Gene Therapy and Cell Therapy Through the Liver

Current Aspects and Future Prospects

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Foreword

This book, entitled *Gene Therapy and Cell Therapy Through the Liver: Current Aspects and Future Prospects*, consists largely of the subject matter of the international session of Japan Digestive Disease Week 2013, held in Tokyo. The planning of this book was initiated by Springer Japan, with the content focused mainly on gene therapy and cell therapy related to liver diseases. In addition, the book includes related topics from legal issues and industrialization to experts in the field as related to the medical application of these treatments. The editors are Drs. Takeshi Suda and Shuji Terai (Niigata University), young and energetic Japanese researchers in this field who co-chaired the internal session of the 17th Annual Meeting of the Japan Society of Hepatology, 2013.

Cirrhosis, especially in the decompensated state, is the main problem leading to death at the end stage for patients with many liver diseases, with such causes as viruses, alcohol, autoimmunity, and congenital gene abnormalities, although the etiology is different. In these conditions, liver transplantation is the fundamental treatment regardless of cause. On the other hand, plasma exchange is a possible tool as a palliative treatment for the purpose of serum protein supplementation and the removal of toxic substances. However, long-term treatment hardly could be expected owing to economic restrictions imposed by medical insurance.

Cell therapy, such as hepatocyte transplantation and hepatocyte-directed gene therapy instead of liver transplantation, may be a promising surrogate treatment for a cirrhotic condition. For the purpose of cell replacement to a damaged liver, mesenchymal cells are also considered. Candidates of cell type to be used may include induced pluripotent stem cells, bone marrow-derived cells, or embryonic stem cells, among others. However, gene therapy is the ideal way for the replenishment of the restored hepatocyte material such as blood coagulation factors, alpha-1 antitrypsin, and several metabolic enzymes in the ornithine cycle. In a cirrhotic condition, dissolution therapy with fiber-degrading enzymes and anti-fibrosis agents are also candidates for the treatment of a liver with an advanced fibrotic change. As a consequence, an increment of portal blood flow with a decrement of portal hypertension and an improvement of esophageal varices are expected.

It is believed that cell therapy by immunocompetent cells is promising for the purpose of enhancement of tumor immunity after curative treatment for hepatocellular carcinoma as well. Of course, it may also prove to be effective for the prevention of a recurrence in patients with hepatocellular carcinoma with viral cirrhosis.

In the practical implementation of these therapies mentioned above, there are many hurdles that must be overcome in Japan. I hope that this book contributes to resolving any technical problems of cell therapy and gene therapy as well as legal problems, with the support of pharmaceutical companies. Then in the near future, cell therapy and gene therapy for inherited and acquired disorders of the liver will no longer be a dream but a reality.

Niigata, Japan Yutaka Aoyagi

Preface

Gene and cell therapy is a challenging medical approach, different from any other options that human beings have taken in disease control. It has never been possible to manipulate directly the code and/or structural unit, gene and/or cell, in clinical medicine. Gene and cell therapy is a medical strategy to recover biological functions in our body by manipulating the code and/or structural unit. As this strategy directly operates on the fundamental elements of the body, an application of the strategy requires a comprehensive framework incorporating not only medical and biological sciences but also a broad range of human activities such as bioengineering, ethical concepts, legislative action, and economics. Furthermore, global agreement is important for efficient administration especially to compensate the donor–recipient imbalance in cell therapy and to share valuable information (Fig. 1). This book is edited on the basis of the international symposium entitled "Gene Therapy and Cell Therapy Through the Liver: Current Aspects and Future Prospects", which was held during Japan Digestive Disease Week (JDDW) 2013, Tokyo, by aiming to enhance cooperation among professionals from different fields.

In this book, the first four chapters focus on cell therapy, which includes not only regenerative medicine but also immunotherapy against hepatocellular carcinoma. In terms of regenerative medicine, macrophages or mesenchymal cells were employed to reestablish functional liver units, while immunotherapy utilized natural killer or dendritic cells to induce antitumor activities. Both strategies have entered a clinical trial stage to establish the soundness of the concept.

Gene therapy relies on vectors enabling efficient gene delivery, and various viral or nonviral gene transfer systems are under development to ensure both efficiency and safety. In more than 2000 clinical trials that have been approved, so far viral vectors were most frequently used due to their efficiency; however, safety concerns are pushing the development of effective nonviral vectors. In this book, adeno-associated viruses, human artificial chromosomes, and hydrodynamic gene delivery are spotlighted as the most promising and advanced approaches in gene therapy.

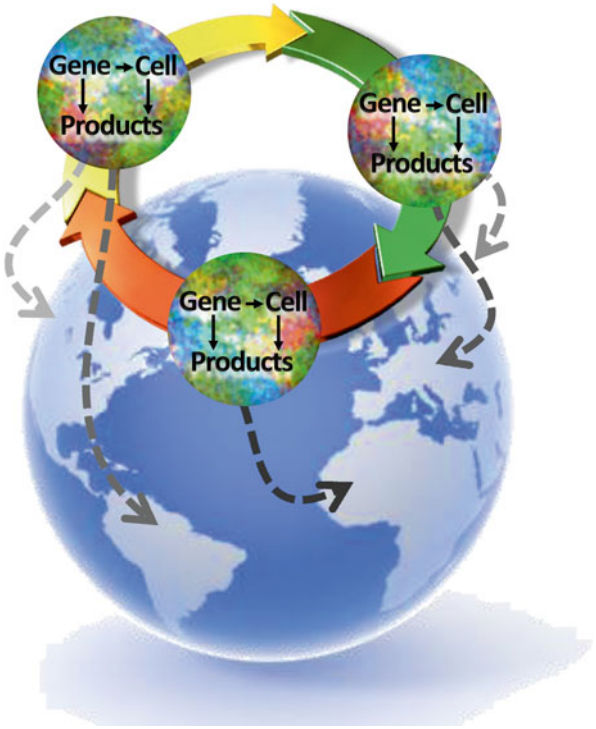

Fig. 1 A colorful round shape represents a legislative embargo, and each color expresses various human activities such as scientific research, clinical medicine, bioengineering, ethical concepts, and economic considerations. In each local area, there is a unique administrative system to provide gene and cell therapy products, and they should be connected to one another to enhance efficient synergistic use of the products and information

Current regulatory aspects in Europe, the United Sates, and Japan are covered in association with gene and cell therapy including induced pluripotent stem cells. Exploring a practical way to merchandise the gene and cell therapy products under good manufacturing practices, Japanese companies were invited to contribute two chapters.

It will be our great pleasure if this book helps achieve the goal of expanding the field of gene and cell therapy to a wide variety of professionals.

Niigata, Japan

Takeshi Suda
Shuji Terai

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Chapter 1
Liver-Targeted Gene and Cell Therapies: An Overview

Mohammad Alsaggar and Dexi Liu

Abstract Until very recently, management of several liver malignancies, viral hepatitis, hepatic cirrhosis, and hereditary metabolic diseases remained unsatisfactory, and thus, efficient therapeutic approaches have always been in need. In parallel with recent advances in molecular biology and recombinant DNA technologies, research in liver diseases and the quest for molecular insights of disease pathology have witnessed remarkable progression, and early and specific detection of genetic, infectious, and malignant liver diseases has become feasible like never before. Several molecular approaches combining genetics, biology, chemistry, and computer sciences have been introduced, and in particular, gene- and cell-based therapies opened up new opportunities that step out beyond classical pharmacology. Gene therapy emerged as promising therapeutic strategy aiming to introduce genetic material into cells to generate curative effects. Gene therapy comprises various methods of gene delivery and innovative overexpression and silencing designs for specific therapeutic needs (Kay MA, Nat Rev Genet 12:316–328, 2011). Cell-based therapies, on the other hand, aim to use biologically active living cells instead of DNA or RNA as treatment modality. Several extracorporeal and implantable cell therapies have been developed, such as bioartificial liver (Baquerizo A, Mhoyan A, Kearns-Jonker M, Arnaout WS, Shackleton C, Busuttil RW et al, Transplantation 67:5–18, 1999) for short-term treatment and liver cell transplantation for permanent liver replacement (Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI et al, N Engl J Med 338:1422–1426, 1998). In this chapter, we will briefly discuss the current theories and potential applications of gene- and cell-based therapies for the treatment and/or prevention of various liver diseases.

Keywords Gene therapy • Liver gene therapy • Cell therapy • Liver disease • Gene delivery

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1.1 Gene Therapies

Liver diseases can be classified into inherited monogenic disorders caused by a single gene defect, such as liver cystic fibrosis, and multifactorial disorders caused by defects of several genes that are acquired and accumulated during lifetime, such as liver malignancies. Most inherited metabolic disorders have an underlying genetic defect in the liver [1–4]. Such defects often result in loss, dysfunction, or accumulation of proteins or metabolites contributing to disease pathology. Gene therapy has evolved as a potent means of treatment of pathogenic gene defects using nucleic acids to alter gene expression and restore pathogenic phenotype into normal status. Importantly, the potential of gene therapy is not limited to monogenic hereditary diseases but also for various multifactorial disorders, such as infections, degenerative diseases, and cancers.

1.1.1 Liver-Directed Gene Delivery

To elicit curative effects, therapeutic genes must be safely and efficiently introduced into liver cells. Importantly, the real target for liver-directed gene transfer is liver parenchymal cells (hepatocytes), which account for 65 % of liver resident cells, rather than sinusoidal cells which constitute the remaining fraction, including endothelial cells, Kupffer cells (liver macrophages), stellate cells or Ito cells, and pit cells (natural killer cells) [5]. Sinusoidal endothelial and Kupffer cells are functionalized scavenger cells for particulate entities and, thus, considered a major challenge for liver-directed gene transfer [6]. Diverse systems have been developed to overcome barriers of gene delivery utilizing biological systems, such as viral and bacterial systems, and non-biological systems, including chemical and physical methods of gene transfer [7]. To date, viral vectors, which are engineered replication-deficient viruses, remain the most efficient delivery systems and the most commonly used in clinic [8]. However, their immunogenicity and insertional mutagenesis are serious hurdles to be overcome. Several types of viruses have been utilized for liver-directed gene transfer, among which adeno-associated virus is the prime vector to be applied in clinic [9], as being highly efficient and nonpathogenic. Non-viral methods of gene transfer comprising synthetic vectors and physical methods evolved as safer alternatives to viral vectors. Yet, the efficiency of non-viral systems is currently a subject of intensive research to be optimized. Synthetic vectors implicate polymers and liposomes as carriers to protect genetic material from biodegradation and to facilitate cell entry via endocytosis [10]. Physical methods preclude the need of vectors by direct gene transfer into cells through physically created transient pores in cell membrane. Different mechanical forces have been applied for liver-directed gene transfer, such as electrical pulses, hydrodynamic pressure, ultrasound waves, laser, and particle bombardment [11]. Hydrodynamic gene transfer is the most commonly used non-viral method for liver-directed gene

transfer because of high efficacy and recent advancement into lobe-specific computerized injection into the liver in rodents and large animals [12].

1.1.2 Gene Therapy Strategies for Liver Disease Treatment

Gene therapy is conducted through various approaches to overexpress or block the expression of certain genes depending on the type of gene defect.

1.1.2.1 Gene Replacement

Liver disorders can be corrected by replacing the defected or inactive gene with wild-type homologue. This approach holds promise for diseases with single gene defect, and indeed it was successfully employed for gene therapy of hemophilia [13] and hereditary tyrosinemia type I [14]. Similarly, gene replacement can be applied for multifactorial liver diseases that are nonetheless characterized by a single gene dysfunction, such as p53 in hepatocellular carcinoma (HCC) [15].

1.1.2.2 Gene Repair

Another approach to correct monogenic liver diseases is to repair defective genes in situ through utilization of innate homologous recombination cells commonly use to repair DNA breaks. The rationale is to use natural or recombinant nuclease enzymes that specifically recognize the mutated genetic sequence and create a DNA break. With proper method of gene transfer, DNA template for the desired functional sequence is provided in diseased cells, and the mutated fragment can be repaired by an exchange of genetic sequences through homologous recombination [16]. Mutated sequences can also be targeted by chimeric oligonucleotides specifically hybridizing mutated genes and successfully applied for gene therapy of Crigler-Najjar syndrome type I [17] and hyperlipidemia type III [18].

1.1.2.3 Gene Augmentation

Boosting gene expression for liver disease management is typically used when a given gene product is absent or insufficient to restore the normal physiologic status. Gene therapy of multifactorial liver diseases, such as HCC, is often explored with gene augmentation approach. Diverse strategies have been evolved for HCC gene therapy, such as suicide gene therapy relying on overexpressing a gene encoding a prodrug-activating enzyme in tumor tissue followed by prodrug administration to spare cytotoxic effects for malignant cells only [19]. Another form of cancer gene therapy is oncolytic viruses, where engineered replication-competent viruses

selectively replicate in malignant cells, resulting in progressive destruction of tumor mass [20]. HCC treatment was also explored with antiangiogenic gene therapy using angiostatin gene [21] and immunotherapy to induce antitumor immune response by means of cytokine gene overexpression [22].

1.1.2.4 DNA Vaccination

Transferring genes coding for tumor-specific surface antigens or viral antigens to elicit durable humoral and cellular immune response has gained increased attention as therapeutic and preventive modalities for HCC and viral hepatitis. Indeed, DNA vaccination with alpha-fetoprotein-expressing plasmid DNA successfully inhibited growth of HCC in mice by triggering antitumor immune response [23]. Likewise, DNA vaccination with plasmid DNA-expressing hepatitis C virus (HCV) genotype *1a/1b* proteins induced potent cell-mediated immune response to HCV in mice and nonhuman primates [24].

1.1.2.5 Gene Silencing

Blockade of specific gene expression or function that is thought to underlie disease pathology is another gene therapy approach to tackle disease progression. Increased expression of pathogenic proteins is often reported with acquired liver diseases like infections and cancers. Different strategies have been developed to silence gene expression to interfere with transcription, RNA transport and stability, and translation. Among these, ribozymes can inhibit gene expression and viral replication by catalyzing sequence specific RNA cleavage [25]. Ribozymes were successfully applied for in vitro inhibition of hepatitis B virus (HBV), but in vivo efficacy is yet to be approved [26, 27]. Oligonucleotides (ON) are also used for gene silencing purposes, and several designs have been introduced such as antisense ON and small interfering RNA molecules, both hybridize to specific regions in the target gene or its corresponding mRNA transcript, respectively. ONs are increasingly used to treat liver cancers [28, 29] and viral infections [30, 31]. Inhibition of gene function at protein level is also achievable using dominant negative mutant method, in which genes coding for nonfunctional peptides or proteins are delivered to diseased cells to interfere with their counterparts [32], and promising potential was demonstrated in the management of HBV infections [33, 34].

1.2 Cell Therapies

Liver transplantation has been the only effective treatment for end-stage liver diseases. However, because of the serious challenges such as shortage of organ donors and the consequences of long-term immune suppression after transplantation [35],

alternative therapies are needed. Cell therapies have evolved as regenerative therapies relying on stem cell and tissue regeneration technologies to rebuild or regrow liver organ to provide the missing product. Theories of cell-based therapies in liver diseases are inspired by a broader understanding of mechanisms underlying the natural ability to regenerate organs in some model organisms in nature [36] and the mechanisms of liver regeneration and repair, including activation of local stem cells [37] and contributions from bone marrow-derived stem cells [38]. Indeed, several innovative studies in preclinical models of liver diseases highlight the remarkable regenerative capacity of hepatocytes in vivo, pointing out the feasibility of cell therapies for rebuilding failed or diseased liver.

1.2.1 Cell Sources

Different types of cells have been utilized for cell therapies, including primary hepatocytes, immortalized cells, and stem cells. Therefore, several issues should be carefully considered when exploring cell therapies. Ideally, cells should be easy to obtain, non-immunogenic and non-tumorigenic, and stably express the desired functional traits [39].

1.2.1.1 Primary Hepatocytes

Primary hepatocytes are mainly obtained from non-transplantable human liver but also obtained from human fetal liver and healthy animal donors (e.g., porcine liver) [40]. These cells are infused via portal vein and rapidly adhered to existing extracellular matrix to repopulate the diseased liver. Despite showing promise in several studies, the reported amelioration of liver function was modest and not durable [41], largely due to substantial variability in functional activity of isolated primary hepatocytes. Also, these cells are not easily maintained in culture for prolonged times, therefore, alternative cell sources have been sought.

1.2.1.2 Immortalized (Transformed) Hepatocytes

The need for functional hepatocytes with high in vitro and in vivo proliferation capacity led to the development of immortalized human hepatocytes, such as tumor-derived C3A cells (hepatoblastoma subclone) that have been explored clinically with bioartificial liver devices [42, 43]. Immortalized normal hepatocytes have also been utilized for liver disease therapy. Immortalized human fetal hepatocytes showed promising results in mice and demonstrated superior curative metabolic activity in comparison to tumor-derived C3A cells [44].

1.2.1.3 Stem Cells

The therapeutic potential of stem cells is based on their capacity of self-renewal and pluripotent differentiation. The activation of liver stem cells to expand and differentiate for liver regeneration has been explored for various forms of liver diseases, using fetal and adult liver stem cells [37]. However, isolation of these cells remains challenging because they constitute lesser than 1 % of liver mass, making them unfavorable for large-scale preparation [45]. The feasibility for regenerating functional hepatocytes was also evident with non-liver stem cells, such as bone marrow-derived stem cells. Specifically, mesenchymal stem cells have demonstrated remarkable improvement of liver function in several studies [46], besides being multipotent and easily accessible. Induced pluripotent stem (iPS) cells have also been assessed for liver regeneration potential and, indeed, successfully generated functional human liver [47]. In fact, iPS cells are superior to other types of cells in precluding ethical concerns of stem cell technology and potential issues of rejection. Other types of stem cells that have been explored and showed promising potential are human placental cells [48] and embryonic stem cells [49].

1.2.1.4 Xenogeneic Cells

Because of the challenges related to the availability and suitability of human hepatocytes, bovine and porcine primary hepatocytes have also been considered for cell therapy of liver diseases [50]. While maintaining liver metabolic activities, the use of porcine hepatocytes, however, is hampered by their immunogenicity and functional mismatch of porcine proteins with the corresponding human counterparts.

1.2.2 Cell Therapy Strategies for Liver Diseases Treatment

Engineering bioartificial liver substitutes have gained increased interest aiming to develop alternative therapies to liver transplantation. Several designs of cell therapies have been developed such as liver repopulation via hepatocyte transplantation, extracorporeal artificial liver devices, and tissue engineering. Such innovations are currently under intense investigation for future applications in liver disease management.

1.2.2.1 Liver Cell Transplantation

Direct hepatocyte transplantation was proposed as a lesser invasive alternative to whole organ transplantation, especially for severely ill and pediatric patients [41]. Typically, isolated hepatocytes are delivered into the liver through portal or splenic

injection. This strategy has been challenged by the modest engraftment efficiency of transplanted cells [51], which limits the applicability of hepatocyte transplantation for inborn metabolic diseases [52] where curative effects are sufficiently driven by minimal recovery of the missing liver function.

1.2.2.2 Extracorporeal Bioactive Liver Perfusion Systems

Attempts to develop artificial liver devices, such as artificial liver support devices (ALSD) and bioartificial liver (BAL) devices, aim to support patients with a liver disease through hemofiltration and detoxification and substituting the missing or insufficient liver function. These devices are often used for short term until the recovery of the transplanted liver or for long term as a chronic supportive therapy. ALSD are non-biological devices similar to renal dialysis units, working through biochemical and biophysical reactions for detoxification when plasma is pumped through cartridges of activated charcoal or immobilized albumin, and hence do not offer metabolic and secretory liver functions [53]. BAL devices are advantageous in providing essential liver functions as they use human C3A or porcine hepatocytes as functional components, which are immobilized on a semipermeable membrane in various configurations such as flat plate or hollow fiber capillaries [54].

1.2.2.3 Liver Tissue Engineering

Liver tissue engineering started in the 1990s when hepatocytes seeded into biodegradable and biocompatible sponges or scaffolds were successfully transplanted into rats [55]. Besides serving as delivery systems, scaffolds are used to provide proper niche for cells to grow. Initial systems suffered from limited survival in vivo because it took a while for the existing vasculature to grow and support implanted cells [56]. Growth factors have been used to activate angiogenesis and to enhance cell survival in newer systems [57, 58]. Alternative to material scaffolds, de-cellularized liver matrix was used to generate three-dimensional implantable grafts. Cells can be removed from organs by various physical, chemical, and enzymatic methods, leaving behind the intact organ extracellular matrix scaffold [59]. Upon implantation, matrix can be efficiently repopulated with adult hepatocytes to generate a functionally competent engineered liver [60].

1.3 Future Perspectives

Molecular biology has become an integral part of experimental and clinical hepatology and increasingly contributes to better understanding of pathogenesis of liver diseases. Several new approaches to illness, particularly gene and cell therapies,

have been inspired by recent advances in molecular biology and stem cell technology and evolved from laboratory curiosities into established therapies for liver disease management.

Despite the stunning increase in the understanding of liver disease pathogenesis and the progress that has been made over the past two decades, clinical application of gene and cell therapies remains elusive. Gene therapy for human diseases has been challenged by the lack of safe and efficient gene delivery methods. Therefore, future research will focus on addressing various cellular and immunological barriers of gene delivery and further optimizing the current viral and non-viral gene delivery methods toward liver-specific targeting and controlled gene expression. Cell therapies, on the other hand, are challenged by the paucity of suitable hepatocyte sources, modest and nondurable engraftment efficiency of transplanted cells, and induced immunogenic reactions especially to xenogeneic cells and tissues. Stem cell technology offers the potential to overcome barriers of hepatocyte availability and suitability, and the extent of repopulation after transplantation, and hence, will undoubtedly drive the future of cell-based therapies. Induced pluripotent stem cells will be of particular interest because of the high capacity of multipotent differentiation and precluding immune rejection concerns. Liver regeneration would be feasible with innovations in bioengineering of de-cellularized matrices and transplantable scaffolds that offer the essential grounding for tissue assembly, regrowth, and function.

In summary, a new horizon has been established with gene and cell therapies, which have become indispensable tools in liver disease management. Given the superb capacity of liver for protein synthesis and secretion, liver-directed gene delivery will certainly be explored intensely in the coming years in gene therapy trials of liver diseases and non-liver diseases cured by circulating proteins. Novel delivery systems are constantly under development to address related challenges, and breakthroughs in delivery methods will pave the way for the application of gene therapy in clinic. In parallel, advancing in stem cell technology and cell-based therapies will be fueled by the massive need for therapies to substitute for challenging organ liver transplantation. We are on the verge of new exciting era in the management of liver diseases. Ongoing basic science and carefully designed clinical trials will ultimately make these new therapies a reality in clinic.

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Part I
Cell Therapy

Chapter 2
Macrophage Therapy for Liver Fibrosis and Regeneration

Atsunori Tsuchiya and Stuart J. Forbes

Abstract The liver has a population of resident macrophages termed Kupffer cells that are phagocytic and aid filtration of the portal blood. Following liver injury both the resident macrophages and circulating monocytes influence both liver regeneration and liver fibrosis. Kupffer cells can stimulate hepatocyte proliferation via the secretion of IL-6; macrophages stimulate a ductular proliferation via TWEAK secretion and also secrete Wnts which stimulate liver regeneration. Macrophages can both promote fibrosis and help resolve fibrosis depending upon the phase of liver injury.

We have been developing macrophage therapy for liver fibrosis and found that injected mature macrophages promote scar resolution in mouse models of liver fibrosis via a number of direct mechanism such as MMP expression but also in indirectly via the expression of chemokines which aid the recruitment of inflammatory cells to the scar area and promote scar resolution. Based on these basic research results, we are planning human studies of autologous macrophage therapy for liver cirrhosis in the near future.

Keywords Hepatic progenitor cell • Macrophage • Liver regeneration

2.1 Introduction

Chronic liver injury causes scarring and eventually liver cirrhosis which results in liver failure with reduced liver function, portal hypertension and an increased risk of liver cancer. Liver cirrhosis is a common and serious clinical problem to resolve, and currently the only cure for end stage of liver cirrhosis is liver transplantation.

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However due to a shortage of suitable donor organs, demand far outstrips supply. Furthermore even putting aside the lack of suitable organs for transplantation, the operation is a serious one with immediate operative complications and long-term potential morbidity associated with the use of immunosuppressants such as cardiovascular disease and an increased risk of cancer. It is important therefore to develop alternatives to whole-organ transplantation for liver cirrhosis. Hepatocyte transplantation is a therapy with reasonable success in the setting of paediatric acute liver failure and metabolic liver diseases where the cell therapy acts as a form of "gene therapy" [1]. Hepatocyte transplantation has also been attempted for the indication of liver cirrhosis, and in this case, the results have been more disappointing. In the context of liver cirrhosis, the gross architectural and vascular disturbance makes hepatocyte cell therapy very challenging. Delivery of hepatocytes via the portal vein, with portal hypertension and coagulopathy, has risks. Furthermore the hepatocyte transplantation does not deal with the underlying architectural issues in the liver such as the excessive fibrosis [2]. Thus the development of alternative therapies is necessary to treat the increasing numbers of patients with end-stage liver disease due to liver cirrhosis.

Understanding the mechanisms that help to control liver regeneration is helpful in order to identify novel therapies. Following partial hepatectomy or acute liver injury, the normal liver regenerates highly efficiently through hepatocyte division. In this setting a rapid series of signalling events take place between the non-parenchymal cells that coordinate and stimulate hepatocyte division. Kupffer cells secrete IL-6, sinusoidal endothelial cells secrete Wnt2 and HGF, and stellate cells secrete HGF and Hh ligand [3]. Following the activation of this regeneration pathway, restoration of liver mass occurs rapidly, in mice and rats taking 10 days and in humans talking approximately 6–8 weeks.

In chronic liver injury, there is a change in the architecture of the liver and the signalling pathways and mechanisms of regeneration. There is activation of stellate cells into activated myofibroblasts and excessive collagen deposition, and the hepatocytes themselves become senescent (unable to divide). In this setting there is activation of the so-called ductular reaction (DR) from the biliary compartment. It is controversial as to whether these ductular cells act as bipotential hepatic progenitor cells in this scenario [4–6]. In mouse models of diet-induced liver injury, lineage tracing of hepatocytes using AAV vectors has led to the conclusion that hepatocytes self-renew in the absence of a contribution from a back-up progenitor population [7]. A recent refinement of this finding has suggested that HPCs can derive from dedifferentiating hepatocytes where injured hepatocytes undergo a ductular change in phenotype [8]. It is however still unclear whether the mouse models used accurately reflect the severely damaged human liver where damage occurs over decades rather than the days tested in these models. Whatever the source of the DR/HPCs, their behaviour and differentiation are affected multifactorially by surrounding components, including a stereotypical niche of cellular components (macrophages, other inflammatory cells and myofibroblasts) and acellular matrix components that surround the DRs [9–13]. In particular we have had an interest in the role of macrophages in promoting liver regeneration in the chronically damaged liver, and

this has led to our attempts to develop macrophages as a therapeutic cell to promote liver regeneration and reduce liver fibrosis.

From 2003, clinical studies of bone marrow cell therapy for cirrhosis patients started and were effective for some patients [14, 15]. However, a recent systemic review of autologous stem cell therapy for liver disease found little evidence of proven clinical benefit in patient groups with chronic liver disease [16]. This in part reflects the lack of sufficiently powered randomised controlled trials in this emerging area, and as such, most studies have been small uncontrolled clinical studies. A further issue worth mentioning is that some studies have used mixed cell populations. This makes it difficult to know whether within this population there are specific therapeutic cell populations or whether more than one cell type contributes to the therapeutic effect [3].

Given that macrophages have a variety of immune, inflammatory, trophic and regulatory actions, we have examined the therapeutic potential of macrophage therapy for liver cirrhosis. Here we introduce our recent basic research in the area of macrophages during liver regeneration and briefly outline our planning of a clinical trial of macrophage therapy for liver cirrhosis.

2.2 Basic Research into the Biology of Macrophages During Liver Injury

2.2.1 The Dual Role of Macrophage During Liver Damage

Macrophage has been proved to have complex roles in both the production of tissue injury and the resolution of tissue injury. An example of this is the lung where alveolar macrophages can either promote the formation of lung fibrosis during injury, yet following the cessation of injury, the alveolar macrophages act to resolve lung fibrosis. Deletion of the alveolar macrophages during the injury phase results in less lung fibrosis; however deletion of the alveolar macrophages during the resolution phase results in an abrogation of the scar resolution [17]. This indicates that any cell therapy utilising macrophages must be mindful of the exact type of macrophage utilised but also the context and timing of the tissue injury.

The role of macrophages in liver fibrosis has been examined using deletional strategies by Duffield et al. To show whether macrophage has dual role during liver injury, a transgenic mouse CD11b-DTR was generated in which macrophage could be depleted after the administration of diphtheria toxin (DT). This mouse was used in a model of liver fibrosis produced by iterative carbon tetrachloride (CCl_4)-induced liver damage. Macrophages were depleted either during the injury phase or during the recovery phase. Macrophage depletion during the injury phase, when liver fibrosis was advancing, resulted in reduced scarring and fewer myofibroblasts. However, macrophage depletion during the recovery phase resulted in a reduction in matrix degradation. These results clearly showed that functional distinct subpopulations of macrophage exist in the same damaged liver and that these macrophages play

critical role in both scar progression and regression [18]. This work has been extended by Ramachandran et al. who have examined the specific phenotype of the macrophages during the injury phase and resolution phase [19]. During the pro-fibrotic injury phase, macrophages associated with the liver tissue scar are predominantly $CD11B^{hi}F4/80^{int}Ly\text{-}6C^{hi}$, whereas following cessation of the injury, during resolution of the scar, the scar-associated macrophages are predominantly $CD11B^{hi}F4/80^{int}Ly\text{-}6C^{lo}$. Interestingly, this phenotypic switch appears to occur in response to phagocytosis and is associated with an upregulation of key mediators of scar resolution, the matrix metalloproteinases MMP-9 and MMP-12. Another key matrix metalloproteinase that is produced by scar-associated macrophages in the liver is MMP-13 which also aids liver scar resolution [20].

2.2.2 Injection of Differentiated Bone Marrow-Derived Macrophages Can Improve Liver Fibrosis, Regeneration and Function in Mouse Models of Liver Fibrosis

The role of monocytes and mature macrophages as a potential cell therapy has been examined in a mouse model of chronic liver fibrosis. To analyse this, mouse bone marrow macrophage precursor cells and differentiated bone marrow-derived macrophages (BMMs) generated by 7 days of culture with colony-stimulating factor (CSF)-conditioned medium were injected into the portal vein in mice that had received liver damage through repeated injections of carbon tetrachloride (CCl_4). The BMMs were grown in low-adherence conditions to allow full maturity and enable ease of removal from the culture containers. This resulted in macrophages that did not conform to either the traditional classically (M1) or alternatively activated (M2) macrophage phenotype. However, the BMM expressed anti-inflammatory (IL-10), antifibrotic (matrix metalloproteinase; MMP-13), pro-regenerative (tumour necrosis factor-like weak inducer of apoptosis; TWEAK) and chemotactic (MCP-1, MIP-1α, MIP-2) mediators. The injection of 1×10^6 wild-type BMMs into the portal vein of recipient mice receiving CCL_4-induced liver fibrosis resulted in significant reduction in fibrosis. In contrast, injection of 1×10^6 BM precursor cells did not significantly reduce fibrosis. Importantly using this model, injection of whole-bone marrow (BM) actually resulted in a significant increase in liver fibrosis. The mechanism of the increased fibrosis is not proven but may reflect the fact that whole BM does contain pro-fibrotic cell populations including BM stromal cells [21]. We further analysed the underlying mechanisms behind the beneficial effects of the mature BMMs. Whilst the engraftment of injected BMMs was transient, the cells released chemokines which attracted host inflammatory cells to the scar areas in the liver (including recipient macrophages) and presumably amplified their effect and accounted for the significant changes seen with such a small number of injected macrophages. The animals that received the macrophage injections had reduced numbers of hepatic activated myofibroblasts, and there was upregulation in the number of hepatic MMP-expressing cells. There was an increase in the

pro-regenerative growth factors and anti-inflammatory cytokines in the liver. All of these effects induced by BMM injection likely contribute to the regression of liver fibrosis and stimulate regeneration [22]. Along with the reduced fibrosis, there was an increase in the serum albumin in the BMM recipient mice.

2.2.3 TWEAK Produced by Macrophage Induces Expansion of the Ductular Reaction Containing Hepatic Progenitor Cells

Bone marrow cell (BMC) therapy has shown promising preliminary results; however no definite mechanism has been demonstrated. One issue is that when injecting cells into the damaged liver there is a large number of "injury and repair signals" upregulated in the liver which makes the analysis of the specific effects of injected cells difficult to determine. To overcome this we analysed the effect of BM injections in the mouse in the absence of any liver injury – so that any signals of regeneration would be specific to the injection of the cells rather than simply a response to the initial injury.

To analyse the direct effect of BMC to the liver, we examined, in a mouse model without injury, a single i.v. injection of unfractionated BMCs. This induced a significant ductular reaction (DR) in healthy liver. Furthermore the livers significantly increased in size, indicating that this was a liver growth stimulating effect. We found this surprising as the DR is often seen in the context of severe and prolonged injury and has been thought to be part of the injury/regeneration response [23]. To examine the effect of macrophage in this process, matured macrophages and whole BMCs from CD11b-DTR mice were injected. Whilst matured macrophages stimulate a similar DR response, BMCs from CD11b-DTR mice that had received diphtheria toxin (DT) to ablate macrophages did not stimulate a DR, suggesting that macrophages are responsible for the stimulation of the DR. An interesting feature of the cell injection was that although the BM cells or macrophages were injected into a peripheral vein (tail vein in mice), the macrophages could then be readily identified in the recipients' liver and were particularly found in the area of the portal tract. The factors that mediate this homing of macrophages to the liver are currently not known, and this is worthy of further study.

We studied what factors made by the macrophages that could have induced the DR. BMC transfer increased the mRNA levels of TWEAK in the liver, and in vitro maturation of BMC to macrophages resulted in a 20-fold upregulation of TWEAK mRNA. We therefore hypothesised that macrophage-secreted TWEAK was stimulating the DR via its receptor – Fn14. To directly test whether TWEAK secretion mediates the DR expansion following BMC transfer, we used two methods: (1) transfer of BMC from wild-type mice into recipient mice lacking the TWEAK receptor Fn14 (Fn14 −/− recipients) and (2) transfer of BMC-lacking TWEAK (from TWEAK−/− donors) into WT recipients. In both of these two experiments, DR stimulation could not be detected proving that in this experimental situation

macrophage-mediated TWEAK signal is critical for stimulation of the DR [24] Of course, macrophages secrete a wide number of trophic factors, and other factors may also have an effect in addition to the TWEAK.

2.2.4 Macrophages Are Key Players in Determining the Fate of Hepatic Progenitor Cells

As discussed earlier, there is some controversy as to the origin and role of the regenerative cells in the liver. The mature hepatocytes can self-regenerate in the mild models of liver injury utilised in the mouse for lineage-tracing purposes, and there is an ongoing debate regarding the contribution to liver regeneration from the so-called hepatic progenitor cells (HPCs). Furthermore the HPCs may arise at least in part from dedifferentiation of mature hepatocytes [8]. Regardless of their origin, their behaviour seems to be markedly affected by their surrounding niche. The HPCs or ductular cells are typically surrounded by a laminin extracellular matrix, stellate cells, myofibroblasts and macrophages [9].

We have found that Notch and Wnt signalling determine the behaviour and fate of the HPCs including their differentiation. In this scenario the cellular niche surrounding the HPCs seems critical for their behaviour. In mice, during activation of HPCs in biliary diseases, HPC expresses the receptors Notch1 and Notch2, and these are activated through interaction with myofibroblast-derived Jagged 1. This Notch high state appears to delineate the default pathway for the differentiation of HPCs into cholangiocytes. This Notch high pathway is inhibited during regeneration of hepatocytes through the ubiquitin ligase Numb. In HPCs during hepatocyte differentiation, Numb transcription is activated by Wnt β-catenin signalling. The Wnt-mediated Numb repression of Notch acts as a node at which the Notch and Wnt pathway can interact to enable lineage specification. In this process macrophages secrete Wnt ligand and as such can act as an inducer of differentiation of HPCs to hepatocytes. We found that when macrophages phagocytise cell debris, they strongly upregulate the production of Wnt3A which then helps to promote the differentiation of HPCs towards a hepatocytes fate [13, 25]. Indeed if macrophages are ablated during the CDE diet-induced regeneration phase in mice, then there is an increase in Notch signalling in the ductules which form into tubular structures.

2.3 Towards Macrophage Therapy

From our work and other group's experiments, we believe that macrophages can stimulate the liver regeneration process through a variety of mechanisms including the promotion of scar resolution and the promotion of HPC-mediated regeneration (Fig. 2.1). Therefore we have developed a protocol of "macrophage therapy" for liver cirrhosis. Our plan is that we collect CD14-positive monocytes from the

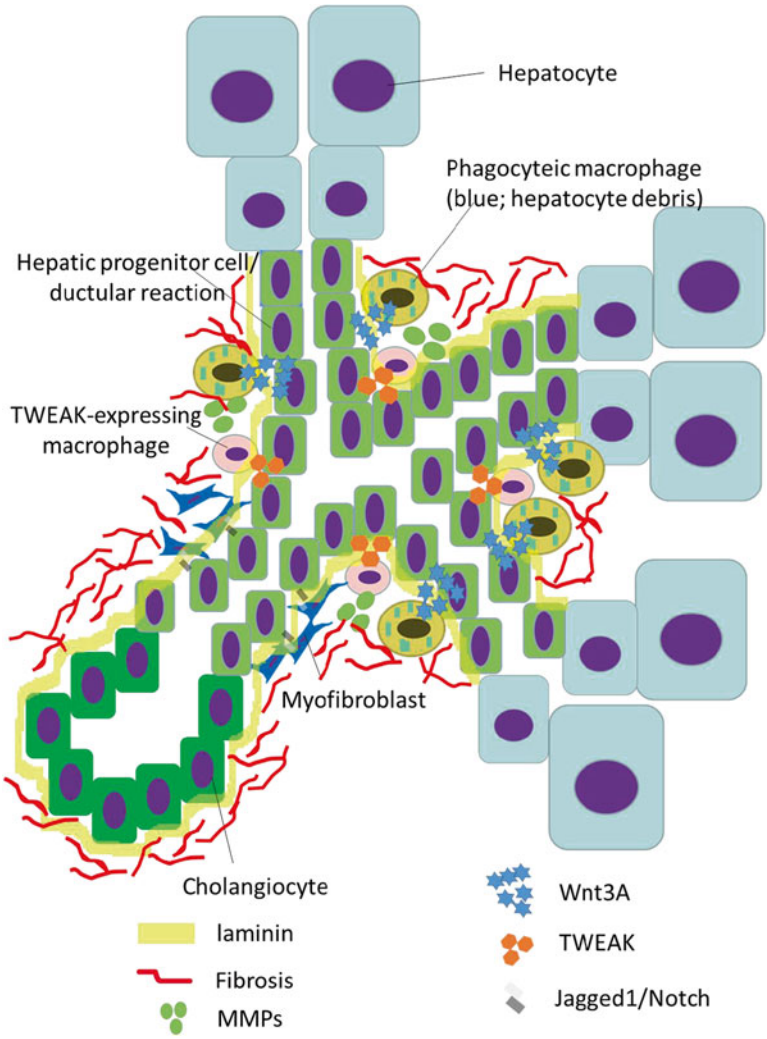

Fig. 2.1 Macrophage has multiple critical roles during differentiation of HPC to hepatocytes. During liver damage TWEAK expressing macrophage stimulates HPC expansion. Hepatocyte debris eating macrophage determine the fate of HPC towards hepatocytes. During this process, macrophage also contributes to the matrix regression by producing MMPs

patient's peripheral blood using a clinical apheresis system, then differentiate the monocytes into "regenerative" macrophages and administrate them to the patient via a peripheral vein infusion. These human macrophages have been tested in immunodeficient mouse models of liver cirrhosis and found to have potential therapeutic effect by reducing CCl_4-induced liver fibrosis. Approval is being sought from the UK regulatory agencies to perform a first in human study of autologous

macrophage therapy for liver cirrhosis. This study will involve a dose-escalation safety study initially followed by a randomised study. The target population would be patients with liver cirrhosis due to a variety of causative factors with the aim of reducing the recipients MELD score.

