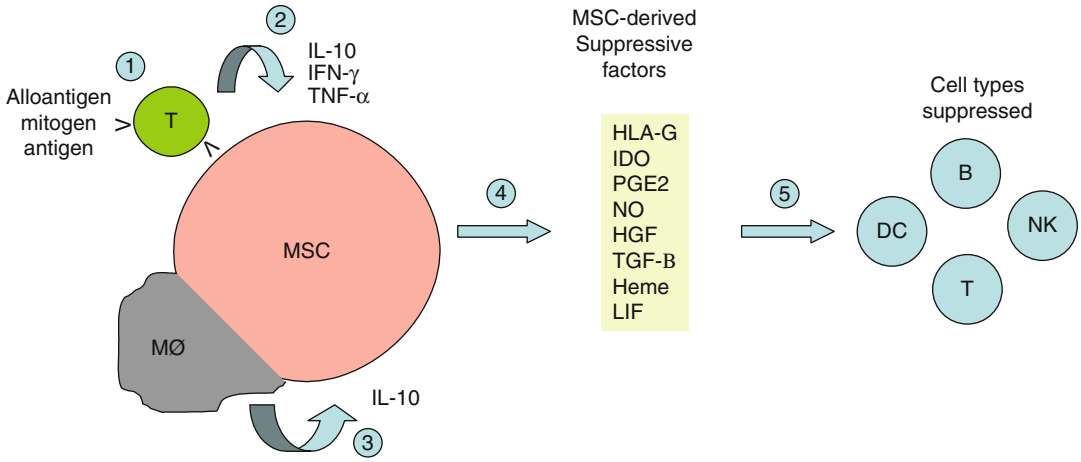

Fig. 7.1 Immune regulation by MSCs. (1) The polarity of MSC immune regulation is modulated by a mixture of inputs. MSCs can be either immune suppressive and stimulatory based upon priming inputs. (2) Exposure to cytokines can also polarize MSCs' immunoregulatory responses and affect MSC MHC expression. (3) Interaction of MSCs with macrophages (*MØ*) or T cells can affect their release of cytokines such as interluekin 10 (*IL-10*), interferon gamma (*IFN-γ*), and tumor necrosis factor alpha (*TNF-α*). MSCs participate in immune regulation, by binding with T cells and inducing T cell anergy due to their lack of expression of co-stimulatory molecules such as CD40, CD80, or CD86. (4) MSCs further participate in immune regulation by expression of a variety of immune-regulating factors such as human leukocyte antigen G (*HLA-G*), indoamine oxidase (*IDO*), prostaglandin E2 (*PGE2*), nitric oxide (*NO*), hepatocyte growth factor (*HGF*), tumor growth factor beta (*TGF-β*), heme and leukemia inhibitory factor (*LIF*). (5) It is through direct contact and indirectly via soluble factors such as cytokines that MSCs produce immune modulatory effects upon T lymphocytes, B lymphocytes, dendritic cells (*DC*), and natural killer cells (*NK*)

- MSCs interact with and impact a variety of immune effector cells including T and B lymphocytes, dendritic cells, and natural killer cells.

Phenotype and Immunogenicity

The immunologic profile of MSCs has been established using in vitro assay methods by evaluating (1) immunologically relevant cell surface molecules by flow cytometry, for example, CD40, CD80, and CD86, for co-stimulatory molecule expression for their ability to block the wind-up of the immune inflammatory response and MHC class I and class II expression to understand their ability to avoid immune surveillance [33, 50–52]; (2) T cell proliferation in cultures containing MSCs and allogeneic T cells [53–55]; and (3) inhibition of T cell proliferation to an activation stimulus. The results of all three analyses demonstrate that MSCs express a non-immunogenic profile. These observations generally hold up when MSCs are transplanted to allogeneic or xenogeneic recipients, although conflicting data are common. Thus, it is not clear whether MSCs will reach their full therapeutic potential when used in an allogeneic format.

Adult-Derived MSCs

As reviewed by Rasmusson [56], immunomodulation by MSCs includes inhibition of dendritic cells (DC) alloantigen processing and decreasing MHC class II expression [57, 58], suppression of T cell responses [59], modulation of Treg cells [60, 61], suppression of B lymphocyte proliferation and antibody production [62], and suppression of the proliferation, cytokine production, and cytotoxicity of NK cells [63]. As illustrated in Fig. 7.1, the mechanisms by which MSCs have their immune effects include both cell-cell contact [59] and (mostly) soluble (diffusible) factors [51, 57, 64, 65]. Bone marrow MSCs (BM-MSCs) express low to negligible amounts of MHC class II

molecules and the co-stimulatory molecules CD40, CD80, and CD86 on their cell surface [66, 67]. Adipose-derived stromal cells (ASCs) express the same profile with the exception that they express CD40 [68]. Treatment of BM-MSCs with the inflammatory cytokine interferon gamma, IFN-γ, has been shown to enhance expression of MHC class I and induce expression of class II molecules on BM-MSCs without inducing the expression of co-stimulatory molecules [66, 67]. MSCs that have been frozen and thawed may show an augmented response to IFN-γ.

Therefore, BM-MSCs and ASCs would be expected to function as deficient antigen-presenting cells (APCs) due to the constitutive lack of MHC class II and co-stimulatory molecules. Indeed, BM-MSCs and ASCs failed to trigger alloreactive T cell proliferation in mixed lymphocyte reaction (MLR) cultures [46, 51, 54, 55, 66, 67, 69], after treatment with IFN-γ [66, 67, 70], and when co-stimulatory molecules were transduced into the cells [66] or if co-stimulation was provided by anti-CD28 antibody. These findings suggest that the active immune suppressive ability of MSCs plays a more important role than the passive lack of stimulatory molecules in determining T cell responses. For these reasons, MSCs have been tested for their effects in a variety of immune diseases such as allogeneic transplantation, tolerance induction, and autoimmune diseases such as Crohn's and multiple sclerosis.

As updated from the review by Toubai et al. [71], many clinical reports describe the use of MSCs (mostly from bone marrow) to limit or treat graft-versus-host disease (GVHD) following allogeneic hematopoietic cell transplantation (allo HCT) [72–82]. In animal studies, MSCs have been used to induce mixed chimerism and improve graft tolerance [83–86].

Transplantation experiments in animals have generally supported the in vitro findings that MSCs are non-immunogenic and suggest that allogeneic BM-MSCs may be transplantable to immunocompetent recipients without the use of immunosuppressive drugs. Our laboratory has not been able to demonstrate T cell priming to alloantigens in rats injected with BM-MSCs [87], in baboons injected with BM-MSCs [88], and in rats injected with ASCs [68, 69]. In all three animal studies, T cells obtained from recipient animals did not respond with accelerated kinetics or higher magnitude of response compared to vehicle or autologous MSC-injected controls when restimulated with PBMCs obtained from the MSC donors. In contrast to the cellular response, allogeneic BM-MSCs induced an antibody response [68, 69, 88] that was found to be, at least in part, specific for fetal bovine serum proteins used in the culture of BM-MSCs [69]. The antibodies were not cytotoxic for ASCs in the presence of complement. In contrast to these studies, Poncelet et al. demonstrated that pigs injected with allogeneic BM-MSCs developed donor-specific cellular and humoral immune responses as evidenced by enhanced MLR responses posttreatment as well as an antibody response that was cytotoxic in the presence of complement [89]. Additional studies have shown that pretreatment with BM-MSCs accelerated rejection of bone marrow allografts in mice [90] and heart allografts in rats [91]. Repeated injection of WJCs in an allogeneic swine model also generated an antibody response [50]. A recent bioluminescence real-time imaging studies indicated that allogeneic MSCs are cleared over about 2–4 weeks following transplantation [92]. In summary, the field is torn between those that see MSCs as universal cells for therapy without matching, those that see MSCs for allogeneic therapy in matched unrelated or haploidentical therapy, and those that see MSC as most useful as autologous cells. We contend that MSCs for allogeneic therapy may make sense only in special conditions. For example, during allo HCT, use of allogeneic MSCs may be acceptable, since the immune system in these conditions is suppressed and the MSCs might have an opportunity to act prior to clearance (see review [93]). Clearly, use of allogeneic MSCs in healthy individuals requires additional considerations, especially in patients with diseases that involve an inflammatory response, such as coronary ischemia, stroke, and Crohn's. In these diseases, immune suppression might be necessary to prevent recipient-derived rejection and to give MSCs a chance to act.

Fetal-Derived or Extraembryonic Tissue-Derived MSCs

MSCs derived from umbilical cord tissue [33, 49, 50], fetal liver [94, 95], and placental tissues [96–98] express the same immunologic phenotype as adult-derived MSCs. One possible exception is that approximately 20 % of human umbilical cord perivascular (HUCPV) cells do not express either MHC class I and class II following successive freeze/thaw cycles [20]. Another significant difference between adult and fetal-derived or extraembryonic tissue-derived MSCs may be their response to IFN-γ. Variable responses have been reported from showing no increase in MHC class II expression [96, 97] to significant upregulation [50, 95]. In an abstract presented at ISSCR 2009, we showed that differences in the IFN-γ response and the change in HLA class I expression may be attributed to use of cryopreserved/thawed/expanded WJCs, since WJCs exposed to IFN-γ responded in the freeze/thaw cells compared to WJCs maintain in culture and never frozen. Otherwise, the difference may be attributed to the tissue source of MSCs: The former studies examined MSCs derived from placenta, whereas the latter studies showing upregulation were derived from umbilical cord and liver. IFN-γ has also been shown to upregulate the expression of PD-L1 (programmed death ligand 1), a cell surface marker that negatively regulates T cell responses on placenta-derived MSCs [97]. It is unknown whether this is a unique response or whether it would be found in other MSC populations.

MSCs derived from fetal liver [95], umbilical cord tissue [33, 49], or placenta [49, 99, 100] did not stimulate allogeneic T cell proliferation in MLR cultures. Pretreatment of MSCs with IFN-γ did not enhance the response [96]. Placenta MSCs fail to induce proliferation from allo T cells in humans [100] or in rats [101]. Human WJCs did not stimulate an immune response when transplanted in either rats or rabbits, and differentiation of the WJCs along the cartilage lineage did not increase immune response [102].

In summary, MSCs from adult or non-adult tissues have many properties that affect the immune response. There do not appear to be clear differences between the sources of MSCs in terms of their immune properties.

Engraftment of WJCs: Comparison to MSCs Derived from Other Tissues

Transplantation studies have shown that xenogeneic WJCs persist for periods of time without evidence of immune rejection. Recently, a study has shown engraftment and production of differentiated progeny that suggests the existence of WJC stem cells [19]. We transplanted pig WJCs into rat brain and found no evidence of immune rejection during the 2–8-week tracking period; in fact, the transplanted cells showed evidence of differentiation and proliferation in situ [103, 104]. There was no accumulation of CD4-, CD8-, CD11b-, or CD161-positive host-immune cells at the injection site. These findings were not due to immune privilege status of the brain since swine WJCs injected IV or IM were recovered 3 weeks later from kidney/muscle, respectively. Fu et al. [105] differentiated human WJCs into tyrosine hydroxylase (TH)-positive dopaminergic neurons in vitro and transplanted the cells into Parkinsonian rats. They showed a significant decrease in amphetamine-induced rotation after transplantation as well as survival of the transplanted TH + cells at 20 weeks. Chao et al. [106] transplanted human WJC-derived islet-like clusters into Sprague–Dawley rats that were pretreated with streptozotocin to induce diabetes. They observed human insulin in rat serum for up to 12 weeks (last time point) and recovered human islet-like cell clusters from the transplantation site in histological analysis. Liu et al. [102] transplanted differentiated and undifferentiated human WJCs into rabbits and rats and found little evidence of immune response and expansion of the human WJCs. Additional studies have shown engraftment in neonatal animal models by MSCs derived from placenta [99, 107] and umbilical cord blood [108]. In contrast to these observations, multiple injections of allogeneic swine WJCs induce cellular and humoral responses in recipient pigs [50]. The allogeneic WJCs induced immune responses when they were enhanced by

exposure to 25 ng/ml IFN-γ prior to injection, or by multiple injections or when they are injected into an inflammatory site. The results of this study emphasize the importance of the microenvironment surrounding the MSCs in determining immunologic outcome.

Potential Advantages of WJCs Over MSCs Derived from Adult Tissues

When confronted with a new cell source such as WJCs, one must make a case whether significant advantages exist for the disruptive technology over the existing and well-studied cell source, such as bone marrow-derived MSCs. From this perspective, one can consider whether the new source can effectively compete. The fundamental differences in how the MSCs are derived from the two sources are an important advantage: WJCs, like umbilical cord blood, are collected from a discarded tissue source at birth, painlessly, safely, and efficiently and from individuals at a young and constant age. Thus, WJCs can be collected without invasive methods or significant risk. The ability to store cryogenically MHC-catalogued units of WJCs, in parallel with umbilical cord blood, offers the advantage of rapid access to MSCs for a variety of applications. For example, WJCs may supplement or co-engraft with autologous cord blood or for other applications such as tissue engineering, facilitating adoptive immunotherapy, and ex vivo stromal support of cord blood for expansion. The advantage of having banked WJCs will only become obvious once the clinical use of MSCs is better understood.

Currently, MSCs are in clinical application for several different indications; of these indications, about one-third involve leveraging the immune modulation properties of MSCs (summarized from clinicaltrials.gov search conducted November 2012). As shown in Fig. 7.2, MSCs, owing to their immune properties, may be used for treating chronic graft-versus-host disease or adoptive immune therapy, for treating inflammatory disease processes such as acute myocardial infarction, or for supplementing umbilical cord blood or for ex vivo expansion of cord blood, for irradiation injury, or for certain neurological disorders such as Parkinson's disease.

The MSCs for clinical use are divided into the autologous source MSCs from bone marrow (usually) or from allogeneic sources. In certain indications, allogeneic MSCs may offer an advantage (as discussed above). However, in other indications such as tissue-engineered heart valve or output track, it would be advantageous to employ autologous MSCs.

WJCs appear to differ physiologically from MSCs from bone marrow in two ways. First, WJCs grow faster and longer in culture compared with BM-MSCs [22]. Second, WJCs have a larger number of colony-forming units-fibroblast (CFU-F) than has been reported for BM-MSCs [19]. Pluripotency or a tendency toward pluripotency has been suggested for WJCs and MSCs due to expression of Oct4 or other early embryonic stage markers such as SSEAs and Nanog [52]. In light of recent work that examined the methylation status of the Oct4 promoter in WJCs [109], the relatively low abundance of Oct4 signal [110], and the possibility of pseudogenes, it is possible that the Oct4 staining in MSCs is a false-positive [111, 112]. We are not convinced that Oct4 or Nanog staining is important for characterizing MSC populations.

In the area of HCT, increasing the number of "stemmy" cells (e.g., CD34 positive) correlates with improved engraftment. In the MSC field, the lack of definitive markers that differentiate stem/progenitor cells has hampered similar evaluations. CFU-F is one of the best surrogate markers for MSC stem cells available currently, although a case may be made for certain markers (CD271, CD146, etc.). Clinical data indicates that higher CFU-F number is associated with improvement following myocardial infarction (discussed above). This result suggests either that increasing the number of stemmy MSCs provides better engraftment or that the stemmy cells produce a better therapeutic effect. Since WJC have more CFU-F than BM-MSC from adults, we suggest that WJCs might have advantages over BM-MSCs in cellular therapy. This hypothesis will require side-by-side comparison between BM-MSCs and WJCs.

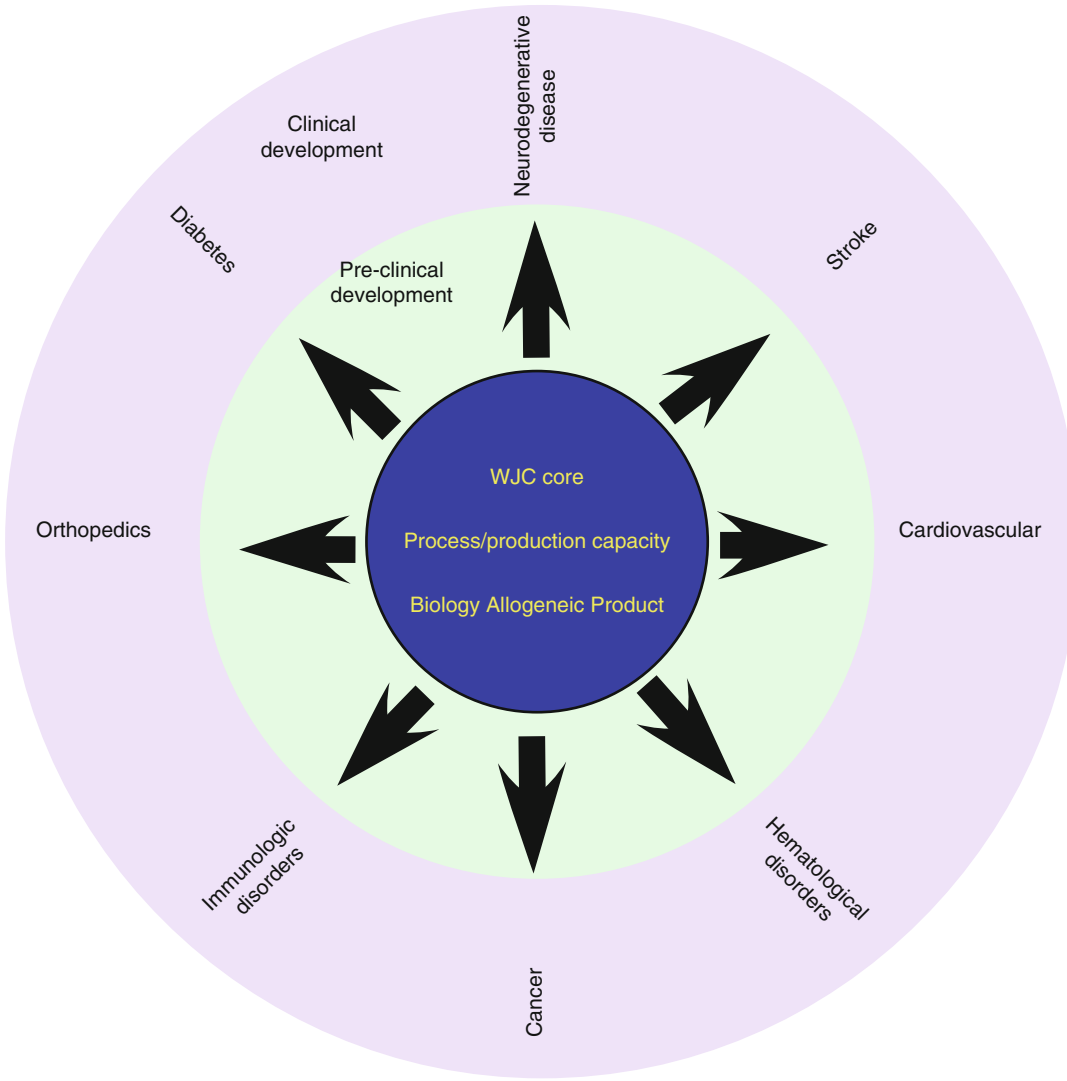

Fig. 7.2 Applications of Wharton's jelly-derived mesenchymal stromal cells (*WJCs*) for clinical use. Clinical translation is predicated upon a core, for example, the ability to produce WJCs to a standard acceptable for use in patients (compliance with good manufacturing practices, GMP). In this regard, WJCs offer an advantage over adult bone marrow-derived mesenchymal stromal cells (MSCs) as an allogeneic cell therapy product since WJCs are collected at a consistent young age and have a high expansion potential in vitro. The next stage of clinical translation is a demonstration of feasibility via in vitro and in vivo preclinical testing. As outlined in the text, MSCs and WJCs have demonstrated potential for clinical development because of positive, reproducible, and preclinical findings

Tolerance Induction

For the purpose of this review, we define tolerance in lymphocytes as an induced, unresponsive state that is antigen specific. Although it may be induced by MSCs, tolerance should be evaluable in the absence of these cells to distinguish it from non-antigen-specific unresponsiveness resulting from suppression. Tolerance has been successfully induced in animals through chimerism, T cell depletion, and blockade of co-stimulation (reviewed by [113]). MSCs would appear to be ideal vehicles for alloantigen-specific tolerance induction as they

can present both MHC class I and II antigens in the absence of co-stimulatory molecules and they may be able to engraft in sufficient numbers to achieve chimerism. The ability to reliably and robustly induce transplantation tolerance using donor MSCs would be a significant achievement in medicine.

Adult-Derived MSCs

Experiments attempting to induce tolerance using BM-MSCs have achieved conflicting results. In cell culture experiments, Klyushnenkova et al. [66] showed that alloreactive T cells cultured with IFN-γ-treated BM-MSCs for 7 days responded normally to restimulation with PBMCs from the BM-MSC donor indicating that donor-specific tolerance was not induced in vitro. Contrasting results were obtained by Batten et al. [114] who showed that purified CD4+ T cells cultured with BM-MSCs for 1 day were not able to respond to restimulation with PBMCs matched at one class II allele. Tregs were induced in this system that could be responsible for the tolerant state. Di Nicola et al. [115] demonstrated that T cells stimulated with PHA in the presence of BM-MSCs were able to respond vigorously to restimulation with DCs, PHA, and IL-2 after the T cells were separated from the BM-MSCs. Although donor-specific tolerance could not be evaluated in this study, it showed that T cell suppression was reversible when BM-MSCs were removed, similar to the results of Klyushnenkova et al. The ability of BM-MSCs to induce tolerance in vivo has been reported, but opposing results have been reported as well [91, 116]. When tolerance was induced, it was attributed to induction of stable mixed chimerism [85, 117, 118], T cell anergy [119, 120], and the induction of Tregs [121]. Further studies are obviously needed to sort out and optimize the parameters required for MSCs to induce tolerance.

Fetal-Derived MSCs

No studies addressing tolerance specifically were found as of this writing. One study showed that human placenta-derived MSCs transplanted to 17-day rat fetuses engrafted at low levels in variety of tissues, differentiated into hepatocytes and hematopoietic cells, and persisted for at least 12 weeks after birth [122]. Although suggestive of tolerance, long-term persistence of MSCs in this model is probably due to the immature state of the recipient's immune system at the time of transplant rather than to tolerogenic properties of the MSCs.

Suppression

MSCs suppress immune responses through multiple mechanisms, mostly mediated via soluble factors. Immunosuppression induced by MSCs affects all cells of the immune system including T cells, B cells, monocytes, dendritic cells, and NK cells. The ability of MSCs to induce immunoregulatory phenotypes from naïve cell populations has been described for monocytes, dendritic cells, and T cells. MSCs prevent differentiation/maturation of APCs which maintains an immature/inhibitory phenotype; they can also induce the differentiation of Tregs from T cell populations.

Adult-Derived MSCs

BM-MSCs suppress T cell proliferation activated by specific or semi-specific stimuli such as alloantigens, protein antigens, and superantigens. They also suppress generalized T cell activation induced by mitogens such as PHA, Con A, and anti-CD3 plus anti-CD28 antibodies. ASCs are able to suppress these immune responses as well in human systems, rats, and canines. Generally, MSCs are able to suppress T cell activation when added at the initiation of culture or when added after the culture has progressed and T cells have been activated, but suppression may be weaker at later time points. Although suppression has been shown to be mediated through transwell membranes, indicating that soluble suppressive mediators are produced by the MSCs, suppression generally appears to be greater when cell contact occurs between MSCs and cells of the immune system. These data support the use of MSCs for immunologic/inflammatory disorders and especially when the MSCs can be given prior to the induction of the inflammatory response.

During the past 8 years, a number of molecules produced by adult-derived MSCs have been

identified as potential suppressor factors. Some of these molecules have been shown to play roles in fetal allograft survival as well as tumor survival, an indication of biological conservation and success (some are listed in Fig. 7.1). Although most of these molecules have not been studied in fetal-derived MSCs thus far, two molecules, HLA-G and IDO, have been evaluated, likely due to their well-known roles in maternal tolerance of the fetus. Of particular interest, these molecules appear to be potent inducers of CD4+CD25+ regulatory T (Treg) cells. Both of these molecules are described below.

HLA-G is a nonclassical HLA class I molecule that has limited tissue distribution including fetal tissues (trophoblasts). Although HLA-G is polymorphic, there are few variants (8) compared to the classical MHC class I and class II molecules which number in the hundreds: HLA-G1 through HLA-G4 are membrane-bound and HLA-G5 through HLA-G7 are secreted and soluble. HLA-G1 has been shown to inhibit the cytolytic function of NK cells and T cells, alloproliferative response of CD4+ cells, and the maturation and function of DC.

Adult BM-MSCs have been demonstrated to secrete HLA-G5 constitutively [123, 124]. There is some evidence that Stro-1+ BM-MSCs may express more HLA-G than the overall BM-MSC population [125]. Similar to what has been described for monocytes [126], IL-10 has been shown to enhance secretion [127]. HLA-G5 suppressed T cell proliferation and induced Tregs (CD4+CD25+FOXP3+). HLA-G5 was shown to inhibit NK cytolysis and IFN-γ secretion. Blockade of HLA-G with Ab resulted in partial recovery of suppression [124].

Indoleamine 2,3-dioxygenase (IDO) is another immunosuppressive molecule produced by fetal tissues that has been proposed to have an important function in preventing rejection of the fetal allograft [128]. This enzyme catabolizes tryptophan to kynurenine resulting in localized depletion of tryptophan and inhibition of cell proliferation. Although tryptophan depletion is typically thought to be the reason for T cell inhibition, increased levels of kynurenine can also be inhibitory when added to MLR assays [129]. IDO inhibition is partially reversed by 1 methyl L-tryptophan (1-MT). As previously described for HLA-G, IDO has a role in the induction of Tregs [130]. IDO is expressed primarily by antigen-presenting cells of the immune system including dendritic cells and monocytes [131]. Human BM-MSCs do not secrete IDO unless stimulated by IFN-γ [59, 129, 132]. TNF-α may also participate in the regulation of IDO [64]. There is evidence that IDO suppresses activated rather than resting T cells [133]. IDO had been shown to suppress proliferation by T cells, B cells, and NK cells [59]. IDO does not appear to be a major participant in suppression in mouse [134, 135] and rat [136] BM-MSC models.

Adult MSCs have been shown to be suppressive in vivo. One of the first studies to show suppression in animals was the demonstration that intravenous injection of BM-MSCs prolonged skin graft survival in baboons [137]. Additional studies have shown BM-MSC-mediated suppression of experimental autoimmune encephalomyelitis (EAE) in mice [120]. Both BM-MSCs and ASCs have been shown to suppress graft-versus-host disease (GVHD) in rodents [70, 138, 139]; however, conflicting results have been obtained as well in which BM-MSCs exhibited no suppression for allograft responses [139–141]. It is worth noting that clinical trials have been initiated to assess the effect of MSCs on GVHD. Preliminary results indicated that BM-MSCs and ASCs are an effective therapy for this disease (data reviewed in [93]), although the final endpoints were not met in the trial.

Fetal-Derived MSCs

Human MSCs derived from fetal liver [142], placenta [143–145], amnion [96, 98], and umbilical cord [33, 146] have been shown to suppress T cell proliferation in MLR cultures and to mitogens. Umbilical cord matrix stromal cells were found to weakly express mRNA transcripts of the HLA-G6 isoform at P4 and P8; no expression of HLA-G5 was found [33]. Similarly, in amnion-derived multipotent progenitor (AMP) cells, HLA-G was detected at low levels by flow cytometry. Expression of HLA-G was greatly increased by treatment of the cells with IFN-γ or after culture in MLR assays indicating that it was inducible [96]. Placenta-derived MSCs have been shown to express intracellular HLA-G [97]. In the

former study [97], blockade of IL-10 partially restored responsiveness of T cell proliferation, consistent with IL-10-mediated enhancement of HLA-G secretion [127].

IDO is expressed by placenta-derived MSCs [97]. The expression of this molecule was enhanced by IFN-γ and blocked by 1-MT [145]. The inhibitor reversed suppression by the MSCs.

Immune Deviation

CD4+ T cells provide helper functions to cells of the immune system to stimulate cellular immunity and antibody production. Two functionally distinct T cell subsets mediate these functions: Th1 cells secrete a pro-inflammatory pattern of cytokines including IFN-γ, IL-1α, IL-1β, TNF-α, and IL-12. Th2 cells secrete an anti-inflammatory cytokine profile that includes IL-4, IL-5, IL-6, IL-10, and IL-13. Th1 and Th2 cytokines cross-regulate each other; that is, Th2 cytokines suppress Th1 responses and vice versa (ref). The production of IFN-γ and IL-10 is of particular interest due to their ability to enhance production of certain immunosuppressive molecules (e.g., IFN-γ induces IDO) and regulatory T cells (see below).

Adult-Derived MSCs

BM-MSCs have a propensity for dampening Th1-mediated, pro-inflammatory immune responses. The MLR is mediated by Th1 cells, and the response is characterized by high levels of IFN-γ and TNF-α. When BM-MSCs are added to MLR cultures, Th1 cytokines are suppressed, whereas the levels of IL-4 and IL-10 are significantly increased [60]. The decreased levels of IFN-γ and TNF-α are likely due to direct immunosuppressive effects on T cells, DCs, and NK cells [60], whereas the increase in IL-10 could be due to immune deviation of T cells toward a Th2 phenotype through production of IL-10 by the BM-MSCs [129] or to effects that BM-MSCs have on antigen-presenting cells, including monocytes and DCs [57, 60]. In studies performed by Aggarwal and Pittenger [60], BM-MSCs decreased TNF-α production by LPS-stimulated DCs when cultured with purified myeloid DC1 cells, whereas BM-MSCs enhanced IL-10 production by stimulated plasmacytoid DC2 cells. PGE2 secreted by BM-MSCs played a role in DC modulation as it was shown that a PGE2 inhibitor significantly enhanced IFN-γ and TNF-α secretion. Studies by Beyth et al. [57] showed enhanced IL-10 production in cultures of BM-MSCs and T cells stimulated with superantigen after addition of monocytes or DCs. Recent studies by Nemeth et al. [147] have demonstrated that BM-MSCs can modulate macrophages through an immune deviation mechanism similar to what was found for DCs. Using a mouse model, these investigators demonstrated that BM-MSCs can interact directly with activated macrophages derived from septic mice. After cell-cell contact, the BM-MSCs subsequently reprogram the macrophages via prostaglandin E_2 to produce large amounts of IL-10 which may prevent egress of neutrophils from blood vessels reducing tissue damage in the infected tissue.

Fetal-Derived MSCs

Studies by Roelen et al. [98] demonstrated that MSCs derived from amnion can shift a response toward an anti-inflammatory Th2 cytokine profile. Whereas fetal-derived MSCs produced IL-10 spontaneously in culture, the levels of IL-4 and IL-10 were significantly increased when the MSCs were added to MLR cultures. The Th1 cytokine, TNF-α, was decreased in these cultures. A role for IL-10 in suppression of MLR responses was shown in blocking experiments using antibodies specific for IL-10.

Generation of Regulatory T Cells (Tregs)

Tregs, existing as naturally occurring cells or induced by antigen, play a major role in the induction and maintenance of immune tolerance (reviewed by [148–150]). Although no single specific marker identifies these cells, they typically express CD4 and CD25 and the transcription factor FoxP3. Induced Tregs referred to as Th3 cells secrete TGF-β, whereas induced Tregs known as Tr1 cells secrete large amounts of IL-10. Tregs induced in the periphery depend on the maturity of stimulating APCs as well as cytokines such as IL-10 and TGF-β. Tregs have

been shown to promote transplantation tolerance in animal models, even at the site of the graft itself (reviewed by [151]).

Adult-Derived MSCs

BM-MSCs can induce Tregs from T cell populations in vitro [57, 60]. The mechanism for induction of Tregs probably involves IL-10 as well as other molecules produced by the BM-MSCs. BM-MSCs have been shown to constitutively produce TGF-β and IL-10 [129], both of which drive the differentiation of Tregs from activated T cells. Elevated levels of IL-10 can also be produced through BM-MSC interactions with DCs that prevent DC maturation and result in the production of Tregs [60, 152]. Similar increases in IL-10 have been reported when activated macrophages are cultured with BM-MSCs [57, 147] and Tregs were induced in this system as well [57]. Finally, Tregs may be induced by IDO and HLA-G produced by BM-MSCs. IDO has been proposed to be involved in the crosstalk between DCs and T cells resulting in the production of Tregs [130]. BM-MSCs secrete HLA-G5 which contributes to the expansion of Tregs (CD4+CD25+Fox3+) when BM-MSCs were added to MLR cultures [127]. Interestingly, HLA-G may be involved in the generation of regulatory T cells through a process known as trogocytosis in which membrane fragments containing HLA-G are transferred to T cells. Trogocytosis has been demonstrated for macrophages [153] but not for BM-MSCs, to our knowledge.

Fetal-Derived MSCs

Placenta-derived MSCs were shown to suppress mitogen- and alloantigen-induced immune responses in vitro with the concomitant induction of Tregs [97]. Tregs could have been induced by IL-10 and TGF-β since suppression was partially reversed by neutralizing antibodies to these cytokines, or by IDO and/or intracellular HLA-G which was expressed by the MSCs.

Summary

MSCs have clear therapeutic use both as a cell therapy and for tissue engineering. MSCs have potential application as autologous cells for tissue engineering or when the patient's immune system is conducive to adoptive transfer as allogeneic cells. Based upon their proliferation potential and expansion potential in vitro, the easy collection from a discarded tissue, the fact that collection is painless and without the potential for donor-site morbidity, and the ability to bank the cells, Wharton's jelly-derived mesenchymal stromal cells have advantages as a source for off-the-shelf allogeneic cell therapy or for tissue engineering. We speculate that banked WJCs will have application as an adjunct therapy for HCT with umbilical cord blood or for prophylaxis or treatment of graft-versus-host disease.

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Pluripotent Stem Cells of the Mammalian Early Embryo

8

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Trophoblast Stem Cells

General Concepts

Throughout very early mammalian embryo development, from zygote to morula, blastomeres maintain totipotency as they eventually give rise to both embryonic and extraembryonic tissues. At the blastocyst stage (day 5 in human embryo development), trophoblast lineage is specified before embryo implantation. This lineage appears as a sphere of epithelial cells surrounding the inner cell mass and the blastocoel. Trophoblast cells will form the part of placenta that is in close contact with maternal blood, providing the main structural and functional components of the placenta [76]. The placenta is a vital organ essential for mammalian embryo development. It acts as an interface between embryo and maternal environments and plays a crucial role in the exchange of gases, nutrients, and waste products. As a result, any genetic alteration or environmental injury to the placental development may result in fetal growth retardation and death. Hence, understanding early trophoblast differentiation events is fundamental to develop potential therapeutic approaches to prevent or treat fetal implantation and placental development–related diseases.

Before implantation, development of mouse and human trophectoderm (TE) is regulated by the transcription factors Cdx2 and Eomes. Cdx2 is expressed from 8- to 16-cell stage embryos predominantly in the outer blastomeres and is essential for the specification of the TE cell lineage [92]. Eomes is expressed from the 1-cell stage in all blastomeres and then in the TE at 3.5 days post-coitum (dpc) in the mouse embryo, but it is required only for proper differentiation of the TE into trophoblast stem cells [54, 79].

After implantation, the inner cell mass (ICM) forms the embryo proper and also some extraembryonic membranes. However, the trophectoderm is restricted to forming the fetal portion of the placenta and the trophoblast giant cells. The polar trophectoderm (in contact with ICM) keeps proliferating and forms the extraembryonic ectoderm (ExE) and the ectoplacental cone (EPC). The rest of the trophoblast loses proliferative capacity and gives rise to primary giant cells [78].

At blastocyst stage, it is possible to derive two types of stem cell lines. From the inner cell mass embryonic stem cells (ESC) are obtained, and from the trophectoderm trophoblast, stem cells (TS) can be derived. Both cell types, although very different in cell identity, exhibit unlimited proliferation in culture keeping their own cell

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lineage identity while also contributing to chimeras [77, 86]. TS cells are characterized by the expression of markers such as Errb, Cdx2, Fgfr2, Gata6, and mEomes (the mouse homologue of eomesodermin) [95]. In mice, TS cells obtained from blastocysts are capable of giving rise to the different cell types of trophoblast lineage, identified by the expression of Errb, Cdx2 and Fgfr2 (ExE specific), and Mash2 (EPC specific), and placental lactogen-1 (giant-cell specific) [95]. This fact means that TS cell derivation and culture is a great tool in the study of the development of trophoblast lineage.

TS cells cultured in vitro need the presence of FGF4 for self-renewal, which is produced by the ICM in vivo, and in the absence of FGF4, they spontaneously differentiate to trophoblast giant cells [95]. This is in agreement with what occurs in vivo, where polar trophectoderm, in close contact with ICM, seems to maintain TS cell phenotype because FGF4 accessible from the ICM. FGF4 binds to Fgfr2 receptor expressed by the trophoblast lineage [3]. TS cells in culture also need fibroblast-conditioned medium, indicating that FGF4 is not the only key-signaling factor crucial in TS cell phenotype maintenance. Further studies are needed to understand the mechanisms involved in the maintenance of TS cell pluripotency state and in the differentiation to the placenta components in order to prevent or to treat several placenta disorders in human beings.

Preclinical Studies in Animal Models

Tanaka's group described the isolation and culture conditions of mouse TS cells for the first time in 1998. At present, there are no preclinical or clinical trials using TS cells, probably due to two main reasons: Firstly, it is necessary to study in depth the molecular mechanisms involved in the processes of determination, differentiation, and maintenance of these cells' pluripotency. And secondly, the main interest of TS cell study is not cell therapy itself, but the use of TS cells to differentiate in vitro the several trophoblast-derived tissues and provide a useful model to investigate these processes in order to understand the mechanisms that occur in vivo in the normal and pathologic placenta development.

The study of the molecular mechanisms involved in trophoblast lineage determination and placenta development implicates difficulties such as: If a gene plays a key role in these processes, the mutant embryo needed for the study dies before it shows an apparent phenotype. Nevertheless, there have been advances in identifying the genes involved in placental development from the analysis of these mutant mice [12]. The genes involved include FGF4, Cdx2, Mash2, and Hand1, which have been identified as trophoblast markers, and suggest key roles in trophoblast development. And also, key genes involved in placenta development not traditionally related to trophoblast have been identified, such as PPARγ (peroxisome proliferator–activated receptor gamma) [5].

Several studies indicate that the main signal between the ICM and trophectoderm are members of the fibroblast growth-factor family. Embryos homozygous for loss-of-function FGF4 die at implantation [24], but ES cells can be obtained from those embryos [108], suggesting a key role for FGF4-signaling pathway in early trophectoderm development. It is known that ES cells produce FGF4 and TS cells produce Bmp4, required for the optimal growth of ICM. Murohashi et al. [60], using mouse TS cells, proposed a paracrine communication in early mammalian embryos between trophectoderm and ICM. In this way, the FGF4 secreted by ICM might act in TS cells through the transcription factor Cdx2 expression (specific marker of TS cells, but not differentiated trophoblast cells) and by promoting Bmp4 expression, required for proper growth of ICM and epiblast. Recently, Parast et al. [70] used a TS cell model for PPARγ to analyze its role in the development of the placenta. They derived TS cells from PPARγ null mice embryos and reported that PPARγ is essential for trophoblast proliferation, differentiation, and invasion, probably through Gcm1 signal, a specific marker of syncytiotrophoblast cells, a differentiated lineage derived from trophoblast, that constitutes an essential and functional layer of the placenta.

Various expression and knockout studies in mice provided evidence that various class B bHLH proteins are essential for placental development. These factors play fundamental roles in the maintenance and differentiation of different trophoblast subtypes. In particular, Mash2 (mouse achaete-scute homologue 2) and Hand1 seem to regulate the development. Homozygous deletion of the Mash2 gene in mice resulted in placental failure and embryonic lethality at 10.5 dpc due to the lack of the particular epithelial cells that constitute the spongiotrophoblast [31]. Mice deficient in the giant cell–specific gene Hand1 arrested at 7.5 dpc due to a reduced number of primary giant cells and the impossibility of normal placenta development [75]. In TS cell in vitro models, it has been reported that Mash2 promotes trophoblast cell differentiation toward trophoblast giant cells. By contrast, Hand1 promotes cell cycle exit and inhibits trophoblast giant cell fate [39].

In addition to the direct study of trophoblast stem cells, human ES (hES) can be also used to generate TS cells. hES cells, in specific culture conditions (presence of BMP4 and absence of FGF2), are also capable of giving rise to trophoblast cells [98]. In these conditions, the cells begin to express markers associated with trophoblast, such as GATA2, GATA3, MX2e, and ID2 [109]. The ability of hESs to differentiate toward trophoblast highlights the utility of human ES derivation as a useful model to study the first steps in the trophoblast lineage determination and differentiation and so might modify the course of pregnancy disorders associated with placental dysfunction.

Embryonic Stem Cells

General Concepts

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass (ICM) of blastocyst-stage embryos [22]. They exhibit the distinct and combined characteristics of self-renewal and pluripotency [77]. Pluripotency refers to the ability of a cell to give rise to differentiated progeny of multiple cell types, in fact all cells of a living organism.

Future medical strategies may aim to achieve replacement or regeneration of malfunctioning tissues and organs. Human stem cells in culture offer an essentially unlimited pool of naive, differentiated, or modified progenitor cells to achieve regeneration by transplantation or graft [64]. As human embryonic stem cells (hESCs) are derived from live blastocysts [97], ethical issues have been raised, and the use of human hESCs for research purposes has been severely limited in several countries.

Recently, it has been shown that somatic cells can be reprogrammed into a state resembling stem cells by forced expression of a defined set of pluripotency- and growth-associated transcription factors [66, 94]. These so-called induced pluripotent stem cells (iPSs) are generated by retroviral-mediated transduction of OCT4, SOX2, KLF4, and, although formally dispensable, c-MYC. Although this process occurs at low frequency, in theory it makes patient-specific stem cells available that can be cured [73, 112] and used for cell replacement therapy or tissue regeneration. Unfortunately, human iPS lines suffer from high levels of variability due to incomplete reprogramming, tissue culture mutations, and epigenetic alterations (addressed by Andrews et al. [4]). For this reason, preclinical characterization of cells for replacement therapy must be extensive and include assessment of correct chromosome number, transgene silencing, differentiation properties in vitro, single nucleotide polymorphisms (SNP) analysis, X chromosome reactivation, and genome methylation both at pluripotency-specific promoters and at repetitive sequences. For the near future, application will also be limited by the elevated cost of individual iPS generation, the necessity to develop therapy grade culture in the absence of animal-derived products, and the propensity of iPSs to generate tumors when engrafted. Prescreening individual lines in nude mice has recently been suggested to discriminate effectively between “safe” and tumorigenic lines [101].

Both hESC and iPS cultures suffer from high levels of variability, also due to inherent cellular

heterogeneity within cultures [21, 45, 91]. Recently, different groups have reported the identification of so-called naïve pluripotent cells or naïve iPSs from hESCs, using forced expression of additional transcription factors or culture in physiological concentrations of oxygen [33, 48]. The reproducibility and quality of hESC (or iPS) cultures may be greatly improved when replaced in the future by these more naïve cells.

Human ES cells and iPS cells can be instructed in vitro to adopt specific cell fates by a variety of treatments including coculture with differentiated cell types, chemical induction, aggregation in suspension, and other methods. Although differentiation into virtually any desired cell type can be achieved, the differentiated progeny obtained is usually heterogeneous, both with respect to the type of cells produced and their proliferation potential [61]. In addition, the inherent cellular heterogeneity in both hESC and iPS cultures contributes negatively to standardization of differentiation protocols. Both the grade of hESC differentiation and cell conditioning before engraftment are important parameters for success in cell therapy.

Preclinical Studies in Animal Models

The graft or transplantation of stem cells has emerged as a promising approach to replace lost or injured cells and tissues. This is especially true for diseases that lack a cure at the present time. Progress in preclinical studies has been limited at times due to a lack of knowledge regarding the favored anatomical locations, the appropriate differentiation state of cells to be grafted, difficulties in standardizing cell quality, and the number of cells applied. Unfortunately, the lack of adequate controls in graft experiments has hampered unequivocal interpretation of results in several studies [59, 63]. Common problems encountered when using stem cells and their differentiated progeny in cell therapy trials include immune rejection, teratoma formation, and tumor formation in different contexts.

Cell therapy is a promising and fast-moving field. Today's results may be improved tomorrow or disqualified because of the side effects observed. Several cell types have been obtained and tested by transplantation in animal models with the promise of cell-mediated regeneration in a wide array of pathologies. Examples listed below are only meant to list some cell types and models under investigation and highlight the underlying concepts that apply in each case.

Heart

Stem cells may be able to replace heart muscle cells (cardiomyocytes) lost during a myocardial infarction (MI). Indeed, different types of stem cells have been tested in transplantation studies in either mice or humans. Human ES cells are a credible source of stem cells for this purpose, as they can differentiate into cardiomyocytes in vitro when stimulated appropriately (growth-factor stimulation, coculture with endodermal cells, or treatment with 5-azacytidine and transforming growth factor–β1). The resulting cardiomyocytes show features typical of fetal cardiomyocytes [105]. Human ES cells can also differentiate into some of the supportive cells including endothelial cells, smooth muscle cells, and fibroblasts that may be required for productive grafts. Typical results from grafting hESC-derived cardiomyocytes in immunodeficient rats or mice indicate they survive for over 3 months and mature to form cardiac syncytium. They do not integrate however in the recipient myocardium due to the presence of an intermediate layer of fibrotic tissue, which may cause arrhythmias [57]. Similar grafts in rodents suffering from induced MI simply ameliorate early damage and offer only an apparent improvement in function in the short term that is not sustained over time [59].

The intrinsic beating frequency of human versus rodent cardiomyocytes is different, and myocardial infarction in humans is not totally comparable to myocardial infarction induced in rodent models. Because of similarities in size and physiology, larger animals, such as pigs, represent better candidates as clinically relevant models for studying cell therapy in myocardial infarction [63]. Potential strategies for improving hESC contribution to damaged heart include the avoidance of the initial inflammatory phase after

infarction for injection and combining stem cell–derived cardiomyocytes with supportive cells such as cardiac fibroblasts. Most importantly, advances in tissue engineering and development of scaffolds may be able to align cardiomyocytes in a way that allows directional contraction and coordination with the regenerating myocardium (reviewed in Mummery et al. [59]).

After initial promising results obtained in mice [68] and positive case reports, clinical trials were initiated using bone marrow–derived stem cells in humans with acute MI. However, it turned out that no major long-term improvements in cardiac function could be demonstrated. Soon thereafter, a trial in which patients received autologous skeletal myoblasts was interrupted once negative side effects in the form of arrhythmias were observed [57]. The modest improvements detected in patients treated with bone marrow–derived stem cells are most likely attributable to paracrine effects rather than cell replacement by grafted cells [46]. The sequence of events highlights the importance of preclinical evaluation of cell-replacement therapies in animal models, the type of model used, and the comparison with appropriate control grafts. Altogether, clinical implementation has been slow to arrive for hESCs in MI and is not called for in the foreseeable future.

Neurodegenerative Disorders

Interest in novel stem cell–based therapies has also focused on Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Alzheimer disease (AD), ischemic stroke, and spinal cord injury [55]. Functional transplantation of mouse ES cells has been achieved more than 10 years ago in the case of glial precursors as a source of myelinating cells [8, 84] and dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease [44]. Clinical application has been hampered, however, by immunological rejection and tumor formation, both of which have been overcome recently in an animal model through the use of iPS cells [101].

"Safe" murine iPS–derived neurospheres, prescreened for absence of tumorigenicity, are capable of turning into functional neurons, astrocytes, and oligodendrocytes in vitro when properly instructed [101]. The therapeutic potential of murine iPSs turned into neural progenitors was examined in a mouse spinal cord injury (SCI) model [101]. When transplanted into the epicenter of the injured spinal cord 9 days after contusive injury, they further differentiated into all three neural lineages without forming teratomas or other tumors. They also participated in remyelination, induced axonal regrowth, and promoted locomotor function recovery [101]. These promising results will undoubtedly boost the quest to obtain similar results using human iPSs.

The use of embryonic stem cells and neural progenitors derived from them also provide a potential remedy for Parkinson's disease. In vitro, dopaminergic neurons can be derived from hESCs by applying exogenous factors, and grafting them has produced functional recovery in rat models of Parkinson's disease (PD) (reviewed in [50]), although low survival rates of grafted cells have limited the efficacy of this cell replacement therapy [71]. Such results have highlighted the extent to which the pathological environment determines the survival, differentiation, migration, and function of grafted cells.

Human embryonic stem cell–derived dopaminergic neurons show poor survival after transplantation in rodent models of PD. However, using genetically engineered mouse ES cells that overexpress a neural adhesion molecule (L1), an improved differentiation protocol is capable of enhancing both neuronal differentiation and migration of grafted embryonic stem cells and also decreasing tumor formation. Dopaminergic differentiation was increased as well as the survival of engrafted cells, causing functional recovery in a mouse model of PD [13]. Improvements engineered in mouse cells may apply to hESCs and thereby allow progress toward clinical applications.

Clinical Studies

Embryonic stem cells, at present, have crucial limitations that mean there are no clinical trials currently open in human patients using undifferentiated

hESCs. The high proliferative capacity and pluripotency of ES cells produce tumor development in several animal models (see above). A possible solution to this problem may consist of the use of predifferentiated ES cells in combination with prescreening as mentioned above.

Several studies in animal models have reported the therapeutic potential of ES-derived cells, including insulin-secreting islets, retinal cells, hepatocytes, chondrocytes, cardiomyocytes, and neuronal cells [6, 27, 40, 51]. Also, these cells have been used in animal models of diabetes, heart ischemia, Parkinson's disease, spinal cord injury, liver disease, macular degeneration, and multiple sclerosis [2, 41, 47, 49, 88, 107] with positive results. Not in all cases however, properties that are fundamental for successful clinical translation have been demonstrated for human embryonic stem cell–derived grafts, such as cell-based effects that promote long-term and substantial improvement of the symptoms in a high percentage of treated animals.

Although human ES cell–derived therapy is most likely a decade away from clinical implementation, the advances in stem cell research in neurodegenerative diseases are already being translated into clinical trials for remyelination in spinal cord injury: On October 2010, the first patient with spinal cord injury was treated with hESC-derived oligodendrocyte progenitor cells (GRNOPC1) that have demonstrated remyelinating and nerve growth-stimulating properties leading to restoration of function in animal models of acute spinal cord injury. In addition, on November 2010, the US Food and Drug Administration (FDA) approved a hESC-based clinical trial to treat the juvenile blindness Stargardt's macular dystrophy (SMD). SMD is characterized by retinal pigment epithelium (RPE) degeneration, and hESs have demonstrated their capacity to differentiate into mature RPE and to improve vision in animal models after transplantation [53]. More recently (January 2011), the same group obtained approval from US FDA to initiate a similar clinical trial in this case to treat "dry" age-related macular degeneration (AMD), the most common form of macular degeneration [53].

Other clinical trials with ESC-derived cells have been proposed for the near future such as ES-derived β islets cells to treat diabetes [100, 107] and for treatment of Parkinson's disease within 5 years [13, 50].

Alternatively, disease-specific hiPS cell lines have been generated from patients with various diseases, including Duchenne and Becker muscular dystrophy. Similar efforts are under way for patients suffering from Fanconi anemia ([73], Bueren and Raya, personal communication, 2011). Although cell therapy using hESCs may translate into future clinical application, the use of patient-specific-induced pluripotent stem cells (iPSs) offers real and present opportunities for modeling and probing human disease.

Epiblast Stem Cells

General Concepts

At the blastocyst stage, when ICM and trophectoderm are established, the ICM forms two layers: the hypoblast that will give rise to the yolk sac and the epiblast that will form the embryo proper. The epiblast cells express markers of pluripotency like SSEA-3/4 (human) [62], Oct4, and Nanog [34].

While the majority of epiblast cells differentiates toward the different somatic cell types, a few cells initiate the germ cell program, repressing the somatic program in order to give rise to primordial germ cell (PGC) precursors. This process includes the reexpression of pluripotency markers such as Nanog and Sox2 and the erasure of epigenetic memory of the somatic program [110, 111].

Previous work suggested that pluripotent cells could only be obtained from the preimplantation embryo, but recent studies show that pluripotent cell lines can be derived directly from mouse epiblast [7, 96]. The EpiSCs (epiblast-derived stem cells) are derived from the epiblast of early postimplantation (embryonic day 5.5–6.5 dpc) mouse embryos. Like mouse and human ES cells, mouse EpiSCs (epiblast-derived stem cells) express transcription factors known to regulate

pluripotency such as Oct4, Nanog, and Sox2, and maintain their genomic integrity. They can be induced to differentiate in vitro into the majority of somatic cell types of the three germ layers, as well as into germ cells. Although EpiSCs have a reduced ability to form chimeras, this data along with their capacity to form teratomas shows that these cells have a wide developmental potency. At present, just rodent and pig EpiSCs have been derived [7, 23, 96]. So far no EpiSCs have been derived from humans, but their derivation will aid the understanding of the precise controls that regulate the transition from pluripotency to specific cell fates.

There are several molecular markers expressed by pluripotent EpiSCs. Some of them are common to other pluripotent cells such as Oct4, Nanog, Sox2, and SSEAS. There are also observable differences distinguishing this stem cell type from germ cells. EpiSCs do not express alkaline phosphatase (AP) activity or Blimp1 and Stella, which are strongly expressed in early germ cells but not in the epiblast, demonstrating that this cell type is not derived from the primordial germ cells [29, 80]. EpiSCs have several properties from epiblast cells, including the X chromosome inactivation in female lines [9], indicating that EpiSCs are more advanced than ES in embryo development.

Interestingly, mouse EpiSCs are more similar to human ES than mouse ES. hESs and mouse EpiSCs depend on the activin/nodal and FGF2 signaling to maintain their pluripotency, whereas mouse ES require LIF and BMP. In addition, the epigenetic status of EpiSCs is closer to hESs than mES [7, 96]. Activin/nodal acts through Smad2/3, which interacts directly with the core transcription factor of pluripotency Nanog. In this way, inhibition of activin/nodal results in loss of Nanog expression and induction of differentiation [104]. In hES, activin/nodal signaling is not enough to maintain the undifferentiated state. Also, FGF2 is necessary to maintain expression of pluripotency markers and to inhibit the cell apoptosis. However, it is believed that the molecular pathway of FGF2 that promotes EpiSCs self-renewal is not through smad2/3, in contrast to hESs [30]. EpiSCs, as well as hES, also show high expression of genes associated with the epiblast and early germ layers such as FGF5, Otx2, Eomes, Foxa2, and Brachyury T [96]. This similar gene expression suggests that EpiSCs are more similar than mES to hESs and that hESs are in a more differentiated state than mESC. Naïve pluripotent cells generated from human ES (mentioned in a previous paragraph) are more similar to mouse ES cells in terms of X chromosome inactivation, absence of epiblast-specific gene expression, and LIF/BMP dependence.

Preclinical Studies in Animal Models

EpiSCs have a limited ability to contribute to chimeras when injected into blastocysts or by morula aggregation. However, EpiSCs are capable of forming teratomas in vivo and embryoid bodies in vitro [96]. Another similarity between hESs and EpiSCs is their capacity to differentiate into trophoblast-like cells in the presence of BMP4, unlike mES which do not have this ability [7, 109]. In addition, EpiSCs could be differentiated into different adult tissues like cardiomyocytes, endoderm-like cells, and endothelial cells [96]. These data demonstrate the high developmental potency of EpiSCs.

The pluripotent epiblast tissue is composed of the most immediate precursors of the early somatic lineages and the germ line, so the stable EpiSC lines will aid in understanding the processes involved in regulating the transition from pluripotent to specific cell fates. The epiblast forms the germ line very early on. Just before the epiblast gives rise to the endoderm, the ectoderm, and the mesoderm, the first morphologically identifiable precursor of PGCs (primordial germ cells) appears in the proximal part of the epiblast [56]. Thus, precursors of PGCs are the first stem cells that are specified in the embryo at the beginning of gastrulation. The emergence of PGCs from EpiSCs in vitro is accompanied by appropriate reprogramming epigenetic events, including X chromosome reactivation, and this model is a great system for investigations into underlying mechanisms such as the erasure of the somatic program that occurs in the germ line [35]. Culture and maintenance of EpiSCs provides

a useful in vitro model to study the early events that occur in the specification of the germ line, as EpiSC culture constitutes a continuous source of PGCs [36].

Embryonic Germ Cells

General Concepts

Embryonic germ cells (EGs) are pluripotent stem cells derived from primordial germ cells (PGCs), the embryonic precursors of the gametes. PGCs are the founder cell population of the gametes and can be identified in humans around day 22 of gestation by expression of tissue nonspecific alkaline phosphatase (TNAP) activity. PGCs become specified in the proximal part of the epiblast at around the third week of gestation in humans, shortly before gastrulation, when the epiblast is about to give rise to the three germ layers ectoderm, mesoderm, and endoderm.

Later on, PGCs migrate to the base of the allantois, at the extraembryonic mesoderm. Afterward, they are incorporated into the epithelium of the hindgut, from which they start to move into the dorsal mesentery at weeks 4 and 5 in human embryos. Next, PGCs migrate from the hindgut, reaching the aorta-gonad-mesonephros (AGM) region around the 5th to 6th week in humans. PGCs finish their migration at the genital ridges that lie on the dorsal body wall at the 6th week of gestation. By 41–44 dpc (Carnegie stages 17 and 18), PGC numbers in males decrease due to testicular cord formation and mitotic arrest [32], whereas PGC numbers in females increase by proliferation in the fetal ovary [102].

PGCs can be isolated and cultured for up to 10 days, maintaining their phenotype, until they are lost by apoptosis [15, 16, 18]. Cultured PGCs depend on several growth factors for survival and proliferation in vitro: SCF (KL), LIF, and BMP4 ([102]; for a recent review, see [14]). PGCs are not pluripotent, as they do not form teratomas when injected into immunodeficient mice, and in vivo will form only gametes. However, PGCs are considered developmentally pluripotent, as they generate the whole totipotent embryo after fertilization. PGCs undergo extensive erasure of imprints during the migration and gonad colonization stages. The erasure of the imprint in early PGCs is one of the mechanisms that shut down PGC developmental pluripotency, making these cells resistant to potential parthenogenesis and teratoma formation in humans [67]. Late germ cells will experience a proper somatic imprint reestablishment so that a fertilized egg will express a developmentally proper somatic imprint of crucial imprinted genes. The epigenetic reprogramming observed in mouse PGCs also occurs in humans, as extensive DNA demethylation is required to erase the imprints and for X reactivation [35].

PGCs can give rise to two types of pluripotent stem cells, the embryonal carcinoma cells (EC) and the embryonic germ cells (EG). In vivo, hPGCs can generate EC cells, the pluripotent stem cells of testicular tumors [90]. In fact, ES cell derivation was based on studies of teratocarcinoma cells. Transplantation of mouse genital ridges into ectopic sites produced teratocarcinomas at a high frequency in mouse strains that did not spontaneously form those tumors [87]. The stem cell of those tumors is the EC cell. The first EC lines were established more than 40 years ago [25, 89] and are considered the malignant counterpart of ES cells, as they share the same pluripotency markers (such as SSEAs, Oct4, Nanog and TNAP) but are usually aneuploid.

In vitro, cultured hPGCs exposed to a specific cocktail of growth factors (KL, LIF, and FGF2) generate pluripotent embryonic germ (hEG) cells [52, 69, 72, 83, 103]. The conversion of PGCs into pluripotent stem cells is a remarkably similar process to nuclear reprogramming after somatic nuclear injection into the egg cytoplasm [17]. EG cells have been derived at different stages of development in humans, always after sex cord formation (5–10 weeks of gestation) due to restrictions of material availability [83, 93, 102]. hEG cells are pluripotent by embryoid body formation [83, 93, 102]. Mouse EG cells have also demonstrated teratoma formation [74], although, intriguingly, hEG have not [82, 102].

The main inducer of this dedifferentiation of PGCs into EG cells is FGF2 [74, 83]. The exact role of this growth factor has only recently started to be elucidated [14, 19, 20] and is related to downregulation of the germ lineage specification transcriptional regulator Blimp1. Importantly, in contrast to hEC cells, hEG cells are euploid, making them more similar to ES cells than to EC cells, and hence feasible tools for use in cell therapy.

Many markers are common to ES, PGCs, EC, and EG cells such as the SSEAs, Oct4, Nanog, and TNAP among others [16, 83, 93]. However, there are several molecular markers that distinguish early germ cells from other pluripotent cells of the early embryo, such as Stella (also known as Dppa3 or PGC7), which helps distinguish germ cells from primitive ectoderm [28, 38, 80]. Other markers, such as TNAP and GCNA, are strongly expressed by hPGCs but are weakly expressed by the epiblast and other surrounding embryonic cells [29]. Only the expression of more mature germ cell markers (such as mouse vasa homolog, Mvh [99]) and the key molecular determinant for germ cell specification, Blimp1 [65, 106], enables in vitro–derived germ cells and EG cells to be distinguished from ES cells themselves.

Preclinical Studies in Animal Models

The pluripotency of EG cells makes these cells attractive for their use in regenerative medicine, as an alternative to their earlier derived counterparts, the pluripotent ES cells. EG cells represent a major alternative to ES cells due to the different epigenetic status of EG cell lines, which may be important for cell therapy applications [1]. Several preclinical studies using these cells in animal models have been performed, as it has been previously demonstrated that hEG cells differentiate into a variety of cell types in vitro [81], such as neurons [69], musculoskeletal cells [43], insulin-producing cells [11], and more recently, adipocytes [37].

Most approaches take advantage of previous EG cell differentiation into embryoid bodies (EB) in vitro, before transplantation of selected cell types into the damaged organ. Remarkably, EB-derived cells from hEG have been demonstrated to restore neurological function in a virus-induced diffuse motor neuron injury paralyzed rat model [42]. Motor recovery in these rats was due mainly to paracrine action of secreted cytokines TGFα and BDNF and not to neuron differentiation from EG cells. In another model of neuronal damage, the NMDA receptor agonist quinolinic acid was injected, which causes excitotoxic brain damage by massive neuronal apoptosis in the neocortex, hippocampus, striatum, white matter, and subventricular zone. EB-derived neural stem cells from hEG were able to partially restore the complement of striatal neurons in brain-damaged mice [58]. In this model, human cells in fact engrafted into the lesioned areas and differentiated into neuronal and glial cells being able to replace lost neurons.

In other fields, EB-derived cells from hEGs were able to induce bladder regeneration in a rat model [26], again probably due to improved intrinsic regeneration conditions brought about by the human transplanted cells. Similarly, hEG-derived cells transplanted into normo- and hyperglycemic mice become insulin and C-peptide immunoreactive and produce plasma C-peptide in response to glucose, providing an exciting first step in the future treatment of type I diabetes mellitus [11].

Clinical Studies

Despite the great promise held by the animal model studies described in the above section, at the time of writing this manuscript, no clinical trials are being performed with human EG cells.

Since submission of the original manuscript, both the risk of arrhythmias and the electromechanical integration following transplantation of HESC-derived cardiomyocytes have been reassessed in a guinea-pig model [85]. Conditions are reported that allow protection against arrhythmias and result in synchronous contraction of grafted cells with the host muscle concomitant with improved mechanical functions.

Also, neural progenitors derived from human embryonic stem cells have been used to repair the auditory nerve in a gerbil model, in such a way that transplanted cells have contributed to improved sensory (hearing) function [10].

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Stem Cells in Fetal Tissue (The Kidney as a Model)

9

Oren Pleniceanu and Benjamin Dekel

Introduction: The Kidney as a Model Organ for Studying Stem Cells and Development

During the process of development, a single cell, the fertilized ovum, becomes an entire functional organism that is made up of a vast number of cell types, each acting within the context of a specific organ or tissue, usually in cooperation with other types of cells.

Long peripheral neurons, generating and conducting electrical current, beating cardiomyocytes, pancreatic cells able to sense glucose levels and secrete insulin in response, and all other cells in our body have the same origin and contain identical DNA. However, they are clearly morphologically and functionally different.

We will try to explain how different groups of cells assume different roles and act in concert to complement one another in order to generate functional organs and ultimately an entire organism.

The separation into different tissues or cell types obviously occurs during embryonic development and can be noted very early in this process.

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Starting from the epiblast stage, regional specification proceeds as a hierarchy, such that each organ or cell type is formed via a sequence of developmental decisions. The process by which cells "decide" in which developmental pathway they shall stride is a very complex one, involving intra- and intercellular interactions, activity of various extracellular factors, and changes in the pattern of gene expression [1].

We will discuss the interplay of all these elements and how they establish the developmental decision-making process.

Stem cells (in this context, they can be referred to as fetal stem cells), which are defined as cells that can self-renew indefinitely and give rise to at least one type of differentiated progeny [1], stand at the base of these processes, representing the source for the various types of mature and functional cells that establish an organ.

By maintaining themselves in an undifferentiated state, while at the same time giving off differentiated cells, they supply the growing organ the necessary cellular substrate for its development.

Importantly, most organs are generated through the combined action of various types of stem cells. Each solid organ, for instance, requires blood supply (necessitating formation of vessels), an interstitial compartment for structural support, and so on. In addition, the parenchyma itself is also often composed of cells derived from different developmental origins, as will be exemplified.

We will discuss the development of the kidney as an example for different aspects of stem cell biology relevant to development and organogenesis

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and describe the current methods for using these insights for therapeutic goals.

The kidney is an excellent model organ for understanding stem cell self-renewal, multi-differentiation, interplay of secreted factors and gene expression, the stem cell niche, temporal and spatial regionalization, and segmentation and branching morphogenesis. We will therefore attempt to highlight these aspects by depicting the exciting journey that starts with a crude mass of cells and ends in one of the more complex organs in the human body, the kidney.

From Mesoderm to Metanephric Mesenchyme (or the Third Time's a Charm)

During the gastrulation stage, three germ layers are formed: ectoderm, mesoderm, and endoderm [1]. The kidney is formed in a specific location along the anteroposterior (AP) embryonic axis, within the middle part of the mesoderm or intermediate mesoderm (IM) [2]. It is clear, therefore, that for the kidney to form in the right location, it is prudent that the correct molecular and developmental events happen within and only within a specific area at both the mediolateral and anteroposterior planes.

In the mediolateral axis, the IM needs to be formed between the lateral part of the mesoderm or lateral-plate mesoderm (LPM) and the medial part of the mesoderm or paraxial mesoderm.

The first evidence of exclusive genetic markers in the IM is seen at about the 4–8 somite stage, when Pax2 and Pax8 genes are activated in a group of cells just lateral to the paraxial mesoderm [3]. Later on, several other genes are expressed, defining the IM, from which will arise the MM, the kidney precursor.

What drives the formation of this group of cells, or in other words, why are certain genes activated? The answer, which will accompany us along this entire journey of kidney formation, is the interplay between secreted factors and the consequent changes in gene expression, leading to phenotypic shifts in the responding cells, allowing them to influence other cells and so on.

In the case of the IM, it has been found that proteins from the bone morphogenetic protein (BMPs) family that are secreted from adjacent tissues are the ones that activate IM-specific genes [4]. Moreover, it has been shown that BMP in low concentrations activates IM genes, whereas higher concentrations activate LPM-specific genes. Thus, the proposed model for IM establishment is that BMPs are secreted from a lateral source, creating a mediolateral concentration gradient of this soluble factor, which is translated into differential gene expression and the subsequent formation of different structures, including the IM, along this gradient [4] (Fig. 9.1). This

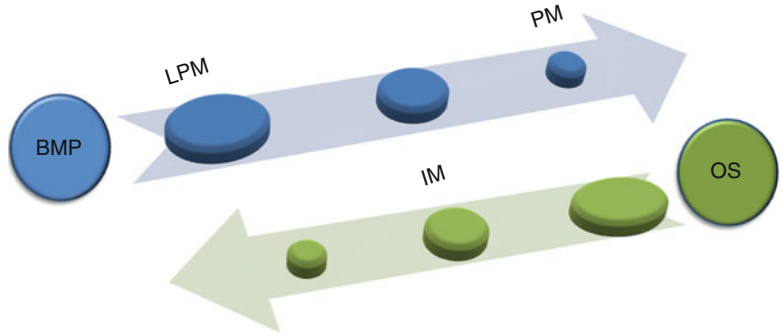

Fig. 9.1 Differential concentrations of secreted factors determine mesodermal-regional patterning: According to the accepted model for mediolateral patterning of the mesoderm, *BMP* (bone morphogenetic protein) is secreted from a lateral source (*left side* of the figure), while BMP-opposing signals (*OS*) are secreted from a medial source (*right side* of the figure), such that a concentration gradient is formed for both types of signals. The balance between these signals upregulates specific genes (e.g., Pax2 and Pax8), thereby inducing the formation of the intermediate mesoderm (*IM*) between the paraxial mesoderm (*PM*) and the lateral-plate mesoderm (*LPM*)

model is most likely too simplistic, as other factors are probably also involved (e.g., activin and retinoic acid) [5], but it is an excellent example for how the developing fetus uses secreted factors to mediate the change of gene expression in order to control developmental processes. In addition, BMP-opposing signals are probably involved as well [6], allowing fine tuning of signal intensity. This balance of activators and inhibitors used for cellular "decision making" is a concept that we will further discuss when we shall describe later stages of kidney development.

Importantly, many factors, including BMP, participate in the development of various organs such as the heart [7] and nervous system [8], reflecting the ability of a certain molecule to lead to different outcomes, depending upon the cellular context, which is defined by the gene expression pattern and the microenvironment of the cell. These variations in cellular response are also reflected by the different role the same factor can have during different developmental stages of the same organ. BMP, for example, is also involved in later processes in kidney formation [9].

As mentioned earlier, the kidney needs to assume its exact position in the AP axis as well. During development, not one, but three pairs of kidneys are successively formed along the AP axis within IM [10].

The first to form (at the 3rd week of human gestation) and the most anterior is the pronephros, which in humans represents a rudimentary and nonfunctional structure that quickly degenerates [10].

A week later, the mesonephros is formed, and although a transient structure as well, it harbors functional tubules.

Finally, at about 5 weeks of gestation and in a posterior location relative to the mesonephros appears the metanephros, which will become the definitive human kidney with hundreds of thousands to millions of functioning nephrons [2].

The sequential formation of three kidneys has made the kidney an excellent model organ for uncovering the mechanisms underlying AP patterning. The main factor that has emerged as crucial in this process is the Hox family of genes.

As previously described, the IM forms within the early mesoderm. However, this only happens in regions of the mesoderm which are posterior to the sixth somite [11], meaning that only a specific region of the mesoderm is capable of responding to the mediolateral-defining signals to become IM. One of the reasons is probably the differential expression of Hox genes along the AP axis. Indeed, the anterior boundary of the IM coincides with the expression boundary of Hox4, and overexpression of Hox4 in more anterior mesoderm regions allows them to become IM as well [12]. Therefore, Hox expression sets the anatomical boundaries of the IM.

However, what defines the metanephros within the IM? Again, the answer resides in Hox gene expression. Specifically, the Hox11 paralogs, Hoxa11 and Hoxd11, have been found to participate in the formation of the metanephric mesenchyme (MM), the precursor of the metanephric kidney [13]. Findings from recent years show that Hoxd11 creates a complex with two other proteins (Pax2 and Eya1) to drive the expression of two crucial factors in kidney development which define the MM, Six2, and Gdnf [14].

Accordingly, when Hoxd11 is ectopically expressed in the more posterior mesonephric tubules, a phenotypic change toward a metanephric one is noted [15].

In conclusion, a complex system of extracellular signals alters gene expression pattern in a way that leads to the specification a certain region of the mesoderm as the IM (mediolateral patterning) and a certain region of the IM as the MM (anteroposterior patterning).

Metanephric Mesenchyme Induces Budding (or to Bud or Not to Bud)

The kidney is eventually formed via reciprocal interactions between two main tissues, the metanephric mesenchyme, or MM, and the Wolffian duct (WD), both of which are derivatives of the IM [16] (Fig. 9.2).

At about 10 dpc (days post-coitum), the MM, previously established from within the IM, "decides" to attract the WD, and via secretion of

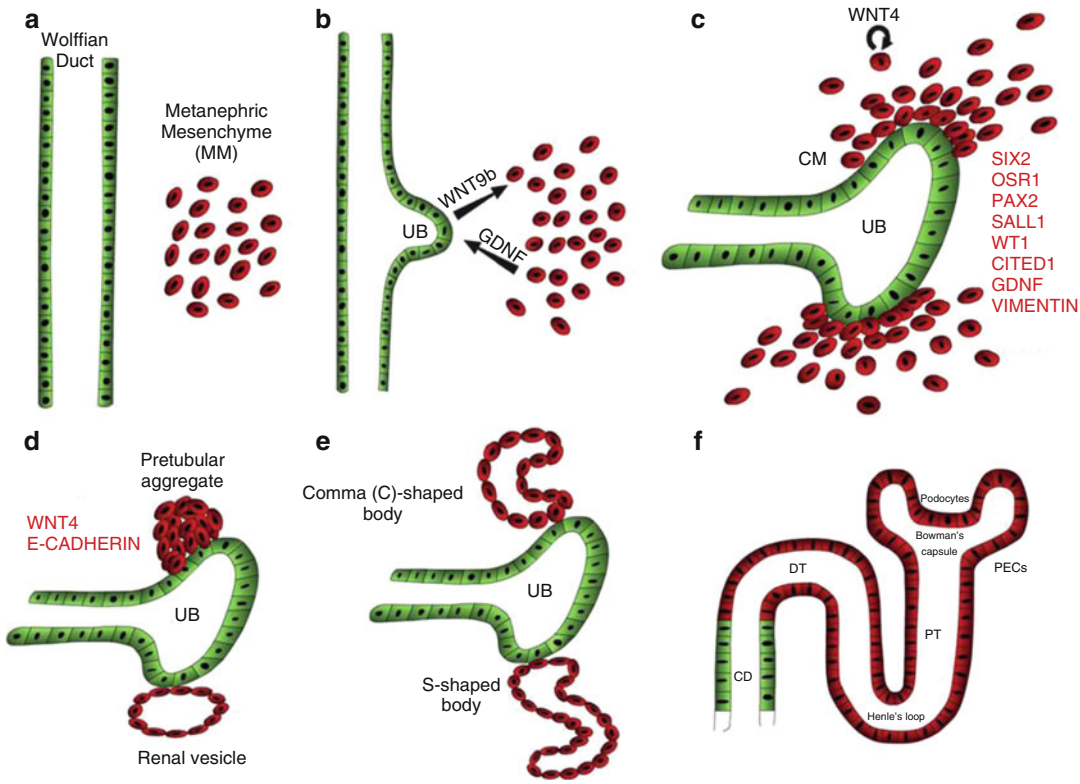

Fig. 9.2 Kidney development: (**a**) The kidney is formed via reciprocal interactions between two precursor tissues derived from the intermediate mesoderm: the Wolffian duct and the MM. (**b**) MM-derived signals, mainly the glial-derived neurotrophic factor, induce an outgrowth from the Wolffian duct, termed the UB. The UB then invades the MM and secretes WNT9b, thereby attracting MM cells. (**c**) MM cells condense around the tips of the branching UB, forming the condensed or CM. The CM expresses a unique combination of genes (*red*) and the mesenchymal marker vimentin. The CM contains the kidney stem cells and is capable of self-renewal. In response to UB signals, CM cells start to produce WNT4, which acts in an autocrine fashion, leading to epithelialization of the cells. (**d**–**f**) The induced cells acquire an epithelial phenotype. This change is accompanied by the shutting down of the major transcription factors described before (**b**) and by the acquisition of the epithelial marker E-cadherin. The cells sequentially form the pretubular aggregate, renal vesicle, C- and S-shaped bodies, and finally the mature nephron. The cells derived from the CM form most of the nephron body (from glomerulus to distal tubule), whereas the UB-derived cells form the collecting duct. *CD* collecting duct, *CM* cap mesenchyme, *DT* distal tubule, *PECs* parietal epithelial cells, *PT* proximal tubule, *UB* ureteric bud (Adapted from [16])

soluble factors, the most important of which is Gdnf (glial cell line-derived neurotrophic factor), induces the formation of a daughter tube from the WD, called the ureteric bud (UB) [17]. The UB, in turn, extends toward the MM and begins a series of interactions, eventually leading to the creation of the kidney.

How do cells "decide"? The decision to secrete Gdnf is, in the simplest manner, the result of the unique combination of transcription factors expressed only in a subset of MM cells, thereby defining them as "UB-inducing cells".

Transcription factors, which are the DNA-binding proteins that regulate gene expression, are probably the single most important determinant of a cells' phenotype. Ergo, when a cell in a developing organ "wants" to carry out an action of some sort (secrete growth factors, divide, migrate, etc.), it has to change the combination of transcription factors it expresses to allow a different set of genes to be expressed, thus changing into a phenotype suiting the specific mission it has to do. When only one or a few transcription factors determine (via their downstream targets)

a specific cellular phenotype, they are called "master regulator gene/s" [18].

If we now reconsider the fetal kidney, no such one master regulator seems to exist. Nonetheless, several important genes lead, directly or via downstream mediators, to the secretion of Gdnf, including Osr1, Eya1, Pax2, and Six2 [16].

However, the question that must arise is why do only few WD cells respond to Gdnf, allowing the formation of only *one* UB? Moreover, how is the specific location of the UB determined?

First, although the Gdnf receptor (Ret) is expressed upon all WD cells, the binding of Gdnf leads to Ret upregulation [19], thereby further increasing the signal for budding, such that the cells closest to the Gdnf-secreting cells (MM cells) are the most likely to respond to the signal.

Second, different factors, for example, BMP-4, are expressed in other regions of the WD, where they act to inhibit budding, thereby preventing ectopic budding [20]. To add to the complexity, Gremlin (*Grem1*), a BMP-4 inhibitor, is secreted in the region of budding to remove the BMP-4 induced inhibition [21]. A good demonstration of what happens when this inducers-inhibitors balance is perturbed is the phenotype of transgenic mice expressing only one copy of the BMP-4 gene, which develop supernumerary and ectopic buds [20]. In contrast, Grem-1 mutations prevent the formation of a UB [22].

Third, the fact that only cells within a specific region secrete Gdnf also contributes to defining one specific area of budding. This exclusivity of Gdnf secretion is also the result of the balance between various factors. While the expression of the transcription factors Eya1, Pax2, and Hox11 promotes Gdnf secretion [14], the transcription factor FoxC inhibits it [23]. Soluble factors are important here as well, as Robo2 and its ligand Slit2 inhibit Gdnf production [24]. As expected, FoxC knockout (KO) leads to the formation of supernumerary UBs [23], while Pax2 [25] or Eya1 [26] KO mice do not develop a UB at all.

An important implication of the reciprocal nature of inducing signals in kidney development is that KO phenotypes are sometimes difficult to interpret because failure of one structure to form can interfere or even completely prevent the formation of another structure, dependent upon signals from the former.

In conclusion, we can once again see that the delicate balance of activators versus inhibitors enables the fine tuning of organ development.

The Ureteric Bud Induces MET (or the Great Invasion)

The newly formed UB is constantly exposed to attracting signals that promote proliferation of cells within it, eventually causing it to invade the MM. It is at this point that the UB takes on an active role and influences MM cells around its tip to condense and form what is known as the cap mesenchyme (CM). This subpopulation of cells is considered as the stem cell population of the epithelial compartment of the kidney or the nephron [16]. CM cells develop into the main nephron body, from podocytes to distal tubule.

Three important processes must happen to allow the formation of hundreds of thousands of nephrons from a relatively limited amount of CM cells.

The first is mesenchymal-epithelial transition (MET) of the mesenchymal CM cells into the epithelial phenotype characterizing tubular cells. This phenotypic change includes acquisition of cellular polarity and establishment of tight junctions between neighboring cells.

The second and third processes, which are also the two defining properties of a stem cell, are self-renewal and multi-differentiation. Clearly, the limited pool of kidney stem cells within the CM must continue dividing while maintaining the same stem cell phenotype and at the same type give off cells that will differentiate into the various tubular cells.

How can we find out which are the factors that enable a selected group of cells to become such stem cells, and how can we follow them as they create mature kidney structures? We will try to give at least a partial answer to this question while at the same time demonstrating some of the modern techniques and challenges of fetal stem cell research.

In order to do so, we must first consider the concepts of cellular lineage and lineage tracing.

Simply put, a lineage is the developmental pathway along which a cell can differentiate, starting with a primitive cell (e.g., a hematopoietic stem cell) and ending in a differentiated or mature cell (e.g., a CD4+ T lymphocyte).

Various techniques have emerged in order to allow us to delineate the differentiation potential of cells, identify the main regulatory factors, and define developmental lineages.

Lineage-tracing techniques involve the tagging of a certain cell type (e.g., a cell expressing a certain gene) and after a given time, after a certain inductive signal or any other intervention, evaluation of the remaining cells for the presence of the marker [27]. Techniques for the assessment of gene function are more complex, but most often, they rely upon the silencing of gene expression and inspection of the resulting phenotype.

The most accurate and advanced of these techniques are carried out in vivo, allowing us to follow both temporal and spatial aspects of lineage development. One such system, applied several times in recent years to reveal some of the landmark findings in developmental nephrology, is the Cre-Lox system.

This system consists of two genetic constructs: the first contains the gene of interest flanked by two short sequences, called LoxP, which upon the presence of the enzyme Cre recombinase are cut, thereby removing the gene present between them. The second consists of the Cre enzyme, usually under the control of a tissue-specific promoter. When transgenic mice, each containing one of the constructs, are bred together, double transgenic mice are created, carrying both constructs [27].

These mice enable both spatial and temporal control of gene expression/silencing.

Gene silencing enables analysis of the role of different genes (KO phenotypes), while gene activation (most often these are marker genes) enables one to perform lineage-tracing experiments.

The spatial control of gene expression is achieved by the tissue-specific promoter. For example, if one is interested in exploring the role of a certain gene in podocytes, then a specific promoter that is active only in podocytes (such as the podocin promoter) can be used.

The necessity of temporal regulation has several reasons. First, as will be demonstrated shortly, the same gene can have different roles at different time points during development, and therefore, the possibility to shut down its expression at a given time is of great value. Interestingly, the fact that most of the information we have about kidney development comes from KO studies is one of the main reasons for the relatively scarce data we have about the roles of genes that control the late stages of kidney development.

Second, serial experiments involving gene KO at successive time points enable one to draw conclusions about the time window during which it is active.

In order to achieve temporal control of gene expression (i.e., the ability to dictate at which time point the gene will be silenced or activated), the Cre recombinase has been modified, such that it is coupled to the estrogen receptor (ER). As part of the nuclear receptor family, the estrogen receptor remains in the cytoplasm until an estrogen agonist (such as tamoxifen) binds to it, causing it to localize to the nucleus. Therefore, in order to silence a gene at given time point, tamoxifen is administered to the animal, where it binds to the CreER complex, which enters the nucleus, enabling the Cre recombinase to excise the fragment present between the LoxP sites [27].

As noted before, this system can also activate a gene of interest. This is done by using a construct containing a “stop” sequence flanked by LoxP sites, upstream of the gene [27]. In this scenario, the gene is detached from its promoter and therefore silenced, and only the activation of the Cre enzyme that removes the stop sequence enables gene expression.

The utility of this system in lineage tracing can be demonstrated by the study performed by McMahon and colleagues that were interested in studying the role of OSR1 in kidney development [28]. The researchers used mice expressing both Cre recombinase under the control of the Osr1 promoter, and a transgene in which the expression of the LacZ gene is activated after Cre recombinase removes poly-A addition sites flanked by LoxP sites. Once this occurs, LacZ is

transcribed in all descendants of the cell in which the recombination took place, regardless of whether Cre remains expressed in those cells, such that each cell in which the promoter was active is irreversibly stained.

By injecting tamoxifen (thereby activating Cre) at different stages of mouse development and examining the kidney (and other organs) at a later stage for LacZ expression, it was possible to precisely define which cells are derived from Osr1+ cells at different stages. For instance, it was found that when injecting tamoxifen at E7.5 (embryonic day 7.5), cells of various lineages, including vasculature and musculature, were positively stained at E19.5. However, when the same experiment was carried out at E11.5, only tubular cells were stained, indicating that Osr1+ cells undergo progressive restriction in differentiation potential during development (Fig. 9.3).

A similar study [29] tested the homeobox transcription factor Six2 and found that cells derived from the early Six2+ population contributed to different cell types of the nephron, deeming it multipotent.

An important point here is the difference between the multipotentiality of a cell population (e.g., the Six2+ population) and the multipotenti-

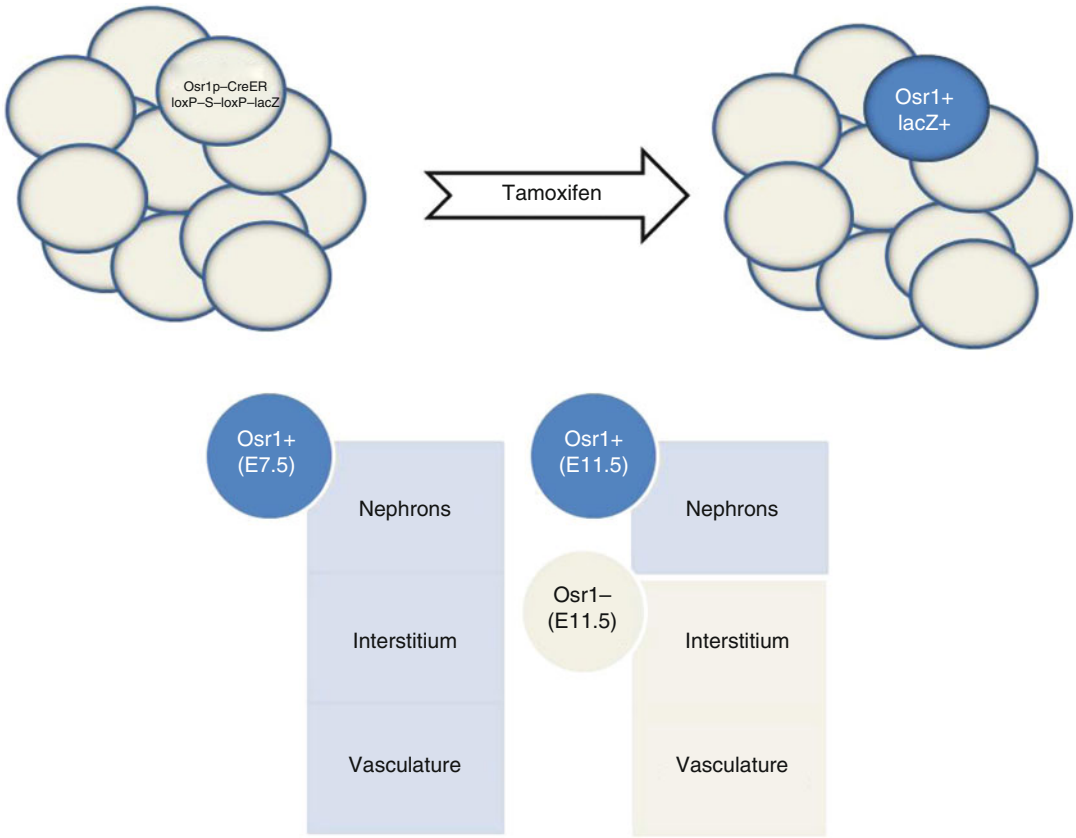

Fig. 9.3 Lineage-tracing studies: A classical example of lineage tracing in vivo is demonstrated. Double transgenic mice carrying Cre recombinase under the control of the Osr1 promoter (Osr1p-CreER) and the lacZ gene, preceded by a floxed stop sequence (loxP-S-loxP-lacZ), were injected with tamoxifen at different stages of fetal development. Tamoxifen injection allowed the Cre enzyme to excise the stop sequence and activate the lacZ gene, thereby irreversibly staining the Osr1+ cells at the time of injection and all their progeny. Later analysis for the presence of LacZ demonstrated the lineage of Osr1+ at different time points of development. For example (*lower panel*), cells that were Osr1+ at E7.5 (embryonic day 7.5) gave rise to nephrons, interstitial cells, and also renal vasculature, while cells that were Osr1+ at E11.5 had a more limited potential, giving rise only to nephrons (Adapted from [28])

ality of a single cell. In the latter case, any single cell has the potential to differentiate into several cell types. In the first scenario, however, the "multipotent" population might be composed of several subpopulations with a more restricted potential, such that the population as a whole can produce all types of differentiated cells. Another option is that only a certain fraction of the population of cells is truly multipotent. In any case, in order to show that a specific cell is indeed multipotent, the *clonal* nature of this property must be demonstrated [27]. In other words, a single cell must be shown to posses the ability to form the entire plethora of cells. This principle is true both in vitro and in vivo (Fig. 9.4).

In the study discussed previously, low doses of tamoxifen were administered in order to label single Six2+ cells and prove their multipotentiality in a clonal manner [29].

After demonstrating multipotentiality, it is prudent to prove self-renewal capacity as well for the cells to be defined as stem cells.

One technique to prove self-renewal in vivo, which was applied by McMahon and colleagues, includes two steps [29]. The first is demonstration that the alleged stem cell population

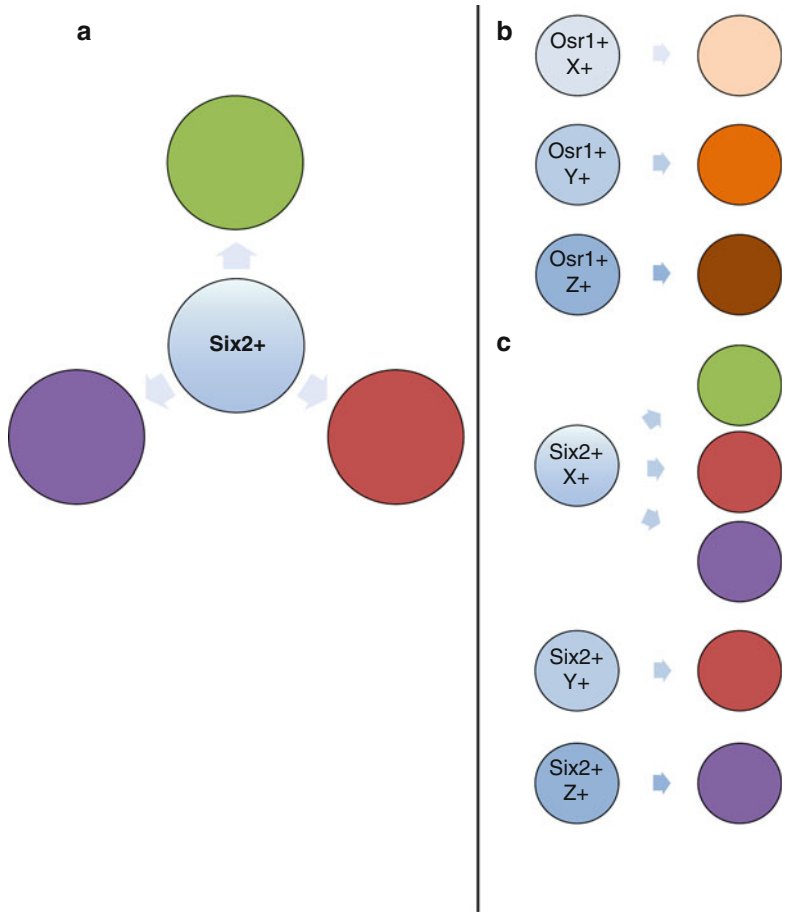

Fig.9.4 Multipotentiality: (**a**) Multipotentiality at the *clonal* level. In other words, *any* single cell (Six2+ cell) can give rise to several types of more differentiated cells. (**b**) Multipotentiality at the *population* level. The Osr1 population as a whole can give rise to three types of more differentiated cells, as three distinct subpopulations (X+, Y+, and Z+) within the Osr1+ cells can each give rise to one type of cell. A colony derived from a single Osr1+ cell will therefore not contain three types of cells, but only one. (**c**) A third scenario in which the Six2+ population is multipotent but at the clonal level, only a specific subpopulation within this pool of cells is multipotent (Six2+X+)

remains in the same undifferentiated state during a period of time. This was shown by using a construct whereby GFP expression was driven by the Six2 promoter, such that all cells that are Six2+ are also GFP+. Indeed, the Six2+ population not only remained intact but actually significantly grew in size during development. This is not enough to prove self-renewal, because Six2- cells could have become Six2+ cells during time; in this case, the increase in cell number would have been secondary to "extrinsic sources," not self-renewal. Therefore, a second step included irreversible marking of Six2+ cells at an early stage and inspection at a later stage. In case self-renewal is indeed the mechanism involved, the population should remain positively stained. If "extrinsic" cells are involved, then the original stained population will become diluted. This experiment showed that Six2+ cells are indeed self-renewing cells and, combined with their multipotent nature, define them as the epithelial stem cells of the kidney. It is important to note, however, that the option that only a fraction of the Six2+ population is a true stem cell population is a valid one.

Several important points must be considered when interpreting such studies regarding the role of different factors, such as Six2 or Osr1, in development.

The first is the difference between a marker and a functional factor.

Whenever confronted with a certain factor active within a population of cells, one must ask oneself whether the factor in question (e.g., Six2) has a direct role in providing the cell with the property in question (e.g., self-renewal) or is it only a *marker* for such cells. In other words, is Six2 the cause for the self-renewal capability, or is it just a bystander, marking this specific phenotype?

The simplest and most common way to answer this question is to deprive the cells of the factor and see whether or not the property is lost. Nonetheless, as previously mentioned, development in general, and kidney development in particular, consists of many interacting cells and tissues, and thus interpretation of findings can be quite challenging.

For example, Sall1 is a transcription factor expressed in the CM, whose KO phenotype includes kidney agenesis [30]. When Sall1+ cells were isolated from mice and cultured, they demonstrated multipotentiality and the ability to form colonies (an important property of stem cells), whereas Sall1- cells were devoid of these properties [31]. However, when the same Sall1+ cells (these were actually cells that had the Sall1 promoter active) were isolated from Sall1 KO mice, they had the same properties, although they did not express Sall1. In this case, Sall1 was only a *marker* of the multipotent population, but not the *reason* for this property.

In contrast, when Six2 expression is lost, the induction of the CM occurs prematurely, the CM population is quickly exhausted, and only few, ectopic tubules are formed [32]. Therefore, Six2 acts cell autonomously to regulate self-renewal.

The second point worth to keep in mind when interpreting studies regarding fetal development is that the vast majority of them used animal models to explore the role of different genes. Therefore, most of the information we have regarding molecular aspects of kidney development (this is true for other organs as well) is derived from nonhuman organisms. Sall1 is a good example of this issue. Mutations in the human SALL1 have been associated with the Townes-Brocks syndrome, an autosomal dominant disease with features of dysplastic ears, preaxial polydactyly, imperforate anus, and, less commonly, kidney and heart anomalies [30]. In contrast, Sall1 deficient mice exhibit kidney agenesis or severe dysgenesis, but not other phenotypes seen in humans. One explanation for this discrepancy is that human mutations result in truncated SALL1 proteins that function in a dominant-negative manner, which leads to more severe phenotypes [30]. In either case, this exemplifies the need to carefully interpret the information retrieved from nonhuman models.

To conclude this part, the CM contains Six2+ stem cells, which are both multipotent and self-renewing, Six2 being responsible for the latter trait. These cells express a wide array

of transcription factors, contributing to their function and defining them as the stem cells of the nephron [16].

The UB Induces the CM (or the Birth of a Nephron)

We saw in the previous section that the UB tip is surrounded by the condensed CM, which contains the stem cells of the nephron.

It is at this stage that the UB tip secretes various factors that cause the CM to condense into a ball of cells, the renal vesicle (RV). This change is induced mainly by members of the Wnt family of proteins [33], which participates in various developmental processes and pathways [34].

According to the simplest model [35], Wnt9b is secreted from the UB and upon contact with CM cells leads to downregulation of Six2 and Wnt4 secretion, acting in an autocrine fashion on CM cells, pushing them to undergo MET.

The switch between mesenchymal and epithelial phenotypes is a cardinal process during both development of many organs and disease states (e.g., EMT-accompanying cancer metastasis) [36].

The signaling produced by Wnt proteins is transmitted via the canonical Wnt pathway, which involves the intracellular action of β-catenin [37] (although a recent study challenges this view [38]). Interestingly, later differentiation of the nephron also involves the Wnt pathway but mostly the noncanonical pathways, highlighting once again the great complexity of development, in which the same factor can act via different pathways and even have several roles in different stages [37].

It appears that here as well, precise molecular interactions govern "decision making." Studies in an organ culture model have demonstrated that Wnt4 alone (without Wnt9b) is sufficient to induce MM cells to undergo MET [33]. In addition, it was shown that cell to cell contact has an important role as well in the communication between the cells involved in the MET. For instance, when Wnt4-expressing cells were physically separated from the un-induced MM by filters (that allowed the secreted Wnt4 to arrive to the target MM tissue), induction did not happen and the MM degenerated [33]. This underscores the importance of cell–cell contact and not only soluble factors in development.

In general, Wnt9b provides an inductive signal for differentiation, while Six2 acts to inhibit this process. Therefore, mice lacking Six2 expression demonstrate premature and ectopic RV formation, while Wnt9b KO mice are unable to form renal vesicles [29]. Interestingly when mice express neither Six2 nor Wnt9b, again no induction occurs, supporting the model described [29]. However, these mice also demonstrate reduced survival of the CM, suggesting that wnt9b might also act to promote survival and proliferation of this population, not only its differentiation. One possible explanation is that it is again the fine balance of factor levels that determines their effect, such that low wnt9b levels promote proliferation while higher levels induce differentiation [29].

Moreover, various other factors have been implicated in determining the proliferation versus differentiation of CM cells. To name a few, BMP7 [39] and TIMP2 (tissue inhibitor of metallo-proteinases) [40] promote survival, while LIF (leukemia inhibitory factor) [41] and TGF (transforming growth factor 2) [42] have been shown to induce differentiation.

To conclude, developmental decisions are determined mainly by the milieu in which stem cells reside. This includes both secreted factors and neighboring cells.

This complex microenvironment represents the stem cell *niche*.

When considering the great complexity of the niche in vivo, it is not surprising that to date, no one has been able to successfully culture CM while maintaining its multipotent nature.

Upon induction, Six2+ stem cells become Wnt4+ RV cells, which begin to undergo a series of changes, giving rise to several successive structures, including the pretubular aggregate, C-shaped body (CSB), S-shaped body (SSB), and ultimately the fully differentiated nephron [16] (Fig. 9.2).

Segmentation of the Nephron (or a Final Touch)

The adult human nephron is composed of at least 14 cell types [43], divided into several segments, including the renal corpuscle, proximal tubule, loop of Henle, distal tubule (all derivatives of the CM), and collecting duct (derivative of the UB). In addition, these segments are positioned in specific locations within the three-dimensional architecture of the kidney.

Therefore, the final stage of kidney development is the segmentation into the various parts of the nephron.

Although morphologically indistinct, when examining gene expression in the RV stage, several "molecular" segments, representative of the future "anatomical" segments, can already be noted. For example, Wt1 is expressed in cells that are the precursors of the proximal segments of the nephron and later on becomes restricted to podocytes, including in the adult human [44].

This is yet another example of how differential gene expression in different groups of cells causes them to differentiate along different developmental pathways.

One important pathway active in the patterning of the proximal parts of the nephron is the Notch pathway, which participates in the development of various tissues, such as blood vessels [45] and the nervous system [46].

This pathway is unique in that it requires cell to cell contact for ligand-receptor binding and signal transduction, thus permitting neighboring cells to directly "communicate" with one other. Among the various members of this family, Notch2 is especially important in determining the proximal fate [47]. The kidneys of Notch2-null mice develop normally until the RV stage, but afterward, no proximal cells types develop [47]. According to this model, in order to "manufacture" proximal cell types, Six2+ kidney stem cells first undergo Wnt4-induced MET and then express Notch2 which pushes them toward the proximal fate. However, when an elegant Cre-lox-based experiment was used to express Notch2 prematurely in Six2+ kidney stem cells, the phenotype was very similar to that of Six2 deficient mice and did not include overproduction of proximal cell types [48].

One possible explanation for this phenotype is that rather than dictating a proximal fate, Notch2 acts to stabilize the proximal phenotype by inhibiting, directly and/or indirectly, Six2 expression [48].

Other Stem Cells in Kidney Development

Thus far, we have limited our discussion to the development of the functional units of the kidney, the nephrons. However, a fully developed and functional organ requires the formation of various types of cells and structures, including vasculature to supply blood necessary for both organ function and for the filtration process, interstitial cells to provide structural support, both directly and indirectly (e.g., by producing the ECM), and resident macrophages (e.g., renal macrophages).

We will present the development of the renal vasculature as an example of the interplay of different cell types, acting in concert to facilitate the formation of an entire organ.

An integral part of the glomerulus is the capillary tuft, providing blood that is filtered into Bowman's capsule, establishing the initial ultrafiltrate. Upon the creation of the comma-shaped body, a "vascular cleft" is formed, marking the future site into which endothelial progenitors or angioblasts will arrive (the origin of renal angioblasts is not yet known and is thought to be either extrarenal or the MM [35]), where they will form the vascular part of the renal corpuscle.

How do these progenitors arrive at the right place? The main factor active here is probably the vascular endothelial growth factor (Vegfa), which acts as an angioblast-attracting agent [35]. Indeed, developing podocytes were shown to express this factor, which attracts angioblasts expressing the Vegfa receptor, causing them to enter the vascular cleft, aggregate into precapillary cords, establish a lumen, and become the foundation of the evolving glomerular vessels.

Endothelial cells alone are insufficient to form stable and functioning vessels, and perivascular support cells are necessary as well. Therefore, endothelial cells express Pdgfb (platelet-derived growth factor b) that attracts Pdgf receptor-expressing mesangial cells, which position themselves at a perivascular niche to provide structural support for the newly formed capillaries [49]. Eventually, capillaries further develop and become the complex network seen in the mature glomerulus.

The Search for Fetal Kidney Stem Cells

Several seminal studies [29, 32, 43] have demonstrated that the fetal kidney indeed harbors multipotent stem cells. The existence of such fetal stem cells has been established in various other organs as well (e.g., the pancreas [50], heart [51], and intestine [52]).

However, several important obstacles stand in the way of those wishing to harness these cells in favor of therapeutic applications.

The first obstacle is the ethical, political, and religious problems surrounding the use of cells derived from fetuses.

The second obstacle is the need to develop methods to successfully isolate the population of relevant stem cells from within the developing fetus. Notably, for some purposes, progenitors may also suffice as a therapeutic tool, as certain pathologies might be limited to only a specific cell type. For example, podocyte loss seen in many glomerular diseases, such as focal segmental glomerulosclerosis [16].

Upon the isolation of the desired cells, a third challenge arises, namely, the need to develop ex-vivo culture conditions to facilitate expansion of the cells into a clinically relevant number. This need is underscored by the limited number of cells which often characterizes the isolation of stem cell populations [16].

Three main approaches have emerged for the purpose of using stem/progenitor cells from human fetal kidney sources [16], which can, in principle, be applied to other organs as well:

Transplantation of Whole Fetal Organs or Fetal Tissue Including of Human Origin

For instance, transplants of whole early human, porcine, and murine embryonic kidneys (tissue transplantation) obtained at early and specific developmental stages and comprised predominantly of nephrogenic zone components can mature into a functional miniature kidney, emphasizing the differentiative potential of embryonic renal progenitors [53, 54]. As nephrogenesis progresses, the relative proportion of the nephrogenic cortex in comparison to the mature cell types decreases but nonetheless, as stem cells are present within the embryonic kidney until relatively late in gestation, this can be exploited for their isolation.

This approach requires a method to precisely define, isolate, and expand the cells in vitro in a way that will allow them to retain their full developmental potential and enhance their regenerative capacities.

Heterogeneous Fetal Cells

Encouraging results regarding the use of cells from developing kidneys came from a report demonstrating that transplantation of a heterogeneous population of dissociated E14.5, and E17.5 rat fetal kidney cells under the kidney capsule leads to formation of renal structures and improves kidney function in a rodent model of kidney injury [55]. In addition, similarly to whole organ transplants [54], the gestational age of the transplanted cells has to be chosen carefully, as early fetal kidney (E14.5) cells differentiated to nonrenal tissues (e.g., bone and cartilage), whereas older cells were unable to form renal structures.

One of the perquisites for using fetal cells is the ability to expand them ex vivo so as to obtain enough cells for transplant while at the same time maintaining their cellular characteristics, which is probably not an easy task. For example, rat FK cells were able to reconstitute kidney tissues upon injection into mice, only when cultured through

passage one as "older" cells experienced proliferation arrest and apoptosis, with concomitant loss of the regenerative potential [56]. This underscores the importance of finding the exact culture conditions to enable in vitro expansion.

Isolated Populations of Fetal Kidney Stem/Progenitor Cells

The ultimate goal of those interested in using fetal cells for transplantation is to establish methods for the *prospective* isolation of defined populations of stem cells.

Several possible methods have emerged for this purpose:

Isolation via Surface Marker Expression: We have discussed some of the major transcription factors active in kidney stem cells. However, the isolation via sorting of such cells requires that we first define those markers that characterize them. Indeed, hampering the identification of stem/progenitor cells in the developing kidney is that as opposed to other organs, specific surface markers have yet to be identified [16]. How can we find such markers?

One approach to marker identification is the use of global gene expression analysis which in the case of the murine embryonic kidney has identified CD24a and cadherin11 as MM surface markers [57].

Work previously performed in our lab analyzed the developing human kidney alongside the pediatric malignancy Wilms' tumor (WT) using microarrays [58]. WT arises from multipotent renal embryonic precursors that undergo a partial differentiation arrest and therefore contains both undifferentiated elements (blastema) and differentiated elements (epithelial and stromal) [59]. Serial transplantations of WT cells into mice lead to expansion of the progenitor blastema at the expanse of differentiated elements, creating WT stem-like xenografts [60]. The latter contain mostly transformed progenitors, thereby combining the properties of both kidney stem cells and tumor cells. By comparing genes with a similar expression pattern in fetal kidney tissue (containing progenitors but also other fetal cell populations) and the WT xenografts (containing progenitors but also other adult cell populations), we identified an overlapping renal "stemness" signature set [61]. This set included a large number of genes from different groups, including renal "progenitor" genes (*PAX2*, *EYA1*, *WT1*, *SIX1*, *SALL1*, *and CITED1*), Hox genes, Wnt pathway, and polycomb group genes and a limited number of surface markers (NCAM1, PSA-NCAM, FZD7, FZD2, DLK1, ACVRIIb, and NTRK2).

The next step was to individually test these surface markers in order to see whether one or more of them can select for the stem cell population.

For this purpose, we defined a set of parameters that should characterize a marker of fetal stem cells [62].

The first is that it should localize to and ideally only to the primitive, undifferentiated structures within the developing organ, which harbor the stem cell population, not to the differentiated ones. Pinpointing these structures can be done by several methods, depending on the organ of interest.

One way is histological identification of the primitive structures. The kidney develops in such a way that histological sections reveal a "differentiation gradient," with the most primitive structures (MM and/or its early nephron derivatives) located at the outer pars and the mature, differentiated ones (formed tubules) at the innermost parts. In addition, the different structures (MM- , C- and S-shaped bodies, differentiated nephrons) are morphologically distinct, allowing identification upon histological inspection.

When assessing areas of expression of the different putative renal progenitor markers that were found in the microarray analysis, we expected the true markers to localize to and ideally only to the primitive structures.

Indeed, NCAM (CD56) as a potential marker of renal progenitors localized to these structures but also to the kidney stroma (which does not contain stem cells of the nephron). Therefore, we had to find a way to isolate the relevant subpopulation out of the entire NCAM+ pool [62].

To our help came the fact that in organs which undergo MET to produce differentiated cells, such as the kidney, the putative marker is expected to

localize to cells of a mesenchymal phenotype. We therefore analyzed the expression of putative markers, including NCAM, with respect to the expression of EpCAM, a surface marker that is upregulated along renal epithelial differentiation [63].

The second way, therefore, for locating the primitive structures, and for choosing a stem cell marker, is to look within the EpCAM– cell fraction, which represents undifferentiated populations that have yet to undergo or undergoing MET.

The third parameter we demanded from the marker is downregulation in the adult kidney, as the kidney stem cell population is thought to be completely exhausted before birth.

The fourth and last parameter is that cells expressing the marker should overexpress the renal "progenitor" genes.

Most of the previously mentioned microarray-predicted markers qualified these definitions, at least in part, highlighting NCAM+ EpCAM- and NCAM+EpCAM+ cells as putative stem/progenitors and representing a framework that can be used to derive human renal stem/progenitors cells from fetal kidney, to be used in the future for regenerative purposes [62].

The general approach described above for selection of cell surface marker for stem cell isolation is summarized in Fig. 9.5.

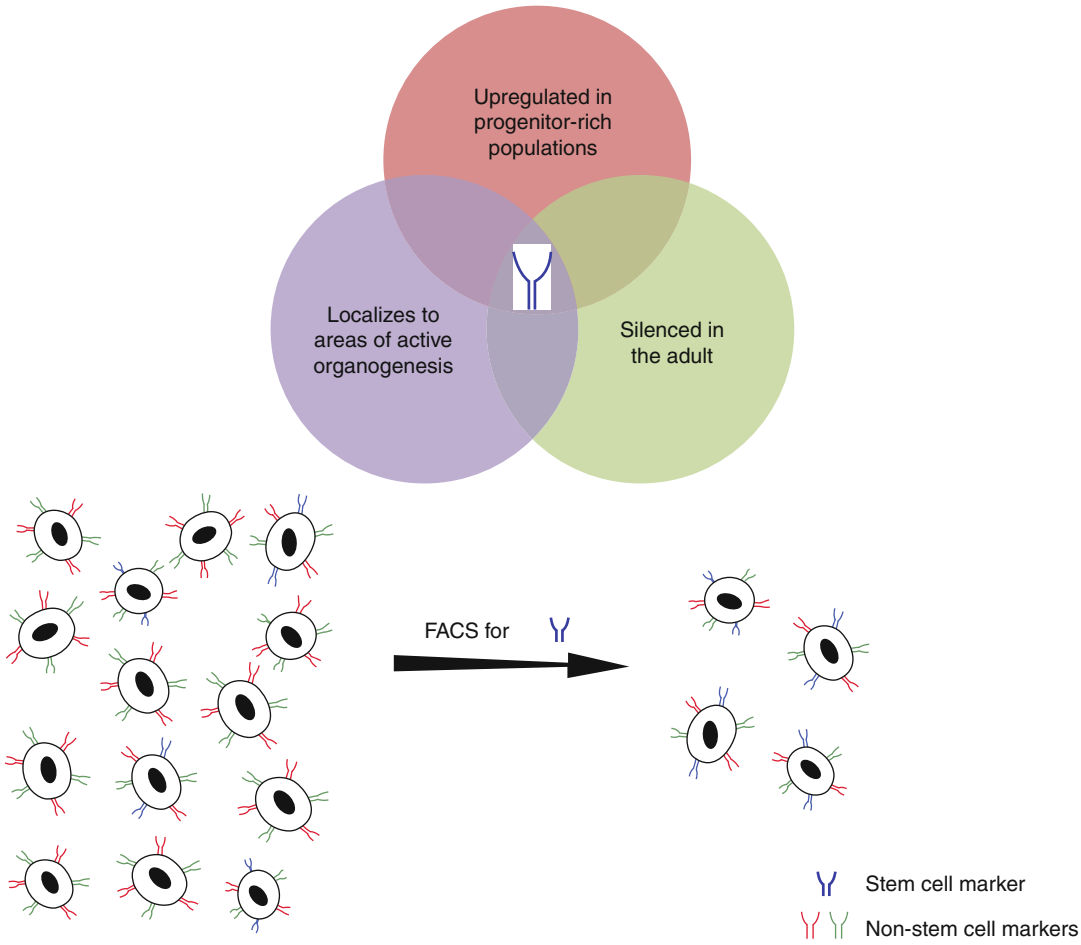

Fig. 9.5 A Strategy for the identification of fetal stem cell surface markers: The ideal marker should be upregulated in cell populations which relatively enriched for progenitors/stem cells (such as fetal organs), be located in primitive/undifferentiated structures within the developing organ and undergo downregulation in adult tissues. The markers established via this strategy can then serve for the isolation (e.g., by FACS) of stem cells from fetal tissues. *FACS* fluorescence-activated cell sorting (Modified from [16])

Interestingly, "universal" stem cells markers such as CD133 and CD24 (not equivalent to the murine CD24a [57]) previously reported to specify renal progenitor cells in both embryonic and adult kidney, mostly appeared as markers for identification of differentiated renal epithelia among human fetal kidney cells, and therefore, their combination will not enrich for a progenitor phenotype. Similarly, another hematopoietic stem cell marker CD34 actually identified the entire endothelial cell network in the developing kidney [62].

Clonogenic Assays: An alternative to the initial step of stem cell isolation via sorting according to specific surface markers is to take advantage of the fact that stem cells are highly clonogenic [16]. This approach which starts from heterogeneous and not enriched cell populations requires assay systems that allow analysis of a single-cell culture, as in the cases of the neurosphere method for neural stem cells and the colony assay for hematopoietic progenitors [35]. Accordingly, as described earlier, Osafune et al. [31] set up an assay using *Wnt4* as an inductive signal, which could identify and characterize the progenitor cells with multipotent differentiation potential from un-induced MM and potentially cells with progenitor characteristics from other sources as well. As discussed previously, they found that only cells strongly expressing *Sall1*, isolated from *Sall1-GFP* mice, formed colonies and that they partially reconstituted a three-dimensional kidney structure, which contains glomeruli- and tubule-like components in an organ culture setting [31].

Genetic Tagging: As previously described, intensive research has come up with several cardinal factors defining the kidney stem cells, including Six2, Cited1, Osr1, Pax2, and others [16]. Therefore, if we could in some way isolate this population, we would have obtained the precursor of the nephron and possibly use it for therapeutic applications. Two major problems currently limit this approach, one concerning the isolation of the cells and the other concerning the in vitro expansion into a clinically relevant amount.

