Cell-Based Regenerative Therapy for Liver Disease

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Abstract The liver can regenerate itself in response to acute liver damage. However, chronically induced liver dysfunction interferes with the liver regeneration process and increases the risk of onset of more severe hepatic failure, including hepatic cirrhosis and liver cancer. To develop more efficient therapeutics for chronic liver diseases, cell-based regenerative therapies using functional hepatocyte-like cells derived from pluripotent stem cells are actively under investigation. In addition to such stem cell–based approaches, recent studies have revealed that direct cell-fate conversion from fibroblasts into hepatocyte-like cells can be induced by forced expression of particular sets of transcription factors in fibroblasts. This phenomenon is known as "direct reprogramming" and is expected to be a complementary or alternative technology to the stem cell-based regenerative therapies. In this chapter, we briefly summarize the recent progress and future perspectives of studies on reprogramming technologies, which are directed at the development of cell-based regenerative therapies for liver diseases.

Keywords Hepatocyte • Direct reprogramming • iHep cell • iPS cell • Regenerative medicine • Cell transplantation

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The Liver and Disease

The liver is the central organ for metabolism, by which it produces energy for life activities and detoxifies various extrinsic and intrinsic harmful substances. In addition, the liver creates endocrine and exocrine materials, such as serum albumin, growth factors, and bile acids. The liver is developed from the foregut endoderm [1] and consists of parenchymal cells, such as hepatocytes, and various non-parenchymal cells, such as biliary epithelial cells, Kupffer cells, pit cells, hepatic stellate cells, mesothelial cells, and sinusoid endothelial cells [2]. Among the diverse kinds of cell populations, the major cell population in the liver is composed of hepatocytes, which play primary physiological roles in this organ.

The liver is one of the few organs capable of regeneration in the body. Since the ancient Greek era, the liver has been known to have a strong intrinsic regenerative ability in vivo. The famous "Prometheus" myth represents the rapid regeneration of this organ [3]. Indeed, deletion of two thirds of the total mass of the liver can be recovered completely in vivo [2]. However, chronic hepatitis generates a highly serious situation for the liver, and often leads to more severe hepatic failure, including hepatic cirrhosis and liver cancer. Thus, various conditions are attributed etiologically to chronic hepatitis. The most common cause of chronic hepatitis is infection by hepatotropic viruses, followed by alcoholic hepatitis, non-alcoholic fatty liver disease, drug-induced hepatitis, and autoimmune hepatitis [4].

Expectations for Cell-Based Regenerative Therapies

Many types of medical treatments have been applied to patients with chronic hepatitis. Although anti-inflammatory drugs, such as ursodeoxycholic acid, glycyrrhizin, and liver extracts, are widely used to treat all types of chronic hepatitis, such treatments are only symptomatic therapies. For patients with viral hepatitis, antiviral drugs, such as interferon, corticosteroid, ribavirin, lamivudine, and azathioprine, have been specifically employed. However, several side effects associated with these drugs, including fever and drug-resistant strains, appear with high frequency [4].

In the treatment of severe liver failure, organ transplantation is the ultimate solution and has a relatively high postoperative survival rate. However, there are many problems to be solved, including a chronic donor shortage, ethics, and immune rejection [4]. Therefore, cell-based regenerative therapies, which employ in vitro-expanded or newly generated hepatocytes, are expected to be the next-generation therapies.

Although hepatocytes have a high proliferative ability in vivo, this potential disappears immediately upon in vitro culture. Consequently, innovative technologies that can expand, maintain, mature, and create hepatocytes in vitro are required for future cell-based therapies for liver disorders.

Pluripotent Stem Cell–Based Approaches to Treat Liver Failure

Among the current technologies aimed toward regenerative therapies for liver diseases, pluripotent stem cell–based approaches could be considered to show the most promise. Pluripotent stem cells can differentiate into any of the cell types responsible for the formation of particular tissues and organs, with the exception of extraembryonic tissues. Embryonic stem (ES) cells have been established as pluripotent stem cells and employed in basic research toward regenerative therapies for liver diseases. Hepatocyte differentiation from murine ES cells is generally induced via methods involving embryoid body (EB) formation [5–8]. Although EB-derived hepatocyte-like cells survive and function in vivo, their low differentiation frequency and associated teratoma formation remain as serious problems [9]. Several studies have tried to purify definitely differentiated hepatocytes or hepatic progenitor cells, using reporter expressions that are regulated by the promoters or enhancers of hepatocyte-specific genes, including *albumin* and *α-fetoprotein* (*Afp*) [10, 11]. Meanwhile, other studies have revealed the molecular mechanisms that regulate hepatocyte differentiation. In particular, employment of a liquidity factor, activin A, was found to efficiently improve both the ratio of hepatocyte differentiation and the quality of parental ES cells [12–14]. Other regulatory molecules required for hepatocyte differentiation have also been discovered [15–17]. By improving the in vitro hepatocyte differentiation of ES cells, many researchers have succeeded in transplantation of mouse/human ES cell–derived hepatocyte-like cells [18–20].