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Chapter 3
Liver Regeneration Therapy Using Autologous Bone Marrow-Derived Cells for Cirrhotic Patients

Taro Takami, Shuji Terai, and Isao Sakaida

Abstract In our animal studies, we have reported that bone marrow cells (BMCs) infused via a peripheral vein efficiently repopulate the cirrhotic liver. Repopulated BMCs produce collagenases including matrix metalloproteinase-9. As a result, we observed reduced liver fibrosis, elevated serum albumin levels, and a significant increase in survival. Based on these data, we have begun "autologous bone marrow cell infusion (ABM*i*) therapy" using non-cultured autologous whole BMCs. This therapy was officially approved as "advanced medical technology B" in Japan. However, ABM*i* therapy involves bone marrow (BM) aspiration under general anesthesia. We therefore developed a less-invasive liver regeneration therapy using cultured autologous mesenchymal stem cells (MSCs) isolated from a small amount of BM fluid aspirated under local anesthesia. We showed that peripheral infusion of cultured human BMCs reduces hepatic fibrosis in immunodeficient cirrhotic mice, consistent with the maintenance of redox homeostasis in hepatic stellate cells and hepatocytes. To evaluate safety using canine models, cultured autologous MSCs were administered to the same subject in approximately three times the quantity and ten times the concentration used in humans. We then constructed a cell-processing facility with a new isolator system to confer protection from hepatitis virus. Here, we summarize the current status and prospects for our liver regeneration therapy.

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Keywords Liver cirrhosis • Liver regeneration • Bone marrow cells • Mesenchymal stem cells

3.1 Background

In patients with chronic viral hepatitis in whom a radical cure had been difficult to achieve, advances in new antiviral drugs and interferon therapy are making virus elimination and a hepatitis cure possible in some cases. However, many patients already had liver cirrhosis before such treatment was possible. The only curative treatment for liver cirrhosis currently available is liver transplantation, but medical issues surrounding this treatment not only include its high surgical invasiveness but also concern about safety in living donors and the absolute deficiency of donors. Thus, the indications for liver transplantation are limited. Given this current situation, there is a need to develop liver regeneration therapy as a new treatment for liver cirrhosis.

3.2 Trends in Liver Regeneration Therapy

In autopsies of female patients with hematological disorders who underwent bone marrow transplantation from male donors, Thiese et al. (2000) reported that fluorescence in situ hybridization (FISH) of chronically inflamed hepatic and intestinal tissue showed Y chromosome-positive cells [1], suggesting the existence of pluripotent stem cells in bone marrow cells (BMCs) [1]. Those case studies demonstrated the plasticity of stem cells to differentiate into hepatocytes and intestinal epithelial cells. Since that time, interest has focused on bone marrow (BM) stem cells as a cell source for use in liver regeneration therapy, and basic and clinical research studies have been conducted [2–4].

Several clinical studies of liver regeneration therapy have been conducted to date. Gordon et al. reported that when CD34-positive autologous peripheral blood cells induced by granulocyte-colony-stimulating factor (G-CSF) are injected into the portal vein or hepatic artery of patients, their serum albumin levels increase [5]. Spahr et al. reported that G-CSF induces proliferation of hepatic progenitor cells in alcoholic liver cirrhosis [6]. However, splenic rupture with G-CSF mobilization in a healthy donor has been reported, so precautions are necessary when using G-CSF in cirrhotic patients with splenomegaly [7].

In five patients waiting for liver transplantation who were infused with autologous CD34-depleted BM-derived mononuclear cells through the hepatic artery, Park et al. reported improvements in serum albumin levels, liver stiffness, and liver volume [8]. In 12 patients with alcoholic liver cirrhosis who were injected with autologous BM-derived mesenchymal stem cells (BMSCs) (cultured from a small amount of autologous BM aspirate) through the hepatic artery, Jang et al. reported improved liver fibrosis and Child-Pugh scores, as well as marked decreases in transforming growth factor-β1, type 1 collagen, and α-smooth muscle actin levels [9].

In a study by Xu et al., 56 patients with hepatitis B virus (HBV) cirrhosis were randomly assigned to an autologous BMSC-transplantation group or control group. Both groups had improved liver function after entecavir treatment, but in the autologous BMSC group, there was further improvement in liver function. They concluded that autologous BMSCs played some role in the regulation of the Treg/Th17 cell balance [10].

3.3 Our Autologous Bone Marrow Cell Infusion Therapy (ABM*i* Therapy) Using Non-cultured Autologous Whole-Bone Marrow Cells (BMCs)

We selected non-cultured autologous whole BMCs for clinical application of liver regeneration therapy for several reasons. No risk of teratoma formation, as seen with embryonic stem cells, has been reported with BM stem cells. These cells are also easier to collect and isolate compared to hepatic stem cells in the liver. In addition, the use of autologous BMCs eliminates problems with potential immunological rejection.

We previously developed a green fluorescent protein/carbon tetrachloride (GFP/CCl_4) murine model to evaluate the differentiation and proliferation of BMCs into hepatocytes for use in basic research [11]. For this GFP/CCl_4 model, CCl_4 (1.0 mL/kg) (total of eight doses over 4 weeks) was injected intraperitoneally into 6-week-old C57BL/6 mice to induce chronic hepatic injury (liver cirrhosis). Next, GFP-positive whole BMCs isolated from the femurs of syngeneic GFP transgenic mice were washed and injected into the tail vein of the recipient mice. Then, CCl_4 injection was continued, and the effectiveness in improvement of liver function was evaluated over time.

Analysis in that model showed that although the infused BMCs did not engraft into mouse livers without hepatic injury, 1 day after infusion into livers with CCl4-induced chronic hepatic injury, the BMCs engrafted along fibers in the periportal region. Week after week, repopulated BMCs were also seen along pre-existing fibers. In addition, during this process, BM-derived GFP-positive cells that engraft in the injured liver produce collagenases such as matrix metalloproteinase-9, hepatic fibrinolysis occurs, and, as a result, serum albumin levels and survival rates significantly improve [12].

Therefore, we began a human clinical research study, "autologous bone marrow cell infusion (ABM*i*) therapy for treatment of liver cirrhosis" in November 2003. In patients followed up for at least 6 months after ABM*i*, serum albumin, total protein, and the Child-Pugh scores significantly improved after 6 months [13]. Moreover, similar improvement was observed in nine patients who were followed up for 15 months. None have developed any problematic adverse events [14]. Therefore, Yamaguchi University conducted a multicenter clinical trial together with Yamagata University and Yonsei University in South Korea. Kim et al. (2011) at Yonsei University reported that with ABM*i* therapy in decompensated HBV cirrhosis, serum albumin levels and Child-Pugh scores improved. Serial liver biopsy findings suggested the possibility that the mechanism involves the activation of hepatic

progenitor cells [15]. Saito et al. at Yamagata University also reported that ABM*i* therapy is effective and safe in patients with alcoholic liver cirrhosis [16]. These findings suggest that autologous whole BMCs are effective and safe for liver regeneration therapy in patients with liver cirrhosis. Based on basic research and clinical studies to date, Fig. 3.1 depicts the proposed mechanism of liver regeneration by ABM*i* therapy [4]. A "study on the effectiveness and safety of ABM*i* therapy in HCV-related liver cirrhosis" as "advanced technology B" was approved in June 2013, and a clinical trial has started. An overview of this clinical trial is discussed below and shown in Fig. 3.2.

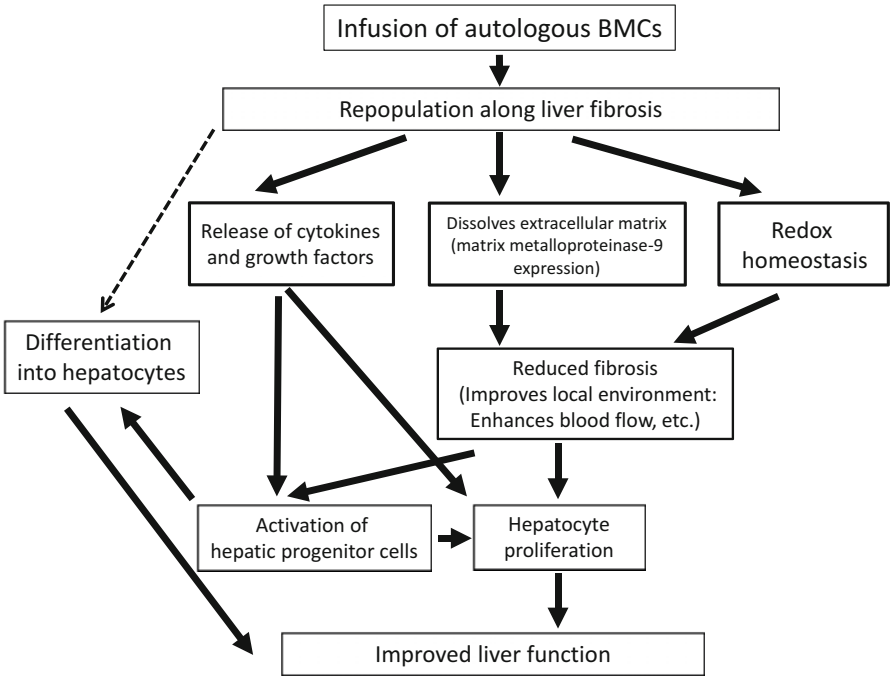

Fig. 3.1 Proposed mechanism of liver regeneration by bone marrow cell (BMC) infusion

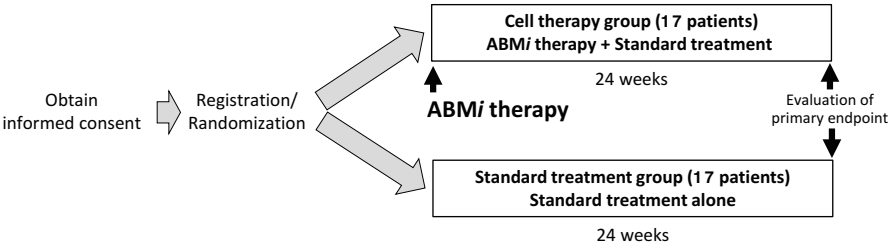

Fig. 3.2 Overview of "study on the efficacy and safety of autologous bone marrow cell infusion therapy (ABM*i* therapy) in HCV-related liver cirrhosis"

3.3.1 Inclusion Criteria

- Patients with hepatitis C virus (HCV)-related liver cirrhosis
- Patients with a Child-Pugh score of ≥7 (Child-Pugh B) at two times at least 90 days apart and in whom no improvement is expected with current medical treatment
- Patients aged 20–75 years
- Patients able to provide informed consent to participate in this study

3.3.2 Exclusion Criteria

- Patients with liver cirrhosis due to another cause other than HCV or in whom the cause of liver cirrhosis is unknown
- Patients with a current or past history of malignant neoplasm
- Patients with gastroesophageal varices at risk of rupture
- Patients with renal insufficiency and a serum creatinine ≥2 mg/dL
- Patients with a hemoglobin <8 g/dL, a platelet count <50,000/μL, or a prothrombin time <40 %
- Patients with a total bilirubin ≥3.0 mg/dL
- Patients with a performance status of 3 or 4
- Patients who refuse to consent to allogeneic blood transfusion
- Patients in whom HBV infection, human immunodeficiency virus infection, viral infection with adult T-cell leukemia, or parvovirus B19 infection cannot be excluded
- Women who are pregnant
- Patients whom their attending physician deems are not suitable candidates for general anesthesia
- Patients with a current or previous severe allergic reaction to a contrast agent
- Any patient deemed unsuitable for study inclusion by their attending physician

3.3.3 Protocol

This study includes 34 patients who were randomly assigned to two groups: 17 patients each to the cell therapy group and standard treatment group. In the cell therapy group, autologous BM aspirates were obtained by a similar procedure as for BM transplants, which are widely used in the field of hematology.

Under general anesthesia, about 400 mL of autologous BM aspirate was obtained, and bone fragments were removed. The BM aspirates were concentrated and washed; then the BMCs were purified and concentrated according to standard operating procedures at the Center for Regenerative and Cell Therapy, which is a completely equipped Good Manufacturing Practice-grade facility. These BMCs were infused through a peripheral vein on the same day they were collected.

For 1 week after this procedure, each patient was in principle hospitalized for strict follow-up observation. If there were no problems, outpatient follow-up was then continued. Patients were followed up at least once monthly over a 6-month period, and treatment efficacy was assessed based on Child-Pugh scores.

3.3.4 Primary Endpoint

The primary endpoint was a rate of improvement of ≥1 in the Child-Pugh score at 24 weeks after cell therapy in the cell therapy group or 24 weeks after enrollment in the standard treatment group.

3.3.5 Secondary Endpoints

The following secondary endpoints were evaluated at 24 weeks after cell therapy in the cell therapy group or 24 weeks after enrollment in the standard treatment group:

1. The rate of no worsening of the Child-Pugh score
2. Changes in albumin levels
3. Changes in serum fibrosis markers
4. Changes in ascites volume
5. Changes in improvement rates and disappearance rates of lower extremity edema
6. Changes in subjective symptoms
7. Incidence of adverse events

3.4 Our Less-Invasive Liver Regeneration Therapy Using Cultured Autologous Bone Marrow-Derived Mesenchymal Stem Cells (BMSCs)

Although studies on the effectiveness and safety of ABM*i* therapy are proceeding, many patients with decompensated liver cirrhosis are not candidates for general anesthesia, and thus many patients do not meet the indications for ABM*i* therapy. For patients with more advanced decompensated liver cirrhosis, less-invasive treatment must be developed.

Therefore, our hospital has conducted research on the use of autologous BMSCs cultured from small amounts of autologous BM aspirates (about 30 mL) obtained under local anesthesia. In our animal studies, the infusion of cultured human BMSCs improves hepatic fibrosis in severe combined immunodeficiency (SCID) mice with induced liver cirrhosis, indicating that clinical application is fully possible with this cell quantity [17]. Efficacy and safety have also been demonstrated in canine studies.

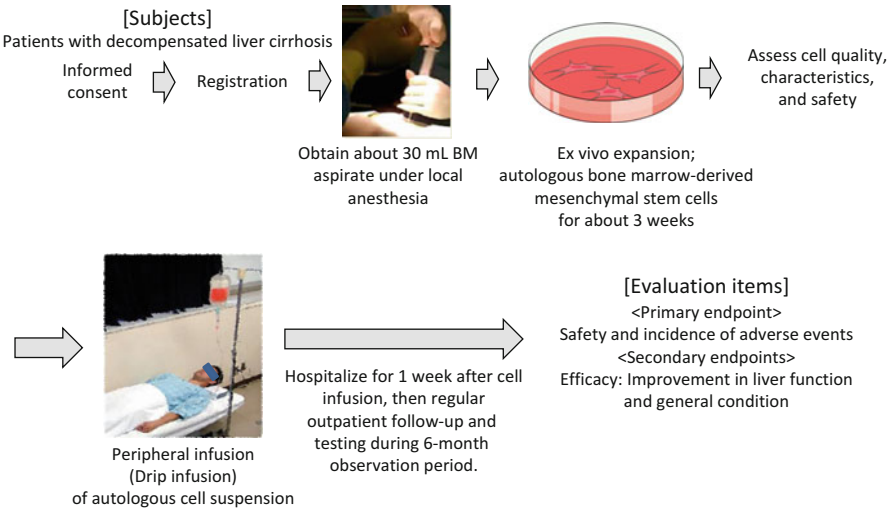

Fig. 3.3 Liver regeneration therapy using cultured autologous bone marrow-derived mesenchymal stem cells

As a result, a "study protocol to evaluate safety of less-invasive liver regeneration therapy using cultured autologous BMSCs in liver cirrhosis" was approved in August 2014. An overview of this clinical trial is discussed below and shown in Fig. 3.3.

3.4.1 Inclusion Criteria

Patients eligible for this study include those with decompensated liver cirrhosis with a Child-Pugh score ≥7 (Child-Pugh B) in whom further improvement with current medical treatment is not expected. Other inclusion criteria are age 20–75 years and a serum total bilirubin of 3.0–5.0 mg/dL, or if the total bilirubin is <3.0 mg/dL, patients who are still deemed unsuitable as a candidate for general anesthesia. Patients must provide informed consent for study participation.

3.4.2 Protocol

The autologous BMCs were obtained by a similar procedure as for BM collection, which is performed in the field of hematology. In brief, about 30 mL of BM aspirate was collected from the bilateral iliac crests under local anesthesia, heparin was added after collection, and the bone fragments were removed. In addition, at the Center for Regenerative and Cell Therapy at Yamaguchi University Hospital, hydroxyl ethyl starch (HES) was added to the BM aspirate to increase precipitation of the erythrocytes, and a nucleated cell fraction was prepared.

Next, a cell suspension was prepared by adding culture medium, and this was inoculated into a culture flask. After subculturing for 3 weeks, the cells were infused through a peripheral vein. About 2×10^7 BMSCs were used, and for cell therapy, approximately 200 mL of the cell suspension was slowly infused into a peripheral vein over a period of 2 h.

3.4.3 Protocol Summary

Study design: single-center, non-blinded, phase I trial (ClinicalTrials.gov; No. NCT02327832)

Target sample size: 10 patients

Three-month observation of the first three patients, and if there are no serious adverse events for which a causal relationship cannot be excluded, registration will proceed with the fourth patient.

3.4.4 Primary Endpoint

The primary endpoint is the incidence of adverse events up to 24 weeks after autologous BMSC infusion.

3.4.5 Secondary Endpoints

The secondary endpoints are the following parameters at 24 weeks or changes in these parameters at 24 weeks after autologous BMSC infusion.

1. Child-Pugh score
2. Albumin levels
3. Serum fibrosis markers
4. Improvement or disappearance of lower extremity edema
5. Subjective symptom scores (SF-36)

3.5 Conclusion

Liver regeneration therapy using autologous BMCs is promising for many patients with decompensated liver cirrhosis, and research is ongoing. More effective therapy, while ensuring safety, should be developed in the future. This should include more efficient large-scale cultures using autologous BMCs, modified culture procedures

such as shorter culture times, methods to improve effectiveness by cryopreservation of cultured cells and repeated infusion, and treatment by other than only peripheral vein infusion (e.g., hepatic artery infusion).

In addition, it may be possible in the future to develop effective treatment not only using autologous (the patient's own) BMCs but also cells from other family members. Furthermore, the use of induced pluripotent stem (iPS) cells for larger quantities of cells suitable for treatment may be promising for effective liver regeneration therapy.

Acknowledgments This study was supported by Grants-in-Aid for scientific research from the Japan Society for the Promotion of Science (JSPS); Ministry of Health, Labour and Welfare, Health and Labour Sciences Research Grants; Japan Science and Technology Agency (JST); and the project of realization of regenerative medicine and highway.

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Chapter 4
Novel Immunotherapy Using Liver-Derived Natural Killer Cells for Preventing Hepatocellular Carcinoma Recurrence in Liver Transplantation

Masahiro Ohira, Seigo Nishida, Andreas G. Tzakis, and Hideki Ohdan

Abstract Tumor recurrence is the main limitation of liver transplantation in patients with hepatocellular carcinoma (HCC) and can be promoted by immunosuppressants. However, no prevention or treatment exists for HCC recurrence after liver transplantation. Here, we describe an adoptive immunotherapy approach that uses natural killer (NK) cells derived from both living and deceased donor liver graft perfusates. Liver NK cells exhibited the vigorous cytotoxicity against hepatoma cell line after IL-2 stimulation through the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-TRAIL receptor pathway. In an in vivo mouse model, adoptive transfer of TRAIL-expressing liver NK cells inhibited the growth of liver tumors. Taken together, these findings suggest that we have established a method of retrieving NK cells from donor liver graft perfusate under current good manufacturing practice conditions for the treatment of liver transplant recipients with HCC. Clinical trials of adoptive immunotherapy with liver NK cells have been conducted in both living and deceased donor liver transplantations.

Keywords Liver transplantation • HCC • NK cell • Immunotherapy

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4.1 Introduction

Hepatocellular carcinoma (HCC) is one of the most common reasons for liver transplantation. However, HCC recurrence remains a serious issue after liver transplantation. The use of postoperative immunosuppression in the transplant recipient poses an additional risk for recurrence and hinders the use of cytotoxic chemotherapy drugs [1–4]. The major problem for HCC recurrence after liver transplantation is that no definitive treatment or prevention modalities exist [5, 6].

Natural killer (NK) cell is a major innate immune component and a first-line defense against invading infectious microbes and neoplastic cells [7]. Functional impairment and decreased number of NK cells have been observed in HCC and cirrhotic patients [8–10]. These functional defects in NK cells might be responsible for the failure of antitumor immune responses after liver transplantation in patients with HCC. Since the immunosuppressive regimen that is currently used after liver transplant reduces adaptive immune components but effectively maintains the innate components of cellular immunity [11–13], augmentation of the NK cell response may be a promising immunotherapeutic approach [14].

4.2 Immunotherapy Using NK Cells

4.2.1 Characteristics of NK Cells

NK cells can destroy many solid tissue-derived malignant cells through two major mechanisms that induce target cell apoptosis, granule-dependent killing, and death receptor stimulation [15]. Granule-dependent killing includes the release of perforin and granzymes [16], while the death receptor pathway is mostly mediated by apoptosis-inducing members of the TNF superfamily such as FasL, TNF-α, and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) [17, 15]. NK cells can also produce high levels of cytokines including IFN-γ, which may play a role in tumor growth regulation [18].

4.2.2 NK Immunotherapy for Cancers

Adoptive cancer immunotherapy was pioneered by Rosenberg and collaborators using cytokine-induced killer (CIK) cells including many NK cell-containing populations [19], but subsequent studies showed that similar antitumor effects could be achieved with high-dose IL-2 alone [20]. Because of the lack of significant clinical effects after treatment with autologous NK cells [21, 20], the focus has more recently shifted toward the potential of allogeneic NK cells in adoptive cell therapy for cancer treatment [22]. Miller et al. reported successful adoptive immunotherapy using short-term ex vivo activated haploidentical NK cells in patients with blood

cancers [13]. In HCC patients, adoptive immunotherapy with cytokine-induced killer (CIK) cells prolonged the overall survival and time to recurrence in some clinical trials [23, 24]. There are few clinical trials that examine adoptive immunotherapy with liver NK cells for HCC because of the limited availability of sufficient NK cells from the liver.

4.3 Cytotoxic Potential of Liver-Derived NK Cells

4.3.1 Liver-Derived NK Cells in Mice

NK cells are quite abundant in the liver of mice, in contrast to the relatively small percentage in peripheral lymphatics [25, 26]. In addition, liver NK cells have been shown to mediate higher cytotoxic activity against tumor cells than those derived from the spleen or peripheral blood in rodents [26, 27]. The antitumor activity of liver NK cells was shown to decrease after partial hepatectomy, suggesting that immunosuppressed patients are susceptible to HCC recurrence after partial hepatectomy or liver transplantation [28–30]. We recently tested this hypothesis using a mouse model [14]. CD69 and TRAIL expression levels on liver NK cells were temporarily downregulated after extended partial hepatectomy (Fig. 4.1). The adoptive transfer of NK cells, including those expressing TRAIL, extracted from the liver perfusate of poly I:C-stimulated C57BL/6 (B6) mice inhibited the growth of liver metastasis in B6 or B6xBALB/c F1 (B6CF1) mice that had undergone hepatectomy and received intraportal Hepa 1–6 injection (Fig. 4.2). These findings suggest that adoptive immunotherapy using activated NK cells extracted from normal liver perfusate may be a novel technique for reconstituting the livers of living donor liver transplant recipients with HCC that are immunosuppressed.

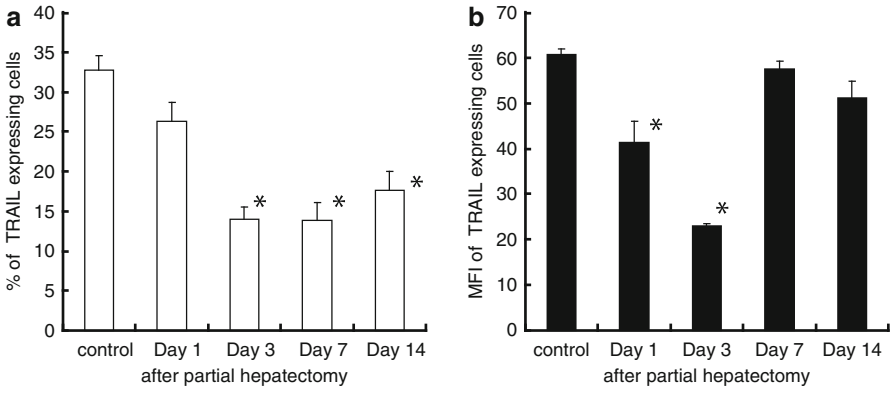

Fig. 4.1 The kinetics of TRAIL expression on liver NK cells after hepatectomy. (**a**) The numbers presented are means ± SEM of the TRAIL-positive cell population in NK cell subsets. *$p<0.01$ for day 3, day 7, and day 14 compared to the control. (**b**) The numbers indicate the mean fluorescence intensity of cells that stained positive for TRAIL. *$p<0.01$ for day 1 and day 3 compared to the control

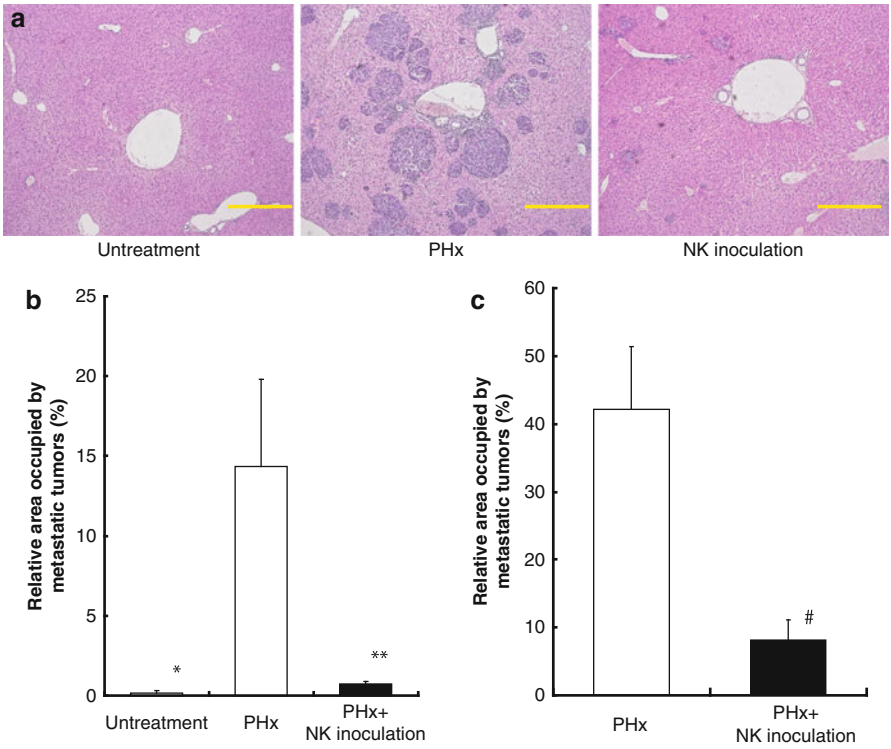

Fig. 4.2 The adoptive transfer of activated liver NK cells inhibited the growth of liver metastasis induced by a portal venous injection of hepatoma cells in extensively hepatectomized mice. (**a**) Representative histopathological findings of the liver specimen. Specimens from the untreated (*left*), partially hepatectomized (PHx; middle), and NK cell-receiving groups that were inoculated after partial hepatectomy (*right*). The adoptive transfer of activated B6 liver NK cells inhibited the growth of liver metastasis induced by a portal venous injection of hepatoma cells (Hepa 1–6; 5×10^5 cells) in extensively hepatectomized B6 mice (**b**) and B6CF1 mice (**c**)

4.3.2 Liver NK Cells in Human

Differences between liver and peripheral blood NK cells have not been extensively investigated in human because of the limited availability of appropriate human samples. We have recently determined the phenotypic and functional properties of NK cells extracted from donor and recipient liver perfusate in clinical living donor liver transplantation [10]. Donor liver NK cells exhibited the most vigorous cytotoxicity against a hepatocellular carcinoma cell line after in vitro IL-2 stimulation, compared to the activities of donor and recipient peripheral blood NK cells and recipient liver NK cells (Fig. 4.3). IL-2 stimulation increased TRAIL expression, which has been shown to be critical for NK cell-mediated antitumor cell killing

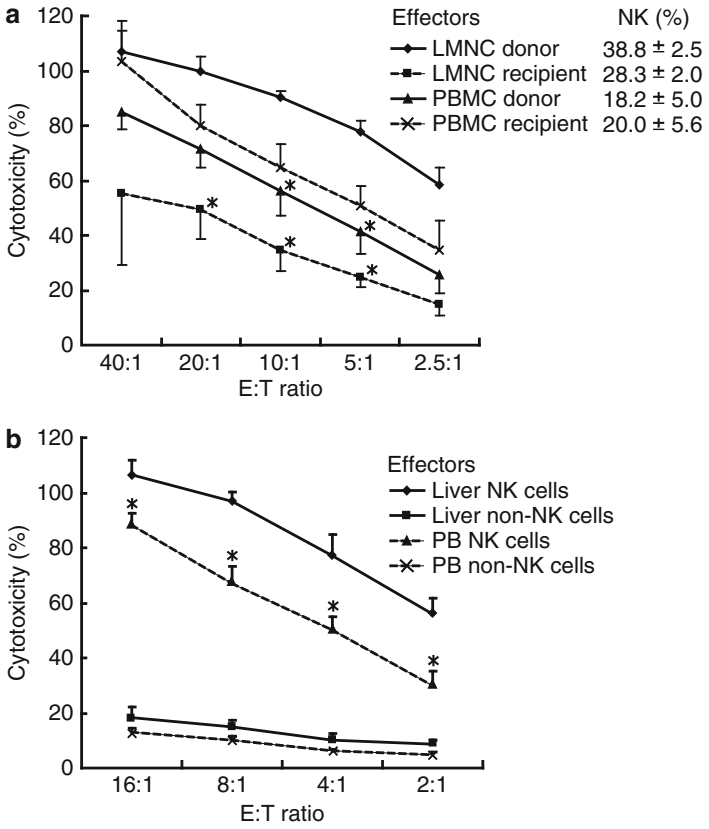

Fig. 4.3 IL-2-stimulated donor liver NK cells show vigorous cytotoxicity against HepG2 cells. NK cytotoxic activities of indicated effectors against indicated target cells were analyzed by ^{51}Cr release assay. (**a**) NK cytotoxic activities of IL-2-stimulated liver mononuclear cell (LMNC) and peripheral blood mononuclear cell (PBMC) populations that were cultivated for 4 days in the presence of IL-2 before the cytotoxic assay. Percentages of $CD3^{-}CD56^{+}$ NK cells in LMNC or PBMC fractions obtained from 4 adult healthy donors and 4 corresponding recipients with cirrhosis are shown in the upper right corner (means ± SEM). (**b**) NK cytotoxic activities of NK and non-NK cells isolated from IL-2-stimulated donor LMNC and PBMC populations against HepG2 target cells

without affecting normal cells, on liver NK cells (Fig. 4.4) [31, 32]. In addition, we have confirmed that death-inducing TRAIL receptors, TRAIL-R1/death receptor (DR) 4, and TRAIL-R2/DR5, which contain cytoplasmic death domains and signal apoptosis, are expressed in HCC [33, 34]. These findings suggest a novel concept to prevent recurrence of HCC after liver transplantation, the adoptive transfer of IL-2-stimulated NK cells extracted from donor liver graft into recipients.

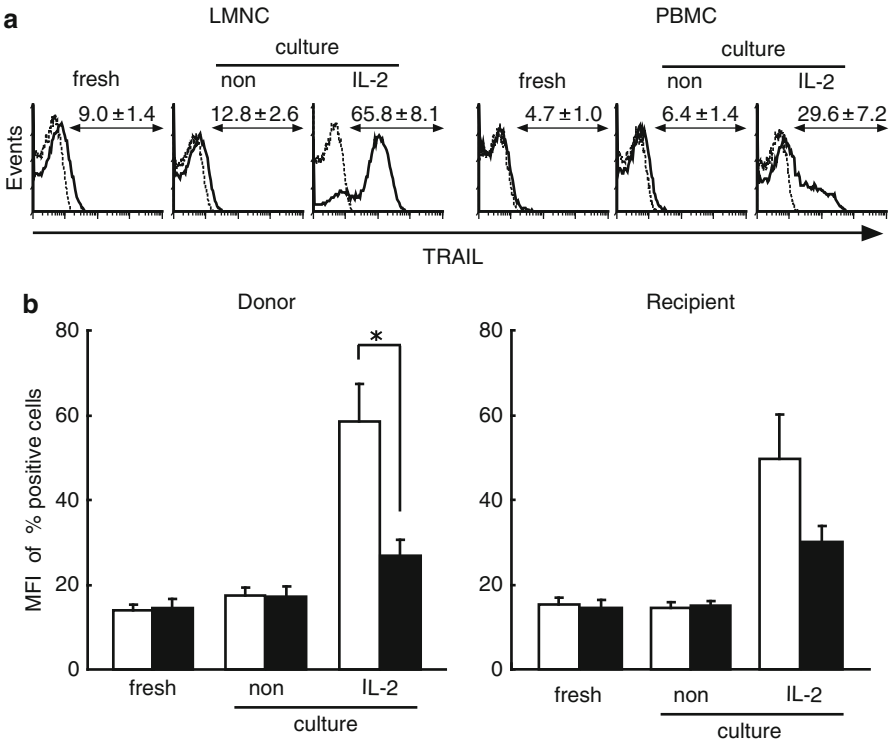

Fig. 4.4 Liver NK cells inductively express remarkable levels of TRAIL, but peripheral blood NK cells do not. Freshly isolated LMNC and PBMC fractions obtained from healthy donors and corresponding recipients cultivated with of without IL-2 were stained with CD3 and CD56 monoclonal antibodies (MAb) together with TRAIL MAb. (**a**) Representative histograms show the log fluorescence intensities obtained upon staining for TRAIL after gating on the $CD3^{-}CD56^{+}$ NK cells subsets of healthy donors. *Dotted lines* represent negative control staining with isotype-matched MAbs. The numbers indicate the percentages of cells in each group that were positive for TRAIL expression. (**b**) The numbers indicate the mean fluorescence intensity (MFI) of cells in each group that stained positively for TRAIL on freshly isolated NK cells cultivated with/without IL-2 (LMNC; open column, PBMC; closed column). The data are presented as means ± SEM. Statistical analyses were performed using ANOVA ($*p < 0.05$)

4.4 Clinical-Scale Isolation of Liver NK Cells from Donor Liver Graft

In order to apply this NK cell immunotherapy to cases of deceased donor liver transplantation, we demonstrated for the first time the phenotypical and functional properties of liver NK cells that were extracted from a deceased donor liver graft perfusate under current good manufacturing practice conditions (cGMP) [35]. First, a large number of NK and NKT cells were verified among liver mononuclear cells, with both subsets possessing characteristics different from those of peripheral blood mononuclear cells. Second, in vitro stimulation with IL-2 induced liver NK cells to

strongly upregulate activation markers, cytotoxicity, and cytokine production while maintaining the expression of inhibitory receptors. These results were compatible with those for a living donor liver graft perfusate [10]. Finally, we confirmed that the final product met the lot release criteria and contained a low T-cell number, thereby reducing the possibility of graft versus host disease (GVHD) in a recipient.

4.4.1 Protocol of NK Isolation

At the time of donor operation, the graft liver was placed in a bag and perfused through the portal vein with 2 L of University of Wisconsin cold storage solution (UW solution) and crystalloids. This perfusate was collected from the hepatic vein and retrieved in our cGMP cell processing facility. Since the UW solution has a high viscosity [36], the perfusate was centrifuged at 2800 × g for 30 min at 4 °C to ensure adequate centrifugation. The cell pellet was then subjected to a Ficoll-Hypaque density gradient centrifugation to separate mononuclear cells. Liver mononuclear cells were cultured with human recombinant IL-2 in complete medium at 37 °C in atmosphere supplemented with 5 % CO_2 for 3–5 days. Anti-CD3 monoclonal antibody was added to the culture medium in order to deplete the $CD3^+$ fraction 1 day prior to cell harvesting. On the day of infusion, the cells were washed twice with 0.9 % sodium chloride and resuspended in 5 % human serum albumin for injection. Testing for lot release included cell counts, viability assessment, Gram stain, and endotoxin analysis (Fig. 4.5).

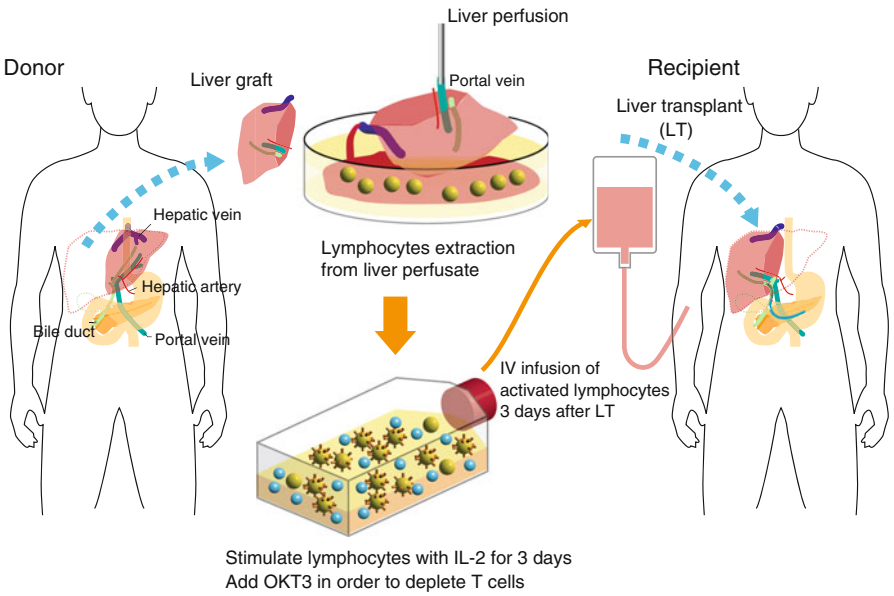

Fig. 4.5 Schematic outline of adoptive immunotherapy with lymphocytes extracted from liver allograft perfusate

4.5 Clinical Trials for Living and Deceased Donor Liver Transplantation

On the basis of these results, we started a clinical trial of adjuvant immunotherapy with activated donor liver NK cells for preventing the recurrence of HCC after living donor liver transplantation in 2006. The lymphocytes in the peripheral blood of liver transplant recipients who received immunotherapy in the early postoperative period showed significantly enhanced cytotoxicity against an HCC cell line (HepG2), compared with those in the peripheral blood of liver transplant recipients who did not receive the therapy in the same period (Fig. 4.6a). Correspondingly, the

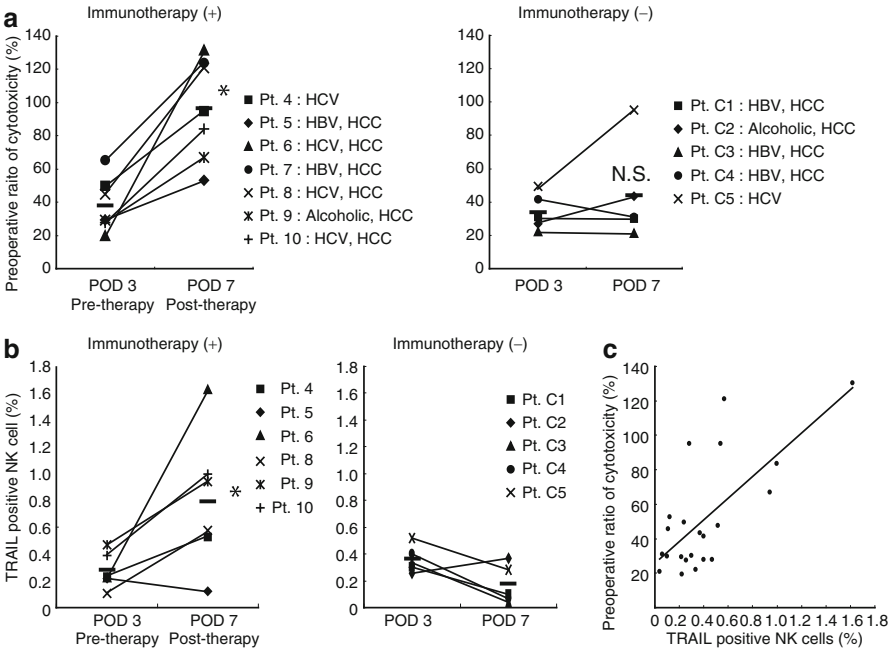

Fig. 4.6 Adoptive immunotherapy with activated donor liver NK cells promoted the cytotoxic activity and TRAIL expression of NK cells in liver transplant recipients. (**a**) The NK-mediated cytotoxic activities of the indicated effectors against their target cells were analyzed by ^{51}Cr-release assay. The dot plot represents the NK cytotoxic activities of freshly isolated peripheral blood lymphocytes obtained from recipients who received immunotherapy (+) and did not receive immunotherapy (−) against HepG2 target cells (effector/target ratio, 40:1) 3 and 7 days after liver transplantation. NK cytotoxic activities are presented as a proportion (percentage) of the preoperative cytotoxicity in each patient. Horizontal lines indicate the mean.*$p<0.05$ for day 7 compared to day 3. (**b**) The frequency of TRAIL$^+$NK cells increased remarkably in the peripheral blood of liver transplant recipients who received immunotherapy. *$p=0.013$ for the immunotherapy group compared to the untreated group on postoperative day 7. (**c**) Correlation between TRAIL$^+$NK cell ratio and NK cytolytic activity after liver transplantation (Spearman rank-order correlation coefficient $r=0.54$, $p=0.01$)

proportions of TRAIL$^+$ NK cells in the peripheral blood of liver transplant recipient who received immunotherapy also significantly increased (Fig. 4.6b). Although the long-term benefits of this approach with regard to HCC recurrence after liver transplantation remain to be elucidated, there were no severe adverse events including GVHD, thus far. With the collaboration of Professors A. Tzakis and S. Nishida at the University of Miami, FL, we successfully performed a phase I study of adoptive immunotherapy using liver NK cells for deceased donor liver transplant recipients with HCC (ClinicalTrials.gov identifier: NCT01147380) beginning in 2009. Both clinical studies are under evaluation and these results will be published soon.