In order to select only the cells expressing the relevant "stemness genes" from the whole heterogeneous pool of cells, we need to manipulate the cells in such a way that the desired ones would "stand out," for example, by expressing a fluorescent protein. One hypothetical way to accomplish this goal is to couple a marker gene to the promoter of the gene of interest and sort out the cells expressing the marker (Fig. 9.6). Safety considerations are highly important to consider in this hypothetical scenario, as genetic manipulations performed on the cultured cells could affect their desired phenotype or, even worse, expose them to the danger of malignant transformation.

Pitfalls and Misinterpretations

Putative stem cells isolated from fetal tissues, the kidney included, need to be tested strictly to ensure that the retrieved cell type is indeed a stem cell and that it is capable of differentiating along the desired lineages.

In this aspect, several pitfalls that must not be overlooked when confronted with a new population of alleged stem cells [16] (Fig. 9.7) are the following:

Isolation of a Resident Progenitor Rather Than an Intrinsic Cell Type

Resident progenitors are defined as cells that do not originate from the within the relevant organ (e.g., the MM in the kidney) and localize to areas outside of the organ parenchyma, such as bone marrow-derived cells [16]. Resident progenitors are less likely to be relevant to organ regeneration, as they most probably cannot differentiate into parenchymal cells, although they sometimes have a therapeutic potential nonetheless, mostly via a paracrine mechanism (i.e., the secretion of various growth factors) [64].

An example for such a putative resident progenitor population is the renal MSCs (multipotent mesenchymal stromal cells).

MSCs, once hypothesized to be responsible for the homeostasis of adult mesenchymal tissues [65], are now considered a subpopulation of perivascular cells (or pericytes), residing in

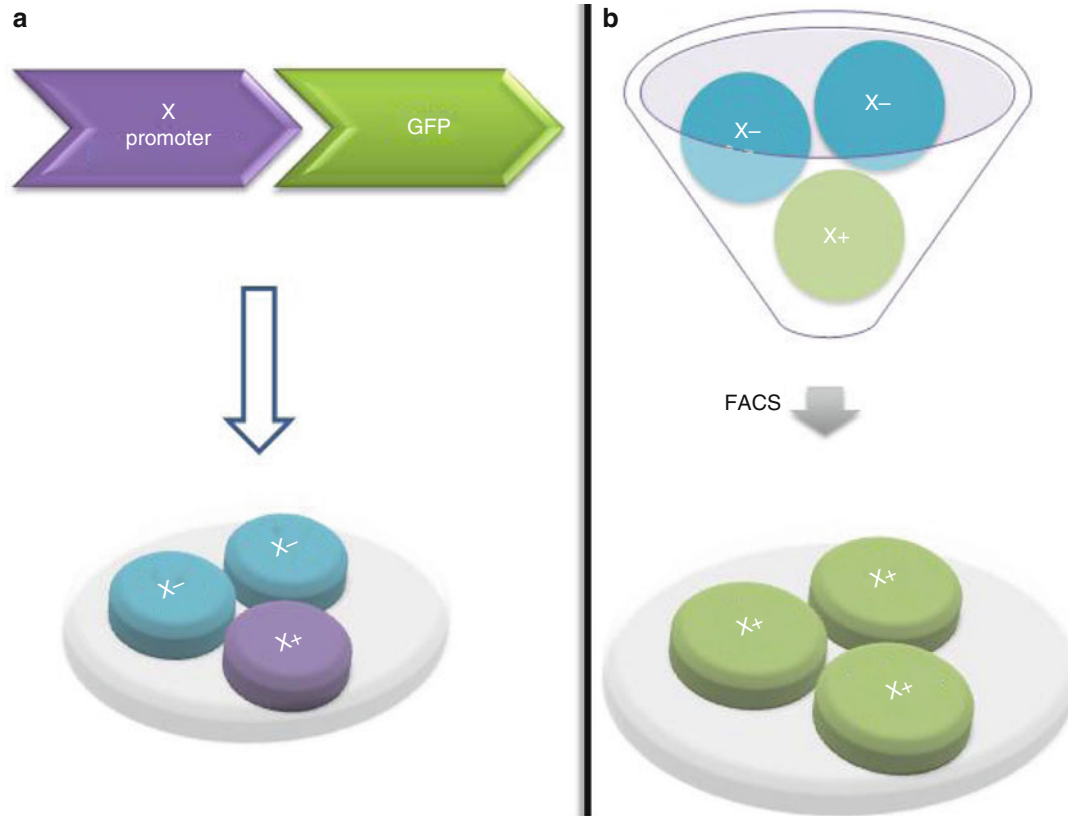

Fig. 9.6 In vitro isolation of stem cells from fetal tissues via genetic tagging: (**a**) Cells retrieved from fetal tissue or organs and grown in culture contain both stem cells, expressing the gene X (X+ cells) and non-stem cells, not expressing this gene (X− cells). The cells are transduced with a construct in which the promoter of X drives the expression of the fluorescent protein GFP, such that all X+ cells express GFP (represented by the transition from *violet* to *green*). (**b**) The cultured cells are then sorted via FACS, allowing isolation of only the GFP+ cells (*green* cells), which are also X+ cells or stem cells. *GFP* green fluorescent protein, *FACS* fluorescence-activated cell sorting

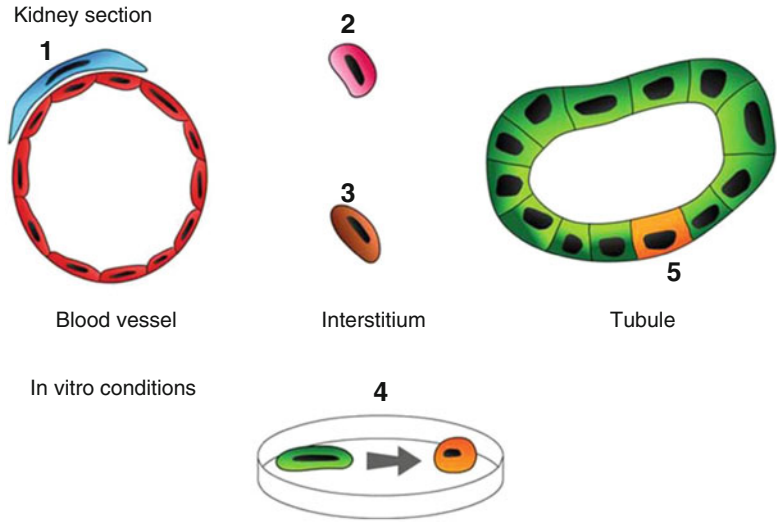

Fig. 9.7 Possible pitfalls in the isolation of stem/progenitor cells from fetal kidneys: (*1* and *2*) Isolation of resident progenitors, for example, kidney MSCs (*1*, *blue*) or hemato-vascular progenitors (*2*, *pink*). (*3*) Isolation of a stromal progenitor cell (*brown*). (*4*) Isolation of a fully differentiated cell type (*green*) that acquires some progenitor properties upon in vitro culturing (demonstrated by the transition in the culture dish into an orange cell type). (*5*) Isolation of tubular progenitors with a more restricted potential (*orange*) (Modified from [16])

virtually every tissue, including fetal tissues [66–68] so that their misinterpretation as stem cells is relevant to practically every organ.

Isolation of Intrinsic Stromal Progenitors

As previously discussed, the developing kidney contains at least two specific progenitor populations [17], that is, the Six2+ nephron progenitors and the Foxd1+ stromal progenitors, which represent mutually exclusive progenitor compartments.

Cells from the latter population should be relatively easy to clone, passage, expand, and differentiate along mesoderm lineages in adhesive cultures, similarly to other stromal populations. Importantly, the Foxd1 stromal population does not give rise to nephron epithelia [69] and lacks nephrogenic potential.

Isolating a Fully Differentiated Cell Type with Some "Stem/Progenitor" Properties

When evaluating newly discovered populations of alleged stem cells from fetal tissues, it is worth remembering that in certain cases, differentiated cells can also present ex-vivo characteristic of stem cells. This is supported by several facts.

First, differentiated epithelial cell types, even when isolated from adult organisms, have been shown to possess clonogenic and self-renewal capabilities, leading to their possible misinterpretation as stem cells/progenitors [70].

Second, ex-vivo growth conditions of cells may result in a nonspecific phenotypic switch of differentiated epithelial cells during epithelial-mesenchymal transition (EMT). Although these cells may show enhanced proliferation and migration and appear in a progenitor state, their nature is mostly fibroblast-/mesenchymal-like, lacking functional relevance [36].

A third reason for this possible misinterpretation is the use of surface markers or functional parameters for isolation that overlap with those of differentiated cell types or that actually mark only differentiated cells. Examples of such cell markers include "universal" stem cell markers such as CD133, CD24, Sca-1, and c-Kit, which have all been shown to be heavily expressed in differentiated epithelia, including renal epithelia [62, 71–75]. Examples of overlapping functional parameters are those used for HSC isolation (label retention and dye efflux capacity) that do not discriminate between progenitors and differentiated cells in other organs [76].

Fourth, the lack of appropriate controls for an alleged progenitor cell fraction can also lead to confusion. Analysis of expression levels of pluripotency or renal developmental markers, clonogenicity, multipotentiality, and in vitro and in vivo differentiation potential in a specific cell type is irrelevant if not compared with a cell not expressing the alleged progenitor phenotype, demonstrating advantageous properties or function [16].

The potential to differentiate into functional cells of the organ should be inherent to the biology of a tissue-specific stem cell. Clearly, this requires a robust in vitro assay to analyze differentiation potential at the single-cell level (as achieved by limiting dilution), as opposed to the often performed mesenchymal tri-lineage (adipocytes, chondrocytes, and osteoblasts) differentiation assay relevant to MSCs (but not to progenitors of different organs) [16]. The kidney is an excellent example for an organ which despite many attempts, lacks such an assay, and thus, there is no definite way to determine the nephrogenic potential of isolated fetal cells.

For such an assay, developmental cues driving nephrogenesis, as stated earlier, are likely to be relevant.

In addition, although in vivo renal potential of isolated fetal cells can be studied in models of renal damage (acute and chronic) or preferably in models of metanephric development in which the microenvironment can support, at least in part, differentiation, one must exclude cell fusion to establish unequivocal renal potential [16].

Isolation of "Partial" Progenitors

Nephrogenesis continues until the 34th week of gestation [17], such that areas in various developmental stages are seen within the fetal kidney. When considering this fact, it becomes evident that different populations with a more restricted differentiation potential than the CM (e.g., a progenitor cell type for proximal tubular cells) probably exist within the fetal kidney and this should be taken into account when attempting to isolate stem cells from fetal tissues.

Conclusion

Although fetal organs and tissues represent an excellent source for stem/progenitor cells as all of them harbor such cells at certain stages of development, there are still many challenges left to overcome in the isolation, expansion, and successful administration of these cells.

We have presented the kidney as a model organ for different aspects of the biology of stem cells in fetal organs, including the different ways cells interact with one another and with their niche and how this interaction actually enables decision making at the cellular and molecular level, culminating in the establishment of an entire living organism.

We discussed several ways in which stem cells can be isolated from fetal tissues, including a general strategy to allow for identification of relevant surface markers in organs in which such markers are not yet available.

These are exciting times in the field of stem cell research. In particular, stem cells derived from fetal tissues hold great promise and will hopefully become a source for starting material for cell therapy that will enable clinicians to treat different diseases and illnesses.

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10 Potential Therapeutic Applications of Placental-Derived Stem Cells to Combat Tissue Inflammation and Fibrosis

Yuben Moodley and Ursula Manuelpillai

Introduction

Lung and liver disease form a major cause of global morbidity and mortality. There is significant overlap in the prevalence of injuries that cause both liver and lung conditions. These include infections, toxins, and genetic factors. The critical step that characterizes major lung and liver disease is progressive and chronic injury. Notably, however, there are no effective treatments to address the ongoing damage to these organs. To this end, there is a suggestion that cellular therapy may indeed assist in replacing damaged cells, dampening inflammation, and thereby reduce the scarring or fibrosis. The plasticity, low immunogenicity, and immunomodulatory properties of placental-derived stem cells may offer an important option for the treatment of these diseases, and studies investigating this avenue are outlined in this chapter.

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Placental-Derived Stem Cells

The placenta is a transient organ that is essential for the growth and development of the fetus providing the required nutrients and carrying out gas and waste exchanges. It is composed of several discrete compartments: the villous placenta, the fetal membranes – amnion and chorion, and the umbilical cord. These compartments arise very early in gestation from fetal-derived cells and are known to be rich sources of stem cells. Mesenchymal stem (stromal) cells (MSC) are readily isolated from the stromal-fibroblast layers of the villous placenta, chorion, amnion, and the Wharton's jelly (WJ), a thick mucopolysaccharide layer which supports and encases the umbilical cord blood (UCB) vessels (Fig. 10.1). Hematopoietic stem and progenitor cells and MSC are routinely harvested from UCB and banked following birth for subsequent autologous use [1]. Studies have also shown that human amnion epithelial cells (hAEC), which form a monolayer lining the amnion membrane, display properties of pluripotent and multipotent stem cells [2, 3]. In addition to placental tissue-derived stem cells, the amniotic fluid (AF), a complex mixture of electrolytes, urea, proteins, carbohydrates, and lipids derived from fetal skin exudates and urine, contains a population of stromal cells that have been ascribed with pluripotential properties [4, 5].

In comparison with human embryonic and adult tissue-derived stem cells, the placenta offers several advantages as a source of cells for

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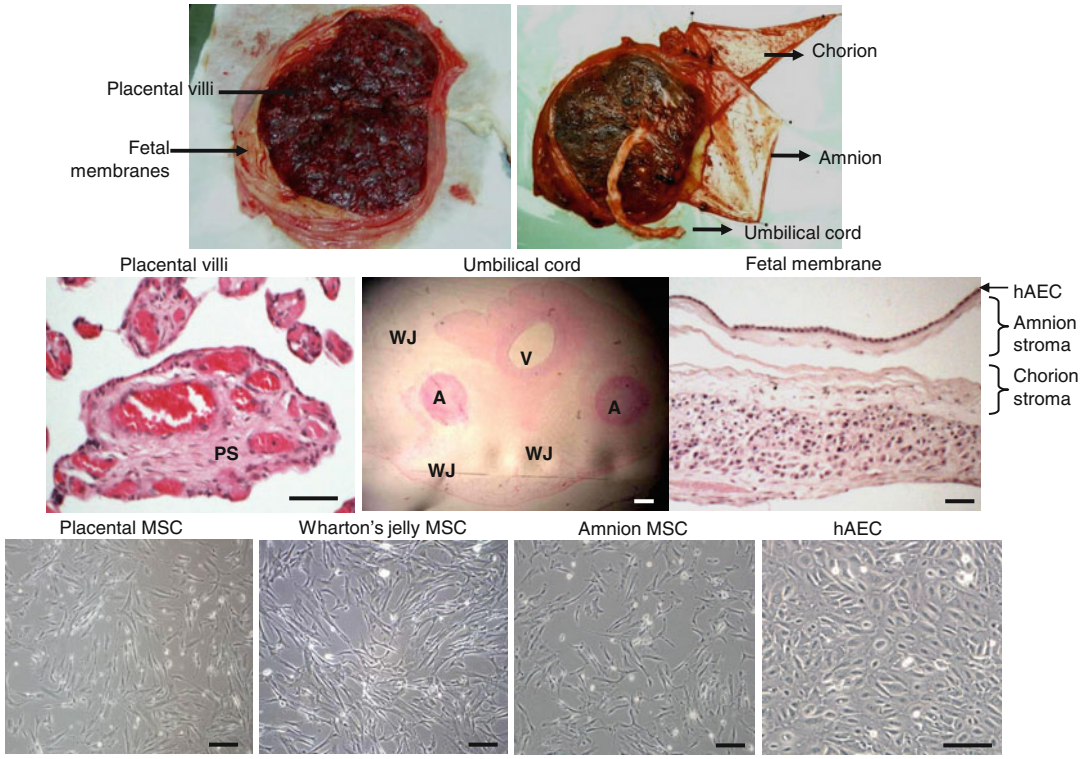

Fig. 10.1 Placental stem cell populations. The maternal and fetal surfaces of the placenta are shown in the *upper panel*. Histological sections of chorionic villi, umbilical cord, and fetal membranes depicting regions harboring stem cells (*middle panel*). *PS* placental stroma, *A and V* artery and vein of umbilical cord, respectively, *WJ* Wharton's jelly of umbilical cord. Cultured mesenchymal stem cells (*MSC*) and amnion epithelial cells (*hAEC*) derived from the placenta are shown in the *lower panels*. Magnification bars = 100 μm

potential therapeutic applications. Spontaneously delivered term placental tissues are unlimited, obtained without costly invasive procedures, and have wide legal, ethical, and community acceptance for the isolation of stem cells and their therapeutic applications. However, stem cells isolated from placentae obtained from women with the pregnancy complication preeclampsia are reported to have altered properties possibly due the changes in placental physiology and/or prolonged exposure to hypoxemia [6]. Preeclampsia affects between 5 and 12 % of pregnancies and is more common in first pregnancies. Similarly, placental stem cells isolated from pregnancies with gestational diabetes and intrauterine growth restriction may have altered properties. The incidence of intrauterine growth restriction and gestational diabetes is 4–7 and 3–10 % of pregnancies, respectively. Due to the risk of harboring infection and as prolonged exposure to inflammation may alter their properties [7], placental stem cells from pregnancies with chronic systemic maternal and localized amniotic fluid infections are also unlikely to be suitable for therapeutic usage.

Properties of Placental-Derived MSC

Stem cells from adult bone marrow (BM) are among the best studied group of stem cells. Their extensive characterization and numerous preclinical studies have led to the testing of BM-MSC for the treatment of a plethora of diseases in autologous and also allogeneic settings in many phase I–III clinical trials worldwide (www.cell-medicine.com). MSC and fibroblasts share many phenotypic similarities expressing cluster of differentiation (CD) antigens and cytoskeletal proteins such as vimentin and actin [8, 9]. Differences in fibroblast specific protein-1 and CD146

between MSC and fibroblasts [9] and changes in CD106 and CD9 expression with cell expansion have recently been noted [10]. However, the International Society for Cellular Therapy was prompted to establish the following minimal criteria for the isolation and characterization of MSC [11]. MSC must:

- Adhere on to plastic culture dishes.
- Express CD73, CD90, and CD105.
- Not express the hematopoietic markers CD14, CD34, and CD45.
- Not express HLA-DR and costimulatory molecules CD40, CD80 and CD86, and CD11b, CD19, and CD79a.
- Differentiate into osteocytes, adipocytes, and chondrocytes in vitro.

Since BM aspiration is invasive, painful, costly, and giving low yields and as cell quality and number decline with age, placental MSC are increasingly being viewed as an acceptable alternative. Placental-derived MSC generally conform to the above-mentioned criteria, and further comparisons have been made with BM-MSC. No significant differences in CD166, CD105, CD90, CD73, Cd49e, CD44, CD29, and CD13 were found between placental, chorion, amnion, and BM-MSC [12–14], but there was high batch-to-batch variability in the percentages of placental cells expressing these markers compared with BM-MSC [14]. Interestingly, significant differences were observed in the capacity of MSC from the various placental compartments to differentiate into myocytes, chondrocytes, osteocytes, and neural cells [14]. Further, several studies have noted the relatively poor ability of all placental MSC subpopulations to differentiate into adipocytes compared with BM-MSC [12, 15, 16]. Thus, notable differences exist between placental derived and BM-MSC. These differences may be due to differences in transcriptional regulation of lineage specification pathways, epigenetic modifications, and ligand–receptor expression and interactions. Intriguingly, a few recent reports have shown that following careful removal of the maternal decidua adhering onto the surface of the term placenta and subsequent protease digestion of chorionic villi release MSC that are karyotypically *female* from pregnancies with *male* fetuses [12, 17]. This finding suggests that the term villous placenta is a hugely abundant source of *maternally derived MSC*. Contrast studies have shown that MSC isolated from placental villi obtained from early gestation in the first trimester of pregnancy are of fetal origin [18]. Whether term placental villi harbors maternal BM, adipose, or possibly uterine endometrial-/decidual-derived MSC, factors regulating their entry into the placenta, the stage of pregnancy when maternal MSC first enter the placenta, and their role in fetoplacental growth remain uncertain. In contrast, we find that karyotyping of amnion and WJ-MSC from pregnancies with male babies show that these cells have the same gender as the fetus.

A key feature of BM-MSC is their low immunogenicity. Like BM-MSC, the placental cells express MHC class IA antigens HLA-A, HLA-B, and HLA-C; lack class II antigens HLA-DP, HLA-DQ, and HLA-DR; and the costimulatory molecules CD40, CD40L, CD80, and CD86 [19–21]. Thus, these cells lack the determinants that would be presented directly or indirectly via antigen-presenting cells to the recipient's T-cells and suggest that transplantation across histocompatibility barriers into allogeneic recipients without immune suppression is feasible. However, whether antibodies are generated against the MSC and effects of subsequent stem cell transplants possibly arising from different donor placentae needs careful assessment.

A major reason for the wide clinical interest in BM-MSC is their ability to modulate immune cell responses and suppress inflammation. Placental-derived MSC have been found to be even more effective in suppressing mitogen-induced T-cell proliferation compared with BM-MSC possibly due to increased interleukin (IL)-10 and vascular endothelial growth factor (VEGF) release compared with BM-MSC [22]. In addition to these cytokines, IL-6, hepatocyte growth factor (HGF), prostaglandin E2, indoleamine 2,3-dioxygenase, and HLA-G have been shown to play a role in suppressing T-cell activation by placental MSC [23, 24]. MSC also modulate antigen-presenting B and natural killer cell activity [25]. BM-MSC are being tested for the treatment of autoimmune diseases, graft versus host disease, and to combat tissue inflammation and fibrosis.

Properties of hAEC and the Amnion Membrane

hAEC display several similarities to MSC. The cells can effectively suppress mitogen-activated T-cell proliferation [26, 27] and modulate NK, monocyte, and B cell function [26]. These effects are likely to be modulated by secreted factors such as prostaglandin E2, HGF, HLA-G, IL-6, VEGF, monocyte migration inhibitory factor, and also by cell–cell contact [26, 28]. Unlike MSC, IL-10 secretion by hAEC is negligible [29]. hAEC also have low immunogenicity and have a similar MHC classes IA and II expression profile to MSC [14]. Indeed, hAEC have been successfully transplanted into allogeneic recipients [30] and during trials for lysosomal storage diseases without adverse effects [31].

Unlike placental MSC that are derived from extraembryonic cells, hAEC arise from the embryonic epiblast prior to gastrulation and are among the first cells to differentiate in the conceptus [32]. hAEC isolated from amnion membranes from term pregnancy retain some of the pluripotency features of their founder cells with ~10 % of hAEC expressing the transcription factors Oct-4, Sox-2, and Nanog [2]. Expression of these factors is linked to teratoma formation by embryonic stem cells and induced pluripotent cells; however, hAEC have not been shown to form teratomas following transplantation [2, 3]. Although the reasons for the lack of teratoma formation remain unclear, this may be due to suboptimal ratios of Oct-4 and Sox-2 expression and high-level expression of the tumor suppressor gene p53 by hAEC [33]. hAEC have been shown to differentiate into multiple lineages derived from the three primary germ layers, and this plasticity coupled with the lack of tumor formation makes hAEC most attractive for cell therapies [2, 3]. While over 100million hAEC can be harvested from a single amnion membrane [34], potentially several billion cells would be needed even for autologous applications. Unlike MSC, hAEC have been shown to have very limited expansion in vitro and display phenotypic changes described as an epithelial to mesenchymal transition during expansion [35, 36]. While this may pose some limitations, the expanded hAEC have not been widely characterized and may still retain key properties of the primary hAEC.

The amnion membrane (AM) which consists of hAEC and amnion MSC has also been shown to possess significant anti-inflammatory and antifibrotic properties that have led to applications in the treatment of ocular disorders [37, 38]. In a seminal study by Solomon et al., the anti-inflammatory and antifibrotic properties were more clearly delineated. The AM-inhibited production of transforming growth factor (TGF)-β, the prototypical profibrogenic factor from fibroblasts that were cultured on AM, and downregulated granulocyte–macrophage colony-stimulated factor and IL-8 [39]. Furthermore, AM reduced fibroblast proliferation that was induced by activated mast cells. Studies have shown that AM also inhibits the expression of basic fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) that lead to fibroblast proliferation and collagen deposition [40]. Possible mechanisms that explain the anti-inflammatory and antifibrotic phenomenon of AM include the suppression of inflammation by the extracellular matrix (ECM) of the AM. In addition, decorin is found in high concentrations in AM and has been shown to inhibit TGF-β production of fibroblasts [41]. Umbilical cord MSC and the AM are also rich in hyaluronic acid which has been shown to reduce TGF-β when applied to a skin wounds [42]. Hyaluronic acid inhibits the expression of NF-κb and reduces IL-1a, IL-6, and tumor necrosis factor (TNF)-α [43]. Further, secreted substances have been reported to downregulate TGF-β signaling and IL-1a and IL-1b levels [44–46]. Collectively, these findings suggest that factors secreted by placental-derived stem cells could also dampen inflammation and fibrosis in organs such as the lung and liver following cell injection. The mechanisms leading to fibrosis in these organs and effects of placental stem cells are outlined below.

Generic Wound Healing and the Evolution to Fibrosis

Human beings are constantly exposed to various environmental insults that may be life-threatening. Over time, evolution has refined

our immune systems to deal with these challenges. Innate immunity has enabled us to respond quickly to potential pathogens while adaptive immunity has refined these responses and provided us with a memory for specific pathogenic responses. Although the immune responses are diverse, there is a generic pattern of injury and repair that is pertinent to most organ systems.

Acute injury generally leads to hemorrhage and extravasation of plasma into injured tissue thereby activating coagulation pathways, fibrin deposition, and the formation of a provisional matrix which becomes a substrate for further inflammation and repair [47]. Platelets degranulate during this acute phase of injury leading to the release of lipid mediators and cytokines resulting in the activation of leukocytes, endothelial cells, fibroblasts/myofibroblasts, and epithelial cells [47].

There is a complex sequence of events that lead to the ingression of leukocytes into the injured organ, the most important being the activation of leucocytes and endothelium that forms the basis for the leukocyte–endothelial cell adhesion and leukocyte diapedesis, along chemotactic gradients [48]. Neutrophils are the first leukocytes at the site of tissue injury, and they function to remove debris and microorganisms [48]. The next wave of leukocytes consists of mononuclear cells such as macrophages that further release mediators of inflammation including cytokines and growth factors. Granulation tissue formation represents the critical step between acute inflammation and extracellular matrix (ECM) deposition forming the basis for tissue repair and angiogenesis; the latter process ensures a continual supply of nutrients to the tissue [49].

With restitution of the epithelium and endothelium, normal tissue repair occurs, and the organ returns its usual physiological state. However, ongoing and progressive injury to epithelial and endothelial cells and irreversible damage to their basement membranes could lead to chronic inflammation and fibrosis [50]. Failure to reconstitute these cells leads to fibroblast/myofibroblast activation and the deposition of collagen (primarily type I collagen) into the wound and to fibrosis. Cytokines play an important role in mediating inflammation and fibrosis. These include IL-1, IL-2, IL-6, and tumor necrosis factor (TNF)-α. TGF-β, PDGF, and IL-1 promote fibroblast/myofibroblast activation and collagen deposition.

Clinical Consequences of Impaired Repair in Lung Diseases

The clinical consequence of chronic inflammation in the lung is the loss of gas-exchange units that are replaced by fibrotic tissue and lead to the morbidity and mortality associated with chronic lung diseases. A prototypical disease of lung inflammation and fibrosis is acute respiratory distress syndrome (ARDS). ARDS is characterized by refractory hypoxemia in patients with bilateral lung infiltrates in the absence of pulmonary edema [51]. An NIH study estimated the incidence of ARDS to be 75 per 100,000 people in the USA [52]. ARDS may be the end result of common direct injuries to the lung, such as pneumonia and aspiration of gastric contents especially in the elderly, H1N1 infection, pulmonary contusion, inhalational injury, and near drowning. Furthermore, indirect lung injury resulting from sepsis, severe trauma, cardiopulmonary bypass, drug overdose, and acute pancreatitis may also culminate in ARDS [53]. Generic injury to the lung results in damage to epithelial and endothelial cells, resulting in a compromised alveolar–capillary barrier and exudation of fluid into the alveolar space, as well as infiltration of inflammatory cells such as neutrophils. The inflammatory process is driven by cytokines IL-8, TNF-α, and IL-1 and a reduction in IL-1 receptor antagonist, soluble TNF receptor, and IL-10. Progression from acute lung injury to fibrosis may be observed at 5–7 days post injury [54]. There is infiltration of the alveolar space by fibroblasts and myofibroblasts, with fibroblastic foci, increased collagen deposition, and disordered lung architecture. The pathogenesis of fibrosis involves several complex mechanisms [55, 56], and this phase of injury predicts a poorer prognosis [57].

Idiopathic pulmonary fibrosis (IPF) is another major chronic lung disease. The condition most commonly associated with extensive interstitial

loss of lung tissue and has a prevalence of 14–42 per 100,000 in the USA [58]. Risk factors include smoking and family history. The pathogenesis of the condition is shown schematically in Fig. 10.2 and is characterized by ongoing damage to alveolar epithelial cells, progressive formation of fibroblastic foci, and destruction of the lung tissue. There is significant loss of blood vessels and in situ thrombi formation. In addition, there are widespread metaplastic changes of alveoli resulting in the formation of bronchiolar cells lining the alveoli leading to the characteristic honeycomb changes [60].

The clinical consequences of these pathological changes are progressive shortness of breath and a restrictive lung function test with a mortality rate of greater than 50 % at 5 years from time of diagnosis. IPF was initially thought to be due to chronic inflammation [60]. However, more recent studies suggest that the onset and progression of IPF are due to ongoing alveolar epithelial injury and abnormal wound repair [59].

Chronic fibrosis and impaired repair characterize other lung diseases such as chronic obstructive pulmonary disease (COPD) which is among the top 5 diseases causing global morbidity and mortality. The mechanisms following smoke inhalation are complex. There is chronic injury to the epithelium, inflammation, goblet cell hyperplasia, mucus gland metaplasia, and decreased Clara cell protein as well as airway remodeling. The end result of this inflammatory process is destruction of the elastin-rich extracellular matrix resulting in irreversible dilation of the distal airspace and a loss of elastic recoil. The loss of gas exchange units results in hypoxia and is the major reason for patient morbidity and mortality [61]. Taken together, all three major lung conditions would benefit from cell replacement and anti-inflammatory and antifibrotic therapy.

Clinical Consequences of Impaired Repair in Liver Disease

The liver responds to diverse factors including alcohol, viruses, drugs, and cholestatic and metabolic disease through a process linking

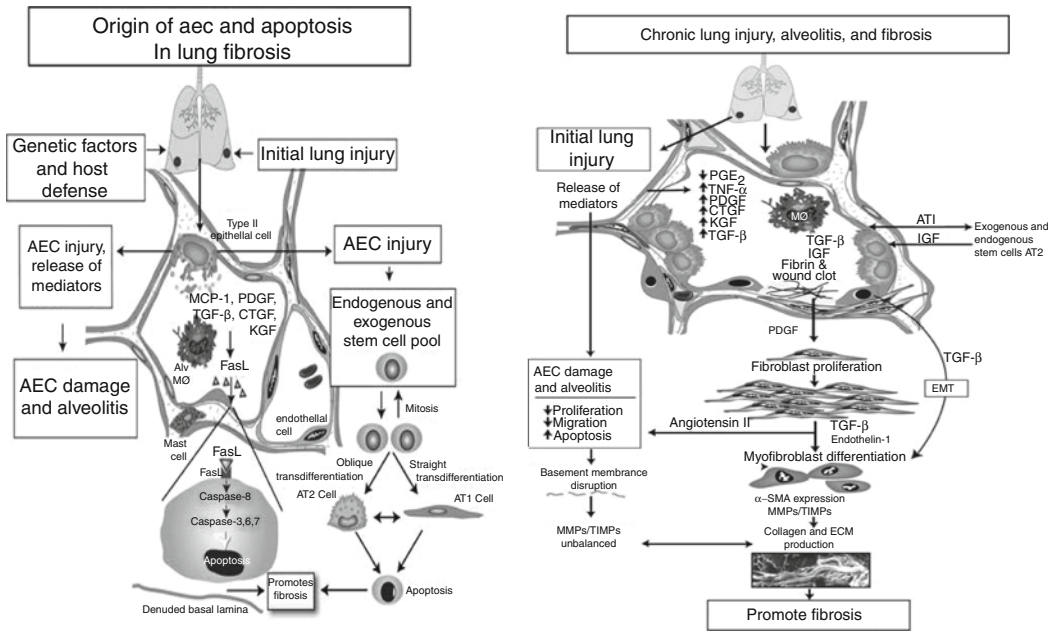

Fig. 10.2 Pathogenesis of idiopathic pulmonary fibrosis (*IPF*). After the initial injury, there is progressive alveolar epithelial damage in IPF. This results in failure of epithelial restitution with accumulation of fibroblasts, myofibroblasts, and collagen type I that is characteristic of IPF (From Gharee-Kermani et al. [59])

inflammation with wound healing and angiogenesis. Injury causes damage to hepatocytes and their membrane components and leads to the recruitment and activation of Kupffer cells (KC), the resident hepatic tissue macrophages and T-cells. Apoptotic hepatocytes, KC, and T-cells release cytokines IL-6, interferon (IFN)-γ, TNF-α, TGF-β, and PDGF that activate quiescent hepatic stellate cells (HSC) into myofibroblasts (Fig. 10.3). HSC are the central mediators of liver fibrosis and adopt an α-smooth muscle actin (α-SMA), desmin, and vimentin-positive myofibroblast-like phenotype. HSC also secrete TGF-β which enhances ECM deposition. The ECM consists predominantly of type I collagen but also includes types III, IV, and V; fibronectin; laminin; and elastin. Further, the activated HSC regulate the infiltration of monocytes and lymphocytes though the secretion of monocyte chemoattractant protein-1 to perpetuate the inflammation [62]. When the injuries overwhelm the capacity of the liver to restitute the hepatocytes and endothelial cell, liver fibrosis occurs in the lung, with extensive ECM accumulation developing into cirrhosis.

The onset of liver fibrosis is usually insidious with clinical complications occurring mainly after the development of cirrhosis. Hepatic cirrhosis is described as a "diffuse process characterized by fibrosis and the conversion of normal liver architecture into structurally abnormal nodules" [63]. The complications associated with hepatic cirrhosis include ascites, portal hypertension, renal failure, and hepatic encephalopathy and is a major risk factor for hepatocellular carcinoma [64]. The World Health Organization estimates that approximately 450 million people are suffering from viral hepatitis and alcohol-related cirrhosis alone [65].

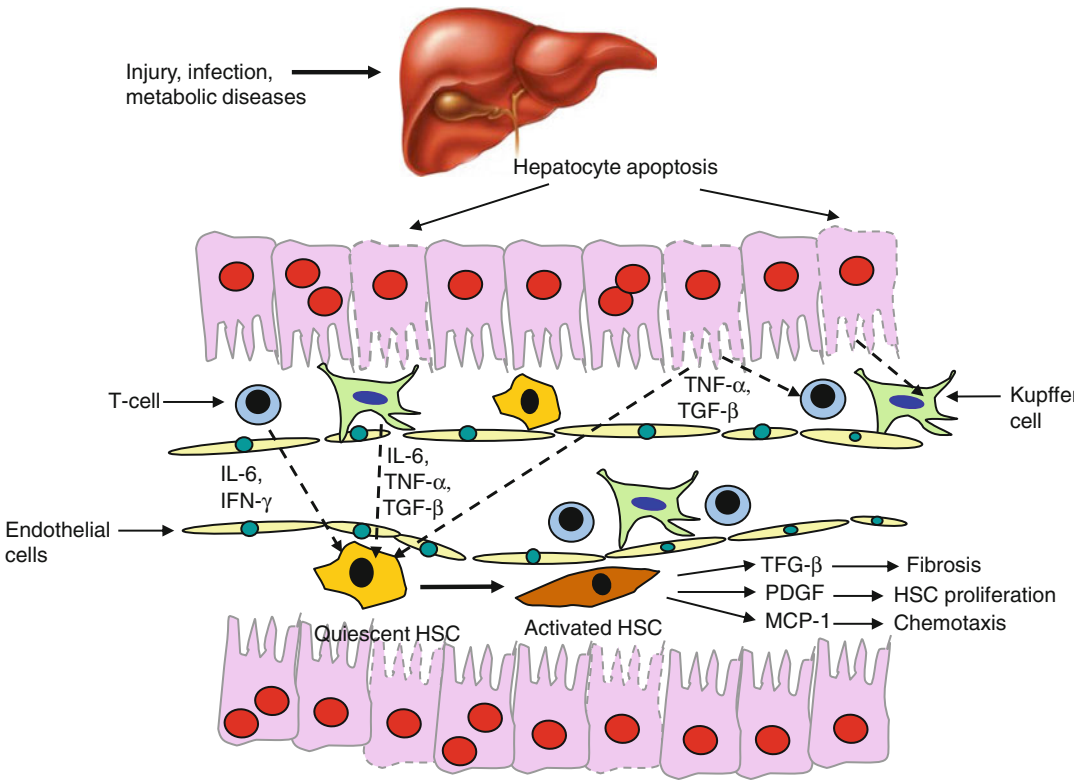

Fig. 10.3 Pathogenesis of liver fibrosis. Schematic showing injury to hepatocytes followed by T-cell and Kupffer cell recruitment and activation. Factors released by the injured hepatocytes and immune cells activate quiescent hepatic stellate cells (*HSC*) into collagen depositing myofibroblast-like cells

The treatment of chronic lung and liver disorders is limited both by therapeutic options and our understanding of the reasons for progressive and ongoing damage in a diseased organ. Most therapies are directed at the acute inflammatory response, namely, with antibiotics and anti-inflammatory agents. To date, there are no truly effective treatments against progressive fibrosis with organ transplantation being the only curative therapy. The restitution of epithelial and endothelial cells, minimizing inflammation, and the resolution of fibrosis are critical components for effective repair. The plasticity and the anti-inflammatory properties of stem cells may offer a viable alternative, and studies have shown that embryonic, hematopoietic, and MSC including placental-derived stem cells can differentiate into liver and lung cells and reduce inflammation and fibrosis in animals with experimental liver and lung injury. Data generated from studies using placental-derived stem cells and limitations are detailed in the following section.

Preclinical Studies Using Placental-Derived Stem Cells for the Treatment of Lung Diseases

Following a groundbreaking study by Ortiz et al. demonstrating the ameliorative effects of autologous transplantation of murine BM-MSC on inflammation and fibrosis reduction [66], human BM-MSC were also shown to reduce IL-1, IL-6, TNF-α and TGF-β, PDGF, insulin growth factor, VEGF and lung collagen, and laminin and hyaluronan content in mice [67, 68]. Further, significant improvement in lung water content, a measure of pulmonary edema and bronchoalveolar lavage protein content, a measure of endothelial and alveolar epithelial permeability were seen following intrapulmonary delivery of BM-MSC [69, 70]. The efficacy of WJ-MSC from the umbilical cord in reducing inflammation and fibrosis has been tested using a bleomycin-induced lung injury model. Intranasal instillation of bleomycin, an antineoplastic antibiotic, is well characterized and a widely accepted model that mirrors the initial inflammation and fibrosis observed in ARDS and IPF. WJ-MSC (1×10^6 cells) were injected systemically following bleomycin injury into adult SCID mice. Cell injection resulted in a decrease in histological evidence of inflammation and fibrosis in the mouse lung [71]. There was also a reduction in the collagen content in lungs as determined by the hydroxyproline assay. WJ-MSC persisted in the lung for up to 2 weeks post injection; however, the effects were seen over the test period lasting 4 weeks even when the WJ-MSC were not evident in the lungs. Possible mechanisms of reducing inflammation and fibrosis identified included a fall in inflammatory cytokines IL-1, IL-6, TNF-α, IFN-γ, macrophage migration inhibitory factor, as well as TGF-β and TGF-β-induced Smad-2 signaling. In addition, there was an upregulation of type 1 collagen degrading matrix metalloproteinase (MMP)-2 in lungs which may have resulted in increased collagen breakdown following fibrosis [71]. Studies using BM and WJ-MSC have shown amelioration following cell injection within 24 h of injury. However, injection of BM-MSC at 5 days after injury demonstrated no effect on collagen deposition. Of note, injection of Flk + BM-MSC at 1 and 2 months post injury *elevated* fibrosis, and the MSC were located in areas of scarring [72]. This suggests that the timing of MSC injection and the lung microenvironment are critical to the outcome. Injection during the early inflammatory stages may reduce inflammation and preserve lung epithelium and endothelium, thereby reducing downstream fibrosis. Late injection may result in the trafficking of MSC to areas of fibrosis and increase in profibrotic mechanisms. Recent studies have shown that MSC can increase fibrosis through the secretion of TGF-β and the Wnt signaling pathway [73] and that MSC can differentiate into lung fibroblasts [74] and lead to ECM deposition. Whether injection of placental MSC into animals with established fibrosis would augment collagen deposition remains unknown.

hAEC have also been shown to reduce inflammation and fibrosis in a bleomycin-induced model of lung fibrosis. Following systemic infusion, hAEC were found 4 weeks post injection in the mouse lung [75]. In addition,

hAEC demonstrated markers of lung alveolar epithelium in both in vitro and in vivo assays. Histological as well as inflammatory (IL-6, TNF-α, and IL-1) and profibrotic cytokines (TGF-β and IL-1) were reduced by hAEC injection. Furthermore, there was a reduction in collagen deposition following hAEC injection. Similar mechanisms to that induced by WJ-MSC were found with a reduction in TGF-β levels as well as possible increased collagen degradation due to enhanced MMP-2 and reduced levels of the MMP inhibitors, the tissue inhibitors of matrix metalloproteinases following hAEC treatment. Notably, however, hAEC reduced fibrosis when injected 24 h following bleomycin-induced lung injury which would then attenuate the inflammatory events as well as 2 weeks post bleomycin which signifies a more established phase of fibrosis. The clinical implications of this finding are significant since most patients present when fibrosis is generally well established.

Cargnoni et al. demonstrated similar findings in that injection of a mixed population of hAEC and amnion–chorion MSC reduced inflammation and fibrosis in bleomycin-induced lung fibrosis [76]. Notably, the positive effects on lung repair were present when cells were injected systemically as well as intratracheally [76]. Furthermore, benefits were observed following allogeneic and xenogeneic cell transplantation which supports other studies demonstrating the low immunogenicity of placental stem cells and their ability to remain engrafted in immunocompetent animals [76, 77].

Differentiation of Placental Stem Cells into Lung Cells

In addition to reducing inflammation and fibrosis, the efficacy of using placental-derived stem cells for replacing lung epithelial cells is being tested. Alveolar type II (AT2) epithelial cells of the distal lung secrete surfactant proteins (SP) that reduce the air–liquid surface tension to facilitate efficient gas exchange by AT1 epithelial cells. Injury from a wide variety of causes such as toxins, pathogens, allergens, dust, asbestos, and ventilators leads to the loss of the epithelial cells. FGF, keratinocyte growth factor, and HGF released by damaged lung cells can induce proliferation, migration, and differentiation of type AT2 into AT1 cells [78, 79]. However, defects in the AT2 progenitor response due to extensive damage and inability to replace injured cells result in grossly reduced lung function and chronic scarring [59]. UCB-MSC/multipotent progenitor cells grown in small airway growth medium (SAGM) were shown to differentiate into AT2-like cells with characteristic features including lamellar bodies and SP-C production [80, 81]. SAGM medium contains several additives including cortisone, epidermal growth factor, insulin, retinoic acid, and epinephrine that are known to induce the differentiation and maturation of lung epithelial cells. Evidence of differentiation following cell transplantation was also demonstrated with SP-C+ cells in lungs following the infusion of undifferentiated UCB-MSC/multipotent progenitors and AF-MSC into NOD-SCID mice and SCID mice with naphthalene-induced injury, respectively [81, 82]. Interestingly, however, WJ-MSC failed to grow and differentiate in SAGM or show evidence of AT2 differentiation following transplantation into bleomycin-injured mice. Recent studies have shown that hAEC cultured in SAGM differentiate into cells with lamellar bodies; produce SP-A, SP-B, SP-C, and SP-D; and respond to dexamethasone by elevating the secretion of SP-D [75]. Further, the transplantation of undifferentiated hAEC into mice with bleomycin-induced lung damage showed that hAEC remained engrafted in lungs during the test period of 4 weeks. Within a few weeks of transplantation, hAEC showed evidence of differentiation and were producing SP-A, SP-B, SP-C, and SP-D [71, 75]. While the preceding cell transplantation studies focussed on AT2 cell replacement in adult mice, the application of exogenous stem cells may also be most useful in the treatment of neonatal respiratory complications. SP production commences in utero at approximately 24 weeks of gestation. Neonatal respiratory

distress syndrome caused by inadequate SP is a major cause of morbidity and mortality in prematurely born infants. Following optimistic results from an initial study using human BM-MSC [83], UCB-MSC cells have been shown to differentiate into SP-C+ cells following intratracheal and peritoneal infusion into hyperoxia-damaged lungs of neonatal rodents [84].

Lung injury also leads to the loss of Clara cells from the small airways. UCB-MSC and AF-MSC have been shown to produce Clara cell secretory protein (CCSP) after culture in SAGM and following transplantation [81, 82]. These studies have also shown that the cystic fibrosis transmembrane conductance regulator (CFTR) protein is produced by UCB and AF-MSC grown in SAGM and following cell transplantation into mice [81, 82]. Cystic fibrosis is an autosomal recessive disease characterized by mutations in the CFTR gene, and these findings imply that placental MSC treatment may be beneficial for treating the disease and/or used as a CFTR gene delivery vehicle using lentiviral transduced MSC [81]. However, a number of factors need to be taken into consideration in applications of placental or indeed other stem cells for the treatment of lung diseases. Following peripheral injection, the stem cells will be rapidly transported and deposited in lung capillaries. MSC have been shown to migrate in response to several chemokines including C5A, stromal-derived factor-α, and monocyte chemotactic protein-1 released by damaged cells [85, 86]. Myristoylated alanine-rich C kinase substrate (MARCKS) protein present in UCB-MSC has been shown to play a role in migratory response to chemokines [85], but whether chemokines and MARCKS play a role in the retention of MSC in the lung remains uncertain. Careful cell dosage determination and slow or multiple infusions would be essential for preventing an embolism. Low retention and engraftment of peripherally injected MSC within a few weeks of administration have been noted in several studies [24, 71]. Although direct comparisons between the different placental stem cell populations have not been made, hAEC appear to have the highest rate of engraftment among placental cells tested constituting ~5 % of alveolar cells in bleomycin-injured murine lungs 2 weeks following transplantation [75]. AF-MSC appears to have a higher rate engraftment compared with UCB-MSC [81, 82]. Intratracheal injections have led to an improvement in the percentage of stem cells being retained in the lungs in comparison with peritoneal delivery [87]. However, whether relatively few numbers of engrafted stem cells could contribute to significant alveolar restitution is questionable. Another concern is that MSC have been found to fuse with recipient cells and contribute to circulating fibrocytes, lung fibroblast, and myofibroblast lineages [88]. Given the pivotal role played by these cells in collagen deposition, potential adverse outcomes need careful assessment. From the clinical perspective, it would be important to determine if transplantation of culture-differentiated AT2 progeny derived from stem cells would lead to a higher rate of engraftment and contribute to alveolar restitution. Further, direct comparisons of the ameliorative effects of placental-derived MSC and hAEC should be made to identify the most effective and safe single or combinational therapy.

Preclinical Studies Using Placental Stem Cells for the Treatment of Liver Fibrosis

BM-MSC, hematopoietic, and embryonic stem cell transplantation have been found to ameliorate hepatic fibrosis in experimental models [89–91]. One of the most widely used models of liver fibrosis is carbon tetrachloride (CCl4) administration. CCl4 causes hepatocyte injury which develops into centrilobular necrosis and ultimately liver fibrosis. BM-MSC are believed to alter the microenvironment of the injured liver, favoring liver regeneration and preventing fibrogenesis through secretion of TNF-α antagonists that would hinder activation of collagen depositing HSC; suppress the secretion of the profibrogenic TGF-β; and/or by increasing IL-10, an antifibrogenic cytokine. Coculture

experiments using BM-MSC and a HSC cell line (LX-2 cells) have shown that activated HSC secrete IL-6, which stimulates BM-MSC to release IL-10, and that IL-10 together with TNF-α inhibit HSC proliferation and collagen synthesis [92]. Further, MSC secrete HGF that promotes HSC apoptosis [92]. In contrast, HGF enhances hepatocyte proliferation, and therefore, MSC transplantation may lead to improved organ function. Albeit limited, studies have demonstrated positive outcomes in treating experimental models of liver fibrosis using placental-derived stem cells. Tsai et al. demonstrated that human WJ-MSC reduced inflammation and fibrosis in a rat model of CCl4-induced liver injury [93]. Following CCl4 administration for 4 weeks, WJ-MSC were injected directly into the liver and CCl4 given for a further 4 weeks. Rats receiving WJ-MSC had reduced inflammation and fibrosis compared to animals treated with CCl4 alone for 8 weeks. There was a reduction in liver enzymes glutamic oxaloacetic transaminase and glutamic pyruvate transaminase in serum signifying improved hepatic function. In addition, WJ-MSC treatment resulted in the fall in α-SMA and TGF-β, implying reduced HSC activation [93]. This group found no evidence of differentiation of WJ-MSC into hepatocytes [93]. However, in thioacetamide-induced injury that leads to extensive hepatic fibrosis, WJ-MSC were not only effective in reducing fibrosis, the transplanted cells expressed the hepatocyte marker albumin suggesting differentiation and HGF and MMP [94]. Similar findings were seen using UCB-MSC in CCl4-treated rodents with antifibrotic effects and differentiation into cells expressing albumin and α-fetoprotein [95]. These studies have also demonstrated engraftment in immunocompetent animals and positive outcomes with xenogenic transfer. However, the expression of a few hepatocyte markers does not indicate differentiation into functional hepatocytes, and the effects of the "differentiated" cells on inflammation and fibrosis, if any, remain unclear. Further, similar drawbacks to that reported in the lung including negligible engraftment, differentiation into HSC, and fusion with hepatocytes to yield dysplastic precursors have been reported using BM-MSC [91, 96–98], and a much more extensive screening of the safety of placental stem cells would be needed.

Manuelpillai et al. demonstrated that hAEC injected systemically into immunocompetent mice with CCl4-induced liver fibrosis demonstrated a reduction in hepatocyte injury, inflammation, and fibrosis [99]. hAEC were transplanted into C57/BL6 mice at week 2 of a 4-week regimen of CCl4. Two weeks following hAEC infusion, intact cells expressing the human-specific marker inner mitochondrial membrane protein and HLA-G were found in mouse liver without evidence of host rejection of the transplanted cells. Human albumin, known to be produced by hAEC, was detected in sera of hAEC-treated mice demonstrating that the engrafted hAEC were still viable. Human DNA was detected in mouse liver and also spleen, lungs, and heart of some animals suggesting that the hAEC have multiple engraftment sites. Following hAEC transplantation, CCl4-treated animals showed decreased serum alanine aminotransferase and reduced hepatocyte apoptosis, compared to controls. hAEC treatment reduced inflammation lowering TNF-α and IL-6 protein levels and elevating IL-10. Mice given hAEC also showed fewer activated collagen-producing HSC and less fibrosis area and collagen content possibly due to reduced hepatic TGF-β levels in conjunction with a twofold increase in the active form of the collagen-degrading enzyme MMP-2 in treated mice compared to CCl4 controls. Importantly, hAEC were found adjacent to HSC and regions of residual fibrosis, and while the mechanisms induced by cell transplantation remains unclear, it is tempting to speculate that cell–cell contact and/or secreted factors play a role [99]. While positive effects were seen in each of the major steps in the hepatocyte apoptosis, inflammation, and activated HSC cascade leading to fibrosis, it is uncertain if hAEC had a direct influence in each of these steps or if the reduction in apoptosis alone was sufficient to block this cascade.

Differentiation of Placental Stem Cells into Hepatocytes

Sustained injury to the liver from a wide variety of causes including drugs, alcohol, viruses, and diet leads to the apoptosis of hepatocytes. Hepatocytes constitute nearly 80 % of liver cells and carry out vital functions including carbohydrate metabolism, protein synthesis and storage, cholesterol and phospholipid synthesis, bile secretion, and detoxification. Liver cirrhosis is characterized by massive loss of hepatocytes that lead ultimately to organ failure. While the focus of studies described above was to investigate the effects of exogenous stem cells on hepatic inflammation and fibrosis, others have examined if differentiation of stem cells could augment hepatocyte numbers in experimental models of hepatic injury. Stem cells from the placental chorionic villi, umbilical cord, and amnion have been shown to differentiate into cells expressing hepatocyte markers in vitro (Table 10.1). Among the placental stem cells, hAEC are noteworthy since the primary undifferentiated cells express several "hepatocyte" genes and, following stimulation, express additional hepatocyte markers including HNF4α; metabolize drugs; store glycogen; and attain ultrastructural features of hepatocytes including the double nuclear envelope [2, 3, 106, 107]. However, hAEC also continued to express genes such as α-fetoprotein characteristic of immature hepatocytes and lacked expression of genes such as tryptophan dioxygenase and ornithine transcarbamylase following stimulation with supplements believed to induce differentiation into hepatocytes [106].

Following transplantation, hematopoietic stem cells from UCB into CCl4-treated animals, albumin, cytokeratin, and α-fetoprotein positive cells were found between 1 and 4 weeks post injection of [108, 109]. UCB-MSC also expressed hepatocyte genes in livers of CCl4-treated mice [111] (Table 10.1). hAEC have been primarily tested as a potential gene carrier into the fetal liver [112, 113]. Although hAEC continue to secrete albumin following transplantation into CCl4-treated mice [99],

Table 10.1 Differentiation of placental-derived stem cells into hepatocytes

Cell type	Hepatocytes characterized by	References
Differentiation in vitro		
Chorionic villus MSC	Cytokeratin-18, albumin, glycogen storage	[100]
Umbilical cord Wharton's jelly MSC	Cytokeratins 18 and 19,albumin, glycogen storage, α-fetoprotein albumin secretion, connexin-32, CYP3 A4, low-density lipoprotein uptake, urea production. Lacked HepPar1 and HNF4α	[101–103]
Umbilical cord blood MSC	Albumin, CCAAT enhancer-binding protein alpha, cytochrome p450 glycogen storage	[104]
Amnion MSC	Cytokeratin 18, albumin, α1anti-trypsin, glucose-6-phosphatase, ornithine transcarbamylase	[105]
Human amnion epithelial cells	Primary cells shown to express several "hepatocyte genes" including albumin, α1-antitrypsin, glutamine synthase, PEPCK, cytochrome P450, and β_2 microglobulin and, following "differentiation," other genes including α-fetoprotein, transthyretin, and carbamoyl phosphate synthetase	[106]
	Albumin, α1-antitrypsin, HNF4α, glycogen storage, drug metabolism via CYP1A, binucleated cells	[3, 107]
	Albumin, hepatocyte growth factor, and ultrastructural studies showing similarities to hepatocytes	[2]
Differentiation in vivo		
Umbilical cord HSC	Cytokeratin-18, albumin, cytochrome P450, α-fetoprotein	[108–110]
Umbilical cord blood MSC	Tryptophan 2,3-dioxygenase, α-fetoprotein, cytokeratin-18, fibroblast secretory protein-1	[111]
Umbilical cord Wharton's jelly MSC	Albumin, α-fetoprotein	[103]

whether hAEC differentiate into mature hepatocytes has not been tested in vivo. Importantly, Kakinuma et al. reported that transplantation of hepatocytes derived from UCB hematopoietic stem cells engrafted and continued to express markers of hepatocytes including cytochrome P450 during the 3-week test period, thus offering another approach [110]. While these studies on hepatocyte replacement by exogenous stem cells are promising, liver regeneration by stimulating endogenous cells in chronic liver disease and following partial hepatic resection by exogenous stem cells may be more beneficial. Hepatocytes generated from placental stem cells may also be more useful in populating in bioartificial support devices in bridging therapies for acute liver diseases [114].

In conclusion, placental stem cells have been found to be as effective as adult BM and hematopoietic stem cells in preclinical models of lung and liver disease and offer exciting prospects for cell therapy. As with the adult stem cells, the mechanisms induced by placental stem cell transplantation in reducing inflammation and fibrosis remain unclear and will be a challenge to unravel. Approvals for the use of stem cells in therapy for these diseases would require a far greater understanding of the mechanisms involved. The influence of the diseased niche, phase of disease, effects on endogenous stem and tissue cells, potential changes in immunogenicity and anti-inflammatory properties, and long-term safety also needs further assessment.

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Part III

Fetal Cell Transplant Experiments in Animal and Human Systems

Experiences with In Utero Transplantation of Mesenchymal Stem Cells

11

Carolyn Troeger, Irina Perahud, Eva Visca, and Wolfgang Holzgreve

Introduction

In utero stem cell transplantation (IUT) has become a valuable therapeutic option in fetuses with congenital immunologic disorders, such as severe combined immunodeficiency (SCID) or bare lymphocyte syndrome [1, 2]. However, other diseases such as thalassemias, storage defects, or osteogenesis imperfecta have either resulted in no detectable engraftment or microchimerism with uncertain effect on the phenotype. Although IUT was performed as early as the end of first trimester, neither bone marrow nor fetal liver cells resulted in relevant engraftment. It can be postulated that the fetal immune system deletes the allogeneic stem cells since several studies suggest that the fetal thymus is colonized in the first third of gestation [3]. A fetal T-cell-mediated alloresponse is evident as early as the second trimester and has cleared most allogeneic cells by term [4, 5]. In principle, IUT could result in long-term chimerism when performed early enough in pregnancy since, for instance, persistent blood group chimerism has been demonstrated for dizygotic twins [6]. But obviously, also the "transmaternal" traffic of cells from a first born to the next infant in a later pregnancy leads to tolerance induction within the T-cell population [7]. The early presentation of allogeneic cells to the developing fetal thymus results in specific tolerance, whereas later appearance (for instance, due to IUT of allogeneic hematopoietic stem cells at embryonic day 14 post conception/E14) leads to clearance of allogeneic cells from the circulation within months by the recipient's immune system [8].

Regarding these immunological concerns against hematopoietic stem cells derived from bone marrow or other sources, mesenchymal stem cells seem to be an ideal candidate for allogeneic in utero transplantation. MSC possess the unique advantage of their immunomodulatory and even immunosuppressive effect [9–11]. Besides, MSC are known to have a wide differentiation and multilineage capacity [12] making them a valuable source for tissue regeneration.

However, there are only few reports on IUT using MSC in both animal models and humans. This book chapter focuses on the experiences that have been made so far.

Problems in Characterization of MSC

The presence of MSC within the bone marrow was first suggested by the German pathologist Cohnheim when he showed that bone marrow-derived fibroblasts deposit collagen as part of wound healing [12]. Friedenstein and colleagues were the first to isolate MSC by plastic adherence and propagate their differentiation into bone and cartilage [13]. Based on this pioneering work, MSC are typically isolated from the mononuclear layer of the bone

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marrow after density gradient centrifugation. MSC are typically defined by their capacity to differentiate into osteoblasts, adipocytes, and chondrocytes in vitro [14, 15]. In addition to bone marrow, MSC have also been isolated from a variety of other tissues, e.g., adipose tissue [16], umbilical cord [17], blood circulation [18], amniotic fluid [19], placenta [20], and fetal blood, liver, bone marrow, and lung [21, 22]. It appears that MSC reside in perivascular niches within most organs [23] making it difficult to isolate these cells from peripheral blood. Possibly in response to signals that are upregulated under injury conditions, MSC can be recruited from these niches for tissue repair; however, the underlying migration mechanisms are not yet fully understood, and results on chemokine secretion and chemokine receptor expression are conflicting [24, 25]. If analyzed at the protein level by either flow cytometry and chemotaxis assay, it has been shown lately that human MSC express functional CCR1, 7, and 9 and CXCR4, 5, and 6 on 43–70 % of cells [26]. In contrast, Ponte and colleagues demonstrated the expression of CCR2, 3, and 4 and CXCR4 [27]. Much of this variability is related to the potential of MSC to home different tissues; thus, results often depend on the MSC source studied. Compared to human MSC, murine MSC demonstrate partly similar selective expression of chemokine receptors [28].

Analogous problems are evident when MSC are characterized by surface markers. It is, however, generally accepted that adult human MSC do not express the hematopoietic markers CD45, CD34, CD14, and CD11. They do not express costimulatory molecules such as CD80, CD86, and CD40 and adhesion molecules CD31, CD18, and CD56. They can express CD105, CD73, CD44, CD90, CD71, and Stro-1, CD106, CD166, ICAM-1, and CD29 [11, 29]. Comparison of the expression pattern of human MSC to that of other species is difficult; however, it is generally accepted that MSC uniformly do not express CD45 and CD31, whereas data on CD34 expression are conflicting [30]. Differences in expression pattern may additionally be influenced by factors secreted in the initial culture passages [31]. Thus, there is not always a correlation between the expression of chemokine receptors and surface markers in vitro and in vivo, and care should be taken in the analysis of results [28, 32].

As stated above, MSC are commonly also characterized by their capacity to differentiate into bone, fat, and cartilage in vitro. Also in this regard, results depend on the stem cell source used. Whereas human bone marrow-derived MSC are capable of differentiating into cells of all three lineages, differentiation capacity is different for MSC from other sources, such as dental [33] or adipose tissue [34]. Similar is true for various mouse strains and fetal versus adult MSC sources [30, 35–37] (see Fig. 11.1).

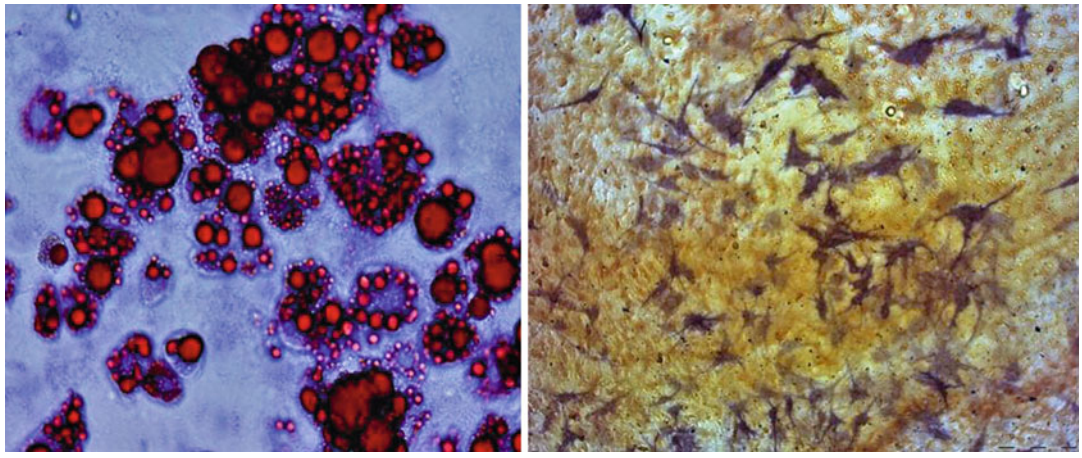

Fig. 11.1 Differentiation of murine fetal liver-derived MSC into osteoblasts and adipocytes

All these variables have a significant impact on the results, and thus, interpretation of most studies using MSC depends on an exact description of the cells used but makes it quite impossible to compare results between groups.

MSC Function and Localization Throughout Ontogeny

It is known for long that bone marrow-derived stromal cells promote survival, proliferation, and differentiation of hematopoietic stem cells in vitro [38]. After cotransplantation, MSC enhance the engraftment of donor hematopoietic stem cells in recipient animals [39]. This regulation of hematopoiesis is most probably mediated by both cell-to-cell contact and the production of growth factors [40].

Whereas the first wave of primitive hematopoiesis in the mouse embryo begins at E7.5 with large and nucleated erythroid clusters in the yolk sac, it is replaced by definitive hematopoiesis by E9.5 using progenitors from the para-aortic splanchnopleural region. Long-term repopulating hematopoietic stem cells then appear in the aorta-gonad-mesonephros (AGM) region by E10.5 to E11.5 [41, 42]. Between E10.5 and E12.0, hematopoietic progenitors amplify within the AGM region and then colonize the fetal liver [43]. Just before birth, hematopoietic stem cells migrate to the bone marrow, which then remains the main site of hematopoiesis throughout life [44].

As stated above, a sufficient hematopoiesis depends on a functional stromal microenvironment. This supportive mesenchymal microenvironment exists in several anatomical sites during ontogeny: AGM, fetal liver, and bone marrow [45]. It has been shown lately that mesenchymal cells from the AGM region are more effective than those from the bone marrow in supporting expansion of HSC, maintaining self-renewal and multilineage differentiation [46]. Although expression patterns of surface markers and differentiation potential of isolated MSC from E12 AGM and E14 fetal liver are similar, MSC are not detectable in E11 fetal liver [36]. When E12 AGM and E14 fetal liver have been compared, the number of osteogenic progenitors significantly increased. Similar is true for adipogenic and chondrogenic differentiation. Probably, MSC migrate at certain developmental stages because progenitors for any of the three lineages were detected in the peripheral embryonic blood at E12 and E14, whereas no MSC were found at E11 and E17. Further experiments on Runx1−/− mice (blocked HSC development with fetal anemia and early lethality at E12.5) revealed that MSC localization and number are not dependent on the appearance and function of HSC.

Rationale for In Utero Transplantation Using MSC

In utero stem cell transplantation has become a valuable therapeutic option in fetuses with congenital immunologic disorders, such as severe combined immunodeficiency (SCID) or bare lymphocyte syndrome (BLS) [1, 2]. However, in other diseases with an intact fetal immune system, such as thalassemias or storage defects, IUT of hematopoietic stem cells has either resulted in no detectable engraftment or microchimerism with uncertain effect on the phenotype. Although IUT was performed as early as the end of the first trimester, neither bone marrow nor fetal liver cells resulted in relevant engraftment. In this context, MSC would be an ideal candidate cell type for in utero transplantation since they seem to suppress T-cell activation and retain their immunomodulatory properties even after differentiation into other cell types [10, 11]. Besides, MSC enhance wound healing and tissue repair. It can be expected that transplanted MSC migrate particularly to sites of injury [37, 47]. Comparing fetal versus adult MSC, it seems that fetal MSC have more advantages: They self-renew faster in vitro, senesce later and retain a stable phenotype, have greater multilineage capacity, and have significantly greater binding to their extracellular matrix ligands than adult MSC [48–50]. They are even less immunogenic than adult MSC since they express lower levels of MHC class I and lack intracellular MHC class II [51]. When gene

therapy is concerned, fetal MSC have a higher transduction efficiency of >95 % using lentiviral vectors with stable short and long-term gene expression [49].

In summary, MSC especially those isolated from fetal sources – although of ethical concern – would be the ideal cell type for prenatal treatment of organ defects that would otherwise cause tissue damage already during pregnancy such as osteogenesis imperfecta [52, 53].

Experiences in Animal Models

In utero transplantation of MSC could be used as a cotransplant to enhance HSC engraftment or to participate in tissue repair [39, 48, 54]. MSC even after xenogenic transplantation into immunocompetent fetal recipients showed long-term engraftment in multiple tissues and organ-specific differentiation into chondrocytes, adipocytes, myocytes, cardiomyocytes, bone marrow stromal cells, and thymic stroma [48, 55, 56]. In the studies using healthy sheep fetuses as recipients, engraftment levels ranged between 0.1 and 3.2 % in various organs such as liver, spleen, bone marrow, thymus, lung, and brain. Interestingly, human MSC that have been transplanted before the fetal immune system is developed lead to engraftment levels as high as 43 % in the Purkinje fibers of the heart, whereas cardiomyocytes of donor origin were only detected at very low levels (0.01 %) [57]. This phenomenon might be explained by the fact that right at the time of transplantation, the Purkinje system due to proliferation is attractive for the transplanted MSC.

It is unclear yet whether even low engraftment levels would be sufficient to cure, for instance, a diabetes mellitus type I since it has been shown by Ersek and colleagues that even low engraftment levels between 0.0008 and 0.001 % of donor DNA in recipient's pancreas are sufficient to secrete detectable amounts of human C-peptide [58]. Similar observations of low-level engraftment have been made in healthy mouse fetuses transplanted at E14 using human MSC from bone marrow. Although long-term engraftment in various organs was achieved, cells could not be detected in peripheral blood after 3 months, and engraftment frequencies decreased. However, no conclusions can be drawn regarding the actual engraftment levels in healthy tissue since no quantitative analysis from the tissue was given [59]. Our attempts to enhance engraftment in this nondefect mouse model (see Fig. 11.2) by transplanting fetal liver-derived MSC as early as E12 resulted in lower engraftment frequencies 4 weeks after delivery. Transplanted cells stayed in the peritoneal cavity for at least 1 week (see Fig. 11.3). After a relevant transplacental loss of MSC into the maternal circulation, the remaining cells primarily migrated to the bone marrow. In contrast to our considerations that an earlier transplantation would increase engraftment, we detected significantly lower engraftment probabilities after earlier transplantation (see Fig. 11.4) [37].

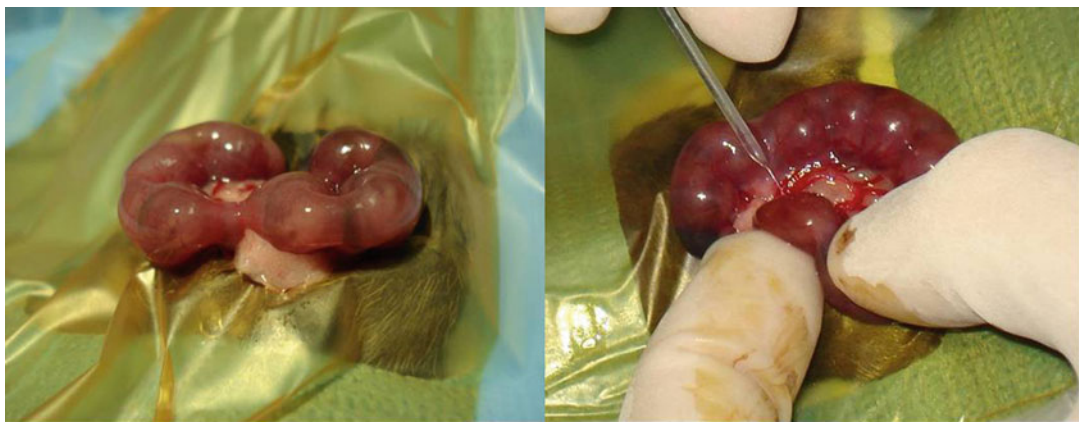

Fig. 11.2 Procedure for intrauterine MSC transplantation in the mouse model

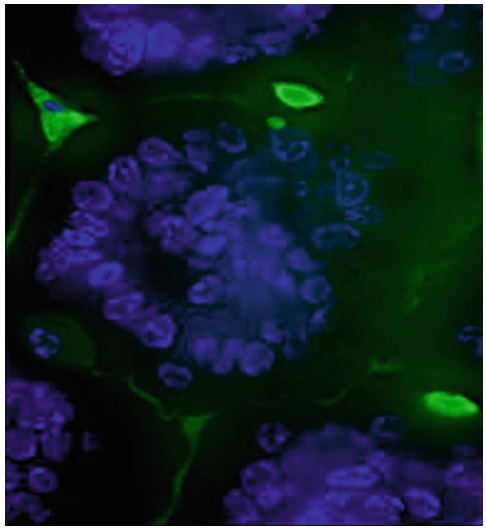

Fig. 11.3 EGFP-positive MSC in the peritoneal cavity of E13.5-transplanted fetuses 1 week after IUT in fluorescence microscopy (magnification 60×)

In contrast to nondisease models, results regarding a curative treatment of inherited diseases by MSC in utero transplantation are promising. Human first-trimester fetal blood-derived MSC transplanted between E13.5 and E15 into homozygous osteogenesis imperfecta (oi) mice resulted in significantly higher long-term engraftment levels in the affected bones (around 4 %) compared to other tissues (around 1 %) [47]. These results indicate that transplanted MSC preferentially migrate to injured organs (fractured bones). The authors of this study could also show that this engraftment leads to less long bone fractures and increased long bone strength and cortical thickness, thus normalizing the phenotype already shortly after transplantation. In contrast, human MSC in utero transplantation into mdx mice suffering from X-linked muscular dystrophy resulted in widespread low-level engraftment with a predilection for muscle tissue (0.71 % vs. 0.15 %).

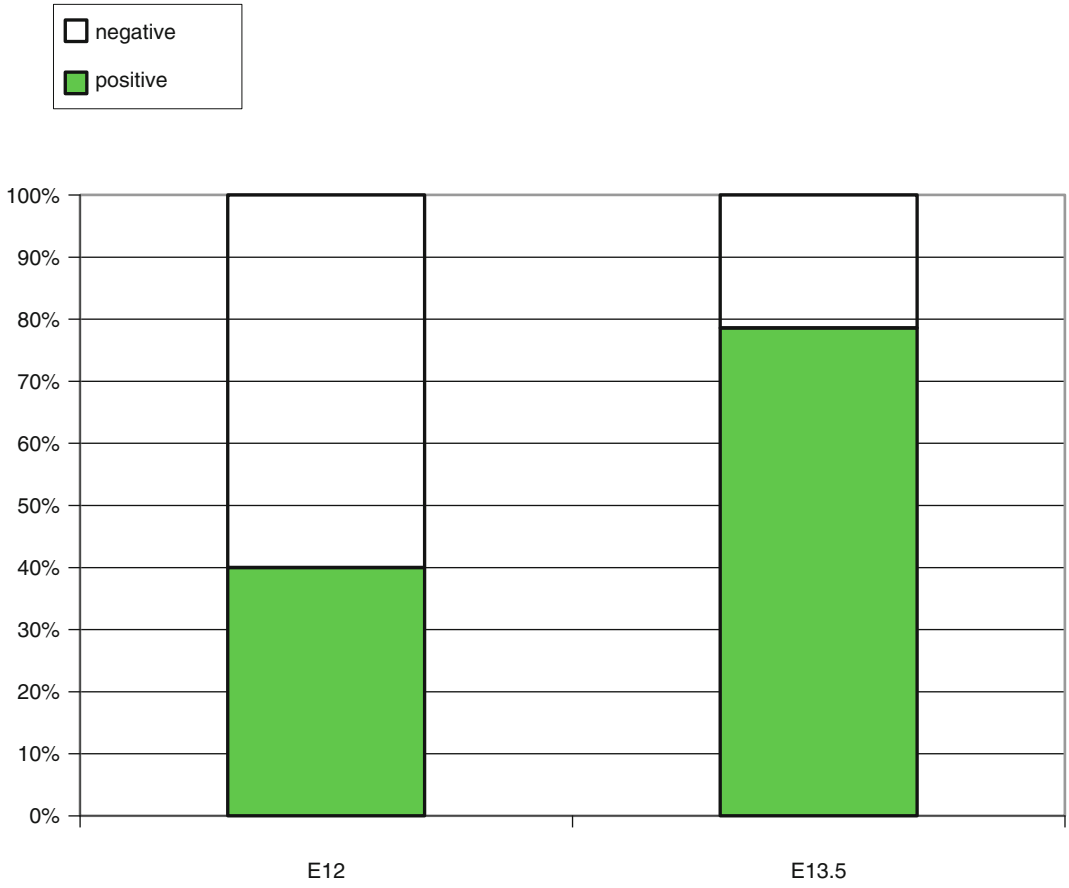

Fig. 11.4 Engraftment of MSC in recipients' bone marrow 1 month after IUT at E12 vs. E13.5

Though muscle fibers contained human nuclei, they did not completely express human dystrophin [60]. This difference can be explained by the relatively mild phenotypic disturbance during fetal life in this disease model compared to the oi mouse. However, all animal experiments demonstrated that in utero transplantation of MSC results in site-specific engraftment and differentiation and might improve the phenotype if organ damage is relevant already before birth.

Experiences in Human Fetuses

Whereas in utero transplantation in human fetuses is widely accepted and successful using hematopoietic stem cells [1, 2, 61], there is only scarce experience when MSC are used. To our knowledge, there is only one case report on a successful allogeneic intrauterine transplantation of fetal liver-derived MSC in a fetal patient with severe osteogenesis imperfecta at 32 weeks of gestation [62]. At 9 months of age, a bone biopsy revealed an engraftment level of 0.3 % using a centromeric Y chromosome probe and 7.4 % using a total Y chromosome probe. It seems that this engraftment had also a positive effect on the recipient's growth. From this single case, it remains unclear how much is enough to cure the fetal patient. However, from the results in postnatal MSC transplantations, it can be concluded that even low levels of donor cell engraftment are sufficient to improve the phenotype. The in utero transplantation reported by the Karolinska group was performed rather late in pregnancy, and delivery was performed 3 weeks later by Cesarean section due to preterm rupture of membranes. Thus, it is unclear whether there is really a benefit from in utero transplantation compared to an early postnatal transplantation. It is also unclear whether other inborn errors such as mucopolysaccharidosis would even more benefit from in utero transplantation. Further studies are needed.

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12 In Utero Hematopoietic Stem Cell Transplantation for Congenital Disorders

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Introduction

Recent advances in stem cell biology, prenatal diagnosis, and fetal surgery have transformed our ability to use in utero stem cell transplantation to cure congenital anomalies. With the recent discovery of inducible pluripotent stem (iPS) cells [1], new opportunities are available to generate patient-matched iPS cells for specific diseases. Advances in prenatal imaging and molecular diagnostics allow us to accurately diagnose congenital hematologic diseases as early as 10–12 weeks of gestation [2]. Furthermore, high-resolution ultrasonography has made it technically feasible to deliver stem cells in the early gestation fetus. Fetal intervention in patients has expanded since its first description in 1982 [3] and is used to treat anatomic anomalies with both conventional and minimally invasive techniques [4]. The improved understanding of stem cell biology, the ability to diagnose congenital diseases that are amenable to prenatal therapy, and the technical capability to deliver cells safely in utero have brought renewed interest and excitement for the promise of prenatal stem cell therapy.

The extensive characterization and understanding of hematopoietic stem cells (HSCs) [5] have made the application of in utero stem cell therapy most promising for congenital disorders of hematopoiesis such as sickle cell disease, thalassemias, and immunodeficiencies. The treatment for these conditions currently involves postnatal bone marrow transplantation (BMT) [6–9]. The therapeutic efficacy of BMT, however, is often limited by transplantation complications such as graft-versus-host disease (GVHD) or graft rejection, by the availability of few HLA-matched donors and by the morbidity of host myeloablation preceding transplantation [9]. The delivery of cells into the early gestation fetus offers the potential advantage of inducing donor-specific tolerance, thus avoiding the toxicity of myeloablation and allowing for postnatal transplantation of allogeneic stem cells or organs [10]. The fetal environment can also promote the proliferation and differentiation of transplanted cells to facilitate widespread engraftment. The promise of in utero hematopoietic stem cell transplantation (IUHSCTx) has been demonstrated in multiple small and large animal models. The clinical application of this technique in humans, however, has been met with limited success. In this chapter, we will review the therapeutic rationale of IUHSCTx and the multiple factors that contribute to its success.

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Therapeutic Rationale

The fetal environment offers numerous advantages for the success of IUHSCTx (reviewed in Ref. [10]). For example, the normal migration of HSC in the fetus may provide an opportunity to engraft allogeneic (foreign) donor HSC and thereby circumvent the need for postnatal myeloablative therapy. HSC originate in the fetal yolk sac, migrate to the fetal liver, and eventually travel to the bone marrow [11, 12]. This relatively large scale cellular migration could provide a favorable environment for donor-cell homing and engraftment. The most compelling reason to perform HSC transplantation in utero is to take advantage of the immature fetal immune system to achieve donor-specific tolerance. Theoretically, introducing allogeneic cells during the period of thymic education to self-antigens should lead to deletion of allo-specific T cells by negative selection [13]. The resulting antigen-specific tolerance that is established in utero can therefore minimize the need for myeloablation during postnatal cellular or organ transplantation [14–16].

The link between donor-cell engraftment in the fetus and antigen-specific tolerance has been best understood through experiments of nature. Dizygotic cattle share placental circulation and have long-lived engraftment of foreign cells from their siblings, resulting in donor-specific tolerance [17]. This phenomenon has also been observed in human twins, where chimerism has led to a lack of alloreactivity between the two siblings [18]. Examples of chimerism in other animals have also been reported [19, 20] with levels of engraftment high enough to be potentially therapeutic for hematologic diseases [10]. An important caveat to these findings is that the constant antigen exposure that begins early in gestation in nature poses challenges when attempting to reproduce this process in the laboratory setting [10]. Nevertheless, these observations have provided support that hematopoietic chimerism established in utero induces immune tolerance.

Even in the absence of hematopoietic chimerism, recent evidence indicates that the fetal environment may inherently promote the induction of tolerance. For example, fetal exposure to non-inherited maternal antigens (NIMA) has been shown to suppress the anti-maternal immune response in mice [21]. This protective effect has been conserved across species. In humans, improved graft survival after haplotype-mismatched kidney transplantation from a sibling has been observed if the donor expresses NIMAs compared to donors that express non-inherited paternal antigens, demonstrating the tolerogenic effect of NIMA exposure [22]. These findings have been corroborated in patients undergoing BMT [23]. Although such tolerance to NIMAs may be secondary to the persistence of maternal cells in the fetus [24], the extent of maternal microchimerism is often quite low and is a proof of principle that tolerance may be achieved even with low levels of engraftment. The mechanism by which the fetus learns to tolerate maternal cells may involve deletion or the creation of regulatory T cells (Tregs) which specifically suppress an anti-maternal immune response [21, 24].

Given the difficulties in achieving clinically significant levels of engraftment after in utero transplantation, the main therapeutic rationale is to establish donor-specific tolerance, such that postnatal "booster" transplants can be performed without myeloablation. Such a strategy has been tested in multiple animal models and will be detailed below. If tolerance to particular antigens is achieved with fetal transplantation, the applications of this strategy will become even broader to include single-gene disorders such as hemophilia [25], whose postnatal treatment is fraught with rejection of the foreign protein [26].

Animal Experience with IUHSCTx

Experimental animal models have improved our understanding of the factors involved with stem cell engraftment after IUHSCTx. The earliest experience with IUHSCTx comes from the seminal experiments of Billingham et al. [27] in which in utero transplantation of allogeneic cells led to donor-specific tolerance to skin grafts in mice. It was soon noted that immunodeficient mice engraft more efficiently, likely secondary to a competitive advantage of the transplanted cells in such an environment [28, 29].

Engraftment of MHC-mismatched donors in immunocompetent, wild-type recipient mice has proven to be much more challenging, and lower levels of engraftment ("microchimerism," detectable only by polymerase chain reaction) were reported [30–33] until recently. These results improved when low levels of chimerism were augmented by postnatal regimens such as donor lymphocyte infusion [15] or non-myeloablative total body irradiation with repeat transplantation of HSC [16]. More recently, modifications to the IUHSCTx technique have allowed for the delivery of higher cell numbers, resulting in macroscopic levels of donor-cell engraftment that are detectable by flow cytometry [34]. The improvements made to the mouse model of IUHSCTx have allowed investigators to demonstrate allospecific tolerance by the lack of host reactivity to donor cells seen in a mixed lymphocyte reaction [35]. The mechanisms by which immune tolerance is achieved include clonal deletion, anergy, or the induction of donor-specific Tregs [31, 35].

Engraftment of allogeneic and xenogeneic HSC has also been achieved in several large animal models including the pig [36], dog [37–39], nonhuman primate [40–42], and sheep [43–47]. The sheep model of IUHSCTx has been of particular interest as high levels of engraftment have been obtained after transplantation of xenogeneic human HSC [46, 47], with engraftment in secondary recipients [45]. Thus, IUHSCTx in this model provides a natural, unperturbed environment in which to study the engraftment and differentiation capacity of human stem cells such as embryonic [45] and mesenchymal stem cells [48]. The idea of establishing donor-specific tolerance with IUHSCTx to allow postnatal solid organ transplantation has also been studied in the pig model. IUHSCTx (using adult bone marrow-derived HSC) in fetal swine led to prolonged survival of a kidney allograft [36], providing experimental support for the use of this strategy in fetuses with congenital renal anomalies. While the human immune system will present its own unique challenges, these large animal models demonstrate the technical feasibility of in utero transplantation and confirm the potential to achieve stable engraftment after IUHSCTx in an immunocompetent host.

Human Clinical Experience

Despite its success in large animal models, the promise of IUHSCTx has not been realized in humans (reviewed in Ref. [2]). The only successful cases of engraftment have been in fetuses with bare lymphocyte syndrome (BLS) [49] and severe combined immunodeficiency (SCID) [50–54]. In these cases, either fetal liver-, paternal BM-, or maternal BM-derived $CD34^+$ cells were transplanted between 16 and 26-weeks of gestation and resulted in engraftment of donor cells at birth. Follow-up at 4 years was available for one of these patients [2] who continued to have cellular reconstitution and intact immune responses to vaccinations.

The lack of engraftment after IUHSCTx in diseases other than SCID has led investigators to study the barriers that limit transplantation success. Identifying common factors that lead to poor engraftment is challenging as there are many inconsistencies among these reported cases. For example, transplantations occurred at different centers, donor cells were obtained from different sources, and the transplantations were performed at varying gestational ages. The inherent variability in these studies has made it difficult to attribute the lack of success to any one specific factor, necessitating the use of animal models to gain insight into the barriers that limit engraftment after IUHSCTx.

Factors Limiting Engraftment After IUHSCTx

Improving engraftment is the most important challenge that must be overcome before IUHSCTx can be used to treat diseases other than SCID, for which there is a clear donor advantage. Several theories have been proposed to explain the poor engraftment seen in humans after IUHSCTx [2]. It is possible that the fetal environment is competitive, and thus, donor cells are at a disadvantage when transplanted into an intact host. Alternatively, there could be a limited number of hematopoietic niches that are available for donor cells. There is also an evidence to suggest that the allogeneic

cells transplanted in utero are susceptible to a host immune response. These theories provide a framework for investigating why IUHSCTx has not realized its therapeutic potential.

The idea of a competitive host-cell environment limiting transplantation success is supported by the observation that conferring donor cells an advantage leads to higher rates of engraftment. When using c-kit knockout mice with a deficiency of host HSC proliferation, full immune reconstitution is demonstrated after the transplantation of only one or two donor HSC [55]. Preconditioning regimens that result in the selective depletion of host HSC result in high rates of engraftment, suggesting that vacating host stem cell niches improves chimerism after transplantation [56]. Furthermore, engraftment after IUHSCTx in an irradiated host is maintained consistently despite a relatively lower number of transplanted cells [57]. Modifications to the host hematopoietic environment that maintain SDF1-α induced migration [58] or inhibit fetal hematopoiesis [59] may improve donor-cell homing and engraftment, but clinical applications will obviously need significant testing to ensure safety.

The number of available niches and the proliferative capacity of the fetal environment add to the competitive disadvantage of donor allografts. Gradually increasing the dose of donor cells results in an eventual plateau of engraftment efficiency in an allogeneic and xenogeneic fetal lamb model, suggesting that the favorable stoichiometry of donor cells cannot overcome the limited hematopoietic engraftment sites that are available [60]. Studies have also demonstrated that the proliferative nature of the fetal environment contributes to the host competitive advantage. Rapid homing of donor adult bone marrow cells to the fetal liver occurs after IUHSCTx and is followed by a decrease of donor-cell engraftment, demonstrating the ability of host cells to outcompete donor HSC [61]. Intrinsic proliferative properties of fetal liver cells likely account for the advantage of the host, thus providing an explanation for the poor engraftment seen when adult BM cells are transplanted in humans [62].

The host immune system also serves as a barrier to IUHSCTx. As reviewed by Perenteau et al., several observations support this conclusion: immunologically active T cells are present in the fetus during early gestation, immunization occurs in fetuses that are exposed to prenatal antigen, and successful engraftment in humans has only occurred in immunodeficiency disorders [63]. There have been several corroborating reports describing lower rates of success when transplanting allogeneic donor cells compared to congenic cells, suggesting there is an adaptive immune response to IUHSCTx [34, 35, 64]. The mechanism by which the adaptive immune response limits engraftment remains unclear but likely involves host T cells, B cells, and NK cells. NK cells have been shown to be critical in limiting transplantation success when donor-cell engraftment is below a specific threshold [64]. It is also possible that microchimerism leads to sensitization instead of tolerance in some cases [65]. Further studies are needed to elucidate the mechanism by which the immune system inhibits donor engraftment.

It is possible that immunomodulatory strategies could be used to improve the success of IUHSCTx. Several studies have demonstrated the use of corticosteroids or antibody-mediated immunosuppressants to inhibit the fetal immune response [66, 67]. While immunosupression of the fetus is an aggressive measure which will need extensive preclinical testing, more recent work by Merianos et al. has demonstrated that the maternal immune response, acting through antibodies transmitted in breast milk, may actually be responsible for donor-cell loss [35]. Thus, transplantation of maternal cells (to which the fetus may already be tolerant) or regulation of the maternal immune response using immunosuppressants may also provide an alternative strategy to enhance transplantation success.

Complications

Fetal stem cell transplantation is technically simpler than many current fetal interventions and is generally well tolerated. The minimally invasive

nature of in utero cellular transplantation decreases the chances for preterm labor or membrane separation, both of which commonly occur after more invasive fetal procedures [68]. While graft-versus-host disease (GVHD) is a common complication after postnatal BM transplantation [9], it is rarely observed when mature T cells are transplanted in utero. In one reported case, a fetus with globoid leukodystrophy was transplanted in utero with a high dose of CD34-enriched BM cells without T-cell depletion (>10^7 $CD3^+$ lymphocytes/kg fetal weight). The fetus died at 20 weeks of gestation, and at autopsy was found to have "overwhelming myelopoiesis," findings that are consistent with GVHD. The possibility of developing GVHD after IUHSCTx has been duplicated in mouse models [69, 70]. Although T-cell-depleted CD34-enriched BM can be used to minimize the occurrence of GVHD, engraftment is often lower with such transplants [36].

Future Directions

IUHSCTx holds great promise for the treatment of congenital hematopoietic diseases by inducing donor-specific immune tolerance. As we continue to hone our understanding of stem cell differentiation, the therapeutic applications of in utero transplantation should expand to include the treatment of non-hematopoietic stem cell disorders such as muscular dystrophy [71]. It is also possible that in the era of "personalized medicine," genetically modified-patient-matched iPS cells can be grown from placental chorionic villus sampling [72]. Given the financial constraints to generating patient-specific HSC, tissue banks containing HLA-matched embryonic stem cells can be used as a source of HSC for transplantation. However, any clinical application of such strategies must overcome the current bottleneck in the differentiation of these cells along the hematopoietic lineage in vivo. Improved insights into fetal immunology will then allow us to surmount the current barriers to engraftment and realize the full potential of fetal stem cell transplantation.

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13 Tissue and Progenitor Cell Transplantation for the Management of Pituitary Disorders: From Harvey Cushing to the Next Frontier

Courtney Pendleton
and Alfredo Quinones-Hinojosa

Introduction

The pituitary gland serves an important function in the regulation of growth, metabolism, and reproduction; in states of dysfunction, in particular, in states of hypofunction, clinical symptoms may cause tremendous effects on patient health and quality of life. Traditional treatments for hypopituitarism are pharmacological and require life-long dosing of multiple medications to maintain physiologic homeostasis. Although pharmaceuticals allow for repletion of pituitary hormones, they offer symptomatic relief rather than a cure for the disease. As the fields of immunobiology, regenerative medicine, transplantation, and stem cell research grow in the twenty-first century, the hope is that hypopituitarism may 1 day be cured through transplantation of pituitary stem cells, which may proliferate and differentiate to form functional pituitary tissue, rather than mitigated through synthetic hormones. Here, we present an overview of the various functions of the pituitary gland, the symptoms and treatments for hypopituitarism, the role of pituitary stem cells in restoring endogenous pituitary function, and a look at the past, present, and future of pituitary transplantation.

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Pituitary Gland Functions

The pituitary gland serves as the master endocrine regulator; it plays a vital role in growth, reproduction, and metabolism. The adenohypophysis, the anterior, hormone-secretion portion of the pituitary, contains five secretory cell types: somatotrophs, corticotrophs, lactotrophs, thyrotrophs, and gonadotrophs. These cells secrete growth hormone (GH), adrenocorticotropic hormone (ACTH), prolactin (PRL), thyroid-stimulating hormone (TSH), and gonadotropins, respectively. The posterior pituitary produces oxytocin and vasopressin.

Causes of Hypopituitarism

Hypopituitarism may be either primary, from etiologies affecting the pituitary gland, or secondary, from etiologies affecting the hypothalamus.

Primary hypopituitarism may be caused by pituitary tumors, infections or inflammation, autoimmune diseases, pituitary apoplexy, or iatrogenic causes (radiation therapy, surgical removal). The most common cause of primary hypopituitarism is pituitary tumors. Secondary hypopituitarism may be caused by hypothalamic tumors, inflammatory disease, head injury, or iatrogenic causes.

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Consequences of Hypopituitarism

Clinical presentation of hypopituitarism is dependent on the specific hormone deficiencies present.

Adrenocorticotropic Hormone

Symptoms of ACTH deficiency may be severe and fatal. Patients are deficient in glucocorticoids, but mineralocorticoids are usually within normal ranges. In acute onset deficiency, as in pituitary apoplexy, patients present with hypotension, shock, hypoglycemia, and dilutional hyponatremia.

Thyroid-Stimulating Hormone

Symptoms of TSH deficiency may demonstrate indolent onset, as hypothyroidism may not manifest for several weeks. Symptoms include fatigue, cold intolerance, constipation, weight gain, dry skin, brittle nails, and depression.

Gonadotropins

Symptoms of gonadotropin deficiency may include amenorrhea or abnormal menses in women and sexual dysfunction in men. It should be noted that hyperprolactinemia alone may cause sexual dysfunction.

Growth Hormone

In children, growth hormone deficiency manifests itself in short stature and slow growth velocity. In adults, symptoms may be nonspecific, including decreased muscle mass, fatigue, and changes in sleep. However, adults with GHD may be asymptomatic.

Prolactin

Prolactin deficiency occurs with complete destruction of the anterior pituitary; it is seen mainly in patients with pituitary apoplexy. Deficiency prevents lactation [23]. However, patients with hypopituitarism from tumor compression of the gland may have symptoms of hyperprolactinemia from compression of the stalk.

Vasopressin

Vasopressin deficiency presents as diabetes insipidus. Patients with craniopharyngiomas, suprasellar germinomas, or pinealomas may present with diabetes insipidus.

Oxytocin

Oxytocin deficiency is generally asymptomatic; the hormone plays a role in stimulating contractions during labor and in lactation.

Treatment of Hypopituitarism

Hypopituitarism is currently treated with pharmacotherapy directed at repleting the deficient hormones. ACTH deficiency is repleted with administration of hydrocortisone daily; additional doses are administered during increased physiologic stress. TSH deficiency is repleted with thyroid hormone replacement. Gonadotropin deficiency is repleted using sex steroid replacement (estrogen or testosterone). In asymptomatic adults, GH replacement may not be required. Deficiency in vasopressin may be repleted with synthetic analogues. Although steroid doses may be tapered over time to avoid supraphysiologic replacement, pharmacologic treatment of hypopituitarism is considered to be a life-long proposition.

Early Proposals of Tissue Transplantation for Pituitary and Other Endocrine Disorders

Endocrine Organ Transplantation

The notion of endocrine organ transplantation is not an invention of the twenty-first century; European surgeons Kocher and Von Eiselberg pioneered endocrine organ transplantation in the late nineteenth century [24, 45]. Across the Atlantic, Halsted demonstrated great interest in parathyroid transplantation, performing the first parathyroid autotransplantation in 1907 [20].

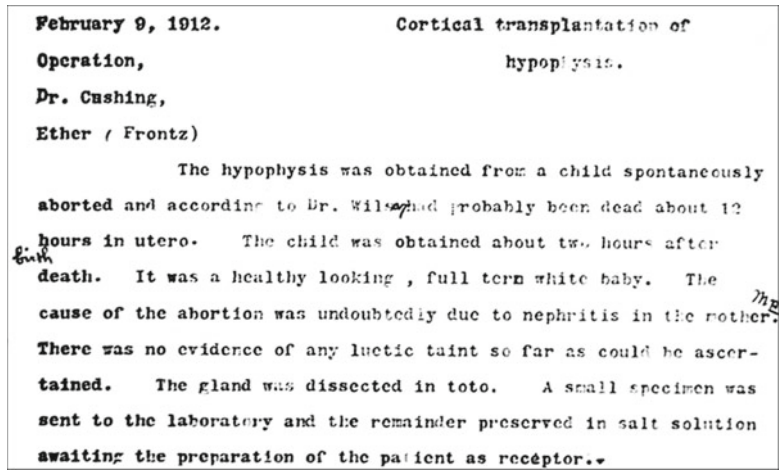
In the midst of these early experiments with endocrine organ transplantation, Harvey Cushing began to pioneer neurological surgery as a viable subspecialty in America. Having completed his residency under the tutelage of Halsted, Cushing was familiar with the tenets of transplantation, as well as the meticulous surgical technique necessary to complete such a procedure successfully [8, 46].

It had already been observed by Kocher that administration of thyroid extract appeared to temporarily resolve symptoms of hypothyroidism, if transplantation of the gland was not performed. Cushing drew on this premise and used pituitary extract to "ward off the characteristic terminal symptoms" of hypopituitarism by administering injections of hypophyseal extract in canine models [12] and patients with hypopituitarism [13]. However, the transient effects of injected glandular extract, coupled with the success of his mentor in transplantation of whole endocrine organs, spurred Cushing to explore the possibility of pituitary gland transplantation [12].

The First Documented Pituitary Transplant

Cushing began his attempts at whole organ transplantation of the pituitary gland with a series of canine studies in 1909; the majority of these experiments involved autologous transplantation; however, one canine model received an allogenic transplant from another canine [12]. These experiments demonstrated that cortical autotransplantation of the pituitary gland into hypophysectomized canines prolonged symptom-free survival [12]. However, Cushing also noted the presence of graft rejection, observing necrotic changes in the transplanted glands [12].

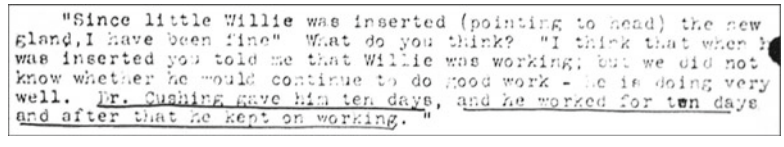
The opportunity to employ a similar technique in a human patient came in 1911, with a 43-year-old male who presented with symptoms consistent with hypopituitarism and a pituitary lesion. Following a series of injections of pituitary gland extract, and perhaps at the insistence of the patient's wife, Cushing brought the patient to the operating room for transplantation of a stillborn, full-term, baby's pituitary gland into the patient's cerebral cortex [33] (Fig. 13.1). The patient remained symptom-free after the operation and discontinued his pituitary gland extract injections (Fig. 13.2). He was discharged from the hospital and remained without recurrence of symptoms for 6 weeks. At that time, his symptoms recurred, and he returned to the Johns Hopkins Hospital for a second pituitary gland transplant, also taken from a stillborn, full-term, baby [33] (Fig. 13.3). The patient's condition deteriorated following the second operation; he received injections of



February 9, 1912. Cortical transplantation of
Operation, hypophysis.
Dr. Cushing,
Ether (Frontz)

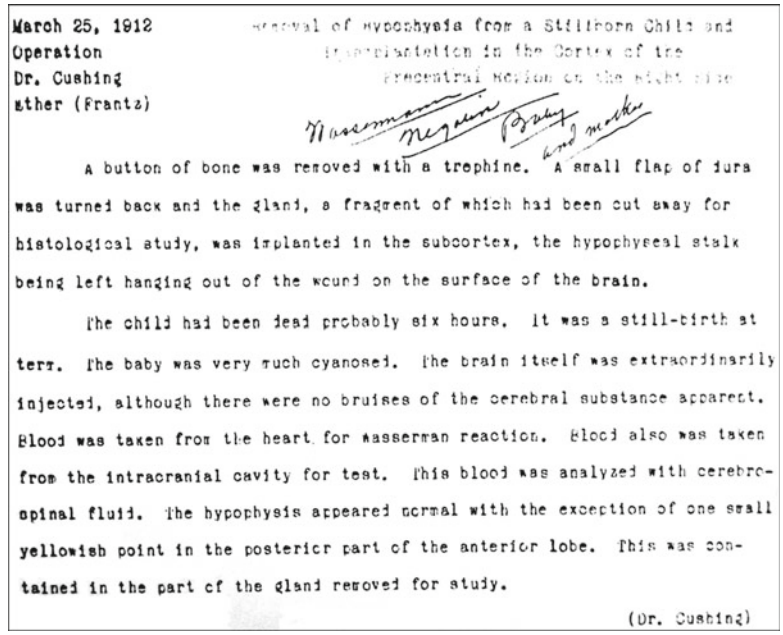
The hypophysis was obtained from a child spontaneously aborted and according to Dr. Wilson had probably been dead about 12 hours in utero. The child was obtained about two hours after death. It was a healthy looking , full term white baby. The cause of the abortion was undoubtedly due to nephritis in the mother. There was no evidence of any luetic taint so far as could be ascertained. The gland was dissected in toto. A small specimen was sent to the laboratory and the remainder preserved in salt solution awaiting the preparation of the patient as receptor.

Fig. 13.1 The operative note from the first documented pituitary gland transplantation, from stillborn infant to an adult male, performed by Dr. Harvey Cushing at the Johns Hopkins Hospital



"Since little Willie was inserted (pointing to head) the new gland, I have been fine" What do you think? "I think that when it was inserted you told me that Willie was working; but we did not know whether he would continue to do good work - he is doing very well. Dr. Cushing gave him ten days, and he worked for ten days and after that he kept on working."

Fig. 13.2 The recipient of the first documented pituitary transplantation describes his transplanted gland ("little Willie") during a postoperative interview documented in the surgical chart


March 25, 1912
Operation
Dr. Cushing
Ether (Frantz)

removal of hypophysis from a Stillborn Child and implantation in the Cortex of the precentral Region on the right side

Wassermann Negative Body and marke

A button of bone was removed with a trephine. A small flap of dura was turned back and the gland, a fragment of which had been cut away for histological study, was implanted in the subcortex, the hypophyseal stalk being left hanging out of the wound on the surface of the brain.

The child had been dead probably six hours. It was a still-birth at term. The baby was very much cyanosed. The brain itself was extraordinarily injected, although there were no bruises of the cerebral substance apparent. Blood was taken from the heart for Wasserman reaction. Blood also was taken from the intracranial cavity for test. This blood was analyzed with cerebrospinal fluid. The hypophysis appeared normal with the exception of one small yellowish point in the posterior part of the anterior lobe. This was contained in the part of the gland removed for study.

(Dr. Cushing)

Fig. 13.3 The operative note from the second documented pituitary transplantation, from a stillborn child to an adult male, conducted by Dr. Harvey Cushing at the Johns Hopkins Hospital

pituitary gland extract and died of pneumonia a month after the operation [33].

Early Discussions of Regenerative Medicine

Cushing was aware of allogenic transplant limitations and contemplated ways to circumvent the issue, drawing on the work of his Hopkins contemporary Ross Harrison, who first cultivated tissue outside a living organism [21]:

> In order to ensure the greatest probability of a successful implantation, it would seem that the best plan of procedure would be […] to secure a growth in vitro of the tissues to be implanted. When a gland is finally secured which can be cultivated in the plasma of the prospective host, the growing fragments may then be injected into the most favorable tissue, possibly even hypodermically, with a probability of "taking." Under these circumstances the survival of the fragments can be foretold with a measure of definiteness which does not pertain to the haphazard implantation of a gland which possibly may find the new soil unfavorable. [13]

Although rudimentary, this proposition is remarkable, given that the field of immunobiology was in its embryonic stages.

Technologic and Pharmacologic Limitations to Early Pituitary Transplantation

Although Eduard Zirm successfully transplanted a human cornea in 1906 [2], the immune-privileged status of that organ allowed for success in the absence of HLA tissue typing and immunosuppressive pharmaceuticals. While Cushing's attempt at human fetal pituitary transplantation did not cure his patient's hypopituitarism, it met with short-term success in symptomatic relief without signs of transplant rejection; this in itself is a remarkable accomplishment, given the undeveloped fields of tissue typing and immunosuppressant therapy.

It was not until the 1940s that the immune system was implicated in the rejection of organ transplants [17, 28], a decade passed between that discovery and the introduction of whole body irradiation or adrenal cortex steroids as potential immunosuppressant for transplant recipients [6, 7, 15, 30]. The possibility of induced tolerance and HLA typing was introduced in 1953 [5], with the specter of graft-versus-host disease, following closely thereafter [4, 40]. Despite Cushing's early efforts at organ transplantation, it was not

until the advent of stronger immunosuppressive agents, such as azathioprine [9, 31] and cyclosporine, that organ transplantation became a viable field.

Contemporary Investigations

Whereas Cushing experimented with whole-tissue transplantation, contemporary physician-scientists have directed their attention to the role of pituitary stem cells in transplantation and regenerating functional pituitary tissue [14]. Cushing's statements in his 1912 monograph, although rudimentary, describe the approach to pituitary stem cell transplantation under investigation today.

Neural Stem Cells (NSC)

It was traditionally held that the adult brain was made up of terminally differentiated cells, without the capacity for renewal. In the 1960s, research discovered neurogenesis within the murine adult brain, primarily in the subventricular zone (SVZ) [1]. More recently, a similar population of astrocytes with stem-like features has been discovered in the human brain, primarily within the SVZ [35, 38].

Pituitary Stem Cells (PSC)

The discovery of NSCs within the adult brain sets off a search for similar stem-like cells within the pituitary. The pituitary gland of newborns already possesses terminally differentiated hormone-producing cells [37, 39], but postnatally, the adenohypophysis undergoes a dramatic growth phase [10, 41]. The adult pituitary gland alters its cellular composition in response to physiological changes; GH-secreting cells double during puberty, and PRL-secreting cells increase during pregnancy and lactation [27]. The plasticity of the pituitary gland throughout adult life has been postulated to be a result of adult stem cells within the pituitary [18, 43].

This theory that stem cell populations may be found in the adult pituitary gland has been supported by evidence that subpopulations of cells within murine pituitary glands express the traditional stem cell markers CD133 [11], nestin [18], and Sox-2 [16]. However, no single cell-type has been defined in the literature as the pituitary stem cell. It has been suggested that these stem cells may be diffusely distributed throughout the gland [26]. Various candidates for pituitary stem cells have been described in the literature; the most commonly discussed is the nonhormone-secreting cells of the adenohypophysis, named chromophobes [14].

The study of chromophobes as a potential pituitary stem cell began in the late 1960s, with reports that these cells could differentiate into acidophils and basophils [32, 47] and form pituitary-like structures [47].

Candidates for PSCs

As only a small subpopulation of chromophobes possessed the ability to self-renew, differentiate, and proliferate to form pituitary-like structures [47], groups began to investigate specific classes of nonhormone-secreting cells of the adenohypophysis [14].

Folliculo-Stellate Cells

These cells were first seen during the early days of electron microscopy [36] and were named for their distinctive star-like morphology and their presence lining the follicles of the adenohypophysis [44]. These cells were proposed as potential pituitary stem cells in the 1960s [47] and were recently shown to possess the ability to differentiate into striated muscle [29], as well as form colonies [26]. These cells have also been shown to express the traditional stem cell marker, nestin [25].

Follicular Cells

Follicular cells are small, irregularly shaped cells found throughout the adenohypophysis [47]. These cells display many similarities to folliculo-stellate cells, and the terms are often

used interchangeably throughout the literature [14]. However, there are distinct differences between the morphology [42] and location [25] of follicular cells, as compared to folliculostellate cells. Follicular cells have also been shown to decrease in number from the embryonic to postnatal stage [48], possible due to follicular cells differentiation into hormone-secreting pituitary cells [48]. In addition, follicular cells have been shown to contain mitotic figures in postnatal stages, unlike folliculostellate cells [22]. Together, these findings make follicular cells an attractive candidate for an adult pituitary stem cell, although there remains no definitive evidence for or against this hypothesis.

Marginal Cells

During development, a portion of the oral ectoderm invaginates to form Rathke's pouch, the embryonic stage of the pituitary gland. In the cleft surrounding the nascent pituitary, the marginal zone contains progenitor cells, or marginal cells [41], which retain selected undifferentiated characteristics into postnatal stages. Murine models have demonstrated cells within the marginal zone which express the traditional stem cell marker nestin [25]. However, no research exists demonstrating marginal cells' ability to self-renew or to differentiate into multiple lineages of hormone-secreting cells.

Side Population

Side population cells are separated from other cell populations using the Hoechst 33342 extrusion first developed by Goodell et al. in 1996 [19]. The presence of side population cells within the pituitary was demonstrated in 2005 [11]; these cells were demonstrated to express the traditional stem cell marker, nestin, as well as Notch 1, a marker expressed during the embryonic stage [11]. Despite these promising results, side population cells remain a heterogeneous class of cells, scattered throughout the pituitary; in addition, no research exists demonstrating side population cells' ability to self-renew or differentiate into multiple lineages of hormone-secreting cells.

Pituitary Stem Cells: A Consensus?

Despite active research in the field of pituitary stem cells, there remains no definitive consensus. Although many lineages of pituitary chromophobes demonstrate expression of traditional stem cell markers, not all these candidates have been shown to self-renew or differentiate into hormone-secreting lineages. While the presence of stem cell markers such as nestin, CD133, and Sox2 may enrich for stem cells within a population, these markers have not been proven to be exclusively expressed on stem cells. As the field of stem cell research develops more specific definitions of stem cells, and more sensitive techniques for isolating these cells, it may be possible to elucidate a single definitive pituitary stem cell.

Risks of PSC Transplantation

Although the potential therapeutic benefits of pituitary stem cells are tremendous, the use of these stem cells is not risk-free; stem cells have been implicated in the formation and propagation of brain cancers, including pituitary adenomas. Therefore, the risks of tumorigenesis must be weighed against the potential therapeutic benefit should pituitary stem cells be considered for clinical usage.

Pituitary Adenomas and Stem Cells

It has been hypothesized that the combination of pituitary stem cells with changes in the pituitary microenvironment may predispose these stem cells to undergo uncontrolled proliferation, thereby forming pituitary adenomas [18, 43]. This theory is further supported by evidence linking neural stem cells and brain tumor stem cells to the formation of brain tumors [34]. Research has demonstrated the presence of cells expressing nestin in regions adjacent to pituitary tumors [18]; it has been hypothesized that these nestin-positive cells are potentially pituitary stem cells and may be linked to tumorigenesis in the pituitary [14, 18, 43]. While this research makes a compelling argument for anatomic proximity of

putative pituitary stem cells to regions of pituitary tumor, a causal relationship has not been established in the literature.

Future Directions

The future of pituitary transplantation lies within the identification of pituitary stem cells and the ability to harness their capacity for differentiation and self-renewal.

Human Pituitary Stem Cells

A substantial portion of the literature regarding pituitary stem cells is derived from animal, particularly murine, models [11, 14, 16, 18, 25, 26]. The presence of human pituitary stem cells has not been definitively demonstrated [16, 18, 27]. Access to human pituitary cells for future research is limited by the challenges to surgically accessing the gland and that access to human pituitary tissue is limited generally to pituitary tumor and the regions of the gland immediately adjacent to the pathology. It has been hypothesized that tissue obtained from or adjacent to pituitary pathology may have alterations in the pituitary stem cell population, as well as the ability of pituitary stem cells to differentiate and proliferate [27]. Although the use of human cadaveric tissue has been proposed, it is probable that postmortem tissue would undergo functional changes that would render it a suboptimal substitute for live human tissue.

Regenerative Medicine

Harvey Cushing suggested in 1912 that future pituitary transplants rely on growing a functional gland from human tissue, preferably fetal or neonatal tissue, and transplanting the gland grown in vitro into the patient [13]. Although this may have seemed an outlandish proposition at the time, contemporary research into regenerative medicine has turned Cushing's proposal into reality; researchers have successfully created whole organs using human stem cells grown on synthetic armatures and transplanted these into patients with reasonable success [3]. Combining research into human pituitary stem cells with the burgeoning field of regenerative medicine may allow Cushing's early experiments with human pituitary transplantation to guide the field in the twenty-first century.

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14 Fetal Cell Therapy and Tissue Engineering for Musculoskeletal Tissues

Nathalie Hirt-Burri and Lee Ann Applegate

History of Fetal Cellular Therapy

Cellular therapy using embryonic and fetal tissue has progressed in the twenty-first century due to the search of new treatments for many accidental and disease states such as Alzheimer's, Parkinson's, diabetes, heart disease, nerve replacement, acute and chronic wounds, and severe burns [1–24]. Already in the 1930s, medical doctors and scientists have used tissue from voluntary pregnancy interruptions not only for understanding cell biology but also for an important entity in the development of vaccines by using defined tissue-derived cell lines. The Nobel Prize for Medicine in 1954 was awarded to American immunologists who developed the polio vaccine based on cultures of human fetal cells. Since this time, many other necessary vaccines (rubella, chicken pox, hepatitis A, etc.) have been developed with the use of fetal cell lines including two primary human diploid cell lines which were originally prepared in the 1960s. The first cell line, WI-38 (Wistar Institute 38) was developed by Leonard Hayflick in 1964 from fetal tissue from a voluntary pregnancy interruption and later given the ATCC (American Type Culture Collection) number of CCL-75. This cell line was used for the historical production of vaccine RA 27/3 against rubella [25, 26].