In 2006, Takahashi and Yamanaka [21] reported epoch-making artificial stem cells, termed “induced pluripotent stem” (iPS) cells. These novel stem cells can be expected to overcome the ethical and immunological problems associated with the use of ES cells [21, 22]. Soon after the publication of their study on iPS cells, many research groups started to employ these novel stem cells for in vitro hepatocyte differentiation using methods and knowledge accumulated in studies on ES cells [23–26]. Most recently, Takebe et al. [27] reported that vascularized and functional human liver bud-like structures could be formed by human iPS cells in culture and regenerate a part of the liver tissue upon transplantation. This technology has brought iPS cells closer to clinical reality. Nonetheless, iPS cells still have some problems to be solved, including tumorigenesis after transplantation and substantial costs. Thus, more vigorous basic studies are required for clinical-level applications using iPS cell technology.

Direct Reprogramming

Development of Direct Reprogramming Technologies

Recently, the strategy of direct cell-fate conversion from one cell type into another cell type, termed “direct reprogramming”, has rapidly expanded worldwide and is expected to be a complementary or alternative technology for future cell-based

regenerative therapies using pluripotent stem cells. This method can generate both mature and progenitor-like cells in specific lineages from other types of cells. Thus, the cells required for treatment of particular diseases could be provided from an alternative source of cells and used as donor cells for transplantation therapy.

The first evidence for direct reprogramming was reported in the 1980s. Davis et al. [28] discovered a single transcription factor, Myod, that could induce fate conversion of fibroblasts into myoblasts. That sensational report encouraged researchers to look for single master transcription factors that could specify and govern the fate of cells in each lineage. However, studies on direct reprogramming subsequently went into decline for a long time, because further master transcription factors were not easy to discover.

In the last decade, the emergence of iPS cells has completely changed the situation for direct reprogramming studies [21, 22]. After the discovery of iPS cells, it was found that sets of transcription factors, rather than single transcription factors, could be successively identified as master regulators capable of inducing fate conversion of cells. At present, various types of cells have been induced from fibroblasts, including neurons [29], cardiomyocytes [30], hepatocytes [31, 32], adipocytes [33], Sertoli cells [34], and chondrocytes [35]. Furthermore, not only fibroblasts but also other types of cells, such as hepatocytes [36], astrocytes [37], Sertoli cells [38], and B cells [39], have been employed as sources of cells for direct reprogramming. These findings imply that direct reprogramming technology will become a universal method for almost all kinds of cells. Although the recent studies on direct reprogramming are summarized in Table 1, studies on direct reprogramming are continuing to increase year by year.

Regarding successful cases of direct reprogramming, three different cell lineages, cardiomyocytes, neurons, and hepatocytes, can be considered to be well-investigated targets for the following reasons: (1) functional failure of these cells is critical for the survival of individuals; (2) a number of patients suffer from diseases associated with malfunction of these cells; and (3) a large number of cells should be prepared in vitro prior to application of these cells for transplantation therapies. Among these three cell types, cardiomyocytes could be considered to be the most widely studied cells in the field of direct reprogramming. Following the publication of the first report by Ieda et al. [30], several groups reported similar methods for cardiomyocyte reprogramming [40–47]. However, the transcription factors used for the induction of cardiomyocyte-like cells were different in each study (Table 1), and the properties of the cells were also inhomogeneous [30, 40–47]. The obtained evidence suggested flexibility in the molecular mechanisms underlying direct reprogramming and a necessity for standardized protocols toward therapeutic applications.

