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A Strategy Using Pluripotent Stem Cell-Derived Hepatocytes for Stem Cell-Based Therapies

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1. Introduction

There are millions of patients suffering from fatal liver disease in the world. Whole-organ transplantation of the liver, such as orthotopic liver transplantation (OLT), improves the survival rate of these patients (Benten et al., 2009). However, the traditional OLT is still limited due to the serious want of viable livers available for organ transplantation. Therefore, cell therapies for the treatment of end-stage hepatic diseases are currently under investigation all over the world (Ito et al., 2009). The simplest method for cell therapies is by transplantation of primary hepatocytes isolated from the donor. Experimental studies on animals have shown that transplanted primary hepatocytes into the spleen or portal vein of host animals repopulated in the liver, suggesting that the primary hepatocyte transplantation may be successful as an alternative to organ transplantation for patients with liver failure (Sutherland et al., 1977; Makowka L et al., 1980; Demetrious et al., 1986; Arkadopoulos et al., 1998; Ribeiro et al., 1992; Ito et al., 2007; Nagata et al., 2003; Kobayashi et al., 2000). However, success in clinical use is limited (Fox et al., 1998; Platt, 1998) and donor human livers to isolate the hepatocytes for hepatocyte transplantations are also limited, since these organs are needed to use in organ transplantation. Furthermore, the primary hepatocytes are cultured in vitro with very limited success due to their slow growth and instability of hepatic phenotype tending to lose differentiation character (Clayton & Darnell, 1983). Thus, although the human primary hepatocytes are ideal cells for cell therapies, an unlimited source of hepatocytes is required. There are two ways to prepare an unlimited supply of hepatocytes: 1) induction of differentiation from pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) and 2) production of hepatocytes from somatic cells by reactivation of mature hepatocytes themselves or reprogramming the somatic cells other than hepatocytes into hepatocytes.
ESC and iPSC are expected to be a promising alternative resources for cell therapy because they are pluripotent, making it possible to produce any type of tissue from a single resource and they are also an infinite resource expanding continuously in the undifferentiated state

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under appropriate conditions (Hanna et al., 2010). A lot of studies have reported the successful induction of hepatic differentiation from ESCs and iPSCs (reviewed in Andersson & Lendahl, 2009; Behbahan et al., 2011; Greenbaum, 2010; Kung & Forbes, 2009). However, undifferentiated pluripotent stem cells possess intrinsic property of teratoma formation in the host after transplantation (Fong et al., 2010; Blum & Benvenisty, 2009; Knoepfler, 2009) and their induction efficiency of hepatocellular differentiation was still not enough so that various types of cells, including undifferentiated pluripotent stem cells, remained at different stages of differentiation (Sasaki et al., 2009; Shiraki et al., 2008; Teratani et al., 2005; Yoshie et al., 2010). Thus, improvement of the induction method for the hepatocellular differentiation to the efficiency of almost 100% and/or development of an efficient selection method for differentiated hepatocytes are needed.

Several strategies for isolation of ES cell-derived hepatic cells have been proposed in the last decade (Basma et al., 2009; Duan et al., 2007; Gouon-Evans et al., 2006; Heo et al., 2006; Li et al., 2010; Soto-Gutierrez et al., 2006; Suzuki et al., 2000; Yin et al., 2002). The method of isolation is based on the sorting system, such as fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) devices and utilizes fluorescent markers or antibodies specific for hepatic cells. To realize enough quality and quantity, we propose in this paper a dual selection method; first the proliferative immature hepatocytes are isolated with a cell surface marker Liv2 antigen and after growing fully in the culture, differentiated mature hepatocytes are purified with indocyanine green (ICG) which is an organic anion that is specifically taken by mature hepatocytes. We suggest that in surface markers only Liv2 antigen is used in the specific isolation of immature hepatocytes from pluripotent stem cells (Takashimizu et al., 2009). Purification with ICG has the two major benefits of being both safe and inexpensive, since the ICG system that we are developing is non-immune based and a non-genetically engineered method for selection of differentiated hepatocytes by using flow cytometry with a newly developed laser beam suitable for excitation of ICG.

Except for cell therapy, human hepatocytes are also useful and necessary for drug development (Greenhough et al., 2010). The liver is the central site of drug metabolism and detoxication and thus liver based toxicological tests for developing drugs are necessary. However, preclinical studies of model animals are inadequate to fully evaluate toxicity due to species variation of hepatic functions, such as cytochrome P450 induction (Lake, 2009). This remark is also true for food development. Therefore, the strategy described here is applicable to a wide area of health science.

2. How to obtain hepatocytes

2.1 Hepatocyte induction by differentiating from pluripotent stem cells (ESCs or iPSCs)

Induction of