There is some confusion between the terminology of embryo or fetal research and the derived stem or fetal cells that can be obtained from these specific tissues. This is understandable since the terms are used in different contexts. In legal terms, "fetus" refers to all prenatal stages, and the term "embryo" denotes the earliest stages following fertilization of an ovum by a sperm (Fig. 14.1). Zygote would include early stage cleavage embryos produced by cell division up to 50–60 cell stage (each cell which is a blastomere) and the blastocyte for the 60 cell stage to the point of implantation at about 2 weeks post fertilization. Pathology would classify the embryonic stage up to 9 weeks of gestation and thereafter as the fetal stage from 9 weeks until birth.

From each of these tissues, different cell lines can be established using tissue culture techniques with varying degrees of complication. Embryonic stem cells are developed from preimplantation embryos from the inner cell mass before the first 2 weeks of development. These cells are frequently obtained from extra embryos developed by "in vitro" fertilization techniques to aid couples for fertility purposes (Fig. 14.1).

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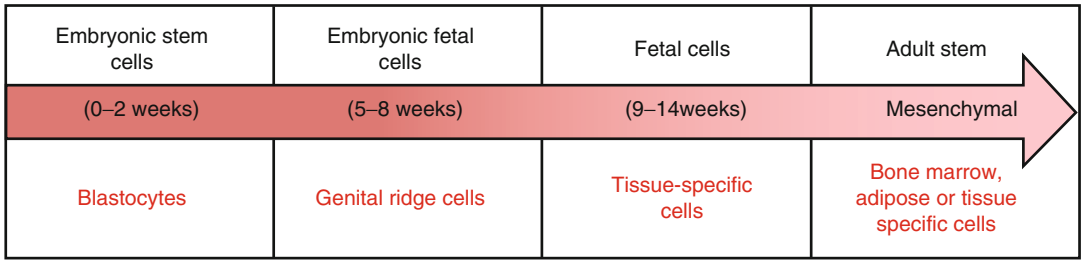

Fig. 14.1 Cellular source can come from human and animals at different stages of development including embryonic, embryonic-fetal, fetal, and adult involving different beginning tissue sources ranging from zygotes to specific tissues (bone marrow, adipose, amniotic fluid, skin, liver, bone, cartilage, etc.)

Most fetal cell research is developed from specific tissues at the latter end of the first trimester (11–14 weeks) following voluntary interruption of pregnancies. This process is considered legal in most countries. Cell lines at this stage are tissue-specific and therefore differentiated with specific functions.

This chapter will address some of the new methodologies related to cellular-based therapeutics using one organ donation of fetal tissue to develop master cell banks and delivery systems that can treat hundreds of thousands of patients for tissue reconstruction and repair particularly for musculoskeletal tissues.

Master Cell Banks Using One Organ Donation

Although the application for fetal cells in tissue engineering could be broad, high levels of safety and consistency in cell banking are common features necessary for their development in the clinic. Figure 14.2 illustrates our Swiss fetal cell therapy platform that was developed for musculoskeletal tissues including skin, muscle, bone, cartilage, tendon, and intervertebral disc. Cell banking is a major advantage for the development of fetal cellular therapies (Fig. 14.2). The mother donor can be assessed for specific viruses and infections by donating blood at the time of her pregnancy interruption and again at 3 months to assure that there would be no seroconversion. The tissue that has been donated by the mother can be put into primary culture as soon as possible (time to transfer to a dedicated culture facility) in order to create a prebank of high quality. For this, fetal biopsies were cleaned of adherent tissue, washed three times for 15 min each in phosphate-buffered saline (PBS – NaCl 6.80 g/l, Na_2HPO_4 1.48 g/l, KH_2PO_4 0.43 g/l), dissected, and put into culture in tissue culture grade plates (60 cm, Falcon). Once the adherent cells reached 80 % confluence, they were trypsinized (EDTA and trypsin solution, Gibco) and amplified, and ampoules of cells in 1 ml of freezing medium (50 % DMEM, 40 % FCS, 10 % DMSO) were stored in liquid nitrogen. Cell banks were stored in the vapor phase of alarm-fitted (to assure sufficient liquid nitrogen) liquid nitrogen storage vessels (Carbagas) rather than in the liquid phase, and portions of each cell bank were split between different vessels in separate locations for security. Data tracking is essential for cell banks including a certificate of analysis for each cell bank giving its designation, number of ampoules prepared, date, tests performed, specification, and results. Ampoules were labeled with the cell bank code and each ampoule individually numbered. Logbooks were used to record all ampoule movements including the initial deposit of the cell bank and also each time an ampoule was removed whether for testing or for experimental purposes. These changes were signed and dated in the cell bank logbook. Prebanks of each fetal tissue type can then be submitted for GMP processing in an accredited facility for master and working cell bank production and subsequent testing.

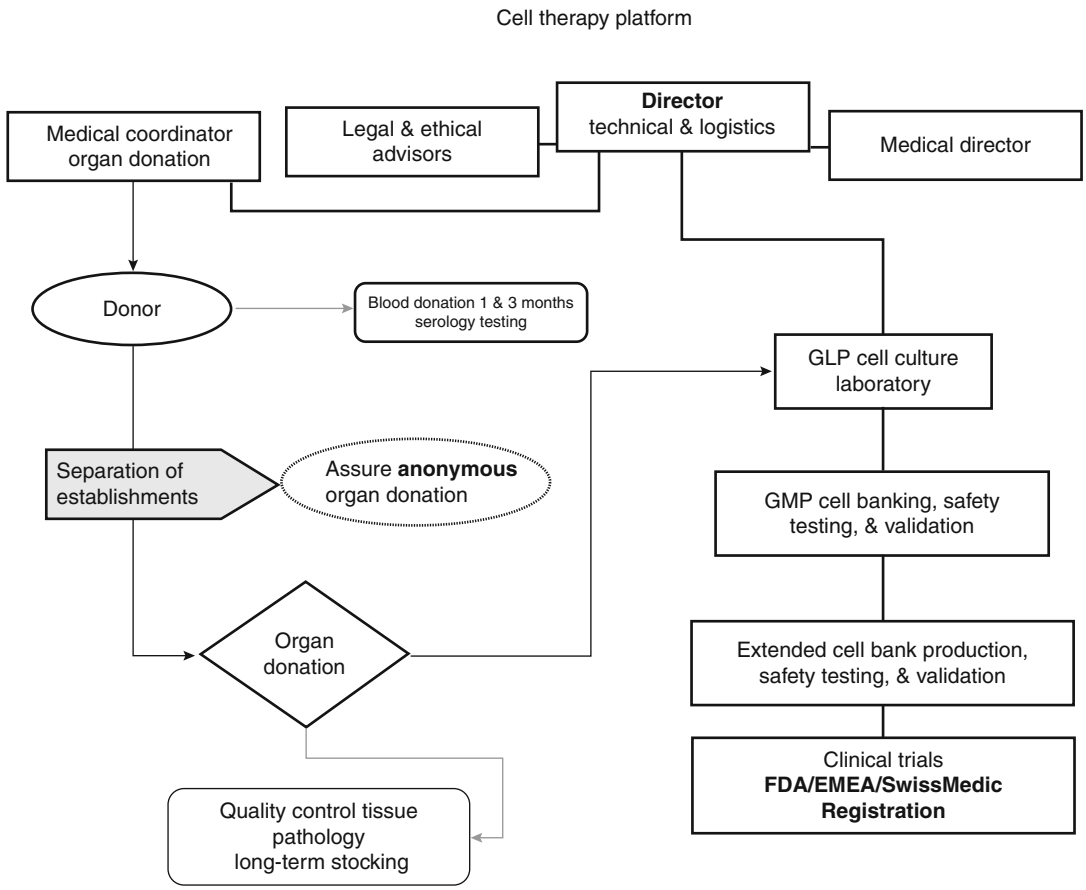

Fig. 14.2 Organization of a cell therapy platform. Director of technical and logistics coordinates the program with essential legal and ethical advisors and a medical director for interpretation of medical quality assurance (serology and pathology reports). The separation of hospitals for the organ donation and all other aspects of the platform including serology, pathology, and the GLP cell culture laboratory can assure complete anonymous and coded organ donations. Importantly, the director of technical and logistics is not involved in any manner in the organ donation process as required by law. Final approval for use of validated cell banks for human therapy is coordinated and approved with both hospital ethics committees and national regulatory agencies (i.e., European Medicines Agency, Food and Drug Administration, and Swissmedic)

The master cell bank (MCB) cell lines destined for clinical use can be characterized and extensively tested (to date, we have used GMP facilities of Bioreliance in Glasgow, Scotland). Testing requirements were based on those required for human diploid cells used for vaccine production and for cell substrates used for biotechnological products. Although the identity of the donor is obviously a human source, isoenzyme testing to show Caucasian human origin is necessary for documentation. Other tests were sterility, mycoplasma, and retroviral reverse transcriptase activity (FPERT assay). Examination for viruses, virus-like particles, mycoplasmas, fungi, yeasts, and bacteria was done with a minimum of 200 cell profiles with quantitative transmission electron microscopy. In vitro testing of picornavirus, orthomyxovirus, paramyxovirus, herpesvirus, adenovirus, and reovirus were accomplished with several control cell lines. In vivo virus testing was completed using suckling mice, adult mice, guinea pigs, and embryonated eggs. Human virus detection was screened using Q-PCR for all of the following viruses: Hep B, Hep C, HIV-1, HIV-2, HTLV-1, HTLV-2, HHV-6, HHV-7, HHV-8, EBV, hCMV, and SV40. B19

parvovirus was also screened with Q-PCR. Safety testing of the working cell bank (WCB) for preclinical and research projects can then be somewhat limited as compared to the MCB safety testing since each MCB ampoule results in a full WCB. Safety testing for laboratory research developed cell banks needs to be continually controlled for mycoplasma and bacterial contamination (Fig. 14.2).

Regarding the use of the cells for only preclinical trials, it will be particularly important to ensure consistency of growth of the cells and consistency of the harvest obtained. High consistency in fetal cell banking can be achieved due to the minimum nutrient requirements of fetal cell cultures. In contrast with mesenchymal stem cells, fetal cells do not require feeder layers for growth nor growth factors for differentiation. For skin tissue engineering, many products to date use a pool of multiple donors for a batch of cells. The differences between donors would not be as important since many donors could be used. There is low heterogeneity between fetal donors for skin cell banks established in the same manner (large-scale expansion). Cell counts, protein determination, and gene expression analysis are useful tools to assess the consistency of the cell banks when there is only one unique donor for fetal tissue engineering. Limits of variation for these assays specific for each cell type will need to be established internally to define the consistency of the process. For therapeutic use, fetal cells can be used up to 2/3 the life-span in an outscaling process. However, if the goal is to develop a "product" used on a large-scale or a multicentric basis, assessment of the cell-derived product should not be restricted to description parameters such as protein concentration but should include a functional assay. Furthermore, activity of the final product is likely to be due to synergistic effects of multiple proteins, which cannot be assessed by individual protein concentration or activity. End of product cell banking would also be an essential step. Fetal cells at higher passage number than the intended clinical use would need to be established in an MCB for extensive testing, including in vivo tumorigenesis testing in nude mice (Fig. 14.2).

Each element necessary to produce a successful cell therapy needs to verify safety and consistency in the development. Cell choice is of utmost importance, and progress to assure the consistency will be necessary before allowing clinical trials. Using only one fetal organ donation, it is possible to develop extensive MCB and WCB. Fetal cells, as already differentiated lineage, need low nutritional requirements (i.e., no feeder layers or external growth factors) to establish a fully defined consistent cell bank and can easily be assessed for safety assuring an interesting cell choice.

Preclinical and Clinical Experience with Fetal Cellular Therapy

Clinical Experience in Skin Tissue Engineering

In the past years, our laboratory has shown that fetal skin cells, more specifically fetal dermal progenitors, offer an ideal solution for effective and safe tissue engineering for wounds of all nature for several reasons including (a) cell expansion capacity from one organ donation, (b) minimal cell growth requirements, (c) adaptation to biomaterials for delivery, and (d) wound-healing capacity. We demonstrate that fetal skin progenitors have extensive expansion possibilities as it requires only one organ donation (1–2 cm^2 tissue) to create enough frozen cells to produce a bank capable of making over 900 million fetal skin constructs (9 × 12 cm Baxter Tissue Fleece equine collagen scaffold) .

We were able to perform two clinical phase I and II studies, and these were accomplished on a total of 20 patients including eight pediatric mild to severe burns (2nd and 3rd degree), two acute wounds, and on ten recalcitrant leg ulcers in elderly adults. All of the clinical results were published in Lancet, Cell Transplantation, and Experimental Gerontology [7, 8, 10, 27].

Preclinical Experience in Bone Tissue Engineering

Human primary fetal bone cells derived from the 12–14 weeks fetal femurs retain several features of

MSCs such as a fibroblast shape, a high capacity to proliferate, and can differentiate into osteoblasts when cultured into an osteogenic medium [12]. They also express typical bone markers such as alkaline phosphatase, Runx-2, alpha-1 chain of type I collagen, and osteocalcin and form a mineralized extracellular matrix. Also, when seeded on a polymer scaffold, these cells can spread on scaffold surface, differentiate, and bone in growth is observed in a rat critical size defect craniotomy model with no inflammation reaction after 4 months [13]. The validation in three different animal models including mice, rat, and sheep has shown not only efficacy but also safety for the use of allogenic therapies in regenerative medicine for bone defect using fetal bone cells. The fate of the fetal cells for in vivo repair could be due the cells acting as a reserve growth factory to simulate host tissue repair or provide a defined extracellular matrix.

Preliminary studies in sheep have shown that fetal bone cells delivered in hydrogels are also able to promote organized bone growth in vivo in short periods of time. Whether this promotion of bone growth is directly related to fetal cell transplantation is not known. There has been some experimental evidence that fetal tissues respond to injury with a more rapid healing response in the absence of scar formation. However, it remains elusive whether the cells themselves have a higher "regenerative" potential or whether the fetal cells promote an environment to more conducive repair through growth factors and cytokines. However, biopsies taken from human patients that had been treated with fetal skin cells 6 months following treatment of severe burns would indicate that it is the latter mechanism which is responsible.

Preclinical Experience in Muscle Tissue Engineering

The proliferation rate of primary fetal skeletal muscle cells is similar to that of fetal skin and fetal bone cells but more rapid than young and adult muscle donor cells [11]. Primary fetal muscle cells present a higher number of cells expressing desmin than did young- and adult-derived muscle cells, and this expression is conserved after long-term cryopreservation. Cell delivery is an important aspect of cell therapy; several hours can elapse from the laboratory to the clinical application with fetal cells. We show that even after 24 h at room temperature in saline solution, 60 % of fetal muscle cells are still viable. After 1 h, our results are comparable with other publications which show 93–97 % viability of the cells after 1 h on ice [11]. In a preliminary *in vivo* study at longer time points, primary human muscle progenitor cells from our working cell bank were cultured, frozen and then thawed at passage 3 to be marked with the PKH26 red fluorescent cell linker kit. Labeled cells were seeded on collagen scaffolds ($1.5x10^6$ cells/cm^2) and the collagen disc contained marked muscle progenitor cells at a dose of $5x10^4$. Normal C57BL6 mouse thighs that had a 4 millimeter diameter dermatology biopsy punch were treated with live cells, collagen alone or no treatment. In our study, we can still observe human fetal skeletal muscle cells 8 weeks after implantation into wounded gastrocnemius C57/bl6 mice. Study described above now.

Preclinical Experience in Spine Tissue Engineering

Fetal spine cells have been shown to have essential high proliferation capacity and differentiation to cartilage-like tissue and may also provide an interesting cell source for vertebral disc degeneration [16].

Delivery Systems

The relatively simple manipulation of fetal cells, related to their collection, culture expansion, and storage, has made fetal cells an attractive choice for cell therapy. Equally important is the delivery system of chosen cells and their interaction with scaffolds to create a three-dimensional tissue. Hydrogels, whether synthetic or naturally derived, have extensive use in medicine, pharmaceutical, and basic sciences. Hydrogels provide a hydrate space and a mechanical carrier for cell transplantation and tissue engineering. The interest in many

studies of hydrogels and collagen-based matrixes is the use of these materials as clinical injectable cell delivery directly within defect areas for tissue engineering to minimize surgical procedures. Collagen has been incorporated into a variety of medical devices and has been used for multiple proposes. For maxillofacial application, resorbable forms of collagen have been used for closure of graft and extraction sites, for delivery of autologous bone and soft tissue residues, to dress oral wounds, and to promote healing. In all, biocompatible biomaterials need to be available in order to provide an extracellular matrix environment for cell differentiation and release. Cell therapy is an alternative with less invasive procedures for replacement of tissues necessary to supply areas for the support of structural tissue (Fig. 14.3).

Conclusions and Perspectives

Fetal cell-based therapies are being developed and introduced for all types of tissue repair including but not limited to, skin, bone, cartilage, muscle, and spine. They offer promise for repairing and/or replacing damaged tissue and restoring lost functionality. One of the major challenges for assuring that more patients will benefit from cell-based therapies in the future will be the optimization of the choice of cell type as well as their isolation and proliferation. Equally important will be the delivery system of the cell choice and their interaction with these scaffolds to assure biocompatibility. The development of master cell banks from the cell choice provides a major advantage for the creation of a therapeutic biological agent. Fetal cells, from the latter portion of the first trimester of gestation, are easily adapted to this type of development since only one organ donation is necessary to develop an MCB from fetal tissue. Fetal cells, unlike many stem cell types, do not need feeder layers nor external growth factors for cell differentiation processes. Fetal cells are differentiated cells since they are derived from specific tissue and organs. Even though stem cells have an advantage to change and differentiate into multiple cell lineages, they are difficult to assure the final cell type differentiation when placed in vivo. Embryonic stem cells also produce tumors in vivo and need to be cloned to assure that this aspect is eliminated or encapsulated before cell delivery.

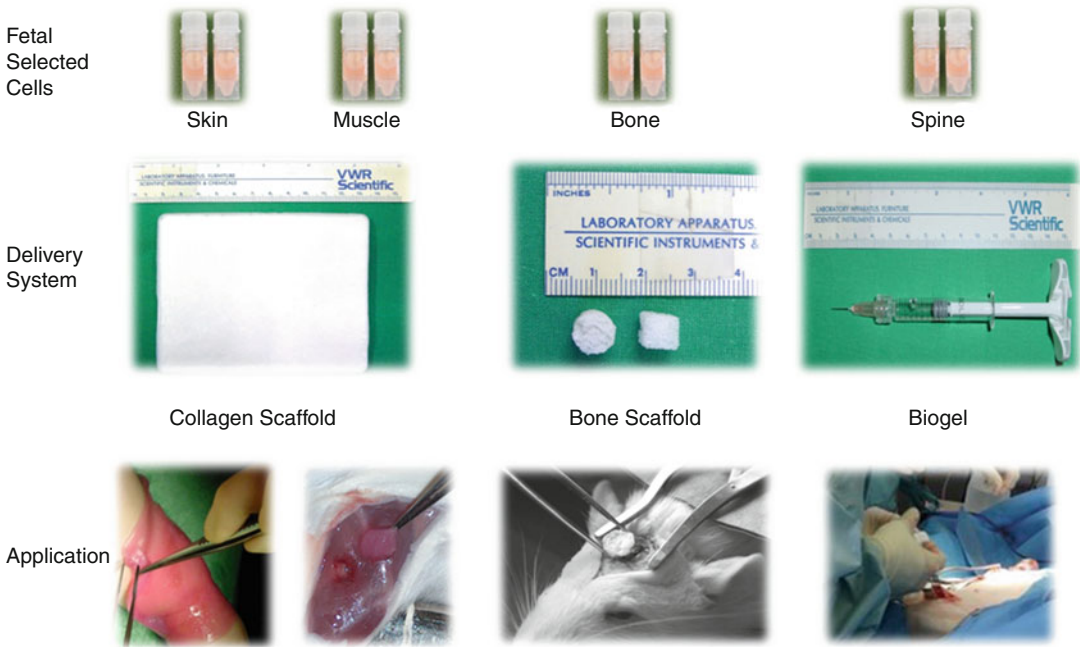

Fig. 14.3 Different types of delivery systems can be used for fetal cell tissue engineering

Once master cell banks can be produced, working cell banks can be produced to establish individual batches of treatments for high numbers of patients (for skin and bone, this can be up to hundreds of thousands of patients from one cell banking process and from one organ donation). Further, these cell banks can be tested completely for safety regarding sterility, pathogens and adventitious agents, and tumorigenicity. Once safety can be assured, efficient cell presentation with biocompatible delivery systems can be assessed for specific tissues. In order to conduct clinical trials, industrialized processes will have to be incorporated and most likely need industry support for success since new laws and restrictions have made it nearly impossible for hospitals to develop advanced cellular therapies.

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Cell Therapy for Parkinson's Disease

15

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In the 1990s, a succession of reports on revolutionary technologies appeared in the field of developmental biology and covered the establishment of human embryonic stem cell (ES cell) lines [1] and the creation of "Dolly," the cloned sheep [2]. Since then, regenerative medicine has been the focus of a great deal of attention, and more recently, the establishment of human-induced pluripotent stem cells (iPS cells) has reinvigorated the field [3, 4].

As neurons are terminally differentiated and do not divide, diseases, with Parkinson's disease and spinal cord injury being the most important, and aging process in the central nervous system have been the major targets for regenerative medicine. Although it has been reported in 1960s that cells surrounding the cerebral ventricles have the capacity to divide and differentiate into neurons [5, 6], this finding went largely unnoticed until studies in which BrdU, a marker for dividing cells, was administered in vivo to adult human in the 1990s [7, 8]. The studies with BrdU demonstrated that neural stem cells reside in the subventricular zone and dentate gyrus and have the potential to form new neurons, namely, adult neurogenesis. Subsequently, a procedure for collecting neural stem cells from the living body and culturing them in vitro as a neurosphere has been established [9]. Methods to enhance endogenous neurogenesis have therefore a great potential in treating central nervous system disorders.

Parkinson's disease has been the leading target condition for cell therapy since human adrenal gland cells were transplanted into the brain of four patients in1987. Soon after, fetal midbrain cells were used for restoring dopamine production in the striatum. Initial open-label studies of fetal cell transplantation for Parkinson's disease showed marked recovery in some patients with abundant supply of donor cells being the only drawback. In this respect, ES and iPS cells, which can abundantly create neurons, seem to provide a groundbreaking solution in cell therapy for Parkinson's disease. Although recent studies have indicated that Parkinson's disease is associated with other factors beside a lack of dopamine and that there is doubt whether cell transplants will ever show a clear benefit beyond what can be achieved by existing therapies, the possible benefits of a novel cell therapy with refined methodology and special stem cells are worth exploring.

We herein summarize the current therapeutic applications for Parkinson's disease, including both standard and cutting-edge methods. We also discuss the research to be taken for stem cells therapy to reach the clinic.

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Parkinson's Disease

Parkinson's disease is a movement disorder common among the elderly [10]. The four main symptoms of this disorder are *tremor* or trembling in hands, arms, legs, jaw, or head; *rigidity* or stiffness of the limbs and trunk; *bradykinesia* or slowness of movement; and *postural instability* or impaired balance. These symptoms usually begin gradually and worsen with time. As they become more pronounced, patients may have difficulty in walking, talking, or completing other simple tasks. Not everyone with one or more of these symptoms has Parkinson's disease, as the symptoms sometimes appear in other diseases as well. Parkinson's disease is a progressive chronic neurodegenerative disorder, meaning its symptoms grow worse over time. During Parkinson's disease progression, there is a loss of neurons in the substantia nigra pars compacta (SNc) that project to the striatum (caudate and putamen), which leads to a severe decrease in dopamine content in the striatum. Although some Parkinson's disease cases appear to be hereditary and a few can be traced to specific genetic mutations, including α-*synuclein*, *parkin*, *DJ-1*, *PINK1*, *and LRRK2* genes, most cases are sporadic. Therefore, Parkinson's disease is believed to be due to a combination of genetic susceptibility and exposure to environmental factors that trigger the disease. Parkinson's disease is the most common form of *Parkinsonism*, the name for a group of disorders with similar features and symptoms. The onset of Parkinson's disease can be at any age between 30 and 80, but it most commonly occurs during middle age or later. The prevalence rate increases with age and reaches approximately 1 % over 60 years old.

Therapeutic Options for Parkinson's Disease

Pharmacotherapy

The onset of motor symptoms in Parkinson's disease is closely linked to dopamine deficiency in the striatum. In the early stages of the disease, the symptoms can be dramatically alleviated by administration of L-3,4-dihydroxyphenylalanine (L-dopa), a precursor of dopamine. L-dopa is converted to dopamine in the brain by the enzyme aromatic L-amino acid decarboxylase (AADC). Although other drugs that stimulate dopaminergic D_2 receptors (D_2R agonists) are clinically available for Parkinson's disease, their potency is relatively low, and L-dopa remains the mainstream pharmacotherapy. As the disease progresses, marked loss of dopaminergic neuronal termini leads to a profound decrease in both AADC activity and the capacity for dopamine storage, resulting in reduced efficacy of L-dopa. Frequent systemic administration of high doses of L-dopa causes oscillation in motor performance and deleterious complications, such as hallucination, due to dopaminergic stimulation of the mesolimbic system. Motor problems with the long-term use of L-dopa include the on/off phenomenon, in which the drug suddenly becomes ineffective; the wearing-off phenomenon, which involves a decrease in the duration of maintenance of efficacy; and dyskinesia, which is characterized by dystonia and choreic involuntary movements. The usefulness of drug therapy is therefore limited, and there is a great demand for development of new treatment methods.

Deep Brain Stimulation

During the past decade, deep brain stimulation (DBS) has rapidly replaced electrocoagulation to become a widely used method for treating various neurological disorders. In DBS, stimulatory electrodes are inserted by stereotaxic surgery and positioned at a specific site in the brain, which is the subthalamic nucleus or globus pallidus in the case of Parkinson's disease, and the thalamus in the case of tremor. Intracerebral stimulation is then carried out using a pulse generator implanted subcutaneously in the anterior thoracic region. DBS has several advantages over classic lesional surgery, in that the effects are reversible, there are fewer complications, and the device can be set so as to be operated remotely: outside the patient's body, enabling adjustment of the stimulation, and

thus achievement of optimal efficacy. In Parkinson's disease, reduced dopaminergic input to the striatum is thought to ultimately result in increased neuronal firing of inhibitory basal ganglia output and disturbed firing patterns with increased synchronization. DBS is believed to alleviate these abnormal neuronal activities, though its mechanism of action remains unclear [11]. High-frequency stimulation of the subthalamic nucleus is effective against tremor, muscular rigidity, short-stepped gait, involuntary movement, hallucination, and moderately stooped posture. DBS increases the "on" time period of good symptom control and constitutes a therapeutic advance for severely disabled patients with Parkinson's disease in whom long-term pharmacological treatment has failed. However, no efficacy has been achieved by DBS against balance disorders, frozen gait, extremely stooped posture, or lateroflexed posture. In addition, DBS may result in neuropsychiatric adverse effects with increased suicide rate being reported [12]. It is also difficult to use DBS in patients who have dementia or serious systemic complications and/or are not less than 75 years old. Therefore, the long-term outcomes of DBS need to be defined.

Gene Therapy

The recent development of viral vectors, especially vectors derived from adeno-associated virus (AAV), has helped move gene therapy for Parkinson's disease from animal experiments into clinical trials. The current clinical trials of gene therapy for Parkinson's disease are based on three major strategies [13]: (1) Local production of dopamine via introduction of dopamine-synthesizing enzyme genes into the putamen. In this fist strategy, AADC gene is transferred into the putamen to efficiently convert orally administered L-dopa to dopamine. The delivery of triple genes including tyrosine hydroxylase (TH), guanosine triphosphate cyclohydrolase I (GCH), and AADC can also be undertaken and is aimed at continuously supplying dopamine into the putamen. (2) Protection of nigrostriatal projections via production of neurturin, a trophic factor for dopaminergic neurons, in the putamen. (3) Modulation of neural activity along the output pathway of basal ganglia by transducing the subthalamic nucleus with vectors expressing glutamic acid decarboxylase (GAD-65, GAD-67), a key enzyme required for the synthesis of the inhibitory transmitter γ-aminobutyric acid (GABA).

Administration of immunosuppressive agents is not necessary for viral vector-mediated gene delivery. Compared with DBS, gene therapy can avoid the risk of infection associated with electrode implantation, and there is no need to replace batteries. The initial results of phase 1 studies using AAV vectors have confirmed the safety of these vectors and have revealed alleviation of motor symptoms associated with Parkinson's disease [14–16]. Following the success of these studies, a phase II double-blind study with a sham surgery control was undertaken for neurturin gene therapy. Based on press-released preliminary data, no benefit on motor symptoms was obtained at 12 months, although some beneficial effects have since been observed in the subjects followed for 18 months under blind conditions. Since placebo effects may be overwhelming in the case of invasive therapies for Parkinson's disease, further studies in this field are necessary.

Cell Therapy for Parkinson's Disease

Target Region for Cell Implant

In ideal cell therapy for Parkinson's disease, dopaminergic neurons are implanted into the SNc, and axonal projections to the striatum are reconstituted. However, in reality, it is difficult to regenerate complex fiber connections between the transplanted neurons and host tissue. To date, most cell transplantation protocols for Parkinson's disease have adopted ectopic implantation of dopamine-producing cells into the striatum, mainly into the putamen. In that situation, grafted cells are expected to achieve functional improvements that could be clinically valuable through mechanisms beyond restoring dopamine production, such as immunomodulation.

Early Stage Clinical Research

Initially, the adrenal medulla or sympathetic ganglia of the patients themselves were used as donor cells since autografting could avoid immune rejection. However, the results of such transplantation were poor probably because the functions of these donor cells were impaired by aging and pathological changes involved in Parkinson's disease, resulting in insufficient dopamine production in the striatum. Clinical studies using midbrain cells from aborted fetuses have been conducted at several institutions, including Lund University in Sweden and the University of Colorado in the USA. In open-label studies with small numbers of patients carried out at four representative institutions, beneficial effects were observed after transplantation, including a decrease of 43–66 % in the duration of L-dopa ineffectiveness (off time), a decrease of 30–40 % in the motor score in the unified Parkinson's disease rating scale (UPDRS, off time), and a decrease of 16–77 % in the necessary daily dose of L-dopa. In addition, positron emission tomography (PET) measurement of [^{18}F]fluoro-L-dopa (FDOPA), a ligand for AADC, showed an increase by 55–107 % in comparison with before transplantation [17]. Furthermore, PET measurement using [^{11}C]-raclopride, a D_2 receptor ligand, showed that the transplanted cells survive for up to 10 years and that these cells respond to methamphetamine by releasing dopamine [18].

Results of Double-Blind Studies

Two double-blind studies have been carried out in the United States (Table 15.1). The studies tested rigorously the efficacy of cell transplant therapy against sham surgery, in which the patients had burr holes on the skull but did not receive the cell transplant during the blind phase. In the study carried out by Freed et al., 40 patients, with a mean disease duration of 14 years, were allocated to the control group and the transplantation group, each containing 20 patients [19]. Midbrain cells collected from four fetuses were cultured for 2–4 weeks and then transplanted to patients in the transplantation group. Immunosuppressive agents were not administered. After evaluation at 1 year, 14 of the 20 patients in the control group also underwent cell transplantation. As a whole, there was no significant improvement in UPDRS score in the transplantation group compared with the control group. However, when only patients of 60 years old or younger were evaluated, significant improvement was observed with 28 % in total and 34 % in motor UPDRS scores (off time). PET measurement showed increase in uptake of FDOPA by 40 ± 42 % in 19 patients in the transplantation group and by −2 ± 17 % in the control group, in comparison with before transplantation. Postmortem examination of the brains of two patients who died of unrelated cause revealed that more than 10,000 tyrosine hydroxylase-immunoreactive (TH-IR) cells were present in the putamen. These cells were localized within 1 mm of the center of the cell injection track, but their projections extended for 2–3 mm. Runoff dyskinesia that persisted after a substantial reduction or elimination of dopaminergic drugs developed in five patients (15 %). Freed et al. continued the analysis and found that mean response time plus movement time were shorter in the transplantation group than in the control group [20]. It is expected that better results could be achieved if patients who showed favorable responses to L-dopa and no involuntary movement were selected. Long-term outcomes from 33 of the original trial participants suggested that clinical benefit as well as graft viability assessed by FDOPA PET could be sustained for up to 4 years after transplantation [21].

In another study by Olanow et al. [22], 31 patients were divided into the following three groups: (1) single-donor group, cells from one fetus were transplanted into each side of the putamen; (2) four-donor group, cells from four fetuses were transplanted into each side of the putamen; and (3) control group, no cells were transplanted. The patients were evaluated 24 months after transplantation. Although no significant difference among the groups was found in the UPDRS motor score, there was a consistent tendency toward improvement in this score during the period of immunosuppressive agent administration. In addition, when evaluation was restricted

Table 15.1: Double-blind study of fetal cell transplantation: Summary of results [13, 14]

Institution	Denver, Colorado	Tampa, Florida
Number of patients	Two-donor group: 20 Control group*: 20 *14 patients received transplants after they had completed double-blind phase, which lasted for one year.	Four-donor group: 12 Single-donor group: 11 Control group: 11
Evaluation (months)	12	24
Outcome:	No significant difference in UPDRS motor score at off time In patients 60 years old or younger: 34% alleviation	No significant difference in UPDRS motor score at off time In patients with mild symptoms (UPDRS motor score ≤ 49): No deterioration in comparison with control group
[18F]fluoro-L-dopa Positron emission tomography	Significant increase in transplantation group	Significant increase in transplantation group
Number of surviving cells per side of putamen	66-year-old female, 7 months after transplantation: Left: 38,392; right: 24,115 71-year-old male, 3 years after transplantation: Left: 36,796; right: 6,840	Four-donor group (two patients): 70,000 to 120,000 Two-donor group (two patients): Approx. 30,000
Run-off dyskinesia	5 of 33 patients who underwent transplantation	13 patients (6 in four-donor group and 7 in single-donor group)

to patients who showed mild symptoms with pre-transplantation UPDRS motor scores of 49 or less, the symptoms in the transplantation groups worsened less than in the control group after 2 years, showing therapeutic efficacy. PET measurement showed significantly increased uptake of FDOPA in both the single- and four-donor groups in comparison with the control group. Autopsy was conducted on five patients who died of unrelated causes within 53 months after transplantation. In the four-donor group, the number of surviving TH-IR cells in one side of the putamen was 70,000–120,000, whereas in the single-donor group the number was 30,000. CD45-positive cells and active microglia were found in the surrounding region of the graft. Runoff dyskinesia was developed in 13 of the 23 patients, and in three of them, the dyskinesia was severe enough to require treatment by DBS.

Lewy Bodies in the Graft

Lewy body is a pathological hallmark of Parkinson's disease, although similar pathological aggregates incidentally occur in about 5–20 % of nonsymptomatic individuals older than 60 years [10]. Recently, Lewy bodies were found in transplanted cells in patients who survived for a long period after fetal cell transplantation. Li et al. examined brain tissues of patients who were operated 11–16 years before death and found some Lewy bodies as well as numerous TH-IR cells [23]. Lewy bodies were found in a small fraction (2–5 %) of presumed dopaminergic neurons [24], and PET measurement using FDOPA and [^{11}C]-raclopride 10 years after transplantation confirmed the synthesis of sufficient dopamine. It is therefore considered that although some cells were involved in the pathological process, most had maintained their functions. Olanow et al. found intracellular inclusions similar to Lewy bodies in the putamen of patients who survived 14 years after transplantation. The graft was positive for α-synuclein and ubiquitin but showed low dopamine transporter expression [25]. They assumed that fetal cells transplanted into the striatum have degenerated after being affected by the pathological process and that it would be difficult for the beneficial effects of cell

transplantation to persist for long-term even after development of novel stem-cell-based methods. On the other hand, Mendez et al. examined transplanted cells in tissues from five patients 9–14 years after transplantation and found cells positive for TH and tryptophan hydroxylase, which is a marker for serotonergic neurons. However, all cells examined were negative for α-synuclein and ubiquitin, and no Lewy-body-like structures were found [26].

As the role of Lewy bodies in cell degeneration in Parkinson's disease has not been elucidated, the long-term efficacy of cell transplantation therapy must continue to be investigated.

Future Cell Therapy for Parkinson's Disease

Technical Problems in Fetal Cell Transplant

Different transplantation techniques are used at individual clinical institutions (Table 15.2). However, several technical problems remain to be resolved. The use of immunosuppressive agents may contribute to variable outcomes. Patients who have been operated on in Lund University continued to be administered cyclosporine, azathioprine, and prednisolone for a mean of 29 months after transplantation, and even after discontinuation of the drugs, no deterioration in the UPDRS motor score or decrease in FDOPA uptake was found. However, involuntary movement was exacerbated after discontinuation of the drugs, and this was probably linked to mild inflammation. In the method of Freed et al., no immunosuppressive agents were administered. In the study carried out by Olanow et al., cyclosporine administration was discontinued 6 months after transplantation. Thereafter, the tendency to the alleviation of symptoms diminished.

Runoff dyskinesia may be associated with uneven distribution of dopamine in the striatum, immune response, and insufficient functional connection between the transplanted cells and the host tissue. However, the exact mechanism remains to be elucidated.

Survival rate of transplanted fetal brain cells is less than 5 %, and four to five abortions are necessary for treating one patient. It is also difficult to standardize the quality of the cells. If donor cells are cultured for several weeks before transplantation, their properties may change. In addition, some surgical approaches with long or multitracks are more invasive than other techniques, and these points should be taken into consideration.

ES Cells as Donor Cells

Stem cells may offer a substitute for fetal midbrain cells because they can proliferate extensively in an undifferentiated state and may provide an unlimited source of dopaminergic neurons. In addition to ES and iPS cells, several types of stem cells are considered as potential donor candidates, including adult stem cells, such as marrow interstitial cells [27]; totipotent stem cells, such as embryonic germ cells derived from fetal primordial

Table 15.2: Double-blind study of fetal cell transplantation: Comparison of transplantation methods [13, 14]

Institution	Denver, Colorado	Tampa, Florida
Fetal age	7 to 8 weeks	6 to 9 weeks
Tissue morphology	Cord-shaped, 200 μm in diameter	Irregular, lumpy
Cell storage	Culture for up to 4 weeks	Used within 2 days
Injection method	Two tracks per side of brain, from front	Eight tracks per side of brain, from above
Injection timing	Same time on both sides	On opposite side after 1 week
Immunosuppressive agents	None administered	Cyclosporine administered for 6 months

germ cells [28]; and multipotent germ line stem cells established from the testes [29]. As immune response to graft is relatively mild in the brain, it is considered feasible to use totipotent stem cells for treatment of Parkinson's disease. Particularly, it is believed that immune response to graft can be largely avoided in Japan if a stem cell bank including 100–200 human leukocyte antigen types common in Japanese can be established.

Recently disease-specific iPS cell lines have been prepared from cells collected from patients [30]. However, patient-derived cells are more susceptible and may be readily affected in the pathological process. Instead of using these cells as donor cells for transplantation, they can be applied to studies for elucidation of disease mechanism and hence to drug development. Although iPS cells can be created with various methods, rigorous characterization of each cell line is necessary [31].

While adult stem cells are still difficult to culture in large quantities, considerable experience in ES cells culture and differentiation techniques has already been accumulated over the past decade. In a rat model of Parkinson's disease, transplantation of dopaminergic neurons derived from mouse ES cells resulted in the electrophysiological and behavioral properties expected of neurons from the midbrain [32–34]. Survival of dopaminergic neurons obtained in vitro from primate ES cells was also reported in primate hosts [35–37] (Fig. 15.1). Much effort has been made for producing neurons that mimic A9 dopaminergic neurons, a subtype of SNc neurons. However, as long as the donor cells are ectopically implanted in the striatum, they do not need to have A9 characteristics. Moreover A9 cells may be more susceptible to the disease process.

Recently we have developed an efficient method to produce many neurons from human ES cells in xeno-free condition. In our method, astrocytes derived from ES cells in a chemically defined medium can be used for continuous generation of neurons [38] (Fig. 15.2).

Prevention of Teratoma Formation

One major problem in using ES cells as donor cells for transplantation therapy is the risk of tera-

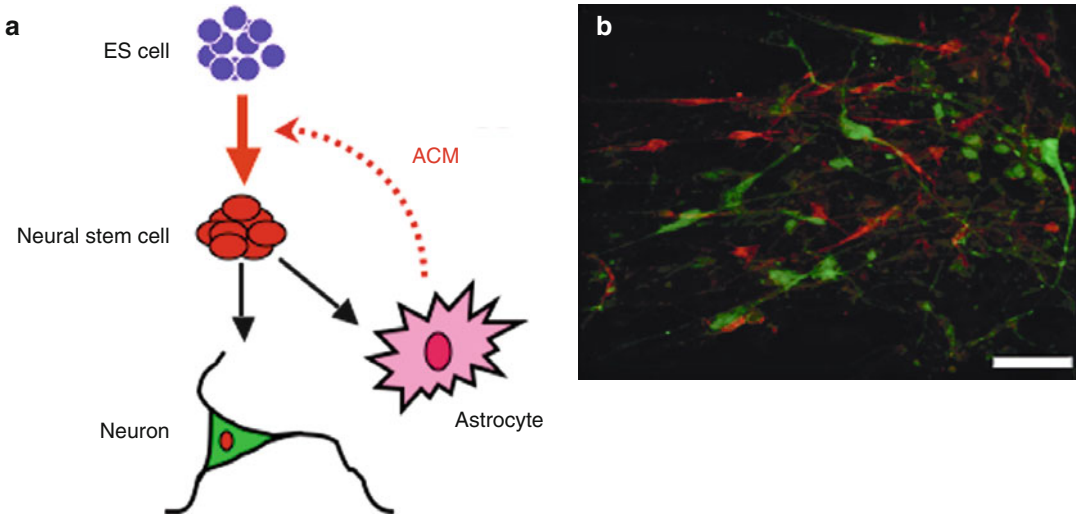

Fig. 15.1 Self-contained induction method and differentiation of human ES cells into dopaminergic neurons in xeno-free condition. (**a**) First, astrocytes are induced from undifferentiated human ES cells in a chemically defined medium. A conditioned medium obtained from the ES cell-derived astrocytes (ACM) is then used for induction of neurons. This self-serving method provides an unlimited source of human neural cells. (**b**) Immunostaining with antityrosine hydroxylase (TH) antibody and expression of green fluorescent protein (*GFP*) show that human ES cell-derived neural stem cells can differentiate into dopaminergic neurons. Expression of GFP (*green*), 4',6-diamidino-2-phenylindole (DAPI, *blue*), and TH (*red*) staining profiles. Bar = 50 mm

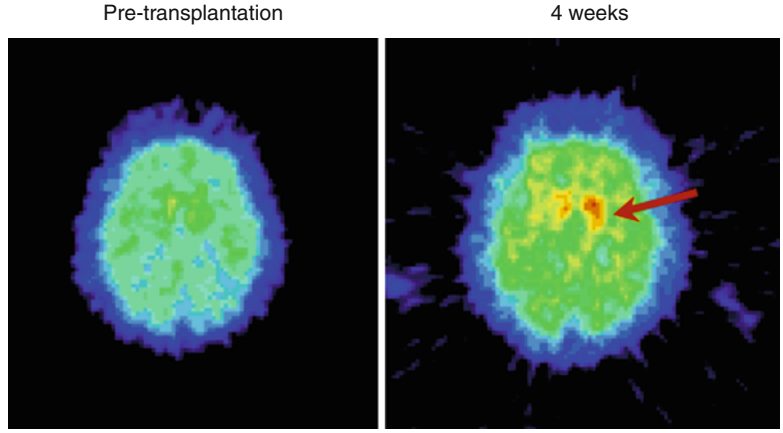

Fig. 15.2 Positron emission tomography images in a monkey model of Parkinson's disease. A cynomolgus monkey model of Parkinson's disease was made by systemic administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (*MPTP*). Neural stem cells derived from cynomolgus ES cells were then implanted unilaterally in the putamen. Four weeks after implantation, increased [11C]-2β-carbomethoxy-3β-(4-fluorophenyl) tropane (dopamine transporter ligand) uptake was detected in the implanted putamen (*arrow*) (Reprinted from [37] with permission)

toma formation. Several recently developed methods have been designed to reduce this risk. Because undifferentiated cells in the graft would be the origin of teratoma, selection of neuronal cells is important. Undifferentiated cells can be eliminated using a marker of specified surface antigen [39]. However, there is, up to date, no report on the use of specified surface antigen on dopamine-producing cells, and thus the so far reported methods use exogenous markers, such as green fluorescent protein, expression of which is driven by specific promoters for dopaminergic neurons during differentiation [40]. Another method to prevent tumor is to use herpes simplex virus type 1 thymidine kinase (HSV1-tk) gene and ganciclovir (GCV) treatment, a widely used prodrug/suicide gene therapy. ES cells expressing HSV1-tk are used as donor cells. If intracerebral tumor is found after transplantation, the tumor cells can be eliminated by GCV administration.

Cell Therapy for Parkinsonism

In Parkinson's disease, there are symptoms that do not respond well to L-dopa, such as dementia, depression, postural instability, autonomic nervous system dysfunction, and frozen gait [41]. These symptoms are intractable to dopamine supplementation and demand other remedies. A study on Huntington's disease demonstrated that cognitive functions related to the neural network between the striatum and the cerebral cortex were improved after transplantation of fetal brain cells into the striatum [42]. Similarly cell transplantation in the striatum may be effective for non-dopaminergic symptoms in Parkinson's disease.

Although Parkinson's disease is the most common form of Parkinsonism, vascular and degenerative lesions in the striatum can also give rise to Parkinsonism. Since gene therapy with dopamine-synthesizing enzymes is aimed at restoring dopamine production in the striatal neurons, it cannot be applied to Parkinsonism where the stiatal neurons are injured. Cell transplantation is probably the appropriate option for Parkinsonism. To reconstruct local neural network in the basal ganglia, graft cells should be differentiated into GABAergic neurons instead of dopaminergic neurons (Fig. 15.3).

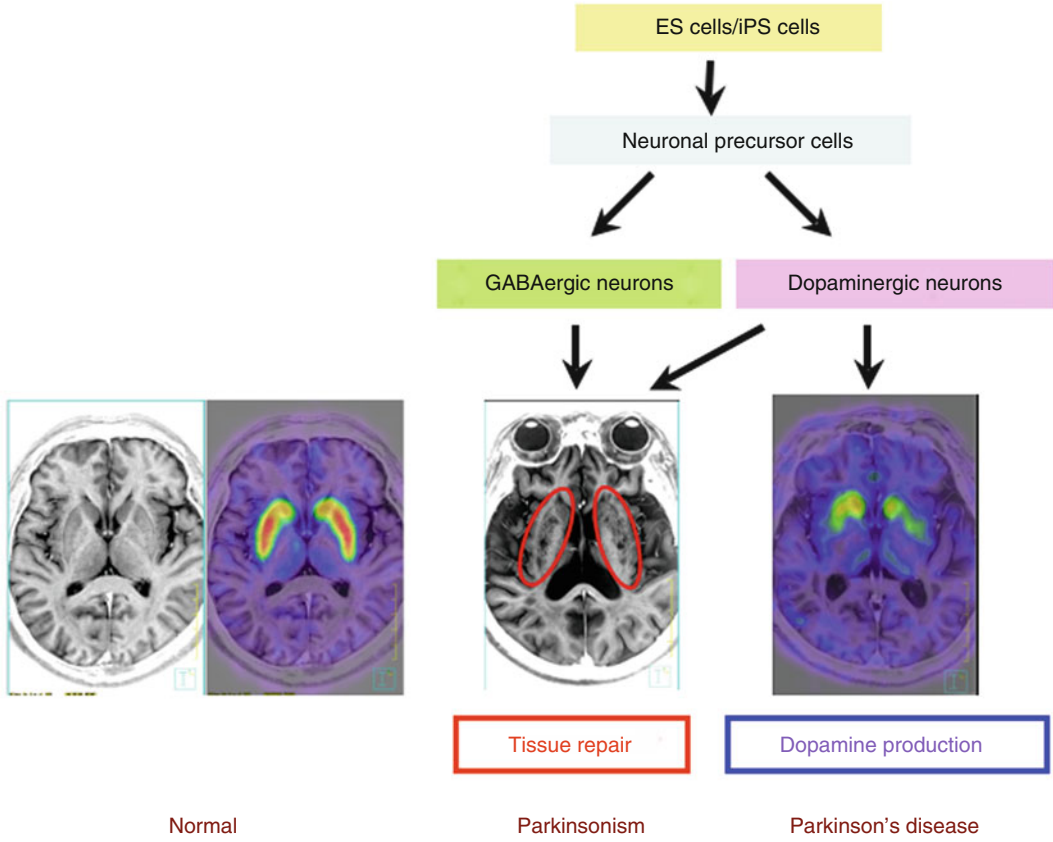

Fig. 15.3 Future strategies for cell therapy. In Parkinson's disease, the main pathology is a loss of dopaminergic neurons that project from the substantia nigra to the striatum with neurons in the striatum remaining intact. Thus, restoration of dopamine production in the striatum is important for functional recovery. In the case of other Parkinsonism where neurons in the striatum are also affected by the disease process, cell transplantation is appropriate for replacing damaged neurons. For this purpose, GABAergic neurons with dopamine receptors should be created. The *black-and-white* images on the *left* of each panel show magnetic resonance imaging of the brain in the horizontal plane. In Parkinsonism, which in this case is cerebral infarction, changes in signal strength were observed in the injured putamen, as shown by the areas enclosed with *red lines*. The colored images on the *right* of each panel are positron emission tomography images with [^{18}F]fluoro-L-*m*-tyrosine, a tracer for aromatic L-amino acid decarboxylase. In healthy people, the tracer accumulates throughout the striatum, whereas uptake is reduced in Parkinson's disease patients

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Transplantation of Human Fetal Liver Cells into Children or Human Fetuses

16

Jean-Louis Touraine

A number of severe congenital disorders and some acquired, especially hematological, diseases can be successfully treated by bone marrow transplantation [1–4]. However, a large proportion of patients have no perfectly matched HLA-identical donor available, and the incompatible transplant is responsible for severe graft-versus-host disease (GVHD). Since GVHD is induced by T lymphocytes which are present in the transplant and react with host tissues [5], transplants from a donor who is not genotypically HLA-identical frequently need to involve T-cell depletion of the donor bone marrow. This maneuver reduces the incidence of GVHD but is associated with increased risks of graft failure as well as incomplete reconstitution, Epstein-Barr virus-induced lymphomas, or leukemia relapse [6, 7].

It is, therefore, of considerable interest that, because of the natural lack of T cells during the early phases of ontogeny, fetal liver stem cells can reconstitute the hemopoietic and lymphopoietic systems of experimental animals and humans without producing severe GVHD, even in cases of full donor-host incompatibility [4, 8, 9]. From the transplanted stem cells, the various cell lineages develop progressively and uneventfully. Many patients can thus be fully cured of their congenital and severe immunodeficiency, or they can have some beneficial effect on their inborn error of metabolism [10].

Since 1988, we have initiated the first in utero treatment, in human fetuses with various diseases, using a similar mode of fetal liver transplantation (FLT) [11, 12]. This prenatal cell therapy has been demonstrated to be feasible and effective in the human fetus, when performed during the early stages of fetal development and immediately after prenatal diagnosis [13]. In this chapter, I shall review postnatal and prenatal transplantation of fetal stem cells, then I shall summarize some immunological implications of these therapies.

Rationale

The human fetal liver, between weeks 8 and 12 postfertilization (i.e., 10 and 14 weeks of gestational age), contains all hemopoietic stem cells and no T lymphocytes. These stem cells have earlier migrated from the yolk sac of the human embryo to the liver. After week 12 postfertilization, the fetal thymus produces thymocytes and T lymphocytes which migrate to the periphery. A few T cells are then present in the fetal liver and in the bone marrow. Later on, stem cells migrate from the fetal liver to the spleen and to the bone marrow, where T cells are always present. Fetal livers cells, obtained during the third month postfertilization, are therefore the only convenient source of a large number of hemopoietic stem cells naturally devoid of GVHD-inducing T lymphocytes.

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The remarkable properties of fetal stem cells include their considerable capacity for proliferation and differentiation, their ability to become tolerant to host antigens and to differentiate normally in a foreign host. They have prompted us to develop the field of human FLT.

Good-quality reconstitution has been obtained in most patients treated postnatally, despite the lack of HLA matching between donor and recipient. The resulting T-cell functions did not appear to be limited in any way [9, 14].

In utero FLT was then developed, and the main advantages of this procedure can be summarized as follows:

1. Increased probability of graft take and chimerism, especially in diseases such as the bare lymphocyte syndrome (BLS) in which residual immunity can induce rejection, and even more so in diseases without immunodeficiency (provided that transplantation is performed very early in gestation, before acquisition of any capacity to amount rejection)
2. Improved isolation at the time of transplant, since the uterus is even better than a sterile bubble
3. Ideal environment for fetal cell maturation, within the fetal recipient

Preparation of Fetal Cells for Transplantation

Donor fetal cells can be prepared in a variety of ways. They can be used fresh or cryopreserved and then thawed before administration. The preparation described below is that employed in our institution from 1974.

Fetal organ procurement was organized in accordance with the recommendations of the French National Committee for Bioethics [15]. A few hours following fetal death, the liver and the thymus were removed aseptically. Only fresh tissues and cells were used for transplantation.

For transplants to immunodeficient patients and to fetal recipients, the ages of fetal donors ranged from 7 to 12.5 weeks postfertilization; for postnatal transplants to patients with inborn errors of metabolism, they ranged from 8 to 22 weeks. The fetal thymus and liver were gently disrupted using a homogenizer, and a single-cell suspension was thus prepared in RPMI 1640 medium supplemented with gentamicin. The fetal liver was mainly a source of stem cells, the fetal thymus of epithelial cells.

The cells were counted and their viability checked using the trypan blue exclusion method. Cell suspensions with insufficient viability (i.e., less than 70 % when fetuses were below 12.5 weeks of age and less than 40 % above this age) were discarded. The total number of living nucleated cells that were transplanted from an individual fetal liver varied greatly with the age of the fetal donor (mean number 8×10^8 cells). Thymuses which contained numerous thymocytes, i.e., 12.5-week-old thymuses, were irradiated with 40 Gy prior to transplantation into patients with SCID, when such thymuses were transplanted together with syngeneic, untreated fetal liver cells.

Maternal serum was tested for hepatitis B antigens and antibodies, and for antibodies against hepatitis C, cytomegalovirus (CMV), human immunodeficiency viruses (HIV1, HIV2), and human T-lymphotropic viruses (HTLV1, HTLV2). Tissue was not used for transplantation when a risk of transmitting infectious disease, e.g., HIV infection, hepatitis B or C, septicemia, or other maternal infections, was identified. The tissues were also discarded in cases of certain tumors or in cases of known chromosomal abnormalities. Bacteriological tests were performed on cell suspensions, but the results were not available until after the transplant itself (in cases of bacterial contamination, antibiotics could then be given to the transplant recipient). In recent years, a spleen cell suspension and a fibroblast cell line were routinely performed to determine the ABO blood group and the HLA phenotype by both serological and molecular methods. Before administration to the patient, the cell suspension was diluted in the appropriate volume of medium for intraperitoneal injection or intravenous infusion.

Postnatal Transplantation of Fetal Stem Cells

Immunodeficiency Diseases

Transplantation of fetal liver cells, with syngeneic thymic cells, was used to treat every severe combined immunodeficiency (SCID) child who

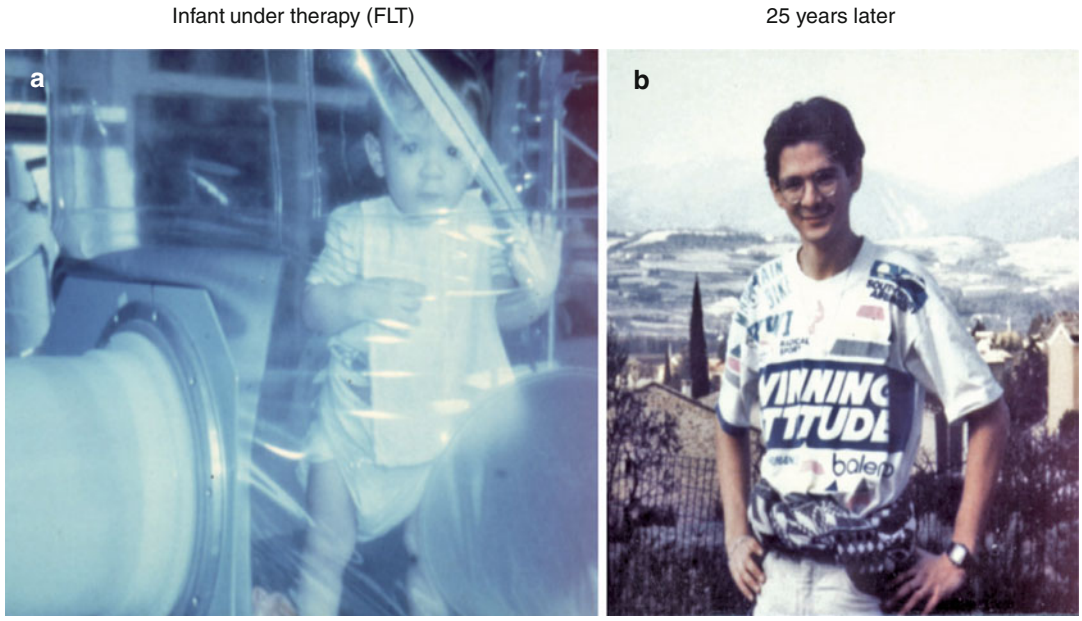

Fig. 16.1 Sergio, first SCID patient cured by FLT: the two pictures show **(a)** the infant in the isolator, a few months after transplantation, and **(b)** 25 years later; he now is perfectly healthy man of 36 years

had no available HLA-identical donor for bone marrow transplantation. The first of these transplants was done using two fetal donors in 1976. In this male infant, immunological reconstitution has been very successful [9, 16, 17], the lethal disease completely cured, and he is now a very healthy adult, living a normal life, without sequelae, and receiving no treatment. He is the oldest patient in the world that has been cured of SCID by fetal liver transplantation. The following year, a female infant with SCID was also fully reconstituted from the immunological standpoint and completely cured of her disease by FLT. Pictures of these first and second patients are presented at the time of FLT and during the following years (Figs. 16.1, 16.2, 16.3, and 16.4). Both patient 1 (Sergio) and patient 2 (Aurélie) are now more than 30-year-old adults. All their T lymphocytes are of donor origin. Their clinical status and immunological parameters are perfect. Aurélie is the mother of two healthy children.

In addition, nine other SCID patients have been treated in our institution (Table 16.1). They include three patients with adenosine deaminase (ADA) deficiency and one patient with bare lymphocyte syndrome [17]. Half of all these patients are completely cured, with a very long-term evaluation and a persistent immunoreconstitution (Fig. 16.5).

Fig. 16.2 Sergio, after he has left isolation

Fig. 16.3 Sergio, out of the isolator, bringing flowers to his "girl friend", Aurélie, still in isolation

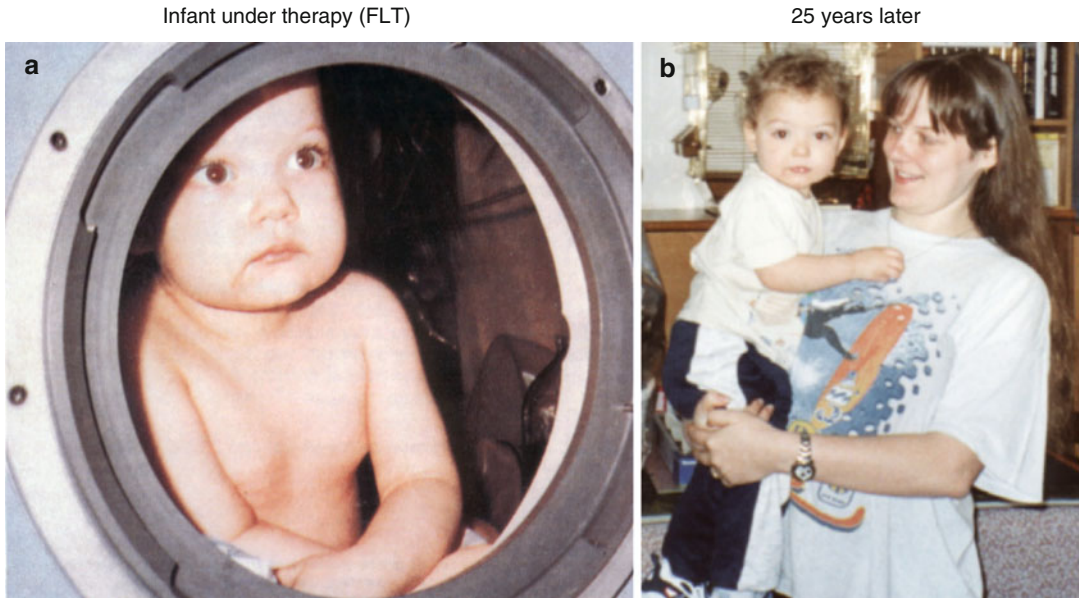

Fig. 16.4 Aurélie, second SCID patient cured by FLT: the two pictures show **(a)** the little girl leaving the isolator after she has been successfully transplanted, and **(b)** 25 years later, being mother of her first child; she now is herself a perfectly healthy woman of 34 years, with 2 healthy children

Table 16.1 Inherited severe immunodeficiencies and inborn errors of metabolism treated postnatally with FLT

Diseases treated	No. of patients
SCID	
With ADA	3
With BLS	1
Other SCID	7
Fabry	6
Gaucher	5
Familial amyloidosis	3
Fucosidosis	2
Niemann-Pick A	1
Niemann-Pick B	1
Niemann-Pick C	2
Glycogenosis	2
Hurler	2
Metachromatic leukodystrophy	2
Adrenoleukodystrophy	1
Morquio B	1
Sanfilippo A	1
Hunter	1
Gangliosidosis (GM2)	1

Inborn Errors of Metabolism

A variety of inborn errors of metabolism (IEM), without associated immunodeficiency, have been treated by fetal liver transplantation, in conjunction with prolonged immunosuppressive therapy at moderate doses comparable to those given in non-severe autoimmune diseases. No adverse effect of the treatment was observed. Thirty-one patients with IEM had transplants, as shown in Table 16.1. Most of them received repeated FLT, every 2 years at a mean.

Most patients are now in relatively good condition and display objective criteria of amelioration [10, 18]. The survival rate at 15 years posttransplant is 75 % (Fig. 16.5). By comparison with children having immunodeficiency diseases treated postnatally by fetal tissue transplantation, patients with inborn errors of metabolism were not completely cured by the fetal transplant, but their disease was stabilized for some time after each transplant. For example,

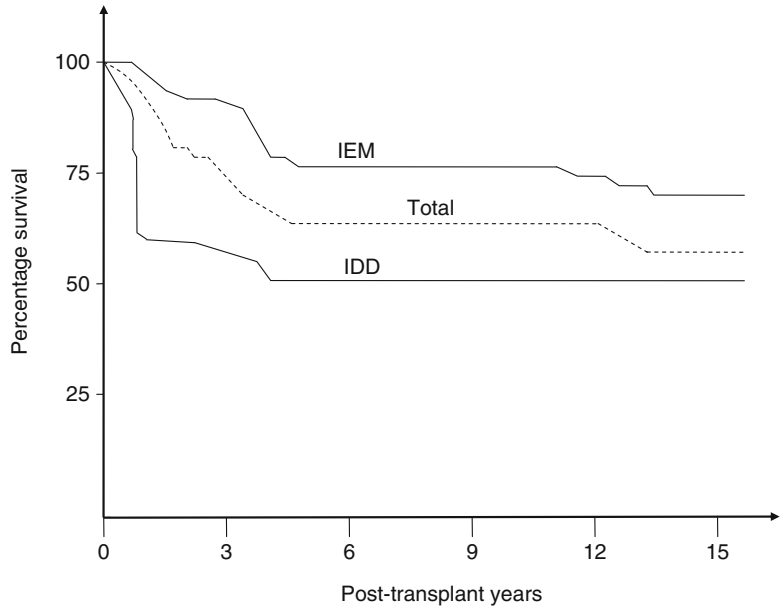

Fig. 16.5 Survival of children treated by postnatal transplantation of fetal tissue

stabilization for 5–10 years in three children with Gaucher disease enabled them to benefit later from glucocerebrosidase therapy. Patients with Fabry disease had diminished symptoms, with slower or halted disease progression. Fetal liver transplantation was repeated to maintain the clinical result, but some patients in an already advanced state of deterioration, especially of the central nervous system, eventually died. The serum levels of the defective enzymes were not increased dramatically, but the various substrates did decrease after FLT and tissue deposits stabilized. Fetal donors for liver transplantation in IEM were relatively more advanced in gestation than those chosen for fetal liver and thymus transplantation in SCID. The respective part played by stem cells and prohepatocytes in the partial improvement seen after transplantation is under investigation.

Overall Results

In both severe immunodeficiency diseases and IEM, fetal tissue transplantation into postnatal recipients has demonstrated beneficial effects. Two-thirds of children achieved cure or significant improvement as a result of treatment, and this effect seems long lasting (Fig. 16.5). Without transplantation, the life expectancy of more than 80 % of the patients was less than 3 years. Nevertheless, almost one-third of patients did not improve. In most cases, failure was the result of one of two factors: first, insufficient graft take in patients capable of rejection and second, late diagnosis of the initial disease with severe infection being present prior to transplantation. It is largely to overcome these difficulties, both in transplant take and in control of infection, that we have more recently developed in utero transplantation. Provided that the diagnosis can be performed at an early phase of pregnancy, such earlier transplants (before immune development and before exposure of the host to microorganisms) would lead to an increased probability of graft take and a lower risk of infection.

In Utero Transplantation of Fetal Stem Cells

Immunodeficiencies

The first two patients treated in utero with a successful engraftment in 1988 and 1989, in Lyon, were fetuses with SCID diagnosed at mid-gestation [11, 12]. A fetal patient with chronic granulomatous disease (CGD) also received in utero transplantation of fetal liver cells in Lyon [13]. At least 20 additional fetuses with immunodeficiency have been treated worldwide with in utero transplants of either fetal liver or T-cell-depleted bone marrow cells and have been reported in the literature ([13, 19–23], F. Porta, personal communication in 2006).

The first fetal patient, designated D.T. in Table 16.2, suffered from bare lymphocyte syndrome (BLS), a genetically transmitted form of combined immunodeficiency disease caused by the absence of expression of HLA antigens [24, 25]. In the particular case of D.T., the gene defect was responsible for a lack of binding of the RFX protein on the X-box, with a resulting absence of adequate transcription of HLA genes.

Infections, especially with opportunistic microorganisms, lead to death in such infants unless they grow up isolated in a fully sterile atmosphere while being successfully reconstituted with stem cell transplants. When carried out postnatally, however, such stem cell transplants, in the form of either bone marrow of fetal liver transplants, are usually associated with graft failure as a result of allogeneic reactions in the host (persisting transplant immunity) and with high susceptibility to infections (defective immunity to infectious antigens). Prenatal diagnosis of BLS can be performed by HLA analysis of lymphocytes in fetal blood.

The first child in this family had previously died of BLS before 1 year of age, despite an attempted stem cell transplant which failed to result in a stable graft take and immunological reconstitution. When the mother became pregnant again, she asked for prenatal diagnosis, which demonstrated type III BLS with a virtually

Table 16.2 In utero transplantation of fetal liver cells (Lyon experience with a follow-up of 15 years)

Patient	Disease	Fetal age[a]		Evidence for engraftment	Correction of initial disease	Clinical status
		Patient	Donor			
D.T.	BLS	28	7,7.5	HLA markers	Reconstitution of immuno-competent T cells	Well for 11 years. Additional BMT at another center, resulting in fatal CMV infection (11.5 years)
M.H.	SCID	26	7.5	HLA markers	Reconstitution of immuno-competent T cells	Well for 9 years then sclerosing cholangitis. Transplant of liver organ. Dead (9.5 years from surgical infectious complications)
M.R.	TM	12	9.5	Y chromosome	Presence of HbA in decreasing amount after 1 year	Alive and well (blood transfusion required)
R.M.	TM	17	11.5	—	—	Bradycardia and fetal death
A.V.	CGD	17,21	13.5,14	—	—	Bradycardia and fetal death
C.D.	N-P(A)	14,16	12,13	HLA markers	Insufficient corrections of neurological symptoms (blood-brain barrier?)	Deceased at 22 months
M.N.	HϕA	13	13	HLA markers	Limited (at present no immunization against factor VIII)	Well

BLS bare lymphocyte syndrome, *SCID* severe combined immunodeficiency, *TM* thalassemia major, *CGD* chronic granulomatous disease, *N-P(A)* Niemann-Pick disease type A, *HϕA* hemophilia A
[a]Weeks postfertilization

complete lack of expression of both class I and class II HLA antigens at the cell surface. Three choices were offered to this family: (a) therapeutic abortion, (b) no treatment before birth and stem cell transplant after birth, or (c) in utero FLT.

The parents were informed that the last option had not previously been attempted and that its efficacy was, therefore, uncertain. The mother and father opted for the earliest possible transplant. At 28 weeks, the transplant was carried out by infusing 7-ml culture medium containing a suspension of 16×10^6 fetal liver cells and fetal thymic epithelial cells into the umbilical vein [11].

The technique for intravenous infusion was comparable to that used for intravascular intrauterine transfusion [26]. Livers and thymuses were obtained from two dead fetuses of 7 and 7.5 weeks. At birth, the diagnosis of BLS was again confirmed in D.T., but some cells with HLA class I antigens became progressively detectable. Ten percent of the lymphocytes had a normal expression of HLA class I antigens at the age of 1 month [11], and these cells were of donor origin since their HLA specificities were of donor type and not inherited from the child's parents. In particular, these cells expressed the HLA-A9 specificity of donor origin, which made transplanted cells readily detectable in the initial test, at birth, and in subsequent tests. These results demonstrated the persisting engraftment of the fetal liver cells infused into the sick fetus. The expression of HLA class II antigens at the surface of resting lymphocytes remained comparatively low. This finding suggested a lesser B-cell than T-cell development from the donor fetal liver stem cells.

As scheduled initially, the newborn was placed in a sterile bubble and, to accelerate reconstitution, he received seven additional FLTs from nine fetal donors. This complementary treatment was carried out following the tests that demonstrated

engraftment of the in utero transfused stem cells. No engraftment of the cells infused after birth could be demonstrated, confirming the "resistance" to transplantation in these patients. However, the number of cells deriving from the in utero transplant increased and was found to be 26 % among peripheral blood lymphocytes (PBL) at 1.5 year of age. Donor-derived T lymphocytes further developed, and their percentage was found to be 28 % at 8 years [13]. In parallel, T-cell maturity and immunological reconstitution could not be considered absolutely complete; the proliferative responses to antigens (Candida antigens, CMV antigens, and tetanus toxoid) occurred and progressed up to normal levels. Serum immunoglobulins remained relatively low, a finding consistent with the limited number of donor-derived B cells. Because of the T-cell reconstitution from the cells transplanted in utero and in view of good health, the child was allowed to leave the isolator at the age of 16 months. For 11 years, the child was well, went to school, and had few infectious episodes. Because antigen-presenting cells remained mostly of host type, therefore defective in HLA molecules, the immune reactions were suboptimal only. Antibody production was quite low, whereas T-cell reconstitution was satisfactory. Unfortunately, this family went to another center where a bone marrow transplant from an unrelated, matched donor was proposed and carried out. As expected, it did not add significant humoral immunity, but it reduced the cell-mediated immunity. It was also responsible for several complications, especially a GVHD, then a severe and prolonged CMV infection, and ultimately resulted in death of the patient.

Earlier, we separated HLA-A9, donor-derived, lymphocytes from the patient's blood lymphocytes, for analysis of their characteristics by cytofluorimetry. All cells had a normal class I expression (W6/32+), and most of them were CD3+ T lymphocytes, while a small percentage of cells were CD19+ B lymphocytes (Fig. 16.6).

The second fetal patient, M.H., was similarly treated, after a prenatal diagnosis of a complete SCID. At birth, this female infant still had immunological manifestations of SCID. She was, therefore, maintained in sterile isolation and received additional infusions of fetal liver and thymus cells, with the aim of accelerating the development of the in utero transplanted stem cells. Cell-mediated immunity progressed sufficiently to allow adequate immunity against microorganisms, so that the child was allowed to leave the isolator.

Engraftment of male donor cells in this female patient was initially detected by the polymerase chain reaction (PCR) gene amplification technique demonstrating Y-chromosome-specific DNA sequences in DNA extracts from PBL. Chimerism was further established by HLA typing of lymphocytes. HLA class I phenotype was determined by serology and class II by molecular methods. At the age of 4 years, 80 % of lymphocytes were of donor origin, expressing HLA determinants of the very donor whose cells were administered prenatally to M.H. (Table 16.2).

The immunoreconstitution progressed up to levels close to normal values: 462 CD3+ T lymphocytes per microliter of blood, three-quarters of which were CD4+. IgG, IgM, and IgA also became progressively normal although a restriction of heterogeneity was transiently detected. T lymphocytes had satisfactory proliferative responses to various stimuli, including phytomitogens and specific antigens.

For 9 years, M.H. was relatively healthy, despite a sclerosing cholangitis. At 9.5 years she received a cadaver liver transplant, in an attempt to treat the progressing sclerosing cholangitis, and unfortunately she had surgical and infectious complications that proved to be fatal.

A further fetus (A.V.) received two fetal liver transplants to treat CGD. An elder brother was known to have the disease, and prenatal diagnosis in the current case was positive. The first transplant was carried out uneventfully by umbilical vein infusion at 17 weeks, but the number of cells available for infusion was considered insufficient. A second transplant was, therefore, attempted at 21 weeks; unfortunately, the procedure resulted in fetal bradycardia (possibly as a result of the relatively rapid infusion of a large number of cells) and led to fetal death within 1 h.

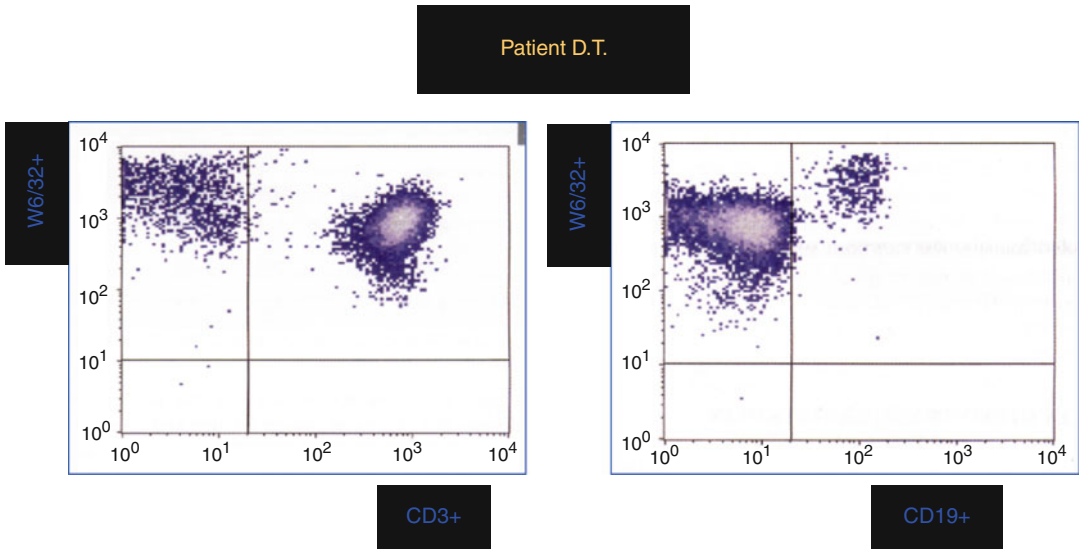

Fig. 16.6 Presence of CD3+ T lymphocytes and CD19+ B lymphocytes, both with a normal expression of W6/32+ HLA molecules, among HLA-A9+ cells (cells of donor origin) separated from blood lymphocytes of patient D.T.

Among the 20 other reported cases, 13 had sustained engraftment together with partial or total immunoreconstitution. It is noteworthy that all but two of the SCID fetal patients, but none of the CGD or Chediak-Higashi, with less complete immunodeficiency, had sustained engraftment of the in utero administered stem cells.

Hemoglobinopathies

The initial results obtained in the first two patients with severe immunodeficiency disease next prompted us to attempt in utero FLT in fetuses with severe nonimmune hematological disorders. In such conditions, however, graft take may be more difficult in a fetal host with an intact immune system and we, therefore, assumed that grafting had to be carried out early, during the first or at the beginning of the second trimester, at a time when normal fetuses have not yet developed very efficient cell-mediated immunity [27].

In one case, designated M.R. (Table 16.2), the mother had a family history of thalassemia and requested early prenatal diagnosis. The fetus was shown by molecular techniques to have β0-thalassemia major. The mother rejected the option of pregnancy termination for religious reasons and, instead, requested an in utero FLT. Transplantation was carried out at 12 weeks by intraperitoneal injection of donor fetal liver cells. Studies performed after birth showed the presence of thalassemic cells, but also of some cells of donor origin: PCR gene amplification techniques revealed Y-chromosome-specific DNA fragments in the PBL of this girl. In addition, hemoglobin (Hb) A was found to account for 0.9 % of all Hb at 6 months. No further transplant was performed in this child who is presently in very good general condition. Investigations have been carried out, 1 year after birth, 3 months after she had received a single-blood transfusion; the total Hb level was slightly below normal and the HbA percentage was 30 %. These data suggest that the engraftment of few donor cells has been followed by some cell proliferation resulting in an improvement and partial correction of the hematological disorder. The number of donor red cells has not, however, reached a level that would permit to avoid blood transfusions. At the age of 4 years, this number had further decreased and donor cells, with the HLA phenotype of the donor, were found only in the bone marrow of the patient. Approximately 0.5–1 % of bone marrow

cells expressed simultaneously the CD34 marker and the HLA-A32 phenotype of donor origin. These low values are obviously not sufficient for a significant clinical benefit, even if they suggest maintenance of tolerance to donor antigens. After the age of 5 years, the patient has regularly received blood transfusions every month, then every 3 weeks, together with deferoxamine.

In a further fetal patient in whom a prenatal diagnosis of β0-thalassemia major had also been made, FLT was attempted by intravascular infusion at 17 weeks. A 4-ml sample of blood was drawn, and 10-ml medium containing fetal cells was infused. Unfortunately, fetal bradycardia, possibly related to the relatively rapid infusion, occurred and led to fetal death within 1 h.

At least 18 additional patients, with α- or β-thalassemia or sickle-cell anemia, have been treated in utero with stem cell transplants and have been reported in the literature [13, 19, 28–31]. Of the total 20 patients, only 6 had a demonstrated engraftment, and no very long-lasting clinical benefit was recorded.

The different evolutions in severe immunodeficiencies and in hemoglobinopathies may result from the addition of two factors: (a) more feasible engraftment by lack of functional T cells and NK cells in the former patients and (b) selective advantage for donor cells over host cells in SCID patients, while the reverse appears to occur in patients with hemoglobinopathies.

Inborn Errors of Metabolism

Seven fetal patients with metabolic diseases have been treated in utero [12, 19], including one of our patients with Niemann-Pick type A disease [12]. Two patients had donor cell engraftment, but none had clinical benefit.

Experimental results in sheep fetuses with ceroid-lipofuscinosis, in goat fetuses with β-D-mannosidosis and in cat fetuses with α-mannosidosis also showed that in utero stem cell transplants had a relatively limited effect on the clinical disease [32–34]. At the present time, it seems to be wise to refrain from in utero transplants in patients with diseases involving the central nervous system, although the blood–brain barrier is more permeable in early fetal life than later on.

Hemophilia

Patient M.N. presented, at the fetal stage of development, with a prenatal diagnosis of severe hemophilia A. One brother had died of hemorrhages. Another brother had severe hemophilia and had developed antibodies responsible for resistance to factor VIII treatment. At the age of 13 weeks postfertilization, patient M.N. received a fetal liver transplant, from a donor of also 13 weeks. The objective was not to provide correction of the disease to the patient but to attempt induction of immunological tolerance to factor VIII by presenting this molecule to the fetus throughout immune development.

As a result of FLT, engraftment of donor cells with the HLA-A31+ phenotype was demonstrated with percentages ranging from 8 to 15 %, at birth and 10 months later. The patient naturally remained hemophiliac, but he appeared to have less severe bleedings than his brother. No significant amount of factor VIII was detected in the blood of the infant. No antibody and no resistance to factor VIII treatment developed, suggesting acquisition of tolerance to factor VIII.

Immunological Considerations

Patients who had SCID and were treated with mismatched stem cell transplants represent human chimeras. Their T lymphocytes derived totally from donor cells, while other cells in the body, including most B lymphocytes and antigen-presenting cells (APC) were of host origin [14]. Despite predictions that T-cell functions would be impaired under such circumstances, due to mismatch with interacting cells in the body [35–38], normal defense mechanisms against various bacterial and viral microorganisms did develop [10].

T lymphocytes (donor type) of the patients responded to tetanus toxoid (TT) antigen presented by the HLA-mismatched APC of the host

type. B lymphocytes (host) also cooperated with the mismatched donor-derived T lymphocytes for the efficient production of anti-tTG antibodies (IgM and, to a greater extent, IgG) both in vivo and in vitro [16, 39]. Similarly, cytotoxic T lymphocytes (CTLs) from these patients recognized and lysed host target cells, which were fully mismatched with the CTLs [40].

Host HLA antigens, rather than self-HLA antigens, were thence demonstrated to act as restricting elements for helper T cells as well as CTLs in HLA-mismatched chimeric patients. The T-cell receptor thus recognizes the antigenic peptide X when it is presented by an HLA molecule identical to that found on the T cell itself as it does under normal conditions (Fig. 16.7). This "allo + X" recognition [9, 41] is probably acquired by positive selection within the host thymus.

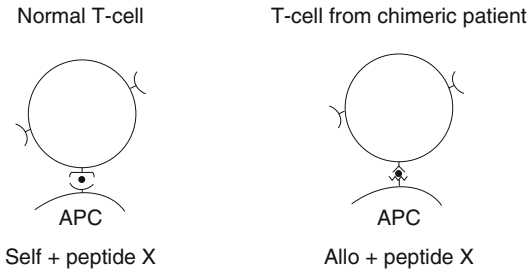

Fig. 16.7 Two distinct varieties of T lymphocyte differentiating in the thymus. On the *left*, a T-cell developed in an HLA-identical thymus of a normal individual; the recognition structure recognizes the X antigenic peptide presented in the groove of a self HLA antigen. On the *right*, a T-cell developed from a stem cell donor, in an HLA-mismatched thymus of a chimeric host; the recognition structure recognizes the X antigenic peptide presented in the groove of an allogeneic HLA antigen of host type

Donor stem cells differentiated under the influence of host thymic epithelial cells, which exhibited host HLA molecules and governed selection and development of thymocytes. Thymocytes that were issued from donor stem cells and differentiated within the host thymus might have initially acquired recognition structures for the various histocompatibility antigens that were possibly associated with a common peptide (Fig. 16.8).

In normal individuals, only T cells that recognize self-HLA antigens are induced to proliferative and develop a second degree of diversity at the level of the T-cell receptor, which leads to "self + X" recognition. In contrast, in chimeric patients, a given set of other HLA molecules is continuously presented to the developing T cell within the thymus.

T cells that recognize the given allo-determinants are then solicited, proliferate, and develop the gene rearrangement that leads to

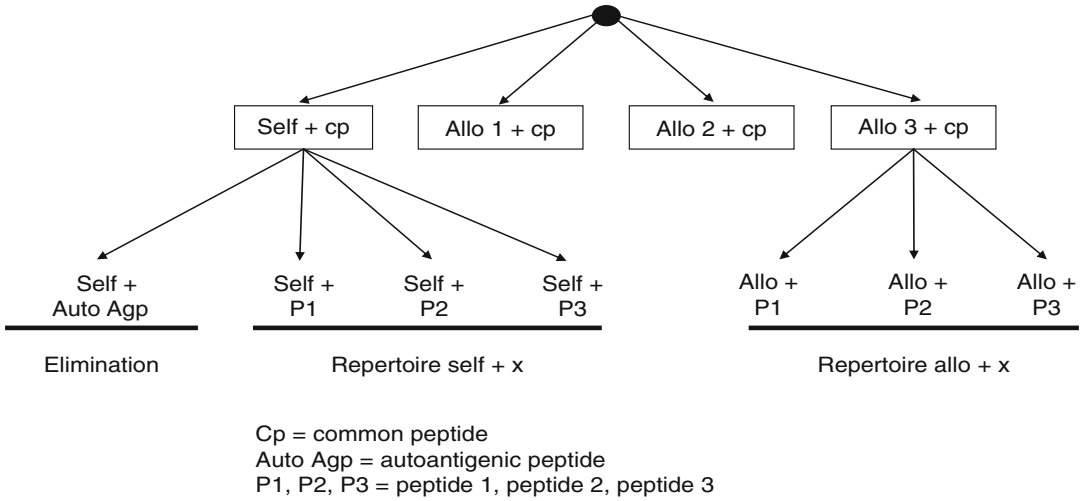

Fig. 16.8 Development and selection of a large variety of T lymphocytes with distinct recognition structures during the differentiation processes from the stem cell, after migration to the thymus. *Cp* common peptide, *auto Agp* auto antigenic peptide, *P1*, *P2*, *P3* antigenic peptides 1, 2, 3

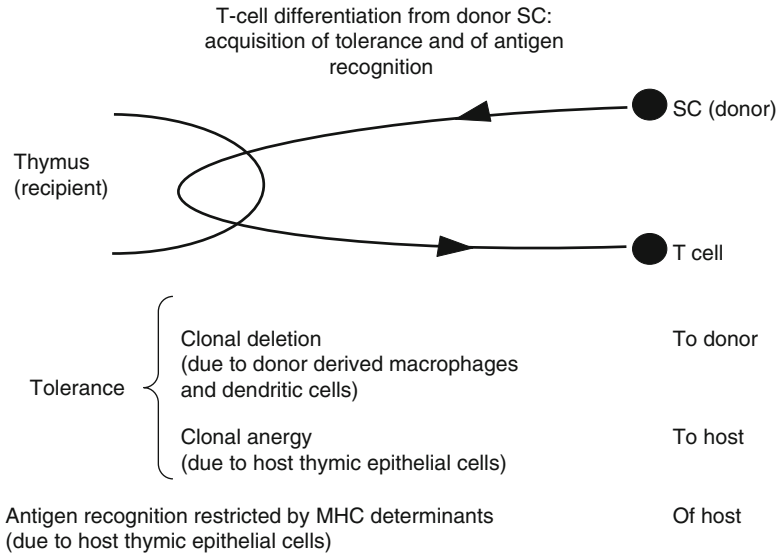

Fig. 16.9 Differentiation of donor stem cells into mature T lymphocytes within the host thymus : acquisition of tolerance and of antigen recognition.
SC stem cells

the expression of T-cell receptors that recognize the various antigens in the context of these allogeneic molecules [14, 41].

With regard to negative selection and tolerance following fetal stem cell transplantation, donor-reactive (but not host-reactive) cells have been shown to be deleted from the T-cell repertoire [42]. Clonal deletion is responsible for tolerance to antigens of the donor and clonal anergy for tolerance to host antigens. "Autoreactive" T-cell clones might be deleted upon interaction with stem cell-derived macrophages or dendritic cells [43], which is also suggested by animal experiments [44]. "Alloreactive" T cells that specifically recognize host determinants are numerous in the patients' peripheral blood, but they are not detrimental, probably because they are suppressed or anergized in vivo.

In brief, progenitor T cells that derive from the transplant are led to maturation in the host thymus that contains (a) positively selecting epithelial cells (of host origin) and (b) deleting cells that were provided by the donor and nested in the recipient thymus at the same time as prothymocytes.

All the accumulated data suggest that T cells derived from donor stem cells have acquired a tolerance to antigens of the donor via clonal deletion (following contact of thymocytes with dendritic cells and macrophages of donor origin) and a tolerance to antigens of the host via clonal anergy (following contact of thymocytes with host thymic epithelial cells).

Transplantation tolerance toward all donor and host antigens is therefore present in these patients as is the capacity of T cells to recognize foreign antigens presented in the context of host allogeneic HLA molecules (Fig. 16.9).

Conclusion

The fetal liver is a very efficient source of stem cells for transplantation. FLT has shown its ability to cure completely, and for the life-long, a significant percentage of immunodeficient patients, either treated postnatally or prenatally, in utero. Early diagnosis is required, as well as optimal conditions for fetal tissue preparation. The hope to treat inherited hemoglobinopathies as efficiently as immunodeficiencies is not yet fulfilled. Ongoing animal experiments should provide the knowledge that will permit to overcome host resistance in non-immunodeficient recipients.

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Fetal Liver Cell Transplantation

17

Suchitra Sumitran-Holgersson, Meghnad Joshi, and Michael Olausson

Introduction

Liver diseases are exceptionally common cause of morbidity and mortality worldwide. The standard death rate from chronic liver disease (CLD) is ranging from 3.1 to 22.6 as per survey. Despite the high incidence of diseases that results in liver dysfunction and failure, acute and chronic liver diseases are still treated with supportive rather than curative approaches. Orthotopic liver or hepatocyte cell transplantation has so far been the only available therapy for patients with end-stage liver failure. Unfortunately, the availability of donor organs is limited, and many patients die each year waiting for liver transplants. Cultured human hepatocytes have extensive diagnostic and clinical applications. Scarcity of donor livers and technical difficulties associated with cryopreservation and culturing of primary hepatocytes prevents a wider use of this technology. Cellular therapy with stem cells and their progeny is a promising new approach to this largely unmet medical need. The mammalian fetal liver contains a compartment of colony-forming cells with high proliferative potential. Thus, generation of human fetal liver cells (hFLC) as candidates for ex vivo expansion which could be an alternative tool for cell therapy and diagnostics.

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Treatment Options for Liver Diseases

Orthotopic Liver Transplantation (OLTx)

The first human OLTx was performed in 1963 [1]. Nowadays OLTx has become the only curable therapy for many end-stage liver diseases, including primary biliary cirrhosis [2], primary sclerosing cholangitis [3], hepatitis B and C, alcoholic liver cirrhosis, autoimmune hepatitis, metabolic liver diseases, and hepatocellular carcinoma [4–6]. The general 1-year survival of OLTx has now reached to 80–90 % [6, 7].

Hepatocyte Cell Transplantation (HCTx)

HCTx has been considered as an alternative to OLTx for the treatment of acute and chronic liver failure and metabolic liver diseases [8–10]. One of the main limiting factors of clinical therapeutic viability is the significant cellular loss after HCTx. The liver has long been considered as an immunologically privileged organ [11, 12] because of the observations that donor liver usually can be accepted by recipient without any immunosuppressive therapy in allogeneic liver transplantation in rodents. In human OLTx, the usage of immunosuppressive agents is often less

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compared with recipients of other organs. Furthermore, some OLTx recipients have successfully withdrawn immunosuppressant without recurrent rejection to allografts. So, it was believed that transplanted hepatocytes also possess the feature of immune privilege as liver. However, it has been confirmed by evidence that this inference is incorrect. Not only have transplanted allogeneic hepatocytes been destroyed by innate immune system (including Kupffer's cells and neutrophils), they have also stimulated the adaptive immune system and been rejected by immune responses mediated by $CD4^+$ and $CD8^+$ cells. Transplanted hepatocytes can only survive for 7–10 days in the absence of immunosuppressive agents [13]. The rejection cannot be inhibited with the immunosuppressive therapies which are often used clinically.

Furthermore, primary human hepatocytes are scarce and undergo maximally one to two cell divisions in vitro and therefore cannot be efficiently expanded. Some transplantation centers have possibility to isolate primary human hepatocytes from discarded liver not suitable for transplantation. However, accessibility of primary hepatocytes at the right time, arrival situation of the patient, and effective cell number are still important drawbacks of this source of cells, especially as long as cryopreservation of human liver cells is insufficient. After thawing and warming, cryopreserved cells show a significant loss of viability and lose significant capacity of attachment to matrices.

Liver Repopulation by Fetal Hepatoblasts

The liver originates from the gut endoderm. On embryonic day (ED) 8.5 in the mouse and 1 day later in the rat, primitive epithelial cells of the foregut contact the cardiac mesoderm and form the liver diverticulum [14–19]. These cells proliferate extensively, invade the septum transversum, begin to differentiate, and, on ED 9.5 in mice and ED 10.5 in rats, acquire the morphological appearance of immature liver epithelial cells (hepatoblasts), expressing first α-fetoprotein (AFP) and then albumin [19, 20]. Following the expression of these and other hepatic markers, including also cytokeratins (CKs), most authors conclude that hepatoblasts are bipotential cells, capable of differentiating along the hepatocytic or bile duct epithelial cell lineage [20–24]. At ED 15–16, the rat liver already contains committed immature hepatocytes and bile duct epithelial cells [7, 8, 25, 26]. In both rats and humans, embryonic hepatoblasts in large vascular spaces also form primitive ductal structures, which ultimately give rise to the intrahepatic bile ducts [27–29].

A number of transcription, signaling, and growth factors have been identified that play an essential role in gut endoderm differentiation and fetal liver development. These include factors that bind to the GATA DNA sequence motif (GATA); signal transducers and activators of transcription (STATs); hepatocyte nuclear factors (HNF)-3-α and -β, HNF-4, and HNF-6; and certain fibroblast growth factors (FGFs) [30–37]. However, the mechanisms by which primitive pluripotential endodermal cells undergo hepatic specification and how bipotential hepatoblasts differentiate further into hepatocytes and bile duct epithelium remain largely unknown.

During embryonic development, the fetal liver bud contains hepatoblasts. These cells express AFP and hepatocyte (albumin) and biliary (CK19) markers and could represent fetal liver stem cells that are capable of regenerating hepatocytes and the biliary system. Several studies have investigated transplantation of fetal hepatoblasts [32–34] (Fig. 17.1).

The mammalian fetal liver contains a compartment of colony-forming cells with high proliferative potential consisting of primitive hematopoietic progenitors and, most likely, pluripotent stem cells [38]. The development of the human hematopoietic system begins in the paraaortic splanchnopleural and/or yolk sac at 4 weeks of gestation. At 5 weeks of gestation, hematopoiesis starts to shift from the yolk sac to the fetal liver (FL) [39]. The FL is the main site of hematopoiesis in the fetus until development of the bone marrow. The first-trimester human FL contains the full hierarchy of several primitive

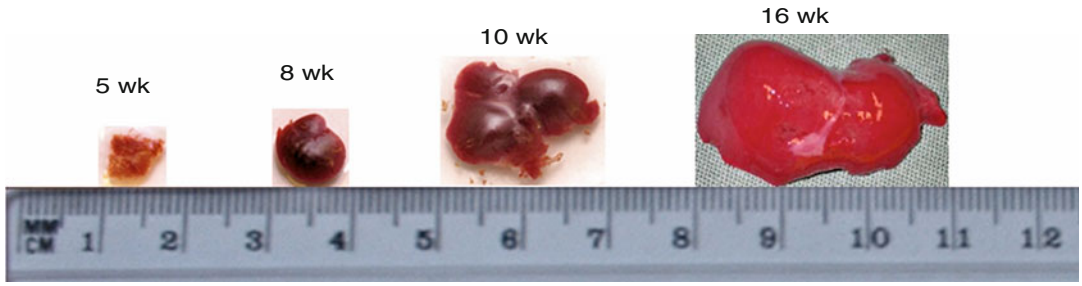

Fig. 17.1 Development of human fetal livers at various gestation weeks

progenitors detectable by their functional endpoints. These include hepatic and hematopoietic stem cells (HSC) [40–43].

It is demonstrated that activation of a hepatic progenitor cell (HPC) population or the so-called oval cells occurs when liver damage is so severe that hepatocytes are destroyed or for some reason prevented from entering the growth cycle by exposure to hepatotoxins or carcinogens [44]. These cells are thought to have both clonogenic and multipotential capacity, i.e., the ability to proliferate and differentiate into hepatocytes or cholangiocytes and under certain conditions can be induced to differentiate into non-hepatic lineages including intestinal and pancreatic epithelium. Using markers originally shown to be expressed on HSC including c-Kit (CD117) and CD34, some groups have identified and isolated hepatic progenitor cells [45, 46]. Using double immunolabeling techniques, some of these cells were found to co-express hepatocytic or biliary phenotypic markers implying lineage progression. The exact origin of these cells is not known [47–49].

Recently [50], using flow cytometry and single cell-based assays, Suzuki et al. identified in the developing mouse liver hepatic stem cells with multilineage differentiation potential and self-renewing capability. These cells could be clonally propagated in culture where they continuously produced hepatocytes and cholangiocytes as descendants while maintaining primitive stem cells. When transplanted, these cells differentiated into hepatocytes and cholangiocytes, as well as into pancreatic ductal and acinar cells or intestinal epithelial cells when transplanted into the pancreas or duodenal wall.

Studies in the adult liver have also provided strong evidence for the existence of putative liver stem cells, i.e., undifferentiated liver epithelial cells that can be activated to proliferate and differentiate into hepatocytes or bile duct epithelial cells [51–53]. These cells are thought to reside within or adjacent to the canals of Hering. Unlike stem cells in other tissues, such as bone marrow, skin, and intestine, which undergo continuous renewal, liver stem-like cells are facultative; they comprise a quiescent compartment of dormant cells that is activated only if the regenerative capacity of hepatocytes is impaired. Attempts have been made to identify their counterpart in fetal liver [53–56], and it has been suggested that the dormant stem-like cells originate most probably from bipotential fetal liver epithelial progenitor cells [53, 57, 58].

Thus, the fetal liver is rich in hematopoietic and hepatic stem cells, as well as cells expressing pancreatic markers and is therefore an attractive source for isolation of hepatic progenitors for transplantation. The developmental relationship between the stem/precursor cells of hepatic and hematopoietic cells at different times during development is fundamental to their biology.

Primary Culture of Fetal Liver Cells

Fetal hepatocytes isolated from liver can be maintained in culture ex vivo for several days and have the ability to organize as pseudo-liver tissue [59]. Animal hepatocyte culture systems also have been shown to supply biologically active substances crucial for the regeneration or the repair of the

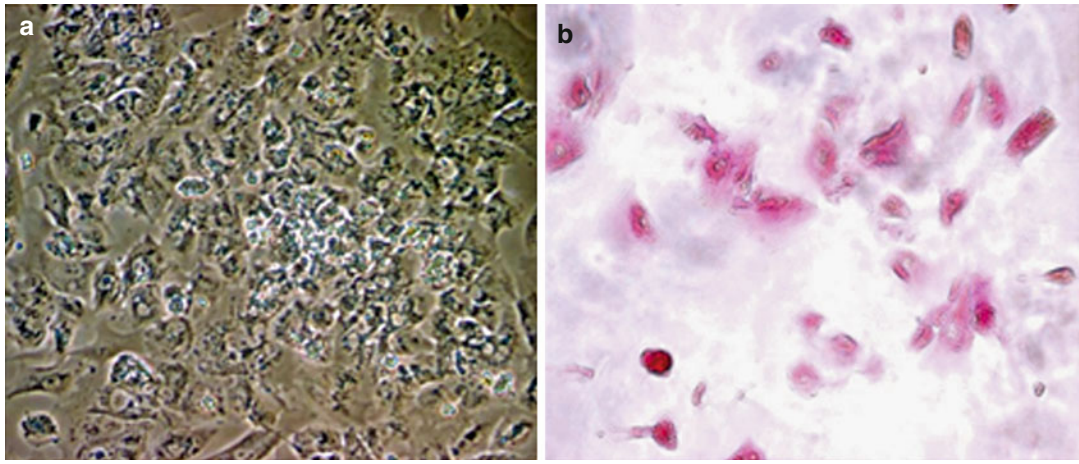

Fig. 17.2 (**a**) Fetal liver progenitor cells (CD117+/CD34+/Lin–) have epithelial-like morphology when grown on collagen-coated plates (**b**) and stain positive for glycogen (*pink*)

damaged liver. In addition, significant progress in cell culture technology allows the assembly of these cells as extracorporeal bioartificial liver support systems, to be used as temporary support devices for patients with acute liver failure. Primary cultures of human hepatocytes transplanted in mice after 48-h culture in vitro did not alter the isolated cell functionality or the expression of liver-specific proteins [60] (Fig. 17.2).

Growth and Expansion of Fetal Liver Cells in Serum-Free Medium

Primary cultures of hepatocytes for use in long-term metabolic, toxicological, or clinical applications are essential. Much effort has been devoted in the past in attempting to develop conditions suitable for the in vitro culture of rodent hepatocytes. However, interspecies differences justify the need to develop long-term cultivation of primary human hepatocytes for evaluation of liver-specific processes in man. Some culture media have been described for sustaining the differentiated state of hepatocytes, but they only maintain function in the short term, maximally 8 days [61, 62]. Alternative approaches to resolving the problem for human liver include isolating and maintaining embryonic liver cells in culture or adult human hepatocytes [63, 64]. Derivation of human hepatocyte lines for therapeutic applications in humans should preferably be done in non-xenogenic culture systems to avoid potential contamination with pathogens. Ideally, the cell culture systems that are constructed for this purpose will minimize exposure to animal cells and proteins. A step toward serum-free culture conditions is the development of chemically defined synthetic serum substitutes. To this medium, one can add defined hepatic growth factors. Thus, this medium would be useful for achieving controlled maintenance of hepatic stem cells in vitro. In addition, because the growth is in a chemically defined medium, it provides a good system in the future to investigate signaling pathways in controlling the hepatocyte differentiation. It will also be a valuable tool for studying the molecular basis of the developmental processes influencing hepatic cells in vitro, bringing us a step closer to establishing a safe and effective stem cell therapy to treat hepatic failure in vivo.

In our recent study, we developed a method to culture fetal liver cells for prolonged periods in serum-free medium with intact capacity to differentiate into functional mature hepatocytes and tested their capacity for engraftment after transplantation. Serum-free FLC obtained from 6 to 10-week-old human fetal livers grew as multiple clusters in suspension and could be subcultured for at least six passages. These cells maintained

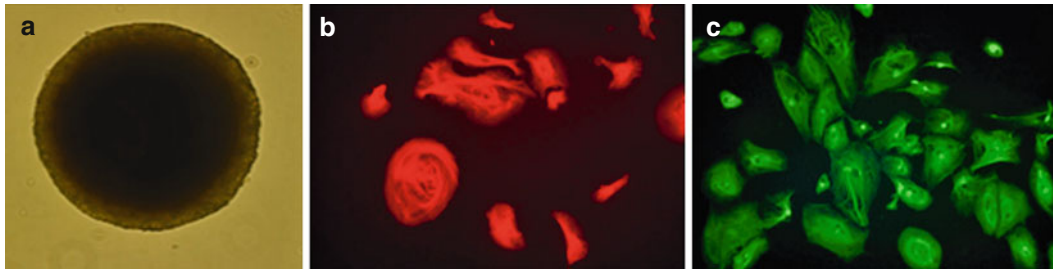

Fig. 17.3 (**a**) Fetal liver cells grow as spheroids or in clusters in serum-free medium (2-month culture). When a cluster of cells is seeded in collagen-coated flasks, the cells attach and migrate out of the sphere. The same cells adhere on the matrix after few days and differentiate into cells expressing cholangiocyte markers (**b**, CK19, *red*) and hepatocyte markers (**c**, albumin, *green*)

stable hepatocyte phenotype and gene expression patterns in culture up to 6 months. When a cluster of these cells in various passages was placed on collagen-coated plates, they formed a monolayer and morphologically resembled hepatocytes. The cells expressed cytokeratin, 8, 18, 19, and albumin. Hepatocyte nuclear factors 4α and 1β and CYP3A4 and CYP3A7 mRNA expression were demonstrated by RT-PCR. Cells in different passages when transplanted into nude mice with liver injury successfully engrafted as detected by in situ hybridization using a human-specific DNA probe. Colonies of human-specific CK8, 18, c-Met nuclear antigen, mitochondrial antigen, hepatocyte-specific antigen, and albumin-expressing cells were present in the livers of recipient animals. We have established at least seven different primary cultures derived from different donors. Thus, these cultures may be used to explore efficient methods of culture and clinical applications of hepatocytes in the future (Fig. 17.3).

Immortalized Fetal Liver Hepatocytes

Early fetal hepatoblasts, found in the developing liver, are good candidates for generation of liver progenitor cell lines by means of conditional immortalization. For cell therapy applications, a temporally controlled expression of the immortalizing transgene would permit reversion of the immortalized phenotype prior to cell transplantation. In most cases, murine hepatic stem cell lines have been used to study immortalization and transformation in vitro. However, characteristics of murine stem cells cannot be extrapolated to their human counterparts; therefore, it is important to establish human hepatic stem/progenitor cell lines to study the molecular events involved in their proliferation and differentiation in vitro as well as their fate in vivo after transplantation. Xenogenic primary cells, human hepatoma cells, immortalized hepatocytes, and stem cells have been used in isolated cell transplantation. However, xenogenic hepatocytes may transmit infectious pathogens and produce immunologic and physiologic incompatibilities between animals and humans. Human hepatocyte cell lines may become widely available and can be obtained using currently available cell cultures and gene transfer technology [65]. A clonal hepatocyte cell line that could be grown economically in vitro and that exhibits a differentiated, nontransformed phenotype after transplantation would be an attractive solution, provided that such cell lines divide indefinitely while maintaining characteristics of differentiated hepatocytes. Different oncogenic, viral, or chemical agents can induce cell immortalization. Hepatocyte immortalization using the Simian virus (SV) 40 DNA does not alter the ability of these cells to express a number of acute-phase genes and to respond to external stimuli [66–70], while drug-metabolizing enzyme activities also are maintained [71]. Immortalized cells promote in coculture survival of both fresh fetal human hepatocytes and adult mouse hepatocytes [72]. Such cells will be of great interest to study the molecular events involved in their

proliferation and differentiation in vitro as well as their fate in vivo after transplantation in the livers of recipient mice.

Introduction of SV40 into primary cultures of human cells with either whole virus or plasmid DNA results in enhanced cell division and prolonged life span and, in a few cases, will lead to the development of an immortalized cell line. It has proven difficult to establish conditions to support long-term primary cultures of adult human liver. Kobayashi et al. established several immortalized hepatocyte lines derived from human fetal or nonhuman adult hepatocytes [73–75]. Their results showed that the immortality of the hepatocytes can be significantly increased by transformation with the cDNA encoding SV40 LT antigen. Immortalized hepatocytes retain some of the differentiated features of normal primary hepatocytes in culture, including the expression of albumin (ALB), transferrin, hemopexin, and glucose-6-phosphatase (G-6-P). Furthermore, these cells do not produce detectable α-fetoprotein or show characteristics of fetal or abnormal liver cells. Similar results were obtained by the Andreas research group [76]. They established two immortalized hepatocyte lines from normal human liver cells following transformation with the SV40 LT antigen. These cell lines, which lacked tumorigenic properties, expressed many mature hepatocyte markers and possessed enzymatic pathways responsible for xenobiotic metabolism.

We recently successfully established a human fetal liver cell line by introduction of SV40 large T antigen into normal primary HFL cells. The newly established immortalized fetal hepatocytes revealed morphologic characteristics of primary hepatocytes in standard culture systems and expressed many liver-enriched markers, such as albumin, HNF-4α, and CYP3A4/7. Flow cytometric analysis showed that in the early passages, these cells were positive for the hepatic stem cell markers, EPCAM, CD133, and CD90, but not CD34 or CD45 indicating the non-hematopoietic origin of these cells. We have previously isolated and characterized hepatic precursors from human fetal livers expressing the hematopoietic markers CD34 and CD117 [64]. However, hepatocytes and cholangiocytes may be obtained from stem/progenitor cells expressing either hematopoietic-associated or non-hematopoietic stem cell markers.

RT-PCR and immunocytochemical assays demonstrated that the transfected cells expressed the genes and proteins (positive cytoplasmic staining for CY3A/7) and the transcription factors HNF 4α and HNF-1α. HNF-4α is required for the PXR- and CAR-mediated transcriptional activation of CYP3A4 and is a transcription factor that is involved in the regulation of the expression of several liver-specific genes. CYP3A4 is believed to be the predominant cytochrome P450s expressed in adult human liver and is involved in the oxidation of the largest range of substrates of all the CYPs. CYPs are the major enzymes involved in drug metabolism and bioactivation. HNF-1α (hepatocyte nuclear factor 1, homeobox B), is a human gene that encodes transcription factor 2, a liver-specific factor. Therefore, these immortalized SV40LT-HLF cells may be useful for the development of diagnostic tools for toxicity studies.

In addition, immunohistochemistry assay demonstrated that the cells expressed liver-specific markers such as glucose-6-phosphatase and glycogen. Importantly, using cancer-associated markers such as Ber-EP4, MOC-31, or p53, we demonstrated that the transfected cells in vitro did not express any of these markers. Furthermore, transplanted cells in vivo did not demonstrate tumor formation at 4 weeks after transplantation. It is important to state that further long-term in vivo experimental studies are required to evaluate the tumorigenic potential of the present cell line. This cell line would facilitate studies on cell engraftment and differentiation within the hepatic parenchyma.

Transplantation with Immortalized Liver Cells

In rats, intrasplenic transplantation of immortalized hepatocytes protects against CCl4-induced acute liver injury [77].

The unlimited growth of hepatocyte cell lines in vivo raises the possibility of a subsequent risk of malignant tumor growth in transplant recipients.

To develop methods to prevent this undesirable possibility, primary Lewis rat hepatocytes were conditionally immortalized with a thermolabile mutant SV40 large T antigen. Such cells proliferate in culture at 33 °C and stop growing at 37–39 °C thus preventing excessive proliferation after transplantation. Intrasplenic transplantation of these hepatocytes in portacaval-shunted rats protects recipients from hyperammonemia-induced hepatic encephalopathy [78]. The same strategy has been shown to improve survival in rats subjected to 90 % hepatectomy [79]. Transfected cells expressing herpes simplex virus thymidine kinase sequences can subsequently be inhibited by ganciclovir [80].

Immortalized primary hepatocytes of Gunn rats, transduced with a recombinant retrovirus expressing human bilirubin-UDP-glucuronosyltransferase, were injected into the spleens of syngeneic Gunn rats and led to decreasing serum bilirubin levels as long as 4 months after cell transfer [81].

These studies and others concerning liver-expressed proteins [82] confirm the possible use of immortalized cell lines for transplantation, at least in animals [83].

Human clonal immortalized cell lines obtained from human fetal hepatocytes transplanted in rats subjected to a 90 % hepatectomy protect animals from hyperammonemia and the associated hepatic encephalopathy [84]. Other immortalized human cell lines have been shown to provide metabolic support during acute liver failure in rats [84].

Cryopreserved Fetal Liver Hepatocytes

Cryopreservation of freshly isolated fetal liver hepatocytes is required for the long-term storage of liver cells. The use of cryopreserved adult hepatocytes for transplantation was first documented in rat model in the early 1980s [85]. Only small numbers of cryopreserved hepatic cells are detected in the host liver after transplantation [86]. Good quality cryopreserved hepatocytes are crucially dependent on the components of the medium in which they are frozen, including the dimethyl sulfoxide (DMSO) concentration needed to avoid osmotic shock and prevent formation of ice crystals within cells.

Studies suggest that freshly isolated and cryopreserved transplanted hepatocytes have similar behaviors after their intrasplenic transplantation [87] and that long-term cryopreservation has no effect on cell growth and the hepatic-differentiated activity of small hepatocytes [88]. Long-term preservation does not alter the ability of the cryopreserved hepatic cells to repopulate the damaged liver [89, 90]. Moreover, in a recent study, no major differences were observed in many hepatic-specific parameters when hepatocytes were immediately cryopreserved or after short-term culture [91]. However, other studies have failed to demonstrate the efficacy of cryopreserved hepatocytes to engraft and to produce albumin [92]. Survival of transplanted cryopreserved hepatocytes is inversely correlated with immunogenicity, as demonstrated in mice [93]. Apoptosis may contribute to cell death in cryopreserved hepatocytes [94], an effect that seems dependent on caspase-3 protease activation [95, 96].

In our own experience, using cryopreserved human fetal liver cells has resulted in comparable engraftment of these cells after transplantation into nude mice as that obtained with freshly isolated human fetal liver cells. Moreover, we found that frozen human fetal liver cells are more resistant to cryopreservation, have better viability and attachment properties after thawing, thus making them attractive candidates for investigating potential clinical applications.

Characterization of Cells in Human Fetal Liver

Studies in human liver development are relatively few in number as they rely heavily on ex vivo liver specimens. These studies are invaluable as not only do they provide direct observations and knowledge of the regulatory factors involved in human liver organogenesis, but their findings could also lead to successful isolation and in vitro propagation of fetal liver progenitor cells suitable for clinical use. Human embryonic stem cells

(ESCs) and induced pluripotent stem cells (iPSCs) hold great promise for a potentially abundant source of hepatocytes; however, directing their differentiation into specific, fully functional adult cell lineages remains a significant challenge. The use of fetal human liver progenitor cells abrogates the issue of forced differentiation, as fetal progenitors have undergone sufficient morphological and physiological differentiation so that they are committed to a hepatic fate, and yet they retain their "stemness" by maintaining their bipotentiality, proliferative capacity, and transplantability.

The phenotype of fetal human liver progenitor cells remains controversial. In humans, cells expressing the c-Kit antigen (CD117+) and in rats the Thy-1 antigen (CD90) are suggested to be precursors of hepatic cells. In our own studies, we found, that the earliest hepatic marker to be co-expressed on CD117+ cells was c-Met (receptor for hepatocyte growth factor), followed by the emergence of cells double stained for CD117/AFP, CD117/albumin, and CD117/CK19. These cells do not co-express CD90.

A range of cell markers based on rodent studies, such as Liv2 [97], E-cadherin [98], and delta-like kinase-1 (Dlk-1) [99] have only been characterized in human livers by immunodetection methods in vitro [100]. Liver progenitors can be isolated from human fetal livers by immunoselection for epithelial cell adhesion molecule (EpCAM)-positive cells [101]. In situ studies reveal that $EpCAM^+$ fetal liver progenitors are located in the ductal plate. Once isolated, these cells are capable of self-renewal and clonogenic expansion, as well as differentiation into both hepatocytic and biliary lineages in defined culture conditions [101]. Moreover, purified $EpCAM^+$ fetal liver progenitors when transplanted are able to engraft the livers of immunodeficient adult mice yielding mature human liver tissue [102].