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Chapter 5
Cell-Based Immunotherapy for HCC: Our Experiences and Future Directions

Noriho Iida, Eishiro Mizukoshi, and Shuichi Kaneko

Abstract One of the most exciting advances in recent cancer treatments is the success of immunotherapy including dendritic cell-based vaccine and antibody treatment against the molecules regarding immune checkpoints like PD-1 and CTLA-4. Because the effect and application of current conventional treatments for intermediate or advanced hepatocellular carcinoma (HCC) including chemotherapy and trans-catheter arterial chemoembolization (TACE) are limited, novel immunotherapy is really desired to develop. We summarized status of completed or ongoing clinical trials of cell-based immunotherapy for HCC. Of those trials cytokine-induced killer cells (CIK) or dendritic cells (DC)-based immunotherapies revealed promising results. Future directions of immunotherapy for HCC are also discussed.

Keywords Dendritic cell • Vaccine • Adoptive transfer • T cell • Clinical trial

5.1 Limitation of Conventional Treatments and Advantage of Immunotherapy for Intermediate to Advanced HCC

Currently, conventional treatments for intermediate or advanced HCC are TACE or chemotherapy, respectively [1–3]. TACE is a procedure to inject an embolic agent with cytotoxic drug, resulting in ischemic necrosis of the tumor. The benefit of TACE in unresectable HCC was demonstrated in two randomized control trials [4, 5]. However, anti-tumor effect of TACE is limited in intrahepatic HCC lesions, and

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extra-hepatic lesions are not applicable for the TACE. Advanced HCCs with extra-hepatic lesions or vascular invasion in the liver are treated by oral molecular targeting drug, sorafenib. Discontinuation of the sorafenib treatment is often necessary due to the reasons other than progression of HCC, like liver dysfunction, skin disease and fluid retention [6]. Arterial infusion of cytotoxic drug, 5FU and cisplatin, is another option as a treatment for intermediate or advanced HCC [7]. Although the conventional treatments prolong overall survival, those are limited in patients with good hepatic reserve. Immunotherapy should be more suitable for HCC patients with liver dysfunction, because the antigen-specific immunotherapy specifically targets cancer cell, not background hepatocytes. The first DC-based peptide vaccine for castration-resistant prostate cancer patients, sipuleucel-T, approved by Food and Drug Administration (FDA) in United States caused no severe adverse effects [8]. Sipuleucel-T is comprised of DC loaded with prostatic acid phosphatase (PAP) peptide and specifically targets prostate cancers. Sipuleucel-T significantly prolonged overall survival (median survival time; MST 25.8 months) compared with control (MST 21.7 months). The success of sipuleucel-T shed a light in efficiency of immunotherapy and possibility of the therapy to prolong survival in other cancers. Because HCC patients often have impaired hepatic reserve, HCC-specific treatments with little adverse effects are really desired. Thus, HCC-specific immunotherapy is one of the promising treatments.

5.2 The Current Cell-Based Immunotherapy Trials

Four randomized control trials (RCT) of cell-based immunotherapy for HCC were reported (Table 5.1). All trials were based on infusion of the lymphocytes stimulated with IL-2 in addition to anti-CD3 or other cytokines. Kawata et al. first reported safety of lymphokine-activated killer cells (LAK) which derived from peripheral blood mononuclear cells (PBMC) and then were stimulated with IL-2, but the infusion of LAK didn't improve both disease-free survival (DFS) and overall survival (OS) [9]. Methods to activate PBMC was then improved by adding various cytokines and anti-CD3, and the activated cells were called cytokine-induced killer cells (CIK) which include activated non-specific αβT cells and $CD3^{+}CD56^{+}$ NKT cells. CIK treatment first showed clinical benefit in the HCC patients after radical haptic resection in 2000. Takayama et al. reported that lymphocytes generated from PBMC by the stimulation with IL-2 and anti-CD3 significantly prolonged disease-free survival compared with control arm [10]. Hui and colleagues also reported infusion of CIK generated by stimulation with IL-2, anti-CD3, IFN-γ and IL-1α increased DFS rate [11]. Weng et al. also demonstrated clinical benefit of CIK after radical hepatic resection of HCC as the prolonged DFS [12]. However, all 4 RCT failed to prolong OS by infusion of cytokine-stimulated lymphocytes including LAK and CIK. Two meta-analyses of adoptive immunotherapy in postoperative HCC also revealed no benefit in OS although did benefit in DFS [13, 14]. Prolongation of DFS by CIK was only achieved after radical resection of HCC. Kawata's study targeted advanced HCC by LAK with adriamycin, resulting

Table 5.1 Randomized control trials of cell-based immunotherapy for HCC

Study	Treatment arm	Control arm	Number of patients	Outcomes	Treatment arm vs control arm (%)	*p*-value
Takayama et al. [9]	CIK	Radical resection	150	5-year DFS	38 vs 22	$p<0.05$
	Radical resection			5-year OS	68 vs 62	$p>0.05$
Weng et al. [11]	CIK	TACE + RFA	85	1-year recurrence	9 vs 30	$p<0.05$
	TACE + RFA			1.5-year OS	100 vs 100	$p>0.05$
Hui et al. [10]	CIK + IL-2	Radical resection	127	5-year DFS	23 vs 11	$p<0.05$
	Radical resection			5-year OS	38 vs 37	$p>0.05$
Kawata et al. [8]	LAK + IL-2	adriamycin	24	3-year DFS	50 vs 25	$p>0.05$
	Adriamycin			3-year OS	71 vs 74	$p>0.05$

CIK cytokine-induced killer cells, *DFS* disease-free survival, *HCC* hepatocellular carcinoma, *IL-2* interleukin-2, *LAK* lymphokine-activated killer cells, *OS* overall survival, *RFA* radio-frequency ablation, *TACE* trans-catheter arterial chemoembolization

in no effect in DFS. Benefit of CIK therapy may be limited in prolongation of DFS of patients who undergo radical resection. Novel cell-based immunotherapy which prolongs OS even in advanced HCC, hopefully chemotherapy-resistant HCC, is really desired to develop.

Clinical trials of immunotherapy using other cells than CIK or LAK are ongoing or were completed according to the databases of clinical trials. According to ClinicalTrial.gov, eight cell-based immunotherapies were conducted as clinical trials so far (Table 5.2). Innate lymphoid cells including NK cells, NKT cells and γδT cells were used as effector cells in 3 trials. No evidence was demonstrated by phase III clinical trials with those innate lymphoid cells so far in any types of cancer, but in theory NK cells, NKT cells or γδT cells can target lack of MHC class I on cancer cells or phosphate antigen induced by abnormal metabolism in the tumor microenvironment. Tumor-infiltrating lymphocytes in 2 trials or dendritic cells in 2 trials were used to target cancers by tumor-specific immune responses.

In Japan, seven cell-based immunotherapies were conducted as clinical trials so far according to UMIN (Table 5.3). Two trials were γδT cell-based immunotherapy. Four trials were DC-based immunotherapy which intended to induce HCC-specific immune responses. As tumor antigen, tumor lysate was pulsed in DC in 1 trial (UMIN000005820). To load more specific tumor antigen in DC, tumor antigen mRNA-encoding DC was used in 1 trial (UMIN000005836). DC stimulated with OK-432, inactivated streptococcus pyogenes, were used in 1 trial to activate DC (UMIN000001701). Thus, various cell types and techniques were examined in the current clinical trials to enhance anti-tumor immune responses against HCC.

Table 5.2 Clinical trials of immunotherapy for HCC registered in ClinicalTrial.gov

Cell type	Cancer	Phase	Facility	ID
γδT	HCC	I	Rennes University Hospital	NCT00562666
NK, IL-2-activated	HCC	I	University of Miami	NCT01147380
NK & NKT	Various cancers including HCC	I	Envita Medical Center, Inc.	NCT00909558
TIL	HCC, nasopharyngeal, breast	I	Sun Yat-sen University	NCT01462903
DC loaded with AFP peptides	HCC	I/II	Nantes University Hospital	NCT01128803
DC	HCC, melanoma, renal cell	II	Clinica Universidad de Navarra	NCT00610389
$CD8^+$ TIL	Metastatic cancers including HCC	II	National Cancer Institute, NIH	NCT01174121
CIK	HCC	III	Sun Yat-sen University	NCT01749865

AFP alpha feto protein, *CIK* cytokine-induced killer cells, *DC* dendritic cells, *HCC* hepatocellular carcinoma, *IL-2* interleukin-2, *NK* natural killer cells, *NKT* natural killer T cells, *TIL* tumor-infiltrating lymphocytes

Table 5.3 Clinical trials of immunotherapy for HCC registered on UMIN in Japan

Cell type	Cancer	Phase	Facility	UMIN ID
Naïve T cells	HCC	II	Kyoto Prefectural University of Medicine	UMIN000003861
γδT	HCC	pilot	Tokyo Medical University	UMIN000004583
γδT	HCC	pilot	The University of Tokyo	UMIN000001418
DC	HCC	pilot	Tokyo Medical University/The University of Tokyo	UMIN000000971
DC, encoding cancer antigen mRNA	HCC, pancreatic	pilot	Yamaguchi University	UMIN000005836
DC, OK432-stimulated	HCC	I/II	Kanazawa University	UMIN000001701
DC, tumor lysate-pulsed	Various cancers including HCC	I/II	Tokyo Women's Medical University	UMIN000005820

DC dendritic cells, *HCC* hepatocellular carcinoma

5.3 DC-Based Immunotherapy for HCC

Among various types of cell-based immunotherapy, sipuleucel-T is the only therapy which was verified to prolong overall survival of prostate cancer in phase III trial [7]. Sipuleucel-T comprises DC pulsed with tumor-specific antigen, PAP. If tumor-antigen

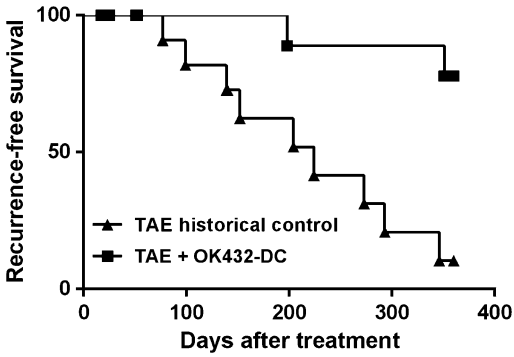

Fig. 5.1 Recurrence-free survival of patients with hepatocellular carcinoma treated with trans-catheter arterial embolization (TAE) with or without infusion of OK-432-stimulated DC. $N = 13$ in TAE + OK-432-DC group and $N = 11$ in TAE historical control group

is known, DC-based immunotherapy is a promising therapy to treat HCC according to the success in sipuleucel-T. Palmer reported that phase II trial of tumor lysate-pulsed DC infusion for patients with unresectable advanced HCC achieved disease control rate 28 % (combined partial response and stable disease) [15]. In the trial, hepatocellular carcinoma cell line HepG2 was used for source of tumor lysate. What is the best to load in DC, autologous tumor lysate, cell line-derived lysate, or HCC-specific peptides, is still controversial and to be determined in future.

We activated autologous DC from cancer patients with OK-432 before the infusion [16]. OK432-activated DC expressed high level of co-stimulatory molecules like CD80, CD83, and CD86. OK-432-stimulated DC also highly produced IL-12p40 and IFN-γ and revealed high tumoricidal activity against HCC cell lines like Hep3B and PLC/PRF/5 in *in vitro* assay. After TAE OK-432-stimulated DC were injected in hepatic artery via catheter. OK432-stimulated DC significantly prolonged recurrence-free survival compared to historical TAE control ($P = 0.0017$; Fig. 5.1). Although IFN-γ responses with PBMC specific to hTERT which is highly expressed by HCC as tumor antigen didn't significantly increase after the OK-432-stimulated DC treatment, serum level of TNF, IL-9 and IL-15 was significantly higher in OK-DC group than TAE control patients. In mouse model of OK-DC treatment for HCC, OK-DC clearly induced tumor-specific immune responses against colon cancer cells [17]. It is still controversial that under which mechanism OK-432-stimulated DC exerts anti-tumor effect, tumor-specific immunity or non-specific direct killing. Although the mechanisms underlying the prolonged DFS caused by OK-432-stimulated DC treatment should still be elucidated, DC-based immunotherapy is a promising treatment for HCC.

5.4 Strategies to Enhance the Effects of DC-Based Cancer Vaccine

In addition to the success in sipuleucel-T for prostate cancer, DC-based vaccines are showing promising results in the clinical trials for other types of cancer. Phase III trial of DCVax-L (Northwest Biotherapeutics), NCT01582672 or NCT01875653

are underway for glioblastoma, renal cell carcinoma or melanoma, respectively [18, 19]. Based on the promising results in DC-based vaccine in various cancers, further strategies to enhance the effect of DC-based therapy are now developing. Combined immunotherapy with DC and CIK for various cancers is also ongoing. Meta-analysis of non-randomized trial demonstrated that chemotherapy + DC + CIK therapy increased the 3-year overall survival rates (RR 11.67, 95 % CI 2.28 to 56.69, $P = 0.003$) and progression-free survival (RR 0.64, 95 % CI 0.34 to 0.94, $P < 0.0001$) in patients with non-small cell lung cancer compared with those treated with chemotherapy alone [20]. Infusing more than 2 immune cells might synergistically enhance anti-tumor immune responses. RCT is required to verify the clinical benefit of DC and CIK combination therapy.

One of the most exciting advances in recent cancer immunotherapy is the success of breaking immune checkpoints to prolong survival of cancer-bearing patients. Development of antibodies against negative regulators of T cell, CTLA-4 and PD-1, induced high clinical responses in various cancers [21–23]. Breaking tolerogenic aspects of tumor-infiltrating T cells might enhance effect of DC-based vaccine to induce tumor-specific cytotoxic T cell populations. Combining DC immunotherapy with anti-CTLA-4 antibody was feasible and well tolerated in advanced melanoma patients and induced high clinical responses compared to either treatment alone [24]. Blockade of PD-1 as well as inhibitory cytokine IL-10 or TGF-β may augment anti-tumor effect of DC-vaccine therapy [25].

Because massive cell death of the cancer releases damage-associated molecular pattern (DAMP) and subsequent acute inflammation accompanied with the production of cytokines and chemokines, it activates tumor-infiltrating DC [26]. This provides a rationale for combining DC-based therapy with radio-frequency ablation (RFA) or TACE which destroy large part of cancer tissues. As support of the idea, we reported that activation of endogenous DC population and subsequent anti-tumor T cell responses are induced by injection of ECI301, an active variant of CCL3, only if the injection was combined with RFA treatment of liver cancer [27]. Moreover, infusion of OK-432-stimulated DC prolonged DFS in combination with TAE in the clinical trial [16] and OS in combination with RFA in murine model [17]. Conventional chemotherapy also augments anti-tumor immune effects by innate cells activated by gut commensal bacteria and probably pathogen-associated molecular pattern (PAMP) [28]. Radiation increases release of lipopolysaccharide from gut to blood, resulting in activation of DC and the enhanced cytotoxicity of adoptively transferred T cells [29]. Thus, combination of DC-based vaccine and conventional cancer therapy including RFA, TACE, chemotherapy and radiation will be tested to see clinical benefit.

5.5 Future Perspective

Although DC-based immunotherapy is the only cell-based immunotherapy which has been approved by FDA so far, whether the infused DC induce effective anti-tumor immunity depends on immunosuppressive background of the host and

strategies to break the tolerant environment [30]. Adoptive transfer of tumor-specific T cells will target cancer independently of immunosuppressive milieu in cancer-bearing hosts if enough number of the T cells is infused [31]. Genetic engineering enabled to induce large number of tumor-specific TCR-transgenic T-cell ex vivo [32–34]. The transfer of tumor antigen-specific TCR-transgenic T cells eradicated some metastatic solid tumors like melanoma in clinical trials. Complete responses or partial responses were observed with gp100-targeting T-cell transfer in 6.3 or 12.5 % of metastatic melanoma patients, respectively [34]. Partial responses were achieved by NY-ESO-1-targeting T-cell transfer in 67 % of synovial cell sarcoma patients, and 27.2 % melanoma patients accompanied with 18 % complete responses [33]. TCR targeting HCC-specific antigen is required for establishment of efficient T-cell transfer therapy for HCC. The emerging development of induced pluripotent stem (iPS) cell technology might further enable to produce large number of tumor-specific TCR-bearing T cells by generating iPS from tumor-infiltrating lymphocytes [35].

Even if "good" tumor-specific antigens are used, DC or tumor-specific T-cell-based immunotherapy cannot overcome heterogeneity in cancer tissues. Because a cancer tissue includes heterogeneous cancer cell populations, some populations should be resistant to tumor-specific immunotherapy. One possible method to overcome the heterogeneity is targeting cancer stem cells (CSC) in the tissue [36]. If antigens specific to CSC are known, DC or TCR-transgenic T-cell therapy might regress whole cancer tissues including heterogeneous populations. In leukemia model, Wilms Tumor 1 (WT1) and cancer testis antigen termed PRAME are potential antigens related to cancer stem cells, and the vaccine using those antigens killed leukemic stem cells [36]. Because CSC-specific antigens are still unknown in HCC, further examination of the antigens is required.

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Part II
Gene Therapy

Chapter 6
AAV Vector-Mediated Liver Gene Therapy and Its Implementation for Hemophilia

Hiroaki Mizukami, Jun Mimuro, Tsukasa Ohmori, Yoichi Sakata, and Keiya Ozawa

Abstract Adeno-associated virus (AAV) vector is widely used in gene transfer purposes. Not only the experimental gene transfer but also applications toward disease therapy are promising. Numerous attempts are ongoing for this purpose. One of the most prominent examples is the sustained clinical benefit in hemophilia gene therapy targeting the liver. These successes have been brought chiefly by the progress in vectorology, especially capsid development. At present, one of the biggest issues is the presence of neutralizing antibody (NAb) against AAV vector capsid. Challenges have been made to conquer this problem. Although there are still some hurdles for wide clinical application, use of this vector will soon be a common practice for the treatment of suitable disease conditions.

Keywords AAV vector • Serotype • Hemophilia • Neutralizing antibody • Empty capsid

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6.1 Preface

AAV vector is one of the most promising systems for human clinical applications. Originally, it was found as an infectious agent from adenovirus stocks and humans. Because the wild-type virus lacks pathogenicity, this vector system is advantageous for administration into humans. In addition to many naturally occurring serotypes, numerous others have been developed and characterized. Recent research has highlighted the tissue tropisms of these serotype-derived vectors, affording a means to select a vector based on the desired target organ. In this review, we will focus on the basic characteristics and applications of AAV in gene therapy with particular emphasis on hemophilia.

6.2 General Features of AAV

The major advantages and disadvantages of this vector system are summarized in Table 6.1. AAV vectors are able to transduce a variety of tissues, and transgene expression can be sustained for many years. Importantly, during this period of persistent transgene expression, the vector sequence exists in an episomal state and is not integrated into the cellular genome. Therefore, nondividing cells are considered prime target, and in the case of rapidly dividing cells, the expression will be diminished after each round of division. When intending supply of a transgene product into systemic circulation, liver and skeletal muscles are preferred targets, although some other organs are also explored for this purpose. In large animals and humans, liver is selected as a target organ rather than skeletal muscle because the procedure is less invasive, as well as less provocative to the immune system. Major clinical trials using AAV vectors are listed as Table 6.2. Numerous successful results have been published, especially in the cases of targeting the brain, retina, and liver.

Table 6.1 Features of AAV vector

Advantages	Disadvantages
Safety based on non-pathogenicity	Limitation of transgene cassette length (up to 5 kb)
Wide range of target tissue	
Long-term transgene expression	High prevalence of neutralizing antibody
Stability of the vector capsid	Complicated procedure for vector production

Table 6.2 Major clinical trials using AAV vector

Target tissue	Diseases	Genes
CNS	Leukodystrophy (Canavan disease)	Aspartoacylase
	Alzheimer's disease	NGF
	Parkinson's disease	GAD, AADC
	Lipofuscinosis	CLN2
	Temporal lobe epilepsy	NPY
Eye	Leber's congenital amaurosis	RFP65
Muscle	Muscular dystrophy	Sarcoglycan
	Cardiomyopathy	SERCA2a
Respiratory	Cystic fibrosis	CFTR
	Alpha-1 antitrypsin deficiency	hAAT
Metabolic	Hemophilia B	Factor IX
Others	Prostate cancer	GM-CSF

Selected from OBA homepage

6.3 A History of AAV Vector Development

6.3.1 Discovery and Vector Development of AAV1–6 (First Generation: Naturally Occurring Serotypes)

AAV was first discovered in adenovirus stocks as a contaminating virus [1]. In the 1960s, AAV2–4 were subsequently discovered and studied intensively. As it appeared to be nonpathogenic, the interest from the virological point had been disappeared. After that, a limited number of groups investigated this virus chiefly as vectors. During that period, AAV5 was discovered from human pathological site [2] and AAV6 from laboratory strain [3]. At that time, early attempts to develop vectors from these serotypes were conducted [4–8].

6.3.2 AAV Serotypes Discovered from Monkey and Human Tissues (Second Generation: Selected Naturally Occurring Serotypes and Clones)

Following the isolation of AAV1–6, significant attempts were made to discover other serotypes. Among these, most influential studies were the development of AAV7–9 [9, 10]. AAV7 and AAV8 were isolated from monkey tissues and AAV9 from humans. As performance of these vectors were tested and selected using mice, in vivo experiments using these serotypes were likely to be successful. Many other serotypes and clones have been reported thereafter. Practically, it is not possible to test the performance of all these derivatives in vivo.

6.3.3 *Development of Novel Vectors Suitable for Specific Purposes (Third Generation: Ongoing)*

In order to overcome the limitations of naturally occurring serotypes and clones, a number of efforts have been made to modify the capsid domains through shuffling or mutation [11]. Although not many of these derivatives have been integrated into the research community, the use of these techniques may eventually lead to the invention of the ultimate therapeutic vector.

6.4 A Brief History of Hemophilia Gene Therapy

6.4.1 *Overview of Hemophilia*

Hemophilia is the largest bleeding disorder of genetic origin. Based on the responsible gene, it is named as A and B, which correspond to factors VIII and IX, respectively. As these genes are located on X chromosome, males are usually affected. Worldwide, an estimated one in every 5,000–7,000 live male births is affected by hemophilia A, while one of every 25,000–30,000 is affected by hemophilia B [12]. The disease severity is classified based on levels of blood coagulation factor (Table 6.3). Gene therapy is often beneficial in severe form patients. Hemophilia is an ideal target for gene therapy, as the supplementation of a single gene product should theoretically improve disease status. In reality, achieving this goal has proved challenging [13].

6.4.2 *Advantages of Gene Therapy as a Therapeutic Modality*

In severe form patients, regular injection of blood products is costly and painful, and such treatments may still not completely prevent further bleeding episodes. In this case, gene therapy may hold the key toward minimizing unwanted aspects while keeping the levels of coagulation factor constant.

Table 6.3 Severity of hemophilia

Disease status	Coagulation activity (% of normal)
Mild	>5
Moderate	5>, >1
Severe	1>

6.4.3 Attempts Using Various Vectors for Hemophilia A

As the size of the cDNA of factor VIII is much greater than the packaging capacity of AAV, early attempts have utilized various vector systems, including plasmid, adenovirus vectors, and retrovirus vectors (Table 6.4).

6.4.4 Attempts Using AAV2 Vectors for Hemophilia B

Early clinical trials have targeted both the skeletal muscle [14] and the liver [15]. Although transient expression was observed in some patients, neither study resulted in significant clinical benefit. The main reason for the insufficient efficacy is now attributed to the incomplete performance of AAV2 vectors in these organs.

6.4.5 Clinical Trials Incorporating the AAV8 Vector

The next clinical trial was conducted for hemophilia B patients utilizing liver-tropic AAV8. The vector was administered intravenously to patients, and sustained level of coagulation factor was observed even the amount of blood product was decreased or discontinued [16]. Three different doses of vector were tested, and the most significant benefit was observed in the highest dose patients. Based on the success of this protocol, several new patients have been enrolled in the study at the highest dose. This success stimulated the field of hemophilia gene therapy, and it is reported that other clinical trials are currently proposed using AAV8-based vectors.

Table 6.4 A list of clinical trials for hemophilia

Type	Target	Vector	Start	Cases	Outcome
A	Fibroblast	Plasmid	1998	6	Low efficiency
B	Muscle	AAV type 2	1999	8	Low efficiency
A	Liver	Retrovirus (MLV)	1999	13	Low efficiency
B	Liver	AAV type 2	2000	8	Transient efficacy
A	Liver	Adenovirus (helper dependent)	2001	1	Toxicity
B	Liver	AAV type 8	2008	10+	Clinical benefit

6.5 Considerations for Optimizing AAV Vector Administration

6.5.1 General Considerations

As in the case with many therapeutics, determining the optimum route of administration has been a point of emphasis for AAV vectors. While simple injection into the target tissue is sufficient in some cases, including the use in the central nervous system [17], there are several other methods and procedures that have been developed and optimized for other target organs.

6.5.2 Liver-Mediated Gene Transfer

To achieve efficient liver transduction, vector injection into the portal vein would be the obvious deliver method. However, injection into the main part of the portal vein is invasive and poses a high risk of development of difficult bleeds. Therefore, injection into a more peripheral portion, such as the mesenteric vein, is safer and preferred. In one clinical trial of hemophilia B, vector was delivered into the hepatic artery [15]. In that trial, some patients showed significant level of expression, although the duration of expression was limited. In the case of more liver-tropic vectors such as AAV8, simple intravenous injection seems to be a sufficient means for liver transduction.

6.5.3 Skeletal Muscle Transduction

6.5.3.1 Direct Injection into Muscle

The natural choice for muscle transduction would be direct injection. Indeed, this has proven efficacious in rodents; however, in larger animals there are complications. First, a robust immune response often results as the tissue injury caused by the needle provokes innate immune responses. Additionally, due to uneven distribution of the vector within muscle tissue, there is often an equally uneven transduction efficiency, which often results in incomplete posttranslational modification. Obviously, it is impossible to transduce entire muscle tissues by this method. In an early clinical trial for hemophilia B, vector was administered intramuscularly at over 100 sites in the highest dose patients [14]. Intramuscular injection was discouraged as a route for clinical use especially after the demonstration of anemia due to immune reaction against erythropoietin, which was the original transgene aiming to promote red blood cell formation [18, 19].

6.5.3.2 Other Methods for Muscle Transduction

Whole-muscle transduction in vivo has been reported using adult mice [20]. This method utilized simple IV injection of AAV6-mediated vectors. At first, VEGF was administered simultaneously to enhance vascular permeability. Subsequently, it was shown that VEGF was not essential if the vector dose was higher by ten times. Detailed analysis revealed widespread transduction of muscle tissues throughout the body. The only drawback of this method was the necessary vector dose (10^{13} vg per mice), which accounts for nearly 100-fold excess of regular applications. Although various attempts have been made to reduce the vector dose, this method still requires much higher vector dose than regular approaches and therefore hard to translate.

6.5.3.3 Limb Perfusion

In the field of cancer treatment, this technique has been developed to maximize therapeutic efficacy by concentrating the delivery of chemotherapeutic agents. A tourniquet is applied to cease blood flow through the local arteries and veins, and then vector is injected intravenously into the isolated area. Histamine or other drugs may be included to promote vessel permeability. This method is effective for the muscles of extremities; however, it is not feasible to apply this technique for muscles of the trunk, head, and neck area [21]. Moreover, as this approach requires intensive exposure of the vector to the blood supply, presence of NAb against the vector capsid also diminishes the efficacy of such a treatment (see next section for details).

6.6 Neutralizing Antibody (NAb) Against AAV Vector

6.6.1 The Impact of NAb Upon AAV-Mediated Gene Transfer

The presence of neutralizing antibodies against vector capsid is one of the most important issues in AAV-based therapies, especially for liver transduction in vivo [22, 23]. The antibody is considered as a consequence of natural infection. As wild-type AAV may distribute on earth, worldwide prevalence of NAb against AAV would be of interest.

6.6.2 Prevalence of NAb Against AAV Serotypes Around the World

The actual procedures for NAb measurement is described in Fig. 6.1. Several studies illuminated prevalence of NAb against various AAV serotypes in different locations around the world [24–31]. The results indicate that a fraction of the population

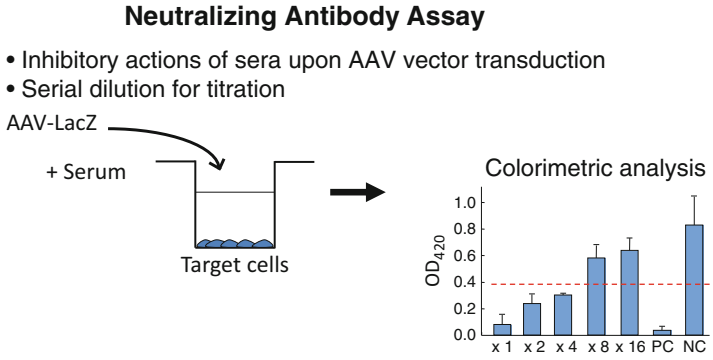

Fig. 6.1 Assay for NAb

possesses NAb against all serotypes tested. Given the complexity of this assay, there is some inherent variability, although it is clear from the findings that NAbs are prevalent and pose a serious challenge for AAV-based therapies in every country tested.

6.6.3 Analysis of Japanese Population

We tested the prevalence of NAb in Japan in both healthy and hemophilia populations [31]. Our results indicate that the overall prevalence in Japanese population is similar level to the rest of the world. There was not a significant difference between the healthy population and the hemophiliacs. Surprisingly, there was a huge difference in the prevalence if the population was classified by age; those who were born before 1970 showed remarkably high prevalence (80 % or higher) in both groups (Fig. 6.2). On the other hand, younger populations showed low prevalence with a decreasing trend year by year. We speculate that wild-type AAV is on the way of elimination, presumably by the improvement of public hygiene status. Our results indicate that the proportion of NAb-negative population will increase over the years, although another round of study is necessary in the different time point to warrant the conclusion.

6.6.4 Approaches for Evading Inhibitory Actions of NAb

Various methods have been developed to circumvent inhibitory actions of NAbs against AAV (summarized as Table 6.5). For example, if a subject shows seropositivity against a specific serotype, the use of alternative serotype can be a solution. In reality, as a different serotype exhibits different tropism, switching to a suitable alternative may not be feasible.

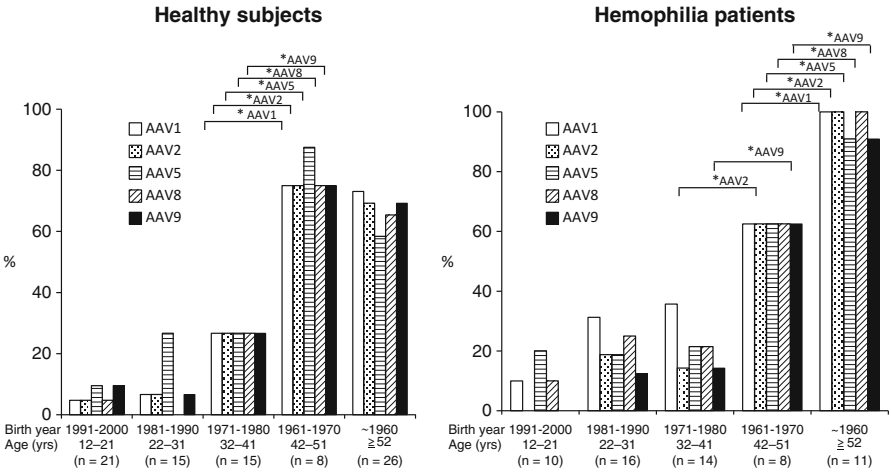

Fig. 6.2 Prevalence of NAb against various serotypes of AAV capsid in Japan by age

Table 6.5 Possible approaches to overcome inhibitory actions of NAb

Approach	Status
Use of alternative serotypes	Not feasible
Use of NAb-resistant capsids	Under development
Chemical modification of capsid	Limited efficacy
Pharmacological intervention	Limited efficacy
Plasmapheresis	Limited efficacy, rebound
Saline flushing	Complex procedure

Use of NAb-resistant capsids originates from the same idea. This method is usually combined with the modification of capsid structure. In the process of building better capsid, capsid gene sequences are modified either by inserting mutation or domain swapping. At the end of the cycle, the pool of candidate capsids is reacted with NAb-positive sera to select a capsid not reactive to the known preexisting immunity.

Plasmapheresis was suggested as a practical method to decrease NAb titer [32]. This method seems effective to reduce the preexisting antibody titer several-fold, but the titer rebounds relatively quickly. Pharmacological intervention using rituximab was reported with some reduction of NAb titer [33].

6.6.5 Saline Flushing

In order to conquer inhibitory actions of NAb, we developed a technique called as saline flushing [34]. The principal idea is to avoid vector contact with blood, before reaching liver tissue. During the prototype experiments, portal vein injection was performed. Later, the method was revised to utilize double-lumen catheter. The

catheter is inserted into a branch of mesenteric vein, and then a balloon is inflated to occlude the main part of the portal vein to ensure the flow of saline into portal circulation. After saline flushing, the vector solution is injected, followed by some volume of saline again, which is intended to retard blood contact to the vector solution and liver parenchyma.

6.7 The Search for the Best Vector for Liver Transduction

6.7.1 Early Results Using AAV2

As AAV2 was considered as a standard and actually the most intensively studied serotype, earlier clinical trials were performed using AAV2. However, as described earlier in this chapter, the performance of AAV2-based vector in liver transduction turned out to be suboptimum. Upon the report of these results, considerable attention was focused on the use of other serotypes.

6.7.2 Features of AAV8 Vectors

One promising candidate was AAV8, as it had been shown to hold significant liver tropism when screened in mice. There seems to be some species specificity in the liver tropism, as the performance of this vector in larger animals, especially monkeys, was less impressive than that observed in mice. Even in the successful clinical trial using AAV8, this may be true, as the initial estimated necessary vector dose proposed to the Recombinant DNA Advisory Committee (RAC) was a significant underestimate due to the inefficiency in Q-PCR steps [35]. After adjustments were made to the reaction conditions, it became clear that the necessary vector dose was actually 10 times higher than the original, which turned out to be the same dose that was previously used in the AAV2 study (2×10^{12} vg/kg).

6.7.3 Revival of AAV3-Based Vectors

AAV3 was developed as vectors next to AAV2 [4]. At that time, the interest to the AAV1 was much lower, as it was considered monkey origin. The utility of AAV3-based vectors were tested and some cell lines showed positive results. Despite these initial findings, interest in AAV3 was modest until a pair of works brought it back to light. One recent study characterizing a series of specific vector mutants found that AAV3 has a robust transduction capacity in human liver cells [36]. Another focused on the development of a vector capsid with superior transduction capacity in the human liver. Shuffling was performed, followed by selection in human liver tissue

transplanted to the immune-deficient mice. After several rounds, the group obtained a vector with significant liver transduction capacity. Sequencing of the capsid revealed high similarity to AAV3 [37]. These independent works highlight the potential of AAV3-based vectors for human liver transduction. Whether these vectors show higher transduction efficiency in human liver awaits further study.

6.8 Issues Regarding Empty Capsid

6.8.1 Characteristics of Empty Capsid

An empty capsid consists of an incomplete length of the vector genome inside a regular capsid structure. These empty capsids are by-products of vector production and are also generated during the course of infection of a wild-type virus. The amount of empty capsid found in a vector preparation depends on a number of factors, especially the mode of vector generation. At present, the precise mechanisms and conditions contributing to the generation of empty capsid have not been well understood.

6.8.2 Impact of Empty Capsids Within Vector Preparations

As empty capsids interfere with transduction, excessive presence within a vector stock results in an overall reduced performance. Moreover, contaminating empty capsids inflates the antigenicity of the vector stock, resulting in an increased immunological burden to the body. From these points of view, empty capsids should be excluded from vector preparations as much as possible. On the other hand, there is a body of work, which indicates advantages of empty capsids. The point is that empty capsid binds to NAb and cancels inhibitory actions of NAb. In one study, in order to reduce the undesirable actions of empty capsid, the capsids are mutated to avoid competition with full particles [38]. One concern is that even if mutated, these empty capsids may be an immunological burden to the body. The optimum ratio of full and empty capsids remains unclear. Further study is necessary to evaluate the positive and negative roles of empty capsid within vector stocks.

6.8.3 Procedures of Removing Empty Capsid

Because empty capsids have unique physical and chemical properties compared to full particle, they can be separated using a number of techniques. One of the most dramatic physical differences between empty and full capsids is their density, and

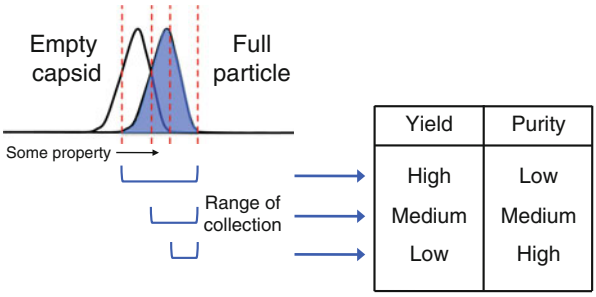

Fig. 6.3 Removal of empty capsid from vector stocks

for this reason, the most efficient isolation method involves the use of density gradient. Although other methods have been identified and applied, the differences are relatively small. Therefore, purity of the vector stock often comes at the expense of the recovery rate (Fig. 6.3).

6.9 Future Perspectives: Hurdles for Application

6.9.1 Difficulties in Translation

Many difficulties arise when developing and applying new techniques. Given the previous clinical success, the focus has now shifted toward improving each step of the process, including the preparation of vector, patient selection, and regulation.

6.9.2 Preparation of Vectors for Human Trials

While it does contribute significantly to cost, it is critical that vectors for human administration be produced and purified under strict GMP conditions. As for the method of production, transient transfection has been a standard method. For large-scale production as is seen in hemophilia gene therapy settings, other methods are preferred like baculovirus-mediated production or adenovirus- or herpesvirus-mediated production.

6.9.3 Standardization of Protocols

Another issue is the standardization of methods dealing with vectors. Although the same methods are utilized, results may vary among institutes. In an attempt to minimize this variability, a study was conducted in which standard materials were shared between 16 institutes, and vector titers and infectious titers were evaluated using

standard protocols [39]. The results indicated that there is some variability among the institutes. The results were processed, averaged, and deposited into ATCC. A similar study was conducted using AAV8, and the findings were consistent with those described above [40]. While these studies have highlighted potential issues that must be addressed, it is clear that the gene therapy has great utility when it comes to treating disease conditions such as hemophilia.

6.10 Concluding Remarks

Half a century has passed since the discovery of AAV, and its utility, as well as drawbacks, has been widely elucidated. There has recently been significant interest in the use of AAV in human clinical use, and while there are a number of challenges to overcome, further study will surely build up the way toward development of novel treatment modality using this vector.