The molecular machinery for the direct induction of neuronal cells in vitro can be considered to be the most deeply investigated example in the field of direct reprogramming. Vierbuchen et al. [29] showed that three transcription factors, Brm2, Ascl1, and Myt1l, induced conversion of mouse fibroblasts into neuron-like cells, designated induced neuronal (iN) cells. iN cells had neuron-specific characteristics, including neurite outgrowth, expression of specific neuronal markers, and electrophysiological activities. In addition to the discovery of iN cells, the same group

Table 1 Recent examples of direct reprogramming studies

Target cell	Source cell	Factors used	Species	References
Neuronal cell	Embryonic fibroblast	Brn2, Ascl1, and Myt1l	Mouse	Vierbuchen et al. [29]
Glutamatergic neuron	Cortical astrocyte	Ngn2	Mouse	Berninger et al. [56]; Heinrich et al. [57]
GABAergic neuron	Cortical astrocyte	Dlx2, or Dlx2 and Ascl1	Mouse	Heinrich et al. [57]
Neuronal cell	Hepatocyte	Brn2, Ascl1, and Myt1l	Mouse	Marro et al. [36]
Dopaminergic neuron	Embryonic/skin fibroblast	Ascl1, Lmx1a, and Nurr1	Mouse/ human	Caiazzo et al. [58]
Neuronal cell	Fetal/postnatal fibroblast	Brn2, Ascl1, Myt1l, and NeuroD1	Human	Pang et al. [59]
Neuronal cell	Neonatal foreskin/ adult dermal fibroblast	Ascl1, Brn2, Myt1l, Lmx1a, and Foxa2	Human	Pfisterer et al. [60]
Neuronal cell	Astrocyte	Brn4	Mouse	Potts et al. [37]
Neural stem cell	Sertoli cell	Pax6, Ngn2, Hes1, Id1, Ascl1, Brn2, cMyc, and Klf4	Mouse	Sheng et al. [38]
Cardiomyocyte	Cardiac fibroblast	Gata4, Mef2c, and Tbx5	Mouse	Ieda et al. [30]
Cardiomyocyte	Cardiac fibroblast	miR-1, 133, 208, and 499	Mouse	Jayawardena et al. [40]
Cardiomyocyte	Cardiac fibroblast	Gata4, Mef2c, Tbx5, and Hand2	Mouse	Song et al. [41]
Cardiomyocyte	Cardiac fibroblast	Gata4, Mef2c, and Tbx5	Mouse	Chen et al. [42]
Cardiomyocyte	Cardiac/embryonic fibroblast	Mef2c, Tbx5 + Myocd or Gata4	Mouse	Protze et al. [43]
Cardiomyocyte	Embryonic fibroblast	Gata4, Tbx5, Hand2, and Myod M3 domain fused with Mef2c	Mouse	Hirai et al. [44]
Cardiomyocyte	Dermal fibroblast	GATA4, MEF2C, TBX5, MESP1, and MYOCD	Human	Wada et al. [45]
Cardiomyocyte	Dermal fibroblast	GATA4, MEF2C, TBX5, ESRRG, MESP1, ZFPM2, and MYOCD	Human	Fu et al. [46]
Cardiomyocyte	Cardiac/embryonic fibroblast	Gata4, Mef2c, Tbx5 + miR133 or Mesp1 and Myocd	Mouse/ human	Muraoka et al. [47]
Hepatocyte	Embryonic/dermal fibroblast	Hnf4α + Foxa1, 2, or 3	Mouse	Sekiya and Suzuki [31]

(continued)

Table 1 (continued)

Target cell	Source cell	Factors used	Species	References
Hepatocyte	Tail-tip fibroblast	Gata4, Hnf1α, Foxa3, and $p19^{Arf}$ KD	Mouse	Huang et al. [32]
Hepatocyte	Fetal limb fibroblast	FOXA3, HNF1A, HNF4A, and SV40 Large T antigen	Human	Huang et al. [53]
Hepatocyte	Embryonic fibroblast	HNF1A, HNF4A, HNF6, ATF5, PROX1, CEBP, MYC, and TP53 KD	Human	Du et al. [54]
Hepatocyte	Neonatal fibroblast	HNF1A + FOXA1, FOXA3, or HNF4A (mRNA)	Human	Simeonov and Uppal [52]
Sertoli cell	Embryonic fibroblast	Nr5a1, Wt1, Dmrt1, Gata4, and Sox9	Mouse	Buganim et al. [34]
Chondrocyte	Dermal fibroblast	KLF4, MYC, and SOX9	Human	Tam et al. [35]
Erythroid cell	B cell	Gata1, Scl, and Cebpa	Mouse	Sadahira et al. [39]
T cell	B cell	Pax5 KO	Mouse	Cobaleda et al. [61]
Monocyte	Skin fibroblast	Spl1, Cebpa, Mnda, and Irf8	Mouse	Suzuki et al. [62]
Macrophage	Pre-T cell	Cebpa or Cebpb	Mouse	Laiosa et al. [63]
Macrophage-like cell	3 T3 cell, embryonic/skin fibroblast	Pu.1 + Cebpa or Cebpb	Mouse	Feng et al. [64]
Dendritic cell	Pre-T cell	Pu.1	Mouse	Laiosa et al. [63]
Multilineage blood progenitor	Adult/neonatal dermal fibroblast	OCT4 + hematopoietic cytokine treatment	Human	Szabo et al. [65]
Megakaryocyte	3 T3 cell, dermal fibroblast	Nfe2, Mafg, and Mafk	Mouse/ human	Ono et al. [66]
Thymic epithelial cell	Embryonic fibroblast	Foxn1	Mouse	Bredenkamp et al. [67]
Vascular endothelial cell	Amniotic cell	ETV2, FLI1, ERG1 with TGFB inhibition	Human	Ginsberg et al. [68]
B cell	Pancreatic exocrine cell	Ngn3, Pdx1, and Mafa	Mouse	Zhou et al. [69]
Brown fat cell	Skin fibroblast	Prdm16 and Cebpb	Mouse/ human	Kajimura et al. [70]
Pancreatic islet cell	Hepatocyte	Ngn3	Mouse	Desgraz and Herrera [71]