Another potential stem cell population, side population (SP) cells, has been found to contribute to hematopoietic and epithelial lineages in the early gestational phase of human liver development [103]. SP cells have been isolated using fluorescence-activated cell sorting based on their ability to efflux DNA-labeling Hoechst dye [104], a phenotype determined by expression of ATP-binding cassette (ABC) transporters encoded by the multidrug resistance (MDR)-1 gene. Their location in situ however remains uncertain, not least because of the widespread distribution of ABC transporters in the liver [105]; clearly the vast majority of cells in the liver expressing ABC proteins are not stem cells.

In our own studies, we found that abundantly occurring cells in the human fetal liver even in early gestation are the red blood cell precursors. A high frequency of endothelial cell precursors expressing Flk-1 is also found early in the developing liver. As seen in Table 17.1, no mature hepatocytes or cholangiocytes were present in the early gestation period between 5 and 11 weeks. The only hepatic marker to be detected in high numbers in FLC was alpha-fetoprotein (AFP) which was detected already at 5 gw and increased rapidly in the subsequent weeks. Albumin was not found to be expressed in the early FLs; however, the expression of this marker too increased rapidly from 14 gw onward. The expression of the other hepatic markers increased gradually over time. Interestingly, a high percentage of cells expressing the histocompatibility markers HLA class I and HLA-DR were found already in the fifth week of gestation. In general, precursors of hematopoietic cells and cells expressing pancreatic markers were present early in gestation, while substantial numbers of cells expressing hepatic markers were detected in the late phase of the second trimester. In contrast, lower numbers of cells expressing endothelial and hematopoietic precursors were detected in adult livers.

The hematopoietic and hepatic lineages in the fetal liver have been considered separate and distinct. However, reports have demonstrated a novel lineage relationship between hematopoietic and hepatic cells [106, 107].

We found that the first-trimester human FLs contained hematopoietic stem cells and other progenitors and the least of committed cells. It is considered that the yolk sac is the origin of all hematopoietic cells and that stem cells derived from it seed the fetal liver [108, 109]. In the mouse, yolk sac hematopoiesis is transient and

Table 17.1 Frequency of cells expressing known hematopoietic, hepatic, and pancreatic cell markers in fetal and adult liver

Antibodies to	Fetal liver gestation week 9 % cells	Adult liver donor age 55 years % cells
CD117	0.9 ± 0.2	0.003 ± 0.001
CD90	0.5 ± 0.3	0.002 ± 0.001
CD34	2 ± 0.3	0.04 ± 0.01
CD123	1 ± 0.05	0.03 ± 0.02
CD133	0.3 ± 0.04	0.002 ± 0.001
Flk-1	10 ± 4	1 ± 0.05
Flt-1	2 ± 0.2	0.002 ± 0.001
Tie-2	0.1 ± 0.02	0
Albumin	0	60 ± 10
Hepatocyte antigen	0	0.004 ± 0.001
HEA-125	0	6 ± 2
Cytokeratin 19	0	6 ± 2
c-Met	0.02±0.01	70 ± 6
Alpha-fetoprotein	9 ± 0.4	0.008 ± 0.004
CD45	4 ± 2.2	20 ± 5
CD14	3 ± 2	3 ± 0.7
Glycophorin A	70 ± 20	10 ± 5
HLA class I	4 ± 2.4	70 ± 5
HLA class II	2 ± 2	10 ± 5
Anti-nestin	4 ± 2.3	2 ± 0.4
Anti-amylase	4 ± 3.5	5 ± 5.6
Anti-insulin	0.5 ± 0.1	0.002 ± 0.001
Anti-pancreatic polypeptide	4 ± 3	6 ± 1
Anti-glucagon	4 ± 3.5	5 ± 3.5

shows a dramatic decline in activity between days 11 and 12 of gestation. This decline coincides with the onset of activity in the developing liver, which then becomes the major site of hematopoiesis until birth [110]. We demonstrated that the fetal liver in the first and second trimester is a site of multilineage definitive hematopoiesis which includes erythropoiesis, myelopoiesis, and lymphopoiesis. Furthermore, our study demonstrated that the fetal liver is not a "hepatic organ" until the second trimester, during which period the numbers of cells expressing hepatic-associated markers increased greatly. Thus, the liver is essentially a "hematopoietic organ" in the first trimester followed by a shift to a "hepatopoietic organ" during the second trimester.

Interestingly, cells of the fetal and adult liver also express many of the pancreatic markers such as nestin, amylase, pancreatic polypeptide, and glucagon. During development, the pancreas is known to emerge from dorsal and ventral domains of embryonic gut endoderm. The emergence of the ventral pancreas has been shown to be related to the emergence of the liver [111]. The common embryological origin of the ventral pancreas and the liver may be reflected in certain evolutionary and pathological states. Primitive invertebrates such as mollusks have a single organ, the hepatopancreas that serves functions of both organs [112]. In addition, the caudal pancreas in the sea lamprey apparently develops by transdifferentiation of cells in the hepatic duct [113]. Thus, the partitioning of liver and ventral pancreas into separate organs may have evolved from a common cell population. In addition, examples of liver cells appearing in the adult pancreas exist [114–116]. All these findings demonstrate that the existence of plasticity among endoderm-derived organs is detected not only during fetal development but even in the adult liver and pancreas.

Currently it is not known whether the pancreatic hepatocytes emerge due to transdifferentiation of pancreatic duct cells or due to an undefined stem cell population.

Aside from answering the basic biological question of whether stem/precursor cells can be found in the developing human liver, their isolation and characterization have significant implications for the advancement of therapeutic approaches to liver disorders.

Fetal liver cells have a high proliferative capacity, are less immunogenic, and are more resistant to injury during cryopreservation and more resistant to ischemic injury. These qualities could enhance their engraftment, whereas their proliferation increases gene transfer efficiency. These cells can be isolated with a nonperfused collagenase method, according to Devirgiliis et al. [117]. In culture, fetal hepatocytes have a high spontaneous proliferative activity compared with adult hepatocytes [118].

Injection Site of Fetal Liver Cells

Orthotopic Injection Site

Because the liver is supposed to be the optimal site for transplanting isolated fetal hepatic cells, hepatocyte suspensions are infused directly via the portal vein or spleen. The success of such procedures is limited by the number of transplanted hepatocytes that can be infused and by the portal hemodynamics.

Intrasplenic Transplantation

In rodents, intrasplenic transplantation of fetal liver cells can recompose liver tissue in the spleen that has similar structure and function as the liver, suggesting that the spleen may contain appropriate factors and environment to maintain viability and functionality of transplanted hepatocytes. Such a model of LCT has remained the most suitable and efficient procedure in animals. Hepatocytes transplanted into the spleen reach the liver via the splenic vein and restore missing enzyme activities [119]. In Gunn rats, intrasplenic hepatocyte transplantation is effective in reducing plasma bilirubin [120]. In anhepatic rats, intrasplenic transplantation of allogeneic hepatocytes prolongs survival, improves blood chemistry, and lowers blood TGF-beta1 levels [121]. In Wistar Furth rats, proliferation of transplanted hepatocytes occurs in the spleen, indicating that this LCT procedure also may be used as a therapeutic application in the treatment of chronic liver disease [122].

Intraportal Transplantation

In the late 1970s, intraportal hepatocyte transplantation was shown to correct hyperbilirubinemia in glucuronyltransferase-deficient Gunn rats [123, 124]. In dogs, portal hypertension represents a limiting factor for the number of hepatocytes that can be used for a single transplantation. Thus, repetitive transplantations are proposed to correct metabolic alterations [125], and this can be achieved via an indwelling catheter. Nagase analbuminemic rats subjected to repeated cell infusions acquire high plasma albumin levels and normal liver histologic findings. Repeated infusions will allow successful engraftment of a large number of fetal hepatocytes [126]. After LCT in the low-density lipoprotein (LDL) receptor-deficient Watanabe rabbit, a dose-dependent reduction of serum LDL is observed that lasts for 2–3 weeks [127]. Recently, Muraca et al. [128] have explored the effects of intraportal hepatocyte transplantation on portal, pulmonary, and systemic hemodynamics in pigs and concluded that as much as 2.4 % of the total hepatocyte mass can be infused in pig model but with a risk of significant thrombotic complications.

Transplantation via Hepatic Artery

Direct hepatocyte transfer via the hepatic artery in the cirrhotic rat model improves liver function, increases the degradation of liver collagen, and reverses liver cirrhosis [129]. In Long-Evans Cinnamon rats, an animal model of Wilson disease, LCT by the arterial route leads to a reduction in hepatic copper deposits [130]. The phenotype

of donor hepatocytes depends on the surrounding environment, and transplanted hepatocytes originally expressing high levels of glutathione S-transferase placental form (GST-P) and low levels of cytochrome P450 turn GST-P negative within 5–10 days and express cytochrome P450 at a level comparable to that in surrounding host hepatocytes after 2 days [131].

Experimental Models of Fetal Liver Cell Transplantation

Immature FL cells in the environment of adult regenerating liver can proliferate, differentiate, and express genes characteristic of adult hepatocytes/bile duct epithelial cells. This strongly suggests the potential use of these cells for transplantation and ex vivo gene therapy. A few attempts have been made to transplant ED 18 and older fetal liver cells into the spleen or on solid supports implanted intraperitoneally [132–135]. In all these cases, fetal hepatocytes engraft, proliferate to some extent, and perform liver-specific biochemical functions. Isolated fetal hepatocytes from late gestation, when transplanted intraportally into Nagase analbuminemic rats, engraft, expand, and give partial correction of serum albumin when a hepatic regenerative stimulus (portal branch ligation) is also applied [136]. Several studies also report successful engraftment and differentiation of early fetal liver tissue or cell suspensions after transplantation into ectopic sites [137]. However, engrafted liver tissue masses at ectopic sites do not expand very much, and it is unlikely that such limited liver transplantation will have broad therapeutic application.

Data suggests that immature FL cells may represent a preferred source of hepatic cells for transplantation compared to adult hepatocytes for the following reasons: (a) FL cells are small (10–12 μm) and their intraportal injection is better tolerated than transplantation of mature hepatocytes (20–35 μm); (b) the number of injected cells used for the experiments is ~5 times lower than the number of adult hepatocytes used for liver repopulation at the same efficiency as other studies; (c) due to their small volume, FL cells are not trapped in the periportal region, where the highest concentration of transplanted adult hepatocytes is observed [138], and they move easily through the sinusoids, reaching zone 3 of the liver lobule. This increases the seeding and repopulating efficiency of the transplanted FL cells compared to hepatocytes; (d) immature FL cells possess sufficiently high proliferative capacity that they can repopulate the normal regenerating liver; and finally, (e) FL cells differentiate morphologically and phenotypically into both mature hepatocytes and bile duct epithelial cells, which are not observed after hepatocyte transplantation. Since early fetal liver epithelial progenitor cells selectively proliferate in the normal liver in response to a regenerative stimulus (or hepatic parenchymal loss), they differentiate into mature hepatocytes and bile duct epithelial cells, and they become incorporated into the host liver lobule as part of normal hepatocytic cords and bile duct structures; this suggests that fetal liver cell transplantation represents an attractive method to restore functional liver tissue (Fig. 17.4).

Clinical Results of Fetal Liver Cell Transplantation

LCT has recently passed from an animal experimental procedure to its first clinical applications, with the aim of treating patients with fulminant liver failure, end-stage cirrhosis, or inborn errors of liver metabolism. The first attempts in humans have demonstrated at least that the procedure is feasible and well tolerated. In a recent study [139], transplantation of human fetal hepatic stem cells in patients with end-stage liver cirrhosis (patients with high mortality and for whom liver transplantation is the only proven treatment) resulted in highly encouraging outcomes. Patients showed marked clinical recovery, and there was no recurrence of hepatic encephalopathy observed, which suggests marked improvement in the functioning of liver cell.

In another study, following transplantation with allogeneic fetal liver, a boy with a severe combined immunodeficiency and adenosine-deaminase (ADA) deficiency developed immunocompetent T and B cells in an orderly manner. Engraftment was indicated by appearance of

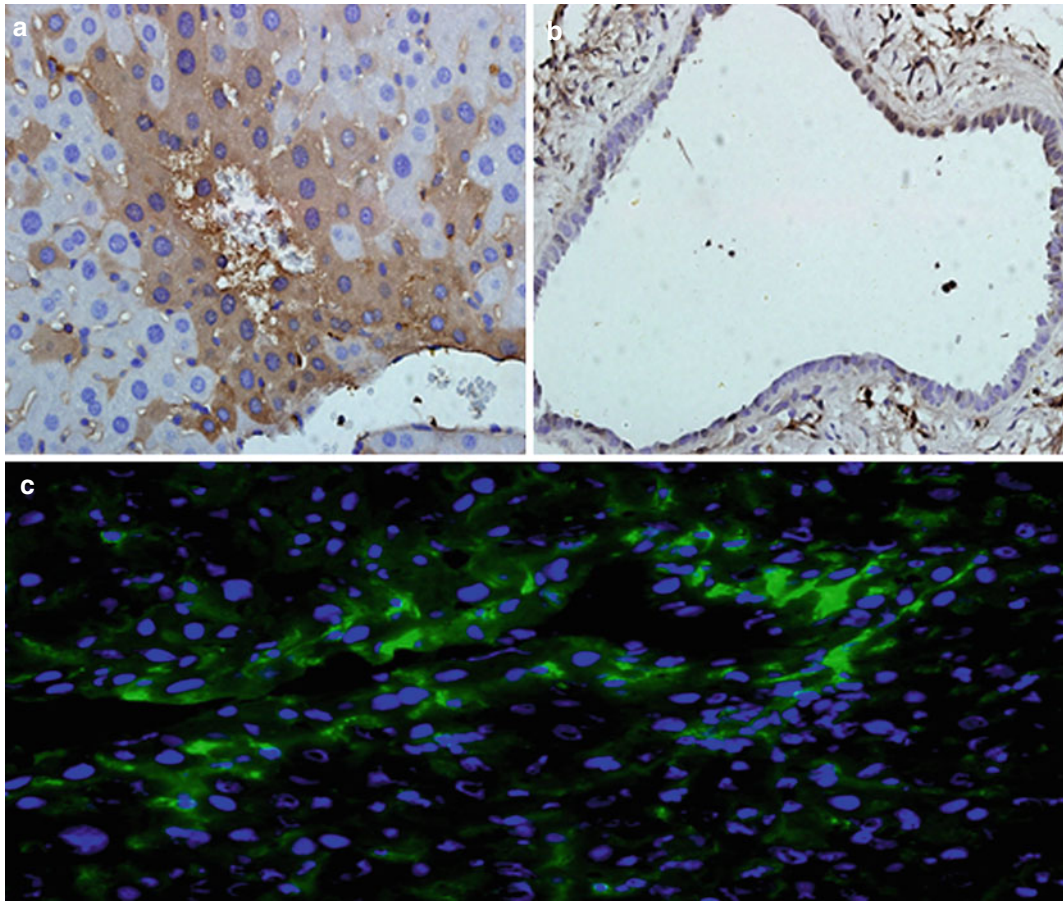

Fig. 17.4 Detection of human hepatic progenitor cells in the mouse liver. (**a**) The human-specific CK18+ antibody stained human cells (*brown*) in mouse parenchyma. Human fetal hepatic progenitor's cells when transplanted into D-galactosamine-treated (GalN) mice showed differentiation into hepatocytes (*brown*). (**b**) The human cells also repopulated areas of mouse bile ducts. (**c**) Human cells stained positive for human albumin+ (*green*). Magnification 60×

ADA activity and donor-lymphocyte and red-cell antigens. The child remained free of major infections until 1 year later when he developed an ultimately fatal nephrotic syndrome associated with immune-complex glomerulonephritis. [140].

Fetal liver transplantation has also been attempted in seven patients with aplastic anemia [141]. Four of these patients showed a partial response as evidenced by decrease in blood transfusion requirements and increase in the peripheral blood counts and hematopoietic cells in the bone marrow. In some cases, bone marrow culture studies revealed evidence of a temporary mixed lymphoid chimerism. While case 1 lived for 16 months, case 3 is surviving at 17 months. None of the patients showed apparent graft-versus-host disease. Increased incidence of infections was noticed. Thus, fetal liver transplant may also be of therapeutic value in management of aplastic anemia.

Summary

Fetal liver cells have a high proliferative capacity, are less immunogenic, and are more resistant to injury during cryopreservation and more resistant to ischemic injury. These qualities could enhance

their engraftment, whereas their proliferation increases gene transfer efficiency. These cells are highly proliferative and have an active DNA synthesis, properties required for gene transfer and for achieving a high degree of repopulation and expression in some liver diseases. Fetal hepatoblasts can serve as efficient vehicles for ex vivo gene therapy. Reconstituting telomerase activity in human fetal hepatocytes has led to immortalization of the cells with no alteration in their liver-specific nature. Thus, fetal liver cells represent an attractive and important alternative source of cells for transplantation. However, fetuses are obtained from abortion, and ethical concerns may limit the use of this material. Nevertheless, fetal liver cell transplantation is a promising new approach for the treatment of liver-based inborn errors of metabolism or secondary liver function impairment, as is seen in fulminant liver failure or end-stage liver disease.

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18 Human Pancreatic Progenitors: Implications for Clinical Transplantation in Diabetes

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Human Pancreas Development

Studies on understanding the developmental biology of human pancreas have been restricted by ethical constraints and access to human fetal tissue. Most of the research related to developmental biology of pancreas has been carried out in mice and other species such as rat, frog, zebrafish, and chick [63, 86, 90, 108, 117, 128]. Observations made in mouse models have highlighted a critical role for several important pancreatic transcription factors such as the pancreas and duodenal homeobox 1 gene (also known as insulin promoter factor 1/ipf1 and formerly recognized as islet/duodenum homeobox-1/IDX-1, somatostatin-transactivating factor 1/STF-1, insulin upstream factor 1/IUF-1 or glucose-sensitive factor/GSF) [2, 3, 68, 88, 111]. It was demonstrated that targeted disruption of Pdx1 causes pancreatic regression soon after bud formation and that PDX1 is not only important in generation of the pancreas but also in achieving enough number of hormone-producing cells in the pancreas [35, 60, 87]. Clinical studies carried out in a loss-of-function mutation in human pdx1 gene have associated these mutations with pancreatic agenesis and maturity onset diabetes of the young (MODY), suggesting a clear role for Pdx1 during human pancreas development and function [110, 111]. Although there are a limited number of studies in understanding the role of Pdx1 in human pancreas development [4, 12, 16, 74, 75, 93, 100, 120, 123], there is a significant amount of information on endocrine pancreas development in lower vertebrates (reviewed in [48, 64, 102, 106]). The studies discussed above confirm the role of Pdx1 in development and function of human endocrine pancreas. However, there exist specific differences in the developmental plan of human and rodent pancreas. Morphological studies carried out during embryonic development have revealed unexpected differences between vertebrates [95], and human development ceases to be a linear correlate of the rodent process during the second and third trimester of pregnancy. Although pancreatic islets form relatively early during human gestation, it is unclear if they become vascularized and express all markers of a mature and functional islet β-cell. Recent work from the group of A/Prof. Manami Hara has helped in achieving a better understanding of the islet cell architecture and function in different vertebrate pancreas [62, 109]. Similar to the rodent developmental plan, human pancreas develops as ventral and dorsal outgrowths of foregut endoderm. The dorsal bud, which appears a day in advance before the ventral pancreatic bud in mouse, is visible at ~25 days post-coitum (d.p.c) in humans. During embryogenesis, these buds extend into the surrounding

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mesenchymal tissue, which provides inductive signals that assist growth (proliferation) and differentiation of the pancreatic buds. The ventral part rotates behind the developing duodenal loop and gives rise to the gallbladder and liver buds. Hormone expression, seen as rare epithelial cells immunoreactive for insulin, is evidenced at 52 d.p.c, which is after around 4 weeks from the appearance of human pancreatic buds. Glucagon, somatostatin, and pancreatic polypeptide (PP) immunoreactive cells are proposed to be appearing only after another week as isolated epithelial cells. A variable number of cells at this time are also reported to coexpress islet hormones [93, 94]. As compared to this, the murine pancreas develops as ventral and dorsal outgrowths of foregut endoderm from embryonic day (E) 9.5 [64, 106]. Hormone expression commences at E9.5–10 with glucagon preceding the appearance of insulin immunopositive cells [114]. Somatostatin and pancreatic polypeptide (PP) mRNAs are also detected at this time [40, 50, 58]. However, somatostatin and PP immunopositivity is only seen from E15.5 and E16, respectively [114]. In mice, functional islets are formed within a few days of birth after a secondary wave of beta-cell differentiation/maturation [106]. Studies carried out in mouse models suggest that such coexpressing cells do not represent progenitor cells in the developing pancreas [42, 43, 49]. Thus, as compared to human pancreas development, where hormone-containing cells are seen to appear during first trimester, rodent pancreatic hormone-producing cells are seen mostly during second trimester. Although the above studies present specific differences in the expression of endocrine pancreatic hormones and transcription factors, the overall developmental plans in generation of human and rodent pancreas are believed to be similar (Fig. 18.1).

One of the other major differences in human and rodent pancreatic cell types is the organization of hormone-producing cells. Human islets tend to contain fewer β-cells and more α-cells compared to rodent islets [62, 109, 113]. The endocrine cells in human islets do not have a distinct distribution as in rodent islets where β-cells form the core of the islet and there is a mantle of other non-β endocrine pancreatic cells. In humans, α-, β-, δ-, and PP-cells appear to be randomly distributed throughout the islet. An adult human islet will contain around 50 % β-cells, 35 % α-cells, 7 % δ-cells, 2 % PP-cells, and ~6 % of other nonendocrine cells [14, 15, 113]. Cabrera reported little difference in the proportion of endocrine cells in islets from different regions of the pancreas [15], although the tail of the pancreas is thought to have more density of endocrine cells as compared to the head or the body of the pancreas [99, 113]. These differences in islet architecture are indicators of the differences in physiology of insulin secretion by pancreatic β-cells and are known to affect the synchronous release of insulin from islets. Although development of the endocrine pancreas has been well studied in several mouse models discussed above, there are only few studies that report development and function of endocrine pancreas in humans [12, 13, 55, 56, 71, 74, 75, 93, 119, 122, 126]. Studies carried out so far on human pancreas development [5, 55, 75, 93, 103] are limited to assessing only snapshots in time, as compared to lineage-tracing capabilities in rodent models [42–45, 112, 127].

Regulation of Gene Expression

All of the studies discussed above point to the intricate temporal and spatial regulation of transcription factors during development and differentiation of embryonic pancreatic precursors/stem cells to hormone-producing cells in the endocrine pancreas (summarized in Fig. 18.1). Such a process of differentiation involves induction of stepwise changes to the genomic structure and organization of cells into functional structures in specified niches that will favor efficient synthesis and release of pancreatic hormones under physiological conditions. It is important to remember that in spite of the diversity in protein expression in different cells of the human body, they are derived from a single fertilized egg. It is therefore essential to

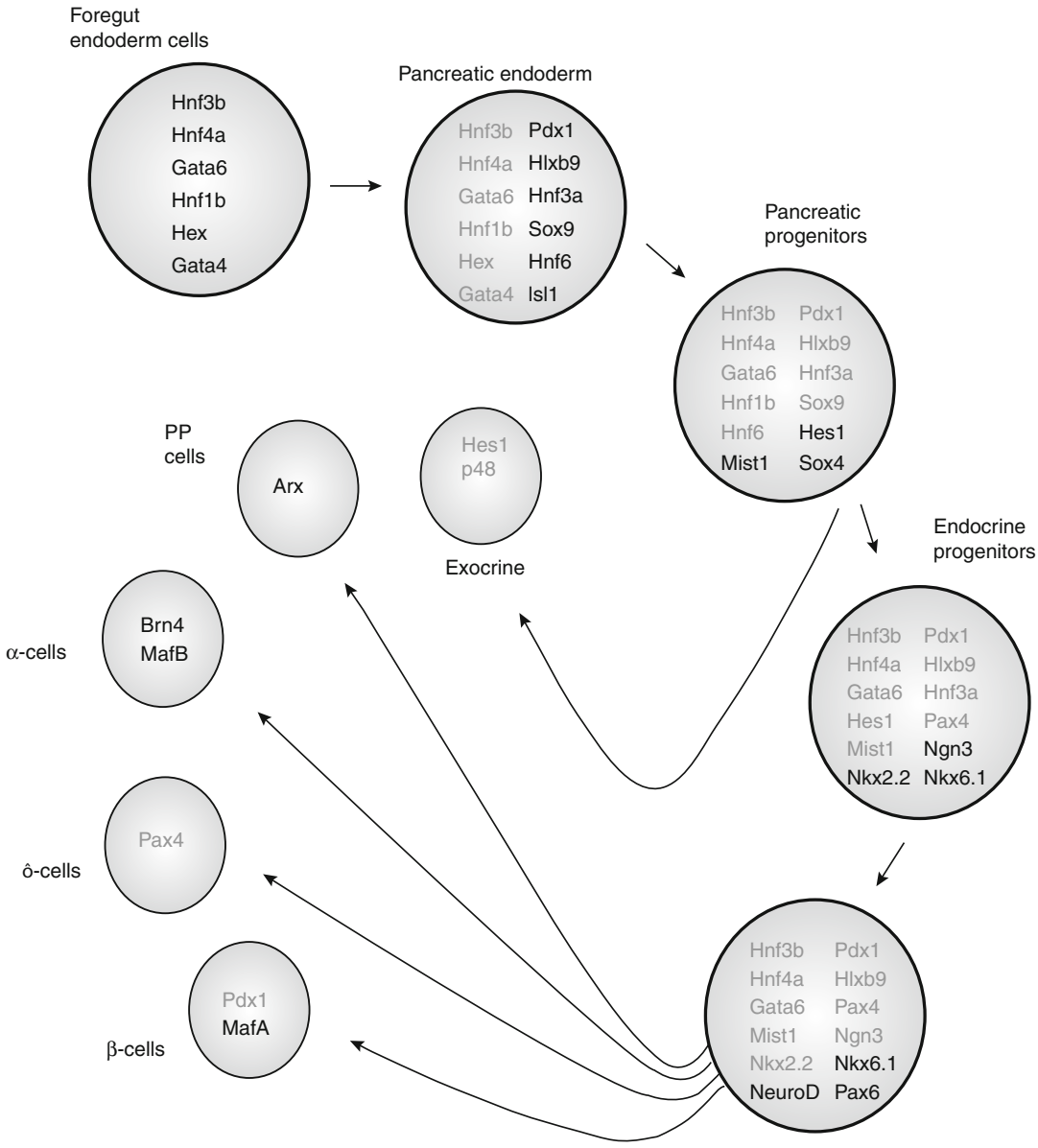

Fig. 18.1 A simplified schematic drawing illustrating the temporal expression of transcription factors during pancreas development. Important transcription factors during pancreas development that have been demonstrated in different original articles discussed in this chapter are illustrated herein. Transcription factors that are specifically expressed in a cell type are shown in *dark black color*, while those that continue to be expressed are shown in *gray color*

understand specific mechanisms that introduce these changes/variabilities in cell types during embryonic development.

The human body is composed of trillions of cells, all of which are the progeny of a single fertilized ovum. After the 3 germ layers are formed, spatiotemporal expression of regulatory molecules, which will be collectively referred to as "growth and differentiation factors"/"GDFs," lead to tissue-specific gene expression. This

process involves selective expression of genes and is carefully regulated by inheritance of epigenetic marks that include specific modification of DNA-binding protein subunits, the histones [39, 51, 129]. Histones are strongly alkaline proteins that are found in all the eukaryotic cell nuclei and help in getting the DNA packaged into structural units called as nucleosomes. Histones are the chief protein components of chromatin and serve as "mini-spools" that wind the DNA inside the nucleus of a cell. This is a very critical step in development of each tissue. For example, in the pancreatic β-cells, the winding of DNA will allow for part encoding the insulin gene to be loosely wound/made accessible to the transcriptional machinery (when necessary). However, this will not be the case for melanocytes/skin cells that transcribe the melanin, but not the insulin gene efficiently. Histones are vital as they allow efficient packaging of the entire human genomic DNA into chromosomes, which would otherwise be very long [10, 33, 41, 91]. Histones are highly conserved and can be grouped into five major classes: H1, H2A, H2B, H3, and H4. The H2A, H2B, H3, and H4 form a class of "core" histones, while H1 is called as the "linker" histone. Two of each of the core histones comprises to form one octameric nucleosome core particle by wrapping 147 base pairs of DNA around this protein spool in 1.65 left-handed superhelical turn. The linker histone H1 binds the nucleosome and the entry and exit sites of the DNA, thus locking the DNA into place while allowing for a physical "space" for DNA-binding protein and transcriptional regulators to interact while maintaining higher-order structure. The assembled histones and DNA is called chromatin. During mitosis and meiosis, the condensed chromosomes are assembled through interactions between nucleosomes and other regulatory proteins [10, 98, 130].

Expression and transcription of eukaryotic proteins is regulated at multiple stages [27–29, 78]. Firstly, the actual physical structure of the chromatin, discussed above, can affect the ability of regulatory proteins and RNA polymerases to access specific genes [36]. Modifications of histone tails as well as CpG methylation affect accessibility of RNA polymerases and transcription factors to DNA. Modification of histone tails and DNA methylation comprise the next level of gene regulation via what are widely known as "epigenetic" factors. Epigenetics refers to heritable changes in gene expression that are not due to any changes in composition of the genome. Next, initiation of transcription is an important process, and in case of insulin gene expression, this is known to be regulated by efficient binding of PDX1 to a binding site that is upstream of the proinsulin gene promoter. Following efficient transcription, processing and modification of the mRNA (capping and polyadenylation as well as removal of introns) is the next major step in regulation. Several genes, such as the pro-glucagon gene, undergo alternative splicing, based on the tissue where it is expressed [25, 34, 54]. Pro-glucagon gene expression in α-cells leads to production of the hormone glucagon, while in L-cells of the intestine, it leads to production of the secretagogue glucagon-like peptide1/GLP1. Once such an RNA is ready, it must be transported out of the nucleus in order to be translated into protein. Transcript stability is the next line of regulation. In case of prokaryotic organisms, it has been observed that the half-life of different transcripts is generally between 1 and 5 min. However, eukaryotic mRNAs are generally very stable [26, 31, 52]. Human proinsulin mRNA has a half-life of around 20 h. A recently demonstrated mechanism of gene regulation was discovered in the beginning of this decade, when small RNA regulatory elements (called as non-coding RNAs/ncRNAs and microRNAs/miRNAs) were shown to affect transcript stability. For review of such regulatory elements in endocrine pancreas development and postnatal regeneration, please refer to Joglekar et al. [57]. Once the mRNAs are available for translation, initiation of translation is the next level of gene regulation. Since mRNAs have several methionine codons, the ability of ribosomes to recognize and initiate synthesis from the correct AUG codon can affect the process of translation. Finally, post-translational modifications such as glycosylation, acetylation, and disulfide bond formations are the limiting steps that may regulate the final

production of a specific protein. The mature insulin protein is a heterodimeric protein consisting of a 21 amino acid long A-chain and a 30 amino acid long B-chain. These chains are linked together by disulphide bridges. The insulin gene actually encodes for pre-proinsulin, which is a much larger molecule (110 amino acids long) and contains a hydrophobic N terminus signal sequence and a 35 amino acid long connecting (C)-peptide that links the C terminus of B-chain to N terminus of A-chain [69, 133]. The signal peptide targets the protein to secretory pathway by targeting it to the endoplasmic reticulum. Following production of insulin, it needs to be packaged into secretory granules and transported close to the cell membrane so that they are ready to be transported out, following increase in glucose concentrations outside the cell.

All the processes discussed above demonstrate the level of complexity of gene expression in islet β-cells. During embryonic development, human pancreatic β-cells are generated following an intricate and orchestrated process that generates these highly efficient factories of insulin-producing cells. We therefore think that generation of such insulin-producing cells from pluripotent/embryonic stem cells will require a high degree of regulation of multiple growth and differentiation factors/GDFs that we are just unable to understand and appreciate. We therefore took a different approach to the generation of islet progenitor cells.

Human Islet-Derived Progenitor Cells

We introduced the concept that lineage-committed mesenchymal cells can be obtained by a phenomenon of epithelial-mesenchymal transition (EMT), where differentiated cells in islets can transition to proliferative populations of mesenchymal-like human islet-derived progenitor cells (hIPCs) [38]. These hIPCs express surface antigens including CD44, CD73, CD90, CD29, and CD105 [19], similar to those seen in bona fide mesenchymal cells such as the bone marrow-derived mesenchymal cells. Single cell suspensions obtained after trypsinization of human islets were demonstrated to undergo similar transition to mesenchymal cells [89]. These investigators presented evidence to confirm that Vimentin (protein)-producing mesenchymal cells were possibly derived from pdx1-producing (coexpressing) cells in culture. As discussed before, pdx1 is an important transcription factor that is restricted only to β-cells in adult pancreatic islets. Thus, presence of pdx1 in vimentin-immunopositive cells indicated that mesenchymal cells obtained in cultures of pancreatic islets originate from pdx1-containing, insulin-producing cells. This idea of proliferation of pancreatic β-cells was not supported by several groups [7, 18, 82, 127] as the initial demonstrations [38, 89] lacked evidence for lineage tracing during mesenchymal transition. However, we [56, 59] and the group of Prof. Shimon Efrat [96, 97] independently demonstrated in the last 2 years that human pancreatic β-cells can undergo EMT to generate proliferative populations of islet-derived progenitor cells. During this transition, β-cells gradually lose key islet proteins as well as (pro)insulin transcripts after ~1,000-fold expansion (passage 10). Such islet progenitors, derived from fetal (fIPCs) or adult (hIPCs) human islets produce several mesenchymal proteins such as vimentin, smooth muscle actin, as well as the intermediate filament protein nestin. To assess if human pancreatic β-cells directly transition to these mesenchymal cells in vitro, Russ et al. used a dual lentiviral system to indelibly mark β-cells and their progeny [96]. They demonstrated for the first time that (labeled) human β-cells proliferate in vitro and undergo epithelial-to-mesenchymal transition (EMT) to generate mesenchymal-like cells [96, 97]. To answer the same issue, we used another approach. This method was derived from the protocol proposed for lineage tracing of mouse β-cells in vivo [115]. Freshly isolated human fetal islets were sequentially pulsed with two different thymidine analogues in vitro. Each of these analogues can be then detected using specific antibodies along with another marker of choice (such as C-peptide/insulin for β-cells). Since all proliferating cells will take up thymidine (or the analogue) for incorporation into DNA, all the proliferating cells will be labeled by either or both the

analogues. Using this method, fetal pancreatic beta cells were found to undergo at least two rounds of replication (labeled with both analogues) in the period of 7 days [56]. Characteristic features of EMT such as translocation of β-catenin to nucleus and loss of E-cadherin immunopositivity were also observed [59]. The group of Prof. Shimon Efrat has also looked at expansion of β-cells that were sorted out from whole islets [97]. Such mesenchymal cells exhibit surface markers similar to those on hIPCs and do not possess multilineage differentiation ability. These β-cell-derived mesenchymal cells thus appear to have restricted differentiation potential and can be considered as endocrine pancreatic lineage-restricted progenitor cells.

Differentiation of Human Islet-Derived Progenitor Cells

Use of stem cells is based on the concept that undifferentiated precursor cells can be expanded exponentially and then induced to differentiate into mature endocrine cells in vitro. There are several different types of precursor cells that could be used in such an approach [11, 37, 66, 72, 76, 79, 92, 107, 118]. It is generally agreed that inducing efficient differentiation of precursor cells into mature β-cells is the most difficult aspect of this approach. We believe that embryonic stem (ES) cells are the most difficult cell types to achieve efficient differentiation to a β-cell type [37]. Embryonic stem cells are pluripotent stem cells that can differentiate into majority of cell types found in the human body [6, 24, 125]. However, nonspontaneous differentiation of ES cells into insulin-producing cells by maintaining a delicate balance of transcription factors and signaling molecules is difficult. Different dose or timing of any single factor may turn the fate of ES cell to a different cell type. Another important source of progenitors is mesenchymal stem cells derived from various adult tissues such as the bone marrow. Several reports indicate ability of these progenitors to differentiate into insulin-producing cells [17, 61, 73, 116, 132]. Apart from such mesenchymal cells, transdifferentiation of liver cells, pancreatic duct cells, as well as acinar cells into endocrine pancreatic lineage is also considered as an approach to generating β-cells in the laboratory [8, 9, 77, 80, 81, 85, 134]. As demonstrated in our initial observation [38] islet-derived mesenchymal cells can be induced to undergo reversible EMT (mesenchymal-to-epithelial transition/MET). Since hIPCs contain progeny of islet β-cells, we proposed that these cells retain epigenetic marks that make up a β-cell and will therefore be able to transcribe insulin.

As described earlier in this chapter, histone tail modifications are one of the critical steps in regulation of gene expression (represented in Fig. 18.2). Certain histone modifications such as methylation of 4th lysine residue on H3 histone (H3K4-Me) or acetylation of H3 and H4 histones (H3-Ac and H4-Ac) are indicators of active chromatin conformation that allows efficient transcription. On the other hand, methylation of 9th or 27th lysine residue on H3 histone (H3K9-Me or H3K27-Me) denotes inactive chromatin that is less accessible to transcription machinery [53, 70, 83, 124]. We assessed insulin promoter region during generation and expansion of islet progenitor cells. We observe that insulin promoter region retains active chromatin conformation even after several 1,000-fold expansion in vitro, indicating that these fetal islet-derived progenitors contain progeny of β-cells [56]. Similar findings were also seen in adult islet-derived progenitors [84]. These data strongly support the thought that progenitor cells obtained from islets are better candidates for replacement therapy in diabetes.

We demonstrated that hIPCs can be induced to differentiate into insulin-producing cells in serum-free media [38]. Under such conditions, hIPCs migrate toward each other and form islet-like cell aggregates (ICAs). This process involves migration of mesenchymal cells into cell aggregates and their transition to an epithelial/islet-like phenotype. Following exposure to different GDFs such as exendin (a GLP1R agonist) and nicotinamide, ICAs transcribe, translate, process, and secrete insulin, as observed in mice transplanted with hIPC-derived ICAs [38]. However, the efficiency of differentiation, as measured by

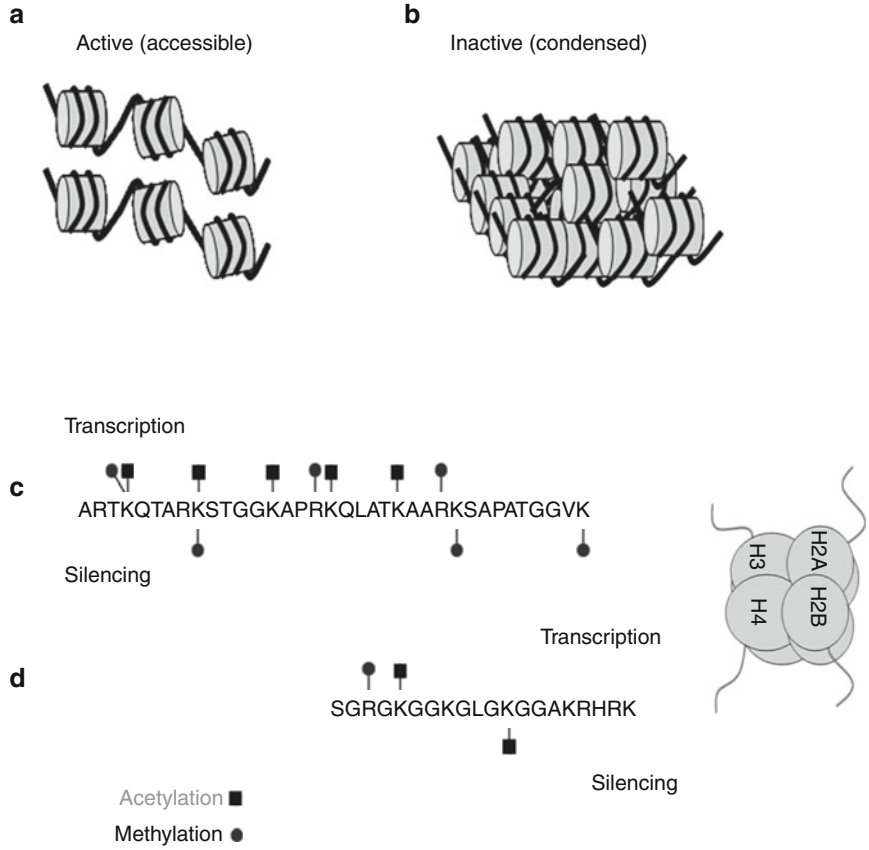

Fig. 18.2 The insulin promoter region of β-cells has an "active" (**a**) promoter region that can efficiently transcribe insulin. Other cells, such as skin cells, have a condensed/inactive chromatin conformation (**b**) at insulin promoter region. Such an "open" or "compact" chromatin conformation is brought about by selective modifications (acetylation, methylation, phosphorylation, etc.) of histone tails. This schematic represents modifications (acetylation and methylation) on histone H3 (**c**) and H4 (**d**) tails. Modifications that are presented above the specific amino acid residue favor transcription, while those, which are shown below the residue, indicate silencing. A *circle* ( ) indicates methylation, while *square* ( ) denotes acetylation

insulin transcript abundance, was found to be less than a fraction of adult human islets. In another study, betacellulin was found to be more potent than exendin-4 or activin A in inducing in vitro differentiation of pancreatic progenitor cells [89]. Several other strategies have also been employed to improve efficiency of in vitro differentiation [1, 67, 131]. It was further demonstrated that similar to fetal (immature) cells [46], hIPC-derived ICAs also mature and differentiate better when transplanted under the kidney capsule of the NOD/SCID animals [19]. All these studies indicate the potential of islet-derived progenitor cells for differentiation into insulin-producing cells.

However, none of the studies mentioned above was able to achieve efficient synthesis and secretion of insulin from differentiated islet-derived progenitor cells so as to meet the physiological demand of adult human body. We observe that the in vitro differentiation potential of hIPCs decreases with increasing number of passages in vitro. We also found that expansion of fetal islet-derived progenitors leads to increase in H3K9 methylation at insulin promoter region [56]. Our observations suggest that islet-derived progenitor cells originate from β-cells as well as non-β-cells within the islets. Although it is demonstrated that human β-cells can proliferate in vitro [56, 59, 96, 97], the proliferation rate of

non-β-cells is much higher than islet β-cells. This can eventually lead to a population of islet-derived progenitors that is dominated by progeny of non-β-cells. This explains the decreased differentiation ability/multilineage capabilities as well as increased inactivation of insulin promoter region in late passage progenitors that are derived from the heterogenous populations of human islets. We propose that progenitor cells obtained after EMT of pure β-cells will be an answer to the above mentioned difficulties. Efficient isolation and expansion of β-cell progeny without any contaminating cell type will help us to achieve a β-cell lineage-committed progenitor cell type that could be easily and efficiently differentiated into insulin-producing cells. It is therefore important to generate clonal populations from β-cells and study their differentiation potential.

Transplantation of Pancreatic Progenitor Cells

Transplantation of whole pancreas or isolated islets is currently the most successful cell-based therapy for diabetes [47, 104]. Transplanted islets are able to keep the individual off insulin for at least 1 year, under immunosuppressive regimen. Immune response of the host to the graft is critical for survival and function of graft after transplantation. Thus, even if a source of β-cells is generated in vitro, it can be successfully grafted only after successful implementation of an efficient immunosuppressive regimen. However, fetal tissue, especially during the first trimester, is believed to be less immunogenic, thereby imparting an advantage for transplantation [13, 105]. The major limitation here is that it lacks the high numbers of mature insulin-producing cells that can secrete physiologically relevant levels of insulin. Different fetal tissues such as human fetal kidneys and skin are shown to have reduced immunogenicity and prolonged survival after transplantation in humanized animal models [21, 30]. Immunogenicity of kidneys is shown to increase during embryonic development [20, 22]. These studies demonstrated that second trimester and adult kidney grafts are rapidly rejected, while the 7–8-week gestational age kidneys showed good engraftment and remained functional. Similarly, first-trimester human as well as pig fetal pancreatic grafts demonstrate less immunogenicity and minimal infiltration of immune cells [13, 32]. However, second trimester fetal pancreas demonstrated increased immune response and are rejected after transplantation [32, 121]. Reducing immunogenicity by altering culture conditions could not result in complete absence of host immune attack. Recent reports demonstrate that mesenchymal stem cells obtained from various adult tissues are immunomodulatory [23, 65, 101]. They are known to regulate immune response at multiple levels, one of them being inhibition of proliferation of allogenic T cells in mixed lymphocyte cultures. We therefore believe that mesenchymal stem-like cells obtained after EMT of fetal pancreatic ICCs will have combined advantages of mesenchymal stem cells and fetal pancreas. These cells can confer the benefits of better survival and function after transplantation.

Concluding Remarks

As of now, the best replacement for β-cells is β-cells themselves. This is unequivocally proved in several successful transplantations using isolated islets from cadaveric human donors. However, their availability and yield are the major constraints. In order to overcome this problem, attempts were done to expand β-cells in vitro. β-cells rapidly lose their identity in culture and dedifferentiate into mesenchymal-like phenotype. Human pancreatic islet-derived progenitors (hIPCs) are believed to be better precursors for differentiation into insulin-producing cells, because they are derived from cells that produce insulin. Since these islet-derived progenitors have epigenetic memory of producing insulin, they can be efficiently differentiated into insulin-producing cells in vitro as compared to other progenitor cells that lack this advantage. Fetal origin of progenitor cells may provide advantages specifically with reference to proliferative ability and reduced immunogenicity. However, all of these studies are not presently at a stage where they can be taken to clinics. Although

human pancreatic islet-derived progenitors appear to be the cells of choice, the field is yet immature in achieving efficient differentiation of these islet progenitor cells. The coming years will only reveal the potential of islet-derived progenitors to differentiate efficiently and be offered for use in cell replacement therapy for diabetes.

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19 Amniotic Fluid Cell Therapy to Relieve Disc-Related Low Back Pain and Its Efficacy Comparison with Long-Acting Steroid Injection

Niranjan Bhattacharya

Introduction

Human intervertebral disc undergoes multifactorial, biochemical, and morphologic degenerative changes during the process of aging. Surgically removed human discs show an active inflammatory process proceeding from the outside-in. The pathogenesis of discogenic acute nonspecific low back pain is mostly considered to be a re-rupture in an asymptomatic ruptured region in the posterior annulus, repaired by granulation tissue, in a moderately degenerated intervertebral disc with a radial tear [1].

The clinical manifestation of the process starts with acute nonspecific low back pain that is characterized by the sudden onset and severe unendurable low back pain without radicular pain or neurological deficit in the lower extremities. The background pathophysiology indicates that degeneration of the painful disc may originate from an injury and subsequent repair of annulus fibrosus. Growth factors, such as bFGF and TGF-beta1, macrophages, and mast cells might play a key role in the repair of the injured annulus fibrosus and subsequent disc degeneration [2].

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Physical therapies should aim to promote healing in the disc periphery, by stimulating cells, boosting metabolite transport, and preventing adhesions and reinjury. Such an approach has the potential to accelerate pain relief in the disc periphery, even if it fails to reverse age-related degenerative changes in the nucleus [3]. The biochemical and molecular background behind the disease process revealed that type II collagen and proteoglycan (predominantly aggrecan) content is crucial to proper disc function, particularly in the nucleus pulposus. In degeneration, synthesis of matrix molecules changes, leading to an increase in the synthesis of collagens types I and III and a decreased production of aggrecan. Linked to this is an increased expression of matrix-degrading molecules including MMPs (matrix metalloproteinases), aggrecanases, and ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) 1, 4, 5, 9, and 15, all of which are produced by native disc cells. Growth factors and cytokines (particularly TNF alpha [tumor necrosis factor alpha] and IL-1 [interleukin 1]) have been implicated in the regulation of this catabolic process. Investigators have shown that in degenerate discs, there is an increase in IL-1, but no corresponding increase in the inhibitor IL-1 receptor antagonist. Inhibition of IL-1 would therefore be an important therapeutic target for preventing/reversing disc degeneration [4]. Another group of investigators have suggested that ADAMTS-5 is probably involved in the process of IVD degeneration and that IL-1β-induced expression of ADAMTS-5 is mediated by NO [5].

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In a nutshell, traumatic or age-induced degeneration is the key for this condition. Treatment practiced globally is analgesic (nonsteroidal anti-inflammatory) drug and its different permutations and combinations with rest initially, followed by physiotherapy by different approaches. The other option is surgical decompression. But in any of the approaches, the core problem, of degeneration, is not solved. Treatment is aimed to treat the effect of degeneration, not the cause. Cell therapy is a modern new approach which can take care of the main issue of degeneration either by injection of autologous mesenchymal stem cells at the degenerated disc site of the bone after its collection from bone marrow avoiding the immunological problems. The other approach of live cell therapy is a simple solution to the degeneration of the disc problem by treatment with freshly collected amniotic fluid, which is a rich source of epithelial progenitor cells and mesenchymal cells apart from its intrinsic antibacterial component like properdin-like substances embedded in it. This amniotic fluid is collected from consenting mothers undergoing hysterotomy and ligation for family planning purpose approved by the Government of India, Ministry of Health.

Materials and Methods

Fresh amniotic fluid was collected from women admitted for hysterotomy and ligation at Bijoygarh State Hospital (2001–2006) and was used for the present study for the treatment of patients with disc-related pain. As per the standing direction of the State Family Planning Department, hysterotomy and ligation may be allowed up to 20 weeks of pregnancy, provided the mother has two or more healthy children. For the present study, 10 mL amniotic fluid was collected aseptically in the OT from each mother undergoing hysterotomy and ligation. The collection of the amniotic fluid is done always from an intact sac after opening the uterus, when the amniotic membrane containing the amniotic fluid generally herniates outside the uterus. The sac was gently punctured, and the amniotic fluid was sucked out aseptically with a wide-bore size 16 needle and syringe. The collection protocol initiates, after getting the donor's consent and the recipient's informed consent and also the approval of the hospital ethical committee.

Initially, 51 patients volunteered for this project of amniotic fluid cell therapy after confirmation of the degenerative lumber intervertebral disc in magnetic resonance imaging (MRI) of the lumbosacral joint with clinical discogenic pain. Four cases were discarded from the study due to advanced prolapsed intervertebral disc (PID) with serious nerve compression sequel who needed immediate decompression. Another five cases were discarded from the study due to the association of neurodegenerative diseases such as parkinsonism, cerebral atrophy with dementia of varying etiology, and other chronic disease burdens.

These patients were randomized for age and sex and eventually divided in two equal groups: Group A received long-acting steroid and Group B received 10 mL of amniotic fluid as a source of cell therapy. The donor of the amniotic fluid were (HIV 1 and 2 and hepatitis B and C negative) mothers carrying pregnancy (14–20 weeks gestation) and admitted for hysterotomy and ligation from the Family Planning Department. Only informed and consenting mothers were enrolled for the procedure after passing through the institutional ethical committee.

Before the procedure, a thorough history of all the patients was taken, i.e., age; sex; height; weight; menstrual history; history of chronic disease like tuberculosis, hypothyroid, frank diabetes, or even altered glucose tolerance; history of diabetes in the family; lipid profile including uric acid level; apart from a history of specific involvement of cancer; systemic lupus erythematosus; and ankylosing spondylitis. Specific rheumatological history with history of oral or intra-articular steroid intake, degree, and pattern of joint involvement with the duration of affection was noted. The discogenic pain was noted on a 100-mm horizontal visual analog pain scale (VAS). The other parameters that were assessed included the distance walked in 1 min (WD) and also a locally modified and local (Bengali)

language-translated modified Health Assessment Questionnaire that was to be filled up.

At follow-up visits (1st–6th, 9th, 12th, 18th, and 24th month), a specialist doctor made an objective assessment of the clinical condition with subjective correlation, as much as it was practicable, for all the patients enrolled for the type of treatment. The idea is to clinically assess the overall status of cell therapy treatment and its comparison with standard long-acting steroid treatment for relief of the discogenic pain.

Pain score (VAS), WD, and HAQ were recorded. Student's paired test (p value) was also conducted. Analysis of variance for repeated measures was used to compare differences that were assessed by simple regression analysis. The differences in patient opinion of overall change and relationship between clinical evidences were calculated by contingency table analysis incorporating mean with standard deviation (SD). Differences that were significant at the 5 % confidence interval are quoted in the follow-up chart record. At the completion of the study after 3 years of follow-up, patients who received cell therapy were offered steroid therapy if they voluntarily requested for that procedure, and vice versa.

In order to quantify the overall impression of improvement or deterioration of the low back pain with the treatment offered, we have a disability scoring system known as the Oswestry low back pain disability questionnaire. This scoring system has ten components like assessment of (1) pain intensity, (2) personal care capability, (3) lifting capability, (4) walking capability, (5) sitting capability, (6) standing capability, (7) sleeping, (8) sex life, (9) social life, and (10) traveling capability. Each parameter has many components, viz., 0 (normal) to 5 (highest pain) when pain totally prevents that specific intended activity (0–5), viz., (a) 0–20 %: minimal disability: The patient can cope with most living activities. Usually no treatment is indicated apart from advice on lifting, sitting, and exercise. (b) 21–40 %: moderate disability: The patient experiences more pain and difficulty with sitting, lifting, and standing. Travel and social life are more difficult and they may be disabled from work. Personal care, sexual activity, and sleeping are not grossly affected, and the patient can usually be managed by conservative means. (c) 41–60 %: severe disability: Pain remains the main problem in this group, but activities of daily living are affected. These patients require a detailed investigation. (d) 61–80 %: crippled: Back pain impinges on all aspects of the patient's life. Positive intervention is required. (e) 81–100 %: These patients are bed bound [6].

Table 19.1 The patients selected for the study (epidemiological profile) ($N=42$)

1. Age of group: 36–82 years
2. Sex: males 21 and females 21
3. Weight: 44.8–112.6 kg
4. Height: 4 ft 6 in. to 6 ft 3 in.
5. Duration: 1–11 years
6. Low back pain not relieved with lumbosacral corset: 16
7. Low back pain with radiation to leg, not relieved with lumbosacral corset: 26
8. Treatment with analgesic including NSAID and physiotherapy: all of them (42)
All cases were randomized and divided equally into Group A ($n=21$) and Group B ($n=21$)

Result and Analysis

In the present series, 42 patients of age varying from 36 to 82 years were enrolled, vide details on Table 19.1. There was definite history of fall or trauma that was present in 7 cases; the residual 35 cases did not have a similar history. These 42 cases, who did not respond to conventional pharmacological or nonpharmacological treatment, were ultimately enrolled for this trial.

The pharmacological treatment had included use of NSAIDs, i.e., naproxen, ibuprofen as well as the cyclooxygenase-2 inhibitor group of drugs like celecoxib with supporting drugs such as glucosamine, chondroitin, and opiates, only to name a few. The nonpharmacological treatment had included special exercises under supervision and lumbosacral support (corset). Those patients suffering from disc-related pain and difficulty in

Table 19.2 The value of the VAS (visual analog pain scale), WD (walking distance in meters), and HAQ (Health Assessment Questionnaire) assessments in steroid (Group A) and cell therapy (Group B)

(Pretreatment mean ± SD) VAS (mm)	(Third month mean ± SD) VAS (mm)	(Sixth month mean ± SD) VAS (mm)	*p* value
Mean Group A values with SD: 29 ± 7.3	19 ± 6.47	22 ± 3.8	($p < 0.02$)
Mean Group B values with SD: 31 ± 6.2	17 ± 3.3	11 ± 4.6	($p < 0.002$)
Walking distance in meters (WD)			
Mean Group A values with SD 36.4 ± 4.8 m	52 ± 3.7 m	55 ± 4.2 m	($p < 0.01$)
Mean Group B values with SD 34.8 ± 3.9 m	58.6 ± 6.9 m	69.4 ± 7.2 m	($p < 0.01$)
Local language Modified Health Analysis Questionnaire (1–11)			
Mean Group A values with SD 2.2 ± 0.2	2.4 ± 0.2	2.3 ± 0.4	($p < 0.002$)
Mean Group B values with SD 2.4 ± 0.3	1.9 ± 0.12	1.6 ± 0.31	($p < 0.01$)

(The *t*-test, one-way analysis of variance [ANOVA], and a form of regression analysis)

walking not effectively reduced with rest, analgesic, and muscle relaxant were divided in two equal groups for inclusion in either of the following protocols of aseptically, intradiscal C-arm-guided injection in operation theater of 10 mL freshly collected amniotic fluid for cell therapy (Group B) or intradiscal instillation of long-acting steroid, i.e., methylprednisolone acetate injectable suspension of 80 mg dissolved in 5 mL water + 5 mL of 1 % Xylocaine is infiltrated slowly at the site of maximum tenderness of the patient in a sterile manner, (USP Depo-Medrol, 40 mg/1 mL) under X-ray guidance (Group A), depending on the patient's voluntary informed consent.

Epidemiological details are noted in Table 19.1. The subsequent follow-up was noted with the response to treatment as seen on the visual analog pain scale (VAS), walking distance in meters (WD), and modified local language Health Assessment Questionnaire (HAQ) in Table 19.2. In Table 19.3, the response to therapy as per Oswestry low back pain disability and follow-up is shown and graphically represented in Graph 19.2. If we study the Tables 19.1, 19.2, and 19.3 and the impact and comparison of the treatment in Graphs 19.1 and 19.2, one conclusion is quite obvious and is statistically significant ($p < 0.01$), i.e., amniotic fluid cell therapy in its own niche is highly effective and much superior to the globally practiced C-arm-guided long-acting steroid in relieving discogenic pain.

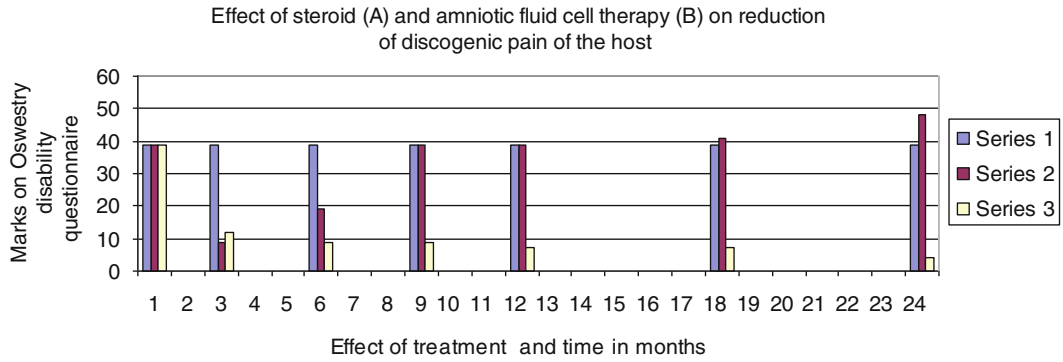
If we study further, the relief with steroid is found to be temporary and there is a great possibility of recurrence; however, cell therapy with its rich epithelial and mesenchymal cell component can help regeneration of the injured tissue with its stem cell-related regenerative potential. Sometimes for immediate pain relief, surgery is effective, but it is highly technical, hence costly. There is also high morbidity and mortality directly and indirectly related to the procedure. Hence, the surgical approach which can have the potentiality for relief is often short lived in most cases, and surgery does not offer a cure or permanent repair for this chronic painful condition.

As a source of cell therapy, amniotic fluid is being studied in great detail by many global investigators. One researcher has analyzed the number and size of viable amniotic fluid cells (AFCs). Small AFCs (SAFCs) and large AFCs (LAFCs) were isolated using a sterile 10-micron pore-size strainer. Flow cytometry analyses showed that cell surface antigen expression on LAFCs and SAFCs was positive for CD29, CD44, CD73, CD90, CD166, and HLA-I, but negative for CD31, CD34, CD45, CD117, and

Table 19.3 The follow-up of Group A and B with the comparison of the results

Group A treated with intra-articular steroid $N=21$	Group B treated with cell therapy $N=21$	Satisfaction after 1 month	Special comment
Assessment after 1 month showed improvement, i.e., mean subjective and objective assessment of definite relief in 20 cases lost follow-up (LFU) = Nil	Assessment after 1 month showed improvement, i.e., mean subjective and objective assessment of definite relief in 18 cases lost follow-up (LFU) = Nil	Group A = 20 Group B = 18	($p<0.01$)
Assessment after 2 months showed improvement, i.e., subjective and objective assessment of definite relief in 15 cases (LFU) = Nil	Assessment after 2 months showed improvement, i.e., mean subjective and objective assessment of definite relief in 18 cases (LFU) = Nil	Satisfaction after 2 months Group A = 15 Group B = 21	($p<0.01$)
Assessment after 3 months showed improvement, i.e., subjective and objective assessment of definite relief in 12 cases (LFU) = Nil	Assessment after 3 months showed improvement, i.e., mean subjective and objective assessment of definite relief in 21 cases (LFU) = Nil	Satisfaction after 3 months Group A = 12 Group B = 21	($p<0.01$)
Assessment after 4 months showed improvement, i.e., subjective and objective assessment of definite relief in 12 cases (LFU) = Nil	Assessment after 4 months showed improvement, i.e., subjective and objective assessment of definite relief in 21 cases (LFU) = Nil	Satisfaction after 4 months Group A = 12 Group B = 21	($p<0.01$)
Assessment after 5 months showed improvement, i.e., subjective and objective assessment of definite relief in 10 cases (LFU) = Nil	Assessment after 5 months showed improvement, i.e., subjective and objective assessment of definite relief in 21 cases (LFU) = Nil	Satisfaction after 5 months Group A = 10 Group B = 21	($p<0.01$)
Assessment after 6 months showed improvement, i.e., subjective and objective assessment of definite relief in 7 cases (LFU) = Nil	Assessment after 6 months showed improvement, i.e., subjective and objective assessment of definite relief in 21 cases (LFU) = Nil	Satisfaction after 6 months Group A = 7 Group B = 21	($p<0.01$)
Assessment after 7 months showed improvement, i.e., subjective and objective assessment of definite relief in 6 cases (LFU) = Nil	Assessment after 7 months showed improvement, i.e., subjective and objective assessment of definite relief in 20 cases (LFU) = Nil	Satisfaction after 7 months Group A = 6 Group B =20	($p<0.01$)
Assessment after 8 months showed improvement, i.e., subjective and objective assessment of definite relief in 5 cases (LFU) = Nil	Assessment after 8 months showed improvement, i.e., subjective and objective assessment of definite relief in 18 cases (LFU) = Nil	Satisfaction after 8 months Group A = 5 Group B = 18	($p<0.01$)
Assessment after 9 months showed improvement, i.e., subjective and objective assessment of definite relief in 4 cases (LFU) = Nil	Assessment after 9 months showed improvement, i.e., subjective and objective assessment of definite relief in 15 cases (LFU) = Nil	Satisfaction after 1 year Group A = 4 Group B = 15	($p<0.01$)
Assessment after 12 months showed improvement, i.e., subjective and objective assessment of definite relief in 4 cases (LFU) = Nil)	Assessment after 12 months showed improvement, i.e., subjective and objective assessment of definite relief in 14 cases (LFU) = Nil)	Satisfaction after 2 years Group A = 4 Group B = 14	($p<0.01$)
Assessment after 24 months showed improvement, i.e., subjective and objective assessment of definite relief in 2 cases (LFU) = Nil	Assessment after 24 months showed improvement, i.e., subjective and objective assessment of definite relief in 12 cases (LFU) = Nil	Satisfaction after 2 years Group A = 2 Group B = 12	($p<0.01$)

Graph 19.1 The comparison and follow-up results of Group A and Group B treatment up to 24 months. *Series 1* is treated with long-acting steroid. *Series 2* is treated with freshly collected second trimester amniotic fluid cell therapy



Graph 19.2 The comparison and follow-up results of Group A and Group B treatment as per scoring by Oswestry low back pain disability questionnaire up to 24 months. *Series 1*: pretreatment. Scoring as per Oswestry low back pain disability questionnaire. *Series 2*: posttreatment. Scoring as per Oswestry low back pain disability questionnaire. Group A (long-acting steroid injection). *Series 3*: posttreatment. Scoring as per Oswestry low back pain disability questionnaire. Group B (amniotic fluid cell therapy)

HLA-II. Importantly, Nanog, Oct-4, ABCG2, and SOX2 expressions in cells were easily detectable among the SAFC population. Expression of Nanog and ABCG2 was not observed among LAFCs [7].

The discovery of amniotic fluid stem cells has initiated a new and very promising field in stem cell research. In the last 4 years, amniotic fluid stem cells have been shown to express markers specific to pluripotent stem cells, such as Oct-4. Due to their high proliferation potential, amniotic fluid stem cell lineages can be established. Meanwhile, they have been shown to harbor the potential to differentiate into cells of all three embryonic germ layers [8].

Amniotic fluid stem cells have more recently been isolated. They represent a novel class of pluripotent stem cells with intermediate characteristics between embryonic and adult stem cells, as they are able to differentiate into lineages representative of all three germ layers but do not form tumors when injected in vivo. These characteristics, together with the absence of ethical issues concerning their employment, suggest that stem cells present in the amniotic fluid might be promising candidates for tissue engineering and stem cell therapy in several human disorders [9].

In recent years, various types of stem cells have been characterized, and their potential for cardiac regeneration was investigated by a prominent group of investigators, who had previously described the isolation of broadly multipotent cells from amniotic fluid, defined as amniotic fluid stem (AFS) cells [10].

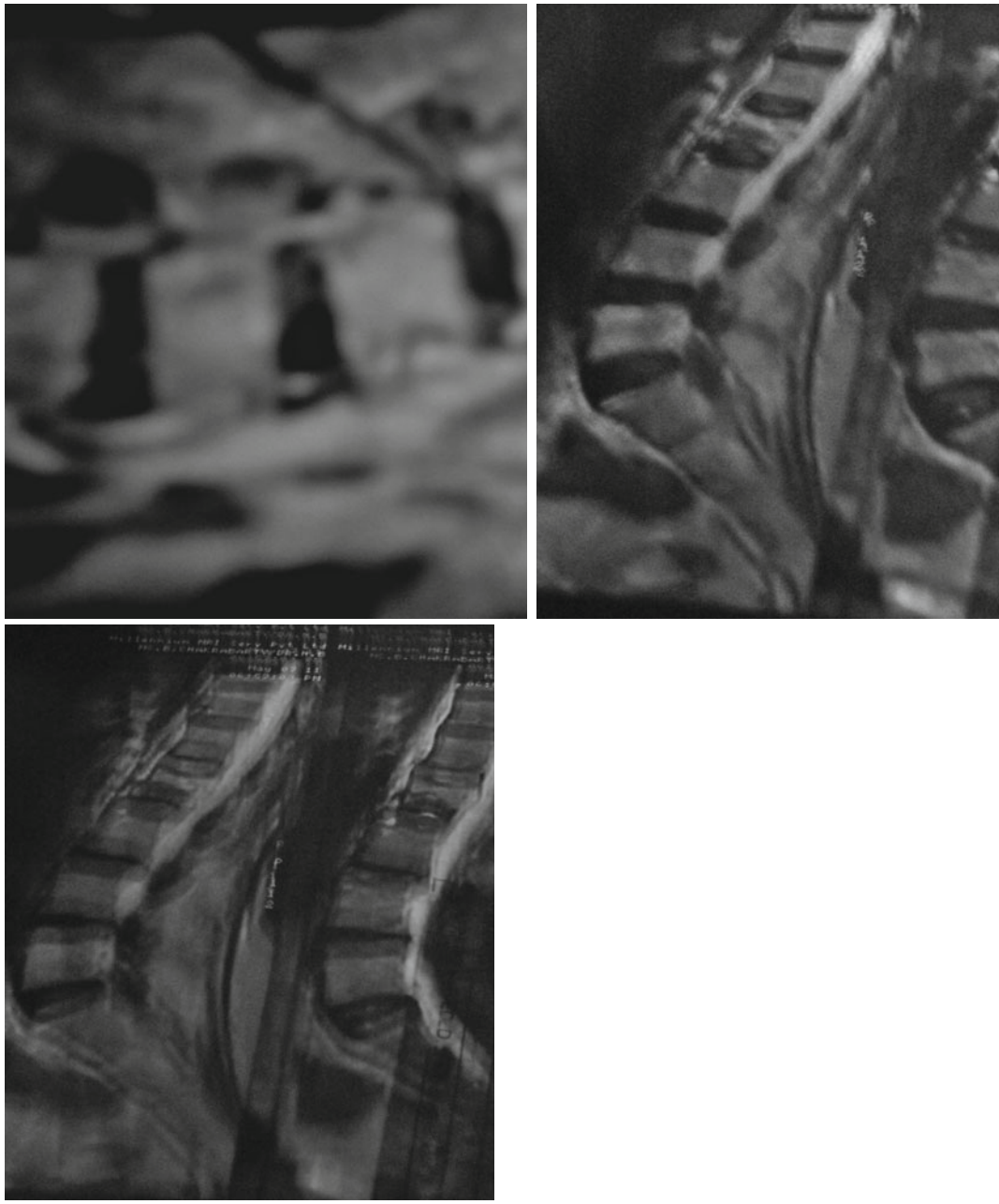
Cell-based therapies for bone regeneration are an exciting emerging technology, but the availability of osteogenic cells is limited, and an ideal cell source has not been identified. Amniotic fluid-derived stem cells (AFS) and bone marrow-derived mesenchymal stem cells (MSCs) were compared to determine their osteogenic differentiation capacity in both 2-D and 3-D environments in one study. It was found that MSCs would be a good choice for immediate matrix production, but the AFS cells would continue robust mineralization for an extended period of time. This study demonstrates that stem cell source can dramatically influence the magnitude and rate of osteogenic differentiation in vitro [11].

The problem lies with the treatment attempt of the patients reporting with degenerated disc with or without disc prolapse, desiccation, bulge, or compression of the adjacent nerves and its implications [12]. Most of the patients with chronic discogenic back pain, without specific history of trauma, are in the geriatric age group. In this age group, low back pain is associated with varying degree of age-induced degenerative osteoporosis, spondylosis, spondyloarthrosis, intervertebral disc prolapse, or even compression collapse apart from other problems like diabetic background, hypertension, ischemic heart disease, chronic obstructive pulmonary disorder, dyslipidemia, and hypothyroidism. MRI presentation of a typical geriatric manifestation of low back pain is shown (Figs. 19.1, 19.2, and 19.3).

If we see the epidemiological profile of the present study groups in Table 19.1, we can see the age of the group (36–82 years), sex of the group suggested (males 21 and females 21), weight of the group (varied from 44.8 to 112.6 kg), and the duration of illness (varying from duration 1 to 11 years). The clinically manifested effects of treatment can be easily noticed from Table 19.2. Here, both steroid (Group A) and cell therapy (Group B) patients showed improvement from the pretreatment value; however, Group B scoring is much better (p, 0.01), as seen and assessed from the value of the VAS (visual analog pain scale), WD (walking distance in meters), and HAQ (Health Assessment Questionnaire) assessments mentioned in the same Table. Further, if we examine the clinical assessment of pain relief and patient's satisfaction as seen from Table 19.3 and Graph 19.1, in case of Group A (long acting steroid group), it was 20/21 cases in the 1st month which became 12/21 in the 3rd month, 6/21 in the 6th month, 4/21 in the 12th month, and 2/21 after the 24th month follow-up. Similarly, in Group B (cell therapy patients), the identical values after the 1st month were 18/21, which became 21/21 in the 3rd month, 21/21 in the 6th month, 14/21 in the 12th month, and 12/24 after the 24th month follow-up. Another globally practiced guideline for pain assessment or scoring for comparison is the Oswestry low back pain disability questionnaire. Here in Table 19.4 and Graph 19.2, we have again compared the effect of treatment of Group A (steroid) and Group B (cell therapy with fresh amniotic fluid) and followed up the results of Group A and Group B treatment as per scoring by Oswestry low back pain disability questionnaire up to 24 months. Here, postinjection with long-acting steroid (Group A) suggested a mean scoring of 9 ± 1.2 % SD after 3 months, which became mean 1 9 ± 1.2 % SD after 6 months, mean 39 ± 9.2 % SD after 9 months, mean 39 ± 8.2 % SD after 12 months, mean 41 ± 7.2 % SD after 18 months, and then ultimately mean 48 ± 12.2 % SD after 24 months.

Similarly, in case of the cell therapy group (Group B), the mean scoring was 11.7 ± 1.6 % SD after 3rd month follow-up which became mean 9.4 ± 0.6 % SD after the 6th month, mean 9.1 ± 0.96 % SD after the 9th month, mean 7.1 ± 0.6 % SD after the 12th month, mean 6.7 ± 0.4 % SD after the 18th month, and ultimately mean 4.1 ± 0.96 % SD after the 24th month follow-up.

If we analyze the results, we can see long-acting steroid, due to its anti-inflammatory and other activities, causes some improvement in the patients; however, it is ill sustained as noted from the follow-up. But freshly collected simple amniotic fluid cell therapy has a much more sustained effect apart from remarkable improvement; the question remains as to why in the long-term follow-up there is a reappearance of pain in some of the victims? Can psychosomatic treatment or recurrent cell therapy or an increase in cell dosage have a more sustained



Figs. 19.1, 19.2, and 19.3 A typical presenting patient with low back pain is noted in the following (Figs. 19.1, 19.2, and 19.3). Here, the patient is 79 years old, female in the background of diabetes, chronic obstructive pulmonary disorder, ischemic heart disease, and aortic and mitral calcification of the valves with resulting incompetence. The MRI (Figs. 19.1, 19.2, and 19.3) suggested: (a) Compression fracture of L2 vertebral body with partial rupture of L2/L3 disc. (b) Disc desiccation at L1–L2 to L5–S1. (c) Disc protrusion causing secondary spinal canal and bilateral neuronal foramina narrowing and thecal compression at L4–L5 level. (d) Mild diffuse disc bulge causing mild thecal compression at L3–L4 level

Table 19.4 The comparison and follow-up results of Group A and Group B treatment as per scoring by Oswestry low back pain disability questionnaire up to 24 months

Group A treated with intra-articular steroid	Group B treated with cell therapy	Pretreatment scoring as per Oswestry low back pain disability questionnaire Group A	Pretreatment scoring as per Oswestry low back pain disability questionnaire Group B	Posttreatment scoring as per Oswestry low back pain disability questionnaire Group A	Posttreatment scoring as per Oswestry low back pain disability questionnaire Group B
$N=21$ $M=12$ Female = 9 Age 38–78 Mean age 48 ±6.4 SD	$N=21$ $M=10$ Female = 11 Mean age 56.4 ± 4.8 SD	12–84 %, mean 39 ± 11.2 % SD	16–88 %, mean 41 ± 9.6 % SD after 3rd month	12–34 %, mean 9 ± 1.2 % SD after 3rd month	6–18 %, mean 11.7 ± 1.6 % SD after 3rd month
Do	Do	Do	Do	22–67 %, mean 1 9 ± 1.2 % SD after 6th month	6–12 %, mean 9.4 ± 0.6 % SD after 6th month
Do	Do	Do	Do	31–69 %, mean 39 ± 9.2 % SD after 9th month	9–11 %, mean 9.1 ± 0.96 % SD after 9th month
Do	Do	Do	Do	32–74 %, mean 39 ± 8.2 % SD after 12th month	6–9 %, mean 7.1 ± 0.6 % SD after 12th month
Do	Do	Do	Do	33–75 %, mean 41 ± 7.2 % SD after 18th month	4–8 %, mean 6.7 ± 0.4 % SD after18th month
Do	Do	Do	Do	26–88 %, mean 48 ± 12.2 % SD after 24th month	4–6 %, mean 4.1 ± 0.96 % SD after 24th month

effect? These are some of the questions for the future investigators in this frontline area of cellular therapy.

From an overall point of view, regeneration can only treat the root cause of degeneration of the whole lumbosacral region. Cell therapy is the only curative approach for such a generalized multisystemic deterioration of the region, and the palliative approach to pain relief with anti-inflammatory drug including steroid is short lived and has longtime use and may lead to drug-induced problems in addition of the recurrence of the symptoms.

Surgical options are mainly aimed at decompression procedure so as to relieve the compression through different neurosurgical and orthopedic combined technical procedures like facetectomy (to remove part of the facet), foraminotomy and laminotomy (to enlarge the vertebral foramen), intervertebral disc annuloplasty (a procedure of heating the disc to 90 °C for 15 min in an effort to seal the disc), intervertebral disc arthroplasty (also called artificial disc replacement), laminoplasty, microdisectomy, percutaneous laser disc decompression, spinal decompression, and spinal laminectomy, only to name a few such procedures which should be individualized for proper application.

Other strategies for disc degeneration have included attempts to upregulate the production of key matrix proteins or reduce the proinflammatory cytokines, interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α) which cause the inflammation. In order to achieve that, protein injection and viral or nonviral gene transfer has been attempted without the desired success. Subsequently, there were attempts to inject anabolic factors or recombinant growth factors, stereotactically at the site of inflammation or injury of the disc, again, without the desired success.

The expression of tumor necrosis factor alpha in adult discs is statistically associated with disc degeneration. Its occurrence in adults of more advanced age suggests that tumor necrosis factor alpha is not involved in the initiation of disc degeneration but may be associated with further promotion of degenerative disarrangement and pain induction [13].

Basic science research has demonstrated that the intervertebral disc is an avascular tissue element occupied by inadequately characterized cells in an extensive extracellular matrix. While the annulus fibrosus is predominantly collagenous, the matrix of the central nucleus pulposus is rich in proteoglycans. A variety of inflammatory mediators have been implicated in the degeneration of the intervertebral disc including nitric oxide (NO), interleukins, matrix metalloproteinases (MMP), prostaglandin E2 (PGE2), tumor necrosis factor alpha (TNF-alpha), and a group of cytokines. MMPs, PGE2, and a variety of cytokines have already been shown to play a role in the degradation of articular cartilage. Nitric oxide is a novel mediator that has recently drawn much attention for its role in disc abnormalities. Elevated nitric oxide production derived from NO synthase activity has been manifested in cerebrospinal fluid in patients with degenerative lumbar disease. However, the regulatory mechanism of NO and its relationship to the clinical manifestations are unclear [14].

Discussion

The investigators mentioned earlier in the chapter have stressed on the effect of disc degeneration and have attempted to understand the process of degeneration in order to find strategies to relieve pain. However, few researchers have tried to attack the root cause of disc degeneration through attempts at regeneration.

Live cell therapy can treat the process of degeneration by reversing it or by an attempt to regenerate those affected cells. Treatment with autologous mesenchymal stem cell from bone marrow, stromal cells from the same source, adipose tissue stem cells, growth factors etc for regeneration of the damaged disc, have been attempted by many investigators with varying degrees of success [15–23].

The present study is the first global report on the treatment of discogenic pain with a biological waste, i.e., amniotic fluid.

The present group of researchers have earlier reported on amniotic fluid cell therapy in advanced arthritis and its regenerative effects [24].

Physical therapies should aim to promote healing in the disc periphery by stimulating cells, boosting metabolite transport and preventing adhesions and reinjury. Such an approach has the potential to accelerate pain relief in the disc periphery, even if it fails to reverse age-related degenerative changes in the nucleus.

Tumor necrosis factor alpha is substantially expressed in disc material of symptomatic patients (surgical specimens) in comparison to samples taken at autopsy. The expression of tumor necrosis factor alpha in early fetal/infantile nucleus pulposus may indicate “physiologic” tissue disarrangement with closure of the blood vessel canals. Human intervertebral disc undergoes multifactorial, biochemical, and morphologic degenerative changes during the process of aging. The frequency of degeneration, especially lumbar degeneration, increases sharply with age and is regarded as a major cause of discogenic low back pain. Since degenerative discs are often asymptomatic, the pathobiology of discogenic back pain remains unclear. Degenerated discs spontaneously produce increased amounts of inflammatory mediators suggesting their role in the degenerative process of the intervertebral disc. However, the relationship between aging, degenerative processes, and actual illness is far from clear.

Randomized clinical trials (RCTs) are regarded as the “golden standard” for providing research evidence for interventions in evidence-based health care [25]. The validity and reliability of trial results are, however, largely dependent on the study design and the methodology in its conduct. Jadad [26] has defined the quality of a trial, with emphasis on the methodological quality, as “the confidence that the trial design,

conduct, and analysis have minimized or avoided biases in its treatment comparisons." In this chapter, an attempt was made to follow the basic guidelines to minimize investigator or other biases as far as practicable. Our subjective assessment of that score in this study is possibly four on the Jadad scale. The present study is the first global report of a clinical comparison of the effect of amniotic fluid cell therapy and the impact of standard intra-articular palliative treatment in case of varying degrees of discogenic low back pain. Under normal circumstances, the fetus and the amniotic fluid-containing sac are immediately disposed for eventual clearance through the incinerator of the hospital. To recapitulate, amniotic fluid is to be found in the amniotic cavity that protects the fetus as a buffer and also helps growth and movement and prevents adherence to the placenta or the surrounding structures. This clear watery fluid is contributed principally from the maternal blood via the amniotic fluid epithelium but freely intermixes with secretions from the fetal lung, kidney, gastrointestinal tract, and the skin; hence, the properties of this specialized fluid compartment are quite complex with contributions from both the maternal and the fetal side. Toward the outside, the amniotic cavity is delimited by the amniotic epithelium, the chorion laeve, and the decidua capsularis. The main constituents are water and electrolytes (99 %) together with glucose, lipids from the fetal lungs, proteins with bactericide properties, and fetal epithelium cells. Pluripotent progenitor cells isolated from the amniotic fluid and the placenta possibly present an exciting contribution to the field of stem cell biology and regenerative medicine. Compared with embryonic stem cells, progenitor cells isolated from the amniotic fluid have many similarities: they can differentiate into all three germ layers, they express common markers, and they preserve their telomere length. However, progenitor cells isolated from the amniotic fluid and placenta have considerable advantages. They easily differentiate into specific cell lineages, and further, they avoid the current controversies associated with the use of human embryonic stem cells. Pregnancy results in the acquisition of specialized and unique cells that may have clinical applications and therapeutic potential. Whether the pregnancy-associated progenitor cells (PAPCs) are hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), or are a new population of stem cells is an unresolved issue. It is also unknown whether PAPCs respond to all types of maternal injury or only those injuries that recruit stem cells. It is possible that these cells, since they are fetal in origin, have a higher proliferative capacity or more plasticity than their equivalent adult (maternal) cells.

In the current debate over the use of embryonic stem cells for treatment of disease, the discovery of a population of fetal stem cells that apparently differentiate from the ones in adult women and can be acquired without harming the fetus may be significant [27, 28]. The growing fetus in the womb is an eternal source of stem cells. Meanwhile, scientists have been able to isolate and differentiate only 30 % of mesenchymal stem cells (MSCs) on an average, extracted from a newborn's umbilical cord jelly-like material shortly after birth. The success rate for amniotic fluid-derived stem cells, on the other hand, is close to 100 %. Analysis of surface markers shows that progenitor cells from amniotic fluid express human embryonic stage-specific marker SSEA4 and the stem cell marker Oct-4 and do not express SSEA1, SSEA3, CD4, CD8, CD34, CD133, C-MET, ABCG2, NCAM, BMP4, TRA1-60, and TRA1-81 [29, 30].

Differentiation of Amniotic Fluid- and Placenta-Derived Progenitor Cells

The progenitor cells derived from amniotic fluid and the placenta are pluripotent and have been shown to differentiate into osteogenic, adipogenic, myogenic, neurogenic, endothelial, hepatic, and renal phenotypes in vitro. Each differentiation has been performed through proof of phenotypic and biochemical changes consistent with the differentiated tissue type of interest. In 2007, Perin et al. showed that AFSC (amniotic fluid stem cells) could be induced to

differentiate into renal cells when placed into an in vitro embryonic kidney environment [31]. In this preliminary clinical study, freshly collected amniotic fluid has been utilized as a source of cell therapy with the hypothetical assumptions that the mesenchymal cells of the AF (amniotic fluid) will participate in the degenerated disc repair process, the viscosity of the amniotic fluid will assist lubrication, and the bactericidal property of the amniotic fluid will guard against inadvertent infection. The idea was to match/compare this new therapeutic protocol (cell therapy for regeneration) with the globally accepted standard protocol of intra-articular injection of long-acting steroid triamcinolone.

New Horizon for Offering a Cure (Repair) for Discogenic Pain with Simple Cell Therapy

In the developing world, surgical abortion as a method of family planning is practiced widely. Hysterotomy and ligation is a standard surgical method of termination in government hospitals in India. Aseptic collection of the amniotic fluid is not a difficult job for experienced gynecologists and obstetricians who perform this simple surgery with skill and dedication. The aseptically collected amniotic fluid can be easily preserved in special containers in the vapor phase of liquid nitrogen chambers or jars. This may work as an amniotic fluid bank that can supply amniotic fluid on demand. Amniotic fluid is a unique fluid made by nature; it is a cocktail of mesenchymal stem cells with antibacterial property, which is used in the present study as the cell therapy source for the repair of damaged cartilage, synovial membrane, supporting muscles, and supporting ligaments, as per the niche provided to these specialized stem cells for regeneration purposes, in advanced and degenerative osteoarthritis with satisfying results. The amniotic fluid, because of its increased viscosity due to protein and other cellular suspension, differs from the steroid-treated fluid (normal saline) and may act as a lubricant that diminishes the irritation at the initial phase; the mesenchymal cells, which do not express HLA antigens, may possibly help in the repair process of the adjacent structures in the joint space as a whole. Though the epidemiological background (Table 19.1) of Groups A and B is grossly randomized, the result of the therapy (shown in Tables 19.2 and 19.3, which is graphically represented in Graphs 19.1 and 19.2) strongly supports the potential of this new form of cell therapy in case of advanced disc-related pain. Lastly, it may be noted with interest that in this simple method of cell therapy, Group B maintained superior patient satisfaction in 12 cases only out of 26 enrolled patients, after completion of the 24-month follow-up period. The corresponding number for the standardized universally practiced protocol of intra-articular long-acting steroid (Group A) therapy for advanced discogenic pain is a pathetic figure of four cases only. The results are further supported by the VAS, WD, and HAQ assessments as mentioned in Table 19.2. The results of these tests reiterated the observation that there was a significant improvement in VAS in the third month and this improvement was sustained at the 6-month interval assessments in both groups but more so in the cell therapy Group B ($p<0.001$). The present treatment proved to be much superior to, and lasted longer than, the conventional widely practiced therapy with corticosteroid instillation at the joint.

Summary and Conclusion

An analysis of the results show that long-acting steroid, due to its anti-inflammatory and other activities, causes some improvement in the disc related pain of the patients; however, it is ill sustained as noted from the follow-up. Freshly collected amniotic cell therapy has a much more sustained effect. However, despite remarkable improvement, long-term follow-up indicates a return of some degree of pain. The issue is whether the symptoms are psychosomatic, or whether other methods like recurrent cell therapy or an increased cell dosage can have a more sustained effect. These questions may perhaps be answered by investigators working in this

frontline area of cell therapy in the future someday.

Regeneration treats the root cause of degeneration in the entire lumbosacral region. While palliative treatment with pain-relieving drugs including steroid has only a short-time effect and can also lead to drug-induced problems, cell therapy has a curative approach for the multisystemic degeneration of the region, which is the actual cause of the pain in most cases.

Discussion and Conclusion

Intradiscal C-arm-guided amniotic fluid instillation is a new method of treatment in advanced disc degeneration-related pain irrespective of traumatic or age-induced degeneration background, especially in cases where the patient is not getting any relief with sustained conventional analgesic and physiotherapeutic support. The long-term follow-up result of this type of cell therapy justifies its procedural superiority over conventionally and universally practiced intra-articular long-acting corticosteroid (methylprednisolone) ($p<0.001$).

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Human Neural Stem Cell Transplants in Neurological Disorders: Current Trends and Future Options

20

Abhijit Chaudhuri and Niranjan Bhattacharya

Introduction

Loss of neurons and glial cells is a common neuropathology in human neurological diseases. From acute stroke to chronic central nervous system (CNS) disorders like Parkinson's disease and Alzheimer's disease, neuronal and glial cell death and damage remain irreparable with existing therapies. During the past decade, cell replacement therapy, gene transfer, and selective repair of injured neural cells in diseased areas of human brain and experimental animal models have become active areas of research with potential for promising therapeutic developments in neurological diseases.

Several sources of stem cells, which have the pluripotency to differentiate into multiple cell types, have already been tested as possible candidates for therapy. These include embryonic stem cells (ESC) from the inner cell mass of blastocysts, embryonic germ cells (EGC) from postimplantation embryos, and induced pluripotent cells (iPC) derived from laboratory-treated adult somatic cell lines such as skin fibroblasts. Tissue-specific stem cells could also be obtained at later stages of embryonic development, although there are unresolved ethical issues surrounding procurement of such material for research purposes. Hematopoietic, bone marrow, and adipose tissue, as well as amniotic fluid and umbilical cord, are generally considered to be ethically acceptable sources of tissue-specific stem cells that could be isolated during late phases of human development.

For the purpose of treating neurological diseases, an ideal candidate would be the neural stem cells (NSC) with the potential for growth and differentiation into neuronal and glial cell lines. In humans, NSCs can be identified in embryonic, developing, and adult (developed) brain. Although there is evidence that new neurons are generated in adult human brain, in reality, the capacity of self-repair in adult CNS to a functional level is virtually nonexistent, which might suggest that the local environment around the area of injured or damaged brain prevents appropriate induction and transdifferentiation of local NSCs. Developing brain tissue has the highest amount of NSCs making human embryonic or fetal brain an ideal source of transplant for NSCs. Besides NSCs, neurons could also be derived from ESCs, EGCs, iPCs, bone-marrow-derived mesenchymal stem

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cells, and umbilical cord hematopoietic cells. Several research and therapeutic studies using non-NSCs are currently underway as possible therapy for neurological disorders. Mensenchymal stem cells are being evaluated as potential therapy in Parkinson's disease, multiple sclerosis, and motor neuron disease.

Parkinson's Disease

In Parkinson's disease, there is gradual loss of nigrostriatal dopaminergic neurons, and current therapies use levodopa or dopamine receptor agonists to raise striatal dopamine concentration. However, there is also degeneration of non-dopaminergic neurons in Parkinson's disease, and response to pharmacological therapies becomes increasingly unpredictable after some years due to changes in dopamine receptor kinetics and sensitivity with continued neuronal attrition. Transplantation of exogenous human fetal ventral mesencephalic tissue into neostriatum of patients with advanced Parkinson's disease confirmed that cell replacement can produce major, long-lasting improvement [1]. Subsequent trials of transplanting dopamine-producing cells derived from ESC, retina, adrenal gland, and bone marrow resulted in less sustained benefit, and none of these protocols was deemed suitable to be of therapeutic value. This observation reopened the debate whether dopaminergic stem cells transplanted into the striatum can effectively reintegrate and reinnervate the striatum to become functional, release dopamine in vivo, and repair or arrest the neurodegenerative process that lies at the heart of the pathology of Parkinson's disease. For dopaminergic neurons generated from the human ESC, survival after transplantation in animal models has been poor [2], and recent reports seemed to confirm that survival of transplanted fetal mesenchymal cells in the patient's brain was very low [3]. It remains unclear why the expansion rate of transplanted NSCs is slow and if this was influenced by the immunosuppressive therapy.

In a prospective study of heterotopic implantation of fetal tissue grafts in patients with Parkinson's disease, significant improvement was observed in the short term, leading to a reduction of pharmacological therapy with dopaminergic drugs in most patients [4]. The experience from this research seems to suggest that fetal tissue may survive in HLA-randomized host without immunosuppressive therapy. There was no change in histology between the fetal brain tissue explanted at the 3rd month and 11th year to suggest graft-versus-host reaction occurring over a period of time, and it has been proposed that fetal tissue transplant in human behaves as a surgical chimera.

Clearly, understanding the physiology of NSCs in the host environment is critical for the success of neural transplants. In an experimental study of stroke model, about a fifth (20 %) of focally brain-transplanted cells had survived at the end of the first month and had migrated to the contralateral hemisphere by the fourth month [5].

Huntington's Disease and Other Neurodegenerative Disorders

Graft of fetal striatal cells in Huntington's disease was associated with functional recovery, but it has been difficult to replicate the study [6]. Outcomes of NSC-based therapy have not been reported in Alzheimer's disease or motor neuron disease (amyotrophic lateral sclerosis). In theory, transplantation of cholinergic neurons can provide symptomatic benefit in Alzheimer's disease, but whether it would prevent progressive cognitive decline is unclear. Prospect for NSC transplant in motor neuron disease seems unlikely in the foreseeable future.

In Pelizaeus-Merzbacher disease, a rare inherited disorder of dysmyelination associated with mutation in human proteolipid protein gene, a phase 1 clinical trial is presently underway in the USA using a patented fetal-derived NSC (HuCNS-SC StemCells, Inc.) following a similar trial in another inborn error of metabolism (neuronal ceroid lipofuscinosis).

Multiple Sclerosis

Transplantation of remyelinating cells represents a possible treatment approach to repair myelin loss in multiple sclerosis. Experimental implantation

of peripheral-nerve-derived Schwann cells has not been reported to be successful. A major problem of NSC-based remyelination in multiple sclerosis is the multiple and disseminated nature of lesions, making heterotopic transplant or systemic administration of NSCs more plausible. In experimental models following systemic administration, NSCs migrated to the areas of demyelinating lesions, where some of the transplanted cells differentiated into oligodendrocyte progenitor cells and remyelinated axons [7].

Stroke

Transplantation of NSCs into brain areas injured by stroke offers a promising strategy for functional recovery in stroke patients. Experimental studies provide strong evidence that intravenously administered NSCs could selectively migrate into ischemic as well as hemorrhagic brain areas and differentiate into new neurons and/or glial cells, leading to functional recovery [8, 9]. Interestingly, there appears to be some capacity of stroke-damaged adult brain for neuronal replacement from its own NSCs [1]. Currently, there is an ongoing clinical trial of fetal-derived conditionally immortalized NSC in stroke in Glasgow, UK (ReN001 – ReNeuron Group plc).

Spinal Cord Lesions

The benefit of transplanted NSCs in injured spinal cord appears to be largely due to release of trophic factors and/or remyelination of axons, and experimental research confirms that implantation of human NSCs into damaged mouse spinal cord can generate new neurons and oligodendrocytes, leading to locomotor recovery [10]. There seems to be good correlation between the number of graft-derived oligodendrocytes, the amount of myelin, and the extent of functional recovery in one study [11], suggesting that transplantation of human NSCs is an attractive therapeutic option for focal spinal cord injury due to trauma and demyelination. Trial of patented human ESC-derived glial progenitor cell (GRNOPC1 – Geron Corporation) is awaiting regulatory approval in the USA.

Conclusion

There is adequate evidence that NSCs can circumvent blood–brain barrier and migrate to specific pathologic brain areas with tropism [12]. This opens the prospect of using heterotopic transplant of fetal or embryonic brain tissue as a therapeutic strategy in a number of neurological disorders. There may be additional benefit of heterotopic transplant from systemic neurotrophic factors and chemokines. Heterotrophic transplant also obviates the need for multiple transplants into different brain areas in diffuse or disseminated brain diseases. The emerging experience from at least one research study [4] indicates that heterotopic NSC transplants may have the potential to succeed without systemic immunosuppression and could still result in functional recovery and therapeutic benefit in patients with Parkinson's disease.

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Umbilical Cord Stem Cells for Pancreatic Regenerative Medicine

21

Hélène Le Roy, Nicolas Forraz, Marcin Jurga, and Colin P. McGuckin

Introduction

Diabetes is an endocrine disease characterized by chronic hyperglycemia. Type 1 diabetes mellitus (juvenile onset), insulin dependent, represents 10 % of all cases of diabetes in the world [1] and affects mainly children [2]. This autoimmune disease is corresponding to destruction of pancreatic beta cells by T cells. The insulin-independent type 2 diabetes mellitus is defined by resistance to insulin with the incapacity of beta cells to secrete insulin. Diabetes mellitus is one of the leading causes of morbidity and mortality in many countries [3] and is considered as one of the epidemics of the twenty-first century [4]. In 2000, there was 2.8 % (171 million) of the worldwide population affected by diabetes (data from 191 countries analysis), and prevalence should increase to 4.4 % (estimated 366 million) by the year 2030 [5]. Diabetes has genetic [6, 7] and environmental origins [8, 9], but the predicted increase is mainly due to modern lifestyle changes (sedentarily, unhealthy diet, and related obesity) [10, 11]. Diabetes has significant impact on public health because of many serious complications [12] including cardiovascular disease [13–15], blindness and retinopathies [16], and kidney failure [17] which can lead to death and affects more and more people in the world.

Different approaches to treat and cure diabetes were evaluated, but the perfect therapy has not been found yet. Current therapies against diabetes are more palliative than restorative [18]. This chapter will describe recent innovative clinical and preclinical applications used for diabetes treatment ranging from insulin injection to newly established cellular clinical trials with umbilical cord stem cells.

Historical Background of Diabetes Treatment

Since diabetes has been formally diagnosed as chronic hyperglycemia, many treatments have been tested on patients [1, 19] (Fig. 21.1). The first exogenous insulin injection took place in 1922 [20]. Today, this therapy is still used and saves lives, but it forces the patient to inject insulin daily. Furthermore, it does not avoid hyperglycemia episodes, anxiety, or even coma and death. This type of treatment is fully symptomatic and does not restore the proper function of the pancreas. In 1966, Dr. Kelly and colleagues performed the first transplantation of whole pancreas and kidney on humans [21]. For this trial, insulin was secreted, but one patient died 3 months later, and the second one needed insulin 4 months after transplantation. Pancreas transplantation has since improved,

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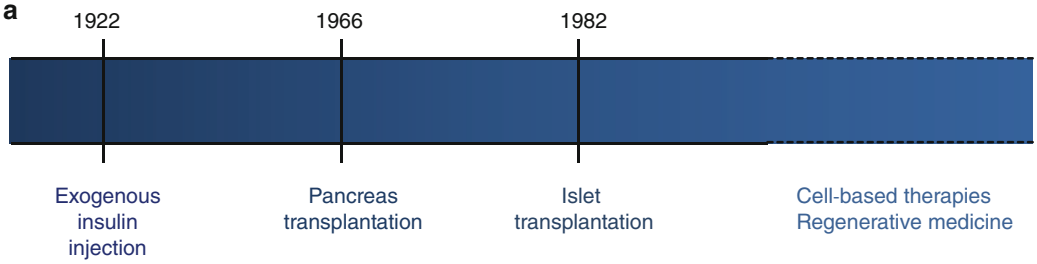

b

Treatment diabetes		
Techniques	**Advantages**	**Complications**
Insulin injection	Lives saved	Sympomattic
Transplantation of pancreas	Good prognosis,longterm success (95 % after 1 year)	Side effects (trombosis, rejection) Shortage of donor
Islet transplantation	58 % patients insulin independent	Shortage of donor immunosuppression
Xenotransplantation		Epidemiological risks

Fig. 21.1 Historical background of diabetes treatment. (**a**) Chronology of techniques used for diabetes treatment. (**b**) Advantages and drawback of each technique

and when applicable, it is the most effective diabetes treatment with 95 % successful outcome, providing stable and continuous normoglycemia. Whole-pancreas-alone transplantation was envisaged, but complications in kidney appeared and were associated with morbidity and mortality [22]. Pancreas/kidney and pancreas transplantations are limited by the need for immunosuppression and the number of donors. In 1982, Lacy collected cadaveric donor pancreatic islet cells and transplanted them to diabetic patients [23]. This technique was first tested in rats [24] and is still in experimental phase due to problems with islet isolation, low success rate, and transient glycemia regulation. In 2000, the Edmonton protocol significantly improved this technique by improving islet transplantation [25] and associated immunosuppressive regimen. However, the shortage of donors and lifelong immunosuppression limit the development of this protocol [26]. In 2007, less than 1 % of diabetic people could receive islet transplants mainly because, on average, 850,000 islets are needed per patient, corresponding to the number of islets of at least two human pancreases. Rejection risk is high as diabetic patients require glucocorticoid-free immunosuppressive regimen. Further to this, the success rate is low with only 10 % of patients improving at 5-year follow-up [1, 27]. Xenotransplantation with porcine islets was envisaged because pigs and humans have physiological similarities and porcine insulin differs from human insulin by only one amino acid [28]. But due to epidemiological risks (foot and mouth disease), such perspectives were given up.

Molecular Markers of Pancreas Formation

Because pancreas transplantation is limited by shortage of donors and efficiency of mature pancreatic cell transplantation, the scientific community has considered the concept of regenerative medicine applied to pancreatic islets [1]. However, the implementation of islet regeneration with cell therapy calls for a thorough understanding of biological mechanisms underlying pancreatic formation and homeostasis.

The cascade of gene expression involved in pancreas organogenesis and islet development is highly complex and not fully understood [29, 30]

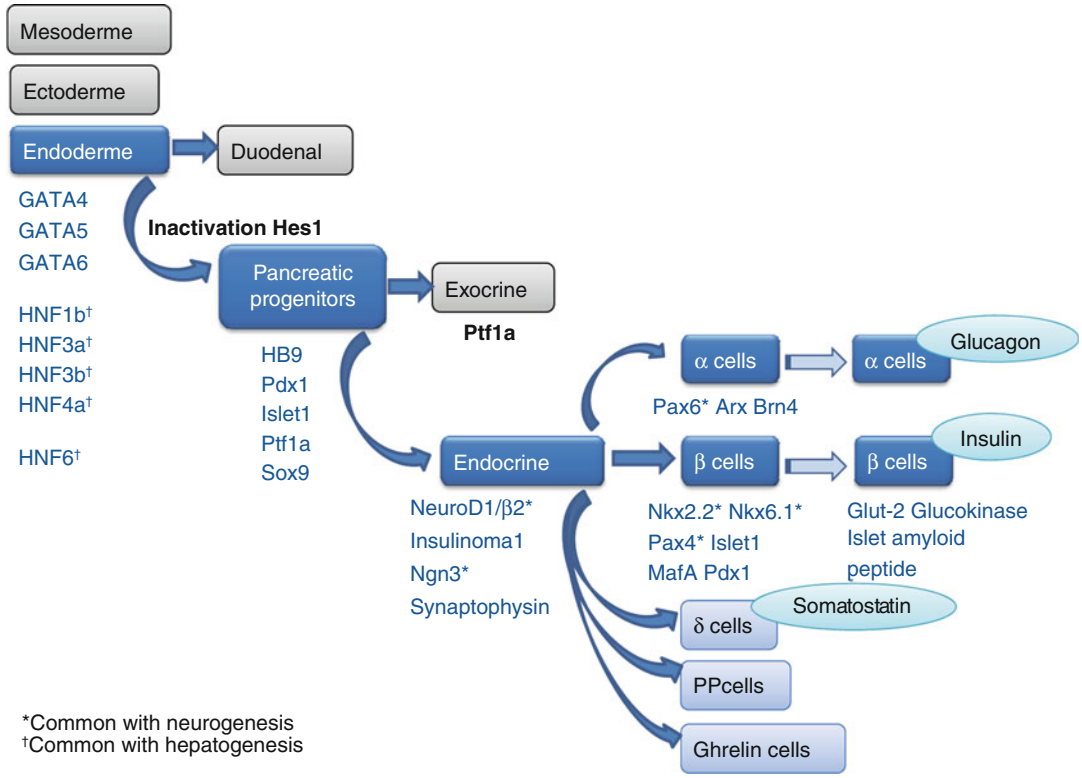

Fig. 21.2 Cascade of pancreatic gene expression during organogenesis. Derived from GATA and HNF endoderm, pancreatic progenitors express HB9, Pdx1, Islet1, Ptf1a, and Sox9. In pancreas, endocrine differentiation is characterized by expression of NeuroD1/β2, Insulinoma1, Ngn3, and synaptophysin. Each cell type from Langerhans expresses different genes: α cells express Pax6, Arx, and Brn4 and secrete glucagon; β cells express Nkx2.2, Nkx6.1, Pax4, Islet1, MafA, and Pdx1 and secrete insulin in the same time as expressing also glut-2, glucokinase, and islet amyloid polypeptide genes; and δ cells secrete somatostatin, whereas PP cells and ghrelin cells are minority and not well known

(Fig. 21.2). Endodermal cells express liver markers, HNF1b, 3a, 3b, 4a, and 6, and zinc-finger transcription factors GATA4, 5, and 6. HNF6 induced Neurogenin3 expression, whereas GATA and HNF factors induce two homeodomain transcription factors: pancreatic ductal homeobox 1 gene (Pdx-1) and HB9. Ngn3, initially described in neuroderm, was shown to be necessary and sufficient for initiating endocrine differentiation in the pancreas [31]. This factor is expressed in the earliest phase of pancreas development and induces NeuroD1/β2 as well as early factors Pax4, Nkx2.2, and Nkx6.1. Ngn3 is not expressed in late development. Interestingly, all these factors are also expressed/involved in neurogenesis even if pancreas and neuron have different embryonic origins [29]. In prepancreatic endoderm, bud formation is initiated by HB9 and needs Pdx-1 for its growth in association with Pbx-1 [32]. Although expressed in ductal cells, Pdx-1 is critical for islet neogenesis [19, 33]. After bud formation, Pdx-1 is downregulated and will be reexpressed in mature cells at the end of beta cell development when late factors Pax6, Islet-1, Brn4, HB9, and Pdx-1 are expressed. Last step of maturation is the transactivation of insulin and somatostatin gene promoters by Pdx-1 and expression of differentiated beta cell genes (glut-2, glucokinase, and islet amyloid polypeptides; prohormone convertases 1/3 and 2; and NeuroD1/β2, Nkx2.2, and Nkx6.1 [34]). Prohormone convertases are enzymes responsible for pre-proinsulin to proinsulin and to insulin cleavage [20]. Glucagon-like peptide (GLP)-1 is produced through post-translational processing

of proglucagon [35]. It promotes beta cell proliferation and insulin production and inhibits apoptosis.

In Vitro Differentiation of Pancreatic and Extrapancreatic Cells to Differentiate into Insulin-Producing Cells

Islet regeneration can be done by stimulation of patient's existing cells from pancreas or extrapancreatic cells [19] with in vitro expansion and differentiation of autologous or donor's progenitors and stem cells.

In Vivo or Ex Vivo Differentiation of Pancreatic Cell Toward Insulin-Producing Cells

Endocrine Cells Expansion

In vivo regeneration of beta cells was shown to be possible using knockout mice for cyclinD2 as stimulator of beta cell proliferation [1]. In parallel, Narushima et al. created an immortalized human pancreatic beta cell line NAK-15 by crelox system. This cell line secretes insulin in response to glucose stimulation and expresses beta cell markers (islet-1, Pax6, Nkx6.1, Pdx1, PC1/3, and PC2). They used Matrigel matrix to facilitate aggregate formation in low-glucose-containing serum-free insulin-free medium. And after injection in STZ-SCID mice, cells controlled blood glucose for a long time without hyperglycemia [36].

Ex vivo expansion of human pancreatic islets could be possible from donor pancreatic tissues [1]. This technique requires specific culture conditions: serum-free medium; matrix; growth factors like FGF, HGF, EGF, and gastrin; and insulinotropic agents like nicotinamide [1]. This technique is binding because production of insulin is limited in the time and needs addition of pharmacological agents (Fig. 21.3).

There is the possibility that islets themselves contain progenitor cells that allow their prolonged survival and continual cellular turnover after exogenic stimulation. The existence of pancreatic "small cells" has been reported [37]. These cells are immature, small in size, and in proportion to mature cells present in islet (1 % of islet cells). They express all four endocrine hormones (glucagon, insulin, somatostatin, and polypeptide) as well as Pdx-1, synaptophysin, alpha-fetoprotein, and Bcl-2. They are able to secrete insulin in response to glucose, but their proliferation rate is not known.

Exocrine Cells Differentiation

Islet neogenesis in the mature pancreas occurs via the ductal epithelium so ductal cells and islet cells may share same factor expression before commitment [38]. The potential role of pancreatic exocrine cells in islet neogenesis is a matter of debate. Arguments for and against pancreatic transdifferentiation are summarized in a review by Bouwens [39]. Islet neogenesis from ductal cells can occur by in vivo ligation of pancreas tail in rats [40]. When gastrin is infused into pancreatic duct-ligated rats, beta cell mass doubled in the ligated versus unligated portion [41]. Viral transduction of early islet developmental transcription factors Neurogenin3, Pdx1, and Mafa [42, 43] leads also to islet neogenesis. Others teams started from murine ductal tissue, but they did not obtain high level of insulin [19]. Islet neogenesis-associated protein (INGAP) induces in vitro differentiation of human pancreatic duct cells into insulin-producing phenotype [1].

Another source of production of islet cells is nestin-expressing cells [44] or exocrine/acinar cells [41]. Long-term culture of acinar cells results in a downregulation of digestive enzymes, cytokeratins, and mucins [45]. Evidence to support the proposal for transdifferentiation is lacking in all of these studies, and alternative explanations may be more attractive.

In Vivo or Ex Vivo Differentiation of Nonpancreatic Cell Toward Insulin-Producing Cells

As ductal cells, liver cells can be transdifferentiated to produce pancreatic cells. Pdx-1 was overexpressed for transdifferentiation of Xenopus tadpoles developing liver cells into pancreatic

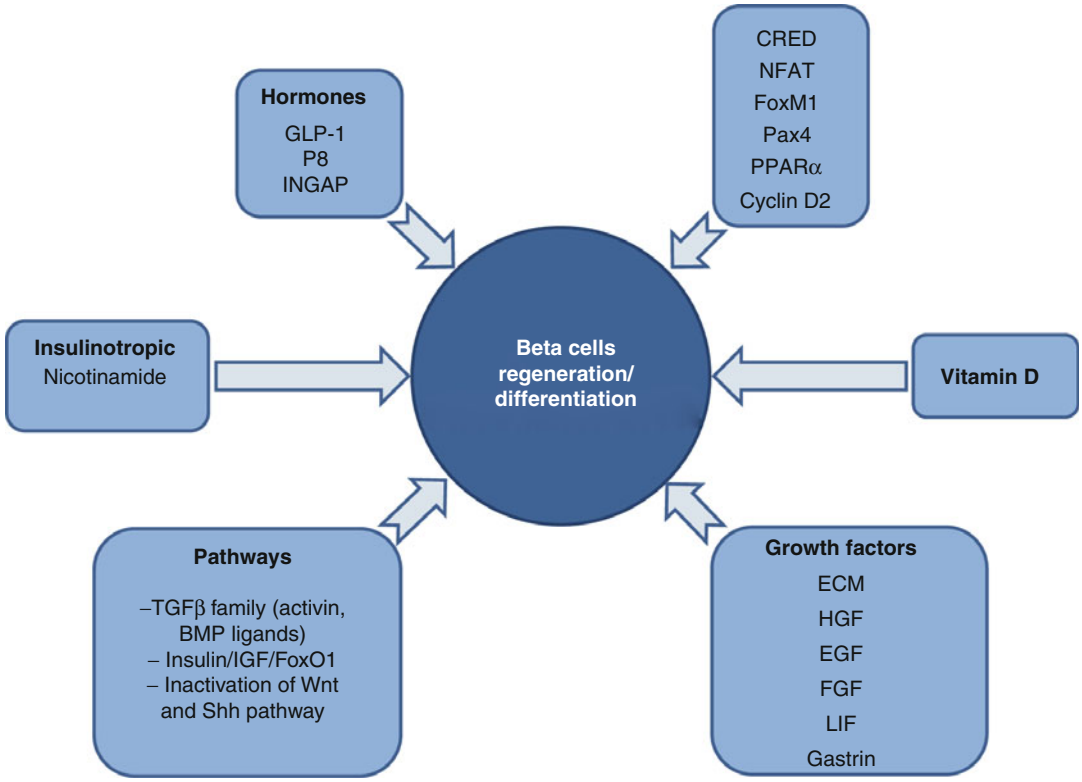

Fig. 21.3 Factors involved in β cells regeneration and expansion. In order to stimulate proliferation of β cells or induce its regeneration, ex vivo combination of hormones, vitamin, growth factors, insulinotropic, and induction of signaling pathways can be applied on β cells or endocrine progenitors

endocrine and exocrine cells [46] and induction of insulin production of adult mice livers [47]. Transcription factors important for pancreas development like Ngn3, NeuroD/β2, Nkx2.2, Nkx6.1, Pax-6, and Isl-1 were also involved in this transdifferentiation. In the adult rat, hepatic oval stem cells were shown to express islet cell characteristics including insulin secretion in response to glucose [48].

Plasmid lipofection of Pdx-1 gene into intestinal-derived cell line stimulates expression of beta cell-specific genes like Nkx6.1 and glucokinase. After transplantation in rat, insulin is expressed and secreted, but these cells were not glucose responsive [49].

Although differentiation of neural stem cells into pancreatic islet cells has not yet been demonstrated, the similarities in developmental pathways and gene expression between islets and other neuroendocrine cells suggest that this conversion could be worth investigating.

In Vitro Expansion of Progenitors and Stem Cells

Recent hypothesis suggested to use stem/progenitor cells as source of cells for regeneration of damaged pancreas [18, 50, 51] and cure diabetes [52].

Stem cells are unspecialized cells which self-renew continuously to maintain a pool and divide infrequently and asymmetrically to produce progenitor cells with the potential to replace cell loss after injury or physiological turnover. Stem cell proliferation and differentiation potential vary according to their source for instance embryonic stem cells (ESC) derived in vitro from blastocysts and adult stem cells (ASC) found into all adult tissues. ASC were described in the spinal cord, fat tissue, bone marrow, placenta, brain, olfactory tissue, and connective tissue of various organs [53]. Neonatal stem cells, albeit being somatic, come from tissues collected after birth such as the placenta and umbilical cord (UC). This substance is

composed of two arteries and one vein surrounded by gelatinous matrix called Wharton's jelly, known to contain mesenchymal stem cells [54]. Cord blood contains mainly hematopoietic stem/progenitor cells and to a lesser extent, mesenchymal stem cells and pluripotent cord blood embryonic-like stem cells (CBEs). CBEs express pluripotency markers OCT-4, Nanog, SSEA-3, and SSEA-4 and can be differentiated into cells from different germ layers. MSCs from umbilical cord and cord blood were shown to differentiate into various tissues like bone, cartilage, muscle, fat, lung, and neural tissue and also toward insulin-producing cells [55, 56].