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Chapter 7
Potential Usage of Human Artificial Chromosome for Regenerative Medicine

Narumi Uno, Yasuhiro Kazuki, and Mitsuo Oshimura

Abstract Human artificial chromosome (HAC) vectors can carry a gene or genes of interest. HACs have been generated mainly by either a "top-down approach" (engineered creation) or a "bottom-up approach" (de novo creation). HACs with one or more acceptor sites exhibit several characteristics required by an ideal gene delivery vector, including stable episomal maintenance and the capacity to carry large genomic loci plus their regulatory elements, thus allowing the physiological regulation of the introduced gene in a manner similar to that of native chromosomes. This mini-review introduces characteristics of engineered HAC and their ability to drive exogenous gene expression in cultured cells via microcell-mediated chromosome transfer (MMCT). A new avenue for regenerative medicine in the future is also proposed.

Keywords Microcell-mediated chromosome transfer • Human artificial chromosome • Chromosome engineering • Regenerative medicine • Gene/cell-therapy

7.1 Human Artificial Chromosome (HAC) as Episomal Vector

Most, but not all, conventional vectors present problems associated with their limited cloning capacity, lack of copy number control, and insertional mutagenesis caused by integration into host chromosomes [1–3]. Human artificial chromosome

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(HAC) displays a number of advantages over conventional vectors, e.g., they do not integrate into the host genome and the size of gene(s) they can carry is not limited.

A novel HAC vector in which known endogenous genes were absent was developed using the top-down approach from human chromosome 21 [4]. This HAC was physically characterized confirming that no known endogenous genes remained in the HAC. Thus, the HAC vector contains four useful features: (1) it has a well-defined genetic architecture; (2) it is present episomally, independent of the host chromosomes; (3) it is mitotically stable in human cells in vitro; and (4) any desired gene can be cloned into the HAC using the Cre-loxP system in CHO cells or by a homologous recombination system in DT40 cells [3–5]. Using the Cre-loxP system, gene cloning can be performed by insertion-type cloning or translocation-type cloning (Fig. 7.1). Circular vectors such as plasmids, P1-derived artificial chromosome (PAC)s, and BACs containing desired genes can be inserted into the HAC vector by insertion-type cloning. Mb-sized genes, which cannot be cloned into the circular vectors, can be cloned into the HAC vector using translocation-type cloning [6]. Furthermore, two different vectors, each containing a desired DNA or gene like an artificial telomere, can be inserted sequentially into the HAC by homologous recombination in DT40 cells. Genome-editing technologies [7] will also enable us to perform this recombination process easily without using DT40 cells. Therefore, any combination of genes, including full-length genomic DNA, can in theory be cloned into the HAC by a combination of these cloning systems and transferred into

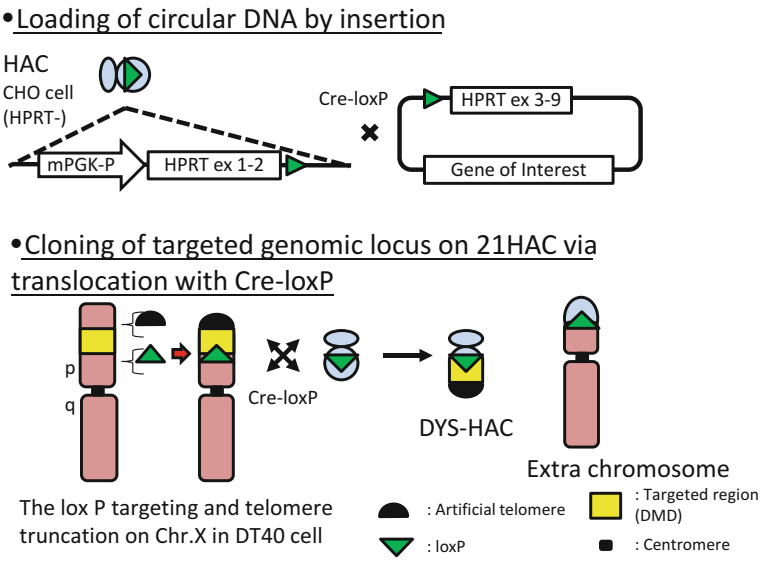

Fig. 7.1 Two types of gene loading to HAC. The gene of interest, isolated in a circular vector, is introduced into the HAC by site-specific insertion (*above*). A megabase-size gene locus, which is above the capacity of circular cloning vectors, is introduced into the HAC by site-specific reciprocal chromosome translocation (*below*)

a desired recipient cell type using the HAC. Thus, the HAC vector may be useful for gene and cell therapies.

As next generation of construction of HAC vector, a novel HAC vector may be generated using the genome-editing technologies in human primary or iPS cells via top-down approach for safe gene and cell therapy, without using intermediate host cells such as DT40 cells although the technologies for transfer of the HAC from normal human cells to desired patient cells without using A9 or CHO cells need to be developed.

One application of HAC vector is transfer of multiple genes into target cells. Since the HAC vector possesses a single acceptor site (loxP), multiple genes should be unified in a donor vector such as BAC or PAC by conventional in vitro recombinant DNA technique. Processing of BAC or PAC for unifying multiple genes is, however, laborious because of their low copy number in host *E. coli* and of their large size beyond the fractionation range in gel electrophoresis.

An alternative to unifying multiple genes in a single donor vector is the increase of acceptor site on the HAC vector by utilizing other recombinase or integrase systems capable of site-specific insertion of donor vector. Application of several integrase systems derived from different microorganisms had been reported in mammalian cells [8]. Yamaguchi et al. made a HAC vector carrying 5 acceptor sites for utilizing FLP recombinase and phiC31, R4, TP901-1, and Bxb1 integrases [9], which was designated as MI-HAC (multi-integrase HAC) (Fig. 7.2a). In the MI-HAC, a pair of promoters for a selection marker gene and an acceptor site for an integrase were tandemly placed, while in a donor vector, a gene of interest was placed along with a promoter-less drug-resistant gene. Theoretically, up to five different genes of interest could be loaded onto the MI-HAC by reconstruction of the selection marker gene by site-specific integration of the donor vector. While the MI system enabled effective loading of the gene of interests, it requires multiple selection markers.

To overcome the problem of this complicated gene-loading protocol on the HAC vectors, a simple method for the simultaneous or sequential integration of multiple-gene-loading vectors into a HAC vector, designated as the simultaneous or sequential integration of multiple-gene-loading vector (SIM) system, was reported [3, 10]. In the SIM system, simultaneous integration is attained by stepwise nested insertion of gene-loading vector by different integrases (Fig.7.2b, left). Sequential integration is attained by the shedding of formerly reconstructed marker gene, caused by targeted insertion of an ensuing donor vector (Fig. 7.2b, right). These are achieved by elaborate placement of target sequence for the integration and smart utilization of splicing acceptor and donor cassettes to splice out the acceptor site for the next reaction embedded between the front and rear half of the selection marker genes. A prominent feature of this system is that multiple-gene-loading vectors can be integrated by the cycling use of only two selection marker genes at most. Thus, the SIM systems on HAC vectors are very useful and are expected to expand the applicability of HAC vectors for multiple-gene expression study, because the SIM systems can be applied to any HAC with a 5′HPRT-type cassette.

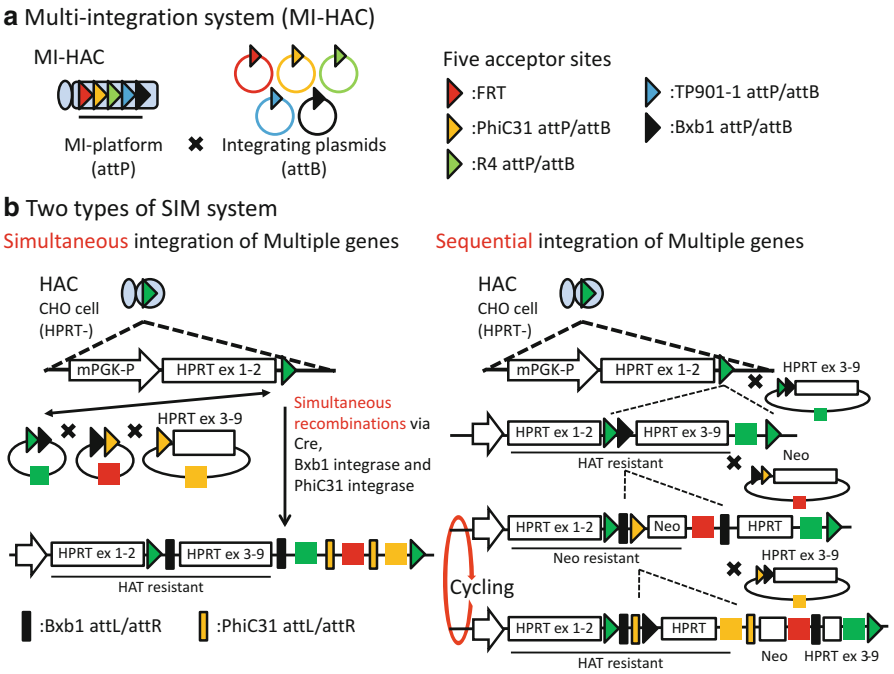

Fig. 7.2 Multiple-gene integration system with multiple-integrase system (MI system) and simultaneous or sequential integration of multiple-gene-loading vector system (SIM system). (**a**)MI-HAC can be inserted to each donor plasmid in each acceptor site. MI-HAC harbors five acceptor sites, which are called MI-platform and include FRT, phiC31 attP, and R4 attP. On the other hand, each donor plasmid contains a companion sequence like FRT or phiC31 attB. Thus, MI-HAC can be inserted to five plasmids in each acceptor site with site-specific recombinase or integrase, respectively. (**b**) There are two types of SIM system. A SIM system (*left*) can insert three plasmids in HAC, simultaneously. These three plasmids are combined via each recombinase and then integrated in HAC, simultaneously. Another SIM system (*right*) can insert several plasmids, sequentially. A first plasmid is inserted in HAC with loxP site via Cre. It also contains selectable marker gene and another acceptor site like Bxb1 attB. Thus, next donor plasmid can be inserted sequentially. Since the next donor plasmids cut in between a selectable marker gene and a promoter, this sequential integration system can need only two selectable marker genes and is theoretically possible to load unlimited number of genes [19]

7.2 Microcell-Mediated Chromosome Transfer (MMCT)

A chromosomal vector including HAC can be transferred into other desired cell lines by microcell-mediated chromosome transfer (MMCT). For MMCT, donor cells (normally mouse A9 cells and CHO cells) are induced to multinucleate their chromosomes (Fig. 7.3). Micronuclei are then forced through the cell membrane to create microcells by centrifugation in the presence of cytochalasin B which disrupts the cytoskeleton [11]. These microcells can be fused to a recipient cell line under appropriate conditions.

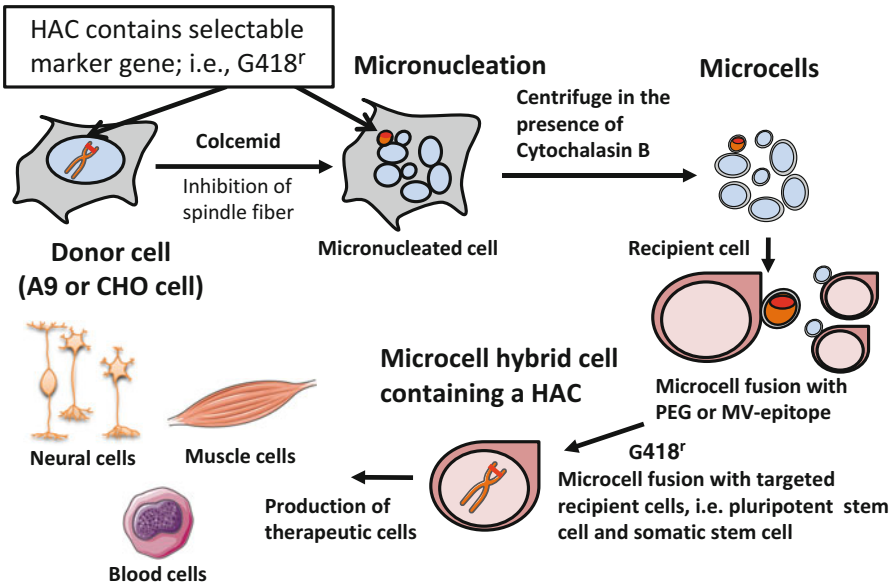

Fig. 7.3 Microcell-mediated chromosome transfer (MMCT) to recipient cells. The figure shows the introduction of the marked human chromosome from the donor A9 or CHO cells to the recipient cells. The procedure can be divided into several parts: micronucleation of the donor hybrids by colcemid treatment, enucleation in the presence of cytochalasin B, purification of the microcells, fusion with the recipient cells, drug selection of the microcell hybrids, identification of the transferred human chromosome by fluorescence in situ hybridization, and DNA analyses. This figure was produced using Servier Medical Art (http://www.servier.com)

MMCT comprises multiple steps: (1) induction of micronuclei in donor cells, (2) isolation of microcells from micronucleated donor cells, (3) fusion of microcells with recipient cells, (4) integration of the transferred chromosome into host cell nucleus, and (5) selection of microcell hybrid cells by drug selection (Fig. 7.3). Eventual efficiency is determined by the summation of contribution from each step. Established cell lines such as pluripotent stem cells including embryonic stem cells, induced pluripotent stem cells, and somatic stem cells have been preferably used as recipient cells. Efficiency of MMCT has been described by the ratio of drug-resistant colony number to recipient cells. Depending on the type of recipient cells, the efficiency is generally 10^{-5}–10^{-6} when using polyethylene glycol (PEG) for the fusion of microcells and recipient cells. With the advent of stem cell studies, the target of MMCT has been extended to primary cells with finite life span, somatic stem cells, and induced pluripotent stem (iPS) cells. Improvement of the efficiency has emerged as an issue to be resolved.

The microcells conventionally have been fused to recipient cells in the presence of polyethylene glycol (PEG) which acts as a dehydrating agent and fuses plasma membranes. Recently, a more efficient MMCT procedure has been developed [12]. During the course of virus particle formation in the infected cells, viral fusogenic

proteins are synthesized de novo, transported to the cell periphery, and presented on the cell surface, followed by extrusion of cell membrane as virus envelope [13]. This well-controlled budding mechanism of envelope virus prompted us to make "a fusogenic microcell" which carries a chromosome to be transmitted and is coated with fusogenic envelope proteins. We chose fusogenic envelope proteins from Measles virus (MV) for which accumulating data have been reported. MV has two envelope glycoproteins, hemagglutinin (H) and fusion (F) proteins, for infection into host cells. Virus particle specifically attaches to the surface of host cells by the interaction between H protein and its receptors on the host cell surface. Binding of the H protein to a receptor triggers the fusion of virus envelope with the host cell membrane by the mediation of F protein. To make "fusogenic microcells," expression plasmids encoding H and F proteins were transfected into CHO cells carrying a HAC vector. Microcells isolated from the CHO donor cells showed fusion ability to recipient human cells that express a receptor protein CD46, leading to successful transfer of the HAC [12]. It was noted that the MMCT efficiency depended on the expression level of CD46 in recipient cells. CD46 belongs to the family of complement activation regulators that prevent self-cell destruction [14]. Overexpression of CD46 is frequently observed in cancer cells to overcome lysis by complement [15]. Indeed, in the case of fibrosarcoma cell line HT1080 which has high surface density of CD46, the MMCT efficiency was 2 orders of magnitude higher than that with PEG fusion. However, in the case of primary fibroblasts that have low surface density of CD46, the efficiency was comparable to that with PEG fusion. An issue with this method is the narrow range of recipient cells that can be used to obtain high-efficiency fusion. The preceding studies that describe the usage of MV for oncolysis by infection have proposed retargeting of MV by engineering the H protein, i.e., addition of single-chain antibody fragment against surface receptors other than CD46 [16, 17]. The addition of single-chain variable fragment (scFv) against transferrin receptor (TfR) improved fusion efficiency to primary fibroblasts [18]. Although high affinity of scFv to desired surface receptor is not always available with ease, retargeting of MV may be an alternative for PEG-sensitive cells.

7.3 HACs and Potential Medical Applications

Gene therapy has been envisioned to provide a direct and permanent correction of genetic defects. To achieve the desired effects, therapeutic genes need to be carried by safe and effective vectors that can deliver foreign genes to specific cells and thereafter sustain their expression in a physiologically regulated fashion. Gene delivery vectors with the following properties may further add to the applications for autologous gene and cell therapies: (1) high transfer efficiency; (2) long-term stable maintenance in host cells without integration into the host genome; (3) appropriate levels of spatial and temporal expression of therapeutic genes in specifically desired cells; (4) no risk of cellular transformation or stimulation of the host's immune system; and (5) a system to safeguard against tumor formation. Although a

number of different approaches have been attempted to achieve efficient gene transfer and long-term gene expression, this challenging task remains unfulfilled because all current methods have certain limitations, including transient expression, consequent toxicity, undesired immunological response, integration of target genes into the host cell genome, and transcriptional silencing.

An alternative solution to these problems could be the use of HAC vectors although all cannot be fulfilled. For example, the advantages of HAC vectors have been demonstrated for reprogramming mouse embryonic fibroblasts (MEFs) into iPS cells [19]. A HAC carrying four reprogramming factors with a p53-knockdown cassette (iHAC) efficiently reprogrammed MEFs. Global gene expression patterns showed that the iHAC generated relatively uniform iPS cells. Under non-selecting conditions, iHAC-free iPS cells were isolated as cells that spontaneously lost iHAC. Analyses of pluripotent markers, teratomas, and chimeras confirmed that these iHAC-free iPS cells were pluripotent. Moreover, iHAC-free iPS cells with a reintroduced HAC-encoding herpes simplex virus thymidine kinase were eliminated by ganciclovir exposure, indicating that the HAC safeguard system functioned in iPS cells [19]. Thus, the HAC vector could generate uniform, integration-free iPS cells with a built-in safeguard system [20]. Similarly, human iPS cells have also been induced from human fibroblasts with a similar iHAC, although the efficiency is still low [18].

Another example is the HAC utility for gene therapy for Duchenne muscular dystrophy (DMD). DMD gene was newly loaded on HAC [21]. DMD is caused by dysfunction of the dystrophin gene [22]. Since some DMD patients show a large deletion in the dystrophin gene, these defects cannot be corrected by exon-skipping approaches [23, 24]. Although several vectors have been developed for DMD gene therapy, no episomal vectors containing the entire dystrophin genomic region have been reported owing to the extremely large size of this region (2.4 Mb) [25]. Thus, a HAC vector containing the entire dystrophin genomic region (DYS-HAC) has been developed for potential application in DMD gene therapy [6]. The complete correction of a genetic deficiency was shown in iPS cells derived from DMD model (mdx) mice and a human DMD patient using the DYS-HAC. In addition, the DYS-HAC isoforms were verified in cardiomyocytes differentiated from iPS cells, which were derived from DMD patients [26]. More details are described in others [20, 21, 24, 27–29].

Finally, advances in the efficiency of methods used for the differentiation and purification of stem cells, including ES and iPS cells, are anticipated, and the application of these methods to ES/iPS cells combined with HAC vector systems may enable the development of more sophisticated gene therapies. Thus, stem cells, potentially derived from multiple sources, combined with HAC-mediated gene delivery, should permit safe treatment of various genetic defects. HACs may be used as a potential platform for regenerative medicine, although there are a number of problems to be resolved before its usage, especially, e.g., “safety and efficiency” of HAC transfer. A combination of chromosome engineering technologies and genome-editing technologies should facilitate the applications to biomedical challenges [7, 30]. The next step in the future of gene therapy is to demonstrate functional restoration and safety in vivo using large animal models such as dogs and monkeys.

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Chapter 8
Image-Guided Hydrodynamic Gene Delivery to the Liver: Toward Clinical Applications

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Abstract Various metabolic and monogenic diseases have genetic defects in the liver and are often caused by lack or overproduction of a critical gene product in its cells. Conventional therapeutic methods are often insufficient or unavailable to manage genetic diseases in the liver; therefore, more efficient strategies are urgently needed. Gene therapy emerged as novel method of treatment relying on liver-directed transfer of a gene-coding sequence to produce the missing gene products or a nucleic acid sequence to inhibit the production of the gene product in excess. Practically, the success of the nucleic acid-based approaches is dependent on the availability of a method capable of delivering the gene or nucleic acid therapeutics to liver cells. Various methods of gene delivery have been developed, including viral methods and nonviral methods comprising synthetic vectors and physical methods. Hydrodynamic gene delivery is among the most efficient and the most commonly used method for liver-directed gene delivery. Here we will briefly summarize the principles and the progress that have been made in hydrodynamic gene delivery toward clinical applications.

Keywords Liver • Gene therapy • Hydrodynamic gene delivery • Nonviral vectors

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8.1 Introduction

The liver is a vital organ for maintaining metabolic homeostasis and sustaining our lives. Numerous inherited diseases have their origin in this organ, including hemochromatosis, hemophilia A and B, alpha-1 antitrypsin deficiency, and Wilson's disease [1]. The field of hepatology has evolved into rapidly growing medicine, comprising substantial therapeutic tools for management of inherited and acquired liver diseases. While gene therapy has demonstrated great promise for liver disease management, efficient and safe in vivo gene transfer into the liver remains a challenge to overcome. Significant efforts have been made in the past to establish a safe and effective procedure for hepatic gene transfer and for treatment of liver diseases. In fact, the liver is an excellent organ for gene transfer because of its large capacity in gene expression and high accessibility for gene delivery. Hepatic gene delivery has been demonstrated from the portal vein, hepatic vein, hepatic artery, and bile duct using viral or nonviral vectors and physical methods [2–6]. Translation of these earlier successes in small animals and development of a safe and efficient method into clinical use has been the focus of hepatic gene transfer in recent years.

Hydrodynamic gene delivery was developed in 1999 [7, 8], and currently, it represents the most convenient and effective method for gene functional analysis in vivo and for establishment of disease animal models by delivering viral genome into hepatocytes in mouse livers [7–11]. Gene therapy studies employing the hydrodynamics-based procedure have also been reported in various animal species [10, 12]. Taking advantage of the simplicity and efficiency of the procedure, efforts have been made to modify the original procedure of hydrodynamic tail vein injection in rodents to meet the requirements of gene delivery in large animals and humans [13–19]. This chapter summarizes the recent progress toward development of a clinically applicable procedure of hydrodynamic gene delivery.

8.2 Image-Guided Hydrodynamic Gene Delivery to the Liver

8.2.1 Principles of Hydrodynamic Delivery

The original procedure of hydrodynamic gene delivery involves a tail vein injection of plasmid DNA solution in mice in a volume equal to 8–10 % of body weight (BW) over 5–10 s [7–9]. A transient elevation of intravascular pressure in the inferior vena cava (IVC), resulting from the large volume and high speed of injection, induces a transient cardiac congestion, accumulation of injected DNA solution in the IVC, and retrograde flow from the IVC into the hepatic veins and liver sinusoids. The elevated intravascular pressure in sinusoids increases the permeability of the endothelium and induces invagination and disruption of the plasma membrane, allowing DNA entry into the cytoplasm and subsequently to the nucleus [20]. Once the DNA

solution enters the cells, cell membranes reseal and the endothelium regains its original structure [20]. Approximately 40 % of hepatocytes were transfected by a single tail vein injection of less than 50 μg of plasmid DNA per mouse [12].

Applications of hydrodynamic gene delivery for studying liver diseases in mice are summarized in Table 8.1. These include the establishment of therapeutic methods and/or the identification of a genetic component for disease pathology, such as nonalcoholic steatohepatitis [21], viral hepatitis [21, 22], fulminant hepatitis [11, 24], liver injury [11, 25], liver fibrosis [26], rejection after transplantation [11], obesity [11, 27, 28], diabetes [11, 29], and alpha-1 antitrypsin deficiency [12] (Table 8.1). Hydrodynamics-based gene transfer has also been used to establish liver disease models in animals, such as hypertriglyceridemia, viral hepatitis, liver fibrosis, and liver cancer using lipoprotein lipase gene [30], viral genomes [31–33], transforming growth factor-β gene [34], and *c-met* gene [35], respectively (Table 8.2). Li et al. have reported efficient development of multiorgan cancer metastasis models by hydrodynamic injection of B16-F1 melanoma, 4 T1 breast cancer, or Renca renal carcinoma cells into mice [36].

Table 8.1 Summary of the applications of hydrodynamic gene delivery

Disease	Reference
Nonalcoholic steatohepatitis	[21]
Hepatitis	[22, 23]
Fulminant hepatitis	[11, 24]
Liver injury	[11, 25]
Liver fibrosis	[11]
Rejection after the transplantation	[26]
Obesity	[11, 27, 28]
Diabetes	[11, 29]
Alpha-1 antitrypsin deficiency	[12]

Table 8.2 The establishment of the animal disease model using hydrodynamic delivery

Disease model	Genes and cells delivered	Reference
Hypertriglyceridemia	Lipoprotein lipase	[30]
Hepatitis B	Viral genome	[31]
Hepatitis C	Viral genome	[32]
Hepatitis D	Viral genome	[33]
Liver fibrosis	Transforming growth factor-beta1	[34]
Liver cancers	c-met	[35]
Multiorgan metastasis	B16-F1 melanoma cells	[36]
	4 T1 breast cancer cells	[36]
	Renca renal carcinoma cells	[36]

8.2.2 *Image-Guided Hydrodynamic Delivery*

The image-guided catheter insertion technique has been employed to achieve liver-directed and lobe-specific hydrodynamic delivery in large animals [15]. The image-guided hydrodynamic gene delivery is performed in a site-specific manner based on the site of catheter insertion in liver. Kamimura et al. reported a procedure in 2009 in pigs [15], and similar studies were also reported by others [13, 14, 19]. At experimental level, a skin incision is made at the neck to expose the jugular vein and to insert a peripheral i.v. catheter. A guide wire is then inserted through the peripheral catheter and later replaced with a short introducer. The guide wire is inserted into the selected hepatic vein, e.g., the right lateral hepatic vein in the right lateral lobe, under X-ray image guidance. An injection catheter with a balloon on its tip is then inserted through the introducer into the targeted liver lobe following the guide wire. Inflation of the balloon is achieved by injecting a small volume of phase contrast medium to block blood flow. The blockade is verified by injecting a phase contrast medium into the vasculature through the injection catheter (Fig. 8.1a). Hydrodynamic injection is then performed directly into the targeted hepatic lobe (Fig. 8.1a). The efficiency of this procedure was demonstrated in pig livers [15, 17]. Because the injection is localized and specifically to a given area in the liver, the injection volume is significantly reduced from 10 % BW in volume of a mouse to 1.25 % BW in pigs [15, 17]. Target-specific gene delivery was confirmed at the area near the injection site (Fig. 8.1b). Gene delivery efficiency, however, is dependent on the position of the catheter insertion in the selected liver lobe, intravascular pressure, and injection volume [15, 17]. Efforts have been made to optimize these parameters for optimal efficiency and safety of the procedure in gene delivery to pig livers and skeleton muscles [16, 17]. The optimum condition achieved so far is capable of achieving expression of the human alpha-1 antitrypsin gene in livers for more than 2 months after gene delivery [17].

To further extend the clinical applicability of this method, the safety of the procedure was also evaluated in dogs [18]. Histological examination and hepatic microcirculation measurements using reflectance spectrophotometry revealed a transient expansion of the liver sinusoids in the injected area with no change in other areas or organs. Serum biochemistry also showed a transient increase in concentrations of liver enzymes and cytokines related to vascular stretching upon injection. Physiological parameters including electrocardiogram, heart rate, blood pressure, oxygen saturation, and body temperature remained normal during and after hydrodynamic injection, and no changes in BW were observed in dogs that underwent three hydrodynamic injections in 6 weeks at 2-week time intervals. Importantly, no transfer of the injected plasmid was observed in other organs. These results suggest that the image-guided hydrodynamic delivery procedure is site specific, safe, and effective for long-term gene expression.

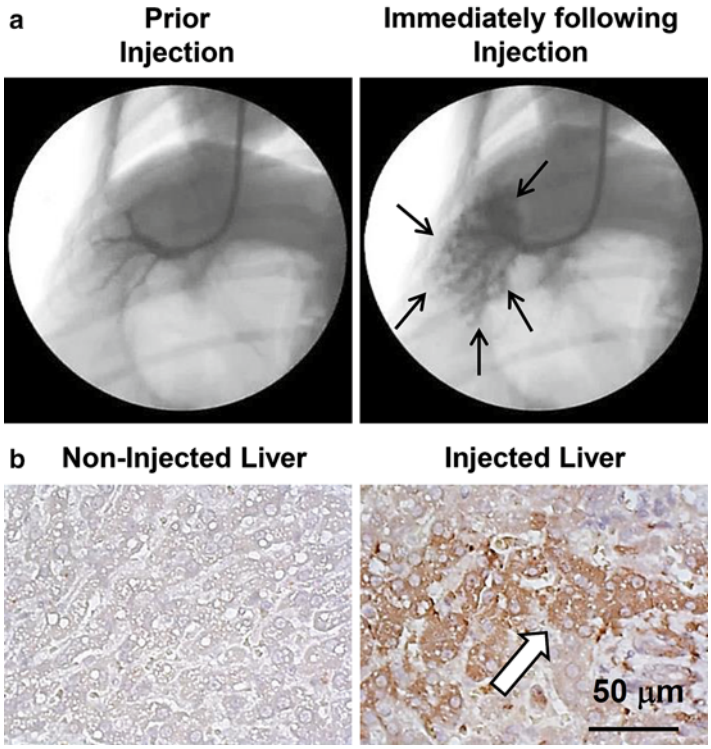

Fig. 8.1 Image-guided, computer-assisted hydrodynamic gene delivery. (**a**) Location of the balloon catheter in the hepatic vein of dog liver. *Black arrows* represent the distribution of the contrast medium in the injected liver lobe. (**b**) Immunohistochemical staining of the liver. Liver samples from the control and plasmid DNA-injected dog liver were stained with anti-luciferase antibodies. Scale bar represents 50 μm. *White arrow* indicates hepatocytes expressing transgene (taken from Kamimura et al. [18] with permission)

8.2.3 Development of Clinically Applicable Injection Device

The first generation of the image-guided hydrodynamic gene delivery system was developed in 2007 by Suda et al. [37] using the pressure of a CO_2 tank to drive the injection. Kamimura et al. have recently reported a different system utilizing an electric power-driven motor as a driving force, instead of pressurized gas [38]. The design of the new system has desirable computerized intravascular pressure profile to guide the injection [38] (Fig. 8.2). Once the injection starts, the electric motor pumps the DNA solution into the target organ and consequently increases the vascular pressure at the tip of inserted catheter. Based on the pressure profile preloaded in the computer, the injection can be self-adjusted based on an algorithm in the computer. The major advantage of the motor-driven system is that it can eliminate the possibility of gas embolism that could occur when a CO_2 tank is used to drive

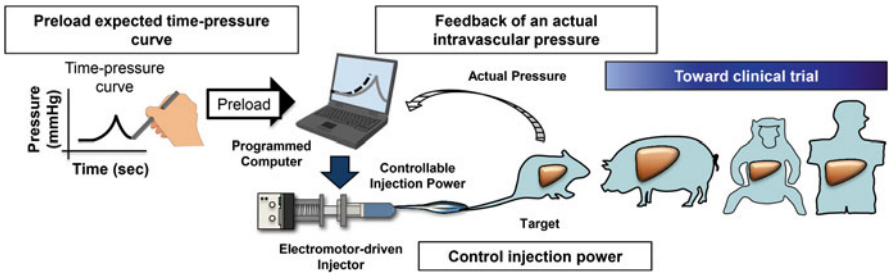

Fig. 8.2 Computer-assisted hydrodynamic gene delivery system (modified from Yokoo et al. [38] with permission). (1) Various time–pressure curves are preloaded to a computer. (2) The electric power-driven injector starts hydrodynamic injection to animals. (3) The actual intravascular pressure in the target organ is transmitted to the computer instantly. (4) Computer self-adjusts motor-driven injection to match the preloaded pressure profiles. Cartoon animals represent the animal species that hydrodynamics-based procedure has been successfully performed toward clinical trial in humans

the injection. Current efforts are to evaluate the efficacy of the new system for treatment of a various diseases, in an animal model [39], and to develop a computer program with optimized injection parameters for a safe and efficient gene delivery under any given defined condition. Clinical trials would be the next logic step bridging the image-guided, computer-assisted hydrodynamic gene delivery to clinical application on human patients.

8.3 Conclusion

Hydrodynamic delivery was originally established for gene therapy studies in mice. It has advanced in the past 15 years to a stage where this technique can be employed to achieve site-specific, safe, and effective gene delivery in combination with other technologies such as imaging, intravascular catheterization, and computer-assisted regulation. It is highly possible that this new technology will proceed beyond nucleic acid delivery into delivery of other pharmaceutics for treatment of diseases that conventional therapeutic methods are insufficient or unavailable.

Conflict of Interest The authors declare no conflict of interest.

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Part III
Legal Regulation

Chapter 9
The Way to Clinical Application of Human Pluripotent Stem Cells

Takashi Aoi

Abstract Human embryonic stem (ES) cells are expected to be a resource for regenerative medicine because of their pluripotency and ability to self-renew. In 2007, human-induced pluripotent stem (iPS) cells were generated from somatic cells by introducing small sets of transcription factors, raising further hopes of clinical applications of pluripotent stem cells. Today, several promising projects using these cells have been launched.

It is necessary to consider regulatory issues regarding iPS/ES cell applications from at least three points of view. First, researchers should pay serious attention to the rights of donors, particularly privacy protection and decision-making in terms of the use of pluripotent stem cells derived from "the pieces of their own body." Second, for iPS/ES cell-based transplantation therapy, patients with target diseases should be protected from the risks of undergoing and not undergoing such treatments. Third, the dignity of life should be carefully considered.

In Japan, there used to be two regulatory frameworks for the clinical application of pluripotent stem cells with respect to clinical studies: the provisions of the Pharmaceutical Affairs Law and Medical Practitioners' Act. Recently, relevant regulatory reforms were made to promote and more rationally regulate the field of regenerative medicine.

In order to expedite the realization of clinical applications of pluripotent stem cells, researchers must follow established guidelines as well as precisely grasp the current trends in corresponding regulations and technologies.

Keywords Pluripotent stem cell • Induced pluripotent stem cell (iPS cell) • Embryonic stem cell (ES cell) • Regenerative medicine • Regulation

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9.1 Pluripotent Stem Cells

Currently, there are two types of human pluripotent stem cells in vitro. The first is embryonic stem (ES) cells derived from fertilized eggs, first generated by Dr. James Thomson in the United States in 1998 [1]. The second is induced pluripotent stem (iPS) cells derived from somatic cells, first generated by Dr. Shinya Yamanaka in Japan in 2007 [2].

ES cells and iPS cells have two common important properties, including the ability of indefinite self-renewal and the ability to differentiate into various types of cells, namely, pluripotency. In addition, iPS cells can be generated from the somatic cells of various individuals with particular characteristics, such as blood and HLA types, whereas ES cells are generated from early embryos and the genetic characteristics of the embryos are unclear.

Based on these properties, the clinical application of ES and iPS cells in the field of regenerative medicine is greatly expected.

9.2 Acceleration of iPS/ES Cell-Based Regenerative Medicine

In order to meet people's expectation for regenerative medicine, the Japanese prime minister made an announcement regarding the availability of significant monetary support for this field: 110 billion yen over 10 years. The government has supported large regenerative medicine-related national projects, such as the "Highway Program for Realization of Regenerative Medicine." [3] Furthermore, a new law regulating regenerative medicine, the "Act for the Comprehensive Acceleration of Policies Promptly to Give the People Regenerative Medicine Free from Danger," was promulgated and enforced in May 2013 [4].

9.3 Promising Projects for iPS/ES Cell-Based Regenerative Medicine

In these following windows, several promising projects have already been launched. For example, Dr. Masayo Takahashi at RIKEN CDB is currently attempting iPS cell-derived retinal pigmented epithelium (RPE) transplantation in patients with age-related macular degeneration [5]. The first transplantation was performed in September 2013 [6]. Furthermore, Professor Jun Takahashi is the front-runner of iPS cell-based therapy for Parkinson's disease and has established technologies for inducing and isolating dopaminergic neurons from human iPS cells. The transplantation of iPS cell-derived dopaminergic neurons into the midbrain in Parkinson's disease model primates has resulted in good engraftment and a significant

improvement in motor behavior, without tumor formation [7]. In terms of spinal cord injury, Professor Hideyuki Okano and Dr. Masaya Nakamura at Keio University have shown significant motor-functional recovery following iPSC-derived neural stem cell transplantation in primate spinal cord injury models [8]. Moreover, there are other promising projects using pluripotent stem cells, such as the applications of iPS cell-derived platelets by Professor Eto at Kyoto University [9], iPS cell-derived cardiac muscles by Professor Sawa at Osaka University [10] and Professor Fukuda at Keio University [11], iPS cell-derived corneal cells by Professor Nishida at Osaka University [12] and Dr. Shimmura at Keio University [13], iPS cell-derived liver organ buds by Professor Taniguch at Yokohama City University [14], and ES cell-derived hepatocytes by Dr. Umezawa at the National Center for Child Health and Development [15], and so on.

9.4 Concerns About the Clinical Application of Human Pluripotent Stem Cells

In contrast, there are quite a few concerns about the clinical application of human pluripotent stem cells. These concerns can be sorted into three categories.

First, it is necessary to protect the donors' rights of privacy protection and decision-making about the use of derivatives of "the pieces of their own body." Technological aspects of information control as well as the ethical and legal aspects of proprietary rights and the right to withdraw should be considered. Second, patients should be protected from the risks of undergoing and not undergoing these treatments. This issue should be mainly discussed from the view of manufacturing, qualification, and clinical practices. The third concern is the "dignity of life," primarily associated with the use of technology for differentiating germ cells from human pluripotent stem cells. In particular, there is concern regarding the ability to conceive babies via the fertilization of iPS cell-derived gametes. Recent progress in research in this field has made this concern more of a reality.

Some of these issues are mainly ethical issues, while others are discussed as regulatory issues. Although ethics and regulation are mutually related, we herein focus on regulations concerning the clinical application of human pluripotent stem cells (iPS/ES cells).

9.5 Past Regulatory Frameworks for Tissue/Cell-Based Therapy in Japan

There were two tracks for cell/tissue therapies in Japan. One was therapy using commercial products developed by companies and approved for manufacturing and marketing through clinical trials according to the Pharmaceutical Affairs Law (PAL) and relevant guidelines. The second was stem cell-based clinical research evaluated

by relevant committees and the Health Science Council within the Ministry of Health, Labour, and Welfare (MHLW) based on the Medical Practitioners' Act and relevant guidelines and regarded as advanced medical treatments in terms of medical technology. The double-track system in Japan is unique among advanced countries, although relatively similar to the "Hospital Exemption" system in the EU and the "Noncommercial IND" system in the United States.

Relevant guidelines on the PAL track focusing on iPS/ES cells came in force in September 2012: "Guidelines on Ensuring the Safety and Quality of Products Derived from Engineered Human Autologous iPS-Like Cells" [16], "Guidelines on Ensuring the Safety and Quality of Products Derived from Engineered Human Allogenic iPS-Like Cells" [17], and "Guidelines on Ensuring the Safety and Quality of Products Derived from Engineered Human ES Cells" [18], together with "Guidelines on Ensuring the Safety and Quality of Products Derived from Engineered Human Autologous Somatic Stem Cells [19]" and "Guidelines on Ensuring the Safety and Quality of Products Derived from Engineered Human Allogenic Somatic Stem Cells [20]."

"Guidelines on Clinical Research Using Human Stem Cells" (MHLW Notification No.425, 2006) relevant to another track were also revised focusing on pluripotent stem cells. These guidelines were amended in full in 2010 (MHLW Notification No.380, 2010) in order to clearly refer to iPS and ES cell-based therapies. In 2013, these guidelines were further amended in full (MHLW Notification No.317, 2013 [21]) to make it possible to conduct research on the establishment and distribution of iPS cells without specified target diseases. The first iPS cell-based therapy developed worldwide by Dr. Masayo Takahashi was conducted according to this track.

9.6 Issues in the Past Regulatory Framework in Japan

Although the regulatory amendment mentioned above accelerated the appropriate development of regenerative medicine, there continue to exist several issues.