revealed that remarkable epigenetic remodeling occurred in iN cells, and that Ascl1 acted as a pioneering factor that could activate closed chromatins during the reprogramming [48]. A better understanding of the molecular mechanisms controlling direct cell-fate conversion could provide great advantages for the development and application of direct reprogramming technology.

Direct Reprogramming of Fibroblasts to Hepatocytes

As mentioned above, functionally mature hepatocytes are strongly demanded for clinical use. To supply hepatocytes safely and stably without any ethical problems, direct reprogramming of patient-derived non-hepatic cells into hepatocytes appears to be a preferable method. Such newly generated hepatocytes could be technologically and economically useful for future cell-based regenerative therapies for liver diseases. The first studies showing direct conversion of non-hepatic cells into hepatocyte-like cells were independently reported by two groups: one was from our group [31] and the other was from Hui's group [31, 32] (Fig. 1). Although both groups induced conversion of mouse fibroblasts into hepatocyte-like cells,

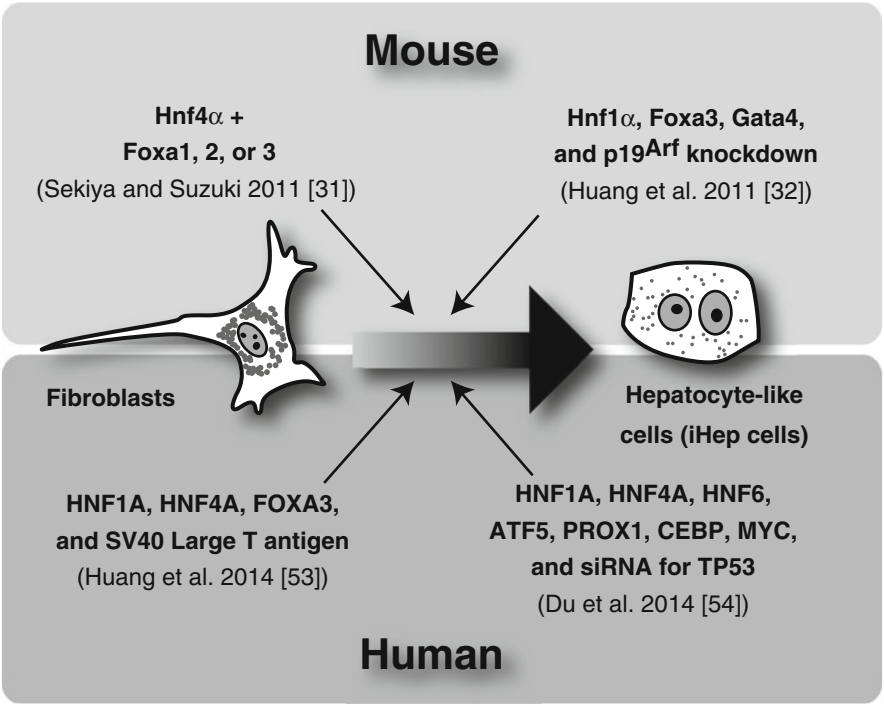

Fig. 1 Direct reprogramming of fibroblasts to iHep cells. iHep cells can be induced by various protocols