For regenerative medicine, mesenchymal stem cells (MSC) from cord blood present advantages on other adult stem cells [57–59]. First of all, it is an easy getting source of multipotent stem cells and with noninvasive method to obtain. This is the richest source of stem cells with more than 100 million births per year worldwide, and actually it is not used for any application. Cord blood stem cells (CB-SC) displayed very low immunogenicity with very low level of major histocompatibility complex (MHC) antigens [59]. First results obtained following study of umbilical cord stem cells (UC-SC) described them as very expansible, cryopreservable, and bankable cells. As these cells are taken in newborn, they are young stem cells, meaning that they are more heterogeneous than ASC. They contain long telomeres and have never seen any pathogens so are mutated as minimum. These characteristics induce a better tolerance for human leukocytes antigen mismatches. Compared with MSC from bone marrow, the number of MSC from Wharton's jelly does not decrease as well as the differentiation potential during increasing donor age. However, by now, some details need to be solved like limited number of stem cells per sample, and collection of umbilical cord is not yet systematically proposed to future parents.

Experimentally Modeling of Stem Cell-Based Protocols

Most protocols described to date follow a three-step experimental plan strategy: (1) in vitro induction/differentiation into insulin-producing cells, (2) aggregation into three-dimensional islet-like structure, and (3) injection in diabetes animal model streptozotocin type 2 SCID mice. To stimulate formation of islet-like structures, differentiated cells are put in presence of extracellular matrix of Matrigel or better in fibrin gels [60–62]. Culture conditions are very important to push cells to differentiate into pancreatic lineage and create islet-like clusters and to regulate blood glucose level. Some elements are helpful like HGF, nicotinamide, and fibrin gel. The first two steps are validated, meaning that in vitro cells would be able to secrete insulin – which is not obtained with all protocols – and prepared cells are injected in SCID mice. To mimic hyperglycemia conditions, animals received glucose analogous by streptozotocin intraperitoneal injection at 200 mg/kg body weight. Secreted insulin must induce perfect control of blood glucose. This result was not obtained by a lot of research teams.

Embryonic Stem Cells Can Produce Insulin-Producing Cells (Table 21.1)

Human embryonic stem cells (ESC), cultured embryoid bodies, can produce partially differentiated cells of all three embryonic germ layers (mesoderm, endoderm, ectoderm) [63]. ESC were shown to be able to differentiate into insulin-producing cells. Lumelsky et al. elaborated a protocol in order to differentiate mouse ESC toward insulin-secreting structures similar to pancreatic islets [64]. This work was the first study describing a several step protocol with formation of cluster (islet-like) structures. Injection of these structures into diabetic mice gave good results in insulin production, revascularization, and maintenance of islet-like organization. Jiang et al. presented also a clear protocol in serum-free condition with different phases following the pathways of pancreatic differentiation from human ESC [65] (Table 21.1).

Although in vitro and in vivo differentiation capacities of ESC into functional β cells seem to be effective and promising, there are limitations of such stem cell sources like inefficient and costly generation of new cell lines. Despite an

Table 21.1 Insulin-producing cells from cells of different origins

	Origin of cells	Pancreatic differentiation protocol	Results	Functional assays	Drawbacks	Reference
Ductal cells	Rat ductal cells	Pancreas tail ligation	BrdU-glucagon- and insulin-positive cells, glut2-positive cells		Low amount of beta cells	Wang et al. [40]
	Adult human pancreatic duct cells	Adenovirus-mediated expression of Ngn3	NeuroD1/β2, Pax4, Pax6, Nkx2.2, Nkx6.1 expression		Low amount of beta cells	Heremans et al. [42]
	Mouse pancreas	Adenoviral mediated delivery of the Pdx-1 gene	Pdx1 endogenous	β cells neogenesis and ductal cell proliferation		Taniguchi et al. [43]
Exocrine cells	Mouse pancreatic exocrine cells	Adenoviral transfection with cocktail of transcription factors Ngn3, Pdx-1, and Mafa	Conversion into β cells (same morphologic properties) insulin, Glut-2, glucokinase, PC1/3, NeuroD1/β2, Nkx2.2, and Nkx6.1 expression	Transfection into diabetic mice	No islet formation	Zhou et al. [34]

continued

Table 21.1 continued

	Origin of cells	Pancreatic differentiation protocol	Results	Functional assays	Drawbacks	Reference
Embryonic stem cells	hES cell line (H9 cell line)	Embryoid bodies formation spontaneous differentiation	Glucokinase and Glut-2 expression	Secretion of insulin	Cell line	Assady et al. (2001)
	ES cells	2-D culture spontaneous differentiation	Pdx-1$^+$/Foxa2$^+$ and Pdx-1$^+$/Isl1$^+$ cells	Transplantation induces cluster-like formation	No secretion of insulin	Brolen et al. (2005)
	Murine ES cells	1. Transfection of gene under control of insulin promoter	Clonal population of insulin-expressing cells		Insulin-induced glucose release abnormal	Soria et al. (2000)
		2. Nicotinamide in DMEM	Formation of clusters	Implantation into diabetic mice	No long effect	
	Murine ES embryoid bodies expressing the central nervous system precursor marker nestin	Serum-free medium	Insulin, glucagon, somatostatin, PP, Pdx-1, Glut-2, IAPP expression	Transplantation into diabetic mice	Production of insulin lower than in native β cells	Lumelsky et al. [64]
		1. Expansion with bFGF in N2 serum-free medium			Not able to correct glycemia	
		2. Differentiation with withdrawal of bFGF and supplementation with B27 and nicotinamide				
	Mouse ES cells	Inhibitor of PI3Kinase	Islet-like insulin, glucagon-positive cells	Transplantation into mice	Size of cells prevents implantation in sufficient quantity	Hori et al. (2002)
			No somatostatin- or PP-positive cells			
	Mouse ES cells	Electroporation with Pdx-1 and Pax4	Nestin-positive embryoid bodies Ngn3, insulin, IAPP, and Glut-2 expression	Transplantation into STZ mice	Experiment done only on short time	Blyszczuk et al. (2003)
	Murine ESC	3-Step approach with DMEM 20 % FBS, induction with activin A and all-trans-retinoic acid	Formation of clusters expressing β cell markers and secreting insulin	Transplantation into STZ mice	Regulation of blood glucose levels	Shi et al. (2005)

hESC and mESC	Differentiation protocol with inhibitor of PI3Kinase	Release of insulin but not c-peptide		Insulin^{+} cells apoptotic or necrotic	Hansson et al. (2004)
hESC	Activin A, low-serum culture	Sox17, GSC, and FoxA2 expression		Just endoderm markers No response to glucose	D'amour et al. (2005)
hESC	1. Coating serum-free system with activin A	Sox17 and brachyury endoderm marker expression			Jiang et al. [65]
	2. All retinoic acid	Early pancreatic marker expression (Pdx-1 and Hlx9)			
	3. Maturation in DMEM/F12 with bFGF and nicotinamide	Islet-specific markers expression (c-peptide, insulin, glucagon, and Glut-2)		Only 15 % of c-peptide-positive cells	
			Transplantation into renal capsules of STZ nude mice	Restoration of stable euglycemia for more than 6 weeks	
hES (embryoid bodies)	1. ITS-fibronectin medium	Insulin, glucagon, somatostatin expression	In vivo transplantation	Failure	Segev et al. (2004)
	2. DMEM/F12 with N2, B27, bFGF	Formation clusters			
	3. Low glucose without bFGF, with nicotinamide	Secretion of insulin			
UCB-MSC	Protocol with ECM	Insulin-positive cells Formation of islet-like clusters	Transplantation into hyperglycemic BALB/C nude mice	Mice remained hyperglycemic	Hu et al. [61]

ongoing phase I clinical trial for spinal cord regeneration in the USA, embryonic stem cell-based therapies still cause ethical controversies. Further to this, histocompatibility and spontaneous teratocarcinoma after ESC injection into animals and genomic instability are significant hurdles for their clinical development.

Adult Stem Cells Can Produce Insulin-Producing Cells (Table 21.1)

Stem cells from adult tissues are more ethically acceptable and accessible in most adult tissues. However, without expansion, access to these cells is limited. Further to this, if autologous transplantation is a viable prospect for regenerative medicine, allogeneic therapies require to match donor to patients' cells and or consider immunosuppression.

Multipotent adult stem cells may prove a viable alternative to induce islet cell formation [66]. Animal-based studies showed that islet neogenesis could be achieved using stem cells derived from bone marrow. In a study by Ianus and colleagues, male mice-derived bone marrow cells, expressing GFP, were transplanted into female recipients. The GFP-positive cells were detected post-transplantation into pancreatic islets of female [67] which secrete insulin. This brings evidence that bone marrow-derived cells can differentiate into functionally competent pancreatic endocrine β cells. Oh et al. further showed that rat-derived bone marrow cells cultured in DMEM supplemented by 1 % DMSO can form cluster-like structures expressing insulin, glucagon, somatostatin, and polypeptide [68]. Upon transplantation into diabetic mice, these cells induced control of blood glucose for at least 90 days. Chen et al. used DMEM supplemented with nicotinamide, beta-mercaptoethanol, and fetal calf serum to obtain islet-like cells from rat bone marrow [69]. They observed insulin secretion and down-regulation of blood glucose level after injection into diabetic rat. Different protocols are proposed from human mesenchymal stem cells from bone marrow differentiation toward insulin-producing cells [70, 71] with or without genetic manipulation. Even if the in vitro results seem to be conclusive, animal experiments stain variable.

Umbilical Cord and Cord Blood Stem Cells for Pancreatic Regeneration

The umbilical cord and the cord blood running through it form a tremendous source of stem and progenitor cells. Although the capacity of MSC differentiation into insulin-producing cells (IPC) in vitro is not yet clear [72], different studies reported the existence of umbilical cord blood (UCB)-derived cells that express marker characteristics of endocrine pancreatic tissues (isl1, pdx1, pax4, ngn3) [1]. Many protocols exist for differentiation of UCB-MSC into IPC (Table 21.1).

Gao et al. achieved pancreatic endocrine differentiation from UCB-MSC following a three-step protocol: recovery phase with glucose and FBS, differentiation and formation of islets with retinoic acid, extracellular matrix (ECM), and nicotinamide, respectively, and maturation with exendin-4 [60].

In this study, spindle shape UCB-MSC became round and epithelium-like culture and subsequently formed islet-like clusters. These cells were insulin positive but were not capable after injection into diabetic mice to regulate glycemia. Many other studies failed in the transition between in vitro and in vivo experiments. It was shown also that UCB-MSC in presence of high glucose and extracellular matrix culture conditions can express pancreatic-specific markers. To induce endothelial differentiation from UCB-SC, CEGF is needed with FBS during 10–14 days [59]. Islet-like cell clusters can be engineered from umbilical cord blood stem cells.

Harris observed that animals with type 1 diabetes treated with cord blood stem cells had lower blood glucose levels, reduced insulitis, and increased life span compared to controlled diabetic animals [18].

In order to prepare protocol for human clinical trials, it is required to work in serum-free conditions of culture and find good conditions for differentiation toward beta cells which is truly a big challenge.

For this reason, our group focused on cord blood stem cell differentiation into artificial islet-like structures in serum-free conditions. This is very important to obtain proper epigenetic

modification and functional signaling pathways active in pancreatic cells.

Our research group identified and confirmed key transcription pathways involved both in physiological development and artificial in vitro stem cell differentiation, including pluripotency (Oct4a, Sox2), endoderm (GATA4), pancreatic stem cell lineage (Islet1, PDX1), and mature islet cell (Pax6) markers. We used embryonic-like stem cells (CBEs) derived from human umbilical cord blood as previously described by McGuckin et al. [56, 73]. The molecular hallmarks of such pluripotent cells are transcription factors Oct4a and Sox2 [74]. At this stage of differentiation, CBEs do not express any other markers of lineage commitment. Most of studies already described for maintenance and expansion of pluripotent stem cells in defined serum-free medium require high dose of EGF. We found that such stimulation results in ectodermal (mainly neural) lineage of development [74].

Based on these results, a new culture media formulation based on stem cell factor and acidic FGF was tested to stimulate endodermal rather than neuroectodermal differentiation. CBEs grown in culture media without EGF upregulated GATA4 expression (Fig. 21.4c), which correlated with Oct4a and Sox2 expression, indicating withdrawal from pluripotent stage (Fig. 21.4a). These refined culture conditions form the basis of our pancreatic tissue engineering strategy. Ninety-eight percent of the resulting cells expressed endodermal-specific markers, whereas less than 2 % of the cells expressed ectodermal markers NF200 (Fig. 21.4b). Differentiation of such cells results in fast (up to 2 weeks) and efficient formation of semi-3-D clusters that resemble pancreatic islet primary cultures (Fig. 21.4). During in vitro differentiation, GATA4 was first expressed, followed by upregulation of Islet1 and PDX1 in 3-D islet-like clusters of committed progenitors (Fig. 21.4). After 2 weeks of differentiation, islet-like cells started to express Pax6 and c-peptide in mature beta cells (Fig. 21.4c). What is very interesting from a tissue engineering perspective is that these cell clusters were composed of both β-like cells (c-peptide/insulin positive) and α-like cells (glucagon positive). It has been proved in several studies that cell–cell communication is extremely important for proper tissue organization.

Wharton's jelly-derived MSC may also be potent for pancreatic tissue engineering as they can form cluster-like structures in nonadherent condition (Fig. 21.4d). In parallel, we developed a 2-D protocol based on activin A, sodium butyrate, and BSA, resulting in glucagon expression of UCB-CB (Fig. 21.4d). Some efforts are done to perform protocol in order to obtain insulin-secreting cells from umbilical cord stem cells.

Clinical Trials of Type 1 Diabetes

In February 2011, as many as 2,803 clinical trials are registered on the clinicaltrials.org website to test therapeutic applications for type 1 diabetes, mostly using drugs targeting autoimmune destruction of β cells.

However, only 40 clinical trials are stem cell based. For cell therapy and regenerative medicine applications, stem cells should be easily accessible, in sufficient numbers, autologous, or matched to the patient and not be tumorigenic. Further to this, the stem cell-based therapy should induce survival and functional maintenance or restore of the damaged organ. To repair any organ, three strategies are possible. Either cells or stem cells are taken in order to colonize and restore function of cells by differentiation. The second solution is to differentiate stem cells toward pancreatic progenitor in vitro and create implant of three or three dimensions and injected after into damaged pancreas to restore the function of organ. The last hypothesis is injection of cells or stem cells which will secrete paracrine signals and regenerate damaged organ by stimulating the process de novo. Stem cells used for diabetes clinical trials are coming from adipose (NCT 00703599), islet cells (NCT 00646724), hematopoietic cells (NCT 00807651), bone marrow cells (NCT 00465478 or NCT 00971503), mesenchymal stem cells (NCT01068951 or NCT00690066), or umbilical cord blood stem cells (NCT 00873925). Since 1999, umbilical cord blood stem cells have

been used for clinical applications mainly to cure hematologic cancer (www.clinicalTrials.org NCT00003335, NCT00003336, NCT00003435, NCT00003913, NCT00003662, NCT00003270). Regarding its therapeutic potential, umbilical cord blood stem cells start to be used to cure many diseases. For diabetes, only three clinical trials using HUC-SC are in process (www.clinicalTrials.org NCT01143168, NCT00989547, and NCT00873925) (Table 21.2).

In Florida, Haller et al. performed clinical trial with umbilical cord blood cells to cure 15 young

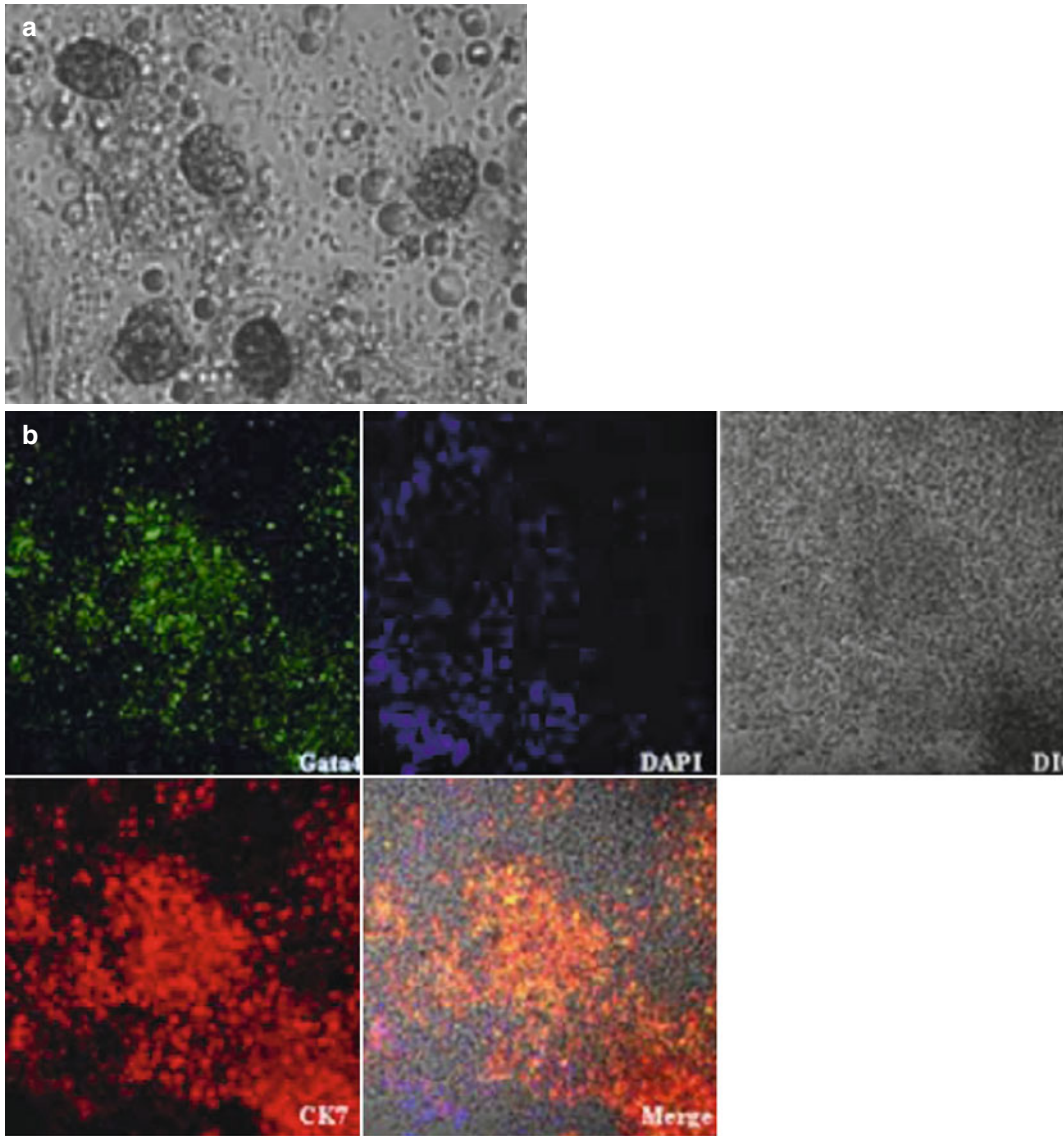

Fig. 21.4 Differentiation of umbilical cord and umbilical cord blood cells toward pancreatic cells. (**a**) Three-dimensional structures generated after maturation in vitro are artificial islet-like tissue. (**b**) 3-D islet-like aggregates show high expression of endodermal lineage markers *GATA4* and *CK7* due to introduction of certain growth factors, which promoted the direction. (**c**) Stem cells generate in vitro semi-3-D islet-like structures within 2 weeks of pancreatic differentiation like clusters express alpha-like cells (glucagon positive in *red*) and beta-like cells (insulin positive in *green*) are present within one 3-D aggregate. (**d**) MSC-WJ can create some islet-like structures, and action of activin A, sodium butyrate, and BSA stimulates glucagon secretion

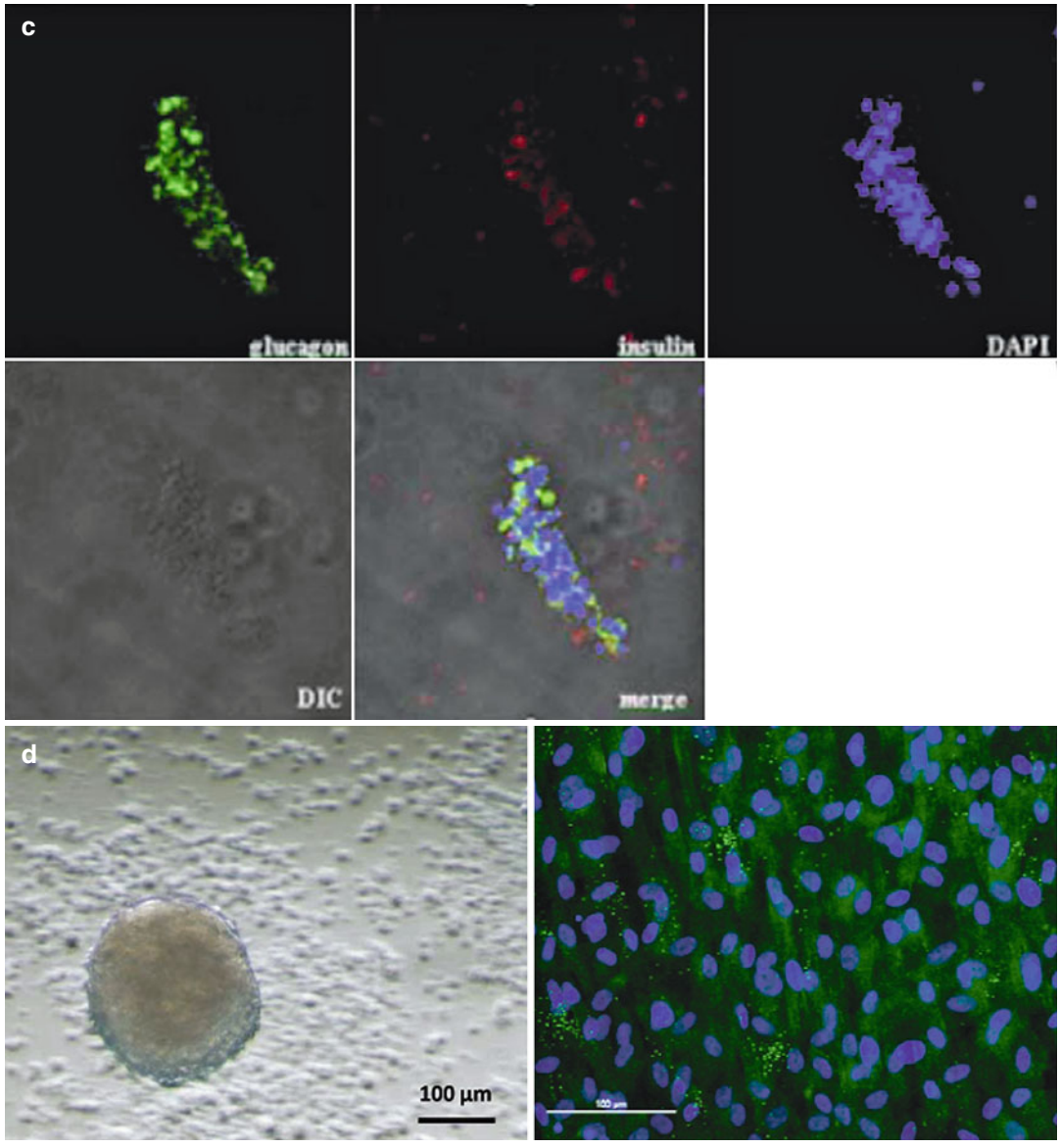

Fig 21.4 (continued)

children diagnosed with type 1 diabetes. Each child received infusion of their own cells. After 3–6 months, results showed that autologous infusion is safe. Loss of endogenous insulin production was slowing and correlated with lowered daily insulin requirements. They also observed some improved HbA1c levels and an increase in regulatory T cells found in peripheral blood of subjects 6 months after infusion. This suggested a potential immune-modulatory effect as a mechanism of action for this treatment [75]. After 1 year, no change in c-peptide endogenous production, insulin requirements, HbA1c levels, T cells phenotypes, and autoantibody titers was observed. These results showed that autologous umbilical cord blood infusion is safe, but effect on c-peptide secretion has to be demonstrated with longer trials and with more patients [76].

Table 21.2 Summary of clinical trials described for type 1 diabetes treatment from stem cells

NCT number	Status	Cell source	Preparation technique	Way of delivery	Outcomes
NCT 00703599	Recruiting participants	Adipose-derived stem cells	100–200 mL lipoaspirate by liposuction of abdominal adipose tissue	Autologous transplantation intravenously	Safety and efficacy
					Exogenous insulin requirement
			Activation of stromal vascular fraction cells		HbA1c and c-peptide levels
					No detrimental change seen in kidney and liver function tests and other hematological parameters
NCT 00646724	Recruiting participants	Islet and mesenchymal stem cells		Cotransplantation of islet of allograft and MSCs of autograft	Safety and efficacy
					Exogenous insulin requirement
					HbA1c and c-peptide levels
					Kidney and liver function
					Autoantibodies
NCT 00807651	Recruiting participants	Hematopoietic stem cells	Mobilization of cells from BM with cyclophosphamide (2.0 g/m^2) and G-CSF (10 μg/kg/day)	Autologous hematopoietic stem cell transplantation (AHSCT) and immunosuppression (cyclophosphamide 200 mg/kg and rabbit antithymocyte globulin 4.5 mg/kg)	Safety and efficacy
					Exogenous insulin requirement
					HbA1c and c-peptide levels
			Collection from peripheral blood by leukapheresis		Anti-GAD titers

Conclusion

Current diabetes treatment is mostly based around daily insulin injection. Pancreas or islet transplantation first appeared as a good alternative, but lack of donor calls for more reliable approaches. UC-SC seem to be a potent source of cells for pancreatic regenerative medicine due to distinct properties in terms of availability, immunological, and ontological status. However, stem cell-based therapies call for a translational approach to integrate anti-inflammatory, de novo and ex vivo differentiation potential, trophic effect for long-lasting glycemia control, and pancreatic function restoration. More preclinical and clinical research is necessary with UC-MSC and other stem cell sources to translate this research into robust therapies for diabetic patients.

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22 Maturation of the Human Fetal Pancreas: A Lesson for Embryonic Stem Cell Differentiation as a Therapy for Diabetes

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Background

The human fetal pancreas is an organ that begins its development at 6 weeks of gestational age, with endocrine cells forming from 8 weeks and exocrine cells from early in the second trimester (>13 weeks). In the early 1980s, its use was promoted as a possible therapy for those with insulin-dependent diabetes. This followed on from some excellent basic research with the rodent fetal pancreas by Jo Brown and colleagues in Los Angeles [1], who demonstrated that transplantation of this immature tissue would normalize blood glucose levels of syngeneic recipients.

The initial studies concentrated on pancreases obtained in the first half of the second trimester at 13–20 weeks of gestational age. Fewer than 8 % of the cells in the pancreas stained for insulin, with the majority being ductal precursor cells which could develop into either endocrine or acinar cells.

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Differentiation of Human Fetal Pancreas

Transplantation of this tissue into immunodeficient rodents, usually in the form of explants, resulted in the selective development over weeks and months of endocrine tissue, with insulin-producing beta cells being predominant [2]. This process of differentiation is accelerated by pretreatment of the tissue with incretins, glucagon-like peptide-1 and cholecystokinin, and nicotinamide [3, 4], by transplanting into a diabetic as compared to a nondiabetic host [5], and by treatment of the recipients with exendin-4 [6]. In addition to beta cells, other endocrine cells were produced, including alpha cells that secrete glucagon, delta cells that secrete somatostatin, and cells that secrete pancreatic polypeptide. Some exocrine cells do persist, but their numbers are relatively small.

In the past decade, human fetal pancreas obtained during the first trimester (up to 13 weeks gestational age) has also been transplanted into immunodeficient rodents with a similar outcome, that is, formation of mostly endocrine tissue [7, 8]. The rate of growth of pancreatic grafts obtained during the first trimester of pregnancy is greater than that of older pancreases [7, 9]. This results in larger grafts in recipient mice many months after small amounts of first trimester pancreas are transplanted, usually beneath the renal capsule.

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Maturation of Human Fetal Pancreas

Fetal beta cells synthesize, store, and secrete insulin but not in response to glucose, the commonest stimulus of the adult beta cell [10–12]. The reason for this appears to be an immaturity of both oxidative phosphorylation which occurs in the mitochondria and the glycerol-phosphate shunt that transports ATP from the glycolytic pathway to the Krebs citric acid cycle [13]. The fetal beta cell is, however, able to secrete insulin when challenged with most other adult secretagogues, including those that act:

- On the membrane to depolarize it, for example, KCl and carbamyl choline [12]
- On secretory granules, for example, agents that increase levels of cyclic AMP, such as glucagon and theophylline [14]
- To increase levels of calcium, for example, calcium ionophores [14]
- To increase levels of protein kinase C, for example, phorbol esters [15]
- To close ATP-dependent K^+ channels, for example, sulphonylureas [12]

Maturation of the ability of the fetal beta cell to secrete insulin when exposed to glucose occurs several months after the tissue is transplanted [16]. Initially, there is a second phase secretion of insulin, and subsequently a first phase also is observed. It is of interest to note, however, that when type 2 diabetes develops, the reverse scenario occurs; that is, the adult beta cell initially loses the first phase of insulin secretion in response to glucose and subsequently also the second phase.

It is not surprising therefore that human fetal pancreas eventually normalizes blood glucose levels when transplanted into diabetic immunodeficient mice and rats [7, 8, 17–19]. This takes 2–3 months to occur, as beta and other endocrine cells develop, and the beta cell matures in its ability to secrete insulin when exposed to glucose. This applies to pancreases obtained from both the first [7, 8] and second trimester [17–19].

Immunogenicity

An advantage of using fetal pancreatic tissue is assumed by many to be its relative lack of immunogenicity. This is not true for second trimester pancreas, in which class II histocompatibility antigens are expressed [20]. However, the situation with 1st trimester pancreas is different. Expression of HLA class II antigens is substantially lower, and this tissue, unlike second trimester fetal pancreas, is not rejected when grafted into humanized mice [8].

Human Fetal Pancreas in Diabetic People

Once the efficacy of human fetal pancreas had been shown in immunodeficient rodents [17–19], it seemed reasonable to attempt transplants with this tissue into people with type 1 diabetes. Screening of the tissue confirmed the safety of this approach, especially if the donors were negative for HIV and hepatitis B. A number of groups principle attempted such transplants including that of the author [21–23]. In some, tissue antigens between donor and recipient were matched, and in others who received a renal transplant, antirejection drugs were administered. In the 1980s when the grafts were performed, the tissue was obtained from the second trimester of pregnancy. Sites of transplantation included the muscle of the forearm, omentum, liver, and renal capsule [21]. A small component of the human fetal pancreatic tissue survived for 12 months [21], and C-peptide production was achieved in some recipients who were previously C-peptide negative [23]. No one, however, convincingly became independent of exogenous insulin administration.

The reason for this lack of efficacy in the clinic can be attributed to immunological rejection of the graft, despite the use of immunosuppressive agents in some recipients that were adequate to prevent rejection of an allografted kidney. It may be that the survival of the renal graft is due to its size with some rejection having little effect on function; the fetal pancreas is much smaller in size, and rejection of even a small amount of tissue would have much greater consequences. Two decades later, we now know that grafts of first trimester pancreatic tissue might result in a better outcome because the

tissue is less immunogenic [8]. Obtaining such tissue can be quite difficult, especially at 8–10 weeks gestation, with the embryo being tiny and the miniscule pancreas quite difficult to identify except for the very skilled. Although the pancreas at this age can normalize blood glucose levels of a recipient diabetic mouse of weight 30 g [7, 8], and diabetic rat of weight 194 g [19] how many pancreases would be required to achieve a clinical benefit in a 70-kg person, even if not rejected, is unknown.

A way of increasing the supply of such tissue is to digest the organ into islet-like cell clusters or islets and culture them in the presence of serum [24]. The epithelial cells grow out into a monolayer, which subsequently differentiates into mesenchymal-type cells that readily proliferate. Reconverting these cells into epithelial clusters that produce insulin can be achieved in vitro, but whether sufficient number of cells can be produced for human application is debatable. That it is possible to improve on the reconversion efficiency by transplanting islets [25] may be helpful for such an application.

Human Embryonic Stem Cells as a Source of Pancreatic Progenitors

A reasonable alternative to human fetal pancreatic tissue would seem to be the even more primitive pancreatic progenitors differentiated from pluripotent human embryonic stem cells (hESC) [26]. These progenitors have recently been derived from hESC by a series of culture steps, which differentiate the tissue initially into definitive endoderm [27, 28], and then primitive foregut before progenitors is derived.

Stimulation of the Nodal and Wnt3 pathways is required to convert hESC into definitive endoderm, which is characterized by the expression of the transcription factors SOX17, FOXA2 (Fig. 22.1), and the factors goosecoid, cerberus, and CXCR4. Less established is the technique to produce foregut and pancreatic progenitors where a precise combination of soluble factors are required. These include inhibition of sonic hedgehog signaling, for example, with cyclopamine; stimulation of notch signaling, for example, with FGF7 and FGF10; inhibition of TGFβ, for example, with noggin; and retinoic acid [26]. The authors have been successful in producing PDX-1 positive cells with this approach (Fig. 22.2) using the following sequence of culture conditions:

Day 1: activin A 100 ng/mL and Wnt3a 25 ng/mL in RPMI without fetal calf serum for 1 day

Days 2–3: activin A 100 ng/mL in RPMI with 0.2 % fetal calf serum for 2 days

Days 4–6: keratinocyte growth factor 50 ng/mL in 2 % fetal calf serum for 3 days

Days 7–9: cyclopamine 0.25 μM, all-trans retinoic acid 2 μM and noggin 50 ng/mL in DMEM containing 1 % B27 for 3 days

Days 10–12: DMEM with 1 % B27 for 3 days

Others add epidermal growth factor [29, 30], including a group from Beijing who have shown that this technique results in pancreatic progenitors from both hESC and induced pluripotent stem cells [30]. Transcription factors expressed in progenitor cells include PDX-1, SOX9, neurogenin 3, NKX2.2, NKX6.1, and HNF6. HNF 1β and HNF4α are expressed slightly earlier, in foregut endoderm.

Maturation of Pancreatic Progenitors Derived from hESC

While it might be ideal to derive fully mature glucose-responsive beta cells from human pancreatic progenitors in vitro, this art has eluded researchers to date. Agents that have been tried to achieve this goal include the incretin, exenatide, nicotinamide, retinoic acid, activin, hepatocyte growth factor, and BMP [28–30]. Insulin production has been achieved, but the levels are lower than those secreted from beta cells/islets dissociated from an adult human pancreas. Glucose responsiveness has been described by some [30], at least in static incubation with 20 mM glucose for 1 h, but whether the cells contain secretory granules, which are the hallmark of endocrine cells, has yet to be shown. Also yet to be described is whether the cells are capable of secreting insulin with the classical first and second phases of insulin secretion. To produce mature β cells might require growing the progenitors on a particular

extracellular matrix, such as laminin-1 [31], since the human fetal pancreas expresses specific integrins, and these will affect its binding to different matrices [32].

One group, Kroon et al. in California, has achieved maturation of pancreatic progenitors derived from hESC [26] but not in vitro. They utilized the knowledge derived from transplanting the human fetal pancreas, namely, that mature beta cells would form after several months [16], and transplanted pancreatic progenitors derived from hESC into immunodeficient mice. Within

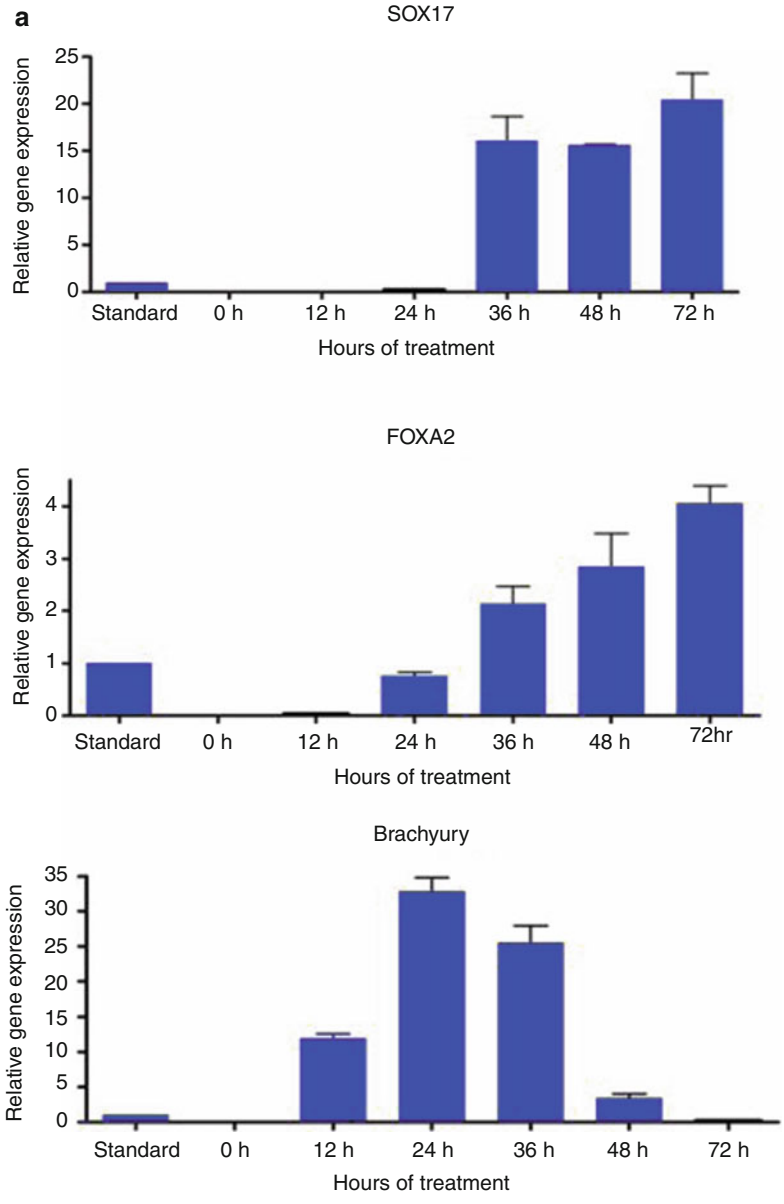


Fig. 22.1 Characterization of definitive endoderm derived from hESC using Wnt3a and activin A. (**a**) Quantitative PCR analysis of the gene expression levels of markers associated with early gastrulation (*Brachyury*) and definitive endoderm (*SOX17* and *FOXA2*) during the course of differentiation. Data presented as mean ± SD. Data were normalized to a standard which consisted of a diluted mix of 18-week human fetal pancreas, liver, and lung cDNA. (**b**) Immunofluorescent staining confirmed that semiquantitatively almost all of the cells co-expressed *SOX17* and *FOXA2* protein at the end of the treatment period. The bottom right panel is a composite picture of *DAPI*, *SOX17*, and *FOXA2*

Fig. 22.2 (**a**) Quantitative PCR analysis of the gene expression levels of markers associated with pancreatic progenitor cells on day 9 and day 12 of differentiation. (**b**) Immunofluorescent staining of PDX-1$^+$ cells on day 12 of differentiation. (**c**) Immunofluorescent staining of SOX9$^+$ cells on day 12 of differentiation

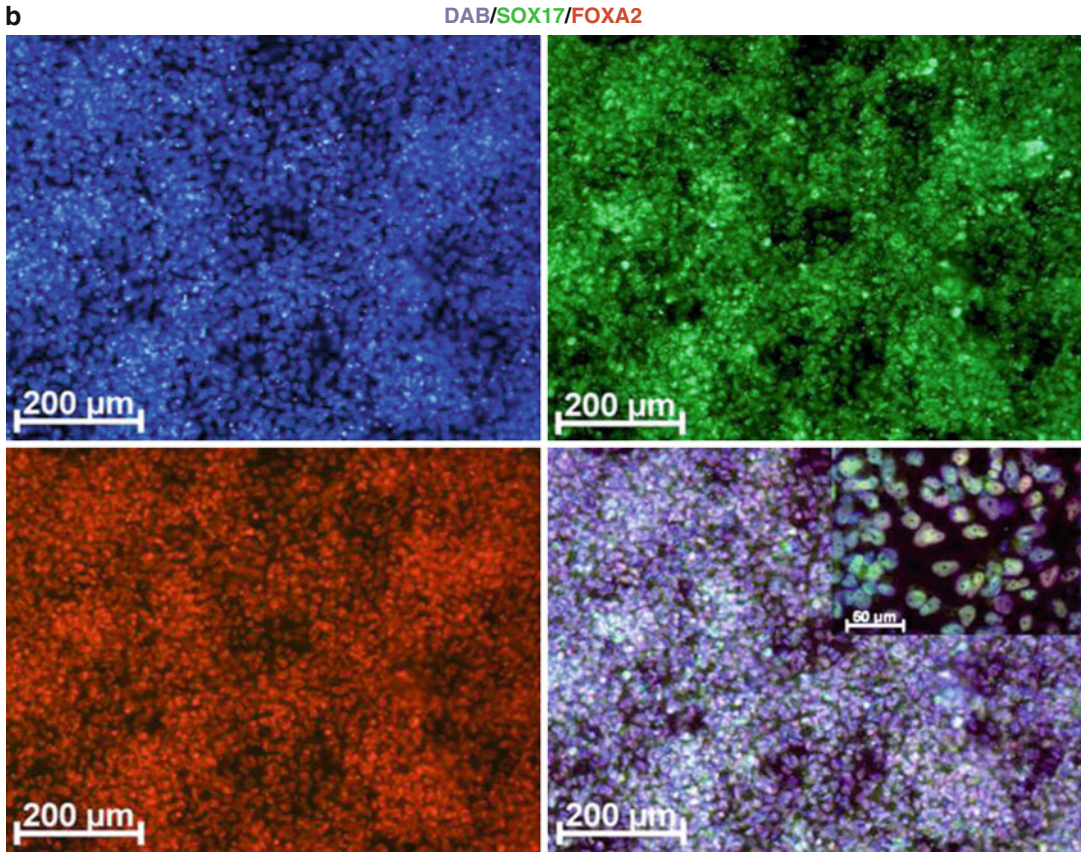
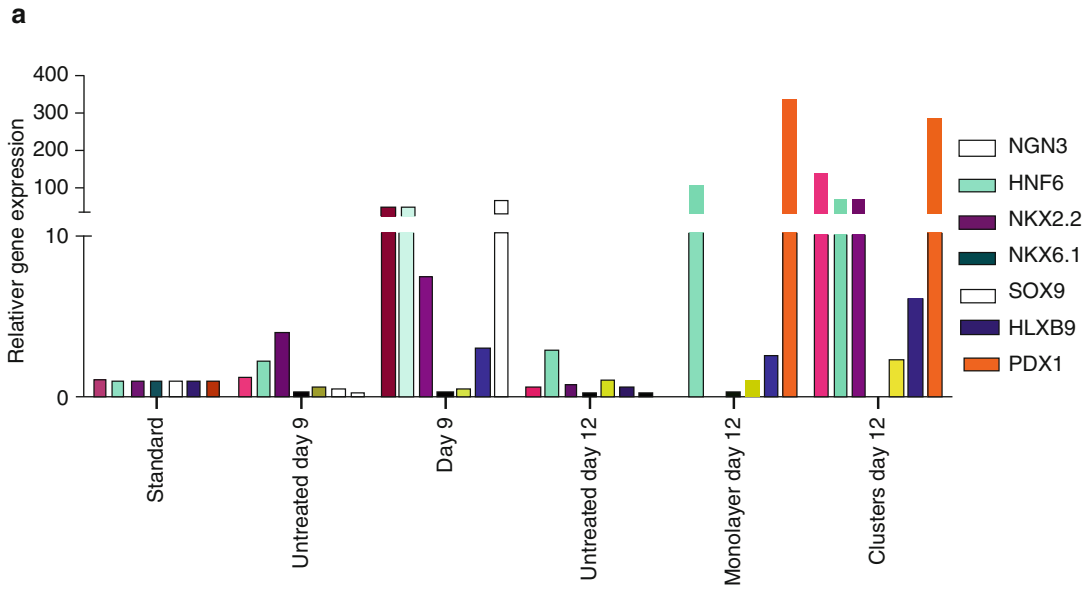


Fig. 22.1 (Continued)



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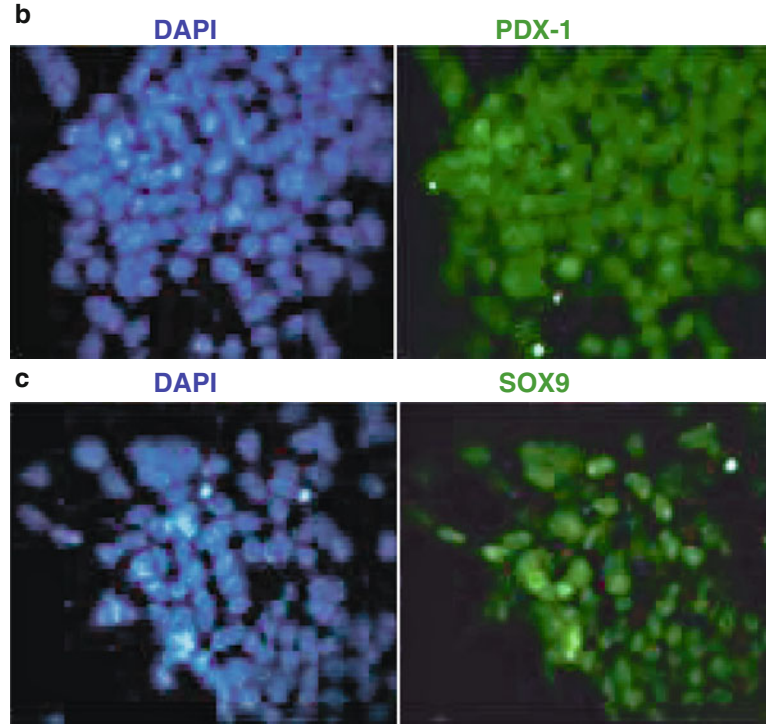

3 months, mature glucose-responsive beta cells had formed. The blood glucose levels of recipient diabetic mice were normalized to the levels found in the blood of humans, as compared to the higher levels that occur in mouse blood [33]. Secretory granules were seen when the tissue was examined by electron microscopy, and insulin levels in vivo rose when the mice were challenged with glucose. Confirmation that normalization of blood glucose levels was a result of the grafts was shown by removal of the grafts, which resulted in the development of hyperglycemia.

It is because of these encouraging results that there is now a move to transplant pancreatic progenitors derived from hESC into the clinic. Indeed in support of this endeavor, the Californian Institute of Regenerative Medicine awarded in 2010 a grant of US$20 million for the company ViaCyte to carry out the necessary tests to obtain approval from the Food and Drug Administration for a phase 1/2a clinical trial [34]. A further $10.1 million was awarded in 2012 to conduct this trial.

There are several matters that need to be addressed in an attempt to deliver these pancreatic progenitors into humans. They include (a) immunological rejection, (b) teratogenic potential, and (c) scale-up to obtain the necessary number of cells that is likely to be needed to normalize the blood glucose levels of a diabetic person.

Delivery Device

While hESC are relatively non-immunogenic by not expressing HLA class II and only low levels of HLA class I, cells derived from hESC are as levels of the HLA increase with differentiation [35]. This is akin to what occurs with the human fetal pancreas during development as was explained earlier. To prevent rejection of these differentiated cells when transplanted into other humans, either the immune system of the recipients will need to be suppressed or the graft will need to be isolated from the immune system. In most people with diabetes, immunosuppression cannot be justified over the administration of

insulin because the risks of infection, neoplasia, and side effects of the medication outweigh the benefits. Tolerance is one method being attempted to effectively isolate the graft from the immune system, but has as yet to be achieved in the clinic. One of the alternatives is to place the grafted cells inside a device that physically prevents immune cells from entering.

There are two main types of such devices – microcapsules [36] and diffusion chambers [37], each of which has pores that are large enough to allow the passage of nutrients and insulin but are too small to permit the entry of cells. Inflammation around the devices needs to be prevented [37] since cytotoxic cytokines and chemokines are produced as a result, and these are small enough to enter.

Microcapsules can be created using either synthetic polymers such as PEG or naturally occurring organic materials such as alginate. They are usually 300–700 μm in diameter and contain 1–2 islets or clusters of pancreatic progenitor cells. Immature fetal pancreatic cells placed in these devices will differentiate into mature functioning beta cells when transplanted into rodents at the same speed as non-encapsulated fetal cells [38]. The use of these capsules in humans is safe, as was shown recently with a phase l/2a trial with encapsulated human islets [39]. A further advantage of these capsules is their ability to prevent teratoma formation in encapsulated pluripotent stem cells that have been transplanted [40] (Fig. 22.3).

Diffusion chambers are a single device that will need to be large enough to contain all of the cells to be grafted. This is different from the microcapsules which each contains only a small number of cells. For use in humans, the number of microcapsules is likely to be in the order of 300,000–500,000 to achieve euglycemia in a diabetic recipient, but this number can readily be accommodated in the peritoneal cavity [36]. As with the microcapsules [39], maturation of fetal pancreatic tissue has been demonstrated inside this device when placed in immunodeficient mice [41]. An advantage of the diffusion chamber is the technical ease of its removal should a problem arise with the encapsulated cells; a disadvantage is its size thereby limiting the sites it can be implanted into.

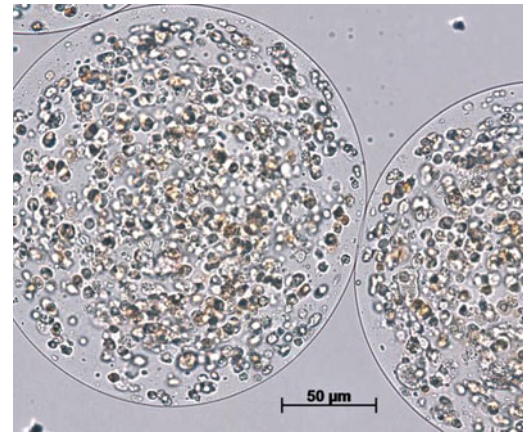

Fig. 22.3 hESC in microcapsules made of barium alginate. These capsules, which are produced with an air droplet generator, have a pore size of 250 kDa [40] (Produced with permission of Wolters Kluwer Health)

Whatever system is chosen to deliver encapsulated pancreatic progenitors to the clinic, their delivery will need to be rapid if large numbers of recipients are to be treated. The authors have experience in delivering up to 300,000 islets inside microcapsules to diabetic people in a rapid manner; 1–2 h from the time the patient arrives until they are discharged. It is to be hoped that a similar degree of efficiency will be achieved with the administration of encapsulated pancreatic progenitors as a therapy for diabetes.

Summary

The lessons learnt over several decades from studying the human fetal pancreas have very recently begun to be of value in differentiating hESC into mature beta cells. While the initial differentiation of the hESC is carried out in vitro, the final stages occur when the tissue is transplanted. The pathways whereby these final stages occur have yet to be elucidated, but it can only be a question of time before this occurs. Finally, the use of a delivery device such as the microcapsule offers a method of administering the pancreatic progenitors to large numbers of people with diabetes without the need for antirejection drugs. When this occurs, insulin administration in the manner

pioneered by Banting and Best in the 1920s with their discovery of insulin will be history for most insulin-dependent diabetic people.

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Part IV

Fetal Tissue Transplant Experiments in Animal and Human Systems

Fetal Neural Tissue Transplantation for Spinal Cord Injury Repair

23

Sankar Venkatachalam

Introduction

Spinal cord being the sole communication link between brain and parts below the neck, its injury invariably results in dreaded sensory and motor control loss. Mankind's recognition of spinal cord injury (SCI) and its consequence dates back to 2500 B.C. evident from vivid descriptions given in Greek papyrus. Despite such early recognition, pathophysiology of SCI and prospective therapies were not identified until early part of previous century. In a review, Osterholm [33] had summarized various pathological events that ensue SCI which ranges from petechial hemorrhage immediately after injury to secondary necrosis and cavitations which take several days to weeks to develop.

Several factors such as Wallerian degeneration [19], axon autotomy [24], slow growth rate of spinal axons [14], glial scarring becoming an impediment to axonal growth [38], axon growth inhibitory molecules [45], and lack of growth/trophic factors [23] were attributed to be the cause for lack of recovery after SCI.

Out of various nonsurgical and surgical methods aimed to treat SCI, transplantation of materials intraspinally gained popularity mainly during the 1970s to late 1990s. Both living and nonliving materials were intraparenchymally transplanted which include bizarre materials like iris and mitral valve [29]. Probably following "an eye for an eye, and a tooth for a tooth" logic, scientific fraternity envisaged the use of "neural tissue" to compensate the lost/injured neural tissue. As parts of adult spinal cord could not be used, attempts were made with embryonic spinal cord strips [1, 3–5, 7, 17, 20, 22, 28, 34, 35, 37, 41, 43] or with embryonic spinal cord neurons [21, 32, 42].

Not only homotopic transplantations but also heterotopic transplantations, in which CNS tissue from other parts such as cerebellar tissue [25, 40], neocortex [13, 18, 21, 35], and locus coeruleus [6, 30], were transplanted for SCI repair.

The intent of this chapter is to analyze the knowledge so far gained along with our lab's limited experience in fetal neural tissue transplantation to appraise the role of such transplantations in SCI repair research. Scope of this chapter is limited to the use of "fetal neural tissue" for SCI, i.e., neural tissue obtained from fetal period of development, and does not include the use of other cells including stem cells of any type. Fetal neural tissue shall contain neural stem cells, progenitor cells, cells of nervous system, viz., neurons and glia in various stages of their development, and probably also other nonneuronal cells like endothelial cells of blood vessels.

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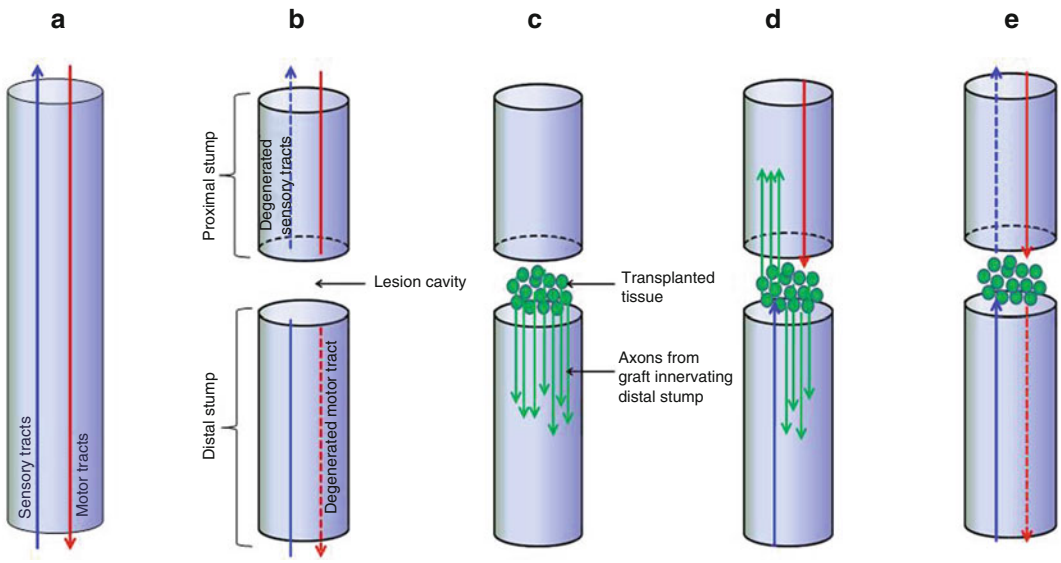

Fig. 23.1 Schematic illustration explaining the concepts of fetal neural tissue transplantation for Spinal Cord Injury. (**a**) Normal spinal cord with descending (*red*) and ascending (*blue*) tracts representing motor and sensory tracts respectively. (**b**) After total spinal cord transaction, both ascending and descending tracts are interrupted. Their distal isolated parts would degenerate. (**c**) Transplantation of Supra-Spinal neurons such as brain stem neurons would innervate distal stump supplying it with the missing neurotransmitters thus might mimic like "local brain." (**d**) Transplanted cell might behave like relay stations for the host's ascending and descending fibers. (**e**) Transplanted cell might favor regeneration by host fibers thus provide a conductive substrate

Rationale for Fetal Neural Tissue Transplantation

Fetal neural tissue had been transplanted under varied conditions. It ranges from allografting to xenografting and homotopic to heterotopic with different rationales [13, 31]. In a nutshell, fetal neural tissue transplantation was aimed to achieve one of the following conditions (Fig. 23.1):

1. *Substituting supraspinal control* (*locally*) – Supraspinal centers, viz., brain stem and brain, are not involved extensively in the locomotion process, rather they only serve to initiate, accelerate, decelerate, and stop the process. Using the minimal control from supraspinal centers, the spinal circuits are able to execute the learned skill of locomotion. This leads to the concept of spinal pattern generators. Therefore, it was considered by transplanting neural tissue from supraspinal sources, the distal spinal cord stump would get the required input to execute locomotion. This paved way for studies like transplantation of brain stem and locus coeruleus neurons to spinal cord (Fig. 23.1c).
2. *Creating* "*spinal relay*" *stations* – It was expected that transplanted tissue would develop into a functional neuronal circuit with input from supraspinal connections and output to distal stump of the spinal cord (Fig. 23.1d). Thus, it can form a "relay station" for the neuronal impulses to get transmitted either way.
3. *Encouraging host regeneration* – It was proposed that transplantation of fetal neural tissue would recreate a developmental stagelike condition wherein cues for proper axonal regeneration and path would be available. Besides that, presence of fetal neural tissue would create a "neuronal milieu" for the host axons [15] to regenerate (Fig. 23.1e).

Our Experience in Neural Transplantation for SCI

In our laboratory, we intended to test the role of fetal neural tissue transplantation for SCI repair using a xeno-heterotopic transplantation model. In above-mentioned three rationales for neural

transplantations, for us, the third one appeared more convincing, i.e., creating a congenial "neuronal milieu" in the lesion site which would encourage host neurons to regenerate across the site. The experimental protocol was approved by the ethical committee of Dr. ALM Institute of Basic Medical Sciences, University of Madras, Chennai.

Primate model of total spinal cord injury was created using bonnet monkeys (*Macaca radiata*). Female bonnet monkeys of body weight 0.75–1.75 kg were used for the study. These animals were procured through Conservator of Forests, Government of Tamil Nadu, after obtaining necessary permission. Under thiopental anesthesia (28 mg/kg), T10–T11 junction of the spinal cord was exposed by adopting standard laminectomy procedure. Dura was longitudinally incised, and spinal cord for about 5 mm was completely excised microsurgically. Care was taken not to injure both anterior spinal artery and posterior median vein. The dura was sutured watertight using 6-0 silk, and the wound was closed in layers. Thus, the lesion created was an isolated neuronal injury without vascular insult.

In transplantation group animals, human fetal neural tissue was transplanted. Human fetuses after medical termination of unwanted pregnancies were obtained after getting necessary consents. The gestational age of these fetuses ranged from 9 to 12 weeks. From earlier studies conducted in our department, it was found that small fragments gave better survival of cells rather than homogenous suspension. Therefore, instead of isolating cells by trypsinization, fine fragments of 2 mm × 2 mm were used for transplantation. The tissue fragments were suspended in Eagle's minimum essential medium supplemented with 10 % fetal bovine serum, antibiotics, and antifungal agents until the time required for transplantation. Following the creation of transection cavity in the spinal cord of bonnet monkeys as described above, these tissue fragments were gently lifted off using small forceps and were placed in the cavity. Microvascular bleeding ensuing transection was found to form a clot with the cells, thus effectively holding them at the transplantation site.

Postoperatively, animals were cared for complications of spinal cord injury. Urine retention was relieved by manual expression of bladder until automatic bladder sets in. Prophylactically, animals received antibiotics and H2 blocker for first 5 days. Analgesia was achieved by pentazocine lactate for first 3 days following surgery. Animals were euthanized after 60 days, and tissues were fixed by vascular perfusion with 10 % formal saline. Spinal cords dissected out were processed for paraffin sectioning, and sections were cut at 10-μm thickness. Sections were stained for neural cell body (CFV), axons (silver staining), and myelin (Loyez method). All the tissue-processing, section-cutting, and staining procedures were carried out as described in standard histopathological textbooks [12].

Histology of lesion site in injury group showed glial scar and connective tissue scar to variable extents, sealing off the cut stumps of the spinal cord and filling up of the entire transection cavity (Fig. 23.2a, b). In fetal tissue transplantation group, the transection cavity was filled with very good neuropil. There was no connective tissue or glial scar at the ends of the spinal cord. Although there were some cavitations seen among the grafts, in general, a good preservation of tissue architecture was seen (Fig. 23.3). Quantification of histological parameters such as area of preserved tissue and neuronal density indicated better preservation in transplantation groups (Fig. 23.4). However, due to limited sample size, statistical significance could not be found out.

Functional recovery in the animals was tested using modified Tarlov's paraplegic scale and also through other tests specific for animals such as contact placing reflex and tendon reflexes. Animals with transplanted fetal neural tissue did not show any significant recovery and were same like that of the injury group animals in their performance in various behavioral tests.

Role of Fetal Neural Tissue Transplantation for SCI Repair

Embryonic/fetal neural tissue transplantations were reported to enhance functional recovery in experimental animals under varied conditions of transplantations [1, 3, 5, 6, 15]. Almost all these

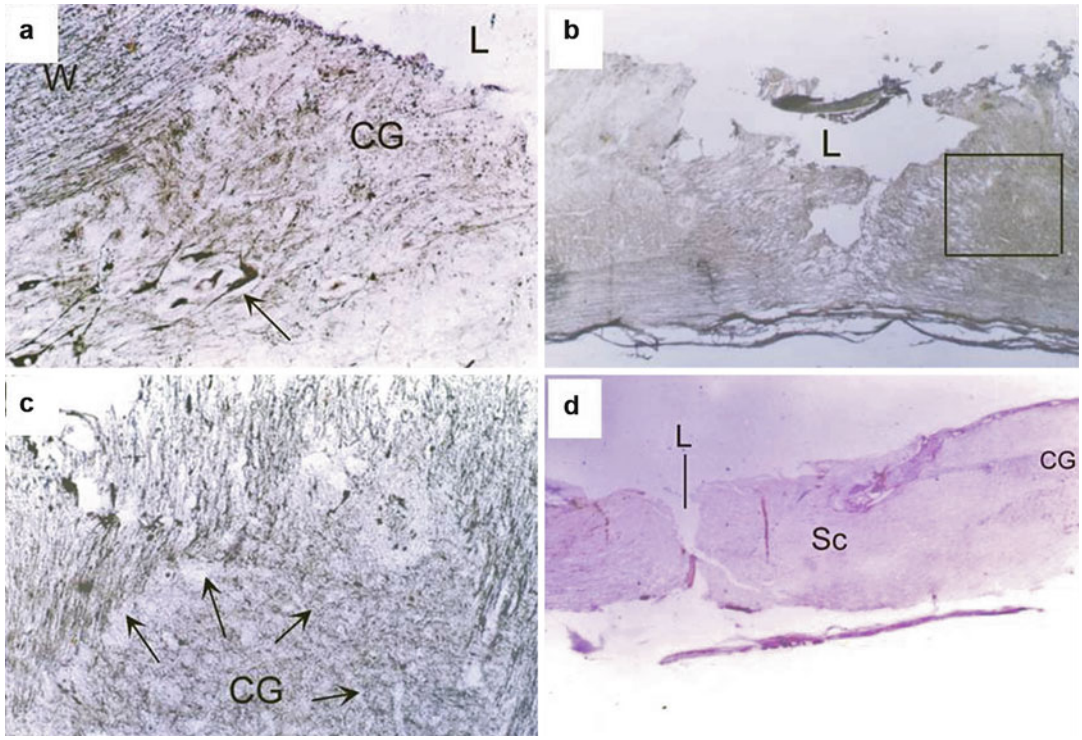

Fig. 23.2 Histology of spinal cord in lesion group animal. (**a**) Lesion site after 3 days. Cut stump were not covered with glial scar. Central grey matter (*CG*) can be seen in contact with lesion cavity (*L*). White matter (*W*) did not show any degeneration signs. *Arrow* indicates host neurons. Silver stain. Magnification 25×. (**b**) After formation of glial scar. Lesion cavity (*L*) got separated from host central grey matter. *Boxed area* enlarged in next frame. Silver stain. Magnification 5×. (**c**) Magnified view of boxed area of (**b**). Central Grey matter (*CG*) was encircled by glial scar (*arrows*) separating it from lesion cavity. Silver stain. Magnification 25×. (**d**) In long term, cut stumps of the spinal cords were completely wrapped in glia-connective tissue scar. Lesion cavity (*L*) can be seen unfilled and scar tissue (*Sc*) separating the central grey (*CG*) from making any contact with opposite side stump

studies were performed in animals lower in phylogenetic scale to primates. Also, total transection of the spinal cord was rarely used. In our experiments, although we observed positive features at histological level like good neuropil formation and enhanced survival of neurons, no functional improvement was observed when compared with lesion group animals. Similarly, in publications about clinical trials performed for spinal cord injury with neural transplantation, functional improvements were not reported [16, 36]. This kind of discrepancy between observed functional recovery in experimental animals and lack of such recovery in primate studies including clinical trials may be due to the higher degree of plasticity in lower animals especially in the event of subtotal spinal cord lesions.

Transplantation of fetal neural tissue for neurological disorders was experimented during the period of the 1970s–2000. Probably, the discovery of embryonic stem cells by Thompson in 1998 [44] and an early very positive report about the usefulness of embryonic stem cells for SCI repair in 1999 by McDonald et al. [27] shift the focus of transplantation approaches to the use of stem cells. In the past decade, there were a lot of reports about the use of different stem cells such as embryonic, mesenchymal, and placental cells for SCI repair.

Much hype about stem cells is based on their ability to form cells of many lineages upon subjecting them to induction methods. It has been shown how successfully stem cells were induced to form different cells such as osteocytes,

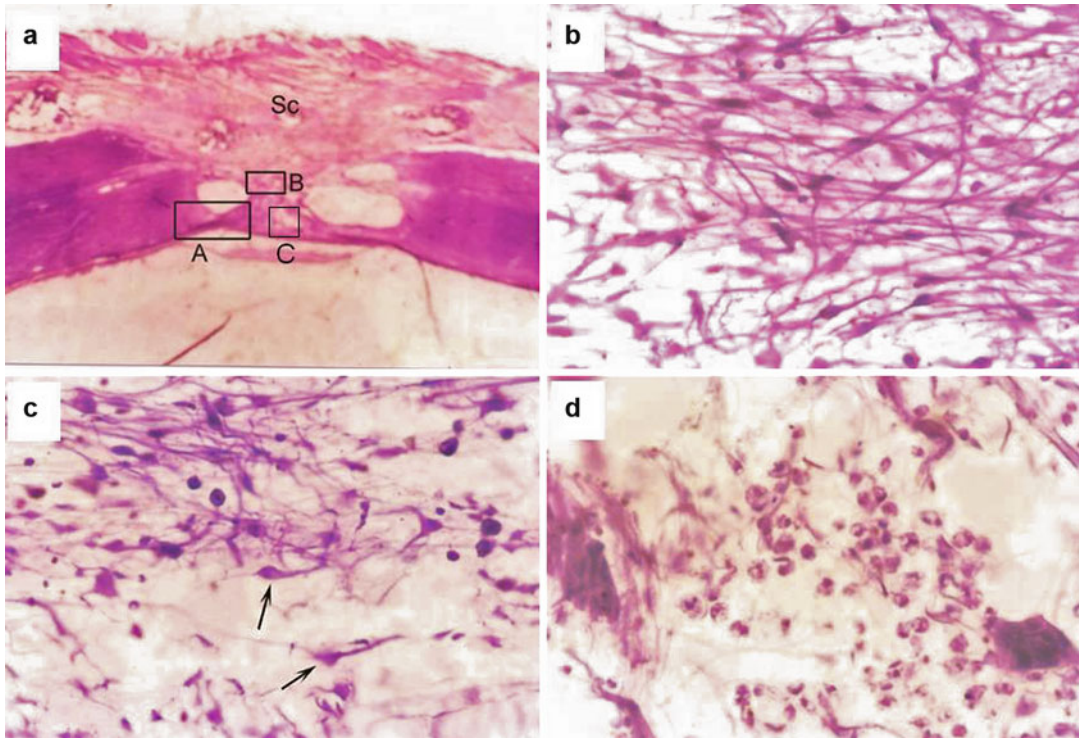

Fig. 23.3 Histology of spinal cord in fetal neutral tissue transplanted group animals. (**a**) In fetal neural tissue transplanted animals, lesion cavity was found to be filled with neural tissue. Connective tissue scar (*Sc*) was found on dorsal aspect. *Box A*, *B*, *C* were enlarged in subsequent frames. CFV stain. Magnification 5×. (**b**) Magnified view of area marked as *Box A* in previous frame. This represents the graft-host junction with good neuropil seen. CFV stain. Magnification 25×. (**c**) Magnified view of *boxed area B* in frame (**a**). This represents graft area not in contact with the host spinal cord stump. Comparatively, the neuropil was not very well formed; however showed developing neurons from the graft. *Arrows* indicate two such cells which start their differentiation evident from the outgrowth of axons from cell bodies. CFV stain. Magnification 25×. (**d**) Magnified view of *boxed area C* of frame (**a**). At this area of the graft, differentiated cells were not seen. Infiltrated mononuclear cells seen; indicating possible immunological attack on the grafted cells. CFV stain. Magnification 25×.

chondrocytes, and adipocytes, including neurons [26]. The central dogma of anatomy is cells-tissues-organs-systems-organism. When it is assumed that stem cells can give rise to any cell, by transplanting stem cells for SCI, we are in stage 1, i.e., replacing/substituting the damaged cells with new ones. It has to go through other stages before it becomes useful functionally. The transplanted cells should successfully form a "neural tissue" (tissue level) and not just "neurons" or "glia" (cell level) alone. Such newly formed neural tissue must first integrate with host spinal cord tissue (organ level) and later must get functionally integrated with supraspinal centers (system level). Only then we can expect such repair be functionally useful (organism-level outcome).

In this context, usage of fetal neural tissue may be considered as commencing the repair process from tissue level but not from cell level as in the case of stem cell transplantation. Therefore, whether the repairing attempt is started from cell level or tissue level, unless functional integration of the transplanted material occurs with host tissue environment, function recovery may be far from reality.

The functional integration of graft with host may be critically important in spinal cord since here the primary problem is lack of connection. Contrarily, in other neurological conditions such as parkinsonism, the mainstay of problem is not lost connections but insufficient production of neurotransmitter. In such conditions, supplementation

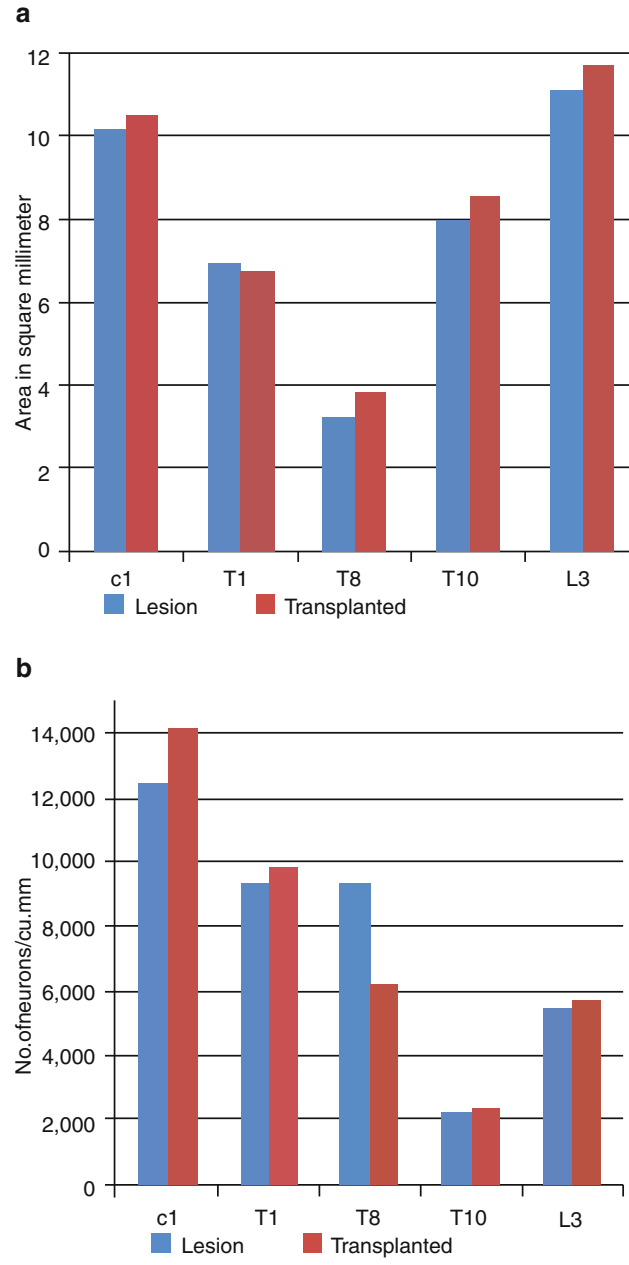

Fig. 23.4 Quantitative histology results comparing lesion and transplantation group animals. (**a**) Histogram showing spared spinal cord tissue at various levels. Results indicate that in transplantation group more tissue was preserved when compared with lesion group. (**b**) Histogram indicating numerical density of neurons present at various spinal levels. In transplantation group, more neurons were seen at various levels when compared with lesion group. Results of quantitative histology present evidence for the possible beneficial effects of transplanting fetal neural tissue after total Spinal cord injury as numerically the values were higher in transplantation group. Values represent mean of each group (Lesion group (n) = 5; Transplantation group (n) = 5). Due to very limited sample sizes, statistical significance of differences could not be worked out. Since the study was conducted using primate animal, unlike rodent studies, number of animals under each group could not be increased beyond this limit

through the grafted tissue/cells might be sufficient and probably may be the reason behind positive clinical reports about fetal tissue/cellular transplantation for parkinsonism patients [10].

From the foregoing discussion, it appears that three rationales on which neural transplantation was carried out may not be viable because, in all the three expectations, the graft-mediated/graft-originated circuits need to functionally integrate with the rest of the circuits, a task which appears in current scenario a difficult one to achieve.

By then, what could be the usefulness of neural tissue transplantation or the currently pursued strategy of stem cell transplantations? The answer is the possibility of neuroprotection, i.e., rescuing the cells/circuits which survive primary injury from death due to inflammation-mediated secondary injury. Now, it is known primary injury

triggers several pathological mechanisms which eventually lead to the devastating results. Thus, by preventing delayed cell death, it may be possible to utilize the plasticity of neural circuits to enhance functional recovery.

Protection of host cells by paracrine effects of secretions from cells transplanted is advocated to be the mechanism behind stem cell-mediated effects. In similar fashion, fetal neural tissue can also rescue cells from secondary damage. It is noteworthy that in our experiments also, although statistical significance could not be worked out, there was numerically more cell survival in transplantation groups. Thus, neural tissue transplantation could serve to protect preserved host circuits rather than enhancing recovery through other means.

Future of Fetal Neural Tissue Transplantation for SCI Repair

The much expected stem cell application did not benefit SCI victims to a great extent. Now, several limitations are known with respect to stem cell usage especially with the observation of tumor formation after embryonic stem cell transplantations [8]. Efficacies of stem cells from alternate sources (mesenchymal stem cells) are controversial [11] with both positive and negative reports about their usefulness [9]. In an unpublished work, in our lab, we observed aberrant differentiation of the transplanted mesenchymal stem cell.

The suggestive method to avoid tumorigenesis or unpredictable behavior of stem cells is subjecting them to neural induction methods [2] before transplantation in vivo. By inducing the stem cells into neural lineage, it is expected to avoid uncontrolled division of them and also preventing the aberrant differentiation into unwanted cell types. Through neurally differentiated stem cells, it might be possible to achieve neuroprotection through paracrine effects.

What could be the advantage of using stem cells induced to form neural cells rather than using neural tissue as such from embryonic/fetal sources? The only answer we could propose is "ethical advantage" especially when the stem cells used are adult-derived ones in an autologous fashion. However, this path does not appear to be without problems. Generating functional neurons from adult tissue-derived stem cells appear to be difficult, and it has been argued that the results of neural induction studies in vitro could be due to stress responses of the stem cells rather than true differentiation into neural cells [11].

Under these circumstances, it would be logical to continue experimental studies with neural tissue transplantations, and once undisputable findings were established about their usefulness, then research on generating neural tissue from adult stem cells could be intensified so that ethical complications could be avoided when large-scale human treatments were carried out.

Transplanted fetal neural tissues serving as relay stations or as suppliers of supraspinal inputs or leading to novel circuit formation through regeneration of host neurons are all less likely. Given that the benefit of such transplantation is limited to the possible paracrine effects of the graft tissue, similar effects could be achieved through other cells not necessarily true stem cells. For example, from a contemporary study, we have already reported that human amniotic epithelial cells offer same effects like that of fetal neural tissue and additionally found to be biologically better and ethically complication-free [39].

With the advantage of fetal neural tissue usage over stem cells usage, leaping progresses, and advancements in neuronal biology around the corner, the future prospects of fetal neural tissue transplantation for SCI repair appear bright.

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24 Fetal Lung Tissue Transplant at a Heterotopic Site in Common Chronic Inflammatory Diseases of the Airways: A Study of 11 Cases

Niranjan Bhattacharya

Introduction

The reported pluripotential capabilities of many human stem cell types have made them an attractive area of research, given the belief that they may hold considerable therapeutic potential for treating a wide range of human diseases and injuries. Although the bulk of stem-cell-based research has focused on developing procedures for the treatment of pancreatic, neural, cardiovascular, and hematopoietic diseases, the potential for deriving respiratory cell types from stem cells for treatment of respiratory-specific diseases has also been explored.

In chronic respiratory disease, selective and super-selective bronchodilators with or without steroid supplementation in inhaler/oral or injectable form along with antibiotics and respiratory physiotherapy may not be able to achieve the desired relief of the respiratory problem in many cases. The patient may progressively become restless, and psychosomatic elements may intervene. There is also a simultaneous predisposition to shift from a pathophysiological background of allergic/infective/obstructive respiratory problem to a restrictive variety, apart from other changes involved in remodeling the airways.

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In order to prevent this deterioration, various forms of therapy have been attempted.

Cell therapy or, to be more specific, stem cell therapy with its transdifferentiation and migration possibility to the site of injury and subsequent repair quality has attracted many investigators around the globe.

It is suggested that stem cell derivatives may be used therapeutically for lung regeneration purposes in a variety of respiratory injuries and diseases including cystic fibrosis, chronic obstructive pulmonary disease, respiratory distress syndrome, pulmonary fibrosis, and pulmonary edema [1]. A group of investigators from Sao Paulo (Brazil) claim that stem cell therapy can attenuate airway and lung parenchymal remodeling. They assert that bone marrow mononuclear cell (BMMC) therapy will mitigate airway and lung parenchyma remodeling in a model of chronic allergic asthma. These investigators evaluated lung mechanics and histology and the expression of insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF) mRNA expression in lung tissue (RT-PCR). They experimented on mice and noted airway resistance (13 %), viscoelastic pressure (51 %), static elastance (27 %), the volume proportion of smooth-muscle-specific actin in distal airways (112 %) and alveolar duct walls (178 %), and the number of eosinophils and collagen fiber content in lung parenchyma and airways and observed that the expression of IGF and VEGF was significantly increased in mice who were sensitized with ovalbumin and exposed to three

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repeated ovalbumin challenges. The control group received only saline for the similar protocol and did not register any change. The treatment with bone marrow mononuclear cell (BMMC) avoided lung mechanical changes, minimized morphometric and inflammatory alterations, and reduced the amount of collagen fiber in airway (18 %) and lung parenchyma (21 %) and IGF (28 %) and VEGF (36 %) expression compared to the OVA group [2].

The understanding of the mechanism of repair, the recruitment of stem cells to sites of injury, and their involvement in tissue regeneration and remodeling is becoming a new and novel therapeutic field for developing more effective treatments against difficult respiratory disorders [3].

Recently it has been discovered that human lungs contain undifferentiated human lung stem cells nested in niches in the distal airways [4]. The present study is intended to examine the therapeutic effect of second-trimester fetal lung tissue transplant, the tissue being collected from consenting mothers undergoing hysterotomy and ligation in accordance with fertility prevention procedures. The idea is to see whether there is any clinical impact of the fetal lung tissue harboring stem and progenitor cells (in remodeling) on the host's airway disease. The fetal tissue is placed at a heterotopic site beneath the axillary skin (under local anesthesia) of a host suffering from common chronic inflammatory disease of the airways (bronchial asthma, chronic obstructive pulmonary disorder) with his/her informed consent.

Materials and Methods

After getting necessary informed consent from all concerned and ethical permission from the institute-based ethical committee, 11 patients were enrolled for fetal lung tissue transplant at a heterotopic site under local anesthesia, that is, at the axilla of the host.

All the patients were given a preliminary screening for hepatitis B and C and HIV 1 and 2 along with hemoglobin, total count, differential count, platelet count, and assessment of ESR. Next, tests were conducted for liver function, urea, creatinine, fasting and postprandial sugar, glycosylated hemoglobin, and lipid profile; then, chest x-ray (DNA PCR in case of suspicion of Koch's infection), ECG (echocardiography in case of ECG problem), and tests for C-reactive protein, antinuclear antibody, anti-dsDNA, T3, T4, and TSH were undertaken to gauge the suitability of the case for the transplantation procedure. Apart from a detailed clinical follow-up, spirometry was advised in all the cases before and periodically after the transplant of fetal lung tissue to see if there was any perceived change from the pretransplant level.

Spirometry is the most common pulmonary function test (PFT) that measures lung function, specifically the amount (volume) and/or speed (flow) of air that can be inhaled and exhaled. Spirometry is an important tool used for generating pneumotachographs which are helpful in assessing conditions like chronic obstructive pulmonary diseases (COPD). The parameters used for comparison of the impact of transplantation are as follows:

(a) Forced vital capacity (FVC) is the volume of air that can forcibly be blown out after full inspiration, measured in liters. FVC is the most basic maneuver in spirometry tests.
(b) Forced expiratory volume in 1 s (FEV1): The average values for FEV1 in healthy people depend mainly on sex and age. Values between 80 and 120 % of the average value are considered normal.

Thus, fresh fetal lung tissue, which was acquired after getting due consent and ethical permission, was sliced serially with a sharp knife and placed immediately at a locally anesthetized (4–5 cc 1 % Xylocaine infiltrated) area at the axilla (2–3 cm length and 2–3 cm in breadth with blunt dissection of the subcutaneous space around the incision). This area was shaved previously and sterilized with Betadine and 100 % rectified spirit solution before the placement of the fresh human fetal lung tissue fragments. The space was subsequently closed with small interrupted (00) atraumatic chromic catgut with cutting needle. No prophylactic antibiotics were given, but the patient received analgesic paracetamol 1–3

tablets/day postoperatively for 2–3 days for symptomatic relief.

Fetal lung tissue contains pneumocytes with different states of growth and differentiation along with connective tissue like elastin, collagen, and matrix. This extracellular matrix (ECM) is a very important component of stem cell niche areas, which regulate the microenvironment of the stem cell pool size and control stem cell mobilization. ECM is a complex interlinked composite of collagenous molecules, noncollagenous molecules, and water-rich mucopolysaccharide ground substance. Cells are integrated to their matrix via integrin and non-integrin receptors, which are utilized in the control of adhesion, migration, division, growth, anoikis, transdifferentiation, and other cellular behavior. ECM provides architecture and strength but also growth factor deposits [5]. Each process is essential for the regenerative process.

After 3 months from the date of placement of the fetal tissue, a part of the tissue was retrieved for cellular study and microscopy with suitable staining. An interaction between the mesenchyme and the epithelium is required for the normal differentiation of fetal lung tissue. This morphogenic interaction may be mediated, in part, by changes in the composition and/or structure of the extracellular matrix. Therefore, the localization and accumulation of extracellular matrix component, during several stages of lung development with other progenitor cells, can provide the niche for the differentiation characteristics of the fetal lung [6].

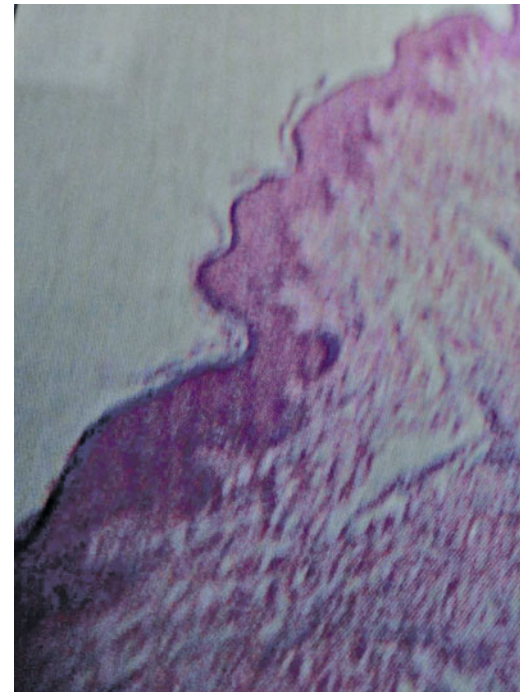


Fig. 24.1 Microphotograph of the fetal lung tissue under the skin as retrieved partially from the host tissue after 3 months and stained with hematoxylin and eosin. What is intriguing is the total absence of any inflammatory or immunological response around the donor lung tissue in the host

Result and Analysis

Eleven patients participated in this fetal lung tissue transplant protocol with consent and safety screening. The age of the volunteers varied from 26 to 64 years, mean 39.4 ± 13.4 years; four patients were male and the rest female. The donors and hosts were all HLA randomized. There was no problem during or after the surgery of the fetal lung tissue for the host. There was no problem or deterioration of the host's pulmonary function as a result of the protocol and subsequent meticulous follow-up of up to 3 months when we retrieved a little tissue from the axilla to study the impact of transplantation at the tissue level. What is interesting is that the donated tissue could be easily traced under the axilla (vide Fig. 24.1) where it can be seen with hematoxylin and eosin stain. What is truly intriguing is the total absence of any immunological (lymphocytic infiltration) or inflammatory cell infiltration (neutrophilic) at the site of the graft under the axillary skin.

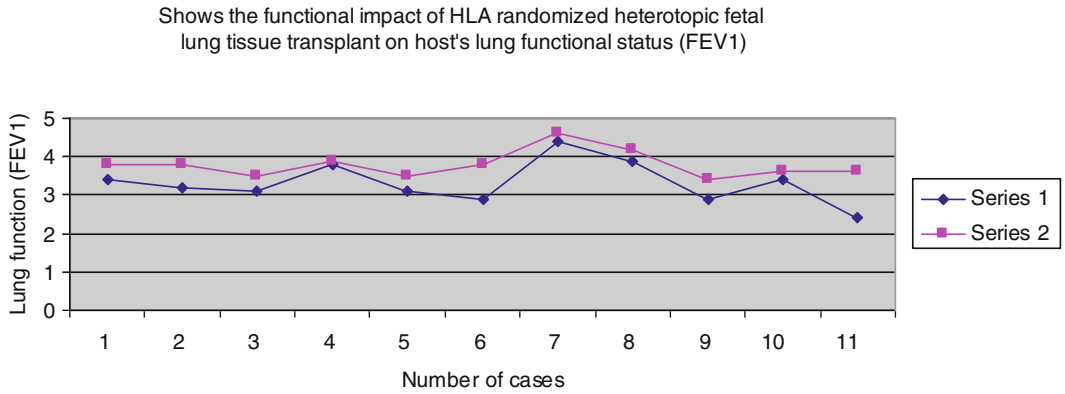
As shown in Table 24.1 (spirometric evaluation), the pretransplant forced expiratory volume was 2.4–4.4, mean 33.18 ± 0.46 L SD, which became 3.4–4.6, mean 37.9 ± 0.66 L SD, after the transplant of lung tissue at the heterotopic site, as revealed (by spirometry analysis) in Graph 24.1.

Similarly, the pretransplant forced vital capacity varied from 2.7 to 4.8 L, mean 37.09 ± 0.54 L SD. This value became 3.6–4.9, mean 40.54 ± 0.76 L SD, after the transplant (vide Graph 24.2).

Table 24.1 The spirometry assessment of pre- and post-heterotopic lung tissue transplant on forced vital capacity and forced expiratory volume in 1 s

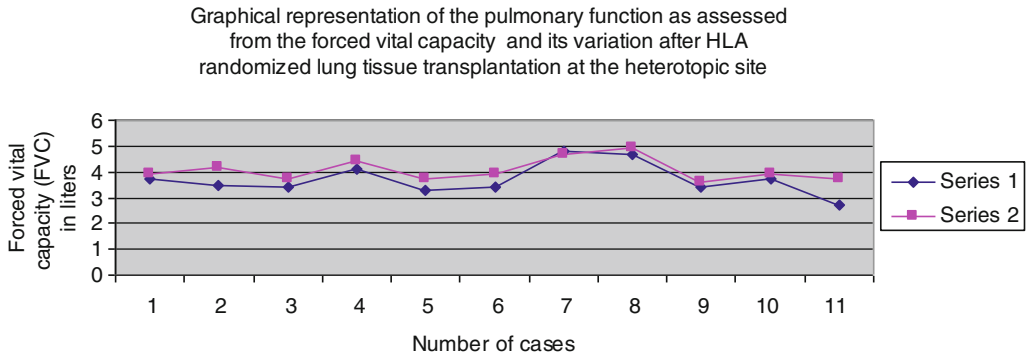
Sr no, name, age, sex	Pretransplant forced expiratory volume (L)	Pretransplant forced vital capacity (L)	Posttransplant forced expiratory volume after 3 months (L)	Posttransplant forced vital capacity after 3 months (L)	Basic treatment offered	Predominant disease	Gestation of fetal tissue
1, AP, 45, F	3.4	3.7	3.8	3.9	A, B, C, D	Asthma with COPD	19-week fetal lung
2, SN, 37, F	3.2	3.5	3.8	4.2	A, B, C, D	Asthma with COPD	14-week fetal lung
3, PT, 39, F	3.1	3.4	3.5	3.7	A, B, C, D	Asthma with COPD	16-week fetal lung
4, KR, 29, M	3.8	4.1	3.9	4.4	A, B, C, D	Asthma	17-week fetal lung
5, UB, 56, F	3.1	3.3	3.5	3.7	A, B, C, D	Asthma with COPD	18-week fetal lung
6, TM, 29, F	2.9	3.4	3.8	3.9	A, B, C, D	Asthma	18-week fetal lung
7, BD, 42, M	4.4	4.8	4.6	4.9	A, B, C, D	Asthma with COPD	18-week fetal lung
8, RS, 35, M	3.9	4.7	4.2	4.7	A, B, C, D	Asthma	14-week fetal lung
9, EL, 26, F	2.9	3.4	3.4	3.6	A, B, C, D	Asthma	15-week fetal lung
10, PM, 64, M	3.4	3.7	3.6	3.9	A, B, C, D	Asthma with COPD	18-week fetal lung
11, CD, 58, F	2.4	2.7	3.6	3.7	A, B, C, D	Asthma with COPD	16-week fetal lung

A bronchodilator from inhaler to tablets and then injection, *B* antibiotics broad spectrum, *C* steroid from inhaler to tablets and then to injection, *D* physiotherapy for lung and other supportive care



Graph 24.1 The functional impact of fetal lung tissue (HLA-randomized) transplant on host's lung function as seen in forced expiratory volume 1 parameter. *Series 1*: pretransplant value of FEV1 in liters. *Series 2*: posttransplant value of FEV1 in liters after 3 months

During fetal development, mesenchymal progenitor (MP) cells are co-localized in major hematopoietic territories, such as the yolk sac (YS), bone marrow (BM), and liver (LV). Studies using mouse and human MP cells isolated from the fetus have shown that these cells are very



Graph 24.2 The functional impact of fetal lung tissue (HLA-randomized) transplant on host's lung function as seen in forced vital capacity (FVC) parameter. *Series 1*: pretransplant value of FVC in liters. *Series 2*: posttransplant value of FVC in liters

similar but not identical to adult mesenchymal stem cells (MSC). Their differentiation potential is usually restricted to the production of highly committed osteogenic and chondrogenic precursors [7].

The lungs of infants, if not mature, become developmentally deficient in a material called surfactant, which helps prevent collapse of the terminal airspaces throughout the normal cycle of inhalation and exhalation. Surfactant is a complex system of lipids, proteins, and glycoproteins which are produced in specialized lung cells called type II cells or type II pneumocytes. The surfactant is packaged by the cell in structures called lamellar bodies and extruded into the airspaces. Pulmonary surfactant protein B (SP-B) enhances phospholipid film formation in vitro and is essential for normal surfactant function in vivo. Four surfactant-associated proteins, SP-A, SP-B, SP-C, and SP-D, have recently been characterized. Recognition of their potential importance in the reduction of alveolar surface tension and in endocytosis and reutilization of secreted surfactant by type II cells has stimulated rapid advancement of knowledge in this field [8].

Discussion

The discovery that adult bone marrow stem cells can contribute to the formation of differentiated cell types in other tissues, especially after injury, implies that they have the potential to participate in tissue remodeling and perhaps regeneration. The current promise of the use of adult stem cells for tissue regeneration, and the belief that once irreversibly damaged tissue could be restored to a normal functional capacity using stem-cell-based therapy, suggests a novel approach for the treatment of diverse chronic diseases.

The basic theory behind cell therapy was stated best by Paracelsus, a sixteenth-century physician who suggested that the heart heals the heart, the lung heals the lung, and the spleen heals the spleen, that is, like cures like. Paracelsus and many other early physicians believed that the best way to treat illness was to use living tissue to rebuild and revitalize ailing or aging tissue. The principle of cell therapy is based on this same old fact [9]. This truth behind this fact appears to be borne out by the present study.

The result of the fetal lung tissue transplantation at a heterotopic site in patients suffering from chronic inflammatory disease of the airways indicates that there is a definite improvement in the spirometric value from the pretransplant level (vide Table 24.1 and Graphs 24.1 and 24.2). The mean value of forced expiratory volume in 1 s was 33.18 ± 0.46 L SD, and the mean value of the forced vital capacity was 37.09 ± 0.54 L SD. Three months after the heterotopic fetal lung tissue transplantation, the mean values became 37.9 ± 0.66 SD liters for forced expiratory volume in 1 s and mean 40.54 ± 0.76 L SD for forced vital

capacity. The ratio of forced expiratory volume in 1 s:forced vital capacity was 88 % pretransplant; this became 91 % in 3 months after transplant as seen via spirometry.

Why Does This Happen?

The improvement of the respiratory function as noted above could be due to a reversal of the remodeling system of the airways as a result of the fetal lung tissue transplantation, as claimed by other investigators [2–4]. The stem cells, progenitor cells, and stem-cell-like cells from the fetal lung tissue may actually migrate to the site of injury and through the process of transdifferentiation may actively take part in the regeneration process.

In this connection, it is worth remembering that chronic inflammatory disease affecting the respiratory system causes many changes in the structure and composition of the airway walls, collectively termed airway remodeling. These include synthesis of increased numbers of mucus-secreting epithelial cells, thickening of the subepithelial collagen layer, and increases in vascularity and smooth-muscle mass around the airways. There is a strong consensus that airway remodeling contributes to the decline in lung function and the development of fixed airway obstruction as seen in patients with chronic, persistent asthma [10].

Fetal tissue contains cells in different states of maturation. The cells include primitive progenitor cells, of different epithelial and mesenchymal contribution depending on the state of gestation apart from connective tissue, as mentioned earlier, like elastin, collagen, and matrix. This extracellular matrix (ECM) is a very important component of stem cell niche areas, which regulate the microenvironment of the stem cell pool size and control stem cell mobilization. As mentioned earlier, there is also surfactant, made from lipid, protein, and glycoprotein (A–D), which helps prevent collapse of the terminal airspaces throughout the normal cycle of inhalation and exhalation. These are produced from specialized lung cells called type II cells or type II pneumocytes, as seen in the fetal lung from the second trimester of pregnancy onward. The surfactant is packaged by the cell in structures called lamellar bodies and extruded into the airspaces. All of them may have a role in regeneration and remodeling, the specificities of which are now under active study by the present investigator. Recently, it has been found that human lungs contain undifferentiated human lung stem cells nested in niches in the distal airways. These cells are self-renewing, clonogenic, and multipotent in vitro. An experiment showed that after injection into a damaged mouse lung in vivo, human lung stem cells from human bronchioles, alveoli, and pulmonary vessels integrated structurally and functionally with the damaged organ [4].

Remodeling of the lungs is a complex phenomenon. Some investigators [11] have concluded that basal SP-B gene expression in epithelial cells of the human fetal lung is regulated primarily at the level of translation or protein stability, whereas glucocorticoids act transcriptionally; thus the SP-B protein accumulates only as type II cells differentiate and acquire lamellar bodies for processing and storage of SP-B. These findings provide evidence of concomitant regulation by glucocorticoids of the phospholipid synthetic enzymes and the major protein of pulmonary surfactant [4]. Surfactant glycerophospholipid synthesis in fetal lung tissue is regulated by a number of hormones and factors, including glucocorticoids, prolactin, insulin, estrogens, androgens, thyroid hormones, and catecholamines acting through cyclic AMP. In studies with human fetal lung in organ culture, it has been observed that glucocorticoids, in combination with prolactin and/or insulin, increase the rate of lamellar body phosphatidylcholine synthesis and alter lamellar body glycerophospholipid composition to one reflective of surfactant secreted by the human fetal lung at term [8].

There are secondary advantages of human fetal tissue transplantation, which are similar to an earlier report by the present researcher on human fetal neuronal tissue transplantation. There, the safety aspects of fetal tissue transplantation [12] have also been mentioned. These nonspecific positive effects are (a) rise of hemoglobin from the

pretransplant level, though there is no concomitant rise of ferritin; (b) there is also a reduction of aches and pain all over the body, which was due to preexisting disease background or was superimposed by different factors either singularly or in combination, viz., viral, bacterial, or fungal, anerobic infection, apart from the sense of well-being which we have studied and reported on earlier.

Malnutrition in many patients, which may have had a contributory role in aggravating the disease, may have been reversed as well; (a) post-transplant weight gain, (b) improvement of appetite, and (c) sense of well-being are universally present among all the transplant recipients in varying degrees, which we will report in a separate chapter in this book. In the present study, the same trends were noted and reverified.

Conclusion

Asthma is characterized pathologically by structural changes in the airway, termed airway remodeling, and clinically presents with features of bronchoconstriction with or without eosinophilic infiltration [13]. This is a very common disease affecting millions of people throughout the world. Treatment with bronchodilator, steroid, antibiotics in various forms, and its permutation and combination may not achieve the desired result of patient satisfaction leading to frustration and intervention of psychosomatic elements of the disease.

In these difficult and refractory cases, cell therapy as an adjuvant therapy has been attempted by many investigators. This therapy has all the potentialities to prevent the remodeling of the lung parenchyma and clinical deterioration and thus retard the progression into restrictive lung disease.

Initially mesenchymal stem cells were attempted with bone marrow from an autologous source. The present work represents the first use of HLA-randomized fetal second-trimester gestational lung tissue placement at a heterotopic site under the axillary skin, as a supplier of stem cells and progenitor cells with the extracellular matrix as a natural niche or microenvironment. This method of fetal lung tissue transplant proved to be very effective as an adjuvant therapy for the treatment of bronchial asthma or COPD to relieve its intractable clinical presentation. This is seen in the post-transplant positive impact of forced expiratory volume and forced vital capacity (spirometry parameters).

Acknowledgments The Department of Science and Technology, Government of West Bengal, supported the investigator with a research grant during his tenure at Bijoygarh State Hospital from 1999 to 2006. The work started at Bijoygarh Government Hospital (1999–2006) and was followed up at Vidyasagar Government Hospital subsequently. The author gratefully acknowledges the support of the patients who volunteered for this research work. The guidance of Prof. K. L. Mukherjee of Biochemistry and Prof. M. K. Chhetri, former Director of Health Services, is gratefully acknowledged.

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25 Treatment by Human Fetal Neuronal Tissue Transplant at a Heterotopic Site in the Axilla in Case of Motor Neuron Disease: A Report of Two Cases

Niranjan Bhattacharya

Introduction

Motor neuron disease (MND) includes a group of diseases that affect motor neurons of the spinal cord. The spinal cord being the sole communication link between the brain and parts below the neck, any injury due to any etiology from autoimmune to excitotoxic stimulus affecting the spinal cord invariably results in dreaded sensory and motor neuronal disease and its loss of control. Mankind's knowledge in the field and recognition of spinal cord injury and its consequences date back to 2500 B.C. as is evident from vivid descriptions given in Greek Papyrus archival material. Despite such early recognition, pathophysiology and prospective therapies affecting the spinal cord or its different constituents and their coordination were not identified until the early part of previous century.

In the United States, MND is known as amyotrophic lateral sclerosis (ALS), or Lou Gehrig's disease, after the baseball player who suffered from it [1]. The overall incidence of MND is approximately 1–5 out of 100,000 people. Men have a slightly higher incidence rate than women. Approximately 5,600 cases are diagnosed in the USA every year. By far the greatest risk factor is age, with symptoms typically presenting between the ages of 50 and 70. Cases under the age of 50 years are called "young-onset MND," while incidence rates appear to tail off after the age of 85.

There are many terminologies around motor neuron diseases, which at times may appear confusing; for instance, in the UK, "motor neuron disease" refers to both ALS specifically (the most common form of disease) and to the broader spectrum of motor neuron diseases including progressive muscular atrophy, primary lateral sclerosis, and progressive bulbar palsy. The diagnosis of MND is a clinical one, established by a neurologist on the basis of history and neurological examination. There is no diagnostic test for MND. Investigations such as blood tests, electromyography (EMG), magnetic resonance imaging (MRI), and sometimes genetic testing are useful to rule out other disorders that may mimic MND. Currently there is no cure for ALS. The pathophysiology has been studied for long in the mouse model, and factors which may trigger this dreadful condition are excitotoxicity and, more controversially, oxidative stress possibly due to mitochondrial dysfunction. Neuronal death by apoptosis has also been suggested [2]. The only drug that affects the course of the disease is riluzole, which blocks the effects of the neurotransmitter glutamate, and is thought to extend the lifespan of an ALS patient by only a few months [3].

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Potentiality of Fetal Tissue for Repair/Regeneration

The present group of investigators has been working on fetal tissue transplantation collected from donor mothers undergoing hysterotomy and ligation for family planning purposes. Second-trimester fetuses were collected from donor mothers after receiving informed consent. The receiver also provided informed and legal consent signed in front of a court of law. The entire procedure for fetal tissue transplantation was free and, further, received approval from the institute-based ethical committee.

The successful development of fetal cell/tissue transplantation in adults has resulted in the possibility of eventual therapeutic solutions in a variety of intractable diseases [4–16]. One reason that the transplant is not rejected and can be implanted successfully is because, during intrauterine growth, the human fetus passes through the preimmune (before 15 weeks) and subsequently through the hypoimmune phases of growth and maturation. The expression of hypoantigenicity of the growing fetus in utero provides an excellent opportunity for fetal tissue/organ transplant, in its pre-HLA state of growth and maturation. The assumption here is that hypoantigenic naive fetal cells will not be targeted by the hosts' HLA system.

A group of investigators in Calcutta, India, worked on the process through which the human fetus acquires immunocompetence from the year 1979. The current investigator was a part of that group and developed concepts on the immunocompetence of the fetal tissue, which was perceived to be safe for transplants. Going forward with this assumption, neurotransplantation has been proposed as a potential treatment for neurodegenerative disorders, from Parkinsonism to Huntington's disease [17–20].

These assumptions are intricately connected with the science of stem cell biology, which is based on three cardinal behaviors of stem cells, i.e., stem cells can easily migrate to a site of injury, it has transdifferentiation properties based on its environment, and lastly, stem cells are immortal due to the telomeric reverse transcriptase activity of the stem cells that prevent the shortening impact on the telomeric end after cell division.

Fetal tissue transplantation is a promising field of medical research. At stake is the source of stem cells – progenitor cells harvested from human fetuses that can differentiate into any cell in the adult human body. Fetal tissue transplants are being investigated as treatments for a wide range of debilitating human conditions. Researchers hope to cure diabetes by regenerating insulin-producing pancreatic cells in diabetics and blindness by regrowing retinal tissue in the eye. Scientists hope to develop better treatments for heart attack victims with the use of fetal tissue to regrow damaged heart muscle. Fetal tissue transplants also look promising for a variety of problems caused by destroyed nerve cells, such as Parkinson's disease, Huntington's chorea, and even spinal cord injuries. The crux of the method is the therapeutic use of fetal stem cells to replace damaged tissue that the body itself cannot repair. For instance, paralysis is currently incurable because, once destroyed, the nerve cells of the spinal cord are not able to grow back. Researchers hope that stem cells can be used to bridge a spinal cord injury in much the same way as skin cells grow back to cover a cut. Although not ready to be tried in people, procedures that inject fetal tissue cells at spinal cord breaks have shown encouraging results in small animals; for instance, in one study scientists were able to get partially paralyzed rats to walk again [5]. Similar experiments to regenerate nerve cells of the brain are also being investigated as cures for Huntington's chorea and Parkinson's disease, two diseases caused when specialized nerve cells in the brain begin to die off. In certain centers of excellence in recent years, pioneering fetal tissue transplants into the brain of Parkinson's disease patients have shown promise in slowing or even reversing symptoms of the disease. In this treatment, cells from the pre-brain structures of 6- to 8-week-old fetuses are injected into the patient's striatum, where if all goes well they grow into a bundle of nerve cells that produce the needed dopamine. Patients with successful fetal tissue transplants have shown remarkable improvement in the severity of tremors [22].

The basic objective of the present study is to verify the fundamental property of stem cells, i.e., migration to the site of injury (homing effect). The study examines whether this homing effect operates in case of fetal neuronal tissue transplant, which is a rich source of neuronal progenitor cells or stem cells, and whether the fetal neuronal tissue helps to restore or repair the neuronal microenvironment directly or indirectly through neurocytokine support and other essential amines necessary for that action. The idea of selecting the axilla for the transplant of the fetal neuronal tissue came from the observation that it is a convenient site from which the tissue can easily be retrieved in both males and females with the use of a little local anesthesia; moreover, it has vascularity, leaves less of a scar, and is therefore aesthetically suitable.

In this study, fetuses were collected from consenting mothers admitted for hysterotomy and ligation at Bijoygarh State Hospital, Kolkata, for transplantation in patients admitted to the same institute. The issue of whether stem-cell-rich spinal cord tissue or the supportive microenvironment of the developing human fetus can retard excitatory oxidative stress and apoptosis of the MND was the main theme behind this research attempt by the present investigator.

Illustrations

Case 1

Mr. A.B., 62 years, Hindu male, a teacher by profession, reported to the OPD of Bijoygarh State Hospital with clinical diagnosis of MND along with suggested recent investigations of EMG/NCV, fresh brain MRI. He completed the clinical proforma, received legal consent, and produced an authenticity certification of his economic condition and residence, etc. as per the hospital norms and rules. His case was passed through the institution-based ethical committee, and after due consent, the first spinal cord neuronal tissue transplantation was done at the axilla on 12.03.2004 under local 2 % Xylocaine infiltration anesthesia. For the purpose of the transplantation, a second-trimester (16 weeks) fetus was collected from a consenting mother undergoing hysterotomy and ligation.

The patient was discharged with advice to report to the OPD for follow-up every third month. He was also advised not to discontinue the standard neurological treatment guidelines that were being continued, i.e., physiotherapy, vitamins, minerals, trace elements, antioxidant supplementation, and riluzole. There was no problem postoperatively. The patient reported every 6th week, and there were progressive signs of slow regeneration features in EMG from the 3rd month onward, and this was sustained for 12 months more with gain in weight, improvement in appetite, loss of weakness and fasciculations, and improvement in walking and dragging feet. Again from June 2005, there was a reappearance of the signs and symptoms with EMG features suggesting recurrence of degenerative features. The patient insisted on a second transplant in November 2005, which could not be arranged. Subsequently, he went abroad in January 2006 hoping for better treatment.

Case 2

Mr. B.M., 45 years, Hindu male, a businessman by profession, reported to the OPD of Bijoygarh State Hospital with clinical diagnosis of MND along with the suggested recent investigations of EMG/NCV, fresh brain MRI, as in the case of the earlier patient. He had a family history of MND with an elder brother and an uncle also suffering from similar disease. He completed the clinical proforma protocol; legal consent was sought and received, and authenticity certification of his economic condition, residence, etc., were also produced. His case was approved by the institution-based ethical committee, and the patient received a spinal cord neuronal tissue transplantation at the site of his axilla on 11.06.2004, under local 2 % Xylocaine infiltration anesthesia maintaining usual antiseptic and aseptic protocol in the OT as was also done in the first case. The second-trimester (14 weeks) fetus that was transplanted was collected from a consenting mother undergoing hysterotomy and ligation.

This patient too was discharged with advice to report to the OPD for follow-up every 3 months. He was also advised not to discontinue the standard neurological guideline he was continuing,

i.e., physiotherapy, vitamins, minerals, trace elements, antioxidant, and riluzole. There was no problem postoperatively. The patient reported every 6th to 8th week for follow-up and was very much satisfied with the improvement he felt with the procedure. Here too there were progressive signs of regeneration features in EMG from the 2nd month onward and was sustained for 24 months more with gain in weight, improvement in appetite, loss of weakness and fasciculations, and improvement in walking and dragging feet apart from improvement in muscle strength. Again from September 2006, there was reappearance of the signs and symptoms of EMG suggestive of the reappearance of degenerative features again. The patient became very upset, discontinued all medical treatment, became very depressed, and lost follow-up.

Discussion

The question is why there is a transient improvement in both the cases. Not only are there clinical improvements, there are EMG-/NCV-supported features of regeneration as well. For theoretical discussion's sake, let us consider that the clinical improvement is due to subjective or objective bias, but the EMG study findings cannot be negated on the basis of a similar placebo effect or an observational error.

This clinical improvement may be the result of the transfer of the neuro-microenvironmental participation which is due to the neurocytokine support for regeneration of the degenerated ant or other horn cells damaged due to the disease process or the participation of the fetal spinal cord's stem cells, stem-cell-like cells, or other specific or nonspecific progenitor cells which might be fighting the excitatory oxidative stress mechanism or the apoptotic mechanism behind this dreaded degenerative disease. Another suggestion could be frequent transplant with an interval of 6–12 months to the same patient till the neuroexcitatory oxidative stress impact is irreversibly combated.

Fetal neuronal tissue is composed of a group of identical functioning cells at different states of maturation and growth surrounded by the extracellular matrix (ECM). This ECM is a unique regulator of the behavior of the cells, group of cells, or even the stem cell or undifferentiated progenitor or stem-cell-like cells. With the help of integrin and non-integrin receptors, the cells get attached with the ECM. These integrin and other receptors control the cellular behavior starting from migration, adhesion, growth, maturation, anoikis, transdifferentiation, and other essential functions of the cells. Apart from this, the structural strength, architecture, and arranging growth factor deposits which proteinases as signaling scissors can release in a site- and process-specific manner. Several processes, like wounds, cartilage, fractures, myocardial infarctions, and tumor growth, are used to exemplify these regenerative processes [21]. More study is needed to confirm or reject this novel concept.

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26 Adjuvant Role of Human Heterotopic Fetal Kidney Tissue Transplant in Reversing the Visible Parameters of Chronic Renal Diseases: A Preliminary Report of 9 Cases

Niranjan Bhattacharya

Introduction

Chronic kidney disease (CKD) is a chronic and progressive loss in renal function over a period of months or years. The symptoms of worsening kidney function are unspecific and might include feeling generally unwell and experiencing a reduced appetite.

Diabetes and hypertension are two important associates of chronic kidney disease. Diabetic nephropathy (DN) is the most common cause of chronic kidney disease [1]. Other important causes are vascular, glomerular, tubulointerstitial, and obstructive diseases affecting the kidney. Blood-pressure-lowering therapy has been shown to reduce cardiovascular events in these patients significantly. Hypertension is common in chronic renal disease and is a risk factor for the faster progression of renal damage, and reduction of blood pressure (BP) is an efficient way of preventing or slowing the progression of this damage. International guidelines recommend lowering BP to 140/90 mmHg or less in patients with uncomplicated hypertension and to 130/80 mmHg or less for patients with diabetic or chronic renal disease [2].

The pathogenesis of hypertensive renal damage involves mediators from various extracellular systems, including the renin-angiotensin system (RAS). Proteinuria, which occurs as a consequence of elevated intraglomerular pressure, is also directly nephrotoxic. Antihypertensive drugs not only reduce blood pressure (BP) but can also have direct effects on intrarenal mechanisms of damage, such as increased glomerular pressure and proteinuria. Intrarenal effects of antihypertensive drugs differ between classes and between individual drugs within certain classes. Angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) have beneficial effects on proteinuria and declining renal function that appear to be mediated by factors additional to their effects on BP [3]. There are many drug combinations to treat diabetes and hypertension, associated dyslipidemia, or associated high uric acid level. Treatment can prevent the pace of deterioration of the renal function; however, stem cell researchers are attempting an approach to regenerate the progressive and chronic degeneration [4].

Current explanation for development of chronic renal injury is the imbalance between the injurious mechanism and its regenerative repair. The possibility that stem cells contribute to the repair of glomerular and tubular damage is of great interest for basic and translational research.