Indeed, there used to be another track for regenerative medicine in Japan. This third track is called "free medical care." In this track, medical activities, including regenerative medicine, were entrusted only to the "discretion" of the physician in the hospital under the Medical Practitioners' Act. "Free medical care" is not covered by public health insurance. Regarding the third track, it is not possible to grasp the actual state of regenerative medicine using this track, i.e., which medical treatments are performed in how many cases and for what diseases. In addition, the safety and efficacy of medical activities provided via this track have not been assured or verified. It was recently reported by the press that one patient who underwent "regenerative medicine" on the third track at a clinic in Kyoto died. It remains unclear whether the medical practices at that clinic were significantly riskier than those performed at clinics and hospitals on the first and second tracks. Regardless, the news evoked public opinion that all types of regenerative medicine should be regulated to protect patients against inappropriate medical practices.

Another issue was the discrepancy between the regulations and current status of research and development of regenerative medicine, especially that using pluripotent stem cells in the non-PAL track. The second track was based on the Medical Practitioners' Act, and relevant guidelines supposed that all processes of regenerative medicine, including tissue collection from donors and cell/tissue processing and administration, are completed at medical institutes. When the method of cell/tissue processing was simple, the regulations fit the practice. However iPS/ES cell-based therapy requires a significant amount of time in addition to labor-intensive processes that are difficult to complete within medical institutes. Therefore, contracts regarding the processes used at processing industries may make these methods more efficient and secure.

There existed several issues in terms of the PAL track as well. The conventional PAL method included two categories, pharmaceuticals and medical devices, as these modalities have different properties and the appropriate regulations for each category differ. Furthermore, products for regenerative medicine in both categories are different from various points of view. Therefore, the same regulations for the existing two categories could not be applied precisely to regenerative medicine products.

9.7 Recent Regulatory Reform of Regenerative Medicine in Japan

Against this background, regulation related to the field of regenerative medicine was revised in November 2014, including new legislation the "Act on the Safety of Regenerative Medicine [22]" and a revision of the PAL legislation.

There are two main purposes of the "Act on the Safety of Regenerative Medicine." The first is to secure the safety of regenerative treatments, including iPS/ES cell-based therapies. This legislation covers all medical technologies using processed cells that are not approved by PAL and includes both previous clinical research under the "Guidelines on Clinical Research using Human Stem Cells" and "free medical care." Under this new act, all iPS/ES cell-based therapies must be approved by a certified committee organized in accordance with the requirements of the legislation.

The second purpose of the "Act on the Safety of Regenerative Medicine" is to promote collaboration between medical institutions and industry from the early stage of development. This new act enables researchers at medical institutes to commission cell-processing methods to licensed industries, i.e., cell processors. If a cell-processing technique is commissioned to a cell-processing center (CPC) outside the medical institute, a license or accreditation by the MHLW is required.

Another pillar of regulatory reform is the PAL revision promulgated in November 2014. The official name of the revised PAL is the "Act on Pharmaceuticals and Medical Devices [23]" (PMD act). The PMD act has an independent chapter for

regenerative medical products as the third category following pharmaceuticals and medical devices. In the PMD act, "regenerative medical products" are defined as processed human cells intended to be used for (I) either (1) the reconstruction, repair, or formation of structures or functions of the human body or (2) the treatment or prevention of human diseases and/or (II) gene therapy.

Furthermore, the PMD act provides an expedited approval system for regenerative medical products to enable the timely provision of safe regenerative medicines to patients, even those based on limited data. In the expedited approval system, the product may be given conditional, time-limited marketing authorization after its safety is confirmed and the results predict likely efficacy.

9.8 Other Activities of Japanese Regulatory Authorities to Facilitate Regenerative Medicine Using Pluripotent Stem Cells

In October 2014, the "Good Cell and Tissue Practice (GCTP)" was issued (MHLW Ministerial Ordinance No.93 [24]). The GCTP indicates quality system requirements for regenerative medical technologies/products considering the characteristics of these products, such as raw materials that cannot be sterilized. The content of the GCTP includes the categories of quality risk management; manufacturing control; quality control, including verification, validation, and quality reviews; and facility requirements. The GCTP is commonly applied for cell processing under both the PMD act and the "Act on the Safety of Regenerative Medicine."

Since July 2011, the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) has provided services of "Pharmaceutical Affairs Consultation on Research and Development (R&D) Strategies" [25], mainly for universities, research institutions, and venture companies that possess promising "seed-stage" research or technologies. As of the end of 2014, 68 introductory consultations, 316 pre-consultations, and 65 face-to-face consultations on regenerative medical products have taken place. Introductory consultations and pre-consultations are free of charge. Face-to-face consultations cost \874,000 or \1,541,600 with a discount of 90 % for academic institutions and venture companies that meet certain requirements.

In May 2012, the PMDA established the Science Board, consisting of external experts, to respond to the rapid promotion of medical innovations and properly address scientific challenges in the field of advanced science and technology. Regarding regenerative medical products, the board issued a statement of "Current Perspectives on the Evaluation of Tumorigenicity of Cellular and Tissue-Based Products Derived from Induced Pluripotent Stem Cells [26]" in August 2013. Since then, the board has been discussing issues related to the manufacturing and quality of cellular products during early development at cell-processing centers.

9.9 International Harmonization

Over the past several decades, international harmonization has been considered to be important in pharmaceutical affairs. Regarding regenerative medicine, including pluripotent stem cell-based products, it has been attempted to build an international consensus on good practices.

One attempt at international harmonization in this area is the implementation of the "International Stem Cell Banking Initiative" (ISCBI). The ISCBI is a working group formed under the International Stem Cell Forum (ISCF) [27]. The ISCF is made up of 21 funders, including Japan, of stem cell research from around the world. It was founded in January 2003 to encourage international collaboration and the provision of funding support for stem cell research, with the overall aim of promoting global good practices and accelerating progress in this vitally important area of biomedical science. The ISCBI published a consensus document on the principles of best practices for centers supplying human embryonic stem cell lines for research purposes [28]. In addition the ISCBI recently released a paper on the use of human pluripotent stem cell banking for clinical purposes entitled, "Points to consider in the development of seed stocks of pluripotent stem cells for clinical applications [29]." This document is not legally binding but useful for developing best practices for quality control of clinical-grade pluripotent stem cells. Therefore, it is efficacious to refer to this publication when trying to develop pluripotent stem cell-based therapy.

In addition to the ISCBI, it is necessary to watch for trends in the activity of the International Alliance for Biological Standardization (IABS) [30]. The IABS is an independent, nonprofit scientific alliance consisting of 250 members in over 50 countries. Since 1955, this organization has been devoted to the scientific and medical advancement of biological agents by facilitating communication among those who develop, produce, and regulate biological products for human and animal health. In the IABS, the Cell and Gene Therapy Committee (CGTC) was first established in 2013 in order to identify issues in the field that could benefit from discussions at conferences with the objective of reaching a consensus on specific follow-up steps that should be taken, and a Japanese scientist, Dr. Takao Hayakawa, was appointed to the chair of the CGTC. The first meeting of the CGTC on "Challenges Toward Sound Scientific Regulation of Cell Therapy Products" was held in Kyoto on March 2014 and the second on "International Regulatory Endeavor Towards Sound Development of Human Cell Therapy Products" was held in Tokyo in February 2015. At both meetings, issues on the clinical application of pluripotent stem cells were discussed as the main agenda.

9.10 Conclusion

Comprehensive measures to facilitate the clinical application of pluripotent stem cells have been taken in Japan as well as other countries worldwide. In order to expedite the realization of such applications, it is necessary to not only follow

established guidelines but also precisely grasp the recent trends in corresponding regulation and technologies as well as have ongoing discussions among various relevant scientists working in research, development, production, standardization, and regulation. Adopting a long-term and broad-ranging perspective is the best way to promote the clinical application of pluripotent stem cells by allowing researchers to obtain sound common sense on these issues and take stock of their own knowledge and experience.

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Chapter 10
Regulation for Gene and Cell Therapy Medicinal Products in Europe

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Abstract An important step in the regulation of cell and gene therapy medicinal products, which are classified as advanced therapy medicinal products (ATMPs) in the European Union, has been made with Regulation 1394/2007/EC. By this regulation a new committee, the Committee for Advanced Therapies, has been established to ensure appropriate coverage of scientific and regulatory aspects of ATMPs. In addition, novel regulatory tools specific for ATMPs such as the classification, certification, and hospital exemption were introduced to support the development of this product class. By nature, ATMPs are a special class of medicinal products with characteristics different to conventional drugs and even other biologicals. Hence, regulatory requirements for manufacturing and clinical evaluation need to be tailored to the type and design of the ATMP, as well as to its manufacturing process and clinical indication. This chapter summarizes the general regulatory pathway for ATMPs as well as the currently applicable ATMP-specific procedures. In addition, the regulatory requirements regarding manufacturing, quality, and nonclinical and clinical testing for cell and gene therapy medicinal products are discussed. Important aspects of the environmental risk assessment, a provision for ATMPs containing genetically modified organisms, are also reviewed.

Keywords Advanced therapy medicinal products • Cell therapy • Gene therapy • Regulatory framework • EU • Clinical trials

10.1 Introduction

The current legislative framework for gene and cell therapy medicinal products was established by Regulation 1394/2007/EC [1] and Directive 2009/120/EC [2] (amending Annex I, Part IV of Directive 2001/83/EC), to harmonize regulation of

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these products in Member States of the European Union. Gene therapy medicinal products (GTMPs), somatic cell therapy medicinal products (sCTMPs), and tissue-engineered medicinal products (TEPs) were legally defined, grouped as "advanced therapy medicinal products" (ATMPs) and subjected to a common and specific regulatory approach as outlined in this chapter. With Directive 2001/83/EC [3] still being valid, the same regulatory principles as for other biotechnological products apply to ATMP but are further adapted and refined by special additional provisions for authorization, supervision, and pharmacovigilance.

To adequately address the specificities of ATMP and to ensure an appropriate evaluation of these products, a specific and dedicated body, the Committee for Advanced Therapies (CAT), was established in 2009. CAT is composed of representatives from all EU Member States, five members from the Committee for Medicinal Products for Human Use (CHMP), and two members each representing clinicians' and patients' associations (1). CAT plays a central role in most regulatory procedures dealing with ATMPs, i.e. classification, certification, contribution in providing scientific advice to applicants, and, most importantly, evaluation of applications for marketing authorization. Since knowledge and feasibilities in the ATMP field are rapidly increasing, Regulation 1394/2007/EC already directed a review, after gaining some experience, of the effect and impact of the regulations on the field of ATMP development in order to modify or adapt as needed. This review has recently been published by the European Commission and identified several areas where an amendment of the regulation may be considered [4]. A specific proposal to further develop Regulation 1394/2007 is expected to be presented by the European Commission in 2015.

10.2 Procedures for ATMPs Within the European Regulatory Pathway

10.2.1 Definitions and Classification

GTMPs contain a recombinant nucleic acid, which is administered to human beings aiming at regulating, repairing, replacing, adding, or deleting genetic information. Moreover, it is essential that the intended therapeutic, prophylactic, or diagnostic effect is directly related to the recombinant nucleic acid or the product of its expression [2]. For example, reprogramming a differentiated cell to a pluripotent stem cell by genetic modification alone may not be sufficient to classify the resulting product as GTMP, as the genetic modification is not necessarily contributing to the therapeutic effect. It is important to note that with the amendment of Directive 2001/83/EC by Directive 2009/120/EC, vaccines against infectious diseases, irrespective whether they are used in a prophylactic or therapeutic setting, are not considered as GTMPs. Thus, for example, a particular active substance could be either vaccine or GTMP depending on whether it is used to treat a virus infection causing cancer or cancer caused by the virus infection.

sCTMPs and TEPs have in common that they contain "engineered" cells (which may be viable or non-viable). In this context engineered means that the cells are substantially manipulated or are not intended to be used for the same essential function in the recipient as in the donor. The latter is commonly referred to as "non-homologous" use. However, sCTMPs and TEPs differ in the purpose of administration. TEPs are administered for regenerating, repairing, or replacing a human tissue. sCTMPs are used for treating, preventing, or diagnosing a disease through the pharmacological, immunological, or metabolic action of the cells. In addition, Regulation 1394/2007/EC defines combined ATMPs as products integrating a medical device into an ATMP that contains viable cells or cells being nonviable but being associated with the primary action of the medicinal product in the human body [1].

Apparently, for classification it is crucial to evaluate whether cells are substantially manipulated or used in a nonhomologous way. To further explain the meaning of these terms and the approach for classification, a "Reflection paper on classification of advanced therapy medicinal products" is being developed as a guidance document [5]. However, in specific cases it still may be rather challenging to unanimously classify a product, especially in case of autologous cells/tissues taken from the patient, prepared in a more or less substantial process and used for not exactly the same purpose as before. Therefore, Regulation 1394/2007/EC in Article 17 implemented a procedure, by which applicants can request from the European Medicines Agency (EMA)/CAT a scientific recommendation whether or not a specific product may be classified as ATMP. Since implementation of the procedure in 2009, more than 120 requests for classification have been submitted to the CAT. Only when classified as ATMP, the CAT is involved in the evaluation process for marketing authorization of the product.

10.2.2 Certification

The certification procedure was implemented as an incentive to support the development of ATMPs by small- and medium-sized enterprises (SME). Since obtaining a European marketing authorization for an ATMP may be a time-consuming and strenuous effort for an SME, certification is intended to provide an intermediate milestone to be achieved. The SME may submit to the EMA/CAT available quality and nonclinical data for scientific evaluation. If the data are in line with the requirements as set out in Directive 2001/83/EC [3], a certificate will be issued stating compliance of data with requirements. For such a certificate it is not mandatory to submit a full quality or nonclinical dossier; a certificate may be achieved also for parts of the data with additional data still needed to be generated. Detailed guidance on the certification procedure and the minimum data set necessary to apply for certification has been provided by EMA [6, 7].

Application for and granting of a certificate was intended to support SMEs in entering into a first dialogue with the regulatory agency and to increase the company's value for potential investors. However, so far only a few certifications have

been applied for. In view of the stakeholders the main disadvantages related to this procedure are the absence of a link between certification and marketing authorization and the exclusion of academia and research groups from certification [8].

10.2.3 Scientific Advice at European and National Level

As for other medicinal products there are several procedures available for applicants to obtain feedback and support from regulatory authorities when challenges or questions arise during the developmental phase of an ATMP or when preparing for Marketing Authorization Application (MAA). Later in development and especially when crucial decisions have to be taken in view of the intended MAA, the EMA scientific advice procedure comes with the advantage of giving the applicant a harmonized view, by regulators throughout the EU, on the specific issues addressed. However, since science and state of the art may develop and progress, the advice given cannot be binding with respect to future evaluations and decisions in the context of an MAA. During early stages of development, advice is mostly sought from national regulatory authorities. This approach may be triggered by various aspects including the national responsibility for the approval of clinical trials and the lower costs and workload associated with a national scientific advice procedure. The latter is an important aspect in view of the fact that ATMPs are quite often developed by academic institutions or SMEs with only limited financial and regulatory capacities. To effectively support applicants in the development of ATMPs, the national advice procedures should be not too formal or associated with an excessive workload but flexible and easy to handle. In Germany, for example, an innovation office was established to serve this purpose for ATMPs.

10.2.4 Marketing Authorization

The centralized procedure for obtaining a marketing authorization for a medicinal product was initially established to ensure a harmonized evaluation and an EU-wide availability of innovative products. In contrast to multifold national applications and authorizations, the centralized procedure is led by the EMA and includes a collaborative evaluation of an MAA by all Member State authorities, resulting in an opinion of the CHMP. Finally the European Commission grants a marketing authorization which is valid throughout all Member States of the EU. This procedure was implemented by Regulation 726/2004/EC [9]. It applies to ATMPs as modified by Regulation 1394/2007/EC, which details requirements for and the procedure of an MAA [1]. While the CHMP is still responsible for issuing the final opinion for granting a marketing authorization, assessment of the application and presentation of the draft opinion are performed by the CAT, based on its ATMP-specific expertise. Accordingly, two CAT members are assigned as the Rapporteur and Co-rapporteur

for the evaluation of the dossier by their own assessment teams. Very recently, a pilot study was initiated by setting up multinational assessment teams with the view of broadening the involvement of EU Member States and to foster inclusion of the best available expertise for evaluation of an ATMP. Beside the central role of the CAT in the assessment of the application, the authorization procedure is quite similar to that for other medicinal products. The Rapporteur and Co-rapporteur perform independent assessments of the dossier, which is presented according to the Common Technical Document (CTD) format, and prepare assessment reports including questions addressing deficiencies and open issues of the application. After discussion between the expert committees and consolidation, a list of questions is issued by CAT and CHMP and forwarded to the applicant in order to resolve the issues raised. The procedure is restarted when the applicant's response document is submitted. The response is jointly evaluated by the Rapporteur and Co-rapporteur and outstanding issues which need to be further addressed and resolved are identified, leading to another cycle of question, response, and evaluation. If the applicant cannot resolve all issues, an oral explanation meeting provides the possibility to directly address and discuss all open questions before the final considerations by CAT/CHMP and the adoption of an opinion. The opinion is drafted by the CAT and forwarded to the CHMP for further discussion and adoption. Marketing authorization finally will be issued by the European Commission. Specific issues and requirements for the scientific evaluation of an MAA for ATMPs are addressed in Sect. 10.3.

10.2.5 Clinical Trials and "Hospital Exemption"

There are several regulatory procedures for ATMPs, which are not in the remit of the CAT but fall under the responsibility of national competent authorities (NCA).

One example is the approval of clinical trials which is performed by each individual Member State, in which the clinical trial is proposed to take place. However, by issuing the Clinical Trials Directive 2001/20/EC [10], the European Parliament and the Council have set the basic rules and procedures for application of a clinical trial and its assessment. As with all Directives, these provisions are not directly legally binding in the Member States but need to be implemented in the national legislation. In Germany, the EU Directive was transposed in the sixth chapter of the German Medicinal Products Act (Arzneimittelgesetz, AMG) [11] and further detailed in the subordinated "Ordinance on the implementation of Good Clinical Practice in the conduct of clinical trials on medicinal products for use in humans" [12].

The national implementation allows some modification and further refinement of the basic principles, and thus some national particularities in the procedure for approval of clinical trials still remain. To facilitate the conduct of multinational trials, a voluntary harmonization procedure (VHP) was implemented by the clinical trial facilitation group (CTFG). This procedure includes a harmonized evaluation of the application by the involved Member States as a first step, followed by the

national implementation of the collective decision as a second step. Since its implementation in 2011, numerous VHP procedures for ATMPs were conducted, which confirmed this approach as a valid tool for facilitating the conduct of multinational trials.

Very recently, in April 2014 a new regulation for clinical trials, Regulation 536/2014/EC, has been adopted and will come into force after a transitional period, but not before May 2016 [13]. This new regulation is, by definition, immediately binding for all EU Member States and does not need to be implemented into national law. The new procedure aims at a continuous facilitation of the conduct of clinical trials through an appropriate and fast evaluation of clinical trial applications by avoiding parallel or separate evaluations in the EU Member States.

A second example for national regulatory procedures for ATMPs is based on Article 28 of Regulation 1394/2007/EC that makes a special provision for a "national" approach to ATMPs, usually called "hospital exemption" (HE) [1]. The term itself may be misleading since it is not restricted to hospitals. Instead, it addresses ATMPs, which are manufactured on a nonroutine basis for an individual patient following an individual medical prescription in a given Member State. It was recognized that for ATMPs with a patient-centered and local perspective, a centralized authorization procedure via CAT and CHMP would not be adequate and may even hinder the availability of new innovative therapies. Thus, such ATMPs are subjected to the national responsibility of the Member States. For implementation of Article 28 at the national level, different approaches were followed. While GMP-compliant manufacturing and the exclusive clinical use of the ATMP in the Member State in which it is manufactured are common principles, the requirements and procedure for granting the HE may differ between the Member States. For example, in Germany a dedicated approval procedure was established which is essentially based on evaluation of the benefit-risk ratio of the product. Compared to a centralized MAA, however, it is taken into account that limited clinical data may be available and thus a higher uncertainty regarding the benefit-risk ratio may be acceptable for granting the HE. It was argued that this procedure might be used by applicants to circumvent the challenges associated with obtaining a centralized marketing authorization. However, this procedure is limited to specific, legally defined ATMPs with a more individual and local perspective and restrictions with respect to commercialization of the medicinal product. Thus, the HE may be more appropriate for these ATMPs, while for others a centralized marketing authorization is more adequate. In line with this, the HE should be regarded as complementing rather than competing with the centralized marketing authorization.

10.3 Regulatory Considerations Regarding Quality, Nonclinical and Clinical Testing

As with all "biologicals", the saying "the product is the process" is highly relevant for ATMPs, indicating that the quality, safety, and efficacy of the therapy strongly relies on a suitable, well-controlled, and consistent manufacturing process of the

ATMP. Data demonstrating or indicating the safety and efficacy of the medicinal product have to be presented to the competent authority when applying for marketing authorization or approval of a clinical trial. These data are derived from nonclinical and clinical studies, in case clinical trials have already been performed. Their extent depends on the stage of the clinical development, the medical need, and the benefit-risk assessment of the product. In view of the specificities of ATMPs, Directive 2009/120/EC introduced the concept of the risk-based approach to help to determine the extent of quality, nonclinical, and clinical data required for an MAA and to justify deviations from the regulatory requirements [2]. The principle of the risk-based approach is based on the identification of risks associated with the clinical use of ATMPs and of risk factors contributing to these risks. By mapping of the individual risk factor-risk combination, a risk profile could be assigned. Detailed guidance how the methodology of the risk-based approach can be utilized has been published recently [14].

10.3.1 Procurement of Tissues and Cells and Traceability

Some characteristics of ATMPs require specific considerations when an ATMP is evaluated in a clinical setting. Therefore, the European Commission prepared detailed guidance on good clinical practice (GCP) specific to ATMPs [15], which is supplementing the detailed GCP guidelines set out in Commission Directive 2005/28/EC [16]. Key aspects are the procurement of tissues and cells, their full traceability, and the requirements for long-term follow-up on safety and efficacy. This latter point is discussed in subchapter Sect. 10.3.4.2.

If an ATMP contains human tissue or cells, the risk of transmitting an infectious disease from the donor to the recipient of the ATMP has to be minimized by careful donor selection and testing of each donation. Therefore, Regulation 1394/2007/EC [1] defines that donation, procurement, and testing of human tissues or cells need to be performed in accordance with the tissue and cell legislation including Directive 2004/23/EC [17], the implementing Directive 2006/17/EC [18], and Directive 2012/39/EU [19] that amends Directive 2006/17/EC with regard to human T-lymphotropic virus type I (HTLV-I) antibody testing. While these directives apply to human tissues and cells including hematopoietic peripheral blood, umbilical-cord and bone-marrow stem cells, reproductive cells, fetal tissues and cells, and embryonic and adult stem cells, blood and blood products are regulated separately in Directive 2002/98/EC [20].

Each ATMP containing biological material from a donor should be allocable from the donor to the individual subject who received the medicinal product and vice versa. This requires that coding systems are introduced that allow anonymized traceability of the ATMP from procurement of the tissue or cells, manufacturing, testing, and storage to distribution and eventually administration to the recipient or disposal. Thereby, the traceability system in place should be complementary to, and compatible with, the requirements laid down in Directive 2004/23/EC [17] and

Directive 2002/98/EC [20] for tissue and blood establishments. To guarantee traceability the following bidirectional links need to be established: a link between the donor source and the donation at the tissue establishment, a link between the donation and the product at the manufacturing site, and a link between the ATMP and the treated individual at the investigator site. All traceability data need to be archived for a minimum of 30 years after the expiry date of the product [21].

10.3.2 Quality and Manufacturing Aspects of ATMPs

The development of the manufacturing process for an ATMP is preceded by accurate planning. Clarity should exist concerning the identity of the product, its mechanism of action, and possible product- or process-related impurities. Based on this knowledge, a manufacturing process is designed, which allows controlled production of a safe and potent medicinal product. During process development, critical process parameters and optimal production conditions are identified, and suitable in-process controls are implemented. Thus, the resulting process should enable manufacturing of an ATMP with high reproducibility, although it is accepted that the degree of consistency is often less than for non-ATMP products due to variations in starting material (e.g., differences between material from different autologous or allogeneic human donors) or due to the complexity of the manufacturing process. As for all medicinal products applied in the EU, the manufacture of ATMPs for clinical evaluation should be in compliance with the principles of good manufacturing practice (GMP).

Starting materials of ATMPs are often inhomogeneous; they often comprise cells from different donors and thus may exhibit different characteristics. Moreover, for GTMPs, it is noteworthy that the material used for production of gene transfer vectors including plasmids and bacterial or eukaryotic cells are defined as starting materials, irrespective of whether they are applied in vivo or used for modification of cells ex vivo [2]. All materials used for production, which comprise all raw materials (in particular if they are derived from human or animal sources), starting materials, and excipients, have to be very carefully qualified to assure a consistent manufacturing process. Most important is the exclusion or minimizing of the presence of adventitious agents such as viruses, bacteria, mycoplasma, fungi, or transmissible spongiform encephalopathies, since in most cases sterilizing or sterile filtration of the final ATMP is not feasible. Besides microbiological safety, the biological activity of the starting materials and of some raw materials is crucial. For example, biological substances such as growth factors are often required for the production of ATMPs and may have a major influence on the potency of the final product.

Characterization of the product includes relevant parameters determining product identity, purity, safety, and potency. In case of cells being the active substance, definition of all cell fractions, contributing to the desired function or regarded as impurity, should be performed. Specific attention should be given to the potential

tumorigenicity of the cells and, if genetically modified, also of the gene therapy vector used for introducing the modifications. The risk of insertional oncogenesis of integrating vectors became apparent in a number of clinical trials using retroviral vectors [22] and may also have to be considered when integrating vectors are used for the generation of induced pluripotent stem cells intended for clinical application.

As for all kind of medicinal products, critical parameters determining identity, safety, purity, and potency identified by characterization studies are included into the specification of the product. Suitable limits should be set based on analysis of a reasonable number of batches produced. The large diversity of ATMPs and their complex manufacturing processes do not allow detailed guidance on release criteria in this chapter. Thus, only a few specific aspects will be highlighted.

A common issue for many ATMPs is the determination of potency. If surrogate markers for potency have to be used, a correlation to the biological activity should be demonstrated, preferably based on clinical data. Furthermore, for some ATMPs quality controls may be performed only to a limited extent and/or completed after the release of the product. For example, genetically modified autologous hematopoietic stem cells often cannot be cryopreserved and need to be applied shortly after manufacturing. Such a limited shelf life may hamper the performance of lengthy quality control/release tests. The number of the cells in the final product may also be a limiting factor. If the cell number at the end of manufacturing is close to the minimal amount of cells required for hematopoietic reconstitution, there may be not enough cells available to perform the entire release testing. In such cases, a thorough scientific justification, supported by meaningful characterization data, has to be provided for the omission of some analyses or for obtaining the test results after administration of the medicinal product only.

Due to the sensitivity of cells, adequately controlled cell storage and shipping conditions need to be established to ensure cell viability, sterility, and function. Finally, adequate stability studies are needed to define the shelf life of products containing viable cells.

10.3.3 Nonclinical Studies for ATMPs

Nonclinical studies in the development of an ATMP are conducted to support the initiation of human clinical trials, i.e., to evaluate the proposed beneficial effects of the ATMP in a specific indication and to obtain an adequate risk assessment for its administration to humans. In this respect a primary objective of nonclinical studies is the demonstration of proof of principle for the ATMP. Furthermore, the determination of pharmacological and toxicological effects that may be predictive for effects in the human body, the establishment of a safe starting dose for starting clinical evaluation, and the support for the intended route of administration of the ATMP are key aspects of the nonclinical testing program. The studies should be performed in relevant animal models, meaning the animal models used should

display comparable characteristics as humans in terms of their physical, mechanical, chemical, and biological properties. In addition, the potential and limitations of the distinct model should be determined. On this basis, the nonclinical studies should identify target organs for toxicity and define the parameters to be monitored in the patients. As ATMPs comprise a diverse and heterogeneous product group with varying biological and technological complexities, a case-by-case approach to the design of the nonclinical program is often necessary to account for product-specific issues that may affect pharmacology and toxicity.

The more specific and complex an ATMP is, the more challenging it is to define appropriate nonclinical studies and to define a relevant animal model. When feasible, nonclinical pharmacology studies conducted in animal models of disease or injury are preferable to studies performed in healthy animals. An animal species or animal model that is anatomically and pathophysiologically similar to the target disease or injury should be selected. Appropriate models may comprise genetically modified animals such as knockout animals, specifically humanized animals or homologous models using animal cells of the respective species. In a homologous model, cells/tissues from the animal are harvested, isolated, manipulated, and applied as similar as possible to the clinically applied ATMP. While homologous models may highly mimic the cellular and tissue environment of the patient, they also integrate several uncertainties. The respective cells in the animal and/or their components are often less characterized than their human homologues. Therefore, these cell preparations may have different functional activities or may be regulated differently in the animal body than anticipated. In addition, although a similar manufacturing process is employed, it may lead to different impurities, which may as a consequence result in a pharmacological and toxicological profile that is different to the one of the actual ATMP. Therefore, a homologous model may be appropriate for only some of the nonclinical questions, while other questions may have to be addressed using an immune-competent or immune-deficient heterologous animal model. Thus, the use of a homologous model may need to be complemented by additional studies in a heterologous setting. For GTMPs, the use of a homologous animal model also refers to the gene product expressed from the recombinant nucleic acid. Ideally, an animal species in which the human protein or expression product is active will be used in nonclinical studies. A homologous setting is needed, if the human therapeutic protein or expression product is known to be biologically inactive in the available animal model. Then, instead of encoding the human sequence, it may be necessary to insert the orthologous gene of the selected animal species as part of the recombinant sequence.

In general, the design of nonclinical animal studies and the criteria on which a particular animal model is chosen have to be scientifically justified. Although the number of animals employed in a study may vary depending on different factors such as the disease model, the test species, and the delivery system, the total number of tested animals per study group has to be sufficient to ensure a biologically significant interpretation of the results. When feasible, it is also important to use animals of both genders and to provide adequate positive and negative controls. In addition, the rationale for the chosen time points, the frequency, and the overall duration of

the monitoring to detect possible adverse effects need to be discussed. In case additional substances are administered together with or as part of the ATMP, such as biomolecules and/or chemical substances, the safety, suitability, and biocompatibility of these materials have to be addressed. Especially for scaffolds, the physical, mechanical, biological, and chemical properties should be considered and the material selection should be justified based on biocompatibility. Scaffolds may be permanent or may be desorbed, they may be two/three dimensional, and all these aspects may influence their interactions with the cell or gene therapeutic agent and its function within the scaffold.

Depending on the mode of application, the cells of a CBMP may be distributed within the whole body either passively by blood or even actively, for example, in the case of multipotent stem cells migrating to sites of injuries. Mesenchymal stromal cells encounter different microenvironments during their biodistribution, which may lead to unintended effects of these cells and adverse events may also be induced by the secretion of biologically active substances. Moreover, biodistribution studies using hepatocytes in different animal models suggest that hepatocytes predominantly stay in the liver sinusoids and are then trapped in the lung capillaries. Thus, in order to predict unintended physiological effects for the patients, it is necessary to study the biodistribution and the fate of such ATMPs in an appropriate model. As for CTMPs, biodistribution of GTMPs also needs to be addressed in nonclinical studies. This is usually done by detecting a specific sequence of the introduced recombinant nucleic acid by quantitative PCR (qPCR). The time points for collecting the various tissues and organs should include early time points, when the distribution of the GTMP is expected to be maximal and later time points to evaluate the clearance of the GTMP. If strong and/or persistent signals are detected in nontarget tissues and organs, expression of the introduced recombinant nucleic acid sequence may need to be addressed in addition. As a first step for evaluating inadvertent germline transmission of the introduced recombinant nucleic acids, the reproductive organs should be included in the biodistribution studies. If no qPCR signals are detected in the gonads, inadvertent germline transmission needs not to be further addressed. Detailed guidance, however, is provided in the EMA "Guideline on nonclinical testing for inadvertent germline transmission of gene transfer vectors" [23].

Toxicity of CBMPs may arise from different factors and, for example, could originate from (1) unintended cellular alterations taking place during manufacturing, such as altered excretion of chemokines, (2) an interaction with components used during the manufacturing process or composing a structural part of the medicinal product, (3) an allogeneic use of the product, or (4) an unintended proliferation of the applied cells with regard to dimension or location. In order to address all of these risk factors, the toxicological studies need to be performed with the final product including all its components. To gain a better understanding of the product, it may also be important to consider analyzing some of the components of the final product individually, for example critical excipients, additional substances, or process-related impurities.

Similar considerations also apply for GTMPs. Toxicities may originate from expression of the introduced recombinant nucleic acid, from the vector backbone, or from any other component of the final product. Therefore, toxicity studies should

be performed with the ATMP that is going to be used in human or with a highly comparable homologous product when using a homologous animal model. Single and repeated-dose toxicity studies may be necessary depending on the intended clinical use of the ATMP. In these toxicity studies the application route and the dosing regimen should reflect the intended use in human. The duration of these studies may need to be longer than anticipated from conventional single-dose toxicity studies.

From a scientific point of view, the risk of tumorigenicity and the need of non-clinical in vitro and/or in vivo studies to address this risk should be thoroughly evaluated on a case-by-case basis. For example, if a GTMP encodes a growth factor, its potential tumor-inducing and/or tumor-promoting effects need to be evaluated. For cell-based products, it is important to determine whether the manufacturing process leads to chromosomal abnormalities. If tumorigenicity studies are performed, the administered cells should ideally be at the limit or even beyond the limit of cell doublings that is routinely used during manufacturing. Tumorigenicity studies may also be needed if an ATMP contains cells with a certain level of stemness. Thereby, analysis of tumorigenicity is of particular relevance if such cells are additionally modified with integrating viral vectors bearing the risk of insertional mutagenesis.

10.3.4 Aspects of Clinical Development of ATMPs

There are a number of challenges in the clinical development of ATMPs, owing to their complexity and novelty. As for all other medicinal products, exploratory and confirmatory studies are expected to address pharmacodynamics, pharmacokinetics/biodistribution, safety, and efficacy, according to Regulation 2001/83/EC [3]. Yet, due to the specificities of ATMPs, their clinical development needs to be adapted to the individual characteristics of the product, also taking into account disease-specific requirements.

10.3.4.1 Cell-Based ATMPs

sCTMPs and TEPs are developed for different therapeutic areas. The main therapeutic areas for TEPs are cardiac and vascular diseases, musculoskeletal disorders, renal and urinary diseases, and eye and skin diseases. sCTMPs are mainly developed to treat malignancies and graft-versus-host disease (GvHD), based on the immune modulating and anti-inflammatory effects of these ATMPs.

Early-phase studies are usually conducted to study the pharmacodynamics and biodistribution in the target indication, to assess the safety of the intended dosing regimen, and to provide a basis for confirmatory studies. These studies are usually conducted in the target population.

It is acknowledged that it is not always possible to perform dose-finding studies with CBMPs. For example, when the product contains a structural component with a fixed number of cells, dose finding is difficult to perform. In cases where proliferation of the cells is expected or has been shown in vivo, dose finding may not be meaningful. In these cases it is suggested to provide a proper justification for omission of dose-finding studies and to provide quality and nonclinical data to justify the selected human dose. Data from literature may in addition be useful to support dosing, provided that the medicinal products are comparable.

Early-phase exploratory studies should be focused on the assessment of the safety of the investigational medicinal product. This is of special importance as the route of administration of CBMPs is highly variable and may include intraparenchymal administration or administration into local vessels. In case specific delivery systems are used, the safety and feasibility of cell delivery should be assessed in preceding nonclinical studies. For example, various ATMPs can be delivered via invasive routes of administration such as surgically placed port catheter systems allowing for intra-portal administration of liver cell suspensions under controlled conditions. In such applications, the safety of placing a portal vein catheter would have to be assessed. The administration procedure would also involve evaluation of the localization and potency of the catheter, as well as assessing portal pressure and flow. Standardization of the administration procedure prior to entering clinical studies is recommended and is expected to facilitate the assessment of the therapeutic procedure as a whole, according to Directive 2009/120/EC [2].

As for other medicinal products, the main points to address in the design of confirmatory studies are the (1) choice of the target population (inclusion criteria) and of a suitable control group, (2) blinding, (3) choice of primary and secondary endpoints, (4) sample size estimation, and (5) statistical design. Evaluation of efficacy is based on valid clinical endpoints. These are usually defined in indication-specific guidance documents. In addition, cell- and tissue-specific endpoints are required to assess structural and functional parameters. The administration of TEPs is often performed within surgical procedures. This implies that it may not be possible to conduct double-blind studies, as blinding of the surgeon is not possible. In such a situation measures to reduce bias, such as performance of blinded endpoint assessment, should be introduced.

10.3.4.2 Gene Therapy Medicinal Products

The broad field of GTMPs has been expected to generate a number of innovative treatment approaches, mainly in the field of hematology/oncology and in the area of monogenetic diseases, implying orphan indications. In malignant diseases, the development of GTMPs often relies on an immunotherapeutic mode of action or on oncolysis by replication-competent (oncolytic) viruses. The proof of efficacy and safety follows in general long-established regulatory principles, while for the development in orphan diseases, new challenges are noted. These relate mainly to the low number of available patients for enrollment into clinical trials. The “Guideline on

clinical trials in small populations" [24] can be consulted for those clinical developments.

First-in-human studies with GTMPs are usually conducted as phase I/II studies in the target population. They aim at supporting proof-of-concept of the therapy, assessing pharmacokinetics and safety, and, as with CBMPs, providing the basis for further clinical development. Information on dosing is usually derived from non-clinical studies. Here the relevance of the animal model in defining the safe starting dose should be taken into account. Specific considerations include the effect on target and nontarget tissues and, in cases where viral vectors are used, the impact of preexisting immunity on the efficacy of the therapy. For confirmatory clinical studies, existing guidelines for the specific therapeutic area are available and should be followed. In hemato-oncological indications, proof of efficacy and safety will generally follow the criteria as outlined in the anticancer guideline [25]. In orphan indications, data from limited patient populations may be sufficient to support the proposed indication. For these cases, the EU regulatory framework has implemented specific types of marketing authorizations, i.e., "Conditional Approval" (Article 14(7) of Regulation EC No 726/2004) and "Approval Under Exceptional Circumstances" (Article 14(8) of Regulation EC No 726/2004) [9].

The active substance of both CBMPs and GTMPs is often intended to persist in the patient. However, this persistence may be associated with side effects such as unwanted immunogenic reactions, autoimmunity, malignancies, or simply a decrease or even loss of efficacy long after treatment. Therefore a long-term follow-up of patients is often inevitable and may include clinical follow-up for the evaluation of safety and efficacy. The need for the duration and the nature of such a long-term follow-up depends on a risk analysis that considers the nature of the ATMP and the current state of knowledge. Detailed guidance on the follow-up of efficacy and adverse reactions and risk management can be found in the "Guideline on safety and efficacy follow-up – risk management of advanced therapy medicinal products" [26] and in the "Guideline on follow-up of patients administered with gene therapy medicinal products" [27].

10.3.5 Environmental Risks of Medicinal Products Containing or Consisting of Genetically Modified Organisms

If a medicinal product contains or consists of genetically modified organisms (GMOs), which is often the case for GTMPs, specific legislation relevant to GMOs has to be considered in the EU. When applying for marketing authorization, Regulation 726/2004/EC requires that an environmental risk assessment (ERA) similar to the procedure described in Directive 2001/18/EC on the deliberate release of GMOs into the environment is performed [28]. The ERA is part of the MAA dossier and is evaluated within the centralized procedure by a qualified assessor. During the marketing authorization procedure, the designated competent authorities for

GMO regulation of all Member States are consulted for review of the ERA. This procedure replaces the notification requested in Part C of Directive 2001/18/EC for placing a GMO or a combination of GMOs as or in products on the market.

The ERA documents that need to be included in an MAA of a GMO-containing medicinal product are listed in detail in Regulation 726/2004/EC [9]. The core document is the actual evaluation of the environmental risks, which should be performed in accordance with Annex II to Directive 2001/18/EC [28]. The objective of this evaluation is to identify the potential harmful effects of the clinical use of the GMO-containing medicinal product for third parties, animals, plants, and microorganisms. The assessment further evaluates the possible consequences and the likelihood that the previously identified harmful effects of the GMO are actually occurring. Based on these considerations, the environmental risk of a GMO-containing medicinal product is either judged as acceptable or as unacceptable. If the risk is initially considered unacceptable, appropriate risk minimization strategies need to be installed to lower the risk for thirds and the environment to an acceptable level. It is noteworthy that the risk associated with a GMO is not only considered for the public in general but also for vulnerable groups such as immunocompromised individuals, pregnant women, and children. Furthermore, the likelihood that a harmful effect is actually occurring is strongly dependent on whether or not the GMO is shed into the patient's secretions and excreta or leaks from the injection site, providing the condition for its transmission to third parties or for dissemination into the environment. Therefore, it is necessary to include shedding analyses into clinical studies of a GMO-containing product. These analyses are not only essential for determining the likelihood for transmission of a GMO to thirds or for dissemination into the environment but also for identifying effective risk minimization strategies and for determining the duration of these measures.