the transcription factors used were different. Our group found that three combinations of two transcription factors, comprising a hepatocyte nuclear receptor, Hnf4α [49], and members of the forkhead domain-containing transcription factor family, Foxa1, Foxa2, and Foxa3 [50], could induce conversion of fibroblasts into hepatocyte-like cells, designated induced hepatocyte-like (iHep) cells [31]. In contrast, Hui's group used a combination of three transcription factors, Gata4, Hnf1α, and Foxa3, with siRNA-based suppression of the expression of a tumor suppressor, $p19^{Arf}$ [32]. Although the reprogramming factors were different, iHep cells could be induced in both methods, and their morphological and functional properties were similar [31, 32]. These findings suggest the presence of lineage-specific core transcriptional networks that can determine the fate of cells.

iHep cells have multiple hepatocyte-specific properties: (1) the morphology and gene expression pattern of iHep cells resemble those of epithelial cells; (2) iHep cells express hepatocyte-specific genes and proteins; (3) iHep cells have functional features of hepatocytes, including glycogen storage, LDL uptake, ammonium metabolism, urea production, cytochrome P450 activity, and drug metabolism; and (4) iHep cells can reconstitute liver tissues, ameliorate hepatic functions of recipient mice, and rescue mice from a deadly hepatic disorder, hereditary tyrosinemia type I, upon transplantation into the liver of a mouse model of the disease. In addition to these features, we recently showed that iHep cells possess the potential to be involved in lipid metabolism, similar to hepatocytes [51]. These findings suggest that iHep cells are useful not only for screening of drugs, but also for the treatment of patients with fatty liver diseases. The morphology and function of iHep cells do not become attenuated during culture or after freeze-preservation, which could be great advantages in the application of iHep cells to cell-based regenerative therapies for liver diseases.

The above-mentioned characteristics suggest that iHep cells could be a potent cell source for future cell transplantation therapies. However, three problems remain to be resolved. First, the level of hepatic function is lower in iHep cells than in primary hepatocytes. Therefore, for use of iHep cells in cell transplantation therapies, the level of hepatic function in iHep cells needs to be improved by inducing the maturation of these cells. Second, viral vectors need to be excluded from the reprogramming procedures, because integration of the virus genomes into the host genomes may induce malignant transformation of cells. Thus, other reprogramming strategies using chemical compounds, growth factors, or non-integrative vectors are expected to be practical. Recently, Simeonov and Uppal [52] demonstrated the potential utility of an mRNA transfection-based technique for the generation of iHep cells. This technology may contribute to safer induction of iHep cells. Third, application of iHep cell technology to human cells is still in the development stage. Recently, two different groups reported data on the induction of human iHep cells [53, 54]. These studies showed that the technology for iHep cell generation in mouse cells could be reproducible in human cells. However, there are some critical differences between the methods for mouse and human iHep cell induction. In the case of human iHep cells, activation of MYC and SV40 large T antigen and suppression of TP53 are included in the methods for iHep cell generation, which have

a risk for inducing malignant transformation of these cells (Fig. 1). Thus, it is expected that human iHep cells capable of proliferation without growth boosters, which can avoid the risk of tumorigenesis, should be generated for the application of iHep cells in transplantation therapies.

Conversely, Zhu et al. [55] reported a completely different strategy for induction of hepatocytes in vitro. They initially used OCT4, KLF4, SOX2, and MYC to induce iPS cells. However, they interrupted the conversion process of iPS cells and continued the culture of partially reprogrammed intermediate cells in the presence of various growth factors and chemical compounds that are known inducers of hepatic differentiation. The resultant hepatocyte-like cells showed hepatocyte-specific features like iHep cells. Thus, this method could be another alternative to stem cell–based regenerative therapies for liver failures, as well as the technology of iHep cell generation.

Conclusions

Cell-based regenerative medicine is one of the most promising clinical technologies in the treatment of liver disorders. However, it has remained unclear whether cell-based therapies are actually available in clinical settings without any risk for patients. To reduce the associated risks, the methods for cellular reprogramming and differentiation procedures should be improved in ES cell, iPS cell, and iHep cell technologies. As one of the important approaches, we need to understand the molecular mechanisms underlying the generation of iHep cells and the differentiation of hepatocytes, with a view to updating the technology for direct reprogramming and actualizing the functional maturation of iHep cells for use in cell-based regenerative therapies toward liver diseases. We believe that many current efforts in basic research will open up a new horizon for next-generation therapies using directly reprogrammed cells.

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