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Fetal renal tissue is a rich source of renal stem cells, both mesenchymal and epithelial, and has other progenitor and unidentified cells in its natural environment. Whether the transplant of a HLA-randomized fetal renal tissue at a heterotopic site improves the host's renal function is the cardinal theme behind the present work.

Material and Method

Eleven patients with chronic renal disease with progressive loss of urine, pedal edema and puffed face including eyelids specially in the morning, hypertension, dyslipidemia and uncontrolled diabetes, anorexia, and anemia were enrolled for the fetal kidney tissue transplant protocol in Bijoygarh State Hospital from 1999 to 2006 and were graded on the involvement of the renal disease as per protocol of the National Kidney Foundation's "K/DOQI clinical practice guidelines for chronic kidney disease" [2]. Patients with stone, tumor (renal malignancy), were excluded from enlistment in this preliminary trial protocol. All enrolled patients were advised with the following: (1) antihypertensive: angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II receptor antagonists (ARBs), (2) insulin: soluble insulin in sliding scale, (3) diet: extra protein and salt-restricted diabetic diet, and (4) replacement of erythropoietin, calcitriol, and phosphate binders apart from aggressively combating hyperlipidemia. Two cases did not present for follow-up and were discarded from the present protocol.

After getting necessary informed consent from concerned patient/guardian, a thorough case history of the patient was taken to identify the trigger for the deterioration of the renal function. They were subsequently advised to request the institute-based ethical committee for the fetal kidney tissue transplantation protocol at a heterotopic site. The donor tissue was arranged from consenting mothers undergoing hysterotomy and ligation for family planning purposes as per the State and the Government of India guideline for birth control.

Each case was thoroughly screened and assessed by the institute-based ethical committee and the medical board for its merits and demerits and the potential advantage of the fetal kidney tissue in reversing the progressive deterioration of renal function. The subsequent procedure was preceded by proper screening of the recipient and donor for hepatitis B and C and HIV 1 and 2. Routine tests for hemoglobin, total count, differential count, platelet count, assessment of ESR, then liver function test, urea, creatinine, fasting and postprandial sugar, glycosylated hemoglobin, lipid profile, chest x-ray (DNA PCR in case of suspicion of Koch's infection), ECG (echocardiography in case of an ECG problem), C-reactive protein, antinuclear antibody, anti-dsDNA, T3, T4, and TSH were undertaken to see the suitability for renal tissue transplantation procedure.

After following the protocol as mentioned earlier, the fetal kidney was collected from mothers undergoing hysterotomy and ligation with their informed consent. The screened and ethically permitted fresh fetal kidney was immediately sliced serially with a sharp knife in a Petri dish containing normal saline. A site in the axilla of the recipient was selected for the placement of the kidney tissue. This area was previously shaved and sterilized with Betadine and 100 % rectified spirit solution. The region was then anesthetized with 4–5 cc 1 % Xylocaine infiltration. An incision was made (2–3 cm length and 2–3 cm in breadth, with blunt dissection of the subcutaneous space around the incision) in the recipient patient with chronic kidney disease, and the fresh human fetal kidney tissue was placed in it in the shortest possible time. The incised and dissected area was closed with small interrupted stitches using (00) atraumatic chromic catgut with cutting needle. No prophylactic antibiotics were given, but the patient received analgesic paracetamol 1–3 tablets/day for 2–3 days for symptomatic postoperative relief.

Fetal kidney tissue contains nephrons and other precursor and progenitor cells in different states of growth and differentiation, along with connective tissue like elastin, collagen, and matrix. This extracellular matrix (ECM) of the growing fetal tissue is a very important component of stem cell niche areas, which regulate the microenvironment of the stem cell pool size and

control stem cell mobilization. ECM is a complex interlinked composite of collagenous molecules, non-collagenous molecules, and water-rich mucopolysaccharide ground substance.

After 3 months from the date of placement of the fetal tissue, a part of the tissue was retrieved for cellular study and microscopy with suitable staining.

Result and Analysis

Nine cases were ultimately studied in this trial of fetal kidney tissue transplantation at a heterotopic site for patients suffering from chronic kidney disease with uncontrolled diabetes, hypertension, and progressive fall of glomerular filtration rate and summation of its clinical manifestation. All patients received the same line of treatment as mentioned in the Materials and Method section above. The age of the patients varied from 39 to 61 year, mean 51.56 ± 8.4 years SD. Three patients were male and six were female. Three cases were suffering from stage 3, and the rest were suffering from stage 2 disease. As mentioned, calculations were made on the basis of GFR calculations for clinical staging of renal disease, vide the British guideline noted below [2]:

Stage 1: Slightly diminished function, kidney damage with normal or relatively high GFR (≥90 mL/min/1.73 m^2)
Stage 2: Mild reduction in GFR (60–89 mL/min/1.73 m^2) with kidney damage
Stage 3: Moderate reduction in GFR (30–59 mL/min/1.73 m^2)
Stage 4: Severe reduction in GFR (15–29 mL/min/1.73 m^2)
Stage 5: Established kidney failure (GFR <15 mL/min/1.73 m^2)

The results of fetal kidney tissue heterotopic transplant on the host with varying degree of renal clinical disease indicate that the improvement of the renal GFR is universal as noted in Table 26.1 and Graph 26.1, and the downstaging of the disease is as per GFR indication, i.e., 4/9 cases (44.44 %) within 3 months. Another 4/9 additional patients returned to normal GFR within 6 months, the rest, i.e., one patient (11.11 %), reverted back to normal GFR within 9 months of the fetal tissue transplant.

The improvement of GFR had the immediate impact of fall of serum urea and creatinine levels as seen in Table 26.2 and Graphs 26.2 and 26.3, justifying the functional return toward normalcy of the host's kidney condition. This indicates the positive impact of the renal tissue transplantation on the host's system. The urea level which was more than 200 mg in 5/9 cases in the pretransplant stage showed a gradual and progressive fall from the third to the ninth month. The trend noted in case of serum creatinine is similar as noted in Graph 26.3.

Another and possibly the most important parameter of renal injury is the loss of albumin in 24-h urine. Here too, as seen in Table 26.3 and Graph 26.4, there is a major improvement in the host's albumin excretion through the kidney, viz., macroalbuminuria (above 300 mg albumin excretion in 24-h urine) was noted in 6/9 cases and microalbuminuria (30 mg–300 mg/24-h urine) in 3/9 cases of the pretransplant patients; this came down in each and every case, abruptly in some and gradually in others, depending on several factors, to normoalbuminuria (less than 30-mg loss of albumin through urine in 24 h) in 6/9 cases after the fetal kidney transplant and microalbuminuria in 3/9 cases, within 9 months from the transplant date.

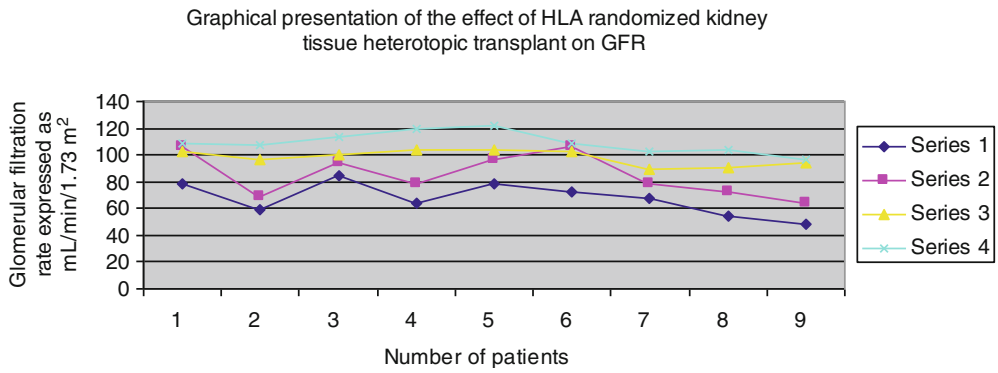
Discussion

Use of bone marrow mesenchymal stem cells for the treatment of advanced renal disease including renal failure has been attempted by many investigators in animal models.

One such experiment shows that systemic administration of one million mesenchymal stem cells not only reduces serum creatinine post-ischemia reperfusion injury, but also that these cells play an antiapoptotic and anti-inflammatory role [5]. Recently, stem cell research has attracted considerable attention because it could be used for the regeneration of damaged organs that are untreatable by conventional techniques, and several stem

Table 26.1 The pretransplant GFR and the impact of HLA-randomized heterotopic fetal renal tissue transplantation on posttransplant GFR as seen on third, sixth, and ninth month follow-up

Case no, age, sex	Existing disease	Basic treatment	Fetal kidney transplant	Pretransplant GFR	Posttransplant GFR 3 months	Posttransplant GFR 6 months	Posttransplant GFR 9 months
1,AK, 56, F	HTN, DM	A, B, C, D	16-week kidney	79 mL stage 2	106 mL (normal GFR)	103 mL (normal GFR)	109 mL (normal GFR)
2,PR, 39, M	HTN, DM	A, B, C, D	16-week kidney	59 stage 3	69 stage 2	96 (normal GFR)	104 (normal GFR)
3,CN, 48, F	HTN, DM	A, B, C, D	16-week kidney	84 stage 2	94 (normal GFR)	100 (normal GFR)	113 (normal GFR)
4,BC, 61, M	Glomerular disease	A, B, C, D	16-week kidney	64 stage 2	79 stage 1	104 (normal GFR)	120 (normal GFR)
5,SD, 44, F	HTN, DM	A, B, C, D	18-week kidney	78 stage 2	96 (normal GFR)	104 (normal GFR)	122 (normal GFR)
6,BP, 56, M	HTN, DM	A, B, C, D	16-week kidney	72 stage 2	106 (normal GFR)	102 (normal GFR)	109 (normal GFR)
7,AM, 59, F	Polycystic kidney	A, B, C, D	18-week kidney	68 stage 2	79 stage 1	89 stage 1	102 (normal GFR)
8,DB, 54, F	Glomerular disease	A, B, C, D	16-week kidney	54 stage 3	72 stage 2	90 (normal GFR)	104 (normal GFR)
9,ES, 47, F	HTN, DM	A, B, C, D	16-week kidney	48 stage 3	64 stage 2	94 (normal GFR)	96 (normal GFR)



Graph 26.1 The impact of kidney tissue transplant on host's glomerular filtration rate (GFR) on host's system. *Series 1*: pretransplant GFR. *Series 2*: posttransplant GFR after 3 months. *Series 3*: posttransplant GFR after 6 months. *Series 4*: posttransplant GFR after 9 months

cells (or progenitor cells) such as endothelial stem cells and neural stem cells have been discovered. Following the progression in this field of research, the potential for stem cell gene therapy has increased and several therapeutic benefits have already been reported. Although this approach was originally investigated for fatal or hereditary diseases, chronic renal failure is also a candidate for stem cell gene therapy. Mesenchymal stem cells should be transplanted, and in contrast, hematopoietic stem cells may be used for gene delivery for diseases, which need foreign cytokines and growth factors such as glomerulonephritis.

Animal experiments and analyses of human renal tissues show that regeneration of degraded renal tubules is caused by adjacent surviving tubules. Differentiation, migration, proliferation, and redifferentiation are regulated by local growth factors. Renal stem cells can also participate in this process. Mesenchymal stem cells play a pivotal role in renal regeneration, and if these are still present in the adult kidney, they could be the source material for repair and regeneration following injury. These mesenchymal cells could be placed in dialysis machines, which could make it possible to improve the quality of filtration and also replace other renal functions. Initial results using this new technique in clinical phase I/II studies on patients with acute renal failure are promising [6].

The risks of renal and cardiovascular disease, conditions that are often associated with long-standing hypertension, are far higher among people of African-American heritage than among those of other racial or ethnic groups according to some studies [7]. But the guideline committees worldwide concur that the blood pressure in patients with diabetes and chronic kidney disease should be kept at 130/80 mmHg or less. In this connection as mentioned earlier, microalbuminuria is predictive of diabetic nephropathy and premature cardiovascular disease.

Diabetic nephropathy is a chronic progressive disease that affects 20–40 % of patients with diabetes mellitus. Clinical trials have shown that strict control of hyperglycemia and hypertension can slow the progression of diabetic nephropathy and that insulin resistance correlates with the onset and severity of albuminuria. This correlation has also been found in normotensive persons who do not have diabetes, suggesting that insulin resistance per se may cause albuminuria [8].

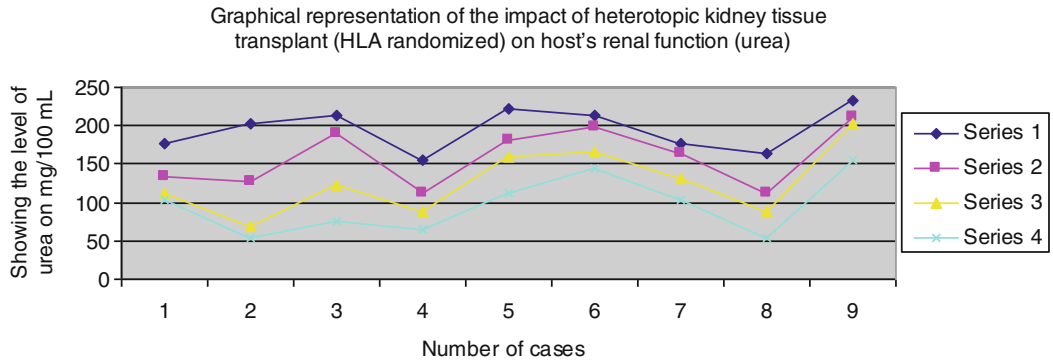
For clinical staging and grading of the disease, repeated estimation of serum urea, creatinine, glomerular filtration rate (GFR), proteinuria, Na, K, Ca, phosphate, and other biochemical parameters and dietary history should be periodically and thoroughly evaluated. This evaluation should be continuous and categorical especially for GFR for overall renal function and its decay or improvement status estimation.

To reverse the appearance of microalbuminuria in patients with type 2 diabetes and to maintain normoalbuminuria are the aims of therapy

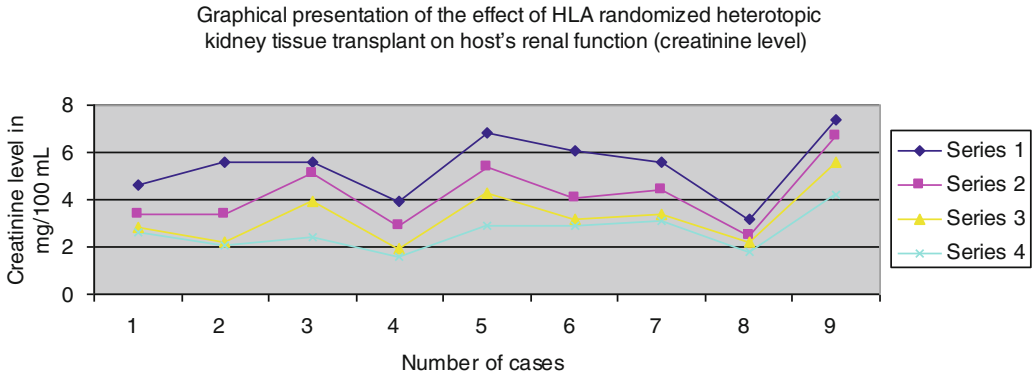
Table 26.2 The pretransplant renal function (urea and creatinine)and the impact of HLA-randomized heterotopic fetal renal tissue transplantation on posttransplant renal function as seen on third, sixth, and ninth month follow-up

Case no, age, sex	Existing disease	Basic treatment	Fetal kidney transplant	Pretransplant urea/creatinine	Posttransplant urea/creatinine after 3 months	Posttransplant urea/creatinine after 6 months	Posttransplant urea/creatinine after 9 months
1,AK, 56, F	HTN, DM	A, B, C, D	16-week kidney	177/4.6	133/3.4	112/2.8	104/2.6
2,PR, 39, M	HTN, DM	A, B, C, D	16-week kidney	203/5.6	128/3.4	68/2.2	54/2.1
3,CN, 48, F	HTN, DM	A, B, C, D	16-week kidney	213/5.6	189/5.1	123/3.9	76/2.4
4,BC, 61, M	Glomerular disease	A, B, C, D	16-week kidney	156/3.9	112/2.9	88/1.9	64/1.6
5,SD, 44, F	HTN, DM	A, B, C, D	18-week kidney	222/6.8	180/5.4	166/4.3	112/2.9
6,BP, 56, M	HTN, DM	A, B, C, D	16-week kidney	213/6.1	198/4.1	167/3.2	144/2.9
7,AM, 59, F	Polycystic kidney	A, B, C, D	18-week kidney	177/5.6	164/4.4	132/3.4	104/3.1
8,DB, 54, F	Glomerular disease	A, B, C, D	16-week kidney	164/3.2	112/2.5	89/2.2	54/1.8
9,ES, 47, F	HTN, DM	A, B, C, D	16-week kidney	233/7.4	212/6.7	202/5.6	176/4.2

A Antihypertensive: angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II receptor antagonists (ARBs) are used, *B* Insulin: soluble insulin in sliding scale, *C* Diet: extra protein and salt-restricted diabetic diet, *D* Replacement of erythropoietin, calcitriol, and phosphate binders and combating hyperlipidemia



Graph 26.2 The impact of kidney tissue transplant on host's renal function (urea level). *Series 1*: pretransplant urea value. *Series 2*: posttransplant effect on urea after 3 months. *Series 3*: posttransplant effect on urea after 6 months. *Series 4*: posttransplant effect on urea after 9 months



Graph 26.3 The impact of kidney tissue transplant on host's renal function (creatinine level). *Series 1*: pretransplant creatinine value. *Series 2*: posttransplant effect on creatinine level after 3 months. *Series 3*: posttransplant effect on creatinine level after 6 months. *Series 4*: posttransplant effect on creatinine level after 9 months

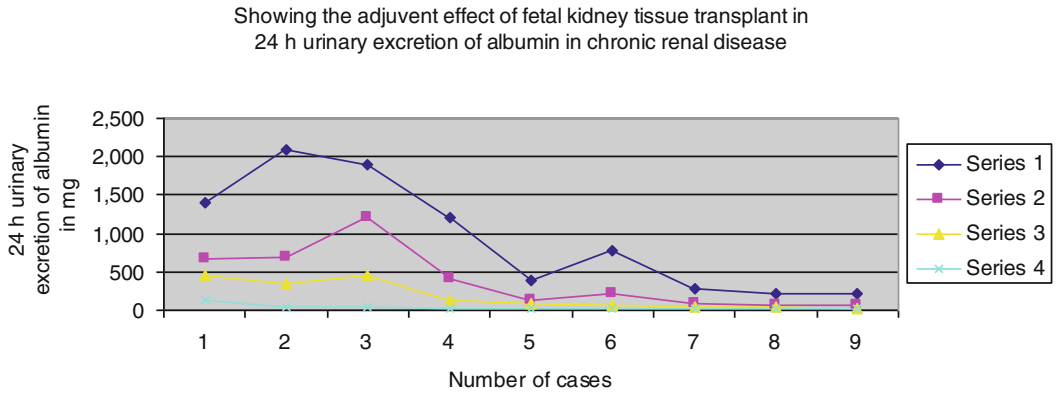
in diabetes. In a recently published article, the investigators suggested that the target blood pressure (<130/80 mmHg) was achieved in nearly 80 % of the patients taking olmesartan and 71 % taking placebo; blood pressure measured in the clinic was lower by 3.1/1.9 mmHg in the olmesartan group than in the placebo group. Microalbuminuria developed in 8.2 % of the patients in the olmesartan group (178 of 2,160 patients who could be evaluated) and 9.8 % in the placebo group (210 of 2,139). Use of olmesartan, an angiotensin II receptor antagonist used to treat high blood pressure, was associated with a delayed onset of microalbuminuria, but the higher rate of fatal cardiovascular events is of concern [9].

In the present study, the result of HLA-randomized fetal kidney tissue heterotopic transplant in the axilla of the host with varying degrees of renal clinical disease shows that improvement in the renal GFR is universal as noted in Table 26.1 and Graph 26.1, and there was downstaging of the disease as per GFR indication, i.e., 4/9 cases (44.44 %) within 3 months. Another 4/9 additional patients returned to normal GFR within 6 months; the rest, i.e., one patient (11.11 %), resumed back to normal GFR within 9 months of fetal tissue transplant.

The improvement of GFR had the immediate impact of a fall in serum urea and creatinine levels as noted in Table 26.2 and Graphs 26.2 and 26.3, implying the functional return toward normalcy

Table 26.3 The pretransplant albumin in 24-h urinary excretion and the impact of HLA-randomized heterotopic fetal renal tissue transplantation on posttransplant albumin in 24-h urinary excretion as seen on third, sixth, and ninth month follow-up

Case no, age, sex	Existing disease	Basic treatment	Fetal kidney transplant	Pretransplant albumin in 24-h urine (range between 30 and 300 mg/24 h)	Posttransplant albumin in 24-h urine after 3 months	Posttransplant albumin in 24-h urine after 6 months	Posttransplant albumin in 24-h urine after 9 months
1,AK, 56, F	HTN, DM	A, B, C, D	16-week kidney	1,400 mg/24 h	670 mg/24 h	450 mg/24 h	130 mg/24 h
2,PR, 39, M	HTN, DM	A, B, C, D	16-week kidney	2,100 mg/24 h	700 mg/24 h	350 mg/24 h	40 mg/24 h
3,CN, 48, F	HTN, DM	A, B, C, D	16-week kidney	1,900 mg/24 h	1,200 mg/24 h	450 mg/24 h	50 mg/24 h
4,BC, 61, M	Glomerular disease	A, B, C, D	16-week kidney	1,200 mg/24 h	400 mg/24 h	120 mg/24 h	26 mg/24 h
5,SD, 44, F	HTN, DM	A, B, C, D	18-week kidney	390 mg/24 h	130 mg/24 h	80 mg/24 h	22 mg/24 h
6,BP, 56, M	HTN, DM	A, B, C, D	16-week kidney	780 mg/24 h	210 mg/24 h	60 mg/24 h	16 mg/24 h
7,AM, 59, F	Polycystic kidney	A, B, C, D	18-week kidney	280 mg/24 h	78 mg/24 h	38 mg/24 h	18 mg/24 h
8,DB, 54, F	Glomerular disease	A, B, C, D	16-week kidney	210 mg/24 h	68 mg/24 h	38 mg/24 h	12 mg/24 h
9,ES, 47, F	HTN, DM	A, B, C, D	16-week kidney	220 mg/24 h	64 mg/24 h	27 mg/24 h	20 mg/24 h



Graph 26.4 The impact of kidney tissue transplant on host's 24-h urinary excretion of albumin. *Series 1*: pretransplant 24-h urinary excretion of albumin in mg value. *Series 2*: posttransplant 24-h urinary excretion of albumin in mg value after 3 months. *Series 3*: posttransplant 24-h urinary excretion of albumin in mg value after 6 months. *Series 4*: posttransplant 24-h urinary excretion of albumin in mg value after 9 months

in the host's kidney condition. This is an example of the renal tissue transplantation's positive impact on the host's system. The urea was more than 200 mg in 5/9 cases at the pretransplant stage; this showed a gradual and progressive fall from the third to the ninth month. Similar is the trend noted in the case of serum creatinine results as noted in Graph 26.3.

Another, and possibly the most important, parameter of renal injury is the loss of albumin in 24-h urine. Here too, as noted in Table 26.3 and Graph 26.4, there was a major improvement in the host's albumin excretion through the kidney: Macroalbuminuria (above 300 mg albumin excretion in 24-h urine) was noted in 6/9 cases and microalbuminuria (30–300 mg/24-h urine) in 3/9 cases of the pretransplant patients; this came down abruptly in most cases and gradually in others, depending on several factors, to normoalbuminuria (less than 30 mg loss of albumin through urine in 24 h) in 6/9 cases after the fetal kidney transplant and microalbuminuria in 3/9 cases within 9 months after transplant. Another interesting observation is the absence of any inflammatory (leukocytic infiltration) or immunological (lymphocytic infiltration) cell migration at the site of the retrieved fetal tissue as seen in the hematoxylin and eosin-stained histology picture (Fig. 26.1).

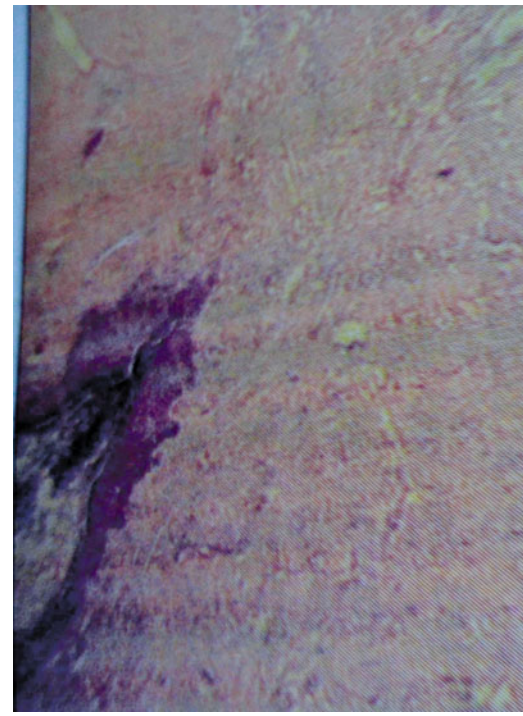


Fig. 26.1 This is a microphotograph of the HLA- and sex-randomized partially retrieved kidney tissue after 3 months from a patient suffering from chronic renal failure. The interesting thing is the absence of any inflammatory (leukocytic infiltration) or immunological (lymphocytic infiltration) cell migration at the site of retrieved fetal renal tissue under the recipient's skin, as seen in hematoxylin and eosin-stained histology picture

The central theme of the present work is that human leukocyte antigen (HLA)-randomized fetal tissue transplantation has many unique properties because of the immaturity of expression of the antigens of the fetal tissue. Fetal tissue is composed of cells with varying degree of maturation, progenitor cells, stem cells, and also stem-cell-like undifferentiated cells with their extracellular support or the matrix. These cells are also in different periods of the cell cycle upregulating and downregulating as per genetic and epigenic environment. These cells are integrated into their matrix via integrin and non-integrin receptors, which are utilized in the control of adhesion, migration, division, growth, anoikis, transdifferentiation, and other cellular behavior. ECM provides architecture and strength and also growth factor deposits [10] which proteinases as signaling scissors and can release in a site- and process-specific manner. All the processes are essential for the regenerative process.

Stem cells have two important properties: one is transdifferentiation and the other is migration to the site of injury to eventually take part in regeneration. Whether the epithelial or mesenchymal component of the fetal kidney tissue transplanted at the heterotopic site is migrating to the site of injured renal tissue in the diseased kidney is an issue to be worked on by future researchers interested in this exciting field of tissue regeneration.

In the case of fetal renal tissue transplant too, the present researchers have noted certain secondary advantages of human fetal tissue transplantation, similar to what they have reported earlier in relation to human fetal neuronal tissue transplantation. The long-term safety of the fetal tissue transplantation has also been reported earlier [11]. The nonspecific effects of fetal tissue transplant are: (1) rise of hemoglobin from the pretransplant level, though there is no concomitant rise of ferritin; (2) reduction in aches and pain all over the body, which had been due to preexisting disease background, or superimposed different factors either singularly or in combination, viz., viral, bacterial, or fungal, anaerobic infection; (3) background malnutrition which may have a contributory role too; (4) posttransplant weight gain; (5) improvement of appetite; and (6) a sense of well-being, universally present among all the transplant recipients, in varying degrees. This will be reported in detail in a separate chapter in this book. We are presently working on analyzing the cause-effect of these symptoms.

Summary and Conclusion

Guideline committees worldwide concur that the blood pressure in patients with diabetes and chronic kidney disease should be kept at 130/80 mmHg or less. As mentioned earlier, microalbuminuria is predictive of diabetic nephropathy and premature cardiovascular disease. With the appearance of microalbuminuria, there is slow but progressive fall of GFR and a slow but progressive rise in urea and creatinine.

On the basis of results and its analysis, we presume that the human fetal renal tissue transplant at a heterotopic site (axilla) may actually reverse the process of degeneration associated with chronic renal disease with diabetes and hypertension. The present study suggests that patients of chronic renal disease who are not responding to the globally standardized method of treatment with soluble insulin, angiotensin inhibitors, dietary regulation of salt and protein, and also replacement of erythropoietin, calcitriol, and phosphate binders and combating hyperlipidemia may respond favorably with HLA-randomized fetal kidney tissue transplant at heterotopic site under local anesthesia if placed under the forearm, i.e., axilla. This improvement is possibly due to the participation of its stem cell and progenitor cell component in the natural niche of the fetal tissue. Both the cytokine network and the stem and the progenitor cells associated with the fetal tissue may actually participate in the process of regeneration of the renal parenchyma thus reversing the impact of degeneration of the kidney by the host's associated disease process. With the coordination of the extracellular matrix (ECM) and its constituent of integrin and non-integrin receptors, the cells get attached with the ECM.

These integrin and other receptors control the cellular behavior starting from migration, adhesion, growth, maturation, anoikis, transdifferentiation, and other essential functions of the cells [10].

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27 Treatment by Human Fetal Neuronal Tissue Transplant from Brain and Spinal Cord at Heterotopic Site in Axilla in Case of Posttraumatic Quadriplegia: A Report of Two Cases

Niranjan Bhattacharya

Introduction

Quadriplegia is paralysis caused by illness or injury to a human that results in the partial or total loss of use of all limbs and the torso of the patient. The loss is usually sensory and motor, which means that both sensation and control are lost. It is caused by damage to the brain or the spinal cord at a high level, C1–C7 – in particular, spinal cord injuries secondary to an injury to the cervical spine. The injury, which is known as a lesion, causes victims to lose partial or total function of all four limbs, meaning the arms and the legs. C1–C4 usually affects arm movement more than a C5–C7 injury; however, all quadriplegics have or have had some kind of finger dysfunction. Typical causes of this damage are trauma (such as a car crash, a fall or a sports injury), disease (such as transverse myelitis or polio), or congenital disorders, such as muscular dystrophy or multiple sclerosis. Although the most obvious symptom is impairment to the limbs, functioning is also impaired in the torso. This can mean a loss or impairment in controlling the bowel and bladder, sexual function, digestion, breathing, and other autonomic functions. Furthermore, sensation is usually impaired in affected areas. This can manifest as numbness, reduced sensation, or burning neuropathic pain. Secondarily, because of their depressed functioning and immobility, quadriplegics are often more vulnerable to pressure sores, osteoporosis and fractures, frozen joints, spasticity, respiratory complications and infections, autonomic dysreflexia [1], deep vein thrombosis, and cardiovascular disease.

Case Studies

Case No. 1

Mr. AM, 18 years, Hindu, male, was crossing a village road near Gobindapur, District 24 Parganas (south) carrying a hay bag on his shoulder. Suddenly a loaded Matador-marked vehicle hit him, injuring his back and causing unconsciousness. He was taken to a nearby nursing home where the attending surgeon arranged CT scan of the brain which did not show any feature of hemorrhage or midline shift or any other specific disorder. The patient was put under IV normal saline, broad-spectrum antibiotics, IV steroid, ranitidine, and analgesic Voveran. His poor father sold his only rickshaw to sustain the treatment for 12 h. The patient was shifted to Bijoygarh Government General Hospital with the recommendation of a minister for free treatment.

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On his arrival, immediate x-ray of the cervical spine revealed fracture without gross dislodgement or misalignment of the cervical spine C4–C5. The patient was immediately provided with a hard cervical collar with minimum mobility. Overall neurological assessment revealed features of quadriplegia as functioning was also impaired in the torso. Furthermore, there was no sensation and severe burning neuropathic pain involving all the limbs. There was also loss or impairment in controlling the bowel and bladder. The patient was catheterized and IV supplementation was started for steroid, mannitol**,** broad-spectrum antibiotics, analgesics, ranitidine with vitamins and minerals, as well as nutritional support.

Next day his father completed the entire clinical proforma, including legal consent, authenticity certification of his economic condition, and residence certification. The patient's condition helped his case to pass through the institution-based ethical committee, and he received the first brain and spinal cord neuronal tissue transplantation under 2 % Xylocaine infiltration anesthesia at the site of the axilla on 23 June 2003 in the O.T., taking all antiseptic and aseptic precautions. The neuronal tissue was taken from a second-trimester (16 weeks) fetus, which was collected from an informed consenting voluntary donor mother undergoing hysterotomy and ligation, after due donor proforma papers had been filled in.

Neurological assessment at this stage of the patient, who had met with a road traffic accident as per suggestions of the American Spinal Injury Association (ASIA) classification, showed that the patient belonged to the grade C category at that stage with autonomic involvement as well. The ASIA scale grades patients based on their functional impairment as a result of the injury, grading a patient from A to D.

The patient started gradually regaining his consciousness 48 h postoperatively and had features of gradual and sustained neurological recovery. Within 2 weeks, the patient started having a remarkable recovery, and gradual physiotherapy was introduced. IV fluid was discontinued after 3 weeks, and all supporting drugs were given through the oral route. The patient left the hospital 6 weeks from his admission with instructions to use a walking stick as support which he ignored citing the argument that it was "not needed any more." The patient came for regular follow-up every 6th week for 9 months until he was advised not to report routinely to the hospital OPD unless it was needed.

Case No. 2

Mrs. KG, 36 years, Hindu female, and mother of two children was suffering from excruciating neck pain and a secondary neurodeficit cervical region tumor not responding to any analgesic or any other support. As per suggestions of a senior neurologist to ascertain the cause of this pain, a CT-guided FNAC was arranged promptly which suggested astrocytoma grade II. She went to a senior neurosurgeon who operated on her and removed the tumor; biopsy reconfirmed the type and grade of astrocytoma. The patient became quadriplegic from the first postoperative day and was provided with appropriate neck support to cover the decompression (laminectomy of the C4, C5, C6); other supportive drugs included steroid, mannitol**,** broad-spectrum antibiotics, analgesics, and ranitidine with vitamins and minerals, as well as nutritional support. As the patient and her guardian could not sustain the economic burden of this treatment in the nursing home, the patient was transferred to a free government hospital for treatment of the postoperative quadriplegia and its complications. Meanwhile, the patient had bedsore which extended through the back region, and features of hypostatic pneumonia were also gradually discernable.

Along with active nursing protocol and physiotherapy, this patient was provided with a 16-week human fetal brain and spinal cord tissue subcutaneously under the axillary skin with local infiltration Xylocaine 2 % in the O.T. on 23 June 2003, following strict antiseptic and aseptic protocol. The transplant decision was taken after getting due legal and ethical permission from the hospital-based ethical committee. Due informed consent was also taken from the donor mother as

noted in Case No. 1. Neurological assessment at this stage of the patient who had postoperative astrocytoma, as per the suggestions of the American Spinal Injury Association (ASIA) classification, showed that the patient belonged to grade D category with autonomic involvement as well.

From the seventh postoperative day, there was gradual improvement of the neurological status of the patient, and there were features of gradual and sustained neurological recovery. Within 3 weeks, the recovery became remarkable, and therefore, gradual supplemental physiotherapy was introduced. IV fluid was discontinued after 3 weeks, and all supporting drugs were given through the oral route. The patient left the hospital 12 weeks after her operation. From the fourth month, she used to come to the OPD for follow-up. By the sixth month, the patient was absolutely free of the quadriplegia state and was ready to take follow-up chemo/radiation as per her oncologist's suggestion.

Discussion

Spinal cord injury (SCI) is mostly an accident or trauma impact problem affecting working-age adults; however, no age is immune to accidents or trauma, and such a problem affects society beyond the victim's family circle. Most of the victims of SCI will never recover completely.

Quadriplegia is often the result of traumatic injury to the spinal cord. Quadriplegia is the medical term used to define paralysis which affects the lower extremities, upper extremities, and most, or all, the trunk from the neck down. So far as the location of the injury is concerned, in general, injuries that are higher in the spinal cord produce more paralysis. For example, a spinal cord injury at the neck level may cause paralysis in both arms and legs and make it impossible to breathe without a respirator, while a lower injury may affect only the legs and the lower parts of the body. Symptoms of cerebral palsy can be as simple as having difficulty with fine motor tasks like writing or using scissors, or as profound as being unable to maintain balance or walk. Severely afflicted patients may have involuntary movements, such as uncontrollable hand motions and drooling [2]. Once a person with a spinal cord injury comes to the hospital, the treatment starts with preventing the neck and head from moving by being placed in a special metal device known as a halo. Medications known as diuretics and steroids are administered to decrease any buildup of fluid in the spine that is caused by leakage from dead cells. After additional therapy, surgery is often performed to remove any pieces of bones and to fuse (combine) together any unstable sections of the bones that surround the spine if it is needed.

Neuropathic pain associated with spinal cord injury is caused by complex neural mechanisms and is often refractory to standard therapy. Salmon calcitonin is already used to help manage pain after limb amputation and also after vertebral fractures, and it is perhaps surprising that it has not been trialed previously for spinal cord injury pain. Calcitonin is thought to exert its effect by modulation of the serotonergic system and is generally well tolerated and convenient to administer [3].

Another drug, gabapentin, can be added to the list of first-line medications for the treatment of chronic neuropathic pain in spinal cord injury patients. It is a promising new agent and offers advantages over currently available treatments [4].

A retrospective study of 106 tetraplegic patients admitted consecutively to the Santa Clara Valley Medical Center (SCVMC) between August 1981 and September 1983 is worth mentioning here. The average age was 28, and 20 (19 %) were female. The majority sustained spinal cord injuries in motor vehicle accidents (65 %) or in diving accidents (19 %). Forty-nine percent (52/106) of these patients had acute surgical intervention. The majority (35/52) had posterior fusion alone. Twelve patients had an anterior fusion (11 at other hospitals) and four had laminectomies [5].

So far as the treatment is concerned for traumatic spinal cord injury, the entire surgical and medical support treatment was aimed mainly at preventing further damage of the residual

undamaged system along with intense counseling and physiotherapy to minimize the lifelong residual impairment of the affected neurological deficit. Hence, any advance in identification of an effective therapeutic target after spinal cord injury has been newsworthy. However, presently apart from methylprednisolone, none of these developments have been clinically proven as effective in double-blind placebo-controlled trials.

A recent trend in many parts of the world may be mentioned here. Proprietary centers now offer autologous bone marrow-derived mesenchymal stem cell transplants and treatment with neuroregenerative substances with claimed reports of neurological improvement. The science behind this stem cell-based therapeutic claim is based on the transdifferentiation, migration to the site of injury of the cells, and the liberation of specific stem cell-dependent neurotropic factors which help neurons and vessels to grow and thus help repair the damage of the injured spinal cord. Fetal neuronal tissue is a rich source of neuronal stem, and other progenitor cells with its natural niche with abundant neurocytokine network which participates in the spinal cord repair process on an urgent basis.

Newer experimental cell therapy for spinal cord injury includes transplant of autologous human bone marrow-derived mononuclear cell therapy through a lumbar puncture site, and the relevant investigator claimed that this process was safe. In his trial, one-third of his spinal cord injury patients showed perceptible improvements in their neurologic status. The time elapsed between injury and therapy and the number of $CD34^{-/+}$ cells injected influenced the outcome of the therapy [6]. Apart from another claim on the efficacy of stem cell-based therapy, all other results of therapeutic permutations and combinations appear to be poor. However, there is an interesting review on the regenerative impact of cell therapy in spinal cord injury in Spanish that is worth mentioning [7].

Spinal neuronal recovery is a long-cherished dream of neurobiologists working on the rodent model. Meheta and his associates worked on stem cell placement in a subarachnoid space in animal model in neurological disorders with positive results [8]. An interesting article was published in a lead journal on the therapeutic potential of combined protocol of cell therapy for chronic spinal cord injury which reported on the electrical and functional recovery of two patients [9]. Hayashi et al. have suggested that there is an increase of sensitivity to mechanical stimulus after transplantation of murine-induced pluripotent stem cell-derived astrocytes in a rat spinal cord injury model [10]. Another positive repair report in spinal cord injury came from Shi et al. on the use of bone marrow mesenchymal stem cell transplantation with sodium alginate gel for repair of spinal cord injury in mice [11]. Alexanian et al. [12] worked on the effect of transplanted neurally modified bone marrow-derived mesenchymal stem cells and noted that there was positive tissue protection and locomotor recovery in spinal cord-injured rats. There are similar interesting contemporary articles on the therapeutic advantages of mesenchymal stem cells and its genetically modified variety on spinal cord repair either directly or through transdifferentiation impact [13–15]. Embryonal stem cells have also been utilized to examine their role in repair of spinal cord injury [16]. Too much dependence on mesenchymal stem cell for therapeutic repair or regeneration for SCI has been cautioned against by one researcher because of the potentiality of mesenchymal stem cell for malignant transformation and potentiating metastasis [17]. Another prominent investigator from China has reported on the role of mesenchymal stem cells in transforming the stem cell niche for vasculogenesis and also on the contribution of the endothelial growth factor gene on spinal injury repair in rats [18]. In another animal study, human keratinocytes have been found to be effective in case of hind-limb paralysis following traumatic spinal cord injury in rats [19].

The present study demonstrates that in patients categorized under the C and D grades of neurological assessment at the time of admission, as per the American Spinal Injury Association (ASIA) classification, heterotopic transplantation of fetal neuronal tissue from the entire spinal cord

and the brain resulted in dramatic improvement of the quadriplegic condition within a span of around 6 weeks.

Conclusion

Quadriplegia treatment caters to the specific needs of each patient and often depends on the nature and severity of a person's condition [20]. Fetal neuronal tissue is composed of a group of identical functioning cell sets with different states of maturation and growth surrounded by the extracellular matrix (ECM). With the help of integrin and non-integrin receptors, the cells get attached with the ECM. These integrin and other receptors control the cellular behavior starting from migration, adhesion, growth, maturation, anoikis, transdifferentiation, and other essential functions of the cells. Apart from this, the structural strength, architecture, arranging growth factor deposits, and other important systems may actively participate in repair of spinal injury through those complicated regenerative processes [21].

Questions may be raised regarding why spinal cord regeneration is considered to be a problem. The plausible answer could be that several factors such as Wallerian degeneration, axon autotomy, slow growth rate of spinal axons, and glial scarring become an impediment to axonal growth. Moreover, axon growth inhibitory molecules' lack of growth/trophic factors may impede spinal recovery.

Scientists have experimented with many materials for spinal cord recovery from iris and mitral valve [22] to "neural tissue" to compensate the lost/injured neural tissue. As parts of the adult spinal cord cannot be used, attempts were made with embryonic spinal cord strips [23]. Homotopic transplantations as well as heterotopic transplantations, in which CNS tissue from other parts such as the neocortex [24], have been tried for SCI repair. For details about the topic, the reader is hereby referred to an interesting article by Dr. Sankar Venkatachalam on "Fetal Neural Tissue Transplantation for Spinal Cord Injury Repair" published in the present edited volume.

Rationale for Fetal Neural Tissue Transplantation

Fetal neural tissue had been transplanted under various conditions. It ranges from allografting to xenografting: homotopic to heterotopic with different rationales like (a) encouraging host regeneration, (b) creating "spinal relay" stations, and (c) substituting supra spinal control (locally). It was proposed that transplantation of fetal neural tissue would re-create a developmental stage-like condition wherein cues for proper axonal regeneration and path would be available.

Clinically, spinal cord injury treatment focuses on preventing further injury and enabling people with a spinal cord injury to return to an active and productive life within the limits of their disability. This requires urgent emergency attention and ongoing care. Conservative treatment of pressure sores includes appropriate wound care, debridement of necrotic tissue, optimization of nutrition, release of pressure, and minimization of muscle spasticity to provide the patient with the best opportunity to heal.

Second-trimester human fetal neuronal tissue if collected aseptically from the operation theater, from the fetal brain and spinal cord, after receiving donor consent, institutional ethical committee, and patient's consent, may be used for heterotopic transplantation purpose, under local infiltration anesthesia, to a patient with recent traumatic injury or an iatrogenic quadriplegic patient for positive end result or speedy neuronal recovery.

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Fetal Liver Tissue Transplant in Alcoholic Fatty Degeneration of the Liver: A Study of 13 Cases

28

Niranjan Bhattacharya

Introduction

Globally, 1.4 million deaths occur annually as a result of chronic liver diseases. The reasons for this high death toll include unavailability of healthy liver donors and highly expensive liver transplantation treatment.

Accumulation of fat (triglyceride) in the liver may be accompanied by a progressive inflammation of the liver (hepatitis), called steatohepatitis. Classically seen in alcoholics as part of alcoholic liver disease, steatohepatitis also is frequently found in people with diabetes and obesity.

Considering the degree of contribution of alcohol, fatty liver may be termed as alcoholic steatosis or nonalcoholic fatty liver disease (NAFLD) and the more severe forms as alcoholic steatohepatitis (part of alcoholic liver disease) and nonalcoholic steatohepatitis (NASH). The criteria used for diagnosis for nonalcoholic fatty liver disease is a daily consumption of ethanol less than 20 g/day for women and 30 g/day for men [1]. It is expected that some of the cases of fatty degeneration of the liver will progress invariably to cirrhosis of the liver, if early and proper intervention is not taken. Hepatic cirrhosis is the end stage of chronic liver disease. Morbidity and mortality from cirrhosis is increasing rapidly in the world. The majority of patients with hepatic cirrhosis die from life-threatening complications occurring at early ages. Currently, orthotopic liver transplantation is the only definitive therapeutic option. However, its clinical use is limited because of poor long-term graft survival, donor organ shortage, and high costs associated with the procedure.

Since liver transplantation is critically limited by so many critical factors, searching for an effective alternative therapy has attracted great interest in preclinical studies.

In one experiment using the rat model, isogeneic (rat) and xenogeneic (swine) fetal liver fragments (FLF) transplantation was done in the omentum of D-galactosamine (D-Gal)-induced acute and carbon tetrachloride (CCl4)-induced chronic hepatic failure in rats. The investigators (from Japan) claimed that recipients who had iso or xeno FLF showed higher survival rates than the non-transplanted controls on a lethal dose (2.6 g/kg body weight) of D-Gal (survival rates: iso 70 %, xeno 80 %, and control 9.1 %) [2]. Fetal liver tissue like the adult tissue contains cells at different states of maturation and division. Fetal liver tissue in addition contains many different progenitor cells, stem cells, and stem cell-like cells, as well as tissues like elastin, collagen, and matrix, which may provide

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the specific microenvironment for the fetal liver, which is essential for specific growth and maturation.

Studies on the isolation of hepatic progenitors have shown promising results in overcoming the limitations posed by nonavailability of fetal liver tissue for transplantation in case of chronic end-stage liver disease. These liver progenitor cells, after their isolation from healthy liver cells, have been found to possess higher proliferative capacity. These cells are less immunogenic and more resistant to cryopreservation and ischemic injury. These properties could enhance their engraftment within the recipient liver after cellular transplantation. The hepatic progenitors may be isolated from intrahepatic sources and extrahepatic sources. Fetal cells are one of the ideal sources of hepatic stem/progenitor cells.

Hepatic regeneration is an interesting field for clinical researchers today. One area of research is cell therapy for hepatic regeneration with autologous bone marrow stem cell transplantation in patients with cirrhosis; this has shown promising results [3].

As a result of the successes in cell therapy mentioned above, stem cell replacement strategies are being investigated as attractive alternative approaches to liver repair and regeneration. Fatty degeneration of the liver due to diet, metabolic, postinfective, autoimmune conditions, or combinations of these is the commonest hepatic injury seen in general practice. Treatment with stem cells which have the potential for migration to the site of injury and the intrinsic transdifferentiation capacity of these cells may effectively participate in the repair of liver injuries. Several types of stem cells including embryonic stem cells, hematopoietic stem cells, and mesenchymal stem cells can be induced to differentiate into hepatocyte-like cells by defined culture conditions in vitro. The mesenchymal stem cells do not express human leukocytic antigens properly; hence, the requirement for immunosuppressant is much less. As such, the transplantation of autologous bone marrow-derived mesenchymal stem cells holds great potential for treating hepatic cirrhosis. Mesenchymal stem cells can also readily differentiate into hepatocytes, thus stimulating the regeneration of endogenous parenchymal cells and enhancing fibrous matrix degradation [4].

Recently stem cell transplantation has been shown to significantly improve liver function and increase animal survival in experimentally induced liver-injury models. Moreover, several pilot clinical studies have reported encouraging therapeutic effects in patients treated with stem cells [5]. In this work, the objective was to verify on the basis of some clinical investigations the therapeutic efficacy and potential of heterotopic human leukocyte antigen randomized second-trimester fetal hepatic tissue transplantation on the host's fatty degeneration of the liver. Human fetal liver tissue contains both epithelial and mesenchymal stem cells with its different progenitor cells and the matrix. The easily accessible vascular site under the axillary skin was selected for the transplantation in the present set of experiments. The volunteers for the experiment were suffering from fatty degeneration of the liver.

Material and Method

After getting necessary informed consent from all concerned and permission from the hospital-based ethical committee, there was a preliminary screening of the selected patients for hepatitis B, hepatitis C, HIV 1, and HIV 2 along with hemoglobin, total count, differential count, and platelet count, followed by assessment of ESR, liver function test, and tests for urea, creatinine, fasting and postprandial sugar, glycosylated hemoglobin, and lipid profile; chest x-ray (DNA PCR in case of suspicion of Koch's infection), ECG (echocardiography in case of ECG problem), and tests for C-reactive protein, antinuclear antibody, anti-dsDNA, T3, T4, and TSH were undertaken to gauge the suitability of individual patients for the transplantation procedure.

Fifteen cases were short-listed for the fetal liver tissue transplant procedure; of these, 13 cases were ultimately recruited for the current protocol. All were diabetic with varying grades of dyslipidemia and hypertensiveness, and 20 % or more did not meet the height-weight index set

by the Indian Council of Medical Research's guidelines for obesity. Informed consent was received from each of the patients, and the hospital-based ethical committee examined and gave clearance for the new procedure to be pursued in all 13 cases.

The 13 patients, who had alcoholic steatosis, were treated with freshly collected fetal liver tissue transplant. The procedure is as follows: the liver was dissected from the aborted fetus of a mother who was undergoing voluntary hysterotomy and ligation for sterilization purpose (donor consent was given beforehand); the fresh liver tissue was sliced serially with a sharp knife; it was then placed immediately at a locally anesthetized (4–5 cc 1 % Xylocaine infiltrated) area of the axilla where a subcutaneous space had been dissected earlier (2–3 cm length and 2–3 cm in breadth). This area had been shaved previously and sterilized with Betadine and 100 % rectified spirit solution before the placement of the fresh human fetal liver tissue fragments. Subsequently the incision was closed with small interrupted (00) atraumatic chromic catgut with cutting needle. No prophylactic antibiotics were given, but the patient received analgesic paracetamol 1–3 tablets/day for 2–3 days for symptomatic relief, postoperatively.

Fetal liver tissue contains hepatocytes with different state of growth and differentiation along with connective tissue like elastin, collagen, and matrix. This extracellular matrix (ECM) regulates the microenvironment of the stem cell pool size and controls stem cell mobilization. ECM is a complex interlinked composite of collagenous molecules, non-collagenous molecules, and water-rich mucopolysaccharide ground substance. Cells are integrated to their matrix via integrin and non-integrin receptors, which are utilized in the control of adhesion, migration, division, growth, anoikis, transdifferentiation, and other cellular behavior. ECM provides architecture and strength but also growth factor deposits [6].

To assess the fate of the fetal tissue, a part of the tissue was retrieved from the recipient's axilla after 3 months of its placement for cellular study and microscopy with suitable staining.

Result and Analysis

Thirteen patients with alcoholic fatty degeneration of the liver were enrolled for this protocol of fetal liver tissue transplantation. Nine of them were male and four were female. The age varied from 38 to 64 years with mean 53.08 ± 15.34 years. The hosts' serum albumin (hepatocyte function) generally correlates with the hepatocytic functional decline in numbers and serum triglyceride value, which were deposited in the liver in case of hepatocytic decline.

These two important parameters were selected to see the impact of fetal liver tissue (16–18 weeks of gestation) transplantation on those values in the hosts' system. The basic objective was to assess the biochemical and metabolic end impact on the host. The pre-transplant values of host's albumin and triglyceride were matched with the posttransplant values at an interval of 3, 6, and 9 months.

The intention was to analyze how the fetal liver fragments, which contain epithelial, mesenchymal, endothelial, and other varieties of stem cells, stroma, precursor, and progenitor cells, as well as unidentified stem cell-like cells, participate in the adult liver parenchymal regeneration process and whether they eventually change the hepatic function-related biochemical parameters. In a nutshell, the experiment was intended to evaluate the effect of the (HLA-randomized) fetal liver tissue transplant (at a heterotopic site under the axillary skin) on the host's hepatic and metabolic system.

In the present set of experiments, the pre-transplant albumin varied from 2.9 to 5.1 Gm % with mean 3.95 ± 1.05 Gm %, which became 4.21 ± 0.55, 4.51 ± 0.92, and 4.85 ± 0.66 in the third month, sixth month, and ninth month posttransplant evaluations. Similarly, the triglyceride value also suggested a similar trend indicating a visible biochemical impact of transplantation justifying the subsequent improvement in hepatic and lipid metabolic parameters. The pre-transplant albumin value was 134–399 mg/100 mL in the blood of the host with mean 257.92 ± 118.58 mg %; this eventually became 219.23 ± 56.66, 193.54 ± 37.68, and 184.23 ± 16.45 in the third month, sixth month,

and ninth month posttransplant evaluations (vide Table 28.1 and Graphs 28.1 and 28.2).

The microphotograph of the partially retrieved tissue after 3 months from transplantation showed groups of cells scattered under the host's skin; however, no inflammatory (leukocytic) or immunological (mononuclear) cellular infiltration was noted in the hematoxylin- and eosin-stained sample tissue (Fig. 28.1).

The usual basic treatment with soluble human insulin in sliding scale, aspirin 50 mg/day, atorvastatin 20 mg/day, ramipril 5–10 mg/day, and exercise under supervision, was continued for all the patients.

Discussion

The liver lobule is formed by parenchymal cells, i.e., hepatocytes, and non-parenchymal cells. In contrast to hepatocytes that occupy almost 80 % of the total liver volume and perform the majority of numerous liver functions, non-parenchymal liver cells, which contribute only 6.5 % to the liver volume, but 40 % to the total number of liver cells, are localized in the sinusoidal compartment of the tissue. The walls of the hepatic sinusoid are lined by three different cell types: sinusoidal endothelial cells (SEC), Kupffer cells (KC), and hepatic stellate cells (HSC, formerly known as fat-storing cells, Ito cells, lipocytes, perisinusoidal cells, or vitamin A-rich cells). Additionally, intrahepatic lymphocytes (IHL), including pit cells, i.e., liver-specific natural killer cells, are often present in the sinusoidal lumen. It has been increasingly recognized that both under normal and pathological conditions, many hepatocyte functions are regulated by substances released from neighboring non-parenchymal cells [7].

Liver failure results in impairment of many functions and dependent organs such as the brain and the kidneys, which also begin to fail, reducing the chances of recovery even further.

Hepatocyte transplantation for the treatment of acute and chronic liver disease and also inherited metabolic disorders has been attempted by researchers in the field, but it has many procedural and technical problems, starting from shortage of good-quality donor living and adequately functioning cells and their availability. To overcome these hurdles, current research has focused on a search for alternatives, for instance, liver progenitors; fetal hepatoblasts; embryonic, bone marrow, or umbilical cord blood stem cells; and conditionally immortalized hepatocytes [8].

Orthotopic liver transplantation (OLTx) is the only treatment that improves the survival rate in patients with liver failure. Liver transplantation (LT), including orthologous liver transplantation (OLT), cadaveric LT, split LT, and living donor LT (LDLT), brings hope to patients suffering from these diseases.

However, some factors such as operative risks and posttransplant rejection are major limitations of OLT. Isolated adult hepatocyte transplantation is emerging as an alternative bridge support till a healthy donor can be arranged. Again, however, mature hepatocytes have several drawbacks such as low proliferation both in vitro and in vivo, low viability after cryopreservation, and requirement of a large number of cells for infusion.

Hepatic transplantation has also many problems like selection of the donor and the recipient, functional characterization, HLA matching, and immunosuppression of the host, just to cite a few.

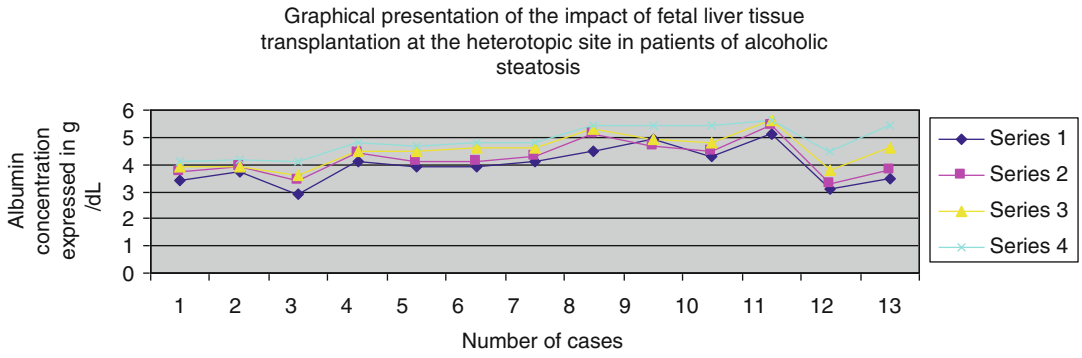
Other investigators have worked on an alternative fetal hepatocyte model, i.e., for effective hepatocyte transplantation. Treating liver disease is largely limited by the availability of useful cells. Human amniotic epithelial cells (hAECs) from term placenta express surface markers and gene characteristics of embryonic stem cells and have the ability to differentiate into all three germ layers, including tissues of endodermal origin (i.e., liver). Thus, hAECs could provide a source of stem cell-derived hepatocytes for transplantation [9].

In this study, the features of fragmentation and cellular migration can easily be traced and assessed in the microphotograph. The researchers are presently working on radioactive tagging in order to view the exact route of the transplanted cellular migration, which would be communicated at a later opportunity. What is exciting is

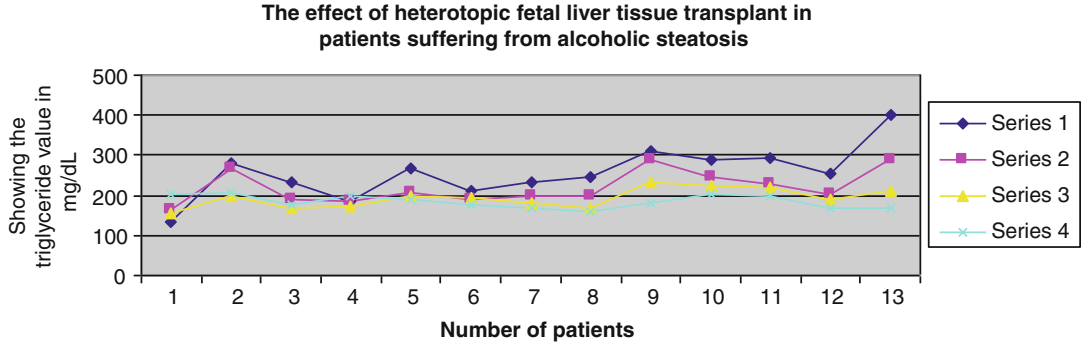
Table 28.1 List of patients who were treated with fetal liver tissue transplantation at a heterotopic site under the skin

Case no., age, sex	Diabetes (a), hypertension (b), obesity (c), and dyslipidemia (d)	Basic treatment antidiabetic (E), antihypertensive (F), antiobesity (G), antilipid (H)	Fetal liver fragments (FLF) transplant	Pre-transplant serum albumin g/dL Triglyceride 150 mg/dL	Albumin (g/dL)/ triglyceride (mg/dL)	Albumin (g/dL)/ triglyceride (mg/dL)	Albumin (g/dL)/ triglyceride (mg/dL)
1, PN, 56, M	a, b, c, d present	EFGH advised	16 week FLF	3.4/134	3.7/164	3.9/156	4.1/204
2, AB, 39, M	a, b, c, d present	EFGH advised	18 weeks FLF	3.7/279	3.9/267	3.9/199	4.2/206
3, CN, 38, M	a, b, c, d present	EFGH advised	18 weeks FLF	2.9/234	3.4/188	3.6/170	4.1/176
4, DC, 51, M	a, b, c, d present	EFGH advised	16 weeks FLF	4.1/186	4.4/184	4.5/174	4.8/200
5, VD, 54, M	a, b, c, d present	EFGH advised	18 weeks FLF	3.9/267	4.1/208	4.5/200	4.7/189
6, CP, 46, M	a, b, c, d present	EFGH advised	16 weeks FLF	3.9/212	4.1/188	4.6/194	4.8/178
7, JM, 59, F	a, b, c, d present	EFGH advised	18 weeks FLF	4.1/234	4.3/198	4.6/179	4.8/168
8, OB, 54, F	a, b, c, d present	EFGH advised	16 weeks FLF	4.9/247	5.1/200	5.3/168	5.4/159
9, RS, 57,M	a, b, c, d present	EFGH advised	16 weeks FLF	4.5/312	4.7/289	4.9/234	5.4/179
10, KP, 56, M	a, b, c, d present	EFGH advised	16 weeks FLF	4.3/287	4.5/244	4.8/222	5.4/202
11, LM, 59, F	a, b, c, d present	EFGH advised	18 weeks FLF	5.1/294	5.4/230	5.6/220	5.6/198
12, RB, 64, F	a, b, c, d present	EFGH advised	16 weeks FLF	3.1/256	3.3/202	3.8/188	4.5/167
13, HS, 57, M	a, b, c, d present	EFGH advised	16 weeks FLF	3.5/399	3.8/288	4.6/212	5.4/169

The patients were all suffering from sonographically proved fatty degeneration of the liver with associated diabetes, hypertension, dyslipidemia, and obesity. All the patients were treated with basic identical drugs and fetal liver tissue transplant under the axillary skin at a heterotopic site and evaluated periodically at 3-month intervals to evaluate the impact of the transplantation on the hosts' system



Graph 28.1 The effect of HLA-randomized fetal liver tissue transplant on the hosts' albumin level. *Series 1*: Pre-transplant value. *Series 2*: Posttransplant value after 3 months. *Series 3*: Posttransplant value after 6 months. *Series 4*: Posttransplant value after 9 months



Graph 28.2 The effect of HLA-randomized fetal liver tissue transplant on the hosts' triglyceride level. *Series 1*: Pre-transplant value. *Series 2*: Posttransplant value after 3 months. *Series 3*: Posttransplant value after 6 months. *Series 4*: Posttransplant value after 9 months

the absence of any inflammatory or immunological cellular infiltration in the adult host's system. The progressive and sustained rise of albumin in the host after the fetal liver tissue transplant actually opens a new therapeutic window for cell and tissue transplantation for future clinical researchers. Similar is the trend of progressive fall in triglyceride value in most of the cases as seen after 3, 6, and 9 months of the transplantation.

Conclusion

Fetal tissue is the richest source of primordial stem cells and has several properties that make it particularly useful for transplantation. It is superior to adult (mature) tissue in certain respects. First, fetal cells are capable of proliferating faster and more often than mature, fully differentiated cells. This means that these donor cells are able to quickly reverse the lost function of the host. In addition, these fetal cells can often differentiate in response to the environmental cues around them. This is because of their location – they can grow, elongate, migrate, and establish functional connections with other cells around them in the host. It has been found that fetal tissue is not easily rejected by the recipient due to the low levels of histocompatibility antigens in the fetal tissue. At the same time, angiogenic and trophic factors are at high levels, enhancing their ability to grow once they are transplanted [10].

Second-trimester fetal liver (HLA)-randomized transplantation at a heterotopic site under the axillary skin has a unique and positive biochemical and metabolic impact on the steatohepatitis-affected

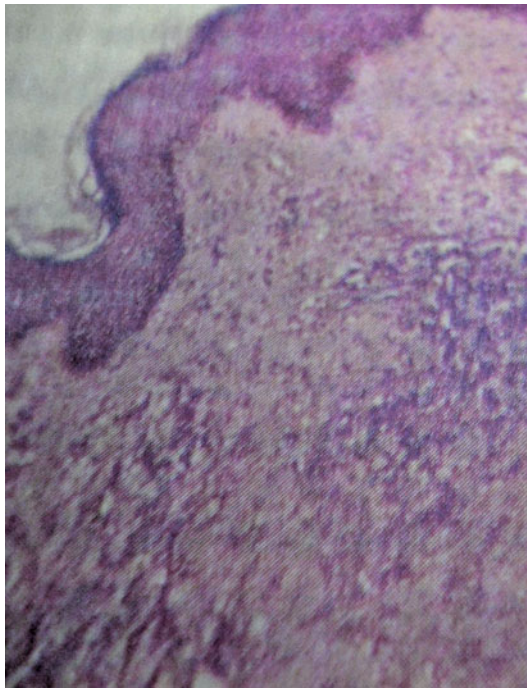


Fig. 28.1 Microphotograph of the fetal liver tissue under the skin as retrieved partially from the host tissue after 3 months and stained with hematoxylin and eosin. What is intriguing is the total absence of any inflammatory or immunological response around the donor tissue in the host

host's system, clinically improving not only the anorexic condition but also improving the hepatocytic structural and functional aspects of the host system; its metabolic implication on reversing the triglyceride value is palpably evident.

There are also secondary advantages of human fetal tissue transplantation as we have reported earlier in relation to other experiments in human fetal tissue transplantation. The safety aspects of fetal tissue transplantation have also been mentioned [11]. The nonspecific effects are the following: (a) rise of hemoglobin from the pre-transplant level; (b) there is also reduction of aches and pain all over the body, which had been due to preexisting disease background or superimposed different factors either singularly or in combination, viz, viral, bacterial, or fungal, anaerobic infection; (c) the background malnutrition may have played a contributory role too; (d) posttransplant weight gain; (e) improvement of appetite; and (f) a sense of well-being, universally present among all the transplant recipients in varying degrees, which we have already reported on earlier [11].

Acknowledgment The Department of Science and Technology, Government of West Bengal supported the investigator with a research grant during his tenure at Bijoygarh State Hospital from 1999 to 2006. The work started in Bijoygarh Government Hospital (1999–2006) and was followed up at Vidyasagore Government Hospital subsequently. The author gratefully acknowledges the support of the patients who volunteered for this research work. The guidance of Prof. K. L. Mukherjee (Biochemistry) and Prof. M. K. Chhetri, former Director of Health Services, is gratefully acknowledged.

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29 Human Heterotopic Fetal Cardiac Tissue Transplant in Patients with Varying Degrees of Cardiomyopathy with Ischemic Heart Disease and Diabetes Mellitus: A Report of 7 Cases

Niranjan Bhattacharya and M.K. Chettri

Introduction

Cardiomyopathy is a difficult condition of the heart muscle when the cardiac cells lose their specialized function and behave like normal muscle. The causes could be extrinsic factors or secondary factors, for instance, drugs (alcohol and others), endocrine, inflammatory, metabolic, nutritional, neuromuscular, and autoimmune, acting either singly or in combination, affecting the myocardium. The other cause is intrinsic (primary), where the problem lies with the functioning of the cardiac muscles itself, with predisposition of genetic (a heritable 25-base pair [bp] deletion from the gene coding for cardiac myosin-binding protein-C (*MYBPC3*) is associated with various *MYBPC3* mutations which predisposes cardiac diseases), acquired, or mixed etiology. From the functional point of view, it could be of a dilated variety, a restrictive variety, and an obstructive variety.

The clinical presentation could be nonspecific to any cardiac disease presentation or even asymptomatic in early cases, but in advanced stages, the clinical features depend on the type of the disease, that is, dilated cardiomyopathy, which is the commonest variety affecting males (age group 20–60 year). It causes valve problem and arrhythmia.

The second variety of cardiomyopathy is hypertrophic cardiomyopathy, affecting both male and female, and can present with sudden cardiac arrest.

The third variety is restrictive cardiomyopathy, which affects mainly aged people. In this variety, there is stiffness and rigidity of the ventricles due to endomyocardial fibrosis. In India today, there is a sea change in socioeconomic and health parameters which has resulted in a slow and steady decline in new cases of endomyocardial fibrosis, especially in the younger age group [1]. This decline parallels the decline of rheumatic fever reported earlier in developed nations. Treatment for cardiomyopathy is aimed to provide symptomatic relief and to retard the progression of the disease with essential medical support and lifestyle change to reduce the cardiac load. If there is no improvement, surgical treatment of ablation and eventual cardiac transplant for end-state cardiac disease has to be considered; however, specific devices like implantation of pacemaker, defibrillators, and ventricular assisted devices may also be helpful in the treatment. Apart from the costly and mostly not so effective surgical procedures, simple alternatives

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like cell therapy have been found to be effective in a number of clinical trials in different animal models.

Prof. Henning, USA, has suggested that cell-based cardiac repair in the twenty-first century will offer new hope for millions of patients worldwide with myocardial infarctions who otherwise would suffer from the relentless progression of heart disease to heart failure and death [2]. Another investigator has suggested that administration of cardiac progenitor cells (CPCs) 4 h after reperfusion ameliorates left ventricular function in rats with acute myocardial infarction (MI) [3].

Research on animal models is focusing on the use of embryonic stem cells which are capable of unlimited self-renewal and have the potential to give rise to all tissue types in the body. Ethical problems and technical hurdles may limit the immediate application of embryonic stem cells. In the meanwhile, fetal hematopoietic stem cells, which have been routinely used to reconstitute the hematopoietic system in man, could present an alternative, owing to their juvenile phenotype and ability to differentiate into vascular endothelial, muscular, and neuronal cell lineages. These observations raise the exciting possibility of using fetal cells as a new way to speed up the healing of damaged tissues [4].

A group of stem cell known as mesenchymal stem cells (MSCs), or multipotent mesenchymal stromal cells as they are also known, has been identified in bone marrow as well as in other tissues of the joint, including adipose, synovium, periosteum, perichondrium, and cartilage. Importantly, MSCs also potently modulate immune responses, exhibit healing capacities, improve angiogenesis, and prevent fibrosis. These properties may be explained, at least in part, by the trophic effects of MSCs through the secretion of a number of cytokines and growth factors. However, the mechanisms involved in the differentiation potential of MSCs, and their immunomodulatory and paracrine properties and potentialities for therapeutic applications, are currently being extensively studied [5].

Materials and Methods

In this study, eight patients were initially enrolled for fetal cardiac tissue heterotopic transplantation under local anesthesia. One patient was found to be HIV positive; he refused to undertake any further surgery. All patients were allowed to continue the drug schedule necessary for their cardiomyopathy status to be kept under control. All the patients were admitted in Bijoygarh State Hospital for treatment in the period 1999–2006.

Ultimately seven patients with diabetic background presenting with cardiomyopathy were enrolled for the fetal cardiac tissue transplant procedure after the completion of a detailed cardiological proforma to assess the stage and grade of illness. After getting necessary informed consent from all concerned and ethical permission from the hospital ethical committee, there was preliminary screening for hepatitis B, hepatitis C, HIV 1, and HIV 2 along with hemoglobin, total count, differential count, platelet count, and assessment of ESR. Various tests like liver function, urea, creatinine, fasting and postprandial sugar, glycosylated hemoglobin, and lipid profile, were also done along with chest x-ray (DNA PCR in case of suspicion of Koch's infection), ECG (echocardiography), C-reactive protein, antinuclear antibody, anti-dsDNA, T3, T4, and TSH. These were undertaken to assess each case for suitability of the transplantation procedure.

These patients were given fresh cardiac tissue transplants collected from consenting mothers undergoing hysterotomy and ligation. The fetal heart is sliced serially with a sharp knife immediately after the hysterotomy procedure and placed at a locally anesthetized (4–5 cc 1 % Xylocaine infiltrated) area at the axilla (2–3 cm length and 2–3 cm in breadth with blunt dissection of the subcutaneous space around the incision) of the patient. This area was shaved and sterilized prior to the procedure with Betadine and 100 % rectified spirit solution before the placement of the fresh human fetal cardiac tissue fragments. Subsequently, the skin was closed with small interrupted (00) atraumatic

chromic catgut with cutting needle. No prophylactic antibiotics were given, but the patient received analgesic paracetamol 1–3 tablets/day for 2–3 days for symptomatic relief, postoperatively. Patients were serially studied pre- and post-fetal tissue transplant for hematological, metabolic, and other safety parameters as mentioned earlier and followed up periodically with echocardiography (2-D study) to assess the cardiological status and its improvement or deterioration, if any.

After 3 months from the date of placement of the fetal cardiac tissue at the heterotopic site, we retrieved a part of the tissue for cellular study and microscopy with suitable staining.

Result and Analysis

Diabetes is very common in India (projected diabetic capital of the world). There are many changes involving the heart in case of diabetes of which ischemic and cardiomyopathic changes are common. One particularity important diabetic cardiomyopathic (DCM) feature is the long latent phase, during which the disease silently progresses. In most cases, there is concomitant hypertension or coronary artery disease. One of the earliest signs is mild left ventricular diastolic dysfunction with little effect on ventricular filling. Diabetic cardiomyopathy is characterized functionally by ventricular dilation, myocyte hypertrophy, prominent interstitial fibrosis, and decreased or preserved systolic function [6] in the presence of a diastolic dysfunction. Microangiopathy can be characterized as subendothelial and endothelial fibrosis in the coronary microvasculature of the heart. This endothelial dysfunction leads to impaired myocardial blood flow reserve as evidenced by echocardiography [7].

Possibly one of the first difference alterations noticed in diabetic hearts was metabolic derangement [8]. The changes seen in DCM are not dissimilar to those of ischemia and are more susceptible to ischemic damage, with associated hyperglycemia, hyperlipidemia, and other metabolic abnormality.

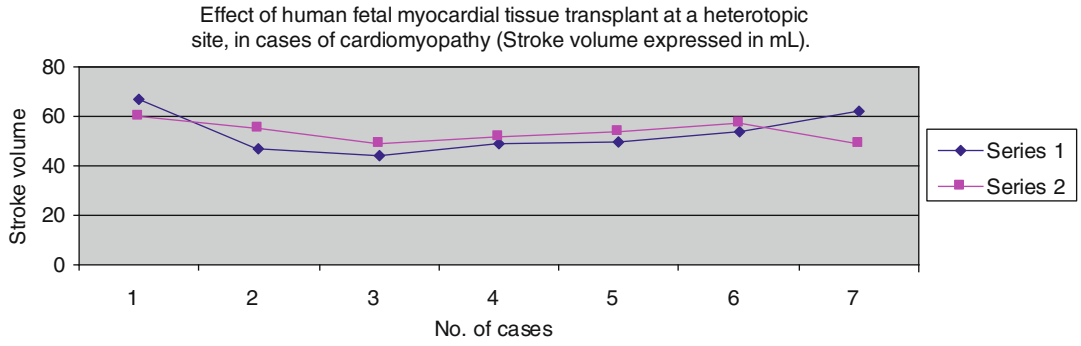
In healthy individuals, fatty acids are the preferred substrate in the adult myocardium, supplying up to 70 % of total ATP (adenosine triphosphate). They are oxidized in the mitochondrial matrix by the process of fatty acid β-oxidation, whereas pyruvate derived from glucose, glycogen, lactate, and exogenous pyruvate is oxidized by the pyruvate dehydrogenase complex, localized within the inner mitochondrial membrane. Cardiomyocytes, unlike endothelial cells, have the ability to regulate their glucose uptake. In order to protect themselves from extracellular hyperglycemia, cardiac cells can internalize their insulin-dependent glucose transporter, GLUT 4 [9].

With this little etiopathogenetic background in mind, if the cases in this study are analyzed, it can be seen that seven patients were treated with the heterotopic cardiac tissue transplant protocol. The age of the patients varied from 35 to 72 years as per Table 29.1. One patient was female in this group, and the rest were male. Two patients were suffering from dilated cardiomyopathy, another two patients were suffering from obstructive cardiomyopathy, two patients were suffering from overt alcoholic/ischemic cardiomyopathy, and one patient was suffering from restrictive cardiomyopathy. All of them were receiving insulin in divided dosages along with cardiac antioxidants, angiotensin-converting drugs, and selective β-blockers in addition to other systemic support and diet regulation to prevent cardiac failure. They were periodically assessed at 3 monthly intervals for echocardiographic values to see whether there was any unpredictable or sudden deterioration of the clinical condition after the heterotopic human leukocyte antigen (HLA)-randomized fetal cardiac tissue transplantation under the axillary skin. The results are shown with graphical representation in Graphs 29.1, 29.2, 29.3, 29.4, 29.5, and 29.6.

Graph 29.1 shows the effect of fetal cardiac transplantation on stroke volume. Graph 29.2 shows the effect of fetal cardiac transplantation on ejection fraction. Graph 29.3 shows the effect of fetal cardiac transplantation on end-diastolic volume. Graph 29.4 shows the effect of fetal

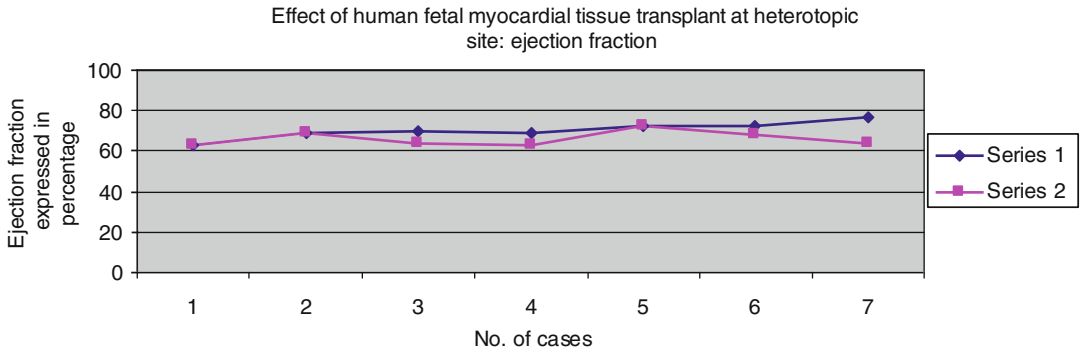
Table 29.1 Medical treatment guidelines for the patients

Serial no., name, age, sex	
1, AK, 35, M, human mixtard (50:50) twice daily + *angiotensin-converting enzyme* (ACE) inhibitors + selective *beta-blockers* + antioxidants and other measures to prevent and combat heart failure	Diabetes mellitus (DM) with dilated cardiomyopathy with diastolic dysfunction
2, SLS, 60, M, human mixtard (50:50) twice daily ++ *angiotensin-converting enzyme* (ACE) inhibitors + selective *beta-blockers* + antioxidants and other measures to prevent and combat heart failure	Alcoholic/ischemic cardiomyopathy with diastolic dysfunction and diabetic and ischemic heart disease background
3, SR, 58, M, human mixtard (50:50) twice daily ++ *angiotensin-converting* enzyme (ACE) inhibitors + selective *beta-blockers* + antioxidants and other measures to prevent and combat heart failure	Diabetes mellitus (DM) with obstructive cardiomyopathy with systolic and diastolic dysfunction
4, PC, 64, M, human mixtard (50:50) twice daily ++ *angiotensin-converting enzyme* (ACE) inhibitors + selective *beta-blockers* + antioxidants and other measures to prevent and combat heart failure	Diabetes mellitus (DM) with restrictive cardiomyopathy and diastolic dysfunction
5, LM, 58, F, human mixtard (50:50) twice daily ++ *angiotensin-converting enzyme* (ACE) inhibitors + selective *beta-blockers* + antioxidants and other measures to prevent and combat heart failure	Diabetes mellitus (DM) with obstructive cardiomyopathy and diastolic dysfunction
6, PH, 72, M, human mixtard (50:50) twice daily ++ *angiotensin-converting enzyme* (ACE) inhibitors + selective *beta-blockers* + antioxidants and other measures to prevent and combat heart failure	Diabetes mellitus (DM) with dilated cardiomyopathy with diastolic dysfunction
7, BB, 39, M, human mixtard (50:50) twice daily ++ *angiotensin-converting enzyme* (ACE) inhibitors + selective *beta-blockers* + antioxidants and other measures to prevent and combat heart failure	Alcoholic cardiomyopathy with systolic and diastolic dysfunction and diabetic background

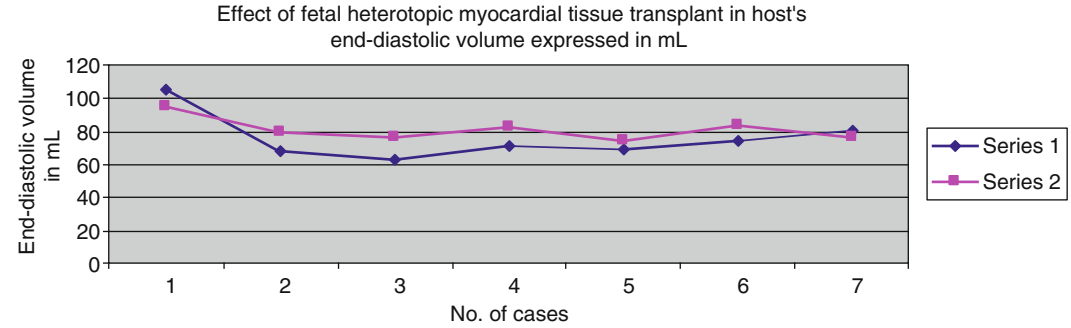


Graph 29.1 The effect of HLA-randomized fetal myocardial tissue heterotopic transplant and its impact on stroke volume as assessed with echocardiography after 3 months. *Series 1*: Stroke volume pre-transplant. *Series 2*: Stroke volume posttransplant as seen after third-month echocardiographical assessment

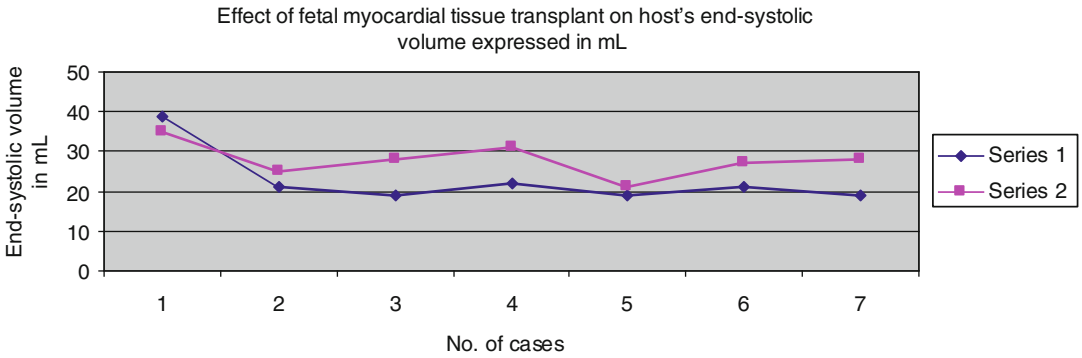
cardiac transplantation on end-systolic volume. Graph 29.5 shows the effect of fetal cardiac transplantation on heart rate. Graph 29.6 shows the effect of fetal cardiac transplantation on cardiac output. A problem with this study is that the number of patients provides too small a sample for exhaustive statistical analysis. Another weakness is that it is not a placebo-controlled double-blind control study. In spite of the weaknesses, it is the first global report of HLA-randomized fetal cardiac tissue transplanted at a heterotopic site under the axillary skin, the results of which suggested a definite trend of 10 to 20 % improvement in the different cardiac parameters from the pre-transplant value (Tables 29.2 and 29.3). This work was undertaken in a state government hospital; the patients admitted there could not afford frequent MRIs or CT scans of the heart which can record the ejection fraction better; they could also not afford the gold standard ventriculography, the



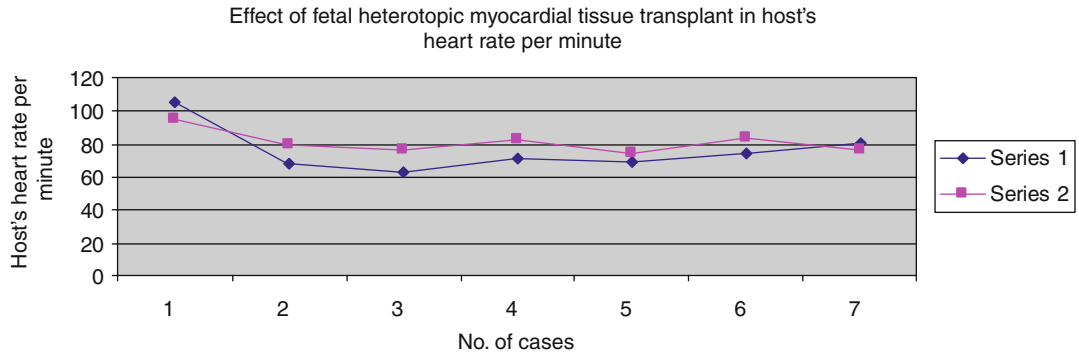
Graph 29.2 The effect of HLA-randomized fetal myocardial tissue heterotopic transplant and its impact on ejection fraction as assessed with echocardiography after 3 months. *Series 1*: Ejection fraction pre-transplant. *Series 2*: Ejection fraction posttransplant as seen after third-month echocardiographical assessment



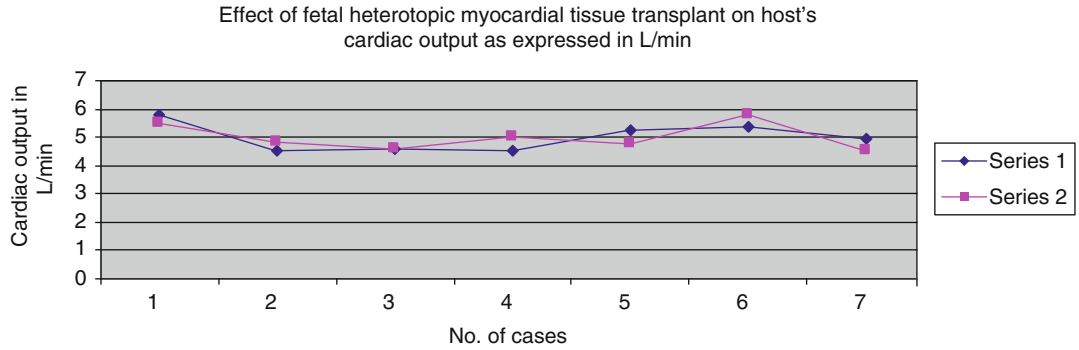
Graph 29.3 The effect of HLA-randomized fetal myocardial tissue heterotopic transplant and its impact on end-diastolic volume as assessed with echocardiography after 3 months. *Series 1*: End-diastolic volume pre-transplant. *Series 2*: Stroke volume posttransplant as seen after third-month echocardiographical assessment



Graph 29.4 The effect of HLA-randomized fetal myocardial tissue heterotopic transplant and its impact on end-systolic volume as assessed with echocardiography after 3 months. *Series 1*: End-systolic volume pre-transplant. *Series 2*: End-systolic volume posttransplant as seen after third-month echocardiographical assessment



Graph 29.5 The effect of HLA-randomized fetal myocardial tissue heterotopic transplant and its impact on heart rate/minute as assessed with echocardiography after 3 months. *Series 1*: Heart rate per minute pre-transplant. *Series 2*: Heart rate per minute posttransplant as seen after third-month echocardiographical assessment



Graph 29.6 The effect of HLA-randomized fetal myocardial tissue heterotopic transplant and its impact on cardiac output in liters/minute as assessed with echocardiography after 3 months. *Series 1*: Cardiac output pre-transplant. *Series 2*: Cardiac output posttransplant as seen after third-month echocardiographical assessment

gated SPECT, or the radioactive MUGA scan to gauge blood flow through the left ventricle.

Discussion and Conclusion

As already mentioned, treatment for cardiomyopathy is aimed to reduce the cardiac load and cardiac discomfort, control arrhythmia, and prevent overt failure of the heart. If there is no improvement with all medical treatment attempts, including lifestyle changes, surgical treatment ablation or injection alcohol at the hypertrophic septum, nonresponders could opt for cardiac transplant for the end-state cardiac disease. There are also other specific remedial measures like implantation of pacemaker, defibrillators, and ventricular assisted devices, which may be helpful in the treatment. These are all very costly procedures but may not be universally effective. Recently scientists all over the world are concentrating on simple alternatives like cell therapy, which have been found to be effective in a number of clinical trials in different animal models.

Cell-based regenerative therapy is based on the concept that replacing dead or deficient cardiac muscle with injected therapeutic stem cells may augment the contractile function of the heart [10]. The efficacy of cardiac stem cell therapy does not rely on cell replacement but could be, and most likely is, mediated by trophic, angiogenic, anti-inflammatory, and antiapoptotic effects. Regardless of this, the concept of "revitalizing" an adult stem cell so as to be able to actually replace cardiac cells is very exciting. This

Table 29.2 Pre-cardiac tissue transplant echocardiological values for the same patients

Serial no., name, age, sex	End-diastolic volume (EDV) (mL)	End-systolic volume (ESV) (mL)	Stroke volume (SV) (mL)	Ejection fraction (E_f) (%)	Heart rate (HR)	Cardiac output (CO) (L/min)
1, AK, 35, M	106	39	67	63	92/min	5.8
2, SLS, 60, M	68	21	47	69	96/min	4.51
3, SR, 58, M	63	19	44	70	104/min	4.57
4, PC, 64, M	71	22	49	69	92/min	4.5
5, LM, 58, F	69	19	50	72	105/min	5.25
6, PH, 72, M	75	21	54	72	100/min	5.4
7, BB, 39, M	81	19	62	77	80/min	4.96

Table 29.3 Posttransplant echocardiological values for the same patients

Serial no., name, age, sex	End-diastolic volume (EDV) (mL)	End-systolic volume (ESV) (mL)	Stroke volume (SV) (mL)	Ejection fraction (E_f) (%)	Heart rate (HR)	Cardiac output (CO) (L/min)
1, AK, 35, M	95	35	60	63	92	5.52
2, FB, 48, M	80	25	55	69	88	4.84
3, SR, 58, M	77	28	49	64	94	4.6
4, PC, 64, M	83	31	52	63	96	4.99
5, LM, 58, F	75	21	54	72	88	4.75
6, PH, 72, M	84	27	57	68	102	5.81
7, BB, 39, M	77	28	49	64	92	4.5

is the reason why DNA methyltransferase inhibitors such as 5-azacytidine were initially added to stem cells before implantation into infracted hearts for regeneration [11]. While various stem cell sources have been studied to induce myogenesis, recent interest has focused on promoting cardiac angiogenesis by proangiogeneic factors [12]. The existence of angiogenic factors such as acidic and basic fibroblast growth factor (FGF1 and 2), VEGF, PDGF, insulin-like growth factor 1 (IGF-1), angiogenin, transforming growth factor (TGF-α and TGF-β), tumor necrosis factor (TNF-α), hepatocyte growth factors (HGF), granulocyte colony-stimulating factor (G-CSF), placental growth factor (PGF), and interleukin-8 to be mitogenic for endothelial cells also participate in the regeneration process of myocardium which are associated with specific stem cell migration [13–15]. Hence, whether the actual migration of different types of stem cells from the fetal cardiac tissue, placed at the axillary site, and its gradual passage to the recipient's heart leads to the regeneration cascade in the myocardium is a valid question. The alternative suggestion could be that the stem cell cytokines which create the microenvironmental niche for the fetal cardiac tissue, are participating in the regeneration process when they reach the adult heart.

The observations in this experimental series involving fetal myocardial tissue subcutaneous heterotopic transplant leading to improvement of different cardiac parameters as seen graphically in Graphs 29.1, 29.2, 29.3, 29.4, 29.5, and 29.6 are not accidental because the improvement is echoed in all the parameters and the patients showed features of improvement and relief.

Fetal cardiac tissue contains cardiomyocyte or cardioblast cells in groups. These are relatively unspecialized mesodermal cells acquiring the specialized structural and/or functional features of a cardioblast, that is, a cardiac precursor cell. It is a cell that has been committed to a cardiac fate, but will undergo more cell division rather than terminally differentiate. There are other primitive and progenitor cells and also mesenchymal and epithelial stem cells, stem cell-like cells and undifferentiated cells. These undifferentiated cells progressively gain functional parameters once they are surrounded by extracellular matrix containing collagen and elastin in a regulated

manner to provide the specific niche or microenvironment for the stem cells and also stem cell-like cells of that system. Fetal cardiac tissue contains cardiomyocytes with different states of growth and differentiation along with connective tissue like elastin, collagen, and matrix.

This extracellular matrix (ECM) is a very important component of stem cell niche areas, which regulate the microenvironment of the stem cell pool size and control stem cell mobilization. ECM is a complex interlinked composite of collagenous molecules, non-collagenous molecules, and water-rich mucopolysaccharide ground substance. Cells are integrated to their matrix via integrin and non-integrin receptors, which are utilized in the control of adhesion, migration, division, growth, anoikis, transdifferentiation, and other cellular behavior. ECM provides architecture and strength but also growth factor deposits [16]. Each of these processes is essential for regeneration.

In fine, the primitive cardiomyocyte and cardioblasts migrate and transdifferentiate after reaching the site of injury, that is, from the heterotopic fetal tissue transplantation site to the host's cardiomyopathic heart, and take active part in the repair or regeneration process either singularly or through coordinated efforts with growth factors and pro-angiogenetic factors as discussed earlier.

There are also some secondary advantages of human fetal tissue transplantation as the present group has reported earlier in relation to human

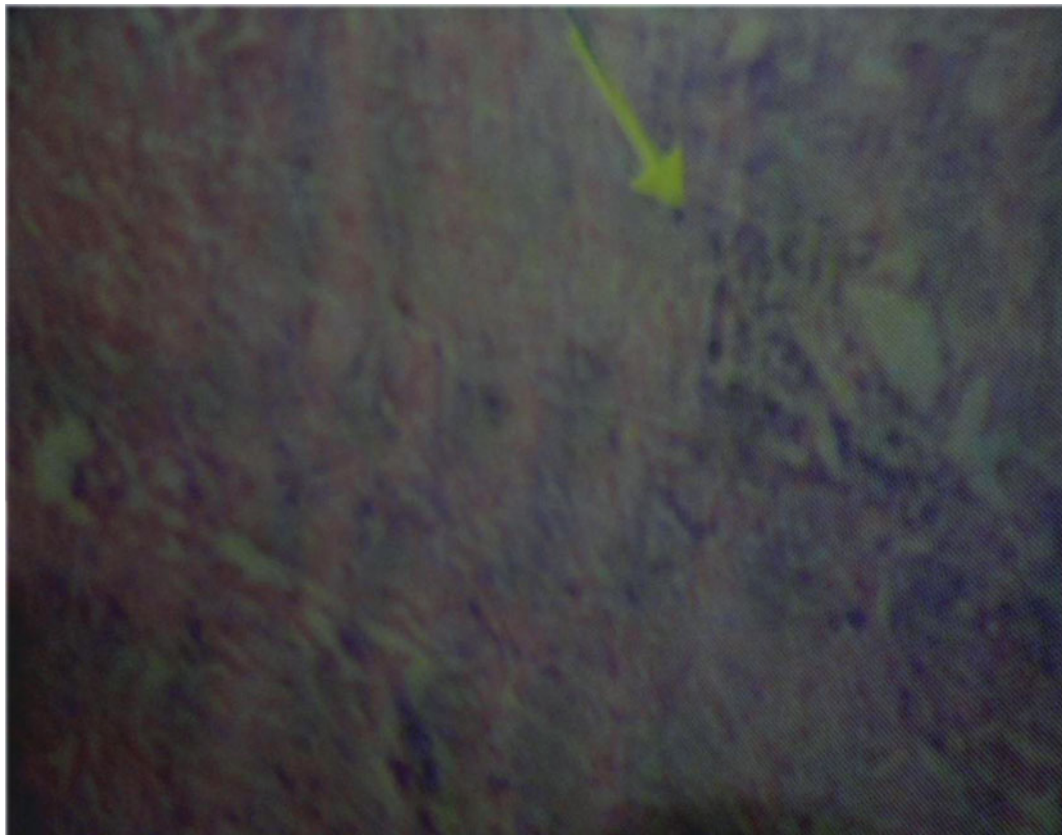


Fig. 29.1 Microphotograph of the partially retrieved fetal cardiac tissue (16 weeks gestational age) collected from the subcutaneous place under the axilla of the host suffering from cardiomyopathy. The *arrow* suggests the fetal cardiac tissue in varying degrees of cluster formation, but the most important thing is the total absence of visible leukocytic or mononuclear tissue around those cells justifying the absence of adequate inflammatory or immunological reaction of the host. This is a typical finding in fetal tissue transplant in adult. It stimulates the investigator to suggest that the fetal tissue creates its own microenvironment for its survival

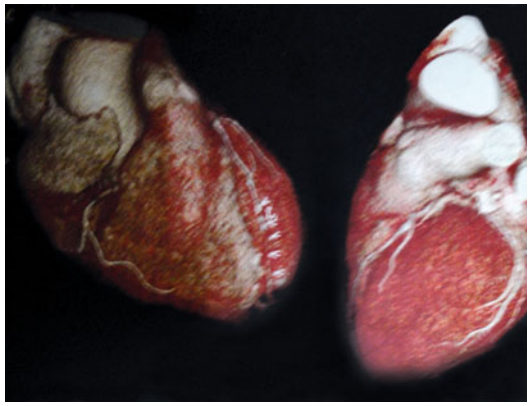


Fig. 29.2 Photograph showing a 64-slice contrast CT scanning of the heart with triple artery disease in the background of diabetes, alcoholism, dyslipidemia, and cardiomyopathy (serial no. 2) of the patient's profile (Table 29.1)

fetal neuronal tissue transplantation. There, the safety aspects of fetal tissue transplantation have also been emphasized [17]. Some of the nonspecific effects are the following: (a) rise of hemoglobin from the pre-transplant level, though there is no concomitant rise of ferritin, (b) there is also reduction of aches and pain all over the body, which is due to the preexisting disease background or superimposed different factors either singularly or in combination, viz, viral, bacterial, or fungal, anaerobic infection, (c) the background malnutrition may have a contributory role too, (d) posttransplant weight gain, (e) improvement of appetite, and (f) sense of well-being universally present among all the transplant recipients in varying degrees, which we will report in a separate chapter in this book. In this study, the same trends were noted (Figs. 29.1 and 29.2).

Conclusion

Though the study hints at the integration and augmentation of the existing cardiac function of the cardiomyopathic heart after the fetal cardiac tissue transplantation, many questions remain unanswered. For example, a recent group of investigators are of the opinion that human fetal and adult heart-derived cardiomyocyte progenitor cells (CMPCs) have distinct preferences to differentiate into mesodermal lineages. Under pro-angiogenic conditions, fetal CMPCs form more endothelial but less smooth muscle cells than adult CMPCs. Fetal CMPCs can also develop toward adipocytes, whereas neither fetal nor adult CMPCs show significant osteogenic differentiation. Interestingly, although both cell types differentiate into heart muscle cells, adult CMPCs give rise to electrophysiologically more mature cardiomyocytes than fetal CMPCs [18].

Tissue and cell therapy or to be more specific organ-specific cell therapy for cardiac regenerative conditions has reached the point where new directions are needed to optimize their effects. Possibilities of next-generation approaches include the use of "in vitro supercharged" cells, combinations of cells and cytokines, and of course combination of cellular therapies. These are currently under intense observation and study, and the subject of speculation at centers of excellence.

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30 A Study and Follow-Up (1999–2012) of Fetal Midbrain Tissue Transplant (Iatrogenic Chimera) at a Heterotopic Site in Axilla as a Treatment Support in Cases of Adult Idiopathic Parkinsonism Patients

Niranjan Bhattacharya and Abhijit Chaudhuri

Introduction

The most advanced research on the use of human fetal tissue has been done in Parkinson's disease, which affects about 1.5 million people in the USA only. Patients with Parkinson's disease experience tremors, slurred speech, and slowness of movement that eventually progress to total paralysis. In this progressive, debilitating illness, the cells in a small part of the brain called the substantia nigra are destroyed, depriving the striatum (the part of the brain that controls movement) of a critical molecule called dopamine. Despite devastating loss of motor control, mental faculties in Parkinson's patients remain intact, and while the disease is in itself not fatal, patients often succumb to complications such as injuries from falls or pneumonia.

During brain development, one of the most important structures is the subventricular zone (SVZ), from which most neurons are generated. In adulthood, the SVZ maintains a pool of progenitor cells that continuously replace neurons in the olfactory bulb. Neurodegenerative diseases induce a substantial upregulation or downregulation of SVZ progenitor cell proliferation, depending on the type of disorder. Far from being a dormant layer, the SVZ responds to neurodegenerative disease in a way that makes it a potential target for therapeutic intervention [1].

For a long time, it was believed that the adult mammalian brain was completely unable to regenerate after insults. However, recent advances in the field of stem cell biology, including the identification of adult neural stem cells (NSCs) and evidence regarding a continuous production of neurons throughout life in the dentate gyrus (DG) and the subventricular zone of the lateral ventricles (SVZ), have provided new hopes for the development of novel therapeutic strategies to induce regeneration in the damaged brain. Moreover, proofs have accumulated this last decade that endogenous stem/progenitor cells of the adult brain have an intrinsic capacity to respond to brain disorders [2]. Newly generated neurons are functionally integrated into the neuronal circuits, which are involved in regulation of brain plasticity. Endogenous neuronal production in the DG and SVZ is expected to provide a continuous source of new neurons that replace degenerated neurons in the injured brain. Recent studies

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indicate that adult neurogenesis is modified by various brain insults including stroke, epilepsy, and neurodegenerative disorders. While upregulation of neurogenesis in these situations may partially contribute to restoration and regeneration of damaged neural tissues, inadequate cell differentiation and/or excessive supply of new neurons should disturb existing neural circuits [3].

There are two populations of neurons which are continually renewed in the adult, the dentate gyrus granule neurons and the olfactory bulb granule and periglomerular neurons. In the dentate gyrus, a secondary proliferative zone termed the subgranular zone is established along the interface between the dentate gyrus and the hilus where granule cells are born throughout life. Olfactory bulb neurons are generated in the anterior subventricular zone of the lateral ventricle and migrate via the rostral migratory stream to the olfactory bulb [4]. In nature, both the degeneration and regenerations within that system are a simultaneous process; hence, the impact of degeneration is clinically visible long after the exhaustion of the reparative mechanism.

Now in a clinically established case of neurodegenerative disease, as seen in Parkinsonism, what are the other options available apart from medical supplementation of L-dopa and other antiparkinsonian drugs?

One remedy could be through cell therapy with autologous mesenchymal cells stereotactically placed at the site of the dopamine deficiency region of the basal ganglion. But the problem with stem cell clinical application is the fact that the behavior of the stem cell in vitro and in diseased in vivo condition is not always identical thus making the therapeutic potential unpredictable.

One of the most controversial areas in medical research today is fetal tissue transplantation. At stake is a source for stem cells, progenitor cells harvested from human fetuses that can differentiate into any cell in the adult human body. This chameleon-like ability of stem cells makes them potentially useful in replacing critical cells in the adult human body that have been ravaged by injury or disease.

Fetal tissue transplants, in which such organ-specific and nonspecific stem cells live in their natural environment and are injected into the failing organs of patients, work on the premise that when placed in the right environment, the transplanted cells take their cues from their surroundings and develop into the needed tissue. Stem cells seem adaptable to such procedures, growing rapidly after transplantation, and secreting hormones and other chemicals that promote tissue growth. As an added bonus, these "master" cells are too undeveloped to be detected by the recipient's immune system and thus often avoid the rejection that plagues normal organ transplant procedures.

The first line of treatment for Parkinson's disease is drug therapy. Unfortunately, L-dopa, a precursor of dopamine which can be absorbed by the brain, helps only as long as there are some substantia nigra cells still alive to absorb the drug. Once that area of the brain is destroyed, L-dopa becomes ineffective, which until recently left the patient without any available treatment for this disorder.

In recent times, in certain centers of excellence, pioneering fetal tissue transplants into the brain of Parkinson's patients have shown promise in slowing or even reversing symptoms of the disease. In this treatment, cells from the pre-brain structures of 6- to 8-week-old fetuses are injected into the patient's striatum, where if all goes well, they grow into a bundle of nerve cells that produce the needed dopamine. Patients with successful fetal tissue transplants have shown remarkable improvement in the severity of tremors and in their ability to move.

With such exciting results and millions of people in India alone suffering from Parkinson's and other diseases that may be helped by fetal tissue transplants, patients and their advocates are urging further research into the use of stem cells. However, currently, the only reliable source of fetal stem cells is selectively aborted human fetuses, collected from abortion clinics with the permission of the mother.

In Calcutta, there is an ongoing research on patients with idiopathic Parkinsonism from 1999 and follow-up till date of subcutaneously placed fetal midbrain at a heterotopic site in the axilla, collected from consenting mothers undergoing

hysterotomy and ligation for family planning purposes only.

This chapter examines all aspects of fetal neuronal tissue transplantation at a subcutaneous heterotopic site under local anesthesia in different patients with severe idiopathic Parkinsonism, not responding to conventional drugs. All the cases passed through the voluntary consent protocol and were cleared by the institutional ethical committee of the hospital.

Fetal tissue is composed of a group of cells with its supporting architecture of collagen or fibrous tissue. Subcortical fetal midbrain tissue is rich in neuronal progenitor cells and a supporting cytokine network.

Whether the placement of the developing human subcortical fetal midbrain tissue at a different heterotopic site outside the brain has any positive impact on the hosts' neurological system, due to migration of the neuronal progenitor or stem cells from the grafted tissue to the site of brain injury, is the principal question behind the present research. The basis for this research lies in the fact that the entire science of stem cell biology is based on three cardinal behaviors of stem cells, i.e., stem cells can easily migrate to a site of injury, it has transdifferentiation properties based on its environment, and lastly, stem cells are immortal due to the telomeric reverse transcriptase activity of the stem cells which prevents the shortening impact on the telomeric end after cell division.

Published Result and Its Discussion

In 2011, the researchers in Calcutta published a report of 48 cases (patients) of heterotopic iatrogenic therapeutic chimera of developing fetal midbrain tissue in cases of advanced idiopathic Parkinsonism not responding to conventional drug treatment [5]**.** During the process of follow-up, all the patients were persuaded to allow partial retrieval of the fetal tissue from the axilla under local anesthesia in the operation theater; however, eight patients refused to allow retrieval because they thought that the improvement was due to the transplant, and as such, if they continued with the transplant, there would not be any further problem. They were further persuaded, and ultimately partial retrieval of the fetal tissue from the last patient was done after more than 10 years from the date of placement of the transplant.

In the present series, the diagnosis of PD remained clinical [6] and was conducted by an experienced consultant to exclude early Parkinson's disease, secondary Parkinsonism, and the Parkinsonism plus group of disorders. Only advanced idiopathic Parkinsonism cases were considered as subjects for the study. The problem of treatment of this disease in developing countries is partially the mind-set which refuses to accept a prolonged treatment which has diminishing returns vis-a-vis the cost of treatment. Our referral service provided the options of stereotactic surgery/ablative or deep brain stimulation procedures in various brain nuclei/dopaminergic cell implantations, which could be conducted in private hospitals in our country or abroad at a prohibitive price. All the 48 patients refused to go for these options. They also refused our suggestion to purchase a peripheral apomorphine pump.

Apart from the clinical profile of the patients who underwent our heterotopic fetal midbrain tissue transplantation surgery, protocol is being followed up including assessment of the mental state (mini-mental state examination), disability assessment, and the mood of the patient to assess depression and prevailing anxiety state. The other studies include assessment of the hematological, immunological, and metabolic parameters to see if there is any adverse impact of transplantation of HLA-randomized neuronal tissue at the heterotopic site in the axilla on the host system. Lastly, the study of a small amount of retrieved tissue, under local anesthesia from the axilla (at 3 monthly intervals randomly from its date of placement, from the consenting volunteers just to assess the fate of the brain tissue), was done under simple microscopy and scanning electron microscopy, to see if there was graft vs. host reaction involving the fetal tissue.

What is significant is the fact that the 5–15 g of fetal tissue heterotopic subcutaneous graft never caused any graft rejection or any other features of acute, subacute, or chronic graft vs. host

reaction in even a single case after a long follow-up till date; nor were there any apparent changes in the biochemical, i.e., hepatic, renal, or metabolic parameters, in the host system. What is also intriguing is the persistence of the fetal tissue, which was not destroyed by the immunoenzyme network of the host defense and surveillance system. In the cases where the graft was removed 10 years after the placement of the tissue, the same survival of the fetal tissue was noted. The conclusions are simple:

1. Fetal neuronal tissue can survive in the adult host where the neuroendocrine, immunoenzymatic, and cytokine regulation is distinctly different from the adult system.
2. There is similarity in the histological and electron microscopic findings of the retrieved fetal tissue from the host adult tissue, from the first month to the tenth year after its placement in the adult host. This is a truly astonishing finding.
3. This observation justifies the hypothesis that there is persistence of the stem cell component of the fetal tissue in the adult host in such a situation. Molecular study of the developing neuronal receptors is needed to confirm the hypothesis.
4. Fetal tissue creates its own microenvironment for its survival.
5. That there is no abnormal growth and differentiation of the fetal neuronal tissue, justifies the observation that the genetic regulation with its apoptosis mechanism is in full operation in the adult host which is ineffective in detecting and destroying the primitive hypoantigenic system existing in fetal tissue.
6. One observation/deduction is that it is possible that this may be one of the mechanisms through which pregnancy and neoplasm induce tolerance of the homograft.
7. A minimum score of 40 points was required for enrollment in the motor portion of the Parkinson's Disease Unified Rating Scale [7]. When the patient had been without medication, scores in this scale varied from 0 to 108. Clinical improvement is rated individually as mild, moderate, and substantial on the basis of objective assessment by the attending doctor.
8. What is exciting is the fact that neuronal progenitor cell-rich fetal tissue transplant definitely improved the state of clinical dis-ability from the pre- to the posttransplant phase.

   In the present study, both subjective and objective improvements of 83.3 % score from the pre-transplant level (to the date of assessment, i.e., 1 month after the installation of the fetal cortical graft) were noted, of which mild improvement was noted in 41.66 % and another 41.66 % patients showed moderate improvement from the pre-transplant Unified Parkinsonism Scoring System [7]; however, 16.66 % of the patients did not show any objective positive response.
9. If the leukocytes value of the pre-transplant level with the posttransplant level, practically minimal or no impact can be observed. metabolic, hepatic, and renal functions, it was observed that there was practically no difference in the urea, creatinine, bilirubin, glucose, and ferritin (Figure 24) levels in the posttransplant value from the pre-transplant levels as noted apart from inflammatory marker CRP and autoimmune marker ANF, dsDNA, etc. Interestingly, there was also a rise of hemoglobin value from the pre-transplant level without any appreciable change in the ferritin value. The cause for the rise of the hemoglobin level could be due to the erythropoietin content impact of the growing fetal brain.

Scanning electron microscopic study of the retrieved tissue from the axilla reaffirmed the presence of fetal neuronal tissue in the background of the host tissue. Investigating further with the electron microscope, the host tissue showed that there were no features of inflammatory cellular reaction when seen in different magnifications. Why there was no obvious inflammatory cellular infiltration or other acute, subacute, or chronic reactions in the host tissue as a result of the fetal tissue transplant remains a scientific mystery to be solved by future researchers. The present study, however, shows a certain degree of inflammatory subcellular cytokine impact on the retrieved tissue. Massive cellular edema can be perceived, which leads to fragmentation and partial loss of collagenous architecture. In fine, the overall

impression of the retrieved tissue through scanning electron microscopy suggests noncellular inflammation sequelae justifying subcellular impact at the nano level.

What has been learned from these histology [microscopy] and electron microscopical results is the fact that there appears to be a kind of insensitivity in the host system to mount any acute, subacute, or chronic inflammatory or immunological reaction. At first, it may appear that the chronic progressive Parkinson's disease itself is causing such an immunological insensitivity in the host system, but in cases of fetal tissue transplant in varying diseases done by the group of researchers in Calcutta, such a condition of host insensitivity or tolerance has been noted and reported on earlier [6–8].

The present study reaffirms the presence of fetal neuronal tissue in the background of host tissue without any further growth and differentiation. If we try to explain this phenomenon on the basis of existing knowledge in stem cell biology, we can accept its feasibility, as seen in case of adult stem cells which remains dormant. Further sophisticated molecular marker studies may explain how fetal stem cells become adult stem cells and reside peacefully in the adult immune system; this phenomenon supports the dictum that the fetal tissue develops its own survival strategy by creating its own microenvironment for its growth and survival.

Mini-Mental State Examination (MIMSE)

Of all the mental status examinations for assessment of patients' concentration and other skills, the most commonly used today is the Mini-Mental State Examination (MMSE). For fetal neuronal tissue transplant, there appeared to be a definite improvement with upgradation (improvement of the status) of the deformity from grade 3 to grade 2 in 42.85 % of the cases. There was also improvement in other grades after the transplantation and the follow-up evaluation of the grade, i.e., in physical disability scoring. Similarly, while assessing the cognitive impairment, the results showed improvement irrespective of the pre-transplant disability grading. This is statistically significant (p value < 0.03). The procedural sensitivity is 71–92 %, and the specificity varied from 56 to 96 % [8–10].

Study of the Mood of the Transplant Patients (e.g., HADS)

There has been considerable controversy regarding the relationship between depression and anxiety. This preliminary validation study of the local Bengali version of the HADS questionnaire showed it as an acceptable and reliable measure of psychological morbidity among Parkinson's disease patients.

Based on Snaith and Zigmond's interpretation of HADS-A and HADS-D scores of 8 or over, patients screened positive for anxiety (72 %) and/or depression (85 %) before the transplant showed an improvement after the transplant from 1 month onward as noted in HADS-A and HADS-D scoring, eventually reaching a lower value of anxiety (32 %) and/or depression (35 %) [11, 12] at the end of 1 year.

Conclusion and Future Directives for Research

Certain questions remained unanswered in this clinical study on fetal neuronal tissue transplantation or to be more specific, fetal neuronal tissue graft at a heterotopic site.