The ERA for GMO-containing ATMPs may be hampered by the fact that Directive 2001/18/EC is focusing on the deliberate release of genetically modified plants and agricultural products. Therefore, some of the information requested in the Directive need to be adapted to GMO-containing medicinal products. Specific guidance on performing an ERA specifically for GMO-containing medicinal products has been provided by EMA in the "Guideline on scientific requirements for the environmental risk assessment of gene therapy medicinal products" [29].

In contrast to the marketing authorization procedure, an application for a multinational clinical trial authorization with a GMO-containing medicinal product may be more demanding. Due to the lack of harmonization between the EU Member States when implementing the Directives regarding contained use (Directive 2009/41/EC) [30] and deliberate release of GMOs into the environment (Directive 2001/18/EC) [28] and the Clinical Trial Directive 2001/20/EC into national law [10], the specific requirements differ significantly. Some Member States consider a clinical trial with a GMO-containing product as a deliberate release and require a similar ERA as needed for marketing authorization. Others consider them as contained use of a GMO with the hospital room serving as containment. Some Member States judge on a case-by-case evaluation whether the trial is classified as deliberate release or contained use. Important criteria for this decision may be whether patients

are hospitalized or treated on an outpatient basis or whether the GMO is actually shed into the patient's body fluids and released into the environment. As a result, for multinational clinical trials with a GMO-containing medicinal product, a dedicated ERA may be needed for each EU Member State in which the clinical trial will be conducted.

10.4 Challenges and Future Directions

As outlined in this article, ATMPs comprise a diverse and heterogeneous group of medicinal products with varying biological and technological complexities, which presents significant challenges for both developers and regulators. As a result, the pharmaceutical, nonclinical, and clinical development of an ATMP and its ultimate evaluation to achieve a marketing authorization are a time-consuming process. Indeed, there are currently altogether five ATMPs approved for the European market, but more ATMPs are in the pipeline and are expected to apply for marketing authorization in the near future. Moreover, science is progressing fast and surely will bring up many more opportunities for developing new approaches and concepts for treatment and ideally cure of diseases which at the moment can be addressed only insufficiently. Many of these products will be based on cells and genes and will thus be classified as ATMPs. It is important that the regulatory framework keeps pace with these developments. Regular review and eventually adaptation of the regulatory procedures and requirements will be crucial.

For example, in the field of gene therapy, important steps have recently been made in clinical development of hematopoietic stem cell (HSC)-based gene therapies. Several publications indicate a clear therapeutic benefit in primary immunodeficiencies (including SCID-X1, ADA-SCID), thalassemia, and leukodystrophies [31–34] as well as in liver-directed gene therapy of hemophilia [35]. Although these studies are still in the phase of clinical evaluation, based on the encouraging results, it is expected that applications for marketing authorization will follow soon. Furthermore, the new Regulation 536/2014 [13] is expected to streamline clinical trial authorization in Europe and to further support the application of multicenter clinical trials with novel ATMP developments across several countries.

The development of such novel therapies, for example, is stimulated by the recent breakthrough technology of induced pluripotent stem cells that carry the promise of a paradigm shift in the wider area of regenerative and personalized medicine. It is now possible to reprogram patient-derived autologous cells to pluripotency, genetically engineer them to express a protein with a therapeutic effect, differentiate them into effector cell types in vitro, and transplant these cells back into the patient. The first-ever clinical trial is currently running in Japan to test the approach with in vitro differentiated retinal pigment epithelial cells for age-related macular degeneration [36], and it is likely that the technology can be extended (but is not limited) to target other tissues and organs, such as the cardiovascular or nervous system, the liver, and the pancreas. The general concerns associated with

the use of iPS cells include quality (difficulties of ensuring and documenting cell identity and quality) and potential safety issues linked to genomic instability and tumorigenic potential of the cell-based product. It will be a joint effort of scientists, the industry, and regulators to define the parameters and requirements for effective and safe applications of these cell-based products.

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Chapter 11
iPSC-Derived Products: Current Regulations

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Abstract Developing clinical grade PSC-based cells for use in patients requires not only careful attention to cGMPs (current good manufacturing practices) but also careful consideration of a variety of issues that include accessing tissue in an ethically appropriate fashion and adhering to the current rules and regulations for specific local, national, and international jurisdictions, which, if not harmonized, will likely hinder progress. Countries have developed different ways to accelerate translation, and in this article, we discuss the issues specific to iPSC-based manufacture and how these issues are being resolved.

Keywords Regulations • Harmonization • Stem cells

Abbreviations

BLA	Biologics Licensing Application
CAT	Committee for Advanced Therapeutics
CBER	Center for Biologics evaluation and Research
CHMP	Committee for Medicinal Products for Human Use
FDA	Food and Drug Administration
GLP	Good Laboratory Practice
IND	Initial New Drug Application
iPSC	Induced Pluripotent stem cells
NACs	National APex
PSC	Pluripotent stem cells
PMDA	Pharmceuticals and Medical Devices Agency

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SME Small Medical entities
HE- Hospital exemption

11.1 Introduction

Pluripotent stem cells are cells that have the capability of differentiating into all somatic cell derivatives and can also contribute to the germ line. This ability combined with the property of indefinite self-renewal provides a unique opportunity for autologous cell-based therapy. Initially, pluripotent lines could only be derived from the fertilized embryo [18], and this work allowed the generation of hundreds of embryonic stem cell lines. Elegant methods were developed to derive lines from different stages of embryonic development and to derive lines without destroying the embryo. However, despite these advances, the ethical and social controversies relating to working with fertilized eggs and the inherent expense and inefficiency of deriving ESC lines. It is probably cheaper to derive an ESC than an iPSC and it is also efficeint. Up to 80 % of good quality blastocysts give a cell line. It's really due to man-made obstacles that ESCs are not derived more often. We also don't mention parthenogenic stem cells that are HLA homozygous that could easily be matched but perhaps there are reasons to not mention these and characterizing them made widespread adoption of this technology difficult [14]. Further, the technology did not allow one to Wouldn't HLA matching also be done after cell derivation using iPSCs? that could obviate the use of immune suppression that is required when cells, tissues or organs are transplanted. Nevertheless, several pioneers initiated the process of clinical development using ESC lines to treat a variety of disorders where no other cell source was available. Companies that have initiated such studies include Geron, ACT, and ViaCyte as well as academic groups led by Dr. Studer, Dr. M. Humayun, and Dr. Kapil Bhatia [10].

In parallel, efforts were made to address the problem of immune matching by using somatic cell nuclear transfer where the nucleus from a cell from a potential recipient of cell therapy is inserted into an enucleated egg and ESCs are derived from this activated egg [12, 20]. Despite initial hiccups and controversies, pioneers have steadily improved the technique and successfully derived human ECS lines from SCNT. Although the technology worked in multiple species and in principle solved the aforementioned immune matching issue, it was nevertheless cumbersome, inefficient, and too expensive for practical use in humans. Additional breakthroughs are required before SCNT is a routine option for personalized medicine. Although SCNT itself is not currently a practical therapeutic option, investigators who developed these techniques [4, 12] have furthered our understanding of the process of becoming pluripotent. This has led to an alternative method of developing pluripotent cell lines that do not require the use of an egg or an embryo and yet provide the ability to make personalized cells from any individual.

Dr. Yamanaka and his colleagues showed that pluripotency and indefinite self-renewal can be induced in most dividing adult cells by the addition of four factors

[16, 17] that activate the innate pluripotency program. Further refinements by numerous investigators showed that different combinations of factors could be equally effective and that mRNA, protein products, or combinations of small molecules, microRNA, protein, or genes that activated the same pluripotency program could be used to generate induced pluripotent cells (iPSCs) relatively inexpensively. Progress in the iPSC field has been rapid with several groups announcing their intention to use iPSC rather than ESCs as the starting material to generate differentiated cells for repair, replacement, or the delivery of trophic molecules. Difference in autologous and allogeneic therapy is summarized in Table 11.1. A variety of targets are being considered. Of particular interest are hepatocytes and the liver where progress has been rapid in identifying stem and progenitor cells in the adult liver and biliary tract (reviewed in [9]). Several trials with promising results have been reported (summarized in [9]). Success has also been achieved in differentiating cells from ESCs and IPSCs into hepatocytes (reviewed in [21]) and more recently using a 3-D culture system to enable mature liver tissue, and it appears that sufficient numbers of cells can be obtained to consider iPSC-based hepatocyte transplant trials.

To develop therapies related to treatment with hepatocytes, three models of iPSC-based therapy have evolved (summarized in Fig. 11.1). The first is the autologous model (one to one model) where cells from the affected individual are isolated and if necessary the gene defect is corrected and the corrected IPSC cell clone is then differentiated into an appropriate phenotype and this differentiated cell product is used for therapy. This autologous model is akin to autologous therapy utilized with sibling-matched bone marrow transplant or autologous cartilage repair or adipose stem cell-based therapy. The model here is that hundreds or millions of iPSC

Table 11.1 The differences between autologous and allogeneic therapies are listed

Autologous	Allogeneic
Donor disease issues may require alternatives	Prospective donor selection can reduce genetic risk
Expensive to manufacture	
Only iPSC-based source	Much cheaper but cost of immune suppression
Much longer to make and so unavailable for acute damage	Alternative tissue sources possible
Regulations may make it impossible	Off the shelf product that fits current business models
Regulatory changes may make it much easier	Available for acute therapy as intermediate product can be stored
Immune matched so immune suppression not required	Regulatory pathway clear
iPSC haplobank strategy may reduce cost	The blood brain barrier may reduce the immune issue even if not matched
Future cost reductions possible with technical breakthroughs	Haplobank strategy can further mitigate the immune issue
Acceptance and perceived value higher	Novel engineering strategies may solve the immune issue

iPSC therapy models	One to One 	One to Many 	Many to Many 
Autologous	Yes	No	No
Donor	Self	Healthy	HLA matched
No of lines	1-3 clones	5-10 lines	>100lines
Regulated as BLA	Yes	Yes	Yes
Multiple products	Yes	Yes	Yes
Fewer tests and tracking issues	Yes	More	Even More
Longer time to therapeutic product	Yes	Much shorter	Shorter
May be GLP	Possibly	Unlikely	Unlikely
HE exemption	Possible	Unlikely	Unlikely
Consent straightforward	Yes	Burdensome	Burdensome
Immune suppression	Unnecessary	High per patient	Intermediate
Cost	High/patient	Lowest	Intermediate

Fig. 11.1 Three models of IPSC-based banking are described and their relative advantages and disadvantages summarized

lines would be made but would be made on a case-by-case basis. Once made, the cells could be stored indefinitely so that if there was a need by the same individual again, then the line could be differentiated into a second type of required cell or even a third if required. While the initial therapy would take time and would be time consuming, subsequent need could be satisfied relatively quickly. Wealthy individuals could have their own iPSC lines made as an insurance akin to what private cord blood and tissue banks do now with the added advantage that iPSC unlike cord blood represents an infinite supply and can make many more derivatives than cord blood. The market has shown that such a model can work though there are some important differences between storing cord blood and making and storing iPSC. Perhaps the first critical difference is the regulations regarding such therapy in the United States. Unlike autologous cord blood transplants, autologous iPSC (even if they are not gene corrected) will be treated as a new biologic and will require an IND process under current rules.

A second model (many to many model) for IPSC-based therapy is to consider HLA matching and make an immune compatible cell. The model here is to identify donors prospectively whose HLA profile is such that they can donate to many individuals. Thus, a much more limited but selected set of iPSC lines will be enough to provide a 6/6 match of the HLA locus and then would require limited immune suppression. Evidence that only limited immune suppression will be required and the drugs that are suitable has come from studies done in bone marrow and organ transplants. The

regimes, while expensive and with side effects, are certainly well tolerated by a large number of individuals. Calculations to determine the number of lines required have been provided by various groups [19], and these suggest that as few as a hundred lines may be sufficient for some homogeneous populations. In any case even in the worst case scenario, the number of lines required is manageable and indeed a haplobank approach has been proposed by Dr. Wilmut and colleagues [19]. The model here is akin to the National Bone Marrow registries where a database of HLA types available worldwide is maintained. A consensus on the collection and derivation of the product by the various national groups is agreed to so that a sample from any bone marrow registry can be sent to any recipient worldwide. A potential recipient or his/her physician searches the repository and identifies a potential match. A criteria is developed for prioritization of recipients and for shipping the sample worldwide.

While the model for iPSC is conceptually similar, there are two important distinctions. One crucial difference is that IPSC themselves are not the final product, but rather they are the starting material to make multiple final products and in the case of a haplobank for multiple individuals. In addition to developing a shipping and storage capacity like the bone marrow banks, one will have to develop a manufacturing capacity to make multiple products. Manufacturing a clinical product adds a separate and additional layer of regulations to those under which registries and banks are governed. It is important to note that there is some but not adequate harmonization of manufacturing regulations that one can reasonably assume that a product manufactured in one country can be utilized worldwide. The second distinction is that there are currently no consensus agreements on what constitutes a good iPSC line and what adequate QC and potency assays are. This is crucial for a haplobank strategy. If the regulators in one country are not comfortable with the approval process in another, then applying the bone marrow model may be impossible. While the regulators may be willing to come to consensus, it is not clear investigators themselves have agreed to either a list of tests or the method of testing or what is an agreed to acceptance and rejection criteria.

A third approach is simply the allogeneic (one to many model) approach with the assumption that cell transplants can work without immune suppression or at most limited suppression in certain regions of the body. Postmortem analysis performed in a limited number of patients showed that the graft survived and integrated. Morizane and colleagues [11] did match and mismatched transplants in primates and likewise showed that while completely matched transplants did better, the mismatched transplants also survived for periods as long as a year (maximum time period examined). There are several reports of organ transplant recipients stopping their immune suppression regimes with organ failure or rejection. Indeed many of the fetal transplants for Parkinson's disease were not immune matched and either limited or no immune suppression was used. There is also hope that as technology advances, other methods of immune regulation will obviate the need for HLA-based matching efforts. In this case, the model would be that a group would identify two or three lines that would be carefully selected from individuals who were healthy, who had no hereditary disease, and who did not carry alleles for increased susceptibility to any common disease and the iPSC clone would be selected for its ability to

grow and differentiate into multiple phenotypes [2]. There are several reasons companies find this model very attractive. One, it fits their current model for manufacturing biologics and for getting approvals through the IND process. Two, it is much cheaper to qualify one line for therapy and then test multiple lines in preclinical studies. Three, since a single iPSC can be used to generate multiple products, one can design manufacturing processes to achieve even greater economies than are possible with adult stem cell populations making iPSC-based cell therapy economically viable. iPSC would still be preferred over ESCs as one could identify healthy individuals and select the best donor which one could not do with ESC lines which are genetically distinct from either parent. Getting approvals/consent is also much easier as case law is quite clear on tissue donation from adults as is ownership of material. Unlike obtaining consent for tissue donation for iPSC generation, neither the approvals nor the consent process is as clear-cut with blastocyst donation for the generation and use of ESCs. Likewise, the patent rules for ESCs ownership are complex and in flux, while those for iPSCs somewhat more clear-cut.

In summary, each of these models appear to be viable, and ones choice depends to some extent on the scientific assumptions one makes and the corresponding manufacturing constraints. If costs can be contained, then true autologous therapy would be a first choice. This could be performed relatively easily, the size of production is small, and the parental line can be stored for future use. There is no ongoing cost or risk of ongoing immune suppression, and the history of autologous transplants being better is generally well accepted. Even in chronic degenerative diseases where the cell product will carry the same genetic susceptibility, one can reasonably argue that the disease process will take long, and as such autologous therapy will work long enough to confer benefit. The advances in zero-footprint gene engineering which allow for integration-free reprogramming using episomal plasmids, *Sendai* virus, or mRNA enable the generation of high-quality iPSCs free from viral integration. The standardization of these methods and reduction in cost allow one to consider autologous cell-based therapy as well. This coupled with advances in gene editing techniques suggests that iPSCs from patients with genetic disorders could be isolated, gene edited, or engineered to provide the missing gene product which permits the treatment of individual patients. These advances, coupled with advances in differentiation, allow us to consider generating "cured cells" for personalized therapy.

The other models previously discussed offer significant cost savings and, in many cases, a time to therapy advantage that is simply not possible for autologous therapy. Thus, there are several acute indications where cells may be required only transiently in which case allogeneic therapy may be the preferred option. Allogeneic therapy may also be the preferred option when cells are required only transiently. Once the cells provide their trophic or immune modulatory or other short-term functions, one can rely on the body's natural immune response to eliminate the mismatched cells in due course. This lessens the risks of long-term integration, potential tumorigenicity, and monitoring. One may also imagine indications where one wants to stimulate the bodies' natural immune response, and in this case, allogeneic cells may be the preferred cell type. It is important not to discount the cost savings as well.

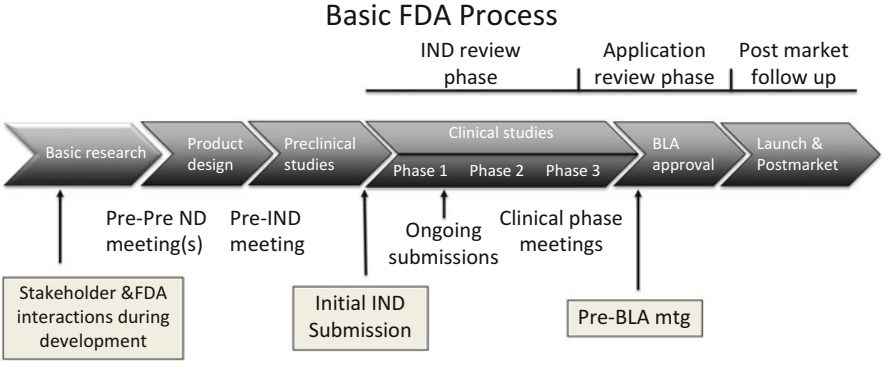
The haplobank combines cost savings with a faster time to delivery with a reduction in the cost of immune suppression, thus allowing cells to be used for both

long-term integration and transient use. While not optimized for either mode of therapy, it provides a significant advantage for approval through the regulatory process though unlike an allogeneic model, it is more complex to develop and requires harmonization and standardization between regulations in different countries to be viable.

To us it appears likely that all three models will coexist and users will have a choice of the optimum cell type they wish to use for a particular indication and regulatory authorities will have to evaluate their regulatory regimes to ensure that choices are available.

11.2 Regulatory Issues with PSC-Based Therapy

Irrespective of which model ultimately prevails or all three models exist side by side, these models share several issues in common. In all cases, donors have to be identified and iPSs generated, iPSCs need to be stored until used, and differentiated products must be derived from them. The end product needs a defined release criteria to ensure quality and consistency. This product then needs to be tested in preclinical studies to demonstrate safety of the manufactured product followed by early clinical studies to demonstrate safety and efficacy of the product. A proposed common framework of how this may be done is outlined in Fig. 11.2, and the US FDA has proactively begun developing regulations for this new class of therapy. What is



- Expertise with autologous and allogeneic therapy and established program for international harmonization
- Standardized process that take 4-8 years to complete
- Multiple opportunities to interact with the FDA/CBER
- Extensive guidance's and webinars and documents available
- Compassionate use exemption
- Orphan and rare disease designation
- Some flexibility on consent, and compensation and reimbursement

Fig. 11.2 The basic FDA processes of evaluating a biologic are summarized. Points where one can interact with the FDA are indicated

clear is that allogeneic, autologous, and cells from a haplobank will be regulated under the same core set of regulations that govern any other manufactured biologic and these issues that are common with other biologics are discussed elsewhere [15]. In the section below, we discuss the efforts made by different regulatory authorities to accelerate the translational efforts of various groups using existing regulations or modifying them to ensure that safety is not compromised while accelerating the translational efforts and reducing the risk burden on providers of this type of therapy.

11.3 The Japanese Regulations

To help accelerate translation efforts for iPSC-based therapy, the Japanese government created new rules. The Pharmaceutical Affairs Act was revised, and the Act for Ensuring Regenerative Medicines was newly enacted in 2013 [8]. These acts were passed by the Japanese Diet in 2014, and at least one iPSC-based application has been approved under these regulations. These acts modified the existing PMDA (Pharmaceuticals and Medical Devices Agency) regulations [6] to say that hospitals and other qualified institutions could introduce experimental therapy provided it was safe and expected to be reimbursed for it during the initial experimental studies in human (see Fig. 11.3). A separate independent body of scientific experts was established to evaluate applications of such products. As part of the act, institutions would have up to 7 years to provide data to the regulatory authorities for a full

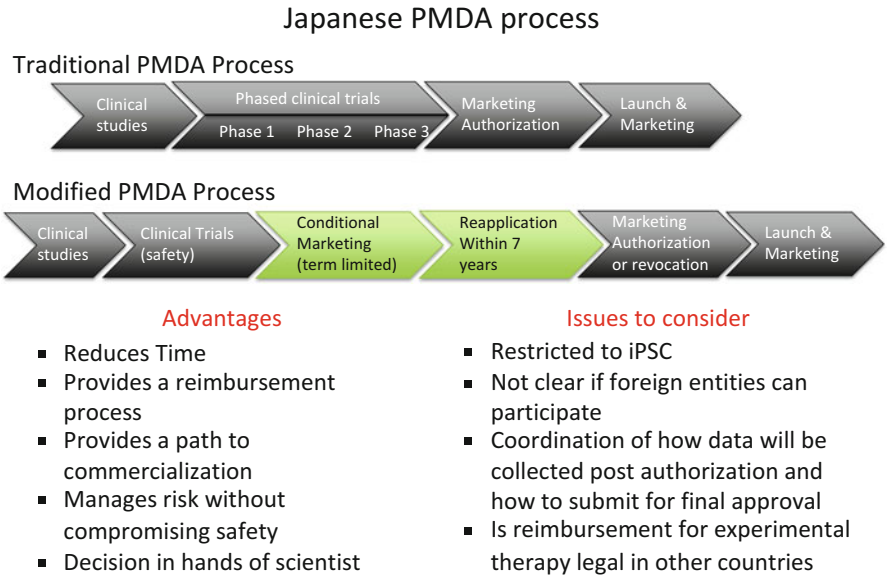


Fig. 11.3 The recently changed PMDA process and its advantages and disadvantages are summarized

commercial license. During this conditional approval phase, the group, company or hospital could charge for this therapy, and insurance companies who cover health benefits would reimburse for such conditionally approved therapy. To ensure widespread participation in the effort, the regulatory authorities made one additional critical change. The authorities permitted the manufacture of the product outside the hospital or institution where the therapy was being instituted. This permitted hospitals to outsource manufacture to cGMP-qualified CMO's and allow one hospital to supply product to multiple other institutes permitting multicenter studies that would not have been permissible under the previous PMDA regulations.

Although the benefits of such an approach are enormous, it is clear that such an approach requires consensus and change in existing rules. Interestingly, Caplan and West have proposed something similar for MSC [5]-based studies arguing that a number of clinical trials that have already been instituted with these cells indicate that these cells are safe. They therefore suggest what they have termed "progressive approval." The EU and United Kingdom have a hospital exemption system not dissimilar to the PMDA system, and the EU has proposed an adaptive licensing system (see below) as well.

11.4 US Efforts to Accelerate Therapy

The United States has not currently changed any of its rules, but has been extremely proactive in helping the stem cell community. The FDA first clarified that the rules for autologous iPSC-based therapy will clearly be those that are for a manufactured product. The FDA recognized that consent for tissue sourcing may occur years, even decades prior to the actual use of tissue, and has developed model consent rules to ensure that ESC and IPSC cells can be used for therapy.

Recognizing that many of the stakeholders advancing cell-based therapy are not the usual "pharmaceutical companies" who are familiar with the regulatory process, the FDA has offered many seminars and web courses to explain the process and modification that are permissible. The FDA has issued several guidances related to the process of tissue collection, banking, release, and distribution of such products and has been liberal in providing input at early stages of the product development process that can reduce the cost and time of development. The FDA, in addition, has recognized that potency and efficacy tests for many of the stem cell products are not available and that there is no consensus on what is a reasonable test of quality. This is true for the entire range of manufacturing whether it is prion protein testing, mycoplasma detection, tests for sterility/asepsis, or more specialized tests such as tests for pluripotency, tests for residual contaminating cells, etc. The FDA, through its own internal research and through its collaborations with NIST and the NIH, has begun developing assays or evaluating existing assays [3] to determine their utility.

The FDA recently also clarified that the cord blood banks that are for public use will have to be licensed and established a process of licensure that most of the major blood banks have successfully complied with. The importance of this effort is that a process for qualifying banks that store other tissues that will be used in the manu-

facture of a product exists, and one can reasonably expect that a haplobank of iPSC will be covered by such a set of regulations. The FDA and the EU have programs for orphan drug designation, accelerated development, and reduction in fees as well as a process for single case exemptions. Each of these processes was developed for biologics other than IPSC-derived products, but the FDA has indicated that these can be used for iPSC products and this has been very useful for the orphan and rare disease programs and potentially for many of the iPSC-based efforts that are currently targeting these diseases.

The NIH recognized that a second hindrance to developing iPSC cell-based applications would be the lack of availability of cGMP grade cells and the cost of developing such lines on a single user basis. The NIH invested in a crowdsourcing model for cell-based therapy [13]. The NIH funded a small bank of 10–12 lines manufactured under a GMPable process that is widely available for preclinical testing and for developing modified differentiation protocols. Researchers can use these lines to test their ability to differentiate, evaluate, and optimize differentiation protocols, develop reporter lines, and perform key preclinical studies with the reasonable expectation that other iPSC lines derived and manufactured with the same cGMP process will behave the same and one will not have to redevelop or requalify QC tests leading to a significant reduction in time and cost to develop an iPSC cell-based therapy. The NIH, recognizing that manufacturing cell-based products may be a bottleneck, also funded hospitals and university centers to set up cGMP facilities to manufacture products for preclinical and early phase I clinical studies. These centers have been very successful in enabling IND studies (Fig. 11.4).

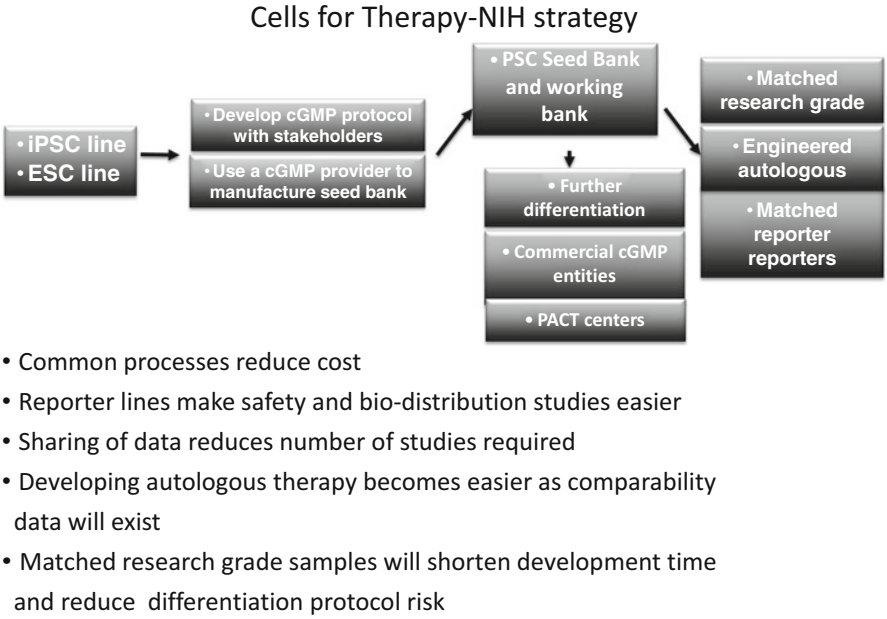


Fig. 11.4 The NIH effort to enable widespread use of PSC is summarized. Note the involvement of private entities and the potential reduction in cost for the individual investigator

Overall, we believe that while the FDA has not been as aggressive as the Japanese regulators in changing rules, their measured approach to facilitating translational work along with the NIH's efforts to reduce cost and the coordination between the various agencies led in part by the NIH and the FDA have facilitated progress. We anticipate additional clarity from the regulators as the research effort matures as to what would be the best way to ensure comparability of lines and the predictive value of the recommended tests which we think would further accelerate the translational process and make the haplobank concept viable.

11.5 European Regulations for Advanced Therapeutic Intervention

The European regulators have realized that there are national regulations that may be different between different countries and may not permit getting a blanket approval for the EU. This may reduce the enthusiasm for developing products in the EU first, and therefore in 2007, the EU introduced the EMA (European Medicines Agency)-based advanced therapy medicinal products (ATMPs), a new class of medicines in the European Union approval process (Table 11.2). The EU has placed iPSC in ATMP class, and the EU has established the Committee for Advanced Therapies (CAT) at the EMA for centralized classification, certification, and evaluation procedures and other ATMP-related tasks [1]. Guidance documents, initiatives, and interaction platforms are available to make the new framework more accessible for small- and medium-sized enterprises, academia, hospitals, and foundations. The CAT provides a centralized cell therapy product evaluation procedure. The CAT formulates a draft opinion on the quality, safety, and efficacy of a product for the final approval by the Committee for the Medicinal Products for Human Use (CHMP). The mandates of the EMA do not cover preclinical development, clinical trial authorization, HE (hospital exemption) authorization, functions of the ethics committee, or pricing and reimbursement. These issues are regulated at the Member

Table 11.2 The EU efforts to standardize approval under various regulations and to accelerate the process are listed

EU efforts to accelerate cell-based therapy
The Regulation (EC) No 1394/2007 established a legal framework for ATMPs that include iPSC and other cell products
The Directive 2004/23/EC helps define the quality and safety standards for the donation, procurement, testing, processing, preservation, storage, and distribution of cells
A centralized marketing authorization procedure established for all EU countries, as well as in Iceland, Liechtenstein, and Norway
A conditional marketing authorization subject to specific post-marketing obligations was set up under Regulation (EC) No 507/2006
Hospital exemption clause clarified and role of NACs and CAT and CHMP clarified
A reduced fee structure for hospitals and SMEs was established
An evaluation, classification, and consulting structure was established

State level by the national competent authority (NCA). Thus, the EMA is not quite similar to the FDA which regulates all of these activities.

There is also a procedure of conditional marketing authorization in the EU prescribed by the Regulation (EC) No 507/2006. This is an authorization to market on condition that the manufacturer undertakes additional studies to verify the clinical benefit. Such a regulatory pathway provides access to treatments for unmet medical conditions and has demonstrated that the benefits outweigh the risks in the clinical trials. This is akin to the Japanese PMDA system but is not codified into law the same way as is in Japan. Adaptive licensing or licensing conditionally could be based on stepwise learning, with iterative phases of data gathering and regulatory reevaluation [7], and a system like the PMDA system could evolve. In addition, the EU and United Kingdom have a hospital exemption (HE) system as well which allows hospitals to initiate cell therapy using a product that is not currently approved. The Regulation (EC) No 1394/2007 also introduced amendments to the Directive 2001/83/EC, and Article 28 of the act added provisions for the hospital exemption (HE, Article 3.7. of the consolidated Directive 2001/83/EC).

In summary, tissue sourcing approval comes from a national authority, and the manufacture, safety, and efficacy of a product are defined by the EMA/ATMP process, but the dossier, which includes the preclinical studies and the clinical data, has to be submitted to each country. Likewise, HE is at the country level as well. Since the EMA works closely with the national agencies, it is expected that the consolidated dossiers will be largely similar and will therefore be expedited.

11.6 Summary

iPSC-based cell therapy is developing rapidly and several models for using iPSC have evolved. The number of potential therapeutic applications continues to grow as people identify novel combinations of cells with scaffold, new methods of engineering, or techniques to build tissues and organs. There is clear consensus on the potential of these cells, and while the potential is exciting, one needs to move forward judiciously to ensure safety of these new products. The regulatory regimes must be modified or altered to ensure the safe development of these products, and the regulators and government agencies in different countries have taken different tacks to advancing this form of therapy. It is important that regulators work together and harmonize regulations to ensure that the therapy is safe and can be used worldwide.

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Chapter 12
Critical Path Initiative for Regenerative Medicine in Japan

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Abstract The Act for the Comprehensive Acceleration of Policies Promptly to Give the People Regenerative Medicine Free from Danger (Act for the Promotion of Regenerative Medicine) was enacted in April and enforced in May 2013, and the enforcement of the Act led to the Act Concerning Safety Assurance of Regenerative Medicine (Act on the Safety of Regenerative Medicine) and the Revised Pharmaceutical Affairs Law (Pharmaceuticals and Medical Devices Law) (enacted in November 2013 and enforced on November 25, 2014). The old Pharmaceutical Affairs Law had only two categories, pharmaceuticals and medical devices, and therefore was an obstacle to the development of regenerative medicinal products under pharmaceutical regulations. It was a great trouble for us in academe, and we had to treat patients in uneasy darkness without the landmarks and lights of regulations. The solutions for the problem are an upgrade to the Guidelines for Clinical Research Using Human Stem Cells to the Act on the Safety of Regenerative Medicine and the introduction of a new category, cellular- and tissue-based products, under the Revised Pharmaceutical Affairs Law or Pharmaceuticals and Medical Devices Law. We have obtained two paths for generalization of regenerative medicine in our society. Among the so-called advanced nations, only Japan has these two paths, and the whole world is watching carefully the start of the grand social experiment for the release of regenerative medicine into society. The selected path would be the answer from the general public to the question of how to establish regenerative medicine in society.

Keywords Act on the Safety of Regenerative Medicine • Pharmaceuticals and Medical Devices Law • Act for the Promotion of Regenerative Medicine

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12.1 Introduction

The Act for the Comprehensive Acceleration of Policies Promptly to Give the People Regenerative Medicine Free from Danger "(Act for the Promotion of Regenerative Medicine)" [1] was enacted in April and enforced in May 2013, and the enforcement of the Act led to the Act Concerning Safety Assurance of Regenerative Medicine "(Act on the Safety of Regenerative Medicine)" [2] and the Revised Pharmaceutical Affairs Law "(Pharmaceuticals and Medical Devices Law)" [3] (enacted in November 2013 and enforced on November 25, 2014). The Act on the Safety of Regenerative Medicine legally regulates the technologies of regenerative medicine collectively that had been categorized into clinical research (with rules such as the Guidelines for Human Stem Cell Research) or non-covered medical services (with no ex ante regulations). The target of the law is regenerative medicine used for clinical research and medical treatment (including non-covered medical service), and the purpose of the law is to make a structure in which the conduct of regenerative medicine is monitored and safety is secured. At the revision of the Pharmaceutical Affairs Law, cellular- and tissue-based products were introduced as a new category in addition to pharmaceuticals and medical devices, and a product approval system with conditions and periods according to the features of the products (which allows special early approval) was stipulated. Also, acquisition of informed consent about the risks from the patient, post-marketing surveillance to ensure safety, and creation and maintenance of records of trade and use of such products were made obligatory. In this section, we will mention the Japanese framework of regulations for regenerative medicine focusing on "the Act on the Safety of Regenerative Medicine" along with the Revised Pharmaceutical Affairs Law (Pharmaceuticals and Medical Devices Law).

12.2 The Pharmaceuticals and Medical Devices Law (Revised Pharmaceutical Affairs Law)

The general purpose of the revision of the Pharmaceutical Affairs Law (legislation of the Pharmaceuticals and Medical Devices Law) in 2012 was to conduct the actions needed to secure the safety and prompt supply of pharmaceuticals, medical devices, etc., and the actions include the institution of the obligation to submit attached documents, expansion of the scope of approval assessed by registered medical devices approval organizations, and establishment of product approval systems with conditions and periods for cellular- and tissue-based products. Regarding regenerative medicine, especially, the formulators intended to create regulations that take into account its features, and therefore they defined a new category—cellular- and tissue-based products—and instituted safety regulations that take into account the features of regenerative medicine. It is worth noting that these regulations concerning inhomogeneous cellular- and tissue-based products enable special

early approval of the manufacturing and marketing of such products with periods and conditions if the products are supposed effective and their safety is confirmed. The old Pharmaceutical Affairs Law also had a clause about a special conditional early approval system; however, it was stipulated for the first time at the introduction of the new category of cellular- and tissue-based products. It is obscure whether *inhomogeneous* cellular- and tissue-based products mean "self-derived cell preparations" or that all cellular- and tissue-based products are not homogeneous. Also, there is a dispute over the interpretation of "supposed effective," for example, whether several certain effective response cases in many cases are assumed to be effective or not. The Q&A notice concerning these interpretations has not been released; therefore, the outlines are expected to be clarified in a future examination process.

12.3 The Act on the Safety of Regenerative Medicine

12.3.1 Circumstances of the Enactment

The Act on the Safety of Regenerative Medicine is established in order to clarify the actions that should be conducted by providers of regenerative medicine, such as ensuring the safety of regenerative medicine technologies used in regenerative medicine and measures for protection of bioethics, and is also aimed at contributing to improvements in medical and healthcare quality through the swift, safe supply of regenerative medicine and the promotion of it by means of establishing systems including approval of production of specific processed cells (Article 1).

The enactment of the Act was originated from accidents, including a fatal one that occurred in a self-professed regenerative medicine clinic. Regulations on regenerative medicine, including medical practices, were required because of these accidents and consequently led to legislation of the Act. Following the formulation of the five-year strategy for healthcare innovation in June 2012 and the Strategy for the Rebirth of Japan in July 2012, the special committee for Safety and Promotion of Regenerative Medicine was constituted within the Science and Technology Group of the Health Science Council on August 20, 2012, and the first meeting of the special committee was held in September 2012. In the initial movement for establishment of the Act, it was not clarified whether revision of the Guidelines for Clinical Research Using Human Stem Cells or establishment of a new law would be adopted for introduction of the new regulations, and the policy was planned for conclusion by the summer of 2013. The news of the awarding of the Nobel Prize to Professor Shinya Yamanaka on October 8 of the same year changed the circumstances. On October 23, interested assembly members from three parties agreed to submit the Bill on the Act for the Promotion of Regenerative Medicine during the next ordinary Diet session, and they discussed preparation of the submission of individual bills concerning regenerative medicine during the next year's ordinary Diet session. In

response to the movement, the special committee advanced its discussion toward enactment of legislation instead of a revision of the guidelines in the second meeting in November. The policy for legislation was reconfirmed in the third meeting in December, and after seven meetings, the report "Building a New Framework for the Promotion and Securing the Safety of Regenerative Medicine" was drawn up in April 2013, and the Bill on the Act on the Safety of Regenerative Medicine was submitted to the Diet along with the Bill on the Revision of the Pharmaceutical Affairs Law in May 2013, and both bills were unanimously approved. We consider the fact that the Act on the Safety of Regenerative Medicine and the Revised Pharmaceutical Affairs Law were enacted on the same day and enforced on the same day to be a clarifying message for Japanese regenerative medicine progress through these two paths for the foreseeable future.

12.3.2 Outline of the Act

The Act on the Safety of Regenerative Medicine has two major features. One is the introduction of compliance obligation to the Standards for the Provision of Regenerative Medicine (Ministerial Ordinance) and of penalties for disobedience to stay, improve, or other orders from the Minister, and the other is establishment of a pre-review system operated by the Regenerative Medicine Committee. Standards for the Provision of Regenerative Medicine are stipulated by Article 3 of the Act, and regarding the details, the standards shall follow the Ministerial Ordinance stipulated by Articles 42 and 44 (the so-called Article 42 standard and Article 44 standard, respectively). Article 3 categorized regenerative medicine into first-class regenerative medicine, second-class regenerative medicine, and third-class regenerative medicine according to the degree of influence on human life and health and defined the required procedures for each class. Explanations for easy understanding are as follows: First-class regenerative medicine includes regenerative medicine using human-derived cells and regenerative medicine using pluripotent stem (PS) cells or PS-like cells; third-class regenerative medicine is regenerative medicine used in cancer immunotherapy or regenerative medicine with minimal manipulation of cells. These ideas were visualized in a flowchart and issued in a Q&A notice (Fig. 12.1).