Though the number of cases are small (n = 48), the results appear positive looking, as suggested by the clinical improvement, both specific (neurological) and nonspecific (for instance, weight gain or improvement of anorexic condition).

However, the fundamental questions that should be addressed in subsequent research in the future are:

(a) Why do the fetal neuronal tissue containing neuronal cells (including stem cells and progenitor cells or other stem cell-like cells) at varying stages and grades of maturation apparently survive at the same site of its placement, without disengagement or disintegration or dysregulated of the surrounding cells and their extracellular matrix (ECM)

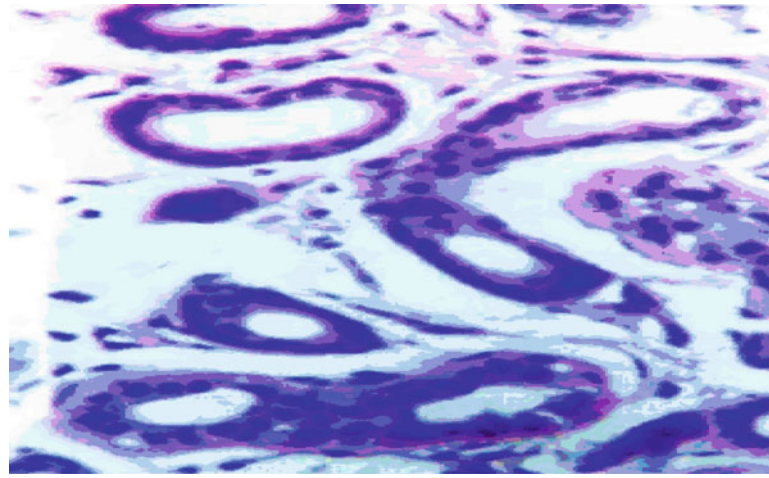

Fig. 30.1 High-power (oil immersion, magnification 375×) microphotograph of H&E-stained 16-week fetal neuronal tissue after its retrieval from the axillary site after 12 weeks

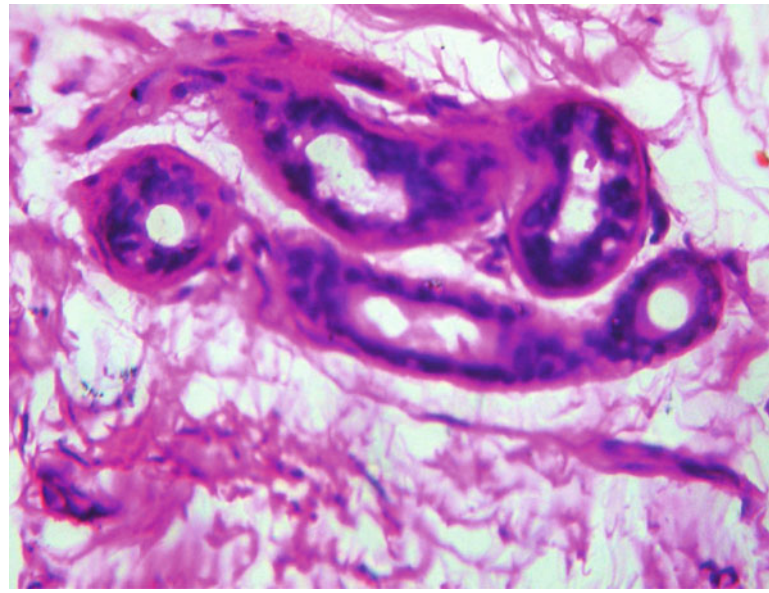

Fig. 30.2 High-power (oil immersion, magnification 375×) microphotograph of hematoxylin and eosin (H&E)-stained 16-week fetal neuronal tissue seen after its retrieval in the tenth year from axillary site

causing a state of iatrogenic chimerism of neuronal cells [14]?

(b) With the coordination of the extracellular matrix (ECM) and its constituent of integrin and non-integrin receptors, the transplanted neuronal cells get attached with the ECM. These integrin and other receptors may control the cellular behavior starting from migration, adhesion, growth, maturation, anoikis, transdifferentiation, and other essential functions of the cells. Apart from this, the structural strength, architecture, and arranging growth factor deposits which proteinases as signaling scissors can release in a site- and process-specific manner.

(c) Is there any strategic functional barrier for the synthesis and turnover of the local neurocytokines for the heterotopic site placement of the developing neuronal tissue?

(d) Are the neurocytokines of the heterotopic site actually helping in the clinical remission and its sustenance in Parkinson patients?

(e) Are the neuronal stem cells actually crossing the blood–brain barrier and migrating to the specific site of damage at the midbrain level?
(f) Do the developing neuronal cells create their own microenvironment for their survival strategy without stimulating the host system.
(g) Fetomaternal cell transfer is a natural physiological phenomenon for fetomaternal well-being and tolerance. Does this iatrogenic chimera act positively for periodical boosting of the host's (Parkinson patient's) neuronal pool by contribution of stem cell or neurocytokine boosting products?
(h) In this connection, it is worth mentioning that in animal studies when neural cells were collected from the entire cerebral cortex of developing mouse fetuses (15.5–17.5 days post coitum) and their nuclei were transferred into enucleated oocytes, 5.5 % of the reconstructed oocytes developed into normal offspring [14]. Presently, there are five common classes of human natural chimeras (cytomictical, whole body, fetal-maternal, germ cell, and tumor chimeras) [15].
(i) The impact of neuronal iatrogenic or therapeutic chimera and its implications have never been discussed in literature; hence, more studies should be undertaken to confirm or reject these exciting questions in the future [16].
(j) Do the fetal tissue's microenvironmental support cause targeted neuroprotection of vulnerable neurons and help in the prevention or retardation of specific neuronal cellular death affecting nigral cells [17]?

Acknowledgment

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2. Investigator 2 was associated from the concept state to its implementation phase including follow-up as technical guide and also actual periodic participation during his visit to Calcutta, India.

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Growing Organs for Transplantation from Embryonic Precursor Tissues

31

Dalit Yutzis-Tchorsh and Yair Reisner

Fetal Tissue Transplantation: The Narrow Window of Opportunity in Embryonic Gestation

Embryonic precursor tissues comprised of various types of lineage-restricted progenitor cells may be ideal for organ replacement, provided that successful organogenesis can be achieved following transplantation. The organ-specific stem/progenitor cells (brain, skin, kidney, liver, pancreas, etc.) found in developing organs are thought to be able of generating many or the entire differentiated cell types in an organ. This approach offers theoretical advantages over transplantation of either pluripotent embryonic stem cells (ESC) or of fully differentiated (adult) organs [1, 2]. Organ precursor tissues, rather than pluripotent ESC, are thought to be sufficiently committed so as to be free of risk for teratoma formation. In addition, the growth potential of cell populations within embryonic organs is superior relatively to those in terminally differentiated mature organs. However, considering the major ethical issues associated with the use of human fetal tissues, as well as the lack of control over donors, timing of cell harvesting, genetic engineering, and quality control of the obtained tissue, we have focused our studies during the past decade on porcine embryonic tissues.

One attractive aspect of fetal xenografts compared to adult tissues is their relatively reduced immunogenicity, likely associated with reduced expression of MHC molecules, and absence of professional antigen presenting cells if harvested prior to their appearance in the tissue [1–8]. Moreover, the working hypothesis behind our studies has postulated that transplantation of pig embryonic precursor tissues which does not require anastomosis between donor and host blood vessels and which can use the host vasculature could potentially evade the humoral hyperacute and acute vascular xenorejection. In addition, fetal tissues are relatively resistant to ischemic damage compared to adult tissues [9, 10].

The major disadvantage in fetal organ transplantation is the latent period between transplantation and functional commencement of the graft, presumably reflecting the differentiation and growth of the grafted tissue after implantation [11]. This delay, however, may be expressed differently in different species and organs and could also be related to the fetal tissue gestational age at transplantation and to the status of stromal elements co-transplanted with the precursor cells.

Obtaining the precursor organs at the "right time" is a major requirement for the use of such tissues for transplantation. The "window" for transplantation is defined as the earliest gestational age at which maximal capacity to grow and differentiate into functional tissue can be achieved, with minimal risk for teratoma formation.

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Table 31.1 Transplantation of human and pig kidney precursors in immunodeficient mice

Gestation age	No. of transplants	Method of transplantation	Graft[a] growth	Graft differentiation[b]		
				Renal	Non-renal	Necrosis
Human						
14w	3	Whole	3/3	None	None	3/3
14w	8	Fragments	7/8	7/7	None	None
10w	2	Whole	2/2	None	None	2/2
10w	6	Fragments	6/6	6/6	None	None
8w	5	Whole	5/5	5/5	None	None
7w	3	Whole	3/3	3/3	None	None
Pig						
8w	7	Whole	5/7	None	None	5/5
8w	6	Fragments	6/6	6/6	None	None
6w	5	Whole	4/5	None	None	4/4
6w	6	Fragments	6/6	6/6	None	None
E27-E28	12	Whole	12/12	12/12	None	None
E24-E25	9	Whole	8/9	5/8	3/8	None
E20-E21	9	Whole	6/9	3/6	3/6	None

Taken from Dekel et al. [2]

[a]Transplant growth and differentiation were assessed at 8 weeks after transplantation

[b]Differentiation was categorized to renal (only nephrons), non-renal (differentiated derivatives other than renal) and necrosis (in addition to nephrons, appearance of necrotic areas mostly in center of transplant)

As a proof of principle, we were able to define initially an optimal gestational "window" required for successful organogenesis of human and porcine metanephroi (Table 31.1). This window was shown at 7–8 weeks and 4 weeks of gestation for human and pig, respectively. Thus, pig embryonic renal precursor tissue harvested at E21 or E24 fails to mature into the desired differentiated cell fate and forms teratomas, while normal growth and development is found at 6 weeks after transplantation of E28 precursor tissue.

Furthermore, pig and human renal precursor tissues harvested at E28 and 7–8 weeks, respectively, were also found to exhibit reduced immunogenicity compared to tissues harvested at late gestational time points. Moreover, markers typical of antigen presenting cells are less frequent in the earlier precursor tissue, suggesting reduced level of donor APC in the E28 metanephroi. This reduced immunogenicity was found to be associated with dominance of host vasculature in renal tissue developing from the early precursor tissue compared to those developing from more advanced tissues, which exhibit vasculature predominantly of donor origin.

While the development of a new source for kidney transplantation is very desirable, the connection of the growing implant to the urinary system represents a major challenge [12]. In contrast, implantation of other embryonic organs, the function of which is to secrete important hormones, factors, or enzymes, could be more easily translated to clinical application. Clearly, the narrow "window" for transplantation of renal embryonic tissue, which depends both on the relative level of pluripotent and committed stem cells in the specific tissue and on the status of stromal elements co-transplanted with the donor precursor cells, might differ greatly between different tissue precursors. Indeed, when we subsequently expanded the scope of this approach by examining liver, pancreas, spleen, and lung precursor tissues at various gestational stages, we found markedly different "windows" in correlation with the emergence of each tissue in normal embryonic development (Fig. 31.1). Thus, optimal growth potential which is free of risk for teratoma for pig embryonic liver, pancreas, spleen, and lung was defined at E28, E42, E42, and E56, respectively [2, 7, 8, 13, 14].

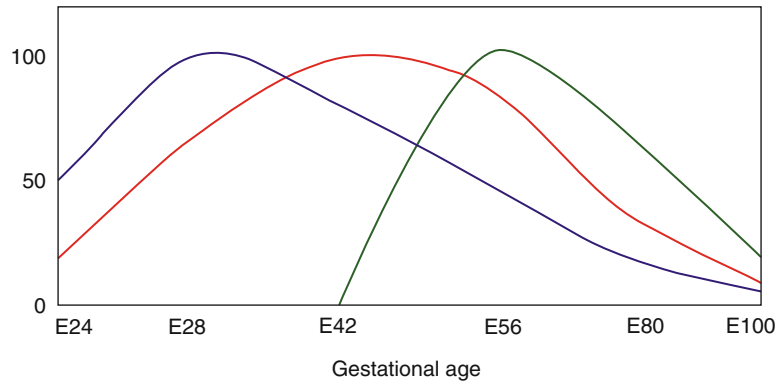

Fig. 31.1 The relationship between embryonic-precursor gestational age and growth potential is schematically presented. Distinct gestational time windows for implantation of liver (*blue*), pancreas (*red*), and lung (*green*) are demonstrated (Taken from Eventov-Friedman et al. [13])

Based on these initial studies, we were able to demonstrate the ability of E42 spleen transplantation for the cure of hemophilia by secretion of factor VIII and the ability of E42 pancreas to reverse diabetes by secretion of insulin.

E42 Pig Embryonic Pancreas Precursor Tissue as an Optimal Source for Transplantation in Diabetes

The use of porcine embryonic tissue might potentially provide an attractive, unlimited source of pancreatic tissue for transplantation. Beta-cell replacement for the cure of diabetes can be achieved by either whole organ-pancreas allogeneic transplantation or transplantation of isolated islet cells. However, statistics show that the transplantation of a whole pancreas achieves a longer graft survival and functionality than isolated islets (67 % after 10 years and 10 % after 5 years, respectively) [15, 16]. This discrepancy could be attributed to the deleterious effects encountered during islet preparation by enzyme digestion, prolonged ischemia time, and the loss of the surrounding mesenchyme and of 30–50 % of islets [17, 18].

The active involvement of the surrounding mesenchyme in pancreatic development and its role in endocrine cell differentiation and proliferation have been documented [19, 20].

Thus, the embryonic pancreatic tissue growing in the context of its own stroma might survive longer than infused neonate or adult islets growing heterotopically in the liver.

In our mouse transplantation model, the embryonic porcine pancreas is extracted under a light microscope and simply placed under the kidney capsule or in the omentum of the host without any further manipulation. The pancreatic precursor tissue contains insulin-secreting cells, endocrine and exocrine precursors, and the supportive stroma. This procedure is very fast and cold ischemia time until transplantation is less than 2 h. Moreover, the surgical procedure is simple and does not include sutures in the kidney capsule or anastomosis of the blood vessels.

As described above, the optimal gestational "window" for porcine pancreas was defined in our lab around day 42 (E42) based on its long-term growth potential, response to glucose challenge, endocrine/exocrine ratio, and reduced immunogenicity compared to adult tissue [8, 13]. The importance of determining the right gestational "window" for transplantation is critical considering the failure of previous attempts to reverse diabetes in diabetic patients in the early 1990s by Groth et al. using E66-81 porcine fetal islet-like cell clusters transplantation [21, 22]. The insulin levels attained in these attempts were too low to maintain normoglycemia in human hosts. Indeed, our results strongly indicate that the growth ability of pancreas extracted on E80 is very poor and able to secrete only low levels of insulin.

Pig pancreatic tissue obtained at the E42-E56 gestational window led to the highest pig insulin blood levels in transplanted mice before and after glucose challenge, 6 weeks after transplantation.

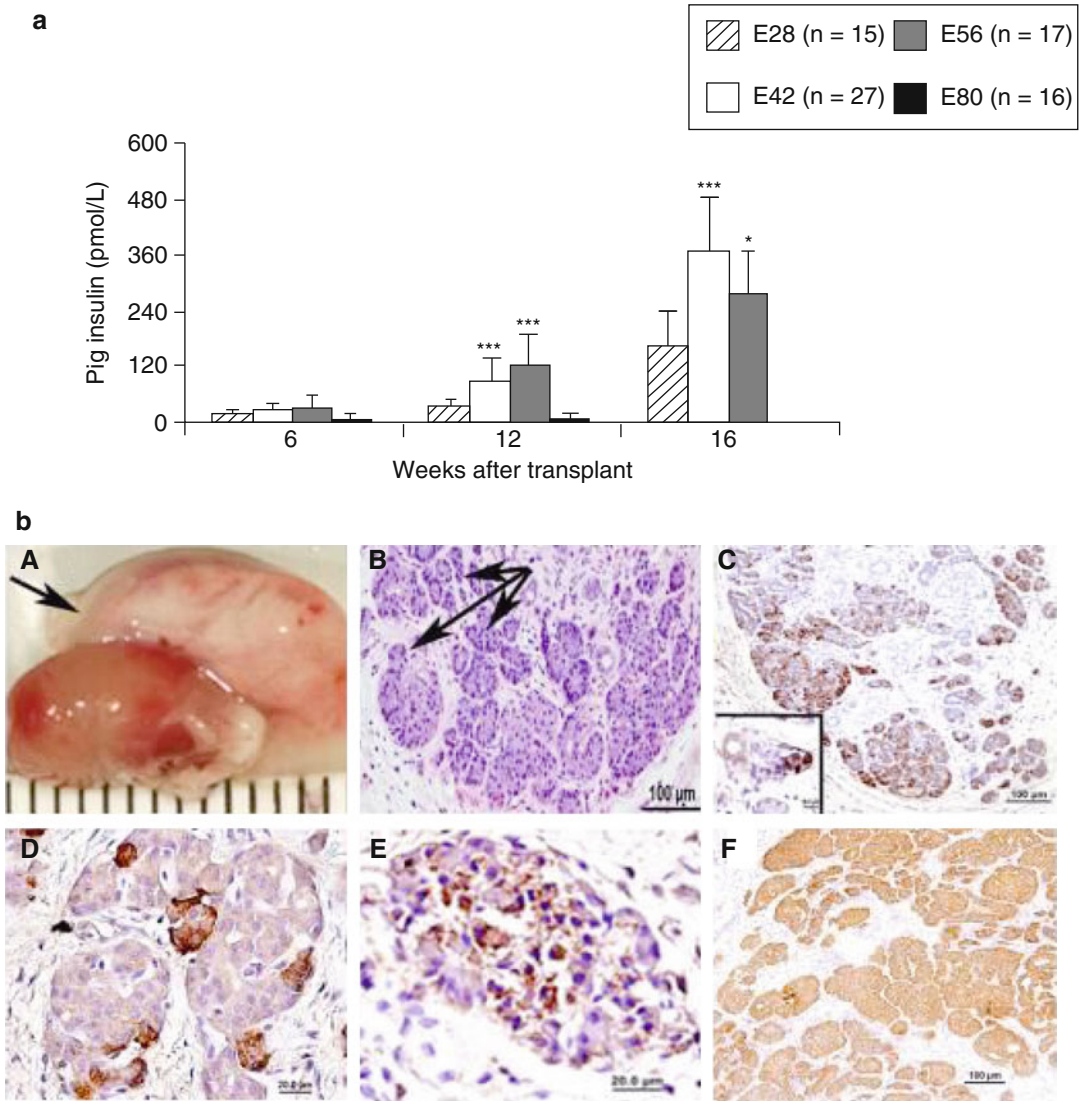

Fig. 31.2 Pig insulin secretion and histological appearance of long-standing embryonic pancreatic grafts. (**a**) Pig insulin levels following transplantation of E28, E42, E56, and E80 pig pancreas tissues under the kidney capsule of NOD-SCID mice. The data are based on average ± standard deviation pig insulin level measured in seven independent experiments, each of which includes comparison among pig pancreatic precursors of two to three different gestational ages (*, $p<0.05$; ***, $p<0.005$; comparing E42 or E56 with E28 pig insulin levels). (**b**) E42 pig pancreatic grafts 5 months after transplantation under the kidney capsule of NOD-SCID mice. Macroscopic appearance reveals a large viable graft that covers the kidney and contains abundant blood vessels (*panel A*); the graft is marked by an arrow. Histological analysis of the grafts demonstrates mainly dense islets of different sizes (*panel B*) (hematoxylin and eosin staining; islets marked by arrows). The ability of these islets to produce hormones is evident by positive staining for insulin (*panel C*), glucagon (*panel D*), and pancreatic polypeptide (*panel E*). Close proximity between islets and ducts is occasionally seen (*panel C*, *magnified inset*). The epithelial cells are widely stained for cytokeratin 20 (*panel F*) (Taken from Eventov-Friedman et al. [8])

Furthermore, as shown in Fig. 31.2, E42-E56 pancreatic tissue was found to secrete more pig insulin 4 months after transplantation than E28 tissue, which was previously advocated [23, 24], or E80 tissue used for harvesting islet-like clusters in the clinical trial [21, 22].

The final choice between E42 and E56 was based on the reduced immunogenicity of E42, compared to E56 pancreatic tissue when transplanted in conjunction with human lymphocytes into SCID mice and when transplanted in immunosuppressed immune-competent hosts.

The growing grafts attain most of their maximal size by the end of the fourth month posttransplant, and 6 months after transplantation, there are no dividing cells in the pancreatic parenchyma. At that point, the transplants reach their final size and stop dividing. This plateau in growth and development is reflected in the insulin blood levels between 6 and 10 months posttransplant.

The relative ratio of endocrine and exocrine elements exhibited by the growing tissue is a major parameter in defining an ideal "window" for implantation of pig embryonic pancreas. Clearly, predominance of exocrine cells that secrete proteolytic enzymes might be destructive for the endocrine tissue. Thus, in contrast to the development in the intact animal, the developing pancreatic graft is composed mainly of endocrine islets expressing insulin with almost undetectable exocrine components, while the adult pancreas consists mainly of exocrine tissue.

Most importantly, the curative potential of E42 pancreas transplantation was evaluated in two different mouse models of hyperglycemia. In the first model, irreversible pancreatic damage was induced by alloxan in long-term engrafted SCID mice. In the second model, streptozotocin-induced diabetic mice were treated by implantation of E42 tissue, and treatment with exogenous insulin was maintained until the grafts were sufficiently large to sustain the mice in the absence of insulin treatment. In both models, a period of 3 months of growth was required to effectively normalize glucose levels, as clearly demonstrated in Fig. 31.3.

Engraftment, Growth, and Development of E42 Pig Pancreatic Tissue in Immune-Competent Mice

While the results in SCID mice are encouraging, the immune barrier to xenografting, even when using early embryonic tissue, represents a major challenge.

It has been shown, by our group and others, that fetal organs are less immunogenic compared to their adult counterpart [1–8]. However, embryonic organs are still prone to immune rejection. In our hands, embryonic pancreases at all gestational ages tested were fiercely rejected upon transplantation into immune-competent mice and rats. The mechanisms underlying xenograft rejection of neovascularized embryonic tissues, such as pancreatic fragments, have not been extensively characterized to date. However, our study showed that upon transplantation into mouse strains with various immunological defects, rejection is primarily dependent on T-cell responses [8]. Other studies describing xenorejection of later gestational age pig pancreas and adult pig islets also support this conclusion [25, 26].

In this context, it is likely that immunosuppressive agents, such as costimulatory blocking agents, directed against T-cell activation and with minimal impact on angiogenesis and/or embryonic growth and development might be effective. Indeed, we were able to show that combined blockade of the CD28-B7 (by CTLA4-Ig) and CD40-CD154 (by anti-CD40 ligand) costimulatory pathways resulted in long-term graft survival with continuous production of insulin accompanied by normalization of glucose levels in diabetic mice. We also developed a novel immunosuppression protocol with anti-LFA1 and anti-CD48, in conjunction with low-dose FTY720 (Fig. 31.4), that prevented rejection of E42 pig pancreas in the C57BL/6 mice for more than 6 months [27]. Furthermore, the treatment with anti-LFA1 and anti-CD48 monoclonal antibodies could be stopped at 11 weeks after transplantation, and the grafts remained functional upon minimal maintenance with FTY720, administered twice weekly.

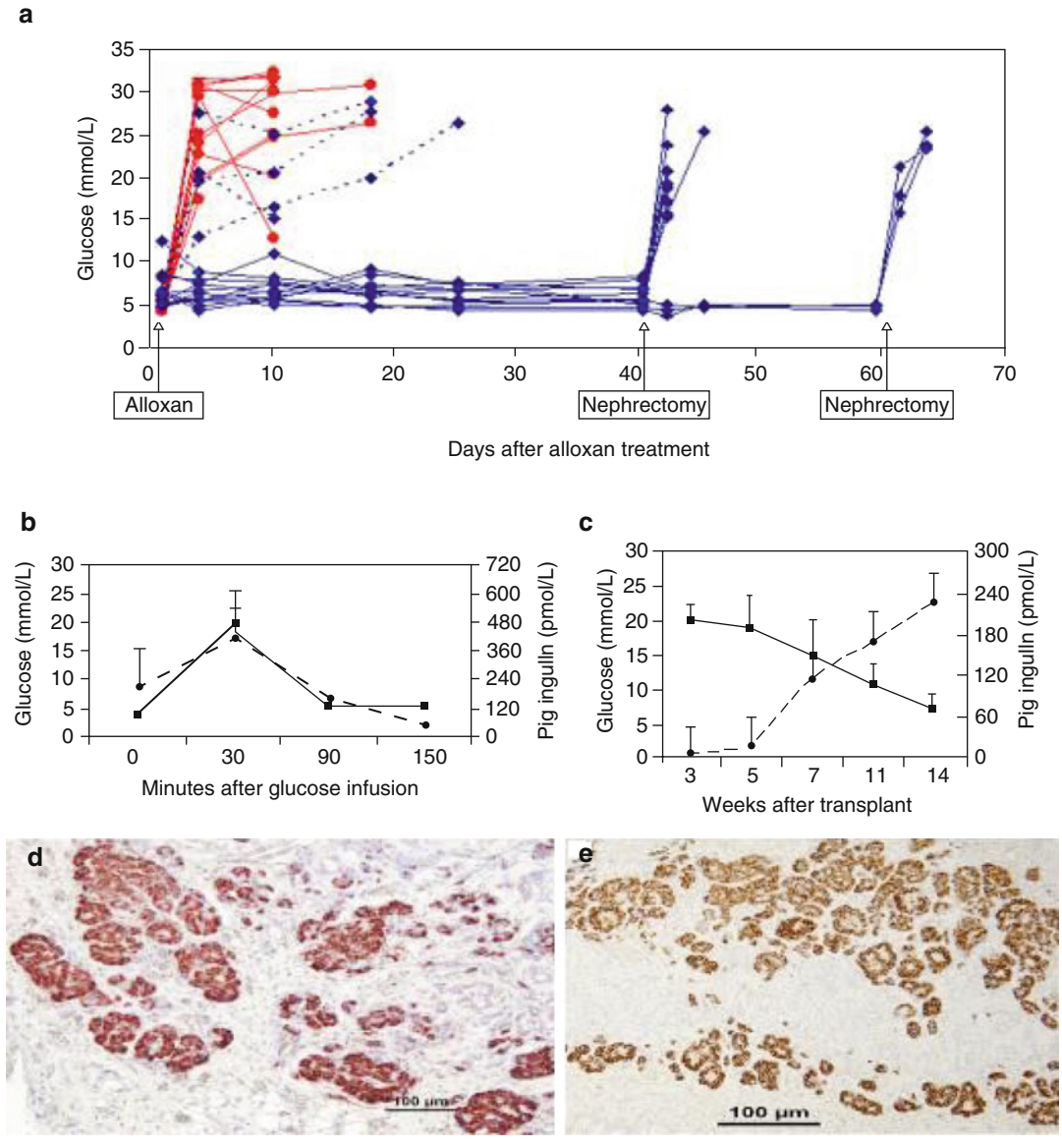

Fig. 31.3 E42 pig pancreatic tissue normalizes blood glucose levels in diabetic mice. (**a**) Glucose levels in durably grafted (E42 embryonic pancreas) and non-grafted alloxan-treated NOD-SCID mice. Grafted (*blue lines*) and non-grafted (*red lines*) NOD-SCID mice were injected with alloxan 4 months after transplantation. All non-grafted mice died within 2–18 days. Grafted mice exhibiting pig insulin levels below 120 pmol/L prior to the alloxan treatment failed to control hyperglycemia (*broken blue lines*); however, grafted mice demonstrating pig insulin levels above 120 pmol/L before alloxan injection maintained their glucose levels within the normal range (*unbroken blue lines*). Removal of the left kidney bearing the pig pancreatic graft at 41 or 61 days after alloxan treatment caused irreversible hyperglycemia. (**b**) Functionality of E42 pig pancreatic grafts in alloxan-treated NOD-SCID mice. After a 10-h fast period, 3 g/kg glucose was administered intraperitoneally. Glucose (*black line*) and pig insulin (*broken black line*) were followed at different time points spanning 150 min. The data represent three experiments and include 15 alloxan-treated NOD-SCID mice grafted with E42 pig pancreas evaluated 4 months after transplant. (**c**) Long-term follow-up of average glucose (*black line*) and pig insulin (*broken black line*) in streptozotocin-treated NOD-SCID mice grafted with E42 pig pancreas. Of 19 animals treated with streptozotocin, 10 survived up to 14 weeks following transplantation and eventually became independent of exogenous insulin. (**d** and **e**) Pig insulin is highly expressed in the alloxan- (**d**) and the streptozotocin-treated (**e**) NOD-SCID mice 4 months after transplantation, as detected by specific staining of nephrectomized kidneys bearing the E42 pancreatic grafts (Taken from Eventov-Friedman et al. [8])

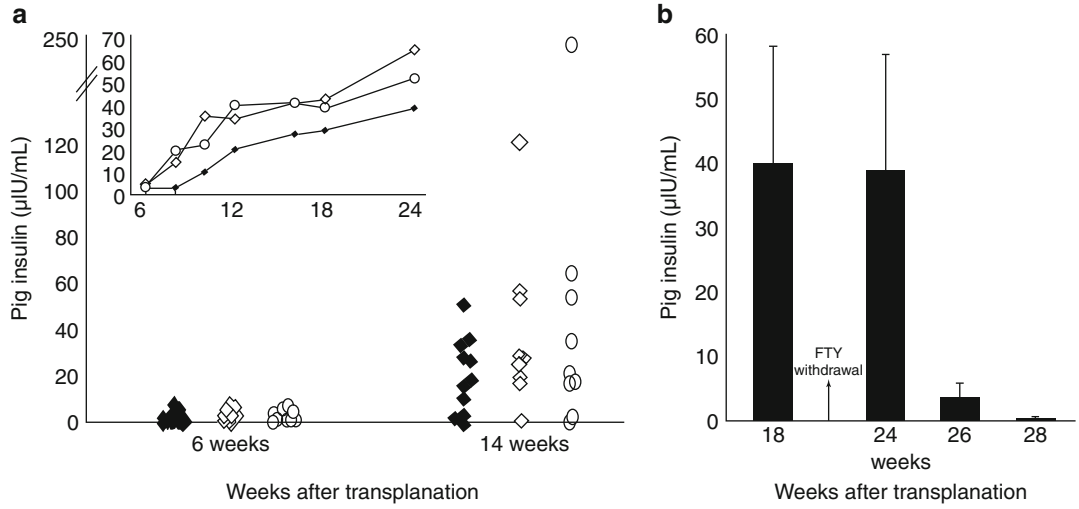

Fig. 31.4 (**a**) Porcine insulin levels in the serum of C57BL mice transplanted with E42 pancreas and treated with costimulatory blockade agents (anti-LFA1, anti-CD48, and ±CTLA4-Ig), FTY720, with (◊) or without (○) debulking, at different time points after transplantation. Treatment with costimulatory antibodies was stopped at 3 months posttransplant, and graft maintenance was continued twice weekly only with FTY720. Insulin levels in the serum of NOD-SCID mice transplanted with E42 pancreas served as a positive control (♦). The *inset* demonstrates average pig insulin levels in transplanted mice over a course of 6 months. No statistical difference could be found between the tested groups. (**b**) Porcine insulin levels in the serum of C57BL mice transplanted with E42 pancreas and treated with costimulatory blockade agents with or without debulking at different time points after FTY720 withdrawal. Data are presented as means ± SE (Taken from Tchorsh-Yutsis et al. [27])

However, as can be seen in Fig. 31.4, cessation of this low-dose FTY720 maintenance led to complete rejection within 1 month, suggesting that immune tolerance had not been attained.

This protocol might offer unique advantages in nonhuman primates and in humans, being free of the thrombotic side effects of anti-CD40L, the toxicity of ATG and rituxan, and the proven deleterious effects of rapamycin on pancreatic graft function and insulin secretion.

When comparing immunogenicity of the embryonic pancreatic tissue harvested at different time points, we demonstrated in various models that the E42 implants were less immunogenic and superior to later gestational tissues which were rejected [8] (Eventov-Friedman et al., unpublished results). Interestingly, in a microarray analysis comparing E56 versus E42 pre-transplant pig pancreatic gene expression, we detected a significant upregulation in the more mature stage of 53 immune-related genes (Eventov-Friedman et al., unpublished results). Of these genes, 26 are directly related to antigen presentation including the development of SLA (swine leukocyte antigen) class II, components of the innate system, and humoral and costimulatory molecules associated with adaptive immunity, all of which may account for the reduced immunogenicity of the E42 developing pancreas.

Vasculature Patterns of the Growing Pancreas

Organs transplanted between phylogenetically disparate species are susceptible to hyperacute (minutes–hours) and acute vascular (days) rejection, leading to thrombosis, interstitial hemorrhage, and severe injury to endothelial cells [28, 29]. Both types of rejection are thought to be initiated by binding of xenoreactive antibodies (Abs) to the endothelium lining the blood vessels of the donor organ and together are now considered the

major barrier to successful clinical application of organ xenotransplantation [28–31].

All mature immunocompetent mammals studied to date have been found to have xenoreactive natural Abs, 85–95 % of them recognize Galα1, 3Gal (Gal), a saccharide expressed on the cells of lower mammals but not humans or apes [32–34].

Recent studies have shown that anti-non-Gal Abs can also activate porcine endothelial cells and may therefore also play a role in xenograft rejection [35]. Moreover, it was shown that both preformed and elicited anti-non-Gal Abs are strongly associated with the pathogenesis of acute vascular rejection in Gal-KO pig-to-primate transplantation models [36–39].

The microvasculature represents the primary target of the immune-mediated injury and also plays a pivotal role in the chemotaxis, activation, adhesion, and emigration of host leukocytes toward the xenografts [40]. Thus, the immunological response to xenografts is conditioned to a large extent by the manner in which the transplant derives its blood supply. In most solid organ grafts (e.g., heart, pancreas, kidney), the vascular system is entirely of donor origin, and nutritive microvascular blood flow is restituted directly after completion of the vascular anastomosis and onset of organ blood perfusion. On the contrary, cell and tissue grafts (e.g., hepatocytes, islets, embryonic pancreatic grafts) require a process

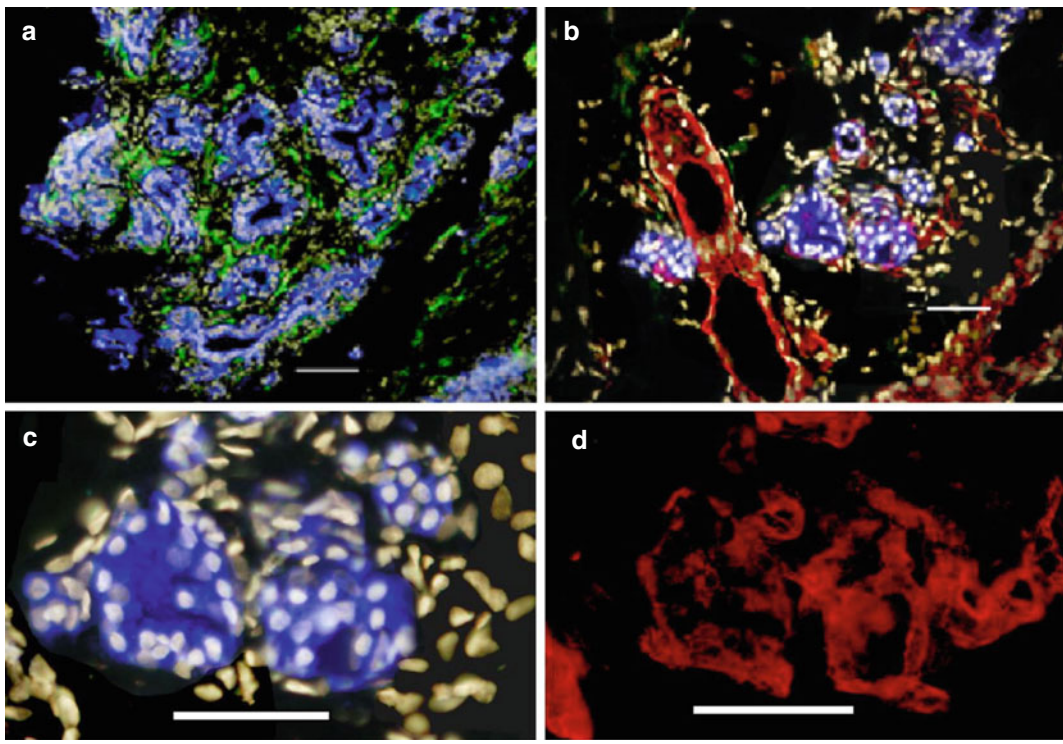

Fig. 31.5 Vascularization pattern of E42 pancreatic tissue. (**a**) Marked expression of α-gal on endothelial but not on epithelial cells in the graft tissue before transplantation, indicated by double staining. Thus, α-gal-positive cells are stained with Banderia simplicifolia isolectin 4 (*green fluorescence*), and fetal epithelial structures are stained with anti-cytokeratin (*blue*). (**b**) Predominant vascularization of the porcine graft by host blood vessels is demonstrated by staining with antihuman CD31 antibody that cross-reacts with monkey but not with pig endothelial cells (*red fluorescence*). Only slight staining of pig endothelial cells was observed with Banderia simplicifolia lectin (*green*). Islet epithelium is outlined by cytokeratin expression (*blue*). (**c**) Higher magnification of anti-cytokeratin-positive islets (*blue*) observed within the marked frame in inset **b**. (**d**) Higher magnification of the vasculature network supporting the islets observed within the marked frame in inset **b**. Double staining with antihuman CD31 (*red*) and Banderia simplicifolia lectin (*green*) reveals predominance of endothelial cells of monkey origin. Nuclei were counterstained by Hoechst yellow (*yellowish*). (Scale bars, 50 μm.) (Taken from Hecht et al. [41])

of angiogenesis and revascularization to reestablish an adequate microcirculation that will provide sufficient and appropriate nutritional blood supply.

Our working hypothesis confirmed in the nonhuman primate model as outlined below (Fig. 31.5) was that the embryonic pancreas, placed without anastomosis, would not be adversely affected by the humoral responses leading to hyperacute and acute rejection.

Engraftment, Growth, and Development of E42 Pig Pancreatic Tissue in Nonhuman Primates: A Proof of Concept

To further assess the curative potential of E42 pig pancreatic tissue, we have evaluated its capacity to correct hyperglycemia under tolerable immunosuppression in an NHP model for diabetes. Considering that xenogeneic transplantation into primates is far more complex than into rodents, due to preexisting anti-porcine antibodies, studies in an NHP model for diabetes were required.

Our group has demonstrated the proof of concept in two diabetic cynomolgus monkeys, followed for 393 and 280 days. Diabetes was induced by the administration of 150 mg/kg of streptozotocin (STZ). About 1 month after STZ administration, E42 porcine tissue was implanted in the omentum. Immunosuppression was induced and maintained using a protocol based on induction with a single dose of anti-CD20 antibody (Rituximab) followed by a short course of ATG and two doses of anti-CD25 antibody (Basiliximab). Immunosuppression was maintained by everolimus, FTY720, and biweekly treatment with CTLA4-Ig (Abatacept).

As can be seen in Fig. 31.5, a marked reduction of exogenous insulin requirement was noted by the fourth month after transplantation, reaching complete independence from exogenous insulin during the fifth month after transplantation, with full physiological control of blood glucose levels. This result is in agreement with our results in the mouse model.

Histological evaluation revealed clusters of islets, with insulin-containing cells, and active proliferating pool of cells within the islets. This observation emphasizes the long-term regenerative capability of the pancreatic tissue, in agreement with the previous mouse studies. Furthermore, only a few incidental lymphocytes were observed, indicating the absence of rejection.

As in the mouse model, the growing tissue was found to be predominantly vascularized with host blood vessels, thereby evading hyperacute or acute rejection, which could potentially be mediated by preexisting anti-pig antibodies. Durable graft protection was achieved, and most of the late complications could be attributed to the immunosuppressive protocol. While fine tuning of immunosuppression, tissue dose, and implantation techniques are still required, our results demonstrate that porcine E42 embryonic pancreatic tissue can normalize blood glucose levels in primates (Fig. 31.6).

Conclusions

Taken together, the tremendous growth capacity, the endocrine predominance, the recruitment of host microvessels, the reduced immunogenicity of the embryonic pancreas and our ability to prevent graft rejection through the use of presently available immunosuppressive agents, and most importantly reversal of diabetes in both mice and NHP strongly suggest that E42 pancreatic tissue transplantation may afford a valuable new option in the field of organ transplantation.

Acknowledgments Y.R. holds the Henry H. Drake Professorial Chair in Immunology and he is the head of the Gabriella Rich Center for Transplantation Biology Research.

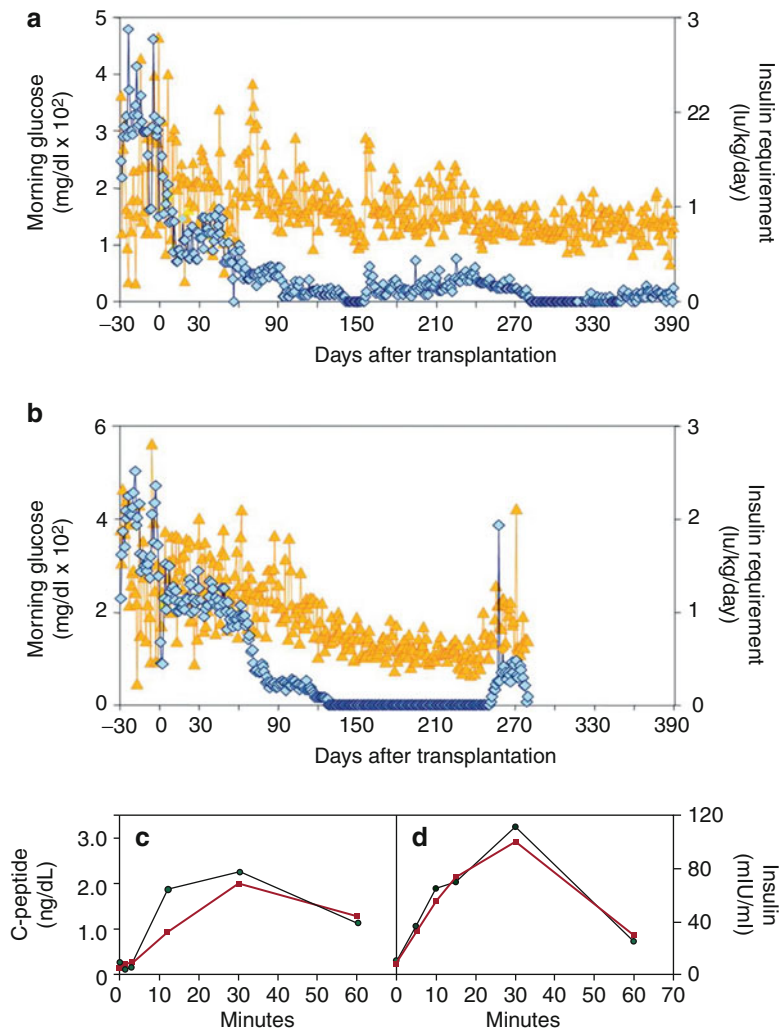

Fig. 31.6 Assessment of E42 fetal porcine xenograft function in streptozotocin-induced diabetic cynomolgus monkeys of cohort 2. Insulin requirement (*blue*) and fasting glucose levels (*yellow*) in monkeys #3 and #4 are shown in panels (**a**) and (**b**), respectively. Specific pig C-peptide (*red*) and insulin (*green*) levels after IVGTT in monkey #3 (**c**) and #4 (**d**) were determined by ELISA specific for pig C-peptide (non-cross-reactive with monkey C-peptide) and for total insulin (using an antibody that does not distinguish between the two species) at 393 and 280 days posttransplant, respectively. *K* values for the clearance of glucose were 1.84 and 2.0, respectively (Taken from Hecht et al. [41])

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Part V

Fetal Organ Transplant Experiments in Animal and Human Systems

Fetal Thymus Transplantation in DiGeorge Syndrome

32

Jean-Louis Touraine

Very soon after the recognition and the description of the two-component lymphoid system [1], fetal thymus transplantation (FTT) has been assayed as a potential therapy for primary or secondary deficiencies of cell-mediated immunity.

This treatment has been felt unsuccessful or giving only partial effects in a number of diseases or conditions, but it has shown a great efficacy in the complete forms of the DiGeorge syndrome [2].

DiGeorge Syndrome

As early as 1965, 4 years after the initial identification of the thymus function in experimental animals [3, 4], Angelo DiGeorge, a Philadelphia endocrinologist, described a rare but most interesting disease [5, 6]. Four infants were reported with congenital hypoparathyroidism, thymic aplasia resulting in faulty cell-mediated immunity, malformations of the aortic arch, and facial abnormalities. This disorder has been shown to be attributable to failure in the normal development of derivatives of the third and fourth pharyngeal pouches.

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Most cases of DiGeorge syndrome are sporadic, but some families have been described with several patients [7, 8].

Various chromosomal abnormalities may be present, including monosomy 10p and 22q11 [9, 10].

Infants with the DiGeorge syndrome have peculiar facial features, with hypertelorism, micrognathia, low-set ears, and shortened philtrum of the upper lip. Malformations of the heart include most commonly interrupted aortic arch, right-sided aortic arch, truncus arteriosus, and tetralogy of Fallot. Hypoparathyroidism results in sometimes severe hypocalcemia. The ossification center of the hyoid bone is absent.

No thymus shadow is seen on chest x-ray. T lymphocytes are very significantly reduced in numbers. Cell-mediated immunity is diminished [11]: peripheral blood lymphocytes respond very poorly to T-cell mitogens, to allogeneic cells, or to antigens. Delayed-type skin hypersensitivity to the various antigens is very low. Infections with microorganisms behaving as "facultative intracellular parasites" frequently develop. Skin allografts have a very delayed rejection. When unirradiated fresh blood is infused into these patients, graft-versus-host disease may occur.

The complete form of the DiGeorge syndrome is relatively rare, but partial forms exist, including some conditions with a mild immunodeficiency, not requiring major treatment.

FTT represents the most efficient treatment in the patients with DiGeorge syndrome when the immunodeficiency is a profound one [2, 12–14].

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It usually results in a rapid restoration of cell-mediated immunity and acquired resistance to infections. By contrast, the patients with a mild to moderate immunodeficiency benefit less from this treatment, probably because they have enough immunocompetent T lymphocytes to reject the transplanted allogeneic thymus. Partial improvement of their moderately altered immunity has seemingly been obtained in a few patients after repeated injections of thymic factors of various kinds [15–17].

FTT in Patient SP

Following the two initial successes of FTT in DiGeorge syndrome [12, 13], we treated one patient in Lyon using fresh fetal thymus tissue sent to us from London [14].

SP was born on May 7, 1974. His parents, his brother, and his sister were healthy. He was admitted in the Department of Pediatrics at 2 days of age because of tetany. Calcemia decreased day after day, down to 50 mg/L. Vitamin D and infusions of calcium were given to the patient.

Rapidly a right pneumonia and a purulent rhinitis were diagnosed and treated with antibiotics.

At 3 months of age, the DiGeorge syndrome was obvious, with peculiar facies including low-set ears, hypoparathyroidism (calcemia 65 mg/L, phosphoremia 70 mg/L, alkaline phosphatases 223 mIU/mL, calciuria 0.5 mg/kg/24 h, phosphaturia 30 mg/kg/24 h, and x-ray manifestations of hypoparathyroidism), infections of the respiratory system, and immunodeficiency (lymphopenia, 15 % of T lymphocytes among peripheral blood lymphocytes, decreased response to concanavalin A 25 % of normal – and to other T-cell stimuli). No thymus shadow was seen on any x-ray.

Fortunately, no significant malformation of the cardiovascular system was present.

Using a test of in vitro differentiation of T-cell precursors into lymphocytes with some phenotypic characteristics of the T-lineage [18], we could demonstrate the normal presence of T-cell precursors in the bone marrow of SP as we did in other patients with complete or partial DiGeorge syndrome [19, 20].

On October 11, 1974, a FTT was carried out. The donor was a female fetus of 12 weeks of age. The fresh organ was provided to us by the London fetal bank (Dr. S.D. Lawler, Royal Marsden Hospital), with respect of ethical considerations. It was flown rapidly to Lyon. Cell viability was found to be 95 %. Small pieces of this fetal thymus were surgically implanted in abdominal muscles of the infant.

Clinical improvement was rapid: after a couple of weeks, infections of the respiratory system disappeared. No side effect of any kind occurred.

Immunological amelioration was also rapid: within 1 month, T lymphocytes reached the normal range and continued to increase to stabilize at 60 % of peripheral blood lymphocytes (Fig. 32.1). In vitro response to concanavalin A increased more progressively over 18 months (Fig. 32.2).

The T lymphocytes of this patient derived from the recipient's precursor cells (with the XY chromosomes) [14]. The differentiation of T-cell precursors into mature T lymphocytes under the influence of the transplanted thymus (and most likely within this thymic tissue) developed following sequential stages [21].

Since this period, SP has been healthy, experiencing no significant infection nor any decrease of the immune reconstitution. A picture of him shows his satisfactory aspect, 1 year after the transplant (Fig. 32.3). The only therapy required was calcium and vitamin D. More than 30 years later, SP remains in good health.

FTT in Other Patients

Additional patients have been reported with successful FTT [2, 12, 13, 22–25]. In all cases, the restoration of cell-mediated immunity occurred rapidly in the months following the transplant. It remained at a normal level in all patients, although a slight decrease in T-cell numbers was observed in some of them at 5 years. Five of these patients have a long-lasting (over 20 years) beneficial effect. In Lyon, we have treated more recently five additional patients with the DiGeorge anomaly [26]. One of them died of cardiopathy and the

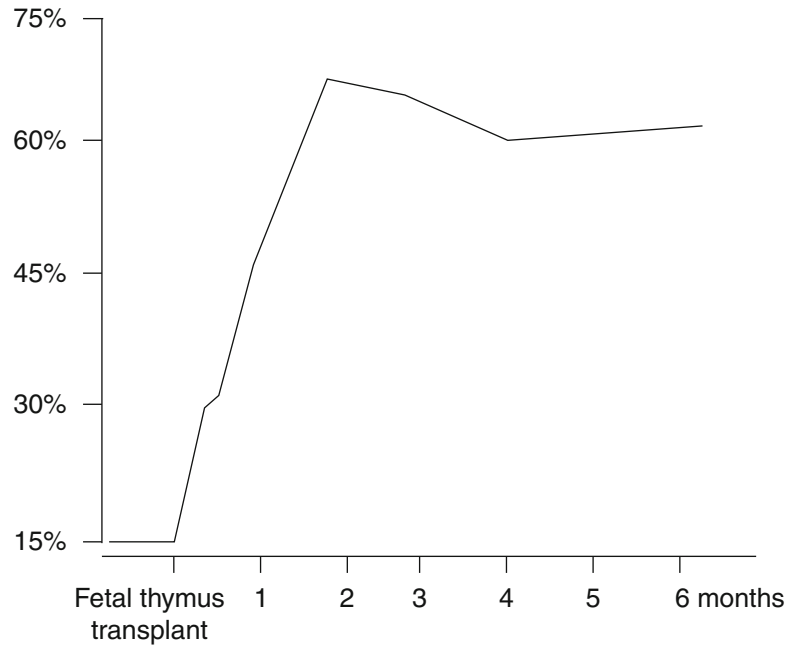

Fig. 32.1 Percentage of T lymphocytes in peripheral blood of patient SP following FTT

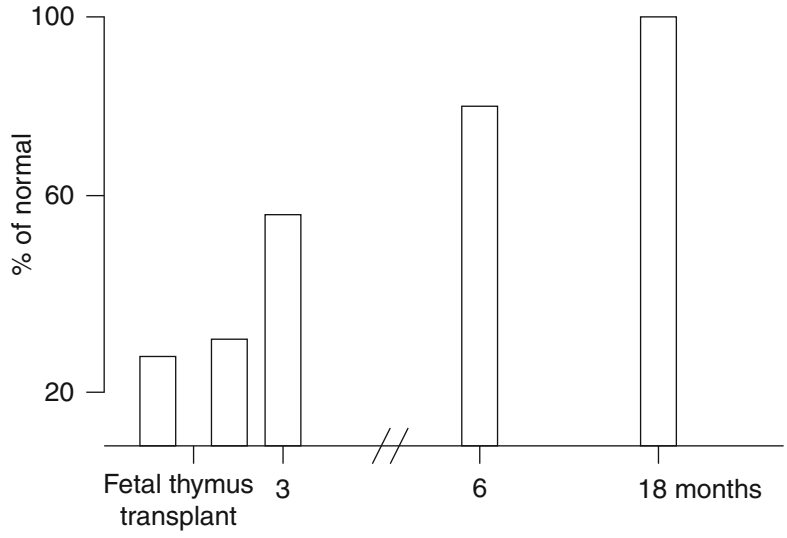

Fig. 32.2 Proliferative response of peripheral blood lymphocytes to concanavalin A, expressed in percent of normal response. Lymphocytes from patient SP were investigated before and after FTT

other patients have been cured of their congenital immune defect.

FTT therefore appears to be the treatment of choice for patients with DiGeorge syndrome, having a profound defect of T-lymphocyte numbers and functions. The beneficial effect is important and long lasting, procuring immune defenses against infections to the patients. The thymus of fetal origin provides the reticulo-epithelial cells that are required for the differentiation of the patient's own precursor cells. Fetal thymuses of the first trimester do not include yet immunocompetent thymocytes, therefore, reducing the risk of graft-versus-host disease that could occur in these patients if more mature thymuses were used.

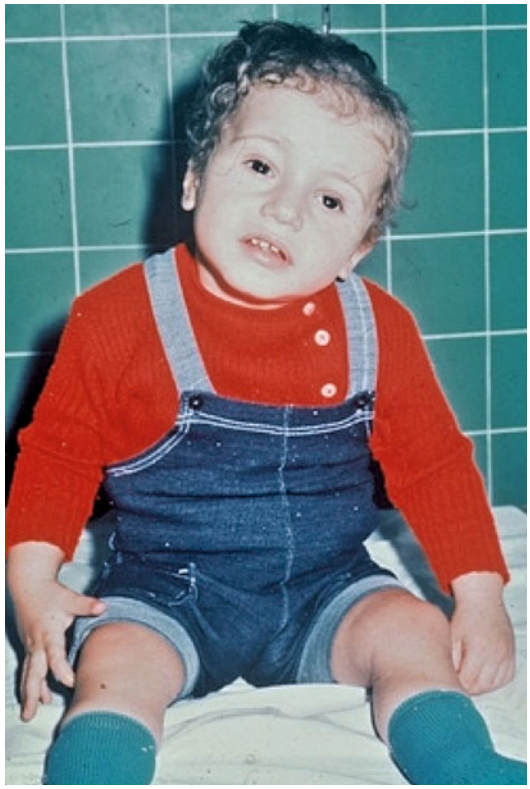

Fig. 32.3 Picture of SP, a patient with the DiGeorge syndrome, one year after FTT

Other Transplants

Besides FTT and administration of thymic factors [15–17], other therapies have been investigated in patients with DiGeorge syndrome. The cell therapies included transplantation of cultured thymus tissue from infants undergoing heart surgery [27, 28], bone marrow transplantation, cord blood transplantation, and peripheral blood cell transplantation [29–31]. Several risks, including graft-versus-host disease, can be associated with these procedures [32]. Measures aimed at decreasing the number of immunocompetent T lymphocytes in the transplants reduce such a risk. A number of successful immune reconstitution have been observed and, in the transplants of cultured postnatal thymus tissue, the absence of graft-versus-host reaction might be related to immaturity of the T cells from the neonatal thymus and/or to the culture period that results in reduction of thymocytes while reticulo-epithelial cells persisted. It should however be noted that circulating donor T cells have been transiently detected in some of these patients [27].

Conclusion

Although several kinds of treatment can be proposed to patients with the DiGeorge syndrome, the most effective and well tolerated is FTT. It represents the correction of the thymic defect; it enables rapid maturation of the patient's own precursor cells, and it has a long-lasting effect on immune reconstitution in these patients.

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33 Clinical Improvement After First-Trimester Fetal Whole Pancreas Transplant at a Heterotopic Site in Uncontrolled Diabetes with Varying Degrees of Skin Ulceration of the Leg and Emaciation

Niranjan Bhattacharya

Introduction

Vascular complications do occur in many patients, despite the best efforts of physicians and diabetic patients in the use of insulin for control of juvenile-onset (insulin-deficient) diabetes. There are several claimed advantages in scientific circles about the transplantation of a whole fetal pancreas as a donor organ in cases of end-state diabetes when the patient is totally dependent on insulin. Other options have also been suggested like the use of fetal stem cells with relatively low immunogenic and tumorigenic potential. These cells are also attractive candidates for transplantation. Apart from this option, use of specifically isolated pancreatic progenitor cells (PPCs) derived from the human fetal pancreas, which are amenable to growth and differentiation into transplantable insulin-producing islet-like cell clusters (ICCs) have been reported recently; however, the immunological nature of these cells has yet to be characterized [1]. The human fetal pancreas has a remarkable capacity to grow and differentiate in vivo and has been shown to reverse diabetes in rodents. However, it is well known that human fetal pancreas obtained from the second trimester of gestation is immunogenic and is rejected after transplantation. Tissue obtained from earlier stages may prove to be immune privileged, as has been shown for other tissues [2].

Streptozotocin-induced diabetes in rats was completely reversed by transplantation of syngeneic fetal pancreases placed beneath the kidney capsule. In xenotransplant model, transplantation of fetal porcine islet-like cell clusters (ICC) was shown to reverse diabetes in experimental animals by Groth et al. [3]. The insufficient supply of tissue, loss due to any reason of transplantation, and limited potential for expansion of β cells restrict the use of islet allotransplantation for diabetes. A way to overcome the supply and expansion problems is to xenotransplant embryonic tissue [4]. Human fetal pancreas in mice model has been attempted earlier by Prof. Tuch, who concluded that only a small component of human fetal pancreas consists of β cells, and yet this tissue is capable of normalizing the blood glucose levels of diabetic recipients when transplanted. The time taken to achieve this goal is several months, during which time the tissue proliferates and eventually differentiates into β cells [5]. The present study is intended to observe whether HLA (human leukocyte antigen)-randomized first-trimester fetal pancreatic transplantation can help to control diabetes through the growth and activity of the B-cell precursors with the assistance of pancreatic

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mesenchymal, epithelial, and other undifferentiated stem cells and also stem cell like primitive undifferentiated cells with the supporting fetal cytokine network. As all the patients in the present study had multiple complications of uncontrolled diabetes, the present investigator wished to attempt control of diabetes through the apparently universal nonspecific positive effects of fetal tissue transplantation as has been discussed in another chapter.

Material and Method

Necessary informed consent from all concerned and ethical permission from the institute-based ethical committee, which was formed as per the guidelines of the Indian Council of Medical Research, was obtained before the actual procedure was begun. Next, the patients were preliminarily screened for hepatitis B and C and HIV 1 and 2 along with hemoglobin, total count, differential count, and platelet count; assessment of ESR, liver function test, and tests for urea, creatinine, fasting, and postprandial sugar were also done; lipid profile, chest x-ray (DNA PCR in case of suspicion of Koch's infection), ECG (echocardiography in case of ECG problem), C-reactive protein, antinuclear antibody, anti-(DS) DNA, and T3, T4, and TSH were assessed as well. These tests were conducted to screen the patient for suitability for the transplantation procedure.

Glycosylated hemoglobin (HbA_{1c}) is a form of hemoglobin which is measured primarily to identify the average plasma glucose concentration over prolonged periods of time. This serves as a marker for average blood glucose levels over the previous months prior to the measurement. Laboratory results may differ depending on the analytical technique, the age of the subject, and the biological variation among individuals. Two individuals with the same average blood sugar levels can have HbA_{1C} values that differ by as much as 3 % points. Results can be unreliable in many circumstances, such as after blood loss, after surgery, blood transfusions, anemia, or high erythrocyte turnover; in the presence of chronic renal or liver disease; after administration of high-dose vitamin C; or erythropoietin treatment [6]. In general, the reference range (that is found in healthy persons) is about 20–40 mmol/mol (4–5.9 %) [7].

In case of long-standing diabetes, there is likelihood of an involvement of the kidney. This is known as diabetic nephropathy where there is albumin present in the urine. This phenomenon can be an indicator of damage to the kidneys. Causes of albuminuria can be graded by the amount of protein excreted. The nephrotic syndrome usually results in the excretion of about 3.0–3.5 g per 24 h. Microalbuminuria (between 30 and 300 mg/24, mg/L of urine) can be a forerunner of diabetic nephropathy. In the present study, the clinical effect of preimmune (first-trimester) fetal pancreatic heterotopic transplant and its effect on glycosylated hemoglobin and loss of albumin are calculated serially to understand the outcome of transplantation on the host.

Sixteen patients (male = 11 and female = 5, age varying from 39 to 72 years, mean 49.6 years 3.4 ± years S.D) were enrolled in the fetal first-trimester (9–12 weeks, mean 10.4 weeks with ±1.2 weeks S.D) whole pancreas transplant program. They had reported to the government hospital for free treatment, with poor general condition, uncontrolled diabetes, gangrene of the leg, and emaciation. After taking high-medical risk consent from the patients'/guardians' and permission of the institution-based ethical committee for both collection of the pancreas and its transplant in the selected patients, fetal pancreases were collected from donors, that is, mothers who already had two or more healthy living children and were undergoing hysterotomy and ligation for family planning purposes.

The fetal pancreas was dissected out within minutes of hysterotomy and collected in a petri dish containing normal saline. This was placed immediately at a locally anesthetized (4–5 cc 1 % Xylocaine infiltrated) area at the axilla (2–3 cm length and 2–3 cm in breadth, with blunt dissection of the subcutaneous space around the incision) of the host who had uncontrolled diabetes and other complications.

This area had been shaved previously and sterilized with Betadine and 100 % rectified spirit solution before the placement of the fresh human fetal pancreas tissue. It was subsequently closed with small interrupted (00) atraumatic chromic catgut with a cutting needle. No prophylactic antibiotics were given, but the patient did receive postoperative analgesic paracetamol 1–3 tablets/day for 2–3 days for symptomatic relief.

After 3 months from the date of placement of the fetal pancreatic tissue, a part of the tissue was retrieved for cellular study and microscopy with suitable staining to examine whether there was any inflammatory or immunological reaction in the host's system as a result of the transplant of the donated tissue.

Result and Analysis

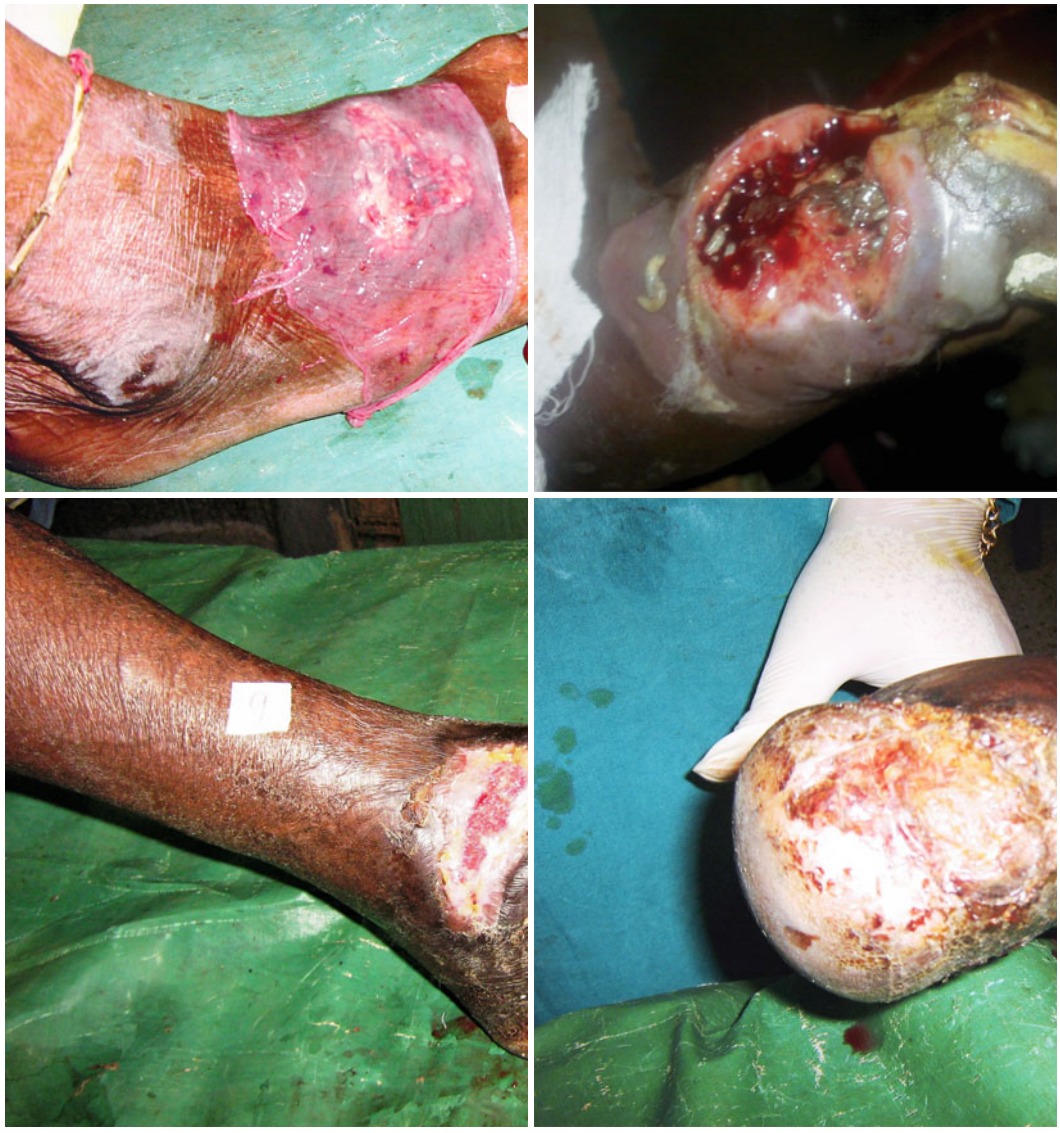
As already mentioned in the "Material and Method" section, 16 patients (male = 11 and female = 5, age varying from 39 to 72 years, mean 49.6 years 3.4 ± years S.D), who reported to the government hospital for free treatment, with poor general condition, uncontrolled diabetes, gangrene of the leg, and emaciation, were enrolled in the fetal first-trimester (9–12 weeks, mean 10.4 weeks with ±1.2 weeks S.D) whole pancreas transplant program.

Questions may be asked regarding why this particular method of fetal tissue transplant was chosen to treat these patients. One answer is that these were very poor patients who could not afford regular insulin treatment following the schedule of two to four times injections daily. They could not also afford the supportive treatment of aspirin, atorvastatin, ramipril, or follow the diet and lifestyle regulations necessary for the control of diabetes (Figs. 33.1, 33.2, 33.3, and 33.4 with wounds in the leg one wound infested maggots are shown to give a visual impression of poor patients. The failure to follow normal treatment procedures or comply with medical advice led to a deterioration of the clinical condition. Moreover, 3 out of the 16 patients had a reactivation of prior tuberculosis conditions, and 2 of the 16 had progressively deteriorating renal function. Since they did not/could not afford to follow normal advice/prescriptions, they were included in the first-trimester HLA (human leukocyte antigen)-randomized pancreatic transplantation, with their informed consent (Tables 33.1 and 33.2).

In the present series the glycosylated hemoglobin varied from 8 to 12 pre-transplant, mean 10.38 ± 1.8 S.D; however, the results came down to 6.8–9.8, mean 8.26 ± 2.1 S.D within 1 month. This came down to 6.3–9.2, mean 7.5 ± 1.6 S.D within 2 months of transplantation. Subsequently, on the third month evaluation, the level came down further to 5.4–7.6 with mean 6.34 ± 1.2 % S.D. The HLA-randomized post-transplant effect in the reduction process of glycosylated hemoglobin was seen to be gradual but uniform.

Higher levels of HbA_{1c} are found in people with persistently elevated blood sugar, as in diabetes mellitus. While diabetic patient treatment goals vary, many include a target range of HbA_{1c} values. A diabetic person with good glucose control has an HbA_{1c} level that is close to or within the reference range. It is formed in a nonenzymatic glycation pathway by the hemoglobin's exposure to plasma glucose. Normal levels of glucose produce a normal amount of glycosylated hemoglobin. As the average amount of plasma glucose increases, the fraction of glycosylated hemoglobin increases in a predictable way.

Similarly, the pre-transplant albumin level in 24-h urine in the present series was within the range of 800–2,100 mg, mean 1,259 ± 247.6 mg S.D. However, the posttransplant value as assessed after 1 month suggested a definite reduction to a range of 400–1,300 mg/24-h urine with mean 789.38 ± 154.4 mg S.D. Subsequent follow-up after the second month suggested the continuation of the same trend with the range of albumin varying from 200–900 mg with mean 565 ± 86.9. This value showed a further reduction, reaching a range of 120–600 mg with mean value 321.25 ± 44 ± 82. Hence, hypoantigenic fetal pancreas transplant in this series suggested a gradual and progressive fall of albumin excretion through urine, thereby justifying a reduction in the nephropathic condition of the host.



Figs. 33.1, 33.2, 33.3, and 33.4 Wounds in the leg with one wound infested with maggots are shown to give a visual impression of poor patients. All wounds of the leg were treated with freshly collected amniotic membrane after cesarean section taking all precautions and safety protocol, as mentioned in other chapters by the same investigator

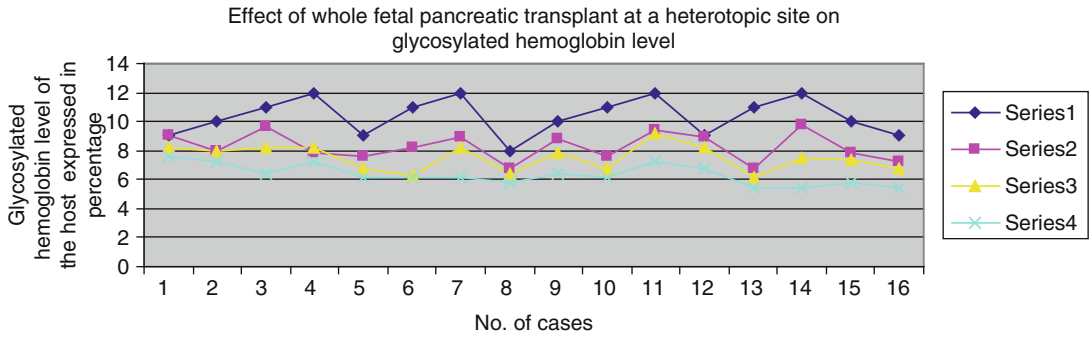
Along with the control of glycosylated hemoglobin or urinary excretion of the albumin, all the patients had a generalized gain in weight and improvement and progressive healing of leg ulceration, though regular and periodic dressing with freshly collected amniotic membrane was used for that purpose (Figs. 33.1 and 33.2). The present researcher has earlier reported on the positive effect of amniotic membrane: it harbors mesenchymal and epithelial stem cells and thus plays a very effective role in healing of burn wounds [9]. A sense of well-being was also noted among the fetal pancreatic recipients. All the patients cooperated with the advice of the consultant in implementing lifestyle modification suggestions gradually for long-term benefit.

Table 33.1 The effect of HLA-randomized first-trimester fetal pancreas transplant on hosts with diabetes and emaciation. The approximate mapping between HbA_{1c} values is given in DCCT percentage (%)

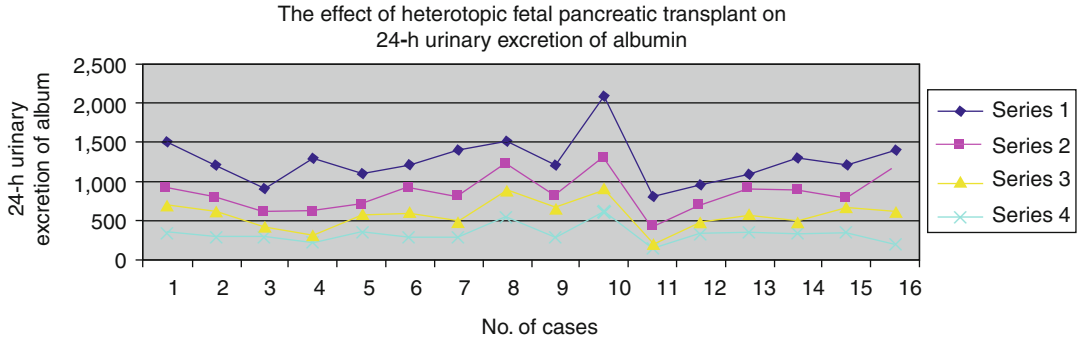
Sr no, age, sex	Basic treatment offered (a): act rapid monocomponent insulin 6 hourly in sliding scale, (b) aspirin 75, (c) atovorstatin 20 mg, (d) ramipril 5–10 mg/day with (e) vitamins, minerals, and (f) diabetic diet	Glycosylated hemoglobin (HbA_{1c}) Pre-transplant (%)	Glycosylated hemoglobin (HbA_{1c}) 1st month after first-trimester pancreas transplant (%)	Glycosylated hemoglobin (HbA_{1c}) 2nd month after first-trimester pancreas transplant (%)	Glycosylated hemoglobin (HbA_{1c}) 3rd month after first-trimester pancreas transplant (%)
1, 52, H, M	a,b,c,d,e,f	9	9	8.2	7.6
2,59, H, M	a,b,c,d,e,f	10	8	8	7.2
3,56, H, M	a,b,c,d,e,f	11	9.6	8.2	6.4
4,42, H, F	a,b,c,d,e,f	12	7.8	8.2	7.2
5,62, H, M	a,b,c,d,e,f	9	7.6	6.8	6.2
6,72, H, M	a,b,c,d,e,f	11	8.2	6.3	6.2
7,52, H, M	a,b,c,d,e,f	12	8.9	8.2	6.2
8,58, H, M	a,b,c,d,e,f	8	6.8	6.4	5.8
9,62, H, M	a,b,c,d,e,f	10	8.8	7.8	6.4
10,48, H, M	a,b,c,d,e,f	11	7.6	6.8	6.2
11,55, H, M	a,b,c,d,e,f	12	9.4	9.2	7.2
12,62, H, M	a,b,c,d,e,f	9	8.9	8.2	6.8
13,46, H, F	a,b,c,d,e,f	11	6.8	6.2	5.4
14,52, H, F	a,b,c,d,e,f	12	9.8	7.5	5.4
15,53, H, F	a,b,c,d,e,f	10	7.8	7.2	5.8
16,39, H, F	a,b,c,d,e,f	9	7.2	6.8	5.4

Table 33.2 The effect of HLA-randomized first-trimester fetal pancreas transplant on hosts with diabetes and emaciation. Microalbuminuria is diagnosed either from a 24-h urine collection (between 30–300 mg/24 h) or more commonly from elevated concentrations in a spot sample (30 to 300 mg/L). Both must be measured on at least two of three measurements over a two- to three-month period [8]. An albumin level above these values is called "macroalbuminuria," or sometimes just albuminuria.

Sr no, age, sex	Basic treatment offered (a): act rapid monocomponent insulin 6 hourly in sliding scale, (b) aspirin 75, (c) atovorstatin 20 mg, (d) ramipril 5–10 mg/day with (e) vitamins, minerals, and (f) diabetic diet	Pre-transplant Albumin in urine/24 h Microalbuminuria 30–300 mg/24-h urine Macroalbuminuria >300 mg in 24-h urine	1st month after first-trimester pancreas transplant (albumin in urine/24 h)	2nd month after first-trimester pancreas transplant (albumin in urine/24 h)	3rd month after first-trimester pancreas transplant (albumin in urine/24 h)
1,52, H, M	a,b,c,d,e,f	1.5 g/24-h urine	900 mg/24-h urine	700 mg/24-h urine	350 mg/24-h urine
2,59, H, M	a,b,c,d,e,f	1.2 g/24-h urine	780 mg/24-h urine	600 mg/24-h urine	280 mg/24-h urine
3,56, H, M	a,b,c,d,e,f	900 mg/24-h urine	600 mg/24-h urine	400 mg/24-h urine	280 mg/24-h urine
4,42, H, F	a,b,c,d,e,f	1.3 g/24-h urine	600 mg/24-h urine	300 mg/24-h urine	220 mg/24-h urine
5,62, H, M	a,b,c,d,e,f	1.1 g/24-h urine	700 mg/24-h urine	556 mg/24-h urine	340 mg/24-h urine
6,72, H, M	a,b,c,d,e,f	1.2 g/24-h urine	900 mg/24-h urine	600 mg/24-h urine	290 mg/24-h urine
7,52, H, M	a,b,c,d,e,f	1.4 g/24-h urine	800 mg/24-h urine	480 mg/24-h urine	280 mg/24-h urine
8,58, H, M	a,b,c,d,e,f	1.5 g/24-h urine	1,200 mg/24-h urine	890 mg/24-h urine	540 mg/24-h urine
9,62, H, M	a,b,c,d,e,f	1.2 g/24-h urine	800 mg/24-h urine	640 mg/24-h urine	290 mg/24-h urine
10,48, H, M	a,b,c,d,e,f	2.1 g/24-h urine	1,300 mg/24-h urine	900 mg/24-h urine	600 mg/24-h urine
11,55, H, M	a,b,c,d,e,f	800 mg/24-h urine	400 mg/24-h urine	200 mg/24-h urine	120 mg/24-h urine
12,62, H, M	a,b,c,d,e,f	950 mg/24-h urine	680 mg/24-h urine	470 mg/24-h urine	320 mg/24-h urine
13,46, H, F	a,b,c,d,e,f	1.1 g/24-h urine	900 mg/24-h urine	560 mg/24-h urine	340 mg/24-h urine
14,52, H, F	a,b,c,d,e,f	1,3 g/24-h urine	890 mg/24-h urine	480 mg/24-h urine	330 mg/24-h urine
15,53, H, F	a,b,c,d,e,f	1.2 g/24-h urine	780 mg/24-h urine	670 mg/24-h urine	340 mg/24-h urine
16,39, H, F	a,b,c,d,e,f	1.4 g/24-h urine	1,200 mg/24-h urine	600 mg/24-h urine	220 mg/24-h urine



Graph 33.1 The effect of HLA-randomized fetal pancreas transplant on the host's glycosylated hemoglobin level. *Series 1*: Pre-transplant level of glycosylated hemoglobin. *Series 2*: 1-month posttransplant level of glycosylated hemoglobin. *Series 3*: 2-month posttransplant level of glycosylated hemoglobin. *Series 4*:3-month transplant level of glycosylated hemoglobin



Graph 33.2 The effect of HLA-randomized fetal pancreas transplant on the host's urinary excretion of albumin. *Series 1*: Pre-transplant level of albumin excretion in 24-h urine. *Series 2*: 1-month posttransplant level of albumin excretion in 24-h urine. *Series 3*: 2-month posttransplant level of albumin excretion in 24-h urine. *Series 4*: 3-month transplant level of albumin excretion in 24-h urine

Discussion

Scientists all over the world are trying to produce an alternative treatment protocol for insulin dependence either by shifting the type of insulin to a long-acting variety or by attempting to improve insulin synthesis or its sensitization. There are also attempts to use insulin in an inhaler mode. However, these attempts may not be sufficient: in order to achieve the goal of lessening insulin dependence, we have to think out of the box.

Lampe et al. in 1975 attempted transplantation of islet cells in an animal model [10]. A registry for human pancreas and islet transplantation has been established subsequently (D. E. R. Sutherland). Between 17 December 1966 and 31 December 1980, 108 pancreas transplantations were performed [11].

Prof. B. Tuch (a contributing author in the present volume) attempted xenotransplantation through the use of a human fetal pancreas in an athymic nude mice model [12]. There are cases of normalization of blood glucose in athymic mice model by transplantation of pig's fetal pancreatic pro-islet or islet cell-like cluster. Identifying a limitless source of β cells that survive transplantation into a neovascularized site and provide normal blood glucose control remains an important goal in the development of pancreatic islet. A recent study has suggested that fetal pig islet tissue has the potential to mature and function normally in a neovascularized site, thereby avoiding the innate immune destruction

that occurs when islet tissue is exposed directly to the circulation [13].

Although this is a significant advance in knowledge, islet replacement therapy is obstructed by a shortage of donor islet cells. Usage of islet cells derived from porcine pancreatic stem cells (PSCs) is currently viewed as the most promising alternative for human islet transplantation. However, PSCs are rare and have a finite proliferative lifespan. In another study, investigators have isolated and established an immortalized mesenchymal stem cell (MSC) line derived from fetal porcine pancreas by attaching human telomerase reverse transcriptase (hTERT) and have named these immortalized pancreatic mesenchymal stem cells (iPMSCs) [14].

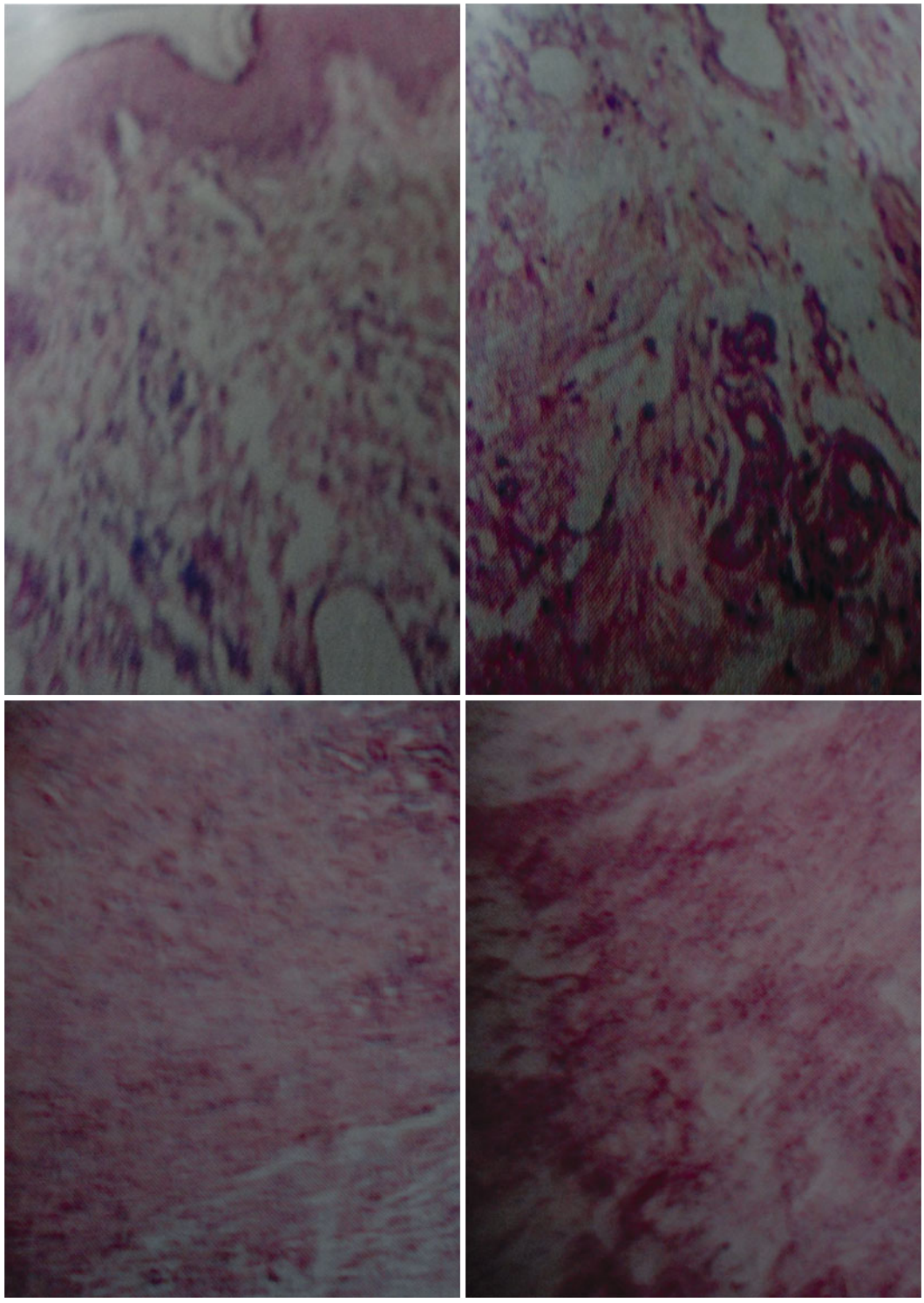
Fetal pancreas tissue contains beta cells and other precursor and progenitor cells at different states of growth and differentiation along with connective tissue like elastin, collagen, and matrix. This extracellular matrix (ECM) of growing fetal tissue is a very important component of stem cell niche areas. ECM is a complex-interlinked composite of collagenous molecules, non-collagenous molecules, and water-rich mucopolysaccharide ground substance. Cells are integrated to their matrix via integrin and non-integrin receptors, which are utilized in the control of adhesion, migration, division, growth, anoikis, transdifferentiation, and other cellular behavior. ECM provides architecture and strength as well as growth factor deposits, [15], which proteinases as signaling scissors and can release in a site- and process-specific manner. Each process is essential for the regeneration.

In the present study, where human subjects have been used, HLA-randomized first-trimester fetal human whole pancreas was freshly dissected and placed immediately in the recipient's heterotopic site under the arm (axilla). Here, 16 patients, who had reported to the government hospital for free treatment, with poor general condition, uncontrolled diabetes and gangrene of the leg, and emaciation (male = 11 and female = 5, age varying from 39 to 72 years, mean 49.6 years 3.4 ± years S.D), were enrolled in the fetal first-trimester (9–12 weeks, mean 10.4 weeks with ±1.2 weeks S.D) whole pancreas transplant program. There was no problem during or after the procedure. There was no infection or rejection of the graft. Histology from the small amounts of tissue retrieved later from the patents' axilla suggested no infiltration of inflammatory cells (neutrophilic) or local immune reaction (lymphocytic), and this can be seen in Figs. 33.5, 33.6, 33.7, and 33.8. On the other hand, there were some positive effects, which are noted below:

(a) As mentioned earlier in the result and analysis section, the glycosylated hemoglobin level showed marked improvement, that is, the pre-transplant mean 10.38 ± 1.8 S.D came down to posttransplant mean 8.26 ± 2.1 S.D within 1 month and the trend continued unabated.
(b) Similarly, the pre-transplant albumin level in 24-h urine was mean 1,259 ± 247.6 mg S.D before the transplant process; the posttransplant value as assessed after 1 month suggested a definite reduction, that is, mean 789.38 ± 154.4 mg S.D. Subsequent follow-up after the second month suggested the continuation of the same trend as mentioned earlier.
(c) Along with the control of glycosylated hemoglobin and urinary excretion of albumin, all the patients had a generalized gain in weight and improvement and progressive healing of the leg ulceration, though regular and periodic dressing with freshly collected amniotic membrane was used for that purpose.

The question that can be raised here is why did these improvements occurred. These are actually secondary advantages of human fetal tissue transplantation, and the present investigators have noted and reported similar improvement in similar exercises involving human fetal neuronal tissue transplantation. They have also reported earlier on the safety aspects of fetal tissue transplantation [16]. These nonspecific effects include: (a) rise of hemoglobin, reduction of aches and pain all over the body, which is due to preexisting disease background, or superimposed different factors either singularly or in combination which we have discussed in earlier papers by the author.

Groth et al. [17] transplanted porcine ICC (fetal porcine islet-like cell clusters) to ten



Figs. 33.5, 33.6, 33.7, and 33.8 This is a microphotograph of a 12-week fetal pancreatic transplant (HLA-randomized), as seen in the partially retrieved tissue from the under surface of the arm (axilla). What is interesting is the absence of any inflammatory (leukocytic infiltration) or immunological (lymphocytic infiltration) cell migration at the site of the retrieved fetal pancreatic tissue, as seen in the hematoxylin- and eosin-stained histology picture

insulin-dependent diabetic kidney-transplant human patients who received standard immunosuppression and, at ICC transplantation, antithymocyte globulin or 15-deoxyspergualin. ICC were injected intraportally or placed under the kidney capsule of the renal graft. Four patients excreted small amounts of porcine C-peptide in urine for 200–400 days. In one renal-graft biopsy specimen, morphologically intact epithelial cells stained positively for insulin and glucagon in the subcapsular space. The authors concluded that porcine pancreatic endocrine tissue can survive in the human body. In another set of experimentation in rat model, the investigators [18] suggested that in streptozotocin-induced diabetes, to accomplish complete reversal of diabetes, four or more pancreases were necessary; three resulted in partial reversal, and two produced a slight but significant effect in some recipients. Removal of the transplants resulted in the prompt return of diabetes. The islets of Langerhans in the transplants functioned homeostatically; this was indicated by regular normal blood glucose values, in addition to normal findings in blood IRI response and glucose disappearance rate after glucose injection.

Another investigator has suggested that after transplantation of one fetal rat pancreas into a diabetic *rat* recipient, maturation and growth of the transplant was adequate for complete reversal of the diabetic state of the recipient. Because of the atrophy of exocrine elements after transplantation of the fetal organ, many of the technical problems inherent in adult pancreas transplants are avoided [19].

Although the human fetal pancreas early in the second trimester of pregnancy contains functional beta cells, its ability to release insulin in response to glucose is either poor or lacking. It is tissue of this age which usually has been grafted into diabetic humans, in unsuccessful attempts so far, to reverse the hyperglycemic state [20].

Summary and Conclusion

On the basis of the results of the study and their analysis, the present investigator suggests that human fetal pancreatic transplantation at a heterotopic site (axilla) may actually reverse the process of degeneration and complications associated with diabetes. This study further suggests that patients of maturity onset diabetes who are not responding to the globally standardized method of treatment with soluble insulin, angiotensin inhibitors if needed, dietary regulation, modification of lifestyle and exercise, etc., may respond favorably to the HLA-randomized fetal pancreas tissue transplant at a heterotopic site.

The improvement recorded in the present study may be due to the participation of the stem cell and progenitor cell component of the fetal pancreatic tissue in its natural niche till there is growth of beta cells or its microenvironment cytokines. Both the cytokine network and the stem and the progenitor cells associated with fetal pancreatic tissue may actually participate in the process of regeneration of the functional pancreatic cells. Thus, it reverses the impact of degeneration which had been caused by the chronic deprivation process of insulin and supporting insulin-like substances in the host The improvement in leg ulceration could also be due to the nonspecific effects of fetal tissue transplantation [16] or due to the properties of the amniotic membrane which was used to dress the ulcers, with the chorionic side of the amniotic membrane helping in the growth of vascularity and the amniotic side helping in the epithelization process through the epithelial stem cell component of the amniotic membrane. The investigators have reported on this aspect earlier [9].

Future Trends

The hallmark of type 1 diabetes is specific destruction of pancreatic islet β cells. Apoptosis of β cells may be crucial at several points during disease progression, initiating leukocyte invasion of the islets and terminating the production of insulin in islet cells. β-cell apoptosis may also be involved in the occasional evolution of type 2 into type 1 diabetes [21]. Nonavailability of organs in adequate numbers is an eternal problem of human islet transplantation. The alternative source could be pancreatic cells from genetically

modified pigs or from autologous stem cells, provided that an adequate microenvironment is created for those cells.

Remodeling of pancreatic duct cells into beta cells could also be a feasible option. In type 2 diabetics, transformation of autologous stem cells isolated from the peripheral blood into HLA-identical islet cells is another option if the autoimmune response is controlled adequately.

For the last several decades, much of the work in the field has focused on the pancreatic epithelium – but in recent years, mesenchyme, a gelatinous mass of cells in the embryo that surrounds the developing pancreas and eventually forms much of the body's connective tissue, has also been an object of focus.

The mesenchyme's role in diabetes has been something of a mystery. Even so, scientists have known for a few years that the mesenchyme provides some sort of chemical signals that drive early development of the pancreas [22].

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34 Experience with Human Fetal Thymus Transplantation In a Heterotopic Site in Patients with Advanced Lymphoma and Leukopenia

Niranjan Bhattacharya

Introduction

Thymus transplantation is a promising investigational therapy for infants born with no thymus. Because of the athymia, these infants lack T cell development and have a severe primary immunodeficiency. Although thymic hypoplasia or aplasia is characteristic of DiGeorge anomaly, in "complete" DiGeorge anomaly, there is no detectable thymus as determined by the absence of naive (CD45RA(+), CD62L(+)) T cells [1]. Research continues on mechanisms underlying immune reconstitution after thymus transplantation. In some infants with profound immunodeficiency and complete DiGeorge syndrome, the transplantation of thymus tissue can restore normal immune function. Early thymus transplantation – before the development of infectious complications – may promote successful immune reconstitution [2]. Complete DiGeorge syndrome is a fatal condition in which infants have no detectable thymus function. The optimal treatment for the immune deficiency of complete DiGeorge syndrome has not been determined. Safety and efficacy of thymus transplantation were evaluated in 12 infants with complete DiGeorge syndrome who had less than 20-fold proliferative responses to phytohemagglutinin. The investigators suggested that thymic transplantation is efficacious, well tolerated, and should be considered as treatment for infants with complete DiGeorge syndrome [3]. In the past 25 years, revelations on the genesis of human cancer have come at an increasing pace. Research on oncogenic infectious agents, especially viruses, has helped us to understand the process of malignant transformation of cells because of the cellular events in viral-driven transformation mirror. Infectious agents, especially viruses, account for several of the most common malignancies – up to 20 % of all cancers.

Lymphoma is a cancer in the lymphatic cells of the immune system. Lymphomas arise frequently in association with infectious agents such as the Epstein-Barr virus, the human immunodeficiency virus, the human herpes virus 8, the Helicobacter pylori, and the hepatitis C virus. Researchers have intensely focused on the association between infectious agents and lymphomas, with a look at the molecular mechanisms they use to disturb cell regulation and eventually result in cancer [4].

Lymphoma causes immunodeficiency which is further aggravated by steroid and chemotherapy/radiotherapy treatment. This leads to frequent leukopenia. Despite its well-known histological and clinical features, Hodgkin's lymphoma (HL) has recently been the object of

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intense research activity, leading to a better understanding of its phenotype, molecular characteristics, histogenesis, and possible mechanisms of lymphomagenesis.

According to the WHO classification, Hodgkin's lymphoma (HL) is subdivided into a classical variant and a nodular lymphocyte predominant variant which are characterized by the presence of Hodgkin's and Reed-Sternberg (HRS) cells or lymphocytic and histiocytic (L&H) cells, respectively. This classification recognizes a basic distinction between lymphocyte predominance HL (LPHL) and classical HL (CHL), reflecting the differences in clinical presentation and behavior, morphology, phenotype, and molecular features [5, 6]. Children with immunodeficiencies who present with HD (Hodgkin's disease) do not have such a favorable prognosis. The investigators propose an alternative treatment to treat children with or without increased DNA breakage so as to improve the outcome of Hodgkin's disease in the subgroup of children with immunodeficiency [7].

Recent advances in the field suggest that Hodgkin's disease is a malignancy of primarily B lymphocytes which is actually responsible for causing immunodeficiency. This disease clearly evades the immune mechanism of the host to avoid self-destruction. In a recent article, the authors have discussed Hodgkin's disease, its association with Epstein-Barr virus (EBV), and the immunodeficiency caused by HD, how tumor immune evasion mechanisms are mediated through its role in altering the functions of the regulatory T cells, cytotoxic T cells, cytokine and chemokine secretion, downregulation of Fas ligand, and indoleamine 2,3-dioxygenase (IDO) secretion [8].

Immunodeficiency may be transient and reversible as seen in malnutrition, steroid, cytotoxic drug effect, radiation, removal of spleen, and certain viruses, just to name a few. But in certain lymphoma, there are structural and functional changes in the receptor and cytokine system which cause immunodeficiency. The main objective of the present work is to assess whether fetal thymus transplantation can combat immunodeficiency caused by any drugs or dreaded disease like lymphoma.

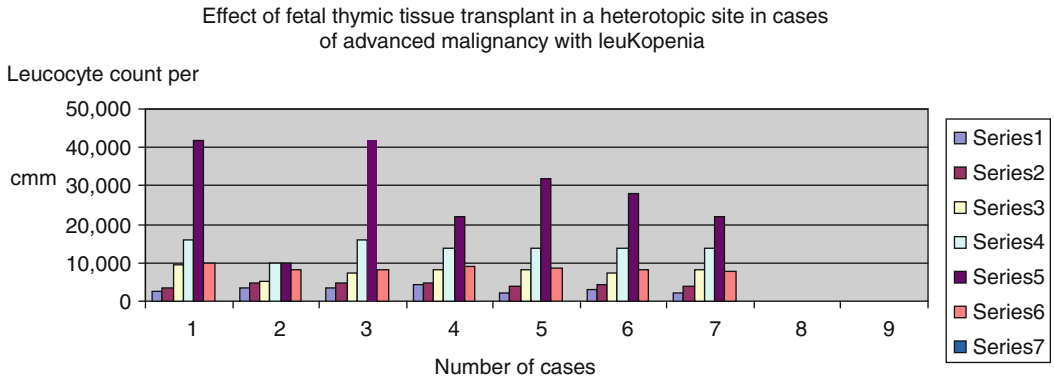
Material and Method

After getting due informed consent from each patient, eight patients were admitted to Bijoygarh State Hospital with refractory advanced cancer and leukopenia between 1999 and 2006 for fetal thymus transplantation. Permission was sought and received from the institute-based ethical committee as per ICMR guideline prevailing at that time for the experimental thymus transplants.

The patients were first screened for hepatitis B and C and HIV 1 and 2 and tested for hemoglobin, total count, differential count, platelet count, assessment of ESR, liver function, urea, creatinine, fasting and postprandial sugar, glycosylated hemoglobin, and lipid profile. X-ray chest (DNA PCR in case of suspicion of Koch's infection), ECG (echocardigraphy in case of ECG problem), and testing for C-reactive protein, antinuclear antibody, anti-dsDNA, T3, T4, and TSH were also undertaken to assess each patient's suitability for the transplantation procedure.

Thymuses were collected from consenting donor mothers who were admitted for hysterotomy and ligation after getting due informed consent from the donors. The freshly collected thymus glands were finely sliced serially with a sharp knife and placed immediately at a locally anesthetized (4–5 cc 1 % Xylocaine infiltrated) area at the axilla (2–3-m length and 2–3 cm in breadth), the subcutaneous space of which had been dissected and prepared for the transplant, that is, shaved and sterilized with Betadine and 100 % rectified spirit solution. After the placement of the fresh human fetal thymus tissue, the space was subsequently closed with small interrupted (00) atraumatic chromic catgut with cutting needle. No prophylactic antibiotics were given, though the patients received analgesic paracetamol, 1–3 tablets/day for 2–3 days, for symptomatic relief, postoperatively.

Fetal thymus tissue contains thymocytes and other precursor and progenitor cells in different stages of growth and differentiation along with connective tissue like elastin, collagen, and matrix. This extracellular matrix (ECM) of the growing fetal tissue is a very important



Graph 34.1 Graphical presentation: showing the effect of HLA-randomized fetal thymic tissue transplant on the host's leukocyte level in cases of advanced malignancy with leukopenia. *Series 1* pretransplant value, *series 2* 7th day posttransplant value, *series 3* 14th day posttransplant value, *series 4* 21st day posttransplant value, *series 5* 30th day posttransplant value, *series 6* 7th day transplant removal value

component of stem cell niche areas, which regulate the microenvironment of the stem cell pool size and control stem cell mobilization. Cells are integrated to their matrix via integrin and nonintegrin receptors, which are utilized in the control of adhesion, migration, division, growth, anoikis, transdifferentiation, and other cellular behavior. ECM provides architecture and strength but also growth factor deposits [9]. After 1 month from the date of placement of the fetal tissue, a part of the tissue was retrieved for cellular study and microscopy with suitable staining [10].

Result and Analysis

The usual treatment of leukopenia is stimulation of the bone marrow with multiple injections of granulocytic/lymphocytic colony-stimulating factor, but the problem with this treatment is the cost factor. Many poor patients cannot afford this line of treatment, particularly because most of them have normally been suffering from the disease for long periods and have exhausted their resources by the time that they have completed chemotherapy and/or radiotherapy. It was these patients who opted for the transplant method of treatment at the government hospital for correction of their hematological profiles.

In the present study, seven patients fulfilled the stringent requirements of the ethical committee. The age of the cancer patients varied from 13 to 64 years. Of the seven patients, two were female and five were male. Two patients were suffering from Hodgkin's lymphoma and the rest, that is, five patients, were suffering from non-Hodgkin's lymphoma. All the patients were suffering from clinical stage IV disease, and the presenting features were leukopenia with poor general condition with frequent bouts of fever, anorexia, loss of weight, etc. The leukopenia varied from 2,200 to 4,400/mm^3. After the transplant, the WBC count started rising to different levels for different patients within 1 month; it reached up to 42,000/mm^3 (serial no. 1 in a case of nodular sclerosis variety of Hodgkin's disease), up to 9,800/mm^3 (serial no. 2 in a case of precursor cell non-Hodgkin's lymphoma), up to 22,000/mm^3 (serial no. 3 in a case of follicular non-Hodgkin's lymphoma), up to 32,000/mm^3 (serial no. 4 diffuse large cell variety of non-Hodgkin's lymphoma), up to 28,000/mm^3 (serial no. 5 in a case of B cell, chronic lymphocytic non-Hodgkin's lymphoma), up to 22,000/mm^3 (serial no. 6 in a case of precursor T cell non-Hodgkin's lymphoma), and up to 24,000/mm^3 (serial no. 7 in a case of mixed cellular Hodgkin's lymphoma) (See Case Histories).

Case Histories

Serial no., name, age, sex, diagnosis	Brief clinical history	Impression vis a vis implication
(1) Miss A.M. (14 years) was suffering for 5 years with nodular sclerosis variety of Hodgkin's lymphoma (Ann Arbor stage IV) and was receiving chemotherapy periodically after completion of her radiotherapy	The disease was showing features of progressive dissemination, and there was an overall deterioration of the patient's condition. After taking proper informed consent from the patient's guardian and the ethical committee, a 16-week hypoimmune (more than 15 weeks on the basis of the last menstrual period [LMP] calculation and ultrasound confirmation) human fetal thymus was transplanted in the patient's axilla Preoperative hematological assessment showed hemoglobin, 9 g%; ESR, 95 mm/h; peripheral blood total count (Tc) before the thymus transplant was 2,400/mm^3 with neutrophil 28 %, lymphocyte 68 %, eosinophil and monocyte 2 % each, and basophil was not present; and the platelet count was 75,000/mm^3 On the 7th postoperative day of placement of the thymic graft, the Tc was 3,600/mm^3 with neutrophil 36 % and lymphocyte 60 %. On the fourteenth postoperative day, the WBC count rose to 9,400/mm^3 with lymphocyte 80 %, which became 86 % with the total WBC count going up to 15,800/mm^3 on the 21st postoperative day of the transplant placement. This ascending trend of the WBC continued unabated and reached 42,000/mm^3 (lymphocyte 86 %) on the 30th day; the total count reached 42,000/mm^3 (lymphocyte 88 % without any blast cell) on the 30th day of the thymic graft placement The thymic graft was removed with the axillary adjacent tissue on the same day, and on the seventh day of the removal of the human fetal thymic graft, the hematological assessment showed hemoglobin, 10.6 g%; ESR, 68 mm/h; and Tc, 9,900/mm^3 with neutrophil 10 % and lymphocyte 86 %, along with eosinophil and monocyte 2 % each without any basophil. The platelet count was 110,000/mm^3	The histology of the thymus after proper processing and staining with hematoxylin and eosin, when compared with the pretransplant (same) thymic tissue, showed growth and proliferation of the thymic tissue with Hassall's corpuscles; scattered and aggregated lymphocytes showed comet-like structures. There were no endarteritis, thrombosis, or any other specific graft vs. host reaction feature. These Hassall's particles were not present in the pretransplant thymic specimen (vide Figs. 34.1, 34.2, and 34.3)

(2) S.R. (13 years) male (precursor T cell lymphoma) was suffering for 5 years with non-Hodgkin's lymphoma (Ann Arbor stage IV) and was receiving chemotherapy periodically after completion of his radiotherapy. However, the disease was showing features of progressive dissemination	There was an overall deterioration of the patient's condition. After taking proper informed consent from the patient's guardian and the ethical committee, this patient received a preimmune (less than 15 weeks) tiny human fetal thymus (12 weeks old) (on the basis of the last menstrual period [LMP] calculation and ultrasound confirmation) which was transplanted in the patient's axilla. Preoperative hematological assessment showed hemoglobin, 8.7 g%; ESR, 45 mm/h; peripheral blood total count (Tc) before the thymus transplant was 3,400/mm^3 with neutrophil 22 %, lymphocyte 74 %, eosinophil and monocyte 2 % each, and basophil was not present; and the platelet count was 77,000/mm^3 On the 7th postoperative day of placement of the thymic graft, the Tc was 4,600/mm^3 with neutrophil 34 % and lymphocyte 72 %. On the 14th postoperative day, the WBC count rose to 9,400/mm^3 with lymphocyte 82 % which became 89 % with the total WBC count going up to 15,800/mm^3 on the 21st day of the transplant. This ascending trend of the WBC continued unabated and reached 42,000/mm^3 (lymphocyte 77 % without any blast cell) on the 30th day of the thymic graft placement The thymic graft was removed with the axillary adjacent tissue on the same day, and on the seventh day of the removal of the human fetal thymic graft, the hematological assessment showed hemoglobin, 9.6 g%; ESR, 58 mm/h; and Tc, 8,200/mm^3 with neutrophil 12 % and lymphocyte 86 %, along with eosinophil and monocyte 1 % each without any basophil. The platelet count was 105,000/mm^3	The histology of the thymus after proper processing and staining with hematoxylin and eosin, when compared with the pretransplant (same) thymic tissue, showed growth and proliferation of the thymic tissue with Hassall's corpuscles; scattered and aggregated lymphocytes showed comet-like structures. There were no endarteritis, thrombosis, or any other specific graft vs. host reaction features
(3) Mr. P.L. (44 years) (*follicular lymphoma*) was suffering for 5 years with non-Hodgkin's lymphoma (Ann Arbor stage IV) and was receiving chemotherapy periodically after completion of radiotherapy	The disease remained unabated, and there was an overall deterioration of the patient's condition. After taking proper informed consent from the patient's guardian and the ethical committee, a 16-week hypoimmune (more than 15 weeks on the basis of the last menstrual period [LMP] calculation and ultrasound confirmation) human fetal thymus was transplanted in the patient's axilla Preoperative hematological assessment showed hemoglobin, 11 g%; ESR, 55 mm/h; peripheral blood total count (Tc) before the thymus transplant was 4,400/mm^3 with neutrophil 32 % and lymphocyte 68 %; and the platelet count was 65,000/mm^3 On the 7th postoperative day of placement of the thymic graft, the Tc was 4,600/mm^3 with neutrophil 32 % and lymphocyte 66 %. On the 14th postoperative day, the WBC count rose to 8,400/mm^3 with lymphocyte 80 % which became 86 % with the total WBC count going up to 13,800/mm^3 on the 21st day of the transplant. This ascending trend of the WBC continued unabated and reached 22,000/mm^3 (lymphocyte 83 % without any blast cell) on the 30th day of thymic graft placement The thymic graft was removed with the axillary adjacent tissue on the same day, and on the seventh day of the removal of the human fetal thymic graft, the hematological assessment showed hemoglobin, 11.1 g%; ESR, 78 mm/h; and Tc, 8,900/mm^3 with neutrophil 12 % and lymphocyte 88 %. The platelet count was 130,000/mm^3	The histology of the thymus after proper processing and staining with hematoxylin and eosin, when compared with the pretransplant (same) thymic tissue, showed growth and proliferation of the thymic tissue with Hassall's corpuscles, scattered and aggregated lymphocytes showed comet-like structures There were no endarteritis, thrombosis or any other specific graft vs. host reaction features

continued

Serial no., name, age, sex, diagnosis	Brief clinical history	Impression vis a vis implication
(4) Mrs. A.M. (64 years) (*diffuse large B cell lymphoma*) was suffering for 5 years with non-Hodgkin's lymphoma (Ann Arbor stage IV) and was receiving chemotherapy periodically after completion of radiotherapy	The disease was showing features of progression despite treatment. After taking proper informed consent from the patient's guardian and the ethical committee, a 14-week preimmune (less than 15 weeks on the basis of the last menstrual period [LMP] calculation and ultrasound confirmation) human fetal thymus was transplanted in the patient's axilla. Preoperative hematological assessment showed hemoglobin, 9.6 g%; ESR, 55 mm/h; peripheral blood total count (Tc) before the thymus transplant was 2,200/mm^3 with neutrophil 18 %, lymphocyte 78 %, eosinophil and monocyte 2 % each, and basophil was not present; and the platelet count was 77,000/mm^3 On the 7th postoperative day of placement of the thymic graft, the Tc was 3,900/mm^3 with neutrophil 26 % and lymphocyte 70 %. On the 14th postoperative day, the WBC count rose to 8,400/mm^3 with lymphocyte 80 % which became 89 % with the total WBC count going up to 13,800/mm^3 on the 21st day of the transplant. This ascending trend of the WBC continued unabated and reached 32,000/mm^3 (lymphocyte 88 % without any blast cell) on the 30th day of thymic graft placement The thymic graft was removed with the axillary adjacent tissue on the same day, and on the seventh day of the removal of the human fetal thymic graft, the hematological assessment showed hemoglobin, 11.6 g%; ESR, 58 mm/h; and Tc, 8,700/mm^3 with neutrophil 14 % and lymphocyte 86 %. The platelet count was 110,000/mm^3	The histology of the thymus after proper processing and staining with hematoxylin and eosin, when compared with the pretransplant (same) thymic tissue, showed similar pattern of growth and proliferation of the thymic tissue with Hassall's corpuscles, scattered and aggregated lymphocytes showed comet-like structures. There were no endarteritis, thrombosis or any other specific graft vs. host reaction features
(5) Mr. C.B. (46 years) (*B cell chronic lymphocytic leukemia/lymphoma*) was suffering for 5 years with non-Hodgkin's lymphoma (Ann Arbor stage IV) and was receiving chemotherapy after completion of radiotherapy	The disease showed features of progressive dissemination; there was an overall deterioration of the patient's condition After taking proper informed consent from the patient's guardian and the ethical committee, an 18-week hypoimmune (more than 15 weeks on the basis of the last menstrual period [LMP] calculation and ultrasound confirmation) human fetal thymus was transplanted in the patient's axilla Preoperative hematological assessment showed hemoglobin, 11.2 g%; ESR, 75 mm/h; peripheral blood total count (Tc) before the thymus transplant was 3,200/mm^3 with neutrophil 18 %, lymphocyte 78 %, eosinophil and monocyte 2 % each, and basophil was not present; and the platelet count was 65,000/mm^3 On the 7th postoperative day of placement of the thymic graft, the Tc was 4,200/mm^3 with neutrophil 22 % and lymphocyte 78 %. On the 14th postoperative day, the WBC count rose to 7,400/mm^3 with lymphocyte 84 % which became 88 % with the total WBC count going up to 13,600/mm^3 on the 21st day of the transplant. This ascending trend of the WBC continued unabated and reached 28,000/mm^3 (lymphocyte 88 % without any blast cell) on the 30th day of thymic graft placement The thymic graft was removed with the axillary adjacent tissue on the same day, and on the seventh day of the removal of the human fetal thymic graft, the hematological assessment showed hemoglobin, 10.6 g%; ESR, 78 mm/h; and Tc, 8,400/mm^3 with neutrophil 12 % and lymphocyte 86 %, along with eosinophil and monocyte 2 % each without any basophil. The platelet count was 114,000/mm^3	The histology of the thymus after proper processing and staining with hematoxylin and eosin, when compared with the pretransplant (same) thymic tissue, showed trends similar to those seen in other occasions of growth and proliferation of the thymic tissue with Hassall's corpuscles; scattered and aggregated lymphocytes showed comet-like structures. There were no endarteritis, thrombosis, or any other specific graft vs. host reaction features

(6) Mr. D.C. (56 years) (*Precursor T cell lymphoma*) was suffering for 5 years with non-Hodgkin's lymphoma (Ann Arbor stage IV) and was receiving chemotherapy after completion of radiotherapy	In spite of the treatment, the disease remained unabated with chemoradiotherapeutic intervention There was an overall deterioration of the patient's condition After taking proper informed consent from the patient's guardian and the ethical committee, an 18-week hypoimmune (more than 15 weeks on the basis of the last menstrual period [LMP] calculation and ultrasound confirmation) human fetal thymus was transplanted in the patient's axilla. Preoperative hematological assessment showed hemoglobin, 7.6 g%; ESR, 45 mm/h; peripheral blood total count (Tc) before the thymus transplant was 2,200/mm^3 with neutrophil 20 %, lymphocyte 78 %, eosinophil and monocyte 1 % each, and basophil was not present; and the platelet count was 75,000/mm^3 On the 7th postoperative day of placement of the thymic graft, the Tc was 3,700/mm^3 with neutrophil 32 % and lymphocyte 64 %. On the 14th postoperative day, the WBC count rose to 8,400/mm^3 with lymphocyte 81 % which became 88 % with the total WBC count going up to 13,800/mm^3 on the 21st day of the transplant. This ascending trend of the WBC continued and reached 22,000/mm^3 (lymphocyte 89 % without any blast cell) on the 30th day of thymic graft placement The thymic graft was removed with the axillary adjacent tissue on the same day, and on the seventh day of the removal of the human fetal thymic graft, the hematological assessment showed hemoglobin, 10.6 g%; ESR, 78 mm/h; and Tc, 7,800/mm^3 with neutrophil 12 % and lymphocyte 84 %, along with eosinophil and monocyte 2 % each without any basophil. The platelet count was 130,000/mm^3	The histology of the thymus after proper processing and staining with hematoxylin and eosin, when compared with the pretransplant thymic tissue, showed identical growth and proliferation of the thymic tissue with Hassall's corpuscles; scattered and aggregated lymphocytes showed comet-like structures. There were no endarteritis, thrombosis, or any other specific graft vs. host reaction features
(7) Mr. A.B. (16 years) (*mixed cellularity lymphoma*) was suffering for 5 years with Hodgkin's lymphoma (Ann Arbor stage IV) and was receiving chemotherapy periodically after completion of radiotherapy	Despite treatment with chemotherapy and radiotherapy, the disease was showing features of dissemination, and there was deterioration of the patient's condition After taking proper informed consent from the patient's guardian and the ethical committee, a 14-week preimmune (less than 15 weeks on the basis of the last menstrual period [LMP] calculation and ultrasound confirmation) human fetal thymus was transplanted in the patient's axilla. Preoperative hematological assessment showed hemoglobin, 11 g%; ESR, 45 mm/h; and peripheral blood total count (Tc) before the thymus transplant was 2,500/mm^3 with neutrophil 22 %, lymphocyte 68 %, eosinophil 8 %, and monocyte 2 %, and basophil was not present. The platelet count was 75,000/mm^3 On the 7th postoperative day of placement of the thymic graft, the Tc was 3,900/mm^3 with neutrophil 16 % and lymphocyte 70 %. On the 14th postoperative day, the WBC count rose to 8,100/mm^3 with lymphocyte 82 % which rose to 88 % with the total WBC count going up to 13,800/mm^3 on the 21st day of the transplant. This ascending trend of the WBC continued and reached 22,000/mm^3 (lymphocyte 78 % without any blast cell) on the 30th day of thymic graft placement The thymic graft was removed with the axillary adjacent tissue on the same day, and on the seventh day of the removal of the human fetal thymic graft, the hematological assessment showed hemoglobin, 11.6 g%; ESR, 78 mm/h; and Tc, 8,900/mm^3 with neutrophil 17 % and lymphocyte 83 %, along with absence of eosinophil and monocyte and basophil in the peripheral smear. The platelet count was 140,000/mm^3	The histology of the thymus after proper processing and staining with hematoxylin and eosin, when compared with the pretransplant thymic tissue, showed identical growth and proliferation of the thymic tissue with Hassall's corpuscles; scattered and aggregated lymphocytes showed comet-like structures. There were no endarteritis, thrombosis, or any other specific graft vs. host reaction features

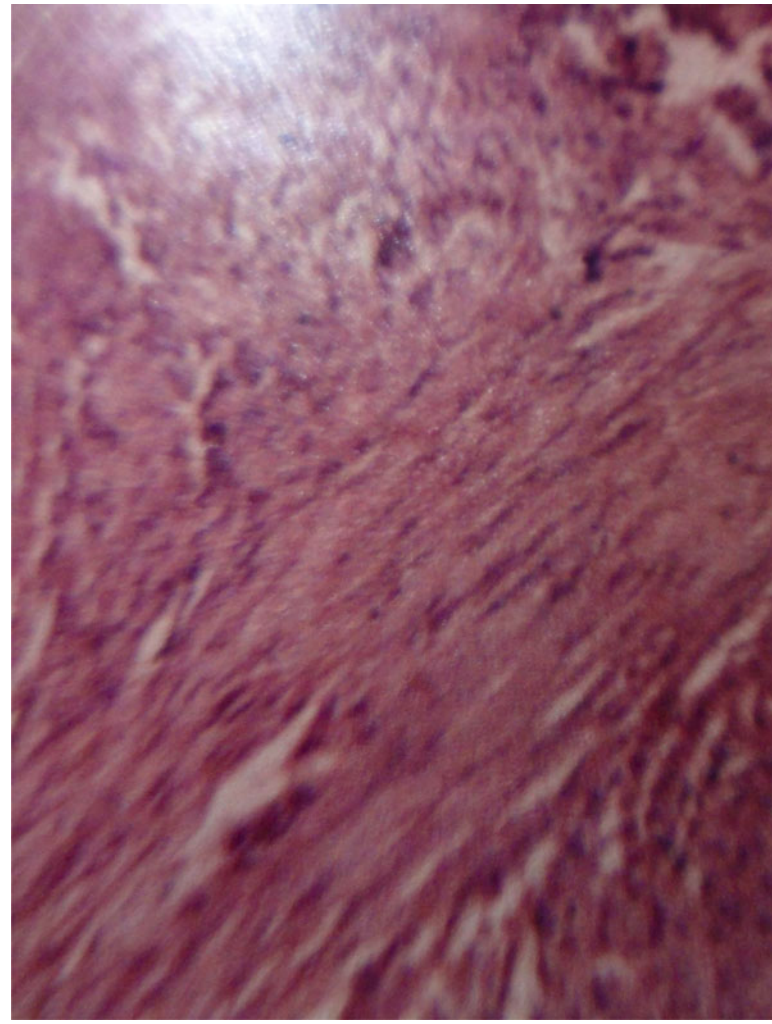


Fig. 34.1 Shows hematoxylin- and eosin stained human fetal thymus at 12 weeks. There is no presence of Hassall's corpuscles as seen in low-power microscope from patient case report (2)

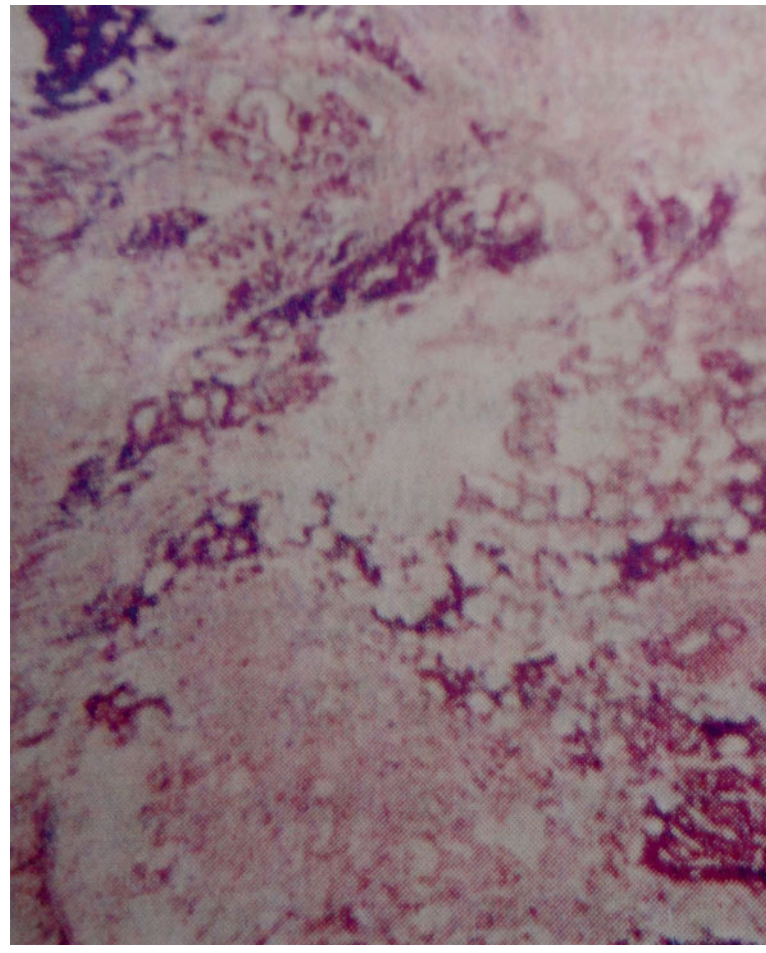
Discussion and Conclusion

Thymic activity is highest during the early years after birth, but it progressively declines resulting in diminished naïve T cell output. The underlying causes of thymic involution may be degeneration of the stromal thymic network, which is providing survival and differentiation factors for developing T cells, or insufficiency of the progenitor cells to home and/or develop in the aged thymus. The reduced thymic output is insignificant in young people since the peripheral T cell compartment is under compensatory homeostatic control. However, in more or less immunocompromised individuals, including aged people and patients depleted of T cells due to conditioning regimens before a bone marrow transplantation or HIV infection, the thymus is necessary to replenish the peripheral T cell component. This may require rejuvenation of the thymus [11, 12]. Reciprocal interaction between bone marrow-derived lymphoid precursor cells and the thymic environment leads, through a series of developmental events, to the generation of a diverse repertoire of functional T cells. During thymopoiesis, fetal liver or bone marrow-derived precursors enter the thymus and develop into mature T cells in response to cues derived from the environment. The thymic microenvironment provides signals to the lymphoid cells as a result of cell–cell interactions, locally produced cytokines, chemokines, and hormones. Developing thymocytes, in turn, influence

the thymic stroma to form a supportive microenvironment. Stage-specific signals provide an exquisite balance between cellular proliferation, differentiation, cell survival, and death [13].