To implement a plan for the provision of first-class regenerative medicine, the provider is required to submit a plan to the Minister of Health, Labor and Welfare after receiving opinions from the Specified Committee for Approved Regenerative Medicine. The Act sets 90 days as the restriction period for the implementation during which the Minister of Health, Labor and Welfare checks the safety of the plan after consulting the Health Science Council. The Act allows the Minister to order modifications to the plan if the plan does not conform to the standards for safety. That is to say, the notable points of the Act are (1) examination by the Specified Committee for Approved Regenerative Medicine, (2) submission of the plan for provision of regenerative medicine to the Minister, and (3) implementation after a

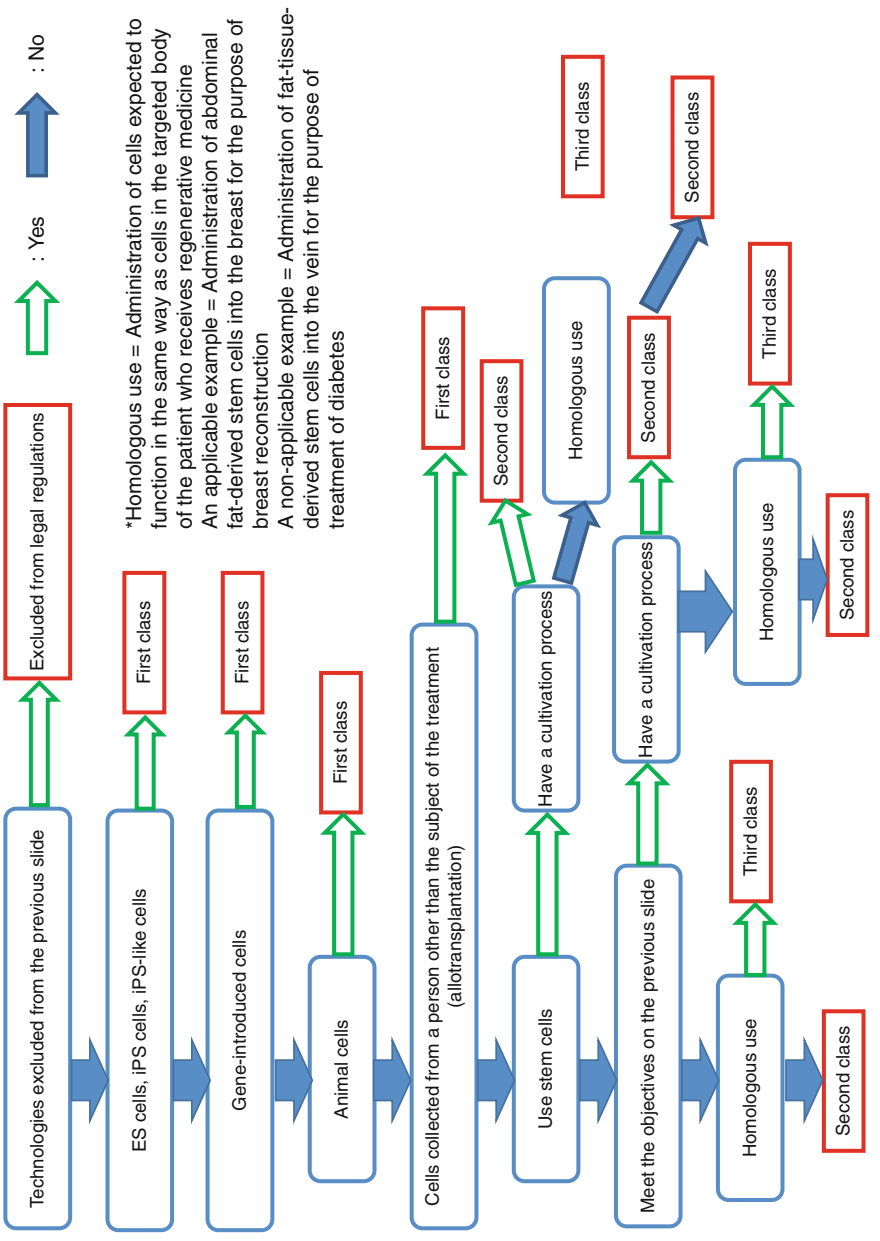

Fig. 12.1 Risk categories of first–third-class regenerative medicine technologies

90-day restriction period (central examination by the Regenerative Medicine Examination Committee of the Health Science Council), and they were not significantly changed from the examination under the Guidelines for Clinical Research Using Human Stem Cells. This is a virtual approval system. To implement a plan for provision of second-class regenerative medicine, the provider is required to submit a plan to the Minister of Health, Labor and Welfare after receiving opinions from the Specified Committee for Approved Regenerative Medicine. The system for second-class regenerative medicine required only the abovementioned (1) and (2) and represents a relaxed version of the current double check system. It is a virtual registration system. On the other hand, securing good examination qualities of the Specified Committee for Approved Regenerative Medicine is an urgent subject; therefore, committees with low examination standards will be eliminated in actual practice. To implement a plan for provision of third-class regenerative medicine, the provider is required to submit a plan to the Minister of Health, Labor and Welfare after receiving opinions from the Committee for Approved Regenerative Medicine. Each medical institution is allowed to constitute the Committee for Approved Regenerative Medicine; therefore, it is very predictable that the examination qualities of such committees will differ greatly. Securing proper examination quality is considered to have a decisive influence on the future of regenerative medicine. The Specified Committee for Approved Regenerative Medicine is defined as a committee that possesses a superior examination capability and objectivity and is not assumed that every medical organization will have it. Specified Committees are assumed to be operated in the way similar to that of the central IRB and to undertake an examination as an onerous contract.

The Act defined measures for the proper provision of regenerative medicine, such as informed consent and measures for personal information protection, and the Act also requires prompt reporting of an adverse event to the Minister of Health, Labor and Welfare on which the Minister takes the needed precautions after consulting the Health Science Council. These are compensating measures for the immaturity of current regenerative medicine. The Act allows the Minister of Health, Labor and Welfare to issue an improvement order when safety measures are required, to restrict provision of regenerative medicine if the provider disobeys the improvement order, and to order emergency measures, such as suspension of the provision of regenerative medicine, when protection against the outbreak and spread of health and hygiene hazards is required. The new medical service law grants the authorities the power to conduct inspections under entrance in consideration of past accidents, including fatal ones, in non-covered medical services.

Concerning production of cell preparations (defined as Specific Processed Cells by the Act), Article 42 of the Act prescribes that the structure and equipment of cell processing facilities shall meet the standards set by the Ordinance of the Ministry of Health, Labor and Welfare. The article manifests that the definition of standards for the structure and equipment of cell processing facilities is committed to the Ministry's ordinance (the so-called Article 42 standards). Article 44 of the Act prescribes that the Minister of Health, Labor and Welfare may, by Ordinance of the Ministry, establish the matters that manufacturers of specific processed cells have to

comply concerning production of specific processed cells in the cell processing facilities, such as management methods of production and quality control, methods of inspection tests, methods of storage, and transportation. The article manifests that compliance by manufacturers of specific processed cells is stipulated by Ordinances of the Ministry (the so-called Article 44 standards). These standards are almost at the same level as the Guidelines for Clinical Research Using Human Stem Cells; therefore, the enforcement of the new Act will not increase the burden on research organizations and medical organizations that have conducted research under the guidelines. On the other hand, the Act on the Safety of Regenerative Medicine allows outsourced production of specific processed cells because installation and maintenance of cell processing facilities require considerable costs. It is based on a consideration that outsourcing cell production ensures more stable provision of steady quality cells for patients. As a result, contract manufacturing organizations are necessarily required to satisfy higher standards; therefore, as a notable consequence, a contract manufacturing organization is licensed through the approval system, whereas a medical organization is licensed through the registration system in the framework of the Act. Also, the Act requires medical organizations to commission an approved or registered manufacturer to provide specific processed cells. Concerning methods of examination and the assumed level of standards for approval of cell processing facilities, the details will be discussed reviewing the consequences after the enforcement of the Act. At this time, it seems that the Act will require standards at the same level as facility standards under the Pharmaceutical Affairs Law because the examination is supposed to be conducted by the Pharmaceuticals and Medical Devices Agency, Japan. Concerning the specific processed cells provided by contract manufacturers, the doctors who use them and the managers of the medical organizations shall take full responsibility because administration of the cells is performed by the doctor. The doctor must assume all civil and criminal responsibility unless he/she can prove a defect in the processed cells. There is a possibility that a company or other institution will try to commission a doctor to provide specific processed cells to avoid pharmaceutical regulations; however, such an act will inevitably be criticized as a violation of the Pharmaceutical Affairs Law.

12.4 Conclusion

The old Pharmaceutical Affairs Law had only two categories, pharmaceuticals and medical devices, and therefore was an obstacle to the development of products for generalization of regenerative medicine under pharmaceutical regulations. Even if researchers try to deal with the products as pharmaceuticals, these products cannot be sterilized and cannot be handled under the regulations set under the old concept of pharmaceuticals. It was a great trouble for us in academe, and we had to treat patients in uneasy darkness without the landmarks and lights of regulations. The solutions for the problem are an upgrade to the Guidelines for Clinical Research Using Human Stem Cells to the Act on the Safety of Regenerative Medicine and the

introduction of a new category, cellular- and tissue-based products, under the Revised Pharmaceutical Affairs Law or Pharmaceuticals and Medical Devices Law. We have obtained two paths for generalization of regenerative medicine in our society. Among the so-called advanced nations, only Japan has these two paths, and the whole world is watching carefully the start of the grand social experiment for the release of regenerative medicine into society. The selected path would be the answer from the general public to the question of how to establish regenerative medicine in society.

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Part IV
Industrialization

Chapter 13
Aseptic Manufacturing of Regenerative Medicine Products Using Isolator Technology

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Abstract Aseptic processing or manufacturing is a method of manufacture in which microorganisms are excluded from the production environment and thereby prevented from entering the product. It has been recognized for decades, and humans working in aseptic environments were the only significant source of contamination and therefore posed the greatest risk to both successful production of heat-labile products and to the patient. Over the last two decades, there has been a technological evolution in aseptic processing which has greatly increased patient safety by dramatically reducing the risk of microbial contamination in aseptic processing. The improvements that have occurred in aseptic processing arise from two principal technological features. The first of these is the use of isolator technology and the second is the introduction of effective machine automation and robotics. Isolators have reduced contamination risk by effectively separating the human technician from the aseptic environment. Because isolators are an unmanned environment, which is much smaller in volume than a conventional manned clean room, they can be decontaminated in a manner that effectively eliminates microbial contamination of all kinds. The isolator after sporicidal decontamination is effectively a microorganism-free environment. The capabilities of the isolator have been further enhanced by the application of robots and other forms of automation. An important recent innovation is the introduction of robotics that could be built into isolator systems and decontaminated in place. These specialized robots along with machine automation have further reduced contamination risk and at the same time eliminated the possibility of technician error. The modern isolator system is very well suited to meeting the cell culture requirements necessary for the production of cytotherapeutics, and as a result cell culture isolators are proving to be the best option for the production of many regenerative medicine products.

Keywords Aseptic processing • Isolator technology • Vapor phase hydrogen peroxide • Decontamination • Robotics

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13.1 Introduction

As the therapeutic use of cell-based products for regenerative medicine continues to expand, it is extremely critical that they be supplied free of microbial contamination. There is only one manufacturing method available for cell-based products and that is aseptic processing. The preferred method for manufacturing of sterile medical devices or drug products is terminal sterilization in which the product is sterilized typically by moist heat, dry heat, or ionizing radiation in its final package. Terminal sterilization cannot be utilized for biological products because they cannot withstand physical sterilization methods such as heat or radiation.

Aseptic manufacturing technologies have been widely applied to the manufacture of heat-labile products in the pharmaceutical and biopharmaceutical industries for nearly a century. Over that time a steady but often slow evolution of aseptic manufacturing technology has occurred. It was recognized from the beginning that the greatest source of risk in aseptic processing was the human technician charged with the conduct of aseptic manipulations. Thus, the evolution has in general involved reducing the risk from human contamination since historically aseptic processing could not be accomplished without significant human activity.

The ongoing evolutionary improvement of aseptic processing followed two different but parallel pathways. The first of these pathways was the direct reduction of human contamination risk through improved gowning material, better clean room design, and enhanced environmental controls. The second pathway was through improved processing equipment design. Improving processing equipment generally required an effort to implement *closed* systems in which all product contact equipment could be sterilized in place, which effectively minimizes human aseptic manufacturing. Closed processing proved to be widely applicable to liquid processing, but there remained other processing activities such as filling and sealing the product into its final dosage form, which had to be done with direct human work activity. Over the last three decades, great strides were made in automating the principal packaging steps, which could not be done in closed environments. The automation applied involved both machine automation and robotics.

A more complete approach to elimination of human contamination was first introduced to the pharmaceutical and biopharmaceutical industries in the mid-1980s. This technology in pharmaceutical applications came to be known as “isolator technology”; other aseptic industries may refer to essentially the same device as an aseptic chamber. For the purposes of this chapter, we will use the pharmaceutical industry term isolator technology or simply isolator to refer to this class of devices. The initial application for isolators in the pharmaceutical industry was microbiological testing and specifically the conduct of sterility tests. With the introduction of isolator, the occurrence of false-positives significantly decreased in sterility test,

encouraging the use in manufacturing field. In 1988, the technology was used for manufacturing sterile drugs that cannot be terminally sterilized in Europe. Pharmaceutical firms in the United States first began to use isolator technology for aseptic packaging by the early 1990s. Isolators were introduced in Japan beginning in the early 1990s as well. At the current time isolators have become not only commonplace in the pharmaceutical industry but perhaps the favored method for aseptic processing.

The reader will note similarity between an isolator used for aseptic handing/processing and *glove boxes*, which have long been used for containment of hazardous materials such as radiation, toxic chemicals, or pathogenic organisms. It is important to note that when isolators are used for containment, they are often operated at a pressure negative to the surrounding room environment. Glove boxes also found application in laboratory microbiology when used to create a near-anaerobic environment. There are however key differences between glove boxes and isolators. An isolator can be defined as a controlled environment that meets ISO 14644-1, 2 Class 5 requirements for particulate air quality and which possesses the following operational attributes:

1. There is no exchange of air with the surrounding environment; all air entering the isolator from the external environment must pass through at least one high-performance particulate air (HEPA) filter.
2. The isolator must be decontaminated with a sporicidal agent with the result being an environment inside the isolator enclosure that is essentially germ free.
3. The isolator when used for aseptic processing must operate at a positive internal air pressure when referenced against the external room environment. (This feature serves as an additional precaution against the entrainment of contaminated air from the surrounding external environment.)
4. The isolator must prevent direct human contact with the aseptic work environment and most specifically with the product. To this end all manipulations required are done using glove/sleeve assemblies mounted on the isolator wall panels. Human contamination risk can be further mitigated by the use of robotics and process automation.

Until about 2004 isolators were used primarily in the pharmaceutical and biopharmaceutical industries for either sterility testing or aseptic manufacturing. However, when the first cell processing isolator was invented in 2004, isolators began to be used as the aseptic environment for cell manipulation with increasing frequency. At roughly the same time, small portable isolators where introduced in Europe and the United States for hospital pharmacy aseptic processing applications as well as for aseptic production of radiopharmaceuticals such as positive emission tomography products. At the present time isolators are widely used for the production of personalized therapies in the hospital setting.

13.2 The Art and Science of Aseptic Processing: A Brief Historical Perspective

As mentioned in "Introduction," healthcare products labeled "sterile," which include small molecular medicines, vaccines, protein drugs, as well as recent cell-derived regenerative medicines that cannot be terminally sterilized in their final containers, must be manufactured utilizing some form of aseptic process manufacturing. Aseptic processing generally requires sterilizing filtration of the liquid product stream as well as utilities such as compressed air (or inert gases). Additionally, all other product contact materials such as product containers including vials, pre-filled syringes, etc., and closures must be sterilized and fed aseptically to the equipment which fills the product and seals the container. All aseptic operations are conducted in an environment in which the room air supply, bulk and materials, facility and equipment, and personnel are controlled according to strict standards in order to control viable and nonviable contaminants, thereby minimizing risk to the product [1]. The main source in conventional human-scale clean room aseptic processing is personnel, and therefore, the engineering of facilities and equipment must focus extensively on the reduction of human interventions.

In the initial era of sterile drugs, aseptic processing in the 1940s consisted of manual filling into vials by personnel wearing a surgical face mask, surgical gown, and simple cap. It is not surprising that the personnel dressed much as they would for a surgical procedure as that was considered the apex of aseptic hygiene at the time. The 1950s saw the development and commercial availability of HEPA air filters. This innovation triggered a revolution clean manufacturing operations. In 1960 American physicist Willis Whitfield designed the first true clean room using HEPA-filtered air at Sandia National Laboratories. The clean room concept was very quickly adapted by for the manufacture of healthcare products and also by the aerospace and microelectronics industries.

Gowning materials evolved along with clean rooms and by the 1980s re-washable gowns of microfiber construction were widely available. Gowning materials and accessories continued to improve as a result of industry demand, and full skin coverage gowns with improved filtration properties along with higher air flow rate clean rooms and automation of operations led to much more reliable aseptic processing. However, it was clear that further improvements could only be made by completely separating human operators from the clean workspace through the implementation of isolators. Over the last 20 years, there has been tremendous effort to improve the human-isolator interface initially through ergonomics alone and more recently through the development of the processing equipment and isolator as an integrated whole. This required the design of isolator-friendly equipment that reduced the need for operators to conduct routine interactions with the isolated process using gloves.

In concert with this global trend, the authors began to promote the use of isolators for aseptic processing and microbiology laboratory operations around 1990 and built and validated the first sterile drug manufacturing system based on isolator in Japan in 1994. The installation of isolator-based production systems has gradually

increased since that time. Currently, isolator systems are often the first environmental control option to be examined in the development of a new aseptic processing manufacturing facility for sterile drugs or biologics. Nearly all new vaccine manufacturing facilities, for example, employ isolator-based aseptic manufacturing systems. Figure 13.1 shows the first isolator system exported from the Japan to the United States, for the purpose of vial and ampoule investigation in new drug manufacturing facility.

13.2.1 Quality Control of Sterile Drugs

When validation was first introduced in the 1970s, the general concept driving the effort was that "quality control testing alone can't secure quality." For example, in sterility test, detection rate is very low because only a limited number of product samples are tested. The number tested, most commonly 20 articles, is not enough to guarantee the sterility of product lots that may consist of 10,000–100,000 or more units. If sterility test uses the samples of 10 ml at 10 locations from each lot, the microbial detection rate of 1 ml is less than 0.0105 unit, which means microorganisms cannot be detected unless there is more than one microorganism in 100 ml [2]. This likelihood of contamination shown in this example would only be true if the limit of detection of the method is one cell, which is very unlikely to be the case. Thus, the sterility test falls far short of being able to guarantee the sterility of products. From this viewpoint, a validation approach focusing on the manufacturing

Fig. 13.1 Investigational New Drug (IND) vial-ampoule manufacturing machine Clinical Scale vialampoule aseptic processing isolator system

facility and aseptic process control strategies is required to ensure lot-to-lot product safety. Furthermore, the current philosophy of the International Congress for Harmonization is one based upon Quality by Design (QbD) This approach to process design means that consideration of microbial contamination control must begin very early in the process by which a new facility is designed or by which a new product is added to an existing manufacturing system. In other words, a facility and process must be carefully designed within the defined design space to ensure product safety under all possible operating conditions. In terms of microbiological safety, this means designing so that the probability of microbial contamination is minimized to the greatest extent possible.

13.2.2 Risk and Sterility

Risk management in aseptic processing consists of two different critical factors. The first of these is "risk evaluation/analysis," the goal of which is risk quantification. The other is "risk mitigation" that considers possibility of contamination during facility design, equipment selection, definition of process, and the operation of process itself. Certainly at the present time, risk evaluation is a significant focus within industry, but risk mitigation is by far the most important of the two factors listed above. Risk mitigation can both indicate the level of need for process improvement and determine the priority that should be assigned to mitigation activities. Risk mitigation as it relates to aseptic processing generally means to properly control human intervention, but ultimately if risk mitigation is to be maximized, personnel must be completely removed from critical area of aseptic processing.

13.2.3 Effectiveness of Gowns

It continues to be widely assumed by some scientists and engineers that aseptic conditions can be adequately maintained if the personnel conducting aseptic processing wear aseptic gowns properly and exhibit sound aseptic technique in conducting their work. However, it is clear from published research data that personnel emit very high levels of microbiological contamination into the environment. This is true even when state-of-the-art gowns are chosen and worn properly. According to Ljungqvist et al., personnel wearing previously unused sterilized gowns emit 1500–3000 cfu/h of microbiological contamination even when conducting limited operations [3]. The microbiological contamination released by personnel can increase to ≥10,000 cfu/h during complex operations that require the execution of many physical tasks.

The total number of aerobic bacteria existing on human skin is normally >1.2 million per square meter. The number of microorganisms existing on a healthy human's hands and arms are in the range 0.9–3 million per square meter [4]. In case

of anaerobic bacteria, such as *P. acnes*, the number of organisms present can be several times more than the quantities shown. Microorganisms are emitted by gowned personnel by a pumping effect of the technician's gown (air coming in and out from, for instance, operator's chest depending on the task) during operation. In recent years, advanced technology has been introduced to reduce the possibility of product contamination caused by personnel in manufacturing site of sterile drugs. These technologies often known collectively as *advanced aseptic processing technologies* include Blow-Fill-Seal and Restricted Access Barrier System, in addition to isolators.

13.3 Isolator Technology

13.3.1 What Is an Isolator?

Isolator is defined as a system having an aseptic processing area that is both physically separated from the environment and does not permit direct interventions by personnel. The system generally has continuous supply of fresh or recirculated air through HEPA or ULPA (ultra-low particulate air) filters. This filtered air supply is maintained at a flow rate that results in a positive air pressure in the isolator being maintained relative to the surrounding environment. Isolators that have a direct opening to the surrounding environment are said to be aerodynamically sealed or sealed by air overspill. This simple concept of aerodynamic separation is a well-proven principle, which has been demonstrated to work well. However, many isolators operate without a direct opening to the external environment [5].

Isolator systems do not allow direct interventions by personnel into the enclosure's aseptic processing area. To ensure that contamination is not introduced into the isolator, it is critical to have means to bring materials into and out of the isolator in a way that presents the transfer of contamination into the isolator. The sterility assurance provided by an isolator is a product of physical separation of personnel, elimination of risk of introduction of airborne contamination, and systems that prevent the introduction of non-sterile materials into the isolator. Safe, contamination-free transfer of materials into and out of the isolator can be assured by the use of devices known as decontamination pass boxes and circular-shaped rapid transfer ports (RTP); also decontamination interfaces for attaching devices to isolators have been developed (Fig. 13.2).

13.3.2 General Features of an Isolator

Aseptic processing that relies on the use of a conventional clean booth or a clean room approach is always at risk from contamination contributed by human operators and technicians. This unavoidable fact makes contamination control difficult. In

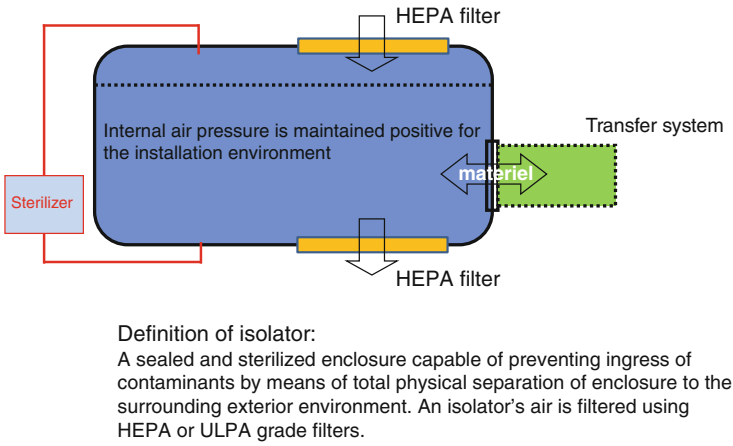


Fig. 13.2 What is an isolator?

reality, effective countermeasures against contamination in conventional clean booth bring with them massive increases in facility and operational costs. In contrast, the most significant advantage of an isolator system is the comparative ease with which an aseptic environment can be maintained. Isolators have the added advantage of minimizing the actual volume of clean space that must be maintained in an essentially sterile manner. This allows a dramatic increase in operating costs through reduced energy consumption and the elimination of a need for consumables such as aseptic gowns.

ISO 5/Grade A environments are required for the critical area of aseptic processing. The critical area in aseptic processing is defined as the part of a facility where product and sterilized components are brought together and filled and/or assembled. It is necessary to install and operate this ISO 5/Grade A critical zone environment within an ISO 7/Grade B environment and, of course, to provide proper gowning and support facilities to ensure safe, contamination-free personnel and component entry. Alternatively, in some modern facilities, the entire aseptic processing room can be designed to ISO 5/Grade A requirements; however, such a facility is both expensive to build and also to operate.

In contrast, an isolator for aseptic processing of pharmaceuticals is installed in a much less expensive ISO 8/Grade D environment; therefore, the strict clean room-style aseptic gowning of personnel is not necessary [1].

Additionally, the surface area in clean rooms is typically about twice as large as that of isolators [6]. Because an isolator is a microbiologically closed environment which can be efficiently treated with sporicidal agents, the inherent risk of microbial contamination is extremely low. The essentially sterile condition inside the isolator is readily maintained by design features such as air filtration and overpressure, which prevent the ingress of contaminations from the surrounding environment. According to authors' experience, the sterile condition inside an isolator can be maintained for one month or more, based on microbial recovery studies done inside

an operational isolator using active air samplers and passive air samplers such as settle plates [5]. In contrast, in unidirectional air flow cabinets (clean booths) or manned clean room facilities, product contamination is prevented by maintaining asepsis using unidirectional flow under HEPA filter and avoiding human intervention in critical area as much as possible. Thus, sterility assurance is depending upon personnel training and maintaining strict control of both air flow and work flow of personnel throughout the workspace.

A potential disadvantage of isolators is reduced productivity owing to restricted or inconvenient work access using gloves. Therefore, it is essential to design and fabricate the isolator system with careful attention to ergonomics. It is always best for complex operations to fabricate mock-up isolators in which human interactions with critical equipment through isolator gloves can be evaluated prior to construction. It can be very difficult to correct ergonomic deficiencies in the field after an isolator is built and installed. With this fact in mind, a careful evaluation of ergonomics often using a mock-up isolator fabricated from wood panels or framework is a necessary design step. Our experience has taught us that compromising on operator access and comfort in the design of an isolator is never a good idea. It can result in designs that are unsafe both to the operator and to the product.

13.3.3 The Isolator: A Very Brief History

Around the end of the 1950s, devices that have similar characteristics to a modern-day pharmaceutical isolator had already been used in European nuclear power industry to protect personnel from risk associated with handling radiation and high toxic materials, such as plutonium. The main function of these isolators was protection of personnel. Later, the technology was utilized in laboratories in which studies were conducted on pathogenic microorganisms, and as previously discussed isolators were then used in the healthcare manufacturing industry in analytical microbiology laboratories for sterility testing.

The first aseptic processing isolator was installed at a European pharmaceutical firm around the end of the 1970s for the purpose of sterility test. In 1981, the second isolator was installed, consequently decreasing false-positives due to personnel significantly, boosting attention of its use for sterility test.

The isolator for manufacturing sterile drugs was first installed at Baxter's Round Lake Factory (United States) and Valencia Factory (Spain), followed by SyntheLabo, Merck, and Novartis. The installation of isolators began to increase more rapidly around 1990, although it can be argued that regulatory compliance uncertainties slowed the pace of implementation until the early years of the twenty-first century.

In Japan, Shibuya Kogyo started to sell isolators in 1992 and installed the first system in Japan in 1994. Then in 1995, the company installed one of the largest and most complex aseptic production isolator systems in the world at a pharmaceutical firm in western Japan. Since that time isolator technology has become widespread in its use in Japan and throughout the developed world.

13.4 Development of Room Decontamination Technology

One of the key operational features of isolators is the ability to create an essentially sterile environment within the enclosure. Both gas and vapor phase sporicidal decontamination agents (sterilants) have been used for such to eliminate bioburden from the isolator enclosure. It is necessary that agents used to decontaminate isolators be both effective sporicides and capable of being used at or very near room temperature. It is important that agents used to decontaminate an isolator desorb readily from the isolator enclosure, equipment, and contents. It is also important that a decontamination agent has a manageable safety profile and that it also is not prone to producing toxic residues. The need for decontamination to be done at generally low temperatures and yet operate quickly and without toxicity has limited the available choices of suitable agents.

13.4.1 Room Decontaminant and Disinfectant

Ethylene oxide was considered a candidate sterilant in the early days of isolator technology but was quickly ruled out because of its toxicity and various safety concerns. Ethylene oxide is both a carcinogenic and mutagenic compound. Formaldehyde was also considered for use with isolators; however, it is also now known to have serious toxicity issues; it is an alkylating agent classified by the IARC as a Group 1 carcinogen. Attempts have been made to use ozone for the purpose of both isolator decontamination and clean room disinfection. Ozone is a powerful oxidant which is a strong sporicide given a high enough concentration and sufficient humidity. Ozone also has the advantage of decomposing rapidly into O_2; however, it also causes system design problems because it is highly corrosive and can adversely affect materials used to construct equipment that is often located inside isolators including metals subject to oxidation.

The agent currently available that has the best set of properties overall for isolator decontamination is hydrogen peroxide which can be utilized either as a fine mist or as a vapor. Hydrogen peroxide is a compound that degrades into O_2 and H_2O, a reaction that can be facilitated by a mixed bed catalyst. Most plastics adsorb H_2O_2 vapor to only a limited degree and outgas rather quickly during aeration using fresh air. Many metals and glass do not adsorb H_2O_2, and since these are common materials of isolator construction, this means reasonable decontamination process times of about 3–8 h can be readily attained. H_2O_2 is not without problems as a decontamination agent; it is corrosive at high concentrations and must be handled and stored properly.

13.4.2 Decontamination by Vapor Phase Hydrogen Peroxide (VPHP)

Sporicidal ability of VPHP was discovered in the late 1970s. The initial research and development was focused on its use as a decontaminating agent of facilities and equipment of hospitals. In the late 1980s, as isolator technology started to emerge in the pharmaceutical and biopharmaceutical industry, experiments were done to determine the suitability of VPHP for this application [5]. Once successes were reported, VPHP began to be used as the primary method for isolator decontamination.

The most common biological indicator (BI) species used to evaluate VPHP decontamination of isolators is *Geobacillus stearothermophilus*. USP General Chapters <1229.5> BIOLOGICAL INDICATORS FOR STERILIZATION. Other microorganisms stated as suitable include *Bacillus subtilis* and *Clostridium sporogenes*, although these are rarely used due to the handling advantages associated with *G. stearothermophilus* and the fact that it reportedly has higher resistance to VPHP. Currently, commercialized BIs typically utilize *G. stearothermophilus* ATCC12980 spores inoculated on stainless steel carriers that are packaged in Tyvek envelopes (see Fig. 13.3).

Hydrogen peroxide is in liquid phase at ambient temperature. Generally, it is supplied as hydrogen peroxide solution and a 35 % hydrogen peroxide solution most commonly used for vaporization

Vapor hydrogen peroxide was, in the early years of its use for isolator decontamination, believed to sterilize in the vapor phase, but in 2002, Watling et al. published a report indicating that microcondensation was a prerequisite for an optimally effective VPHP process [8]. Decontamination systems that purport to operate only in the vapor phase and other systems that rely on condensation on surfaces are marketed. Systems of each type have been successfully installed and validated.

In both cases, decontamination was achieved by diffusing heated and vaporized hydrogen peroxide into the isolator. It is considered that kill effect toward virus and microorganisms is achieved by the formation of the OH radical. Hydrogen peroxide is an extremely powerful oxidant and is capable of causing irreparable oxidative damage to fatty acids, biological membranes, proteins, and DNA. It is listed and approved as a decontaminating agent of isolator in various international guidelines and standards. At liquid phase of solution, it is relatively stable chemically; however, in the vapor phase, it degrades rather quickly, although this too is subject to debate. The half-life measured by some authors was about 10 min [9], although Watling et al. have suggested that the half-life can be as much as several hours once material condenses on surfaces. Given the decomposition of H_2O_2 into water and oxygen, it can be considered an environmentally friendly decontaminating agent.

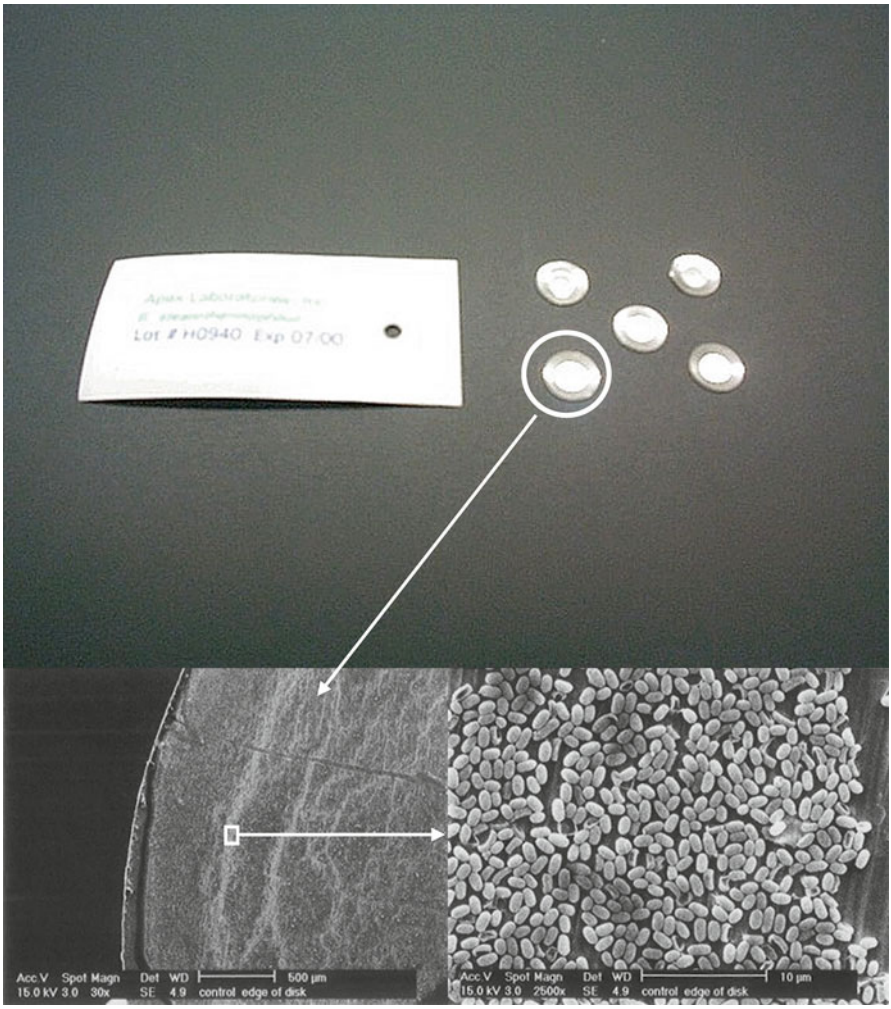


Fig. 13.3 Biological indicator for VPHP decontamination

13.4.3 Development of Vapor Hydrogen Peroxide Decontamination System

The authors of this chapter have done independent studies on VPHP which resulted in the development of a commercial VPHP decontaminating system. The typical approach to VPHP decontamination at the time was to dehumidify isolator enclosure typically to 10–30 %RH, supply vapor hydrogen peroxide continuously, and by so doing attempt to maintain a defined vapor concentration. This decontamination approach took a relatively long time and used considerable hydrogen peroxide for each decontamination operation. We were able to successfully develop a new

approach to VPHP decontamination by 2002 and were able to introduce this system commercially shortly thereafter [9].

Using the process we developed, we found that in most cases, it was possible to complete the decontamination cycle in about a half of the time required for the conventional cycle. This newer decontamination process consists of three phases: (1) rather rapid injection of a suitable predetermined volume vaporized hydrogen peroxide shown to be sufficient to achieve decontamination, (2) an adequate hold time, and (3) aeration.

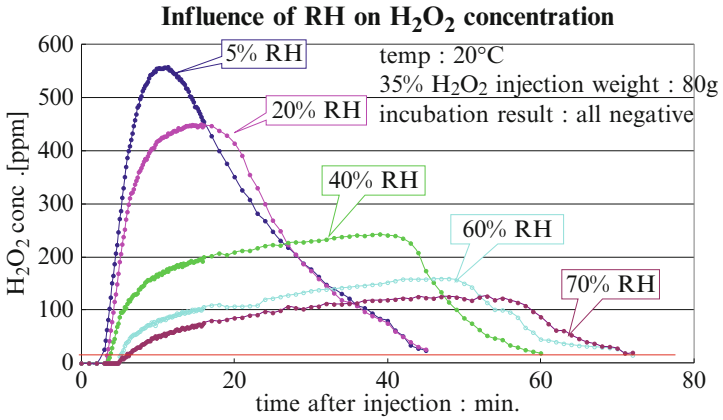
In the conventional approach, humidity control was necessary, but the new approach does not require dehumidification or ongoing humidity control. We found that this process was suitable for use at the standard temperature and humidity conditions present in most production facilities or laboratories (Table 13.1). Figure 13.4 shows that unlike in the conventional approach to VPHP decontamination, it is not necessary to maintain a high concentration of H_2O_2 in air in order to inactivate a 10^6 population of *G. stearothermophilus* spores. These results are consistent with the deposition of vapor onto surfaces through condensation and are similar in that

Table 13.1 Influence of injection weight and relative humidity on decontamination effect

＜Test temperature : 20°C＞

R.H / Injection weight	70%RH	60%RH	40%RH	20%RH	5%RH
100g			0/30		
80g	0/30	0/30	0/30	0/30	0/30
60g	22/30	6/30	1/30	3/30	17/30
40g	28/30	27/30	13/30	26/30	28/30

D value of BIs = 1.6 min positive/total number of BIs
RH: Relative Humidity
BIs: Biological Indicators



RH: Relative Humidity

Fig. 13.4 Relative humidity at the start of a vapor phase H_2O_2 process as a process variable

regard to the process conditions described by Watling et al. It also showed that dehumidification is not necessary before injecting vapor hydrogen peroxide into the isolator at the normal temperature and humidity conditions found in the room or clean room where it is normally installed. The elimination of the dehumidification phase and the reduction in the volume of hydrogen peroxide solution used (typically about 50 % of that used by the conventional process) result in a decontamination process time that is roughly 30–50 % less than that observed using other methods.

13.4.4 Decontamination Efficacy

In the pharmaceutical industry, it is expected that the isolator enclosure should be subjected to a decontamination process capable of achieving a four- to six-spore log reduction of *G. stearothermophilus* biological indicators (BIs). The same decontamination requirement applies to the materials introduced to isolator enclosure. To verify the decontamination process, BIs with an initial population of 10^6 are utilized. A six-spore log reduction is the decrease of the initial spore population to $10^0 = 1$. Total kill of all spores is not required to demonstrate a six-spore log reduction. PIC/S's recommendation describes a verification method using BIs as follows [10]: "…if there are 2×10^6 spores in the BI to start with then there will be 2 surviving spores after a six log reduction. If there are no survivors, then a six log reduction is confirmed and there is an additional safety margin the size of which is not known. If there are other ways to verify delivery of the gassing process to all the target surfaces, supported by a well established mechanism of lethality, these may be considered." In other words, if BIs exposed to a decontamination process are incubated in liquid media, no growth is observed, and a minimum of a six-log reduction of decontamination efficacy is confirmed.

It should be noted that the decontamination expectations for isolators used for pharmaceutical manufacturing are very conservative. Since it is known that most vegetative flora are far less resistant to VPHP decontamination than *G. stearothermophilus* spores and the bioburden in an isolator prior to decontamination is likely to be no more than 50 CFU, it is clear that it is not necessary to kill 10^6 spore populations to have a safe and effective process. It is likely that for many, if not most aseptic isolator applications, a spore log reduction of four or even three logs would sufficiently demonstrate that there would be no suriviving bioburden.

13.5 Application of the Isolator to Cell Culture Facilities for Regenerative Medicine

In November 2013, the new Act for the Promotion of Regenerative Medicine and Revised Japanese Pharmaceutical Law were approved at the Japanese Diet with a scheduled enforcement in November 2014. As a result of this legislative action, it can be expected that the use of regenerative medicine therapies will increase rapidly

in Japan [11]. Against this backdrop, the world's first clinical trial using induced pluripotent stem cells (iPS) is under way in Japan. Over the next decade, there is every reason to expect an increased industrialization and commercialization of regenerative medicine. The others developed the world's first half-suit-type tissue engineering isolator system in 2004 to apply isolator technology in the area of regenerative medicine. This chapter describes the authors' efforts in the development of isolator systems for regenerative medicine as well as the developed of automated cell culture system which can be installed in isolators.