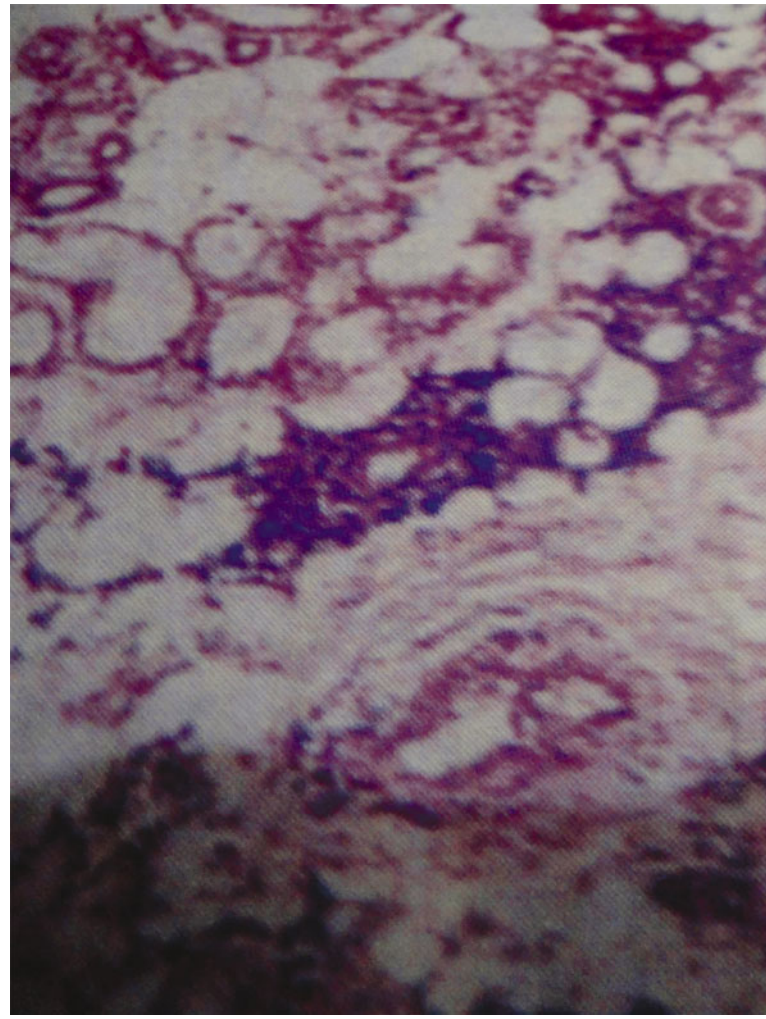
After recapitulating the background of the significance of the thymic role in immunocompetence and its development, let us focus on the findings of this chapter. The first point concerns the technique of thymus grafting – experiments have shown that thymic fragments, in which the structure is preserved, are more effective than dissociated thymic cells in the restoration of functions [14]. For example, if fragments of one thymus are placed in a muscle and then compared with injection of thymic cell suspension in another muscle, the surgically placed pieces show better survival [15]. The technique that was used in the present experiment utilized quasi-total and total human fetal thymuses between 12 and 18 weeks.

In the first case, a little tissue was taken out for histological comparison from the quasi-total human fetal thymus, 16 weeks old; in the second case, the total thymus, 12 weeks old, was taken to avoid trauma and injury effect on this tiny thymic tissue. This simple method of human fetal thymus transplantation under local anesthesia in advanced cancer patients without matching the HLA (human leukocytic antigen) showed that the graft is not rejected in the course of 1 month (period of observation). There is no graft vs. host (GVH) reaction noted clinically or histologically, in either the case of non-Hodgkin's lymphoma (case 1) or other cases (2–7). However, the pretransplant leukopenia was grossly overcorrected on the impact of the 16-week fetal thymus that



Figs. 34.2 and 34.3 Some thymic tissue was retrieved from the patient no. 1 after 1 month from its heterotopic subcutaneous placement in the axilla and seen with hematoxylin- and eosin-stained section. What is exciting is the presence of Hassall's corpuscles both seen in high-power (Fig. 34.2) and more distinctly with oil immersion lens (Fig. 34.3). A simple conclusion may be drawn from this event that a specialized tissue like the thymus can grow and mature in heterotopic nonpregnant HLA-randomized environment and express Hassall's corpuscles at around 16 weeks (from the date of fertilization). Hence, the fetal thymic tissue may possibly create its own microenvironment for its survival strategy and growth

Fig.34.2 and 34.3 (continued)



was transplanted; the leukocyte count returned to normal levels after the removal of the transplant in the case of non-Hodgkin's lymphoma, whereas the impact on the peripheral leukocyte count was less pronounced in other cases of lymphoma, though the histological evidences in all cases showed growth, proliferation, and differentiation of the fetal thymus in the new host site.

Fetal growth is dependent upon a unique symbiotic environment where the mother provides all the necessary factors for the growth and differentiation of the growing fetal organs. The fetal microenvironment is distinctly different from the adult microenvironment [16]. It appears, therefore, that the developing fetal organ prepares/(?) changes its own microenvironment in an altered metabolic situation taking (?) advantage of its hypoimmune and/or preimmune status to survive, grow, and differentiate. The thymus supports the development of T cells throughout life from (through?) hematopoietic progenitor cells migrating from the bone marrow.

These are some secondary advantages of human fetal tissue transplantation, and these have been reported earlier in relation to other human fetal tissue transplantations. The safety aspects of fetal tissue transplantation have also been noted in these articles [17]. The nonspecific effects of human tissue transplantations are as follows: (a) rise of hemoglobin from the pretransplant level; (b) there is also reduction of pain all over the body, which had been due to the preexisting disease

background or superimposed (different) factors either singularly or in combination, namely, viral, bacterial or fungal, or anaerobic infections (background malnutrition may have a contributory role too); (c) posttransplant weight gain; (d) improvement of appetite; and (e) a sense of well-being, which was universally present among all the transplant recipients in varying degrees, which we will report in a separate chapter in this book. In the present study, the same trends were noted.

Conclusion

It is well known that tumor-bearing mammals, including humans, show decreased T cell function due to involution of the thymus. This decrease results in faster tumor growth, susceptibility to infection, and reduced life expectancy. Thus, the best strategy to restore T cell function might be to transplant the thymus from the fetus or newborn, which has been proved in murine model, and some investigators have suggested that in humans, thymus transplantation in conjunction with bone marrow transplantation could become a valuable strategy for suppressing tumor growth, thereby prolonging survival [18].

The present work suggests that fetal thymus can grow in sex- and HLA-randomized host with varying degrees of immunosuppression due to chemotherapeutic drug, radiation, or due to the disease process itself due to lymphoma background. This growth of the thymus can help in the expression of immunocompetence of the growing thymic cells, that is, appearance of Hassall's corpuscles and thus help in the immunoresistance against lymphoma with immunotherapeutic potential.

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35 Human Fetal Adrenal Transplant at Heterotopic Site as an Adjuvant for Treatment of Excruciating Pain in Cases of Arthritides

Niranjan Bhattacharya

Introduction

Arthritides is the plural of arthritis. There are over 100 types of arthritis, and someone can have more than one of them which can present with excruciating pain and features of inflammation.

Arthritis is always associated with severe pain. This pain is one of the most primitive human feelings and is a physical and mental response to stimuli and may be protective in nature. Therapies in medical science are intended to provide some relief of pain and restore normalcy. In a way, respect for the medical profession may be directly proportional to the degree of relief it can provide to the distressed.

Here, the subject of study is some relief of excruciating pain, which is a complex, chronic pain state that usually is accompanied by tissue injury. With neuropathic pain, the nerve fibers themselves may be damaged, dysfunctional, or injured. These damaged nerve fibers send incorrect signals to other pain centers. The impact of nerve fiber injury includes a change in nerve function both at the site of injury and areas around the injury. In neuropathic pain, there is an imbalance in endogenous excitatory and inhibitory spinal systems that modulate sensory processing. Current pharmacological therapies are often ineffective over time for the greater number of patients [1].

In recent years, there has been the development of effective cellular strategies that could replace or be used as an adjunct to existing pharmacological treatments for neuropathic pain. Cell therapy studies for pain relief have tested adrenal chromaffin cells from rat or bovine sources, placed in the subarachnoid space, near the spinal cord pain-processing pathways. These grafts functioned as cellular mini pumps, secreting a cocktail of antinociceptive agents around the spinal cord for peripheral nerve injury, inflammatory, or arthritic pain [2]. The most effective form of cell therapy is the use of different progenitor or immature cells, that is, fetal cell therapy, because these cells, during the developmental process, pass through the preimmune (before 15 weeks) and, subsequently, the hypoimmune phases of growth and maturation.

In these phases of human fetal growth and maturation, there is a lack of proper antigenic expression of the growing fetus in utero, which provides an excellent opportunity for fetal cell/tissue/organ transplantation because of the pre-HLA state of growth and maturation. The assumption is that hypoantigenic naïve fetal cells would not be targeted by the hosts' HLA system. In this context, another interesting phenomenon must be noted, and that is the "homing" behavior of the transplanted cell, which, if seen from the positive angle,

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implies that fetal premature cells (stem cell component of the growing organ) with a high telomerase ribonucleoprotein complex have the potentiality to replace damaged cells or cells in their senescence and thus augment the overall state of health of the tissue or organ in question. The present author leads a group of investigators working since 1979 on the process through which the human fetus acquires immunocompetence [3–10].

It has been demonstrated that transplantation of human dopamine neurons in the brain (to be more precise, the CT-guided stereotactic placement of embryonic mesencephalic tissue in the putamen or putamen and caudate region) has resulted in a marked clinical improvement in patients suffering from Parkinsonism [11]. Adrenal cortical cells' xenotransplantation has also been attempted earlier in SCID mice with amazing results (severe combined immunodeficiency): they eventually got vascularized and secreted steroid that replaced those from the animals' own adrenal glands, which were removed during the transplantation surgery [12]. Cell transplantation techniques have now provided researchers with a way to comprehend the regulation of cell proliferation in the same cell type, in cell culture, or in a vascularized tissue structure in a host animal. The contrasting role of p57(KIP_2) and p21(Waf_1/CIP_1/SDI_1) in transplanted human and bovine adrenocortical cells has also been reported [13]. In Addison's disease, there is a report of a successful allotransplantation of embryonal adrenal tissue [14]. There are also interesting reports on adrenal medullary transplant or purified adrenal chromaffin cells' transplant for the management of chronic pain [15] and pain of central origin after spinal cord hemisection [16] and in cases of intractable cancer pain [17, 18]. Hence, the successful development of fetal cell/tissue transplantation in adults has improved the possibility of eventual therapeutic solutions in a variety of intractable diseases.

Materials and Methods

Twenty-two patients who were admitted with varying degrees of neuropathy in the background of advanced arthritis (suffering from 5 to 15 years) in the government hospital, were enrolled in the present treatment protocol. They presented with at least four of the seven 1987 revised criteria of the American College of Rheumatology for diagnosis of arthritis [19]. These patients were enrolled for the present study on fetal adrenal transplant and its effect on the host's neuropathic and arthritic pain and its clinical visibility; the impact would be studied using the local language modified, and short form of the McGill questionnaire for pain and its relief assessment.

The ultimate goals of therapy in arthritis are relief of pain, reduction of inflammation, protection of the articular structures and their functions, and, finally, control of systemic involvement. Most of the patients enrolled for the study had additional neurological problems.

After getting necessary informed consent from all concerned and ethical permission from the hospital-based ethical committee, the patients underwent preliminary screening for hepatitis B and C and HIV 1 and 2 along with hemoglobin, total count, differential count, platelet count, assessment of ESR, liver function, urea, creatine, fasting and postprandial sugar, glycosylated hemoglobin, and lipid profile. X-ray chest (DNA PCR in case of suspicion of Koch's infection), ECG (echocardiography in case of ECG problem), and tests for C-reactive protein, antinuclear antibody, anti-dsDNA, T3, T4, and TSH were also undertaken to see if the patients could tolerate the transplantation procedure.

Informed consent was also taken from the patients' guardians, and further, the institutional ethical committee discussed the pros and cons of the use of electively aborted fetal tissue and advised us to continue the research within the framework of legal and ethical safeguards. The committee also discussed the possibility of developing clinical benefits from this research for this intractable disease, giving due consideration to privacy, safety, and eliminating the possibility of secondary gain as a reason to decide in favor of an abortion.

It should be noted at the beginning that all the patients admitted for treatment had earlier been treated with (a) nonsteroidal anti-inflammatory drugs, (b) disease-modifying antirheumatic drugs, (c) antibiotics like minocycline, and (d)

glucocorticoids before their enrollment into the fetal adrenal transplant program.

After due consent and ethical permission, adrenal glands were collected from aborted fetuses and sliced serially with a sharp knife and placed immediately at a locally anesthetized (4–5 cc 1 % Xylocaine infiltrated) area at the axilla (2–3 cm in length and 2–3 cm in breadth) of the patient, with blunt dissection of the subcutaneous space around the incision. This area was previously shaved and sterilized with Betadine and 100 % rectified spirit solution before the placement of the fresh human fetal adrenal tissue fragments. The incision was subsequently closed with small interrupted (00) atraumatic chromic catgut with a cutting needle. No prophylactic antibiotics were given; however, the patient received analgesic paracetamol 1–3 tablets/day for 2–3 days for symptomatic relief, postoperatively.

Fetal adrenal tissue contains primitive cells in different states of growth and differentiation along with connective tissue like elastin, collagen, and matrix.

In case of transplantation of the fetal organ, the vascularity is not well developed, but the cells are mostly resistant and can survive even in a relatively hypoxic condition unlike adult cells, the metabolic requirements of which are more stringent. In fetal organs, the fetal cells are in varying states of maturation and growth. These cells are covered with extracellular matrix (ECM), which is a very important component of a stem cell niche because it regulates the microenvironment of the stem cell pool size and controls stem cell mobilization. ECM is a complex interlinked composite of collagenous molecules, noncollagenous molecules, and water-rich mucopolysaccharide ground substance. Cells are integrated to their matrix via integrin and nonintegrin receptors, which are utilized in the control of adhesion, migration, division, growth, anoikis, transdifferentiation, and other cellular behavior. ECM provides architecture and strength but also growth factor deposits [20], which proteases as signaling scissors and can release in a site- and process-specific manner. Each process is essential for the regenerative process.

After 3 months from the date of placement of the fetal tissue, a part of the tissue was retrieved from the transplant site in the axilla for cellular study and microscopy with suitable staining. The impact of fetal adrenal transplantation on pain relief was assessed by the specifications of the local language-modified version of the short form of the McGill questionnaire [21]. The impact of age differences on the quality of chronic pain was also taken into account [22].

Result and Analysis

The developing fetal adrenal gland has two components known as adrenal medulla and adrenal cortex. The adrenal medulla contains neural crest cells which migrate toward the coelomic cavity wall during development and eventually help in forming the adrenal medulla. These cells are also named as chromaffin (chromaphil) cells (originally named because of their staining (yellow) affinity with chromium salts).

The adrenal cortex develops from coelomic epithelium (mesothelium) cells in the sixth week, which proliferate, initially forming small buds that separate from the epithelium. These mesothelial or mesenchymal cells first form the fetal adrenal cortex which will be later replaced by the adult cortex.

Fetal adrenal tissue contains primitive cells in different states of growth and differentiation along with connective tissue like elastin, collagen, and matrix. There are also the neuronal crest cells of the adrenal medulla and the mesenchymal cell buds of the primitive adrenal cortex.

Neuropathic pain, often caused by nerve injury, is commonly observed among patients with different arthritic diseases. Because its basic mechanisms are poorly understood, effective medications are limited. Previous investigations of basic pain mechanisms and drug discovery efforts have focused mainly on early sensory neurons such as dorsal root ganglion and spinal dorsal horn neurons, and few synaptic-level studies or new drugs are designed to target the injury-related cortical plasticity that accompanies neuropathic pain.

In this study, the effect of human fetal adrenal (within 20 weeks, i.e., the legal limit of hysterotomy and ligation) transplant in patients suffering from advanced arthritis/arthropa-

thy with intractable pain, which had not been relieved by nonsteroidal inflammatory drugs and disease-modifying antirheumatic drugs like D-penicillamine, methotrexate, gold compounds, antimalarials and sulfasalazines, dapsone, and/or glucocorticoids, was examined. The objective was to assess how far the transplant of the human fetal adrenal gland was effective in relieving pain of inflammatory and neuropathic origin in cases of long-standing rheumatoid arthritis.

Apart from osteoarthritis, the most important painful condition is rheumatoid arthritis. Our understanding of the pathophysiology of arthritis in general is rapidly changing. There is a growing realization that damage occurs early, and early treatment is a critical determinant in long-term therapy [23]. The last decade has also witnessed the rebirth of steroid [24] and nearly universal acceptance of combination therapy [25]. There is also a recent emphasis on biological therapy, for instance, TNF-alpha inhibition [26] has been a breakthrough discovery in the clinical approach to remission. Glucocorticoids provide a definite relief in arthritic neuropathy for pain and mobility in the early stages of the disease. Between 15 and 40 % of the patients suffering from arthritis take glucocorticoids at any given time in many North American and European clinics [27]. Hench et al. [28] first wrote about the therapeutic use of glucocorticoids to combat arthritis in general. According to them, both acute phase response and the severity of the disease activity were controlled by glucocorticoids for the initial 3–6 months. It is possible that the benefit of glucocorticoids is masked by an increasing response to other antirheumatoid therapy. It should be noted here that the American College of Rheumatology (ACR) [29]-revised criteria for treatment are well-meaning and helpful in treatment guidance, but strictly speaking, the criteria are not optimal in distinguishing early rheumatoid arthritis from undifferentiated polyarthritis and systemic lupus erythematosus. As per the suggestions of the ACR, 1–3 years of the disease process is considered as early disease.

The problem is somewhat different in developing countries due to the poor socioeconomic and educational backgrounds of the majority of the patients. Here, for the most part, noncompliance with the suggested drug starts as soon as there is some relief. We frequently come across poor patients with intractable pain due to the progression of rheumatoid arthritis, with the involvement of the inflammatory and neuropathic components of the disease.

The ultimate goal in the therapy of arthritis is, firstly, reduction and relief of the pain and inflammation and, secondarily, maintenance of the functions and protection of the articular structures and systemic involvement. Twenty-two cases, all of whom were suffering from intractable pain and inflammation not responding to standard antiarthritic therapy as mentioned above, were included for the present study.

In Tables 35.1 and 35.2, the clinical effects of human fetal adrenal transplant (up to 20 weeks, viz., the legal limit for medical termination of pregnancy) in patients with long-standing rheumatoid arthritis, who did not respond to medical treatments done earlier, have been indicated. The study revealed that there was universal pain relief (of varying degrees) as perceived from the first month follow-up after transplant.

The transplant results suggested a definite improvement of the excruciating pain in 82 % of the cases; this was sustained, and the record of pain relief improved further in the second month. In the third month posttransplant follow-up, 91 % of the patients had no pain, while 9 % of the patients had some residual pain.

The next important characteristic in arthritis is swelling of the joints. In the present series, 18 % of the patients had huge swellings of the joints while the rest, that is, 82 %, had mild to moderate swelling. However, evaluation of the 1-month postadrenal transplant results revealed that there was reduction of swelling (mild to moderate) in varying degrees (total reduction of swelling was observed in 77 % of the cases after the third month; reduction was observed in 73 % after the second month and 68 % after the first month of the adrenal transplant). In four cases of huge joint swelling, posttransplant evaluation revealed no swelling in three cases (75 %) and partial reduction in one case. This nonadequate improvement may have been due to bony and fibrous ankylosis

Table 35.1 Assessing the clinical impact of fetal adrenal transplant in cases of arthritis with varying degrees of neuropathy

Serial number, age, name	Pain pretransplant of adrenal	Pain posttransplant of adrenal, 1 month later	Pain posttransplant of adrenal, 2 months later	Pain posttransplant of adrenal, 3 months later	Swelling pretransplant of adrenal, 1 month later	Swelling posttransplant of adrenal, 2 months later	Swelling posttransplant of adrenal, 3 months later
1, 56 HA	EP	PR	CR	CR	MS	CR	CR
2, 20 AR	EP	PR	CR	CR	MS	CR	CR
3, 28 PKM	EP	PR	CR	CR	HS	PR	PR
4, 20 HM	EP	PR	CR	CR	MS	CR	CR
5, 8 HM	EP	PR	CR	CR	MS	CR	CR
6, 75 OM	EP	NR	PR	PR	MS	PR	PR
7, 18 AR	EP	PR	CR	CR	MS	CR	CR
8, 33 BB	EP	PR	CR	CR	MS	CR	CR
9, 13 HD	EP	PR	CR	CR	MS	CR	CR
10, 20 PR	EP	PR	CR	CR	MS	CR	CR
11, 50 KB	MP	NR	PR	CR	MS	CR	CR
12, 65 SB	MP	PR	CR	CR	HS	CR	CR
13, 30 HM	EP	PR	CR	CR	HS	PR	PR
14, 20 BM	EP	PR	CR	CR	MS	CR	CR
15, 18 AR	EP	PR	CR	CR	MS	CR	CR
16, 70 DM	EP	NR	PR	PR	MS	PR	PR
17, 28 GPP	EP	PR	CR	CR	MS	CR	CR
18, 43 DS	EP	PR	CR	CR	MS	CR	CR
19, 33 AH	EP	PR	CR	CR	MS	CR	CR
20, 20 PDA	EP	PR	CR	CR	MS	CR	CR
21, 40 BB	MP	NR	PR	CR	MS	CR	CR
22, 63 LJ	MP	PR	CR	CR	HS	CR	CR

In all the cases mentioned above, pain was gauged on the basis of assessment according to the basics of the short form of the McGill questionnaire. In assessing mobility and swelling, both subjective (patient) and objective (medical consultant) criteria have been incorporated. The dosage of the pain-relieving drugs was reduced as soon as the patient started to feel better *EP* excruciating pain, *MP* mild to moderate pain, *HS* huge swelling, *MS* mild to moderate swelling, *PR* partial response/relief/reduction, *NR* no response/relief/reduction, *CR* complete response/relief/reduction

Table 35.2 Assessing the clinical impact of fetal adrenal transplant in cases of arthritis with varying degrees of neuropathy

Sr no., age, religion, sex	Mobility pretransplant of adrenal	Mobility posttransplant of adrenal, 1 month later	Mobility posttransplant of adrenal, 2 months later	Mobility posttransplant of adrenal, 3 months later	Weight pretransplant of adrenal (kg)	Weight posttransplant of adrenal, 3 months later (kg gain)	Sense of well-being pretransplant of adrenal	Sense of well-being posttransplant of adrenal, 3 months later	Weeks of gestation of the adrenal collected from fetus (weeks)
1, 56 HF	PLM	PR	PR	PR	55	4	Absent	Present	18
2, 20 HF	PLM	PR	PR	PR	41	5	Absent	Present	18
3, 20 HF	PLM	PR	PR	PR	39	6	Absent	Present	18
4, 20 HM	PLM	PR	PR	CR	71	2	Absent	Present	16
5, 8 HM	PLM	PR	PR	PR	56	4	Absent	Present	16
6, 75 HM	TLM	PR	PR	PR	55	4	Absent	Absent	16
7, 18 MM	PLM	PR	PR	CR	49	4	Absent	Present	16
8, 33 HM	TLM	PR	PR	PR	75	2	Absent	Present	16
9, 13 HM	PLM	PR	PR	PR	78	4	Absent	Present	16
10, 20 HF	PLM	PR	PR	CR	62	2	Absent	Absent	16
11, 50 HF	TLM	PR	PR	PR	60	2	Absent	Present	18
12, 65 HM	PLM	PR	PR	CR	38	4	Absent	Present	20
13, 30 HF	PLM	PR	PR	PR	39	6	Absent	Present	18
14, 20 MM	PLM	PR	PR	CR	71	2	Absent	Present	16
15, 18 HM	PLM	PR	PR	PR	56	4	Absent	Present	16
16, 70 HM	TLM	PR	PR	PR	55	4	Absent	Absent	16
17, 28 MM	PLM	PR	PR	CR	49	4	Absent	Present	16
18, 43 HM	TLM	PR	PR	PR	75	2	Absent	Present	16
19, 33 HM	PLM	PR	PR	PR	78	4	Absent	Present	16
20, 20 HF	PLM	PR	PR	CR	62	2	Absent	Absent	16
21, 40 HF	TLM	PR	PR	PR	60	2	Absent	Present	18
22, 63 HM	PLM	PR	PR	CR	38	4	Absent	Present	20

TLM total loss of mobility, *PLM* partial loss of mobility

involving multiple joints. These cases were referred for vigorous physiotherapy and a decision on follow-up surgery after the third month of evaluation.

The third important point is restoration of mobility of the joint in the 22 patients of the present adrenal transplant protocol. Total loss of mobility was present in 27 %, and partial loss of mobility was noted in the rest of the group (73 %). Posttransplant restoration was noted in the first and sustained in the second month, and the cumulative improvement of the pretransplant status was present in all the cases with complete restoration of mobility in 36 % of the cases; the rest – 64 % – had satisfying partial recovery which was sustained.

Another interesting observation was the sense of well-being and weight gain in 50 % of the patients, with a gain in weight (4 kg or more) in another 36 %, who had a gain in weight of 2 kg or more as seen during the posttransplant evaluation of the weight gain after 3 months. The present researchers are following up on the diurnal variations of cortisol and variations of epinephrine along with its urinary metabolites and also the excretion of the Na and K in the 24-h urine of the recipients of the fetal adrenal transplants. Thorough clinical monitoring and follow-up of all vital clinical, biochemical, and endocrinological parameters have also been meticulously done; these will be communicated at an appropriate time later. Whether fetal tissue with its many unique properties has a growth-promoting role is also being investigated through a serial estimation of p21 and p27 levels on the recipients of the transplanted fetal tissue.

Though the fetal tissue was transplanted in sex- and HLA-randomized adult axillas without concomitant immunosuppressive or radiation support to blunt the hosts' immune response, serial estimation of the peripheral blood of the hosts did not show any gross leucocytosis or lymphocytosis within the first 6 weeks of the observation period. The site of transplantation was also found to be healthy in all cases. Further, the retrieved tissue histology showed the proliferation and growth of the fetal tissue in the adult axilla, while at the same time, any feature of inflammation or immunological rejection, namely, mononuclear invasion, thrombosis, endarteritis, justifying graft vs. host reaction, was conspicuously absent (*vide* Figs. 35.1, 35.2, and 35.3).

Discussion

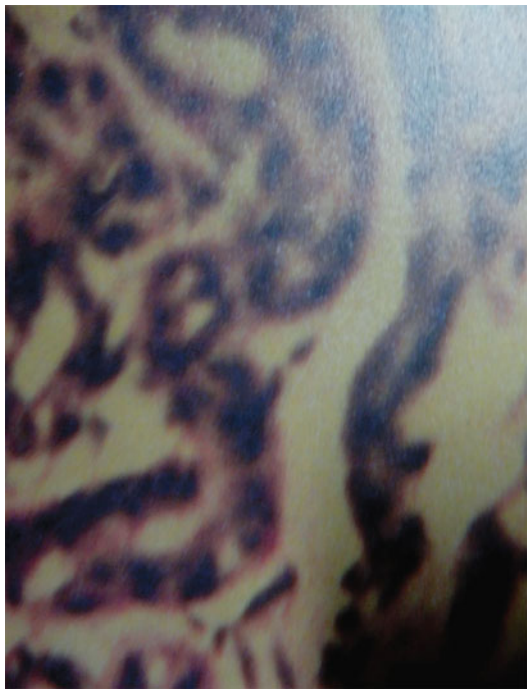
Chronic pain of different etiologies affects brain structure and function. Fetal adrenal transplantation-induced pain relief could be due to analgesic effects similar to neuraxial drug delivery pumps such as the secretion of pain-reducing neuroactive substances at optimal sites and constant levels, while overcoming problems of pump refilling and maintenance.

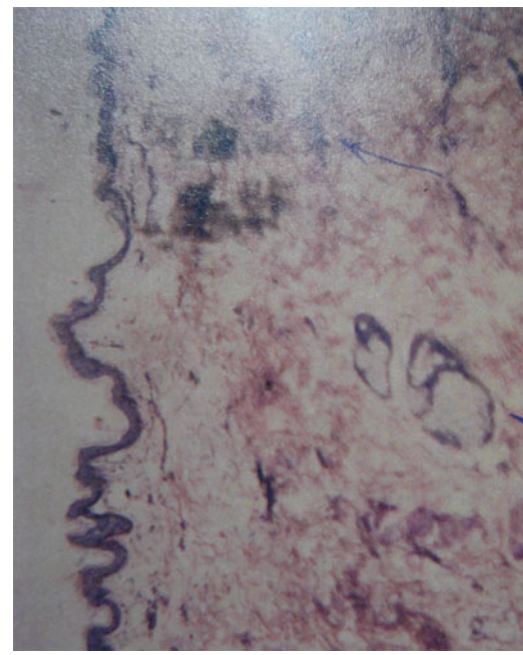
Arthritis is a serious chronic disorder affecting 1 % of the population at large [30]. Clinical researchers have tried to achieve pain relief and remission of the disease with various permutations and combinations of steroid, nonsteroidal anti-inflammatory drugs, antimalarials, and different disease-modifying drugs. However, according to a clinical investigator, the efficacy of the drug becomes progressively modest in advanced rheumatoid arthritis [31]. Therapies such as use of minocycline to combat infective etiology of arthritis and gene therapy have been attempted by scientists working in the field [32]. In a phase I study, researchers tried to introduce interleukin-I receptor antagonist in a cDNA-encoding mechanism into the knuckle joints of a patient with advanced rheumatoid arthritis, with the idea that genes could serve as a biological delivery vehicle for the products they encode, and thus, it would be a targeted delivery system for the proteins and RNA and improve their efficacy while providing a longer duration of effects and potential safety.

A pertinent theoretical question here is whether the structural and functional damage of the patients' own adrenal gland with long-standing arthritis is directly the result of the disease process or is it an indirect result of the steroid-induced suppression of the adrenal gland in the case of patients who took glucocorticoids for remission. Cellular/tissue adrenal medullary and cortical

transplantation may have a role in replenishing the damaged adrenal medullary and cortical cells through the positive intrinsic homing effect of the transplanted cell. It is known that DNA damage of an organ is common in sepsis, shock, and other critical medical conditions. In case of DNA damage, there should be either activation of apoptosis or potential repair of the damage by the activation of cyclin-dependent kinase inhibitor p21 [33]. Investigators have shown that chemical perfusion injury/infection/sepsis can cause damage to the adrenal cortex as shown by labeling 3′ termini of single-strand breaks with terminal transferase in a rat model [34]. Analyzing the findings of the present study, it is interesting to note that the main objective of pain relief was consistently achieved on the 30th day of transplantation with 60 % of the patients reporting complete relief of the pain and 40 % reporting partial relief. There was also improvement in the mobility of the patients in 80 % of the cases (complete remission in 20 %) and reduction of swelling in 100 % of the patients, with 60 % complete reduction. This relief of pain, reduction in the swelling, and improvement in mobility (due to reduction of pain) actually started within 15 days. In all the cases, the transplant scar remained healthy, with no irritation or hypertrophy of the scar. Although in patient no. 4 the transplanted tissue was partially retrieved more than 3 months later and in patients nos. 5 and 6 tissue retrieval was done after two and a half months, the clinical remission continued in all the patients.

The most important and fundamental question to which there is yet no answer, however, is that there was no inflammatory or immunological reaction, as assessed by the hosts' blood sequential study report of total count (Tc) and differential count (Dc). The histology of the retrieved tissue also did not show any inflammatory or





Figs. 35.1, 35.2, and 35.3 Histology of the retrieved tissue of the fetal adrenal gland in high-power microscope (Fig. 35.1), in low-power microscope (Fig. 35.2), and in oil immersion view under the microscope (Fig. 35.3) showing the axillary transplanted fetal adrenal gland in a patient suffering from intractable neuropathic pain. This fetal adrenal transplantation produced substantial relief of the neuropathic pain. What is interesting is the total absence of inflammatory or immunological reaction in any view of the microscope, and there is also no clinical feature of graft vs. host reaction in any of our patients in the present series

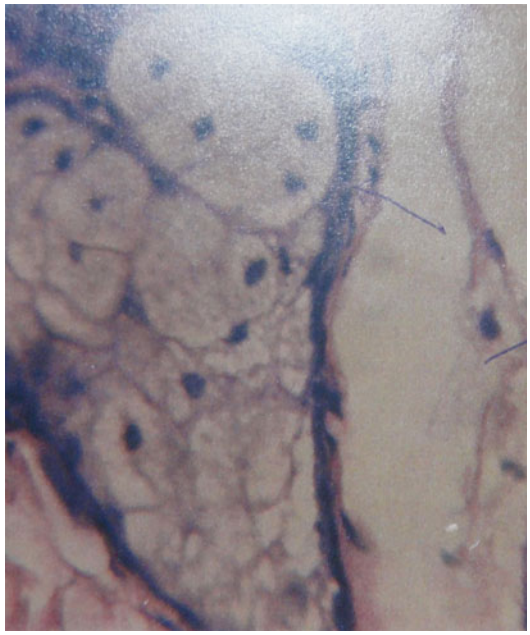


Fig. 35.1,35.2, and 35.3 (continued)

immunological reaction see microphotograph. The present group of investigators have had similar experiences with other fetal tissue transplants, like umbilical cord whole blood transfusion [35, 36], thymus transplant [37], and lung, heart, and pancreas transplants [38], which were, in each case, transplanted in the vascular subcutaneous axillary fold for the treatment of different intractable diseases. Hence, we are of the opinion that nonrejection of the transplanted fetal tissue (up to 20 weeks) is universal. It is a known fact that certain tissues in the human body enjoy a certain degree of immunological privilege, for instance, the cornea, the decidua of the uterus, cartilage, and the brain to some extent. But, the human axilla has never been cited as an immunologically privileged site. A possible cause for the nonrejection may be speculated here. It is generally believed by biological scientists that human fetal growth is dependent on a unique symbiotic environment where the mother provides all the necessary support for human fetal growth, proliferation, and differentiation. The fetal microenvironment is distinctly different from the adult neuroendocrine and metabolic microenvironment [39]. Therefore, it is possible that the transplanted fetal adrenal tissue adjusts its own microenvironment to an altered metabolic and immunological situation, using its naïve preimmune or hypoimmune status to prevent immune recognition of the host. Why and how the fetal adrenal tissue in a sex- and HLA-randomized nonprimed (no immunosuppressive support or radiation) host escapes the immunological or inflammatory recognition system and becomes a human homologous chimera is a mystery to be solved in the future. However, on the basis of our direct experiences as mentioned earlier, we strongly believe that the hypoantigenicity of the fetal tissue is the most important factor in preventing the hosts' recognition of the fetal tissue, which thus escapes rejection.

Furthermore, a question may be raised as to why there is pain relief in cases of advanced arthritis with inflammation and neuropathy after transplanting fetal adrenal tissue. The answer to this question may lie in the fact that the survival of the fetal adrenal tissue in the host, with its medullary component, contributes to the release of endorphin and other related compounds which help in lessening the pain [40–42] The transplanted young cortical cells may actively participate in steroid synthesis and release [43–46] and thus eventually play an anti-inflammatory role, the details of which are now being studied by the present researcher.

These are some of the secondary advantages of human fetal tissue transplantation as we have reported earlier in relation to human fetal neuronal tissue transplantation. The safety aspects of fetal tissue transplantation have also been reported on earlier [47].

Conclusion

Generally, arthritic patients, after a few years, will have pain relating to any or all of the components, like varying degrees of (a) synovitis, (b) muscle weakness involving different joints, and with (c) associated osteoarthritis, only to name a few. It is also important to realize that the cause of pain may differ between joints within an individual patient.

Current pharmacological therapies are often ineffective over time for the greater number of

patients in achieving effective neuropathic pain relief especially in case of advanced arthritis. Although there are a variety of useful surgical and pharmacologic interventions particularly for intractable pain (including electric stimulation, implantable mechanical pumps, and a myriad of drugs for pain relief), pain relief is not always present as per expectations.

Freshly collected and immediately transplanted fetal adrenal transplant for intractable and refractory arthritic conditions in addition to the standard guideline for treatment as an adjuvant has been found to be extremely effective clinically in this study, possibly because of the role of the constituents of the adrenal gland in the synthesis and uptake of catecholamines, opioids, and steroids. This may play an important role in nociceptive responses by the chromaffin cells, neural precursor cells, and mesothelial stem cells.

This effective transplant model for pain relief probably operates through the descending inhibitory of sensory information of the periaqueductal gray, reticular formation, and nucleus magnus project to the dorsal horn. Intrathecal transplantation of these cells induces analgesia in animal pain models.

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Part VI

Biobanking

36 Biobanking and Cryopreservation of Obstetrical Cell Sources for Cardiovascular Tissue Engineering: Implications for Future Therapies

Steffen M. Zeisberger, Benedikt Weber, and Simon P. Hoerstrup

Biobanking

Biobanks are central to translational research and have contributed to frequent advancements in our understanding and treatment of disease. As described by Watson et al., biobanks are collections of biospecimens and patient data. Biospecimens, for example, blood and body fluids, tissues, and their derivatives, collected for therapy and/or research are usually obtained from the public who become patients in the health-care system [1]. These patients donate biospecimens during hospitalization, which accrued by biobanks are processed and preserved in a variety of ways to support different research and/or, at a progressive rate, therapeutic applications. Annotation encompasses documentation of the biospecimens' composition associated with the patient history, circumstance, cure, and outcome. To insure the integrity of the banked specimens, three criteria must be the following: (1) purity/authenticity, ideally confirmed before and after asservation; (2) assignment of patient history, which has to be updated with prospective hospitalization; and (3) constancy, including repeated sample validation and quality control [2–4]. Generally, if material is used for therapy, principles of current good manufacturing practices (cGMP) are applied for the entire process from collection to freezing, storage, transportation, and thawing of the material [5]. Sample purity and authenticity is essential to evade incorrect data or performance of the sample. Authenticity is usually determined by testing stable phenotypic or genotypic characteristics. The value of each collected sample is strongly reliant on the associated dataset, that is, the health history of the donor. Consequently, the database collection should be based on a health history with continuous updates. It is a serious concern as cell cultures and cell lines are known to undergo irreversible changes if cultured, especially in unproven maintenance media for long periods [6]. It is essential that biobanks sustain quality control to ensure that stored and distributed samples maintain their characteristics [7] (for protocols see [3]). The precise quality control procedure varies depending on the particular characteristics of the type of culture. This can be achieved through the adoption of working practices including establishment of master and working banks. These requirements have been outlined in best

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practice guidelines for culture collections such as ISBER's "Best Practices for Repositories: Collection, Storage, Retrieval, and Distribution of Biological Materials for Research" [8].

Cryogenic Approaches

Cryopreservation by freezing or vitrification is a reasonable option and usually the method of choice for biobanks to preserve samples, an approach based on the principle that biological, chemical, and physical processes are effectively preserved at cryogenic temperatures [4, 9]. The difficulty of developing high-viability cryopreservation procedures becomes apparent when one considers the hostile environment to which cells and tissues are subjected during freezing. When the temperature drops from +37 to −196 °C, over 95 % of cell water can be lost. The electrolyte concentration inside and outside the cells can increase by several orders of magnitude relative to isotonic conditions. Concentrated organic solvents in the freezing media permeate the cells, and ice crystals intercalate the tissue and mechanically deform cells. In addition, ice may form inside and outside cells, disrupting intracellular structures and cell membranes, respectively. The key factors influencing the degree of dehydration achieved include the rate of cooling, the temperature of ice nucleation, and the terminal transfer temperature. Successful traditional and controlled rate freezing requires the careful application and choice of colligate cryoprotectants which reduce the "solution" effects, resulting in a lower optimal cooling rate and generally increasing the maximum survival levels obtained [9]. Although chemically defined nontoxic cryopreservation media should be used, the benchmark method for human cell cryopreservation employs either media containing human or fetal bovine serum or serum alone, as well as cell-permeating cryoprotective molecules such as dimethyl sulfoxide (DMSO) at high concentrations. DMSO is an amphipathic molecule and, besides causing adverse effects and toxicity to patients [10], may cause unexpected changes in cell fate [11]. It is well established that DNA methylation and acetylation control mammalian development and cellular differentiation [12]. DMSO most likely affects these epigenetic changes by acting on one or more DNA methyltransferases and histone-modifying enzymes [13]. A nontoxic and nonpermeating alternative cryoprotectant, especially for prospective human therapeutic interventions, is trehalose, a glucose disaccharide which has been shown to improve the stability of cells during freezing [14]. The major problem in using disaccharides is the impermeability of the plasma membrane, leading to considerable difficulties in introducing high concentrations of the polymer into the cell cytoplasm [15]. To increase the intracellular trehalose content, anionic liposomes have been used as a vector vehicle [16, 17]. The mechanism by which these cryoprotective agents protect cells against stress encountered during cryopreservation is not known, but it is assumed that permeating cryoprotectants reduces cell injury due to solution effects by reducing potentially harmful concentrations of electrolytes in the cell [9]. Furthermore, protection against intracellular ice formation has been attributed to the colligate effects of cryoprotectant chemicals [18]. In high concentrations, cryoprotective additives result in an increased viscosity of the extra- and intracellular solutions and, thus, dramatically reduce the rates of ice nucleation and crystal growth. This is the basis for vitrification protocols which employ very large concentrations of additives (6–9 M) in order to suppress ice formation during cryopreservation [19]. Vitrification is increasingly applied as an alternative cryogenic approach to conserve cell and tissue samples. Vitrification occurs when the viscosity of any solution reaches a sufficiently high value that causes the suspension to behave like a solid but without crystallization, that is, to become an amorphous glass. The elevated viscosities may be achieved by evaporative desiccation with osmotic dehydration and the use of penetrating cryoprotectants. Vitrification is critically dependent upon the formation of a stable glassy state on both cooling and thawing. Recurring thawing steps, in particular during sample loading processes, can be a significant problem on storage if cryostat temperatures cannot be guaranteed [20–22]. In some

cases, spontaneous devitrification with ice formation can occur at temperatures above −135 °C. In general, statements regarding unaltered cell-viability of cryopreserved samples are often based on an assumption that their storage temperature will be constantly maintained at the temperature of liquid nitrogen (−196 °C). Firstly, material stored in the vapor phase of nitrogen, of the most frequently used cryocontainer to avoid cross-contamination, does not necessarily achieve this temperature or exhibit a varying temperature gradient. Secondly, individuals searching for stored material very seldom move preserved samples into ambient temperatures even for brief periods, which, if repeated over time, leads to loss of viability. In order to assure the long-term viability of stored cells and tissues, it should be imperative that storage areas are technically well endowed and controlled, which guarantees a sustained cryogenic cooling-chain.

Private and Public Biobanking

Biobanks differ in concept and clients, from private to public and from clinical, including pathology archives, to research cohorts. Research biobanks exist in many formats from population biobanks to disease-focused biobanks. An escalating demand for biospecimens is resulting in the transformation of biobanking from conducted by individuals to a complex institutional activity. The emerging field of tissue engineering has identified product storage as a prerequisite for commercialization of tissue-engineered cell-based implants [23]. The market biobanking has expanded to embrace a range of specialized components including economic models, bioinformatics, equipment, policies, and personnel with defined functions. This means that biobanking, which was once an activity mostly limited to individuals, has now evolved largely outside clinical departments as a self-contained authority [4]. However, although appropriate staff training is very important, the implementation of the occupational area "biobanker" is still in the state of infancy. Since the 1990s, public and commercial blood banks cryopreserve cord blood units for different purposes worldwide [24]. Umbilical cord blood, which will be donated to public blood banks, as with the private storage, will be prepared, cryopreserved, and stored. The exact properties of the "drug" umbilical cord blood – such as tissue compatibility, cell amount, cell viability, and other – are fed into a worldwide computer network and can be retrieved by the recipient hospitals and compared with the data of potential recipients. If umbilical cord blood is donated to public blood banks via a global distribution system (e.g., The Netcord Foundation [25] or Bone Marrow Donors Worldwide (BMDW) [26]), it is available for any diseased child, also for patients, which have stored their umbilical cord blood privately. The BMDW organization combines participants of 43 cord blood banks from 25 countries. The current number of banked cord blood units in the BMDW database is reaching almost 0.5 million units. In contrast to commercial blood banks, public blood banks may struggle with organizational difficulties, resulting from a lack of financial resources. While private companies finance production, transportation, and storage of cord blood specimens with their income, nonprofit blood banks are restricted to public funding and donations. Therefore, donation is only possible in few, mostly to public cord blood banks affiliated hospitals due to high costs of transportation and sample processing. Commercial companies offer families the preservation and storage of cord blood during 20–25 years within a range of private financing of EUR 800–2,500. It is estimated that more than two million umbilical cord blood units are banked privately worldwide [24], which is mainly a result of an efficient logistic – and an intensive advertising system. Commercial companies advertise with the argument of the "private provision" or the "biological life insurance" for the individual storage. If a sibling suffers from leukemia or similar disease, privately stored cord blood can be used therapeutically. Cord blood can then be used as an allogeneic, means exogenous "directed donation" for the sibling [24]. Further, commercial blood banks advertise that private storage will render the possibility of treatment of rising numbers of diseases with cord blood stem cells in

the future, based on prospective establishments in the field of regenerative medicine. Regenerative medicine comprises both cell therapy and tissue engineering. Attempts to use stem cells, for example, similar to leukemic therapy, focus on the transfusion of systemic administered cells via the bloodstream, in order to make their way into the diseased organs and replace or support the damaged cells. The wish is to heal many diseases in the future: (1) In diabetic patients, the insulin-producing pancreatic cells could be renewed by new autologous functional pancreatic-like cells. (2) Paraplegics could be injected with new nerve cells, thus renewing the damaged spinal cord. (3) Another approach is the bioengineering of organ-like or tissue-like implants in the laboratory, a procedure known as tissue engineering [27]. Many strategies have been applied in recent years to replace, for example, cartilage [28, 29], bone [30], or skin [31] using cell-based constructs. Using viable autologous replacement materials for the repair of congenital cardiovascular malformations will improve the current used synthetic prostheses, which are associated with adverse side effects such as lifelong anticoagulation therapy, increased risks for infections, and thromboembolism. One of the most disadvantageous side effect for infants and children is that synthetic implants lack the possibility of remodeling and growth. Particularly, pediatric cardiovascular tissue engineering represents a promising approach focusing on the pre- or perinatal fabrication of viable autologous materials with growth, repair, and regeneration capabilities ready for use at or after birth to prevent secondary damage to the immature heart [32, 33]. Pre- or perinatal autologous cell sources, for example, derived from amniotic fluid [34], chorionic villi [35], amniotic membranes [36], or umbilical cord blood [37, 38] are prerequisites for these prospective therapies. Most likely, data from clinical trials will be available in 5–10 years, so the assessment of many scientists. However, it has to be proven whether the long-term storage of cord blood stem cells has an influence of their survival. Mesenchymal stem cells could not very restrictively be isolated from cryopreserved umbilical cord blood in contrast to hematopoietic stem cells or endothelial progenitor cells [39, 40].

There exists a public discussion which is focused on the question: Should cord blood stem cells be stored individually and used for private purposes only or should the public cord blood be donated to the principal benefit of all people demanding it for health reasons? How can this conflict be solved?

The current boom of private cord blood banks has rather parallel developed in the 1990s into a social situation characterized by severe cuts in healthcare and the privatization of social responsibility. The promise of a “biological life insurance” or a “vigilance for life” meets the reasonable desire of young parents to protect the life and health of their child the best possible way and partly on the comprehensible apprehension that the statutory health insurance will not meet and guarantee complete protection. The donation of umbilical cord blood is in the literature considered as medically appropriate [24, 41]. Unlike privately autologous stored umbilical cord blood, allografts are used under certain conditions as a therapeutic alternative to bone marrow transplants or transplants from peripheral blood. The specific characteristics of allogeneic cord blood stem cells – they are not burdened with pathogens (e.g., cytomegalovirus or Epstein-Barr virus) and, on the other hand, to possess the ability to destroy leukemic cells of the recipient’s body – make them interesting for leukemic therapy and the treatment of other immune and blood diseases in children. Its therapeutic limitations of poor engraftment as well as its scarcity restrict broader use of umbilical cord blood-derived stem cells to their application in pediatric care used for less than 2 % of stem cell transplantations. Side effects, including the delayed growth, lead to almost twice as high healthcare-associated mortality and morbidity for cord blood transplants compared to stem cell transplants from bone marrow. However, it should be noted that transplantation of allogeneic umbilical cord blood is under certain conditions the only option.

Cardiovascular Tissue Engineering

In 1993, Langer and Vacanti defined the term “tissue engineering” as an interdisciplinary field, applying the principles and methods of engineering

to the development of biological substitutes that can restore, maintain, or further improve tissue formation [27]. According to this predefinition, two principle strategies have been established to create living autologous heart valve replacements. One requires an in vitro conditioning phase generating the valvular substitute ex vivo [27]. This traditional tissue-engineering paradigm comprises the isolation and expansion of cells from the patient, subsequent seeding onto an appropriate scaffold matrix, in vitro tissue formation, and, finally, reimplantation into the donor patient. This paradigm, further referred to as the in vitro tissue-engineering concept, is being employed as the principal approach for heart valve tissue engineering and is aimed at full development of the tissue substitute ex vivo. The second approach of in situ heart valve tissue-engineering circumvents the in vitro tissue culture phase by straight implantation of natural tissue-derived heart valve matrices, aiming at potential cell ingrowth and ongoing remodeling in vivo [42] (reviewed by [43]).

According to the approach of in vitro tissue engineering, the successful ex vivo fabrication of autologous living heart valve replacements similar to the native counterpart is supported by three main elements: (1) Autologous cells that resemble their native prototype in phenotype and functionality are isolated and expanded using standard cell culture techniques. (2) These cells are seeded onto a temporary biodegradable supporter matrix fabricated in the shape of a trileaflet heart valve, termed the *scaffold*, which promotes tissue strength until the generated ECM (extracellular matrix) guarantees functionality on its own. (3) In order to promote tissue formation and maturation, the seeded scaffolds are then exposed to stimulation transmitted via a culture medium (biological stimuli) or via mechanical "conditioning" of the tissue in a bioreactor system. This aims at adequate cellular proliferation, differentiation, and ECM formation to form a living tissue model, called "the construct." This construct is subsequently implanted orthotopically as a valvular substitute, and further in vivo remodeling is intended to recapitulate physiological heart valve architecture and function [32, 33, 44].

The attempt to develop a scaffold for heart valve tissue engineering has proceeded along two fronts: (1) a biological matrix material and (2) a fully synthetic scaffold [45]. Regardless of the material of the scaffold matrix, the design of a scaffold capable of supporting cellular growth and of withstanding the cardiovascular environment, while forming a tight seal during leaflet closure, is critical to the success of the tissue-engineered construct. Besides meeting all the standard design criteria of traditional tissue valves, in which durability and biocompatibility are effectively passive attributes of the underlying materials, and selecting the optimal scaffold material, it requires consideration of the active cellular behavior in the regulation of tissue growth, remodeling, and homeostasis for fully laying the foundation for clinical application of the concept. Taken as a whole, the major goal displays the in vitro generation of a living autologous tissue-engineered heart valve with structural differentiation, anatomically appropriate and high quality extracellular matrix (ECM), viable valvular interstitial cells available to adapt to varying physiological needs and to repair structural injury by remodeling ECM as well as the capacity to grow with the patient (*reviewed by* [46, 47]).

Besides creating living autologous valvular replacements, an unmet medical need can be seen in creating growing replacement materials for vascular structures, which could potentially overcome the limitations of contemporary artificial vascular prostheses. In particular, the fabrication of tissue-engineered conduits for the pulmonary outflow tract would offer beneficial options for several patient populations, that is, those requiring Fontan procedures. Several studies have been performed, using different cell sources as well as graft diameters [48, 49], demonstrating the principal feasibility of creating autologous tissue-engineered vascular grafts (TEVG). In 2006, Hoerstrup et al. demonstrated the efficacy and safety of using autologous vascular cell-based TEVG, using a long-term ovine animal model up to 100 weeks follow-up [50].

Obstetrical Cell Sources

Cardiovascular tissue engineering aims at the fabrication of viable autologous cardiovascular substitutes with a thromboresistant surface as

well as a viable interstitium with repair, remodeling, and growth capacities. For the repair of congenital heart defects, the tissue-engineered construct would optimally be available when the first corrective intervention has to be performed to avoid harmful reoperations. For many pediatric patients, this optimal time window is located at or shortly after delivery. In today's clinical setting, congenital heart defects are often already detectable prior to birth by ultrasound examination performed on a routine basis. Therefore, the perfect pediatric tissue-engineering paradigm would comprise an antenatal cell harvest, providing time for the in vitro fabrication of autologous living implants. Ideally, these constructs would then be ready to be used directly or shortly after birth to prevent secondary damage to the immature heart. Consequently, after a prenatal sonographic detection of a congenital cardiovascular anomaly, the autologous fetal cells have to be harvested pre- or perinatally to enable the early fabrication of a tailor-made living autologous cardiovascular replacement. Besides umbilical cord tissue, chorionic villi as well as amniotic fluid-derived cells could be used for these applications (Fig. 36.1) [51].

Prenatal Cell Sources

Amniotic Fluid-Derived Cells

Amniotic fluid represents a highly attractive fetal cell source for the pediatric tissue-engineering concept as it enables easy prenatal access to fetal progenitor cells from all three germ layers using a low-risk procedure [52, 53]. Many studies have proven the principal feasibility of using human amniotic fluid-derived cells (AFDCs) for non-cardiovascular tissue-engineering applications [54–56].

Schmidt et al. demonstrated for the first time the feasibility of creating living autologous heart valve leaflets in vitro, using human amniotic fluid as a single cell source [34]. Both cell populations required for the in vitro fabrication of heart valves, namely, mesenchymal-like CD 133^{-} progenitor cells as well as endothelium-like 133^{+} progenitor cells, have been successfully isolated and expanded. With regard to the expansion of the versatility of these cells also for adult application, cryopreserved AFDCs have also been investigated as a potential lifelong available cell source. In 2008, Schmidt et al. showed the successful fabrication of viable heart valve leaflets in vitro using AFDCs as a cryopreserved cell source [57]. Importantly, it has also been shown that the differentiation potential as well as stem cell phenotype is not impaired by the cryopreservation procedure (Fig. 36.2).

Chorionic Villi-Derived Cells

The human placenta, particularly its fetal portion of the chorionic villi, provides extraembryonically situated fetal mesenchymal cells, including adult stem cells. Samples of the chorionic villi are routinely obtained for prenatal genetic diagnostics by biopsy using a low-risk procedure. These cells might also present a highly attractive cell source for pediatric tissue-engineering applications as indicated by recent investigations [58]. In particular, the high amount of cells gained from the donor organism seems to be a major advantage of this source. Schmidt et al. first demonstrated the feasibility of this approach in cardiovascular tissue engineering by using chorionic villi-derived cells, combined with umbilical-cord-blood-derived EPCs, for the fabrication of viable valvular leaflets in vitro [35].

Perinatal Cell Sources

Endothelial Progenitor Cells

It has been shown that the presence of endothelium on cardiovascular surfaces significantly reduces the risk for both coagulation and inflammatory complications (reviewed by [59]). Aside from these common endothelial properties, recent studies have also indicated a specific role of valvular endothelial cells in regulating the mechanical properties of heart valve cusps, suggesting a fundamental importance of endothelial cells for optimal valve function [60]. Therefore, to improve the functional capacities, the tissue-engineered heart valve constructs are usually covered with a layer of autologous human

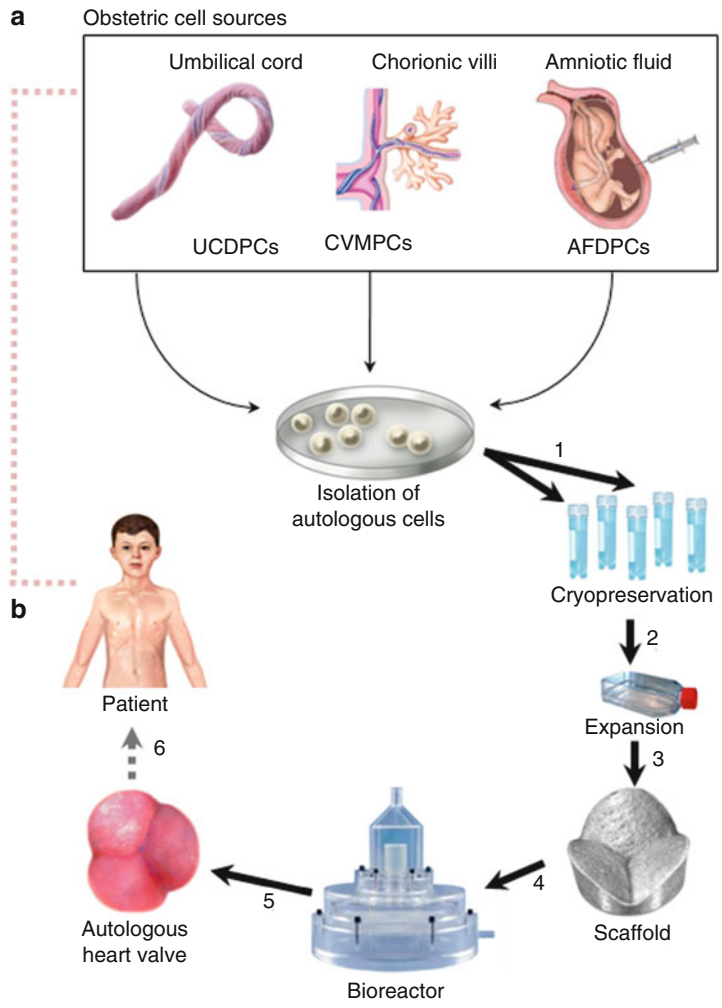

Fig. 36.1 (**a**) Obstetrical cell sources for cardiovascular tissue engineering. Umbilical cord-derived progenitor cells (*UCDPCs*), chorionic villi-derived mesenchymal progenitor cells (*CVMPCs*), amniotic fluid-derived progenitor cells (*AFDPCs*). (**b**) Implementation of cryopreservation in cardiovascular tissue engineering. Autologous cells are harvested from the patient, expanded in vitro, and cryopreserved (*1*). When the optimal time point for the surgical intervention is reached, cells are thawed and expanded (*2*). When sufficient numbers are reached, cells are seeded onto a biodegradable scaffold (*3*). After a short static phase, the constructs are positioned in a bioreactor (*4*) and conditioned. When optimal tissue formation is achieved, tissue-engineered constructs (*5*) are ready for implantation (*6*)

endothelial cells (ECs) as an antithrombogenic lining [61]. Fully matured ECs have been isolated from different vascular donor sources, demonstrating promising results in heart valve tissue engineering [55, 62–64]. However, the expansion rate of these harvested ECs is comparatively slow and the proliferation capabilities are limited, requiring a large number of ECs to be harvested for therapeutic use [65]. In addition, tissue-specific phenotype expression in ECs varies tremendously from one tissue type to another [66], which might interfere with the appropriate function of ECs. Beyond that, the harvest of ECs from donor vessels requires an invasive procedure, which is usually associated with a substantial risk for the donor. Therefore, finding a source of rapidly proliferating and ready-to-use ECs, lacking a tissue-specific phenotypic expression, is of critical importance. Endothelial progenitor cells (EPCs), first discovered in human peripheral blood by Asahara et al. in 1997, have been explored as possible sources of ECs. These blood-derived endothelial progenitor cells constitute a highly attractive alternative cell source of ECs, circumventing the harmful isolation of vascular-derived cells [67].

Theoretically, these cells can also be isolated already prenatally using the well-established method of ultrasound-guided cordocentesis, providing an elegant method of antenatal EPC harvest. However, more common seems to be the perinatal isolation of EPCs from umbilical cord blood [68].

Fig. 36.2 Macroscopic image of a *tissue-engineered heart valve* fabricated from human prenatally harvested amniotic fluid-derived and -cryopreserved progenitor cells [34]

Multiple groups have established isolation procedures for umbilical blood-derived EPCs and characterized these cells according to their angiogenic as well as colony-forming potential [69].

In general, EPCs display a rare heterogeneous population of mononuclear blood cells. Controversy exists with regard to their phenotype, origin, and identification. On the whole, there is consensus that EPCs can derive from bone marrow. However, several other possible sources of EPCs have been discussed critically, including myeloid cells, hematopoietic stem cells, and other circulating progenitor cells, and circulating mature endothelial cells shed off vessel walls (reviewed by [70–72]). Since their discovery, the therapeutic application and potency of EPCs for cardiovascular regeneration have been the subjects of intense clinical and experimental investigation. EPCs have shown to exhibit the potential to differentiate into mature endothelial cells and to show distinct regenerative features (reviewed by [73]). They have been successfully used for the repair of injured vessels, neovascularization or regeneration of ischemic tissue in preclinical

models [74–76], as well as, more recently, early-phase clinical trials (reviewed by [77, 78]). In addition, the ability of EPCs to promote revascularization has also been used for coating of synthetic vascular grafts [79], for endothelialization of decellularized grafts in animal models [80], and for seeding of hybrid grafts [81].

Schmidt et al. demonstrated the feasibility of using human umbilical cord blood-derived EPCs for the creation of constant neo-endothelial phenotypes in tissue-engineered cardiovascular replacements [82–84]. Recently, they also demonstrated the successful fabrication of biologically active living heart valve leaflets using prenatally available human umbilical cord-derived progenitor cells, including EPCs, as the only cell source [37]. EPCs, which were isolated from peripheral blood, showed stabile phenotypes when they were cocultured with non-endothelial cells as well as when exposed to mechanical stimuli. The extracellular matrix production of undifferentiated EPCs proved to be insufficient, even if their differentiation into endothelial cells on biodegradable scaffolds was observed [85].

Overall, EPCs represent a promising cell source for endothelialization of engineered cardiovascular replacements, such as heart valves and vascular grafts. Despite these pioneering studies showing great promise, the application of EPCs in tissue engineering is still in its infancy [86]. In the pediatric setting, the easy accessibility of umbilical cord-derived EPCs represents a major advantage for future therapeutic concepts. That is why current research also increasingly aims at their transdifferentiation into a mesenchymal, myofibroblast-like phenotype in an effort to provide new strategies to guide tissue formation in engineered tissues [87] and to ultimately enable blood as a single cell source for cardiovascular tissue engineering.

Umbilical Cord-Derived Cells

In order to provide tissue-engineered constructs for congenital heart defects including heart valve disease, alternative cell sources have been investigated, with particular attention to preserving the intact vascular donor structure of the newborn patients. With regard to this approach in the pediatric population, the umbilical cord may serve as an optimal perinatal autologous cell source for tissue engineering of cardiovascular constructs.

In general, the human umbilical cord, as a crucial part of the embryonic circulation, is composed of two arteries and one vein embedded in mucoid embryonic connective tissue, called the Wharton's jelly [88, 89]. Isolated cells of the umbilical cord represent a mixed cell population derived from these tissues [90, 91]. Interestingly, Kobayashi et al. demonstrated that all three of these cell types exhibit myofibroblast-like characteristics by co-expressing α-SMA and vimentin [92]. Additionally, Wang et al. identified cells within the Warton's jelly expressing significant amounts of mesenchymal stem cell (MSC) markers, indicating the existence of mesenchymal progenitor cells [89]. Ultimately, the presence of these MSCs, termed umbilical cord matrix stromal cells (UCMSCs), including their potential of multilineage differentiation and specific immune properties, has been confirmed by several groups, suggesting that UCMSCs represent a promising cell source for mesenchymal cell-based therapies comprising tissue engineering [93–97]. Taken as a whole, the umbilical cord contains several cell sources that can be utilized for heart valve tissue engineering: (1) SVECs (standard vascular endothelial cells) including HUVECs (human umbilical cord vein-derived endothelial cells) and HUAECs (human umbilical cord artery-derived endothelial cells), (2) HUCMF (human umbilical cord-derived myofibroblasts), (3) HUCB-EPCs (human umbilical cord blood-derived endothelial progenitor cells), and (4) HUCB-MSCs (human umbilical cord blood-derived mesenchymal stem cells).

At present, only sporadic experience exits with umbilical cord-derived cells for cardiovascular tissue engineering. In 1996, Sipehia et al. first described the use of HUVECs for creating a cell monolayer on artificial vascular prostheses [98]. In 2002, human umbilical cord-derived myofibroblasts were established as a new promising cell source for cardiovascular tissue engineering and used for the in vitro fabrication of pulmonary conduits using a biomimetic culture

environment. The morphologic and mechanical features of the engineered constructs approximated the native human pulmonary artery, and the human umbilical cord cells (UCCs) demonstrated excellent growth properties in culture [90]. A comparative study of vascular myofibroblasts isolated from umbilical cord artery (UCA), umbilical cord vein (UCV), whole umbilical cord (UCC), and saphenous vein segments (VC) revealed similar cell growth, morphology, and tissue formation with regard to the cardiovascular tissue-engineering approach, implying a comparable applicability of all cell sources investigated [91].

In 2004, Koike et al. created a network of long-lasting blood vessels with human endothelial cells derived from the umbilical cord vein in a three-dimensional fibronectin-type I collagen gel connected to the mouse circulatory system, demonstrating its in vivo function up to 1 year [99]. Schmidt et al. used differentiated human umbilical cord blood-derived endothelial progenitor cells (EPCs) seeded on vascular scaffolds for the formation of vascular neo-tissue in both biomimetic and static in vitro environment [83]. Next, the successful in vitro generation of living autologous cardiovascular replacements (patches) was based on myofibroblastic cells derived from Wharton's jelly and EPCs derived from umbilical cord blood demonstrated [84]. Recently, we were able to optimize the usage of umbilical cord-derived cells for cardiovascular tissue engineering by generating functional tissue-engineered blood vessels [82] and by ultimately fabricating biologically active heart valve leaflets in vitro [37]. Sodian et al. demonstrated the use of cryopreserved human umbilical cord cells (CHUCCs) for the in vitro fabrication of tissue-engineered heart valves [100]. Importantly, Wharton's jelly-derived myofibroblasts (WMFs) of the engineered valve leaflets exhibited phenotypic profiles of a fibroblast-myofibroblast lineage, indicating substantial similarity to native valvular interstitial cells [101]. Additionally, WMFs revealed excellent ECM (extracellular matrix) production, in amounts comparable to that of native tissue and similar to other cells that have been successfully used for heart valve tissue-engineering applications [62, 102], thus making UCCs, as WMFs combined with umbilical cord-derived EPCs, a highly attractive sole cell source for pediatric heart valve tissue engineering [37].

Overall, the major advantage of UCCs, as an autologous cell source, is deemed to be the avoidance of invasive harvesting of intact vascular structures from pediatric patients and the availability of approximately 25-cm vascular tissue sections, which allow the isolation of a large amount of juvenile, fast growing cells for the generation of a sufficient cell number for scaffold seeding in a short period of time [103]. Additional advantages include the possibility to preserve the postnatal cords by standard cell and tissue banking technology to obtain an autologous cell pool for the patient's lifetime [41, 102] and the feasibility of isolating EPCs from the cord vessels [104] to create an antithrombogenic endothelial layer on the tissue-engineered constructs, which may be crucial for their long-term function. Moreover, the presence of mesenchymal progenitor cells in the Wharton's jelly of human umbilical cords with multilineage potential [89, 95] and the possibility to obtain these cells prenatally using ultrasound-guided sampling technology make this cell source even more attractive [89, 105]. Recently, Sodian et al. demonstrated the use of cryopreserved umbilical cord blood-derived $CD133^+$ cells as a single cell source for tissue engineering of heart valves by differentiating them into myofibroblastic and endothelial lineages [106]. Even if the differentiation of MSCs into endothelial cells has been reported [107], research on this topic is still rare. Therefore, it seems questionable whether these $CD133^+$ cells represent 133^+ endothelial progenitor cells transdifferentiating into mesenchymal cells or whether they simply represent highly multipotent 133^+ MSCs having the ability to differentiate into endothelial lineages.

In the end, despite these beneficial properties of UCCs and the achievements of recent investigations, the fabrication of autologous pediatric cardiovascular constructs, such as heart valves and vascular grafts, from umbilical

cord tissue is still at a very early stage of development, and a number of issues remain to be investigated.

Outlook

The feasibility of using cryopreserved cells for cardiovascular tissue engineering has been shown as previously described with amniotic fluid-derived stem cells. However, certain stem cells and cell strains still cannot be maintained, cryopreserved, and unaltered. This has been observed in human cell preparations for biomedical research and applications, for example, in cord blood banking or bone marrow preservation. While certain cell types, for example, endothelial progenitor cells and mesenchymal, and hematopoietic stem cells, can be propagated from fresh cell preparations, mesenchymal stem cells appear to be lost post-thaw. Thus, recovered cell cultures could display altered characteristics which may diminish their value in research, medicine, and industrial applications. Not all cell cultures, for example, hematopoietic stem cells, are scalable for biomedical research or therapeutic use. Mesenchymal stem cell preparations may suffer from lack of homogeneity and techniques for reliable expansion assuring the undifferentiated state. Repeated partial freeze-thaw steps during sample processing and tank-loadings do not guarantee a continuous cooling-chain of collocated samples and unpredictably affect their long-term viability. Due to long production cycles for larger 3D tissue-engineered grafts, preservation is essential in order to ensure quality control and the off-the-shelf availability to clinicians. With regard to the clinical realization of the tissue-engineering concept, the cryopreservation process must require minimal post-thaw processing by the end user (clinician). Therefore, xeno-free or chemically defined cryomedia should be used instead of animal-derived material, and cryoprotective agents such as DMSO should be used sparingly or even substituted by nontoxic agents. Vitrification requires the use of rapid freezing and thawing for preservation of cells and tissues. Its boundaries are often found in cryopreservation of larger 3D structures, for example, by the limitation in temperature transmission. Traditional cryovials do not directly enclose the corresponding information of the donor, which makes mistakes possible during sample processing. Cryogenic microchips are beginning to enable new technologies for noninvasive interrogation of inventories of stored materials. Biobanks have to provide specialist advice and training; further governmental and industry support is vital for prospective developments and expansion of cell-based therapies in the field of regenerative medicine. Clinically applicable cryopreservation and biobanking of therapeutically relevant cells and materials will offer unique opportunities to advance the potential uses and widespread implementation for clinical applications. To combine the possibilities of private and public umbilical cord blood banking and to support flexible use, the concept of hybrid banking is used more frequently, for example, in England and Germany. Under this concept, the umbilical cord blood is collected privately and released only on demand if a sample is required by a diseased child. The parents or the donor itself frees the sample and gets the storage fee reimbursed including interest from the recipient's health organization. This might cause prospective tremendous amounts of privately banked umbilical cord blood units to be already used in the near future and account for private-banked units to be also available for the public as it is valid vice versa already.

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Part VII

Ethics of Fetal Tissue Transplant

Of Bioethics, Stem Cells, and Tissue Transplants

37

Sanjukta Banerji Bhattacharya
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I

The concepts of tissue transplant, autograft, allograft, and the use of redundant material for regeneration existed prior to their scientific discoveries in the West and the formation of terminology to describe them. The issue of bioethics was not raised in the societies where these things were practiced because the culture of these societies saw the cure of diseases and preservation of life as a primary focus of medicine, and their philosophical bases were humanistic and benevolent toward all living beings.

Morality and the issue of right and wrong (which may vary from culture to culture and country to country) are at the center of ethical thinking. Bioethics concerns issues that arise from the relationship between medicine and the life sciences and religion, philosophy, law, and also politics. While bioethics as a discipline is relatively new, ethics in medical treatment can be traced back to the earliest religious literatures of all ancient civilizations, be it Jewish, Christian, or Hindu. The Talmudic tradition speaks of a "compassionate God," and there is the parable of the Good Samaritan in the New Testament, both of which imply kindness and care for those suffering from disease. Medical lore in Hinduism was encased in a philosophy that emphasized the transcendent character of human life, the duty to preserve individual and communal health and the duty to rectify imbalances in nature that threatened the life and well-being of both humans and nonhumans, so much so that an entire Veda was dedicated to medicine as then known [1]. Medical ethics in the ancient world meant, if one can draw inferences from the religious texts, care for the sick and cure when possible because life was held in high esteem.

Medical experimentations and the introduction of new ideas in curing people or attaining longevity are described in ancient texts [2]. Hindu scriptures and epics also emphasized procreation and propagation of life as a central tenet, and in this context, it is interesting to note that the births of quite a few important characters in the epics, some of whom have been attributed as gods, were through means other than normal, for instance, through the "swallowing" of "thickened sweetened milk" (*payasam*). Recent scholars have tried to rationally interpret such stories in the light of modern developments in artificial insemination. Religious lore also speaks of the transplantation of an elephant's head on a god, after he had been decapitated. More to the point, the ancient Indian surgeon, Sushruta, apparently demonstrated how to reconstruct facial wounds through skin transplants (reconstruction rhinoplasty) by using the patient's own skin from another part of the body, way back around 600 B.C. [3]. The birth of

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Buddha was apparently a Caesarian birth if one were to interpret the lore. China too has a long history of medicine, and here, medical ethics was framed by Confucianism and Buddhism, both humanist and compassionate at the core. However, traditional Chinese medicine uses matter which may not be acceptable to the West or other societies, for instance, the dried human placenta for regeneration/revitalization purposes. Some of these practices may have raised questions of ethics in contemporary Western religions.

Modern bioethics has a more recent history. In the West, ethics in medicine can be traced back to the Hippocratic Oath. The first book dedicated to medical ethics appears to be *Conduct of a Physician*, written by Ishaq bin Ali Rahawi of Al Raha in northern Syria in medieval times (circa 854–931) [4]. Medical ethics emerged as a more specific discipline only in the eighteenth and nineteenth centuries with authors such as Thomas Percival who is credited with coining the term "medical ethics" [5]. The American Medical Association adopted its first code of ethics, largely based on Percival's work in 1847. While initially the Catholic faith influenced medical ethics, in the early twentieth century, this was substituted by a distinct liberal Protestant approach. By the 1960s and 1970s, liberal theory and procedural justice helped to reshape medical ethics into bioethics [6].

The emergence and development of modern bioethics were in response to blatantly harmful experiments on human beings, particularly belonging to racial minorities and/or underprivileged groups, first in Nazi Germany and later in the United States (USA), when humans were coerced into becoming participants in dangerous nontherapeutic research. Some of the more prominent examples are the Tuskegee syphilis study (1932–1972) and the Willowbrook hepatitis study [7]. In these and other experiments, the interests of the subjects were overtly disregarded. In the late 1960s, rights-based movements like the civil rights and feminist movements were also conducive to the emergence of ethical principles that were patient-centric and emphasized the rights of human subjects. In the United States, the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research produced the *Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Biomedical and Behavioral Research* in September 1978. The underlying ethical principles highlighted by this report are beneficence, autonomy, and justice, that is, the concept of doing no harm to the research subject while, at the same time, maximizing the benefits of the research study; informed consent and fairness; and nonexploitation in the administration of costs and benefits to potential research participants. If one looks back at age-old principles underlying medical treatment, these do not appear to be novel; in fact, they are derived from these same ancient practices of doing no harm while doing good to the individual's and the community's health. In the US, the Office of Human Research Protections was set up, and in 1991, various federal departments and agencies joined together to frame what came to be known as the "Common Rule," based on the Belmont Report to guide research on human subjects. In Europe too, the Council of Europe adopted the Convention for the Protection of Human Rights and Dignity of the Human Being with Regard to the Application of Biology and Medicine, which came into force in December 1999. This Convention lays down a number of principles and prohibitions concerning bioethics, medical research, consent, rights to private life and information, organ transplantation, etc. The Convention is underpinned by the concept that the interests of human beings come before those of science. As such, among other stipulations, it prohibits the creation of human embryos for research purposes and requires adequate protection of embryos where countries allow in vitro research. It also prohibits the removal of organs and other tissues which cannot be regenerated from people who cannot give consent, the only exception being regenerative tissue at the time of birth (under certain conditions), especially bone marrow, between siblings [8].

The main issues that concerned bioethicists in past decades were matters like euthanasia and cloning as well as abuse of human rights through uninformed consent or no consent at all for medical research. The Belmont Report

appears to have had these in mind as well as the conduct of medical practitioners in imparting medical treatment. However, with the turn of the century, many new issues have come to the forefront as new vistas have also opened up with research on the human genome, cloning, and stem cells for regenerative purposes. Questions have been raised regarding such research from the point of view of religion, ethics, law etc. At the same time, the new research promises to produce enormous benefits in patient care particularly in intractable diseases. In response to the issues raised, several methodologies have been advanced by different experts on the subject to incorporate bioethics into clinical practice [9]. The first of these approaches focuses on providing a theoretical framework for matters such as what would make an action good or a policy right [10]. The second approach, on the other hand, seeks to provide ethical guidance on clinical procedures through a certain set of moral principles, which are expected to provide the right answers regarding what the clinician ought to do. The best example for this kind of reasoning is provided by Beauchamp and Childress, who stress the principles of autonomy, beneficence, justice, and nonmalfeasance [11]. Theology-based principlism also comes within this category.

The third approach is a case-based one which addresses ethical issues by guiding clinicians via paradigm cases that have come up in clinical practice [12]. The fourth approach is a combination of techniques for identifying and resolving ethical issues using conflict resolution methods like negotiation, mediation, and arbitration, reasoning that resolving ethical issues can improve clinical practice [13]. An excellent small textbook by Jonsen and colleagues helps clinicians make more ethical choices in managing individual cases [14]. However, none of these approaches cover all the practical issues that may come up in modern clinical research. Moreover, different cultures, as noted earlier, have different approaches to the same issues, and therefore, opinions may vary regarding what actions will benefit humanity as a whole and are socially (morally?) acceptable. Some societies may permit certain types of research, whereas others may consider them to be against their religious beliefs and, therefore, their moral and ethical values.

II

One major subject of controversy is the use of stem cells for research. The present book focuses on fetal tissue transplant; however, the rationale behind such transplantation is the potential regenerative impact of the stem cells in the fetal tissue on damaged or degenerated adult organs/tissues. Today, donated organs and tissue are often used to replace ailing or destroyed organs and tissues, but the need for transplantable tissues and organs is much more than the supply. Moreover, the possibility of organ rejection and the use of supportive therapy to ensure nonrejection have their own hazards. Stem cells, which can be directed to differentiate into specific cell types, offer the possibility of a renewable and potentially large source of renewable cells and tissues and thus, again potentially, provide therapies not only for damaged organs which can now be replaced by organ transplantation but also for diseases which have so far been seen as intractable or incurable; these include Alzheimer's disease, Parkinson's disease, spinal cord injury, stroke, severe burns, heart disease, diabetes, osteoarthritis, and rheumatoid arthritis, to mention a few.

While stem cell-containing fetal tissue has the apparent capacity (potentiality as proved in animal experiments) to cure hitherto intractable diseases in adult human beings, the use of fetal tissue for medical research has led to a debate underpinned by several different types of arguments ranging from the religious to the legal and even the political. However, induced abortion is central to many of these. If a woman has the right to decide whether she wants to have a baby or not, medical researchers may also have the right, with informed consent of the donor and the recipient, to use fetal tissue from the discarded fetus, which in any case will serve no further purpose, for clinical research and practice.

Before going into the issue of abortion, it is important, at this point, to highlight the potential

benefits accruing from the use of stem cell-rich fetal tissue in the cure of various intractable diseases. Stem cells are undifferentiated cells that are able to differentiate into specialized cell types. Stem cells can be embryonic stem cells or adult stem cells, but the focus of this chapter is on the former and not the latter. The embryoblast of the blastocyst contains the group of cells that will differentiate to form all the structures of an adult human being. This is the source of embryonic stem cells or, rather, totipotent cells, that is, cells that have the total potential to become any cell in the body. The first few cells that result from the division of the zygote are considered to be totipotent, while embryonic stem cells are considered to be pluripotent because they have the ability to differentiate into almost any cell type but do not have the ability to become part of the extraembryonic membranes of the placenta. There are also multipotent stem cells that are present at later stages of embryological growth when the blastocyst develops into a fetus. An example would be mesenchymal stem cells (MSCs), which can differentiate into osteoblasts (bone cells), chondrocytes (cartilage cells), and adipocytes (fat cells). MSCs can be derived from umbilical cord blood, Wharton's jelly, amniotic fluid, adipose tissue etc. [15]. In fact, in the last 10–12 years, researchers have derived/extracted stem cells from many hitherto unknown sources. In 1998, John Gearhart of Johns Hopkins University extracted germ cells from fetal gonadal tissue (primordial germ cells); in 2003, Dr. Shonstak Shi of NIH discovered a new source of adult stem cells in children's primary teeth [16]; in 2007, researchers at Wake Forest University led by Dr. Antony Atala and Harvard University reported a new type of stem cell in amniotic fluid [17]; in 2008, Sabine Conrad et al. at Tubingen, Germany, generated pluripotent stem cells from spermatogonial cells of the human testes by culturing the cells in vitro under leukemia inhibitory factor (LIF) supplementation [18]; and in 2008, Monya Baker reported embryonic-like stem cells from a single human hair [19].

The reason that stem cells have become a source of intense research is because a stem cell is a father cell – or a mother cell, as one would have it – from which all tissues, organs, etc., that is, the entire adult body, emerge. Stem cells can differentiate to form different structures – in fact, they create the whole human baby. While they are available in the adult as adult stem cells, these do not have the kind of potency that embryonic or fetal stem cells have, that is, they can be multipotent but are not pluripotent. The argument in favor of embryonic or fetal stem cells is that since they can differentiate into all kinds of structures, they have the potential to regenerate almost any damaged structure. Allogenic adult stem cells have been used for many years in the treatment of leukemia and related bone/blood cancers through bone marrow transplants. In 2008, a report was published about the first successful cartilage regeneration of the human knee using autologous adult MSCs [20]. Although these cells have good potential for treatment of specific problems, these cells are lineage restricted. It is believed that pluripotent stem cells have immense potential therapeutic value, and diseases ranging from cancer to spinal cord disease and Parkinson's disease, to name a few, may be cured with stem cell therapy.

With embryonic and fetal stem cells being considered as extremely significant for clinical research into new cures for intractable diseases, the issue is whether it is feasible to acquire such cells without destroying the embryo – and this is where the crux of the ethical problem lies. In fact, so far, there have been two main sources: human embryonic cells (ES) and induced pluripotent cells (iPSC). Both have raised controversies. However, there is a third source of such cells whose procurement need not destroy an embryo or fetus, that is, amniotic fluid-derived stem cells (AFSC). Modern research has also indicated yet other sources such as umbilical cord blood and menstrual blood, from which stem cells can be isolated.

Human embryonic cells (ES) were first derived in 1998 [21], although mouse ES cells were reported to have been derived in 1981. Human ES cells are derived from embryos produced by in vitro fertilization (IVF). More embryos are produced through IVF than are needed for the treatment of infertility, and while some are frozen

for later use, many are discarded. It is from these discarded IVF embryos that ES cell lines were produced. In the United States, President Bush's administration took a policy decision in August 2001 to allow federal funding of this research (under certain conditions) for the first time. Since then, over a hundred cell lines have been developed in different parts of the world. Human ES cells are important for clinical research, for instance, testing of new drugs prior to testing them on humans. Moreover, they have the potential to provide an unlimited amount of tissue for transplantation therapies for a range of degenerative diseases that are caused by the death or dysfunction of one or a few cell types, for instance, insulin-producing cells in diabetes or dopaminergic neurons in Parkinson's disease. Human ES cells could be transplanted to replace these dysfunctional cells and thus provide lifelong treatment and cure for these disorders. Transplantation of ES-derived cells in animal models has already provided promising results [22].

Another technology is to produce pluripotent stem cells artificially from nonpluripotent cells by inducing a "forced" expression of specific genes. Induced pluripotent stem cells (iPSC) are actually adult cells that have been genetically reprogrammed to an embryonic cell-like state. Mouse iPSCs were first reported in 2006, and human iPSCs were reported thereafter in late 2007 [23]. These cells are already useful tools, like ES cells for drug development. They also hold out promises for transplantation medicine. For instance, tissues derived from iPSCs are expected to be a near identical match to the cells of the cell donor and if transplanted into him will probably not be rejected by the host's immune system. These bioengineered stem cells have so far appeared to have had beneficial effects on transplantation in model systems of sickle cell anemia, Parkinson's disease, hemophilia A, and ischemic heart disease [24].

When human ES cells were derived from IVF embryos, intense controversy was triggered. Several questions surfaced centering on the basic question: when does life begin – at fertilization, in the womb, or at birth? Is a human embryo equivalent to a human child? Does a human embryo have rights? May the destruction of a single embryo be justified if it provides a cure for many patients? Since ES cells can grow indefinitely in a petri dish and can, in theory, still grow into a human being, is the embryo really destroyed? The argument against the use of ES cells is that something which may have life and could have the potential for eventually becoming a human being, is being destroyed [25, 26]. Moreover, the premise here is that the life of human individuals is intrinsically and equally valuable at all stages of life beginning with the embryo, and therefore, destroying it is *pro tanto* intrinsically wrong.

These issues can be countered by utilitarian arguments, for instance, embryos are not equivalent to human life; they only have the potential for life outside the womb. More than a third of zygotes do not implant after conception [27], and therefore, more embryos are lost to chance than are proposed for use in stem cell research. Some believe that life begins only when the heartbeat is first detectable which is during the fifth week of pregnancy or when brain activity begins, which would be around the 54th day [28]; IVF produces many more embryos than are actually utilized; embryonic stem cells are superior to adult stem cells in their ability to differentiate; the potential for the occurrence of twinning and chimera formation until the 14th day of development justifies the lack of personhood status afforded to the embryo at least to that point. The potential for major medical advances justifies the use of ES cells for research – in fact, there are many more arguments used in favor of research using ES cells which will not be outlined here [29–31]. According to many researchers in the field, the destruction of embryonic cells, whatever their moral status as a potential human person (and this is itself contested as there is an issue regarding the right of a frozen human embryo to gestation), is acceptable, keeping in mind the best interests of the community of human beings, particularly if the embryos would be discarded anyway.

In contrast to ES cell research, induced pluripotent cell-based research has produced less controversy because iPSCs do not involve the destruction of an embryo. They are reprogrammed

cells created from adult cells like adult skin cells, which appear to have the properties of embryonic cells, that is, they have been reprogrammed back to a pluripotent state. As they do not require the destruction of the embryo, they could be the perfect alternative to therapeutic cloning or somatic cell nuclear transfer (SCNT). SCNT creates a cloned embryo that would be destroyed for the pluripotent stem cells inside: it requires human eggs, and a cloned embryo is created and destroyed [32]. While SCNT technology is hailed as holding much promise because the pluripotent cells thus created would have a genetic match with the patient, iPSC technology also produces a genetic match because the adult cell is from the patient. The advantage is that this technology does not require eggs or cloned embryos that would have to be destroyed. As such, it provides little ground for ethical arguments against it. Some have claimed that iPSC technology is possibly a superior alternative for future medical research because it obviates many ethical and resource-related concerns [33].

However, even iPSCs have raised certain issues in institutions like Johns Hopkins where they have formed an oversight committee to oversee all stem cell research (Stem Cell Research Oversight Committee or ISCRO). One such issue is donor consent. iPSCs can be generated from any adult cell, for instance, a blood sample or a skin biopsy, and can then be used for a variety of research projects or therapies. Ethically, there should be a patient donor consent to such uses. Another issue is genetic privacy. The iPSC will contain genetic information of the donor. Ethicists feel that protection of the donor's privacy should be ensured [34]. Apart from that, there is a question as to whether iPSCs are truly equivalent to ES cells. Moreover, certain futuristic scenarios have been described where ethical issues could become relevant. For instance, Denker has pointed out that viable individuals can be cloned from iPSCs (as from ES cells) by the direct cloning procedure of tetraploid complementation (TC), a method that does not require using oocytes [35]. In this method, an embryo can potentially be created from an iPSC by transferring it to a peculiar microenvironment [36]. Wernig et al. have already demonstrated that TC works well with iPSC in the mouse [37]. Experts feel that it would be also possible in the human although no such cloning has been reported so far. This, however, raises further ethical questions for the future of iPSC research.

III

ES cells and iPSC are not the only sources of stem cells for clinical research. In 2007, scientists from the Wake Forest School of Medicine and Harvard University reported that they had isolated stem cells from amniotic fluid [38]. The team isolated stem cells via amniocentesis as well as from the placenta after birth. They were able to get the amniotic fluid-derived stem cells (AFS cells) to transform into many different types of tissue found in fat, blood cells, liver, muscles, and bone as well as the central nervous system – which comprise all three embryonic germ layers: the mesoderm, the progenitor of bone, muscle, and connective tissue; the endoderm, which develops into digestive organs as well as the lungs; and the ectoderm, which becomes nerves, skin, and the brain. According to the lead scientist, Prof Atala, AFS cells are "truly pluripotent," and their major advantage is that after 2 weeks of culturing, they expand quickly, doubling every 36 h so that they are in large supply. When compared with embryonic stem cells, AFS cells have two main advantages: first, no embryo needs to be harmed in harvesting the cells, sidestepping that major, hot-button political issue. Also, as Atala points out, AFS cells will not form tumor cells, as the considerably more raw embryo-derived cells can. He further noted that AFS cells lie between embryonic and adult stem cells in that the former expand quickly but can develop into tumors, whereas the latter will not become cancerous, but grow exceedingly slowly [39].

AFS cells were hailed as a breakthrough in finding an "ethical" source of stem cells, with even the Vatican calling it a "turning point we have waited for." Cardinal Javier Barragan, the head of the Pontifical Council for Health,

noted that "what has been discovered is a leap forward, a very significant one because it does not harm any organ or discriminate against life" [40]. However, some critics have raised ethical issues even regarding AFS cells on the ground that amniocentesis should not be done for stem cell harvesting since it could lead to miscarriage; it should only be done for a sound medical reason [41]. The second issue revolves not around ethics but the "flexibility" of the stem cell line in question: while AFS cells are certainly multipotent, the question is whether they are actually pluripotent.

There are many other sources of stem cells, for instance, cord blood, the placenta, and fetal tissue apart from adult sources like bone marrow, body fat, menstrual blood, and peripheral blood to mention a few. Umbilical cord blood (UCB) stem cells are more primitive than bone marrow or peripheral blood-derived (adult) stem cells. They are also easy to obtain without any harm to a developing fetus or a newborn baby because the cord blood is collected after the birth of the baby and after the umbilical cord has been severed. Although the issue of ethicality surrounding UCB appears to be moot, controversy has certainly arisen regarding cord blood banking from governments and nonprofit organizations particularly vis a vis private banking. For instance, the European Union Group of Ethics' *Ethical Aspects of Umbilical Cord Blood Banking* (2004) noted that "[t]he legitimacy of commercial cord blood banks for autologous use should be questioned as they sell a service, which has presently, no real use regarding therapeutic options. Thus they promise more than they can deliver. The activities of such banks raise serious ethical criticisms" [42]. Moreover, according to the World Marrow Donor Association (WMDA) Policy Statement for the Utility of Autologous or Family Cord Blood Unit Storage, the use of autologous cord blood cells for the treatment of childhood leukemia is contraindicated because preleukemic cells are present at birth. Autologous cord blood carries the same genetic defects as the donor and should not be used to treat genetic diseases [43].

The placenta is perhaps an even better source of stem cells since it apparently contains ten times more of such cells than cord blood. Placental cord blood is rich in hematopoietic stem cells, and the advantage is that there is no risk to the donor or the neonate. However, the safety and efficacy of umbilical cord-/placenta-derived stem cells are not yet foolproof, and as such, the US Food and Drug Administration (FDA) has produced an elaborate regulatory document to bring products derived from such biological matters under a legal framework [44]. In fact, developed countries, in general, have legislated frameworks for stem cell research, whether they be ES cells, IPSCs or AFS, UCB, or placenta-derived cells that generate fewer ethical controversies.

IV

While developed countries have the funds and the infrastructure to separate stem cells from various biological products and also induce adult cells to transform into cells that have properties of embryonic cells, less developed countries do not have that kind of funding to develop similar infrastructure. In these countries too, scientists have been working on therapies for intractable diseases using whole biological products, that is, products like umbilical cord whole blood, fetal tissue, and placental tissue, in their entirety without separating the stem cells. The rationale is that these substances contain stem cells and additional material which have the potential to stimulate recovery; for example, umbilical cord whole blood contains not only stem cells but growth factors and cytokines and has more oxygen-carrying capacity, and therefore, transfusion of this blood may have a better long-term effect than adult blood. Similarly, placental or fetal tissues contain more specialized properties that could add to the attributes of embryonal stem cells. While umbilical cord whole blood or placental tissue may raise less controversy, the use of fetal tissue again raises questions regarding life and death and the rights of the unborn. The ultimate question is, of course, the right of a woman to decide whether she wants or does not want a baby, that is, abortion [45]. Some analysts believe that the US government has restricted funding to fetal tissue research

despite other countries' reported advances in the field, not because of moral convictions but because of the politics of abortion.

Actually, fetal tissue research began in the middle of the twentieth century even in an advanced country like the USA. Fetal tissue research for biomedical purposes increased as the restrictions on the availability decreased. Such research played an important role in the diagnoses and treatment of fetal diseases and defects as well as in the development of amniocentesis as a diagnostic tool. Fetal tissue is more flexible than other human tissue and is less likely to be rejected by the immune system, and it is therefore seen as a useful alternative to adult tissue in transplantation. Researchers have already had limited success in the treatment of diseases like Parkinson's disease, Alzheimer's disease, and other illnesses through the use of fetal tissue. Sass and colleagues in a series of publications beginning in 1988 have reported that human fetal mesencephalic tissue could be obtained at suction abortion at 9–12 weeks' gestation, cryopreserved, tested to rule out infection, and eventually be stereotactically implanted into the caudate nuclei of people with advanced Parkinson's disease [46]. Many of the subjects then showed clinically important improvement in memory.

In the US and other developed countries, normally specific relevant fetal cells are injected directly to the diseased organ. However, in developing countries like India, the relevant fetal tissue has been placed at a heterotopic site from where it can be quickly recovered if required, and the resultant effects have been entirely beneficial to the patient [47]. This also perhaps suggests that there is a homing effect, that is, the necessary fetal cells travel to the diseased/damaged organ and regenerate it. Many scientists feel that fetal tissue research holds great promise in the treatment of many debilitating and intractable diseases.

At the same time, scientists also warn that the use of fetal tissue must be strictly regulated to avoid ethical issues. In fact, fetal tissue research became a matter of controversy following a 1973 US Supreme Court ruling in Roe vs. Wade which protected the right of a woman to abortion in the first two trimesters of pregnancy, which potentially made fetuses available for research. In 1974, the US National Research Act created a national commission to oversee research involving fetuses which drew up a guideline placing restrictions on certain types of fetal tissue research [48]. Later, President Reagan's administration issued a temporary moratorium on federal funds for fetal tissue transplantation research because of the link with abortion. In subsequent years, efforts to overturn this moratorium failed repeatedly until President Clinton ordered its end in 1993, and the US Congress passed the NIH Rehabilitation Act (1993) which permitted the use of tissue from any type of abortion to be used for fetal tissue research. The law however criminalized the sale or purchase of fetal tissue and the designation of the recipient of fetal tissue. The controversy again became spotlighted during President HW Bush's administration when federal funding was severely limited for embryonic stem cell research [49].

The arguments against fetal tissue research range from the moral to the legal. The moral argument holds that the fetal tissue researcher is complicit in the destruction of the fetus [50], and fetal tissue research will create incentives for more abortions [51]. The legal argument encompasses the view that a woman who has aborted her fetus has abdicated her parental responsibility/authority through the act of abortion and therefore cannot legally authorize research on the aborted fetus [52]. Other authors are of the opinion that the use of aborted fetal tissue is the first step toward an abyss at the bottom of which are the Nazi experimenters, that is, researchers without scruples and without concern for the research subject or the dead fetus [53]. There is also the issue of guilt by association, that is, complicity in a manner of speaking, which would make not only the mother and the researcher but also the funding agency guilty of funding or encouraging an immoral act [54]. Some critics, however, have noted that fetuses from ectopic pregnancies or spontaneous abortions can be used for research. The ignorance of human reproductive biology displayed by this suggestion is startling. To find an ectopic with a formed fetus is a rare event, and most miscarriages are anembryonic or represent fetal death of several weeks duration.

The issue of fetal rights is intertwined with women's rights and needs explanation at this point. The term "fetal rights" came into usage after the Wade vs. Roe case giving American women the right to abortion in the first trimester and limited rights to abortion in the second trimester. In ruling, the Supreme Court made the point that a fetus is not a person under the terms of the 14th Amendment to the US Constitution. However, the court also maintained that the state had an interest in protecting the life of a fetus after viability (the point at which the fetus becomes capable of living outside the womb). As a result, abortion itself became an impassioned issue raising strong sentiments and lobbies on both sides at the national level, and "fetal rights" became part of the lexicon of those who were against abortion to promote their case. However, many feminists became suspicious of fetal rights because these could seriously diminish women's rights to self-determination and bodily autonomy [55].

The abortion issue, particularly in the West and certain religion-based cultures, has been a contentious one because it involves the issue of "personhood," that is, when does a fetus become a person – and it should be remembered that a *person* has rights as well as *duties* which he must also be viable enough to perform – and whether man has the right to terminate the life of a "person." The argument in favor of right to life holds that a fetus is a *potential* human being, and therefore, the unborn has a right to life from the earliest stage of development. However, others hold that this "potential" argument is flawed: for instance, a child is a potential adult, but that does not give the child the same rights as an adult. Thus, many of the rights of a potential person are potential rights; they become actual rights only when the potential person becomes an actual person.

In the context of women's rights, the abortion issue has often been presented, however, not as a moral issue, that is, whether abortion is ethically wrong, but as a political one – should women be prevented from aborting an unwanted pregnancy? The women's rights argument in favor of abortion places an appropriate value on the lives and freedom of women, which would also imply that they should not be forced to abort a fetus if they do not have family support in continuing the pregnancy. Therefore, while feminists have been portrayed as being pro-choice, it actually means that what they want is the freedom to have the choice to abort or not to abort.

Most traditional religions also do not condone abortion, whether it be Christianity, Buddhism, Hinduism, or Islam, because these religions have sanctity of life at their base. However, on the issue of saving the mother's life, these same religions provided exceptions. Today, many countries allow abortions for other reasons as well, some giving priority to women's rights over their bodies, others placing emphasis on birth control to stem overpopulation, etc. It is interesting to see how religions have coped with the issue; in fact, it emerges that traditional religions are more practical in shaping themselves to practical needs. To cite an example, in Iran, a theocratic Islamic state, the Grand Ayatollah Yousef Sanei, while admitting that abortion was generally forbidden in Islam, issued a fatwa in 2000 stating that "Islam is also a compassionate religion and if there are serious problems, God sometimes doesn't require his creatures to practice His law. So under some conditions – such as parents' poverty or overpopulation – then abortion is allowed" [56]. Traditional Buddhism too rejects abortion if it involves deliberate destroying of life, but now that possible birth defects may be detected before birth, the Dalai Lama has said that "I think abortion should be approved or disapproved according to each circumstance" [57]. Japan, which is predominantly Buddhist, allows abortion for birth control purposes. Even modern Catholic theologians have an interesting take on this issue. To cite Cardinal Bernadin, "if one contends, as we do, that the right of every fetus to be born should be protected by civil law and supported by civil consensus, then our moral, political and economic responsibilities do not stop at the moment of birth. Those who defend the right to life of the weakest among us must be equally visible in support of the quality of life among the powerless among us: the old and the young, the hungry and the homeless, the undocumented immigrant

and the unemployed worker. Such a quality of life posture translates into specific political and economic positions on tax policy, employment generation, welfare policy, nutrition and feeding programmes, and health care. Consistency means we cannot have it both ways. We cannot urge a compassionate society and vigorous public policy to protect the rights of the unborn and then argue that compassion and significant public programmes on behalf of the needy undermine the moral fiber of the society or are beyond the scope of governmental responsibility" [58]. This brings the ethical debate on abortion to a different ethical level altogether wherein the government is given the moral responsibility of taking care of and providing for the amenities and a certain quality to life to the teeming millions that will be born as a result of a public stand defending the rights of the unborn.

Many countries have legalized abortion in the first trimester or until viability or even beyond in the case of serious maternal risk or fetal malformation. For instance, the Medical Termination of Pregnancy Act (1971) of India allows abortion by certified doctors for several reasons which include failure of other methods of family planning but does not imply abortion "on demand" and is further subject to time limits based on the period of gestation. The Act was amended to make safe abortion accessible all through the Medical Termination of Pregnancy (Amendment) Act, 2002, and Rules and Regulations 2003. Other countries have also passed legislations to permit abortions under certain circumstances. In fact, although the exact number of abortions all over the world per year is not known, expert estimates placed the total of induced abortions at 42 million in 2003. The abortion rate per 1,000 women aged 15–44 years was estimated at 29 in the same year. Most countries of the world have approximately the same rate when safe and unsafe abortions are totaled. The exceptions are North America which had a rate of 21 and Western Europe which had a rate of 12 in 2003 [59]. In India, it has been computed that about six million abortions take place every year of which four million are induced and two million are spontaneous [60]. However, the statistics may be grossly understated because hospitals record only legal and reported abortions.

Whatever the case, what is of significance here is that despite religious and ethical views, a large number of pregnancies in both the developed and the developing world end in induced abortions. Though the abortion rates per 1,000 reproductive age women are similar throughout the world, given the huge populations of Asian countries, the number of abortions is much larger in Asia than in Western developed countries [61]. China itself is estimated to have about nine million abortions each year particularly because of its one-child policy [61]. The fetuses which are aborted are destroyed in any case in an incinerator or are otherwise disposed of and do not serve any purpose whatever. If with due informed consent from the donor and the recipient, tissue from the fetus which was aborted by the mother for her personal reasons could be used for the purpose of research which has the potential to do good without doing any harm and is for the benefit of the human community, bioethicists should have little to complain about. Given the statistics of abortions that are induced in both the developed and the developing world, there is no dearth of aborted fetuses, which would otherwise be sent to the incinerator. These and other biological waste could serve the purpose of science instead of remaining just that – waste. Here, the question is not of abortions for the sake of fetal tissue or organ donation and therefore the destruction of a fetus for the sake of clinical research. In countries where women have the choice to abort within the limits of their municipal laws, the otherwise useless aborted fetuses that are slated for total annihilation lest they pollute the premises, could be used for research under stated guidelines.

V

Research in human stem cell biology is providing unprecedented opportunities for understanding human embryology and studying new methods of therapy and cure for debilitating diseases that are

a source of fear to people of all ages, particularly to the aging generations. Although it is too early yet to predict the outcomes of basic ongoing research, there is great hope that such research offers a real possibility of finding a treatment and cure for many diseases for which adequate therapy is not yet available. Evidence from animal studies already indicates that stem cells can be made to differentiate into cells of choice, and these cells will act properly in their transplanted environment. In human beings, transplants of hematopoietic stem cells following treatments for cancer have also been done for years. In fact, transfusion of umbilical cord whole blood which contains these cells has also been done, and its safety and effectiveness have been tested. Fetal cell transplants have also been done for Parkinson's disease and have shown promise. In developing countries, less sophisticated methods like the application of placental tissue in burn patients or fetal organ/tissue transplant in intractable diseases have also shown promising results. Such research has indicated the safety of the procedures and indicated that they hold great promise for therapy and cure of diseases like Parkinson's and Alzheimer's. However, the true promise will unfold only through controlled scientific research.

Innovative technologies and new research always raise controversies, ethical issues, and policy concerns. When it comes to stem cell or fetal tissue research, since the subject involves human embryos, the controversies are bound to be even more virulent involving not only the ethics of medical research but also philosophy, religion, and the rights debate. However, it may be noted that all religious traditions have a commitment to healing and relieving suffering caused by injury and illness. Because of this commitment, many religious communities applaud the promise of stem cell research for enhancing scientific understanding of human development; for probing the cellular origins of cancer, diabetes, spinal cord injury, arthritis, and a host of other lethal or disabling illnesses and conditions; for developing more effective pharmacological drugs; and for pursuing successful tissue and organ transplant technology. The main issue at the moral level is the right of a fetus to life. This question, of course, is very important, but in countries where abortion is legal (under certain strict guidelines) and aborted (first trimester) fetuses are available, these could be used for research without bringing in the moral question because these fetuses would have been aborted in any case.

However, there is certainly a need for oversight and regulatory mechanisms to ensure that there is no malpractice or research that is ethically unjustifiable. Most countries in fact have such guidelines in place. In India, there is the Ethical Guidelines for Biomedical Research on Human Participants [62]. The therapeutic potential for further research is often used as the justification for requesting permission and funds for such research. It is perhaps necessary to ensure therefore that the benefits of this research are available to all with little regard to the ability to pay. According to the WHO, "justice demands equitable access to genetic services." It has also stated that "genetic services for the prevention, diagnosis and treatment of disease should be available to all, without regard to ability to pay, and should be provided first to those whose needs are greatest" [63]. In developing countries, the donors are mainly from the lower-earning sections of society who wish to control the size of their families. They, too, should not be deprived of the benefits of this research through the emergence of a big private sector in the area to the near exclusion of the government sector which could provide these benefits at little cost. This will be in tune with the basic principle of medical ethics to do good and provide medical services to all. Finally, the point of ethical oversight should also include donor consent and the right to privacy of both the donor and the recipient.

To end, given the fact that there can be no end to the debate surrounding bioethics, stem cells, and fetal tissue transplants in current times, that is, not till such research produces significant therapies, a quotation from Confucius appears to be appropriate here:

> To see what is right and not to do it is want of courage [64].

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