13.5.1 Development of Cell Culture-Related Technologies

An important development which has helped make automated cell culture in isolators possible was the world's first VPHP-resistant aseptic robot in 2003. The robot was developed not for the purpose of cell manipulation, but rather for automation of environmental monitoring in isolators. The robot, which could resist VPHP decontamination, was capable of conducting all commonly used forms of environmental monitoring including passive air sampling, active air sampling, and surface sampling using a swab. This VPHP-resistant robot was installed in a vial filling isolator system. This automatic environmental monitoring system attracted the interest of USFDA, but did not lead to integration in an actual filling facility. This robot, however, was used to conduct various basic tests, which played a vital role in the development of automatic cell culture system after that. One of the tests done using this specially designed sterilizable robot was an experiment to examine the reproducibility of surface monitoring using swabs. The following table (Table 13.2) presents part of these test results.

A certain number of microorganisms were inoculated on 25 square centimeters of stainless plates to test detection of swabbing. The result showed that robot had very high detection rate when compared with those obtained by human samplers.

Figures 13.5 and 13.6 compare the pressure force applied by human samplers to that of the robot on stainless steel plates. The pressure pattern, which was found to be extremely consistent with the robot, but not surprisingly, varied greatly with human samplers. Different samplers are included by red and blue colors on the figure and demonstrate the variability observed among operators. The consistency and reliability of the robot at performing a repetitive sampling task was outstanding and provided convincing evidence that robotics in isolators could be a promising technology for both aseptic production and laboratory operations.

In 2006, we developed a versatile glove-type cell culture isolator system which was equipped with an integrated developed vapor hydrogen peroxide generator (see Fig. 13.7). This isolator was employed at hospitals that conduct regenerative medical treatment based on the Medical Practitioners Act, in addition to research institutions including universities. This isolator-based cell culture system operated very successfully in the field and again confirmed the benefits of isolator technology combined with robotics. Isolators with integrated VPHP decontamination systems and equipped with pass boxes are typically portable units mounted on wheels so that

Table 13.2 Swab efficiency of robot vs. manual operation
Table 13.2 shows a comparison of swab surface sampling performance in environmental monitoring between aseptic robot and operators
This is a table comparing the performance of environmental monitoring swabbing by aseptic robot with manual operation by human operators
This part indicates the test condition
A certain number of microorganisms were inoculated on stainless steel plates (25 cm^2) to test detection by swabbing
The incubation results are indicated in percentages. As you can see, the results show that the detection rate is much higher with robot and quite low with human operators

	Number of CFU included			Incubation result	
	Mean	CD	CV%	Positive/total	%
Robot	29	8.01	27.78	17/20	85
	29	6.25	21.94	18/20	90
	28	6.56	23.16	13/20	65
Manual	22	6.11	27.36	0/10	0
	13	5.35	41.19	2/10	20
	28	6.14	22.32	1/10	10
	23	6.55	28.18	3/10	30

Microorganisms; *M. luteus* NBRC 13867
Carriers: Small stainless steel plate (25 cm^2)
The carrier surface was polished to 320 grid

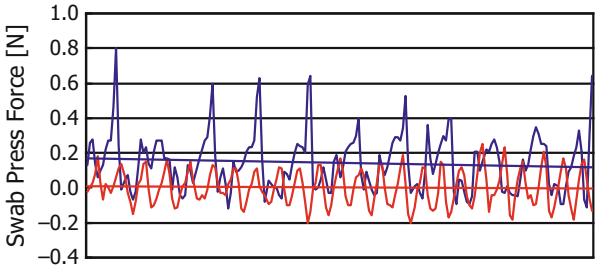


Fig. 13.5 Swab press force variation (manual)

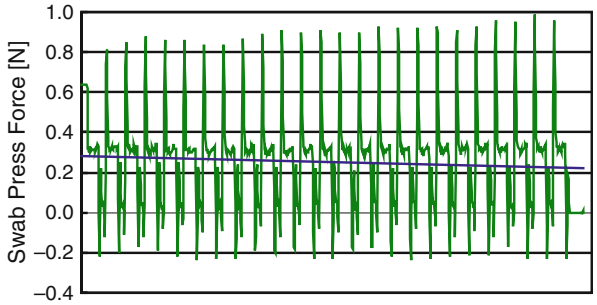


Fig. 13.6 Swab press force variation (robot)

Fig. 13.7 Cell processing isolator

they can be relocated within a facility as required. In the authors' opinion, these isolators have a great deal to offer both research laboratories and healthcare facilities working in the fields of cytotherapy, radiopharmaceuticals, or hospital pharmacy. These systems are capable of providing an aseptic environment of the highest quality without the need for high-cost surrounding facilities. They also obviate costs associated with consumables such as gowns as well as the high ongoing energy costs typical of human scale clean rooms.

13.5.2 Three-Dimensional Cell Incubation

Human and animal cells can be expanded in number through cell culture, but historically it has not been possible to culture cells in a manner that produced the three-dimensional characteristics that exist in human organs and tissues. An important issue in regenerative medicine is developing cell culture approaches that result in three-dimensional tissues. Currently, employed methods to achieve three-dimensional cell culture include porous property carriers, stacked cell sheets, spheroid formation, and airborne incubation using a magnetic field. Generally, collagen, hydrogel or surface-treated PET nonwoven fabrics are used to form a porous carrier. Flat cell sheets, which can be stacked and incubated in Petri dishes, can also be used to achieve three-dimensional cell growth characteristics. Spheroid formation is done by growing cells on small spheres generally in the >100 um range. There are

now several commercial systems to grow cells in 3D spheroids, including systems that are compatible with high-throughput screening (HTS) instrumentation.

Against this backdrop in 2010, Mr. Koichi Nakayama, an assistant professor of Department of Orthopaedic Surgery Graduate School of Medical Sciences, Kyushu University (currently a professor of Graduate School of Science and Engineering, Saga University), requested our collaboration to design and build a system in which Dr. Nakayama could grow three human tissues (organs) through the use of stacking spheroid cultures. The result of this collaboration was the world's first 3D bioprinter (Fig. 13.8). This system recognizes spheroids (sized from 0.2 to 0.5 mm) sunk at the bottom of 96-well plate and is able to manipulate the spheroids using a fine needle which can pick and place a single sphere using vacuum. The printer can then build a three-dimensional structure by placing the spheroids onto needles in water. This is commonly called "kenzan" (spiky frog in English) because the shape of needles mounted on a plate is similar to a "kenzan," which is a special flower holder for Japanese flower arrangement. The configuration of the three-dimensional structure built here can be preprogrammed into the equipment control system. The 3D printer system can readily handle several different types of spheroids. Because this system requires a very large number of cells, we developed an automatic cell processing system (CellPRO) for mass cell culture in 2012.

The 3D printer system can create different types of three-dimensional structures and received considerable media coverage; Prime Minister Abe was among the visitors who got a firsthand look at the system.

Fig. 13.8 Bio 3D printer

13.5.3 Development of Cell Incubation Isolator, CPi, and CellPROi, an Automatic Cell Incubation System

The CellPRO system first developed in 2012 did not utilize isolator technology; it was a unidirectional air flow hood or clean booth ISO 5/Grade A system for animal testing. In 2014, we completed an isolator-based cell culture isolator known as CPi (Fig. 13.9), which was a manual system relying on human technicians working with glove access. Also, in 2014 we introduced CellPROi, which was designed and engineered to handle cell culture for cytotherapeutic products in clinical trials. CPi is a manual system utilizing a hybrid design that blends the functions of clean rooms and isolators. CPi has proven to be an extremely capable system, which can produce very high levels of sterility assurance. The CellPROi is an automated isolator-based system capable of the highest level of process safety and sterility assurance.

The left chamber serves as the workstation, while two chambers shown on the right are pass boxes that are used for decontamination and aeration of materials that will be introduced into the isolator. If the system is used as a clean room, these two chambers can be used as ISO 6/Grade B or ISO 7/Grade C clean rooms. A manual isolator like CPI is highly flexible and allows considerable unrestricted access when operated as an open clean room-like system. This type of system is quite suitable for the product research phase, but for the product commercialization phase, the potential for product loss is a considerable economic risk. Some of the factors affecting

Fig. 13.9 CPi (cell processing isolator)

the product cost are facility cost for cell manipulation, the facility's operating expenses, consumable material cost including media, and labor costs associated with scientific and technical personnel. In order to minimize overall costs while at the same time minimizing risk of product loss, the authors chose the automated isolator-based cell culture system using dual-arm robot. The basic specifications for system development included an isolator system, 6 LRV decontamination capability, dual-arm robot system, manual operation for component/supply introduction, as well as the ability to add fully automation to the system at a subsequent time. CellPROi was designed and developed around this fundamental specification (Fig. 13.10).

Although CellPROi is an automatic system, it is not limited to single production requirement or strategy. CellPROi is designed to be flexible enough that various cell production strategies and programs can be readily accommodated. On the left, there are two incubators that can be aseptically connected and disconnected. CellPROi and CPi are installed at the Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, which is assisting in the optimization CellPROi and CPi performance. At the time this chapter is being written, the team is undertaking process optimization while conducting full-fledged clinical trials to deepen the autologous bone marrow cell infusion therapy for the treatment of hepatic disease under the leadership of Professor Isao Sakaida.

Fig. 13.10 CellPROi

13.6 Summary

Isolator technology was first introduced in the country of Japan in the early 1990s, and large-scale isolator systems had been installed and validated by 1995. Beginning in about 1995, there has been continuous increase in the number of isolators installed in Japan and throughout the developed world. Isolators are now widely used in all aspects of aseptic drug and biologic and medical device products. Isolators are also widely used in active pharmaceutical ingredient manufacturing and bulk sterile biologic manufacturing. Additionally, isolators are now widely used in microbiological testing of products as well as in research and development. There is ample data to confirm that isolators are highly reliable systems, which can be used to create optimal aseptic conditions.

At the present time work is ongoing to further expand the use of isolators into other areas of healthcare that are not presently benefiting from the contamination control capability of these systems. At the same time efforts are under way to further automate processes operated in isolators to improve both functional and economic efficiencies.

One of the most promising new developments in healthcare is the development of regenerative medicine products. The authors believe that isolators provide an ideal environment for the manufacture of these and all cytotherapeutic products. We have already found that the isolators offer both a product safety and operational economy advantage in the production of regenerative medicine products. Japan has taken a leadership role in the commercialization of regenerative medicine products. The world's first clinical trial using iPS is under way, and work on products using stem cells of varying origin is under way in Japan and around the world. Collectively this work has enormous potential and is thought by many scientists to be capable of completely revolutionizing medicine.

We have entered an era in which translational medicine is pointing the way to the future. Translational medicine calls for the progress of promising new therapies from the research bench to the clinic as rapidly as possible and without undo regulatory delay. This approach to bringing new therapies and medicines to commercialization will require a new approach to facility design and aseptic operations because many new products will be made in clinics and delivered directly to the patient. In many cases these will be products arising from autologous cells or customized specifically to meet the treatment requirements of a single patient. Isolator systems operating under a defined specification will help ensure that in this era of cell-based custom treatments, we are able to assure the greatest-level sterility assurance with a high degree of reliability and robustness. Intelligent use of isolator systems and customized automation to suit specific processing requirements will facilitate the implementation of translational medicine and the safe administration of a new generation of healthcare products.

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Chapter 14
Cell and Vector Production Facility for Gene Therapy and Cell Therapy

Junichi Mineno

Abstract Approximately 25 years has passed since the first clinical study of gene therapy was performed, and many good results have been reported in the last 10 years. However, Japan shows a remarkable delay compared with the West in the clinical application of gene therapy, and one reason is that organizations manufacturing bioproducts for conducting high-quality clinical studies have not been fully established in Japan. Recently, the Japanese government introduced the Regenerative Medicine Promotion Acts for accelerating the implementation of "Regenerative Medical Products" including gene therapy and cell therapy. These Acts allow the outsourcing of the production of drug products used for clinical research and also the cell processing which were previously only permitted to do at clinics. We have now constructed a large-scale facility for cell and vector production for gene therapy and cell therapy as a CDMO (Contract Development and Manufacturing Organization), which provides clients with comprehensive services from drug development through manufacture, based not only on Japanese GMP but also on cGMP, EU GMP, and other related regulations and on the Cartagena Act to prevent the dispersal of living modified organism.

Keywords GMP manufacturing • CDMO • Gene therapy • Cell therapy • Regenerative Medical Products

14.1 Introduction

In November 2012, researchers studying gene therapy received excellent news: the first gene therapy product had been granted marketing authorization by the European Commission, allowing it to be sold throughout the EU. Since 1990, after the first gene therapy clinical study "ADA-SCID ex vivo stem cell gene therapy" was

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performed at NIH in the USA, more than 2000 protocols have been performed worldwide (http://www.wiley.com/legacy/wileychi/genmed/clinical/), mainly in the USA and Europe. During the past decade, many positive results about clinical studies have been reported for diseases including progressive metastatic melanoma (TCR gene therapy), Leber's congenital amaurosis, chronic lymphocytic leukemia (CAR gene therapy), and metachromatic leukodystrophy and about clinical trial of Glybera (Table 14.1) [1–16]. Glybera, which is a lipoprotein lipase (LPL) gene-modified AAV vector for LPL deficiency, is the first gene therapy available in Western countries, whereas there are already four products approved in more local governments such as in China (Gendicine and Oncorine, both of which are adenoviral vector-based products), the Philippines (Rexin-G, recombinant gamma-retroviral vector), and Russia (Neovasculgen, recombinant plasmid vector) in 2003, 2005, 2007, and 2011, respectively. In concert with the recent successes of clinical studies, major pharmaceutical companies have expressed interest in gene therapy and have acted. GlaxoSmithKline announced a strategic alliance with the Telethon Foundation and the San Raffaele Foundation to research and develop novel treatments to address rare genetic disorders using gene therapy performed on stem cells taken from the patient's bone marrow (http://www.gsk.com/media/pressreleases/2010/2010_pressrelease_10113.htm). Novartis and the University of Pennsylvania formed a broad-based R&D alliance to study CAR gene therapy for the treatment of cancer (http://www.novartis.com/newsroom/media-releases/

Table 14.1 Examples of gene therapy clinical trials in the recent years

Disease	Trial site	Published year	Reference
ADA-SCID gene therapy	NCI, Hokkaido U	1990	[1]
X-linked chronic granulomatous disease (CGD)	NIH, Frankfurt, Seoul	1977	[2]
Gene therapy of X-SCID disease	INSERM	1999	[3]
Progressive metastatic melanoma (TCR gene therapy)	NCI	2006	[4]
HIV infection	U Penn	2006	[5]
Leber's congenital amaurosis (LCA)	U Penn, UCL	2008	[6]
X-linked adrenoleukodystrophy (ALD)	INSERM	2009	[7]
Parkinson disease	Jichi MU, UCSF	2010	[8]
β-thalassemia	INSERM	2010	[9]
Wiskott–Aldrich syndrome	Hannover MS	2010	[10]
Lipoprotein lipase (LPL) deficiency	AMT	2010	[11]
Hemophilia B	UCL, St. Jude CH	2011	[12]
Chronic lymphocytic leukemia (CAR gene therapy)	U Penn, MSKCC, NIH	2011	[13]
Metachromatic leukodystrophy (MLD)	TIGET	2013	[14]
Acute lymphocytic leukemia (CAR gene therapy)	MSKCC	2013	[15]
Choroideremia	U Oxford	2014	[16]

en/2012/1631944.shtml). Baxter acquired Chatham Therapeutics' gene therapy technology for Hemophilia B (http://www.baxter.com/press_room/press_releases/2014/04_02_14_chatham.html). Amgen acquired BioVex, which had been developing an oncolytic vaccine OncoVEX (GM-CSF) to treat melanoma and head and neck cancer (http://www.amgen.com/media/media_pr_detail.jsp?releaseID=1519312). More than 2000 gene therapy clinical study protocols have been performed worldwide, but only approximately 45 of these have been performed in Japan, including both clinical research and clinical trials (http://www.nihs.go.jp/cgtp/cgtp/sec1/gt_prtcl/prtcl-j3.html). There are several reasons for this remarkable delay in the clinical applications of gene therapy in Japan compared with the West despite the presence of high-level basic research. Major examples include the following: (1) the drug approval process in Japan is complicated, and the guidelines are not fixed; (2) support organizations manufacturing viral vectors and gene-modified cells for performing high-quality clinical studies are not well established in Japan; and (3) the public research spending on gene therapy has been deficient, and the supporting basic research has become weak in Japan. We, Takara Bio Inc., have a long history in the gene therapy field. In 1995, we developed RetroNectin, which is a fusion protein containing the C-domain, heparin-binding domain, and CS-1 site of human fibronectin. We found that it significantly enhanced the transduction efficiency of lymphocytes and stem cells, which express integrin alpha4beta1 (very late antigen-4) and alpha5beta1 (very late antigen-5), using retroviral vectors [17], and it quickly became a standard method for gene transfer by retroviral vector worldwide. We have since been developing our own gene therapy clinical studies in both Japan and the USA. During these 20 years, we have constructed four facilities, one RetroNectin GMP facility, one viral vector GMP facility, and two GMP cell processing centers. Recently, we have constructed a new, large GMP facility (Center for Gene and Cell Processing, CGCP) for "Regenerative Medical Products" including gene therapy and cell therapy to provide clients with comprehensive services from drug development through manufacture as a CDMO (Contract Development and Manufacturing Organization).

In this chapter, we will describe the construction of the facility for manufacturing "Regenerative Medical Products" to help address reason no. 2 above.

14.2 Worldwide State of Biologics Manufacturing Facilities

14.2.1 USA

The Association of Academic Biologics Manufacturers (AABM) has been established, and AABM-registered facilities offer manufacturing services for drug products such as viral vectors, cells, vaccines, and recombinant proteins. According to their website, their mission is "to establish a cooperative network of non-profit

academic based biologics manufacturers as a national resource to bring together clinical investigators with manufacturing capacity and expertise, develop and share facility operations expertise amongst members, provide quality, manufacturing and regulatory training opportunities, and hold annual meetings" (http://www.aabmonline.org/). Forty-six facilities are registered, and researchers who would like to perform clinical studies with their seeds can search for facilities that fit their purposes. For example, 15 facilities are registered for manufacturing retroviral vectors, including the Indiana University Vector Production Facility, which is one of the most experienced veteran facilities, and the Center for Cell and Gene Therapy at Baylor College of Medicine, which is one of the largest facilities in the USA and contains 22 ISO 7 clean room suites. Only two facilities are registered for human iPS cells, the Cincinnati Children's Hospital Translational Core Laboratory (CCHTCL) and PLACEMA. However, they cannot manufacture human iPS cells under the GMP. Instead, CCHTCL provides iPSC characterization assays including flow cytometry for markers of pluripotency and differentiation, teratoma formation analysis, and DNA methylation analysis; there is no detailed information about PLACEMA, but the AABM website states that they provide research-grade material.

14.2.2 Europe

There is no consortium, but some NPOs and companies such as Genethon in France, MolMed in Italy, and Oxford BioMedica in the UK actively offer gene therapy services. Genethon, the AFM-Telethon (the French muscular dystrophy association) laboratory, is an NPO, and their mission is "to design gene therapy products for rare diseases, to ensure their pre-clinical and clinical development as well as their production in order to provide patients with access to these innovative treatments" (http://www.genethon.fr/en/). Genethon has one of the largest capacities for drugs for gene therapy in the world. MolMed and Oxford BioMedica are CMOs (Contract Manufacturing Organizations), and they have also been developing their own gene-based medicine pipelines, such as genetic engineering of T cells from an HSC donor to express an HSV-TK gene for high-risk leukemia (MolMed) and a lentiviral vector expressing two genes encoding antiangiogenic proteins for age-related macular degeneration (AMD) (Oxford BioMedica).

14.2.3 Japan

There are many universities with cell processing centers, but few facilities can be maintained adequately, largely because of limited budgets. Further, there are few facilities offering the services of a CMO, to the best of our knowledge.

14.3 Construction of the Facility for Gene Therapy and Cell Therapy

14.3.1 Steps for Constructing the Facility

The steps for constructing the facility for gene therapy and cell therapy drug products are as follows:

1. Establishing a user requirement specification (URS)
2. Making a validation master plan (VMP)
3. Designing the facility
4. Design qualification (DQ)
5. Construction
6. Installation qualification (IQ)
7. Operational qualification (OQ)
8. Performance qualification (PQ)
9. Process validation (PV)

Between the steps, system impact assessment (SIA) and qualification risk management (QRM) will be performed on a case-by-case basis.

In the URS, all the requirements of the facility are recorded, including manufactured products, the processes for manufacturing each products, the basic plan for flow lines of materials, products and personnel, the conditions for each suite, the requirements for each room, requirements for utilities and air-conditioning, equipment for each production line, electrical equipment, and requirements for monitoring systems and manufacturing execution systems, and applicable regulatory requirements (e.g., laws, regulations, guidelines) are defined.

Our facility is multipurpose, with the capacity for manufacturing several types of cells (lymphocytes, iPS cells, iPS cell-derived differentiated cells, genetically engineered cells), vectors (plasmid vectors and viral vectors such as adenoviral, gammaretroviral, lentiviral, AAV, HSV, and HVJ), recombinant *E. coli* for plasmid vector production, and recombinant protein production and also for protein purification, aseptic filling of bags and vials, lyophilization, and QC. The manufacturing area is physically separated from spaces such as the *E. coli* culture area, the protein and plasmid purification area, the viral vector production area, the cell processing area, and the aseptic filling area. In particular, the *E. coli* culture area should be completely separated from other areas both with independent air-conditioning systems and independent water supply and drainage systems, as well as independent personnel movement. As a general outline in the URS of our facility, the following matters were emphasized: prevention of cross-contamination and mix-up and thorough pest control, with a one-way system for flow lines of materials, products, and personnel, is adopted; biohazard products are thoroughly contained; a backup power supply system is included for constitutive production and quality control, measures for energy conservation are promoted; and the facility must ensure sufficient space for maintenance.

The VMP puts forth the validation plan, including the applicable scope and implementable scope. The validation organization is defined, and applicable regulatory requirements are defined again. In our case, the facility meets Japanese GMP, ICH GMP, and the following laws and guidelines:

- Pharmaceutical Affairs Act
- Standards for Manufacturing Control and Quality Control of Investigational Products (Investigational Products GMP) (PFSB Notification No. 0709002)
- Ministerial Ordinance on Standards for Manufacturing Control and Quality Control for Drugs and Quasi-drugs (MHLW Ministerial Ordinance No.179)
- Regulations for Buildings and Facilities of Pharmacies etc. (MHLW Ministerial Ordinance No.10)
- Guide to Good Manufacturing Practices for Medical Products (2013 PIC/S)
- Current Good Manufacturing Practices for Finished Pharmaceuticals (Code of Federal Regulations title 21, part 211)
- EU Guidelines for Good Manufacturing Practices for Medicinal Products for Human and Veterinary Use
- Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Cartagena Act)

The drug products should be made in compliance with GMP, and the gene therapy and cell therapy products for clinical research performed under the Medical Practitioners Act should be produced in compliance with Investigational Products GMP and the Guidelines for Human Stem Cell Clinical Research in Japan (2006/09/01). Because the products we plan to manufacture in the facility include gene-modified organisms such as viral vectors for gene therapy that are then used for making genetically engineered cells, we should consider making the facility (or those areas) comply with the Cartagena Act. Furthermore, because the products will be used in the international clinical trial, the ICH GMP, cGMP, EU GMP, and PIC/S (Pharmaceutical Inspection Convention/Pharmaceutical Inspection Co-operation Scheme) have been selected as applicable guidelines.

DQ verifies that the proposed design of the facilities, equipment, or systems is suitable for the intended purpose.

IQ verifies that the equipment and utilities have been installed as directed in relevant design specification in accordance with written procedures.

OQ verifies that equipment and utilities have the performance capacity required by their specifications. It should be verified that the required cleanliness in the aseptic processing areas is maintained throughout operation or use.

PQ verifies that the equipment and ancillary systems, when operating together, can perform effectively and reproducibly based on the approved process method and specifications.

14.3.2 Designing the Facility

The manufacture of sterile products should be conducted in clean areas.

The facility should be designed to prevent from cross-contamination and mix-ups.

To achieve these goals, regulations such as PIC/S GMP guidelines and Japanese guidelines describe many things, from which some points are combined with additional information about our specific needs, as follows.

14.3.2.1 The Basic Perspective

The flow and control planning of materials, products, and personnel within the areas needs to be fully considered; flow of humans and goods should be separated, one-way flow is better, and a system of interlocking pass boxes and pass rooms should be established. In our case, *E. coli* culturing, vector production, aseptic filling, and cell processing areas employ the one-way system. Purification areas do not, because it is operationally difficult to maintain one-way flow, but the changing rooms for entering and leaving the area are separate. Further, the strict rules for material handling and administration procedures are documented to prevent confusion between clean and dirty or sterilized and non-sterilized apparatuses and utensils.

Clean areas should be well separated from other areas and from outside, biohazard area should also be separated, and clean areas for the manufacture of sterile products are classified according to the required characteristics of the environment.

14.3.2.2 For Maintaining the Cleanliness of the Facility

Walls, floors, and ceilings should be designed to be easily cleanable. Their surfaces are polished, dust will not accumulate on them, and they are resistant to water and chemicals, especially cleaning agents and disinfectants, and impermeable. The corners between floors and walls should be rounded so that dust can be easily wiped off. Particular consideration should be given to seals and packing of interior materials to keep rooms tightly closed. Sometimes, the seals around screws to hold doors, gas lines, plugs, and similar structures are insufficient, and adequate verification is needed at the inspection.

The location of equipment in the areas and rooms should also be carefully planned to minimize crossing of personnel, product, and material flows. We selected fixtures such as stainless steel rustless worktables and shelves inside the clean rooms, with round legs and without drawers for preventing the accumulation of dust.

Air showers before entering the clean area are no longer recommended because they constitute a projection hazard and cause cross-contamination; the current trend is control of the cleanness through a gowning plan and differential pressure between the areas.

Drains and sinks should be prohibited in the aseptic processing area. If drains are placed in indirect support areas, drains should be fitted with traps or water seals that are easy to clean and to disinfect to prevent contamination by backflow. We will not place drains and sinks inside the production areas; all the materials used for production, including fluid materials such as medium, are sterilized by the one-way autoclave with a door separating the inlet and outlet, and they are discarded into sinks placed outside the production area. All the drainages from the sinks are connected to kill tanks and sterilized again to ensure that biohazardous wastes are not removed without sterilization.

An appropriate pest control program should be conducted to prevent contamination of sterile products by insects and other vermin such as mice and birds. Walking and flying insect traps should be placed to prevent pest infestation and to monitor the direction of infestation and the source of contamination.

As much as possible, paper should not be brought into clean areas to reduce the dust arising from paper during GMP manufacturing, so the SOPs and records, such as instrument control logs and batch records for manufacturing biologics, should be made on dust-free paper. Where feasible, paperless systems should be used: all manufacturing components, such as raw materials, process, sampling, intermediate products, drug substrates, drug products, and inventory, are recorded and controlled on computers. Unlike the production of small-molecule drug products, manufacturing of gene therapy, cell therapy, and regenerative medicine is complex, and it can be difficult to fit these manufacturing processes to the manufacturing execution system. In our case, we could not find a good system for the bioproducts we produce. Only a system for small-molecule products or uniform biological products such as antibodies or vaccines could be found. Therefore, we need a customized system for the manufacture of recombinant proteins and regular viral vectors and will not use the facility for cell processing or contract manufacturing until a suitable system for bioproducts becomes available.

14.3.2.3 For the Operators

To suppress mold generation, humidity should be maintained between 30 and 60 % (varying with the season). Sometimes, the products are susceptible to heat, such as gamma-retroviral vectors, which degrade to half infectious activity after about two weeks even when kept at 4 °C. Because lower temperatures are better for filling heat-labile products, the filling space should be designed for approximately 4 °C, not room temperature. However, this introduces the risk of condensation in the room, which increases the likelihood of mold. To protect against condensation at temperatures below the dew point, the filling environment should be dry. Furthermore, we limited the low-temperature space for filling to laminar hoods,

safety cabinets, and isolators to minimize the physical burden on operators. In consideration of operators' health, we placed many windows on the walls in the working area, which is independent from the outside. Of course, multiple emergency exits are necessary in all areas, and internal locks should be released during disasters. We placed video cameras in several aseptic processing areas. In the cold room for purification, a convenient video camera is placed to monitor the purification equipment from an outside room that is not cold. We placed the visitor routes such that visitors and auditors can see the inside and operating processes without entering the production areas, but because some rooms are located further inside and cannot be seen from the visitor routes, we fitted the ceilings of those rooms with video cameras that can make a 359° turn, move up and down, and zoom. They can also be used for checking the safety of the operators.

Of course, periodic inspection is important for verifying that the facilities are maintained as originally designed. Other requirements for manufacturing of sterile pharmaceutical products can be found in the guidance and guidelines.

14.4 Air-Conditioning Requirements

Air-conditioning is the most important factor in maintaining the aseptic processing area and controlling the quality of the sterile products. We must consider that maintaining a comfortable environment results in the improvement of quality. There are several standards for air control in medical production facilities. Table 14.2 shows a classification of air cleanliness described in ISO (ISO-14644-1 "Cleanrooms and associated controlled environments"), FDA guidance (Guidance for Industry Sterile Drug Products Produced by Aseptic Processing – Current Good Manufacturing Practice), EU guidelines (EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use), PIC/S GMP guidelines (Annex 1 "Manufacture of sterile products"), and Japanese guidance (Guidance on the Manufacture of Sterile Pharmaceutical Products by Aseptic Processing).

The areas are classified as grades A, B, C, and D depending on the nature of the operation to be conducted. All the guidelines and guidance (PIC/S, FDA, EU, and Japan) have the same requirements for each grade, as follows:

Grade A: critical and/or high-risk operation area where sterilized products and materials as well as their surfaces are directly exposed to the environment, e.g., filling, aseptic connections, stopper bowls, open ampoules and vials, and sterile ingredient additions

Grade B: direct support and/or supporting clean area that is a background environment for the grade A zone, e.g., working areas for personnel who operate machines installed in the critical area and also routes for the transfer of sterilized products, materials, and equipment to the critical area or for moving sterilized products from the critical area and where non-sterile components, formulated products, in-process materials, equipment, and container/closures are prepared, held, or transferred

Table 14.2 Classification of air cleanliness specified in ISO, FDA, EU, PIC/S, and Japan guideline

ISO			FDA		EU, PIC/S, JP				
			In the vicinity of exposed materials/articles during periods of activity			At rest		In operation	
ISO Class	0.5 mm particles/m^3 of air	5 mm particles/m^3 of air	0.5 mm particles/ft^3 of air	0.5 mm particles/m^3 of air	EU Grade	0.5 um particles/m^3 of air	5.0 um particles/m^3 of air	0.5 um particles/m^3 of air	5.0 um particles/m^3 of air
5	3,520	29	100	3,520	A	3,520	20	3,520	20
6	35,200	293	1,000	35,200	B	35,200	29	352,000	2,900
7	352,000	2,930	10,000	352,000	C	352,000	2,900	3,520,000	29,000
8	3,520,000	29,300	100,000	3,520,000	D	3,520,000	29,000	Not defined	Not defined

Grades C and D: indirect support areas and/or clean areas for carrying out less critical stages, e.g., preparing drug solution prior to sterilization, washing and cleaning sterilization equipment and apparatuses, and weighing and preparation processes

Figure 14.1 shows a typical cell processing room. Our facility was designed based on the regulations above. The room for the sterilization of filling materials such as vials, stoppers, and aluminum seals by autoclave or dry heat is Grade A. Cell processing rooms, including supporting rooms such as the clean corridor, 2nd gowning area, and air lock and pass room, are Grade B, and Grade A safety cabinets are installed in the Grade B working rooms. Filling areas including supporting rooms are Grade B, and they include Grade A laminar flow hoods, isolators, or safety cabinets.

It is important to achieve a proper airflow from areas of higher cleanliness to adjacent less-clean areas to maintain aseptic conditions and cleanliness. Aseptic

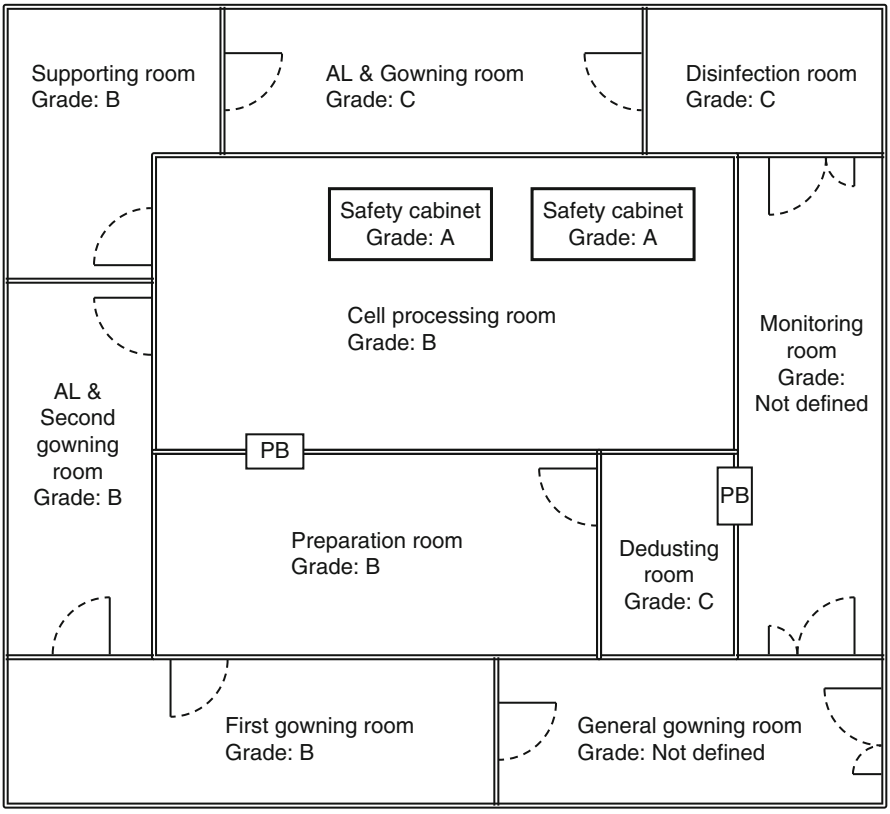

Fig. 14.1 Typical cell processing room. *PB (Pass Box)*

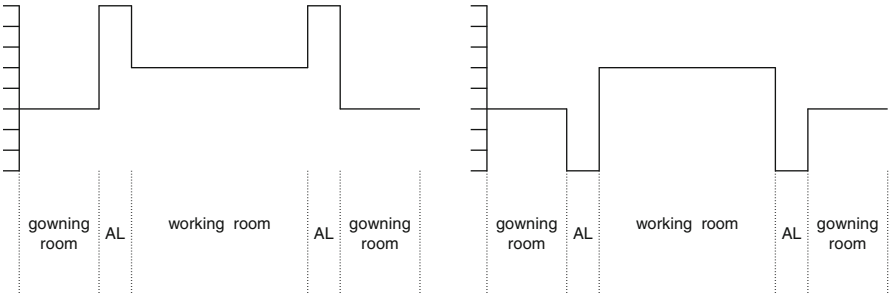

Fig. 14.2 Two types of AL (Air Lock): one is high pressure (*left*) and the other is low pressure (*right*). Vertical-axis is air pressure

processing areas are designed with the highest air pressure in the facility to protect these areas from adventitious contamination under the general GMP manufacturing guidelines. In the facility for gene therapy, however, both GMP and the Cartagena Act should be addressed, and the clean area should be designed not only to protect from adventitious contamination (requiring high air pressure) but also to consider the containment of gene-modified organisms (requiring low air pressure). To achieve a proper airflow with this conflicting air pressure, the design of appropriate pressure differences between rooms, including their supporting rooms, is very important. An air lock (AL), which is a small room composed of interlocking doors, is a key room for maintaining both protection from adventitious contamination and containment of gene-modified organisms. Figure 14.2 shows two types of AL: one is high pressure, and the other is low pressure. The high-pressure AL is a wall for protection, and the low-pressure AL is a trapping trough to prevent the outside air from contaminating clean area and to prevent air from moving from the clean area to outside; these two types of ALs are sometimes used in combination. Figure 14.3 shows a design for strict control, with paired high-pressure and low-pressure ALs.

14.5 Conclusions

Because of the recent attention paid to regenerative medicine after Dr. Shinya Yamanaka and Dr. John Gurdon were awarded the Nobel Prize in 2012 for the discovery that mature cells can be reprogrammed to become pluripotent (iPS cells), the Japanese government has introduced the Regenerative Medicine Promotion Act, including "revision of Pharmaceutical Affairs Law" and "enactment of Act on the Safety of Regenerative Medicine," toward pursuing the development of "Regenerative

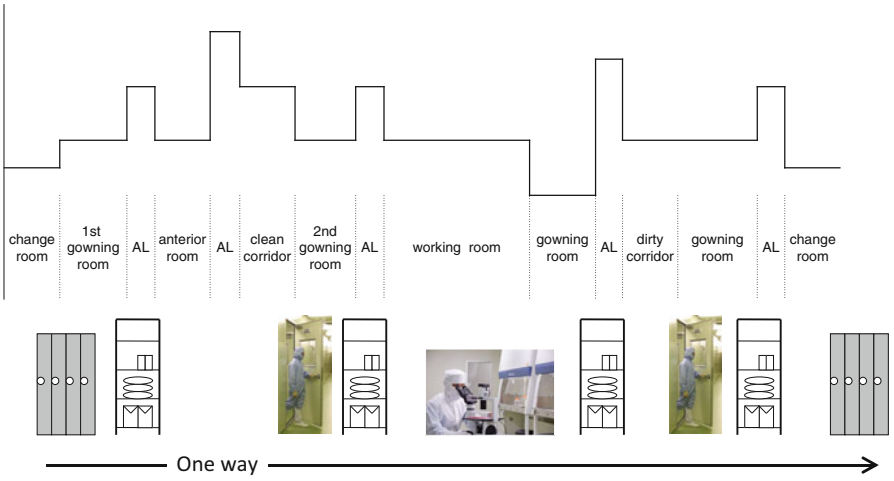

Fig. 14.3 A design for strict control with paired high-pressure and low-pressure ALs. X-axis is air pressure

Medical Products" including gene therapy and cell therapy. Regarding the drug examination system for "Regenerative Medical Products," the Pharmaceutical and Medical Device Agency (PMDA) has a plan to increase the inspection personnel from the current approximately 30–1000 persons by the end of 2019, which will address reason 1 in the Introduction. Recently, the GCTP (Good gene, Cell, and Tissue Practice) ministerial ordinance became effective in Japan on 25 November 2014 (Ordinance of the Ministry of Health, Labour and Welfare, No. 93, 2014), providing requirements for quality systems for regenerative medical technologies and products, considering the characteristics of these products, such as raw materials that cannot be sterilized. It is comparable to "Good Tissue Practice + GMP/QMS," and there appear to be similar regulations in the USA (HCT/P (human cells, tissues, and cellular and tissue-derived products)) and the EU (ATMP (advanced therapy medicinal product)). These Acts allow the outsourcing of the production of drug products used for clinical research and cell processing for which safety and efficacy have not yet been established and previous regulations only permitted production at the clinic site. Of course, as GMP and GCTP describe, both hard and soft, i.e., total quality system should be achieved such as quality control, personnel education and training, raw materials control, storage and transport, environmental monitoring, sterilization processes, manufacturing control, aberration control, document control, change control, validation, and verification. Lastly, our new facility is introduced in Fig. 14.4 and Table 14.3, and we expect that this facility will support the development of "Regenerative Medical Products" made in Japan.

Fig. 14.4 Center for gene and cell processing

Table 14.3 Center for Gene and Cell Processing, outline of the facility [18]

Floor	Function	Cleanness	Containment measures	Number of suites
1	*E. coli* culture and banking	Grade C	GILSP	3
	QC test (sterility, mycoplasma)	Grade C	Category 1	3
	Cell bank storage	–	–	5
2	Protein purification	Grade C	–	2
	Cell culture, banking, and viral vector production	Grade C	Category 1	6
	Aseptic filling	Grade A, B, C	Category 1	3
3	Cell processing	Grade B, C	Category 1	5 (+3)
	QC test (test for cells and viruses, qPCR, bioassay, etc.)	–	Category 1	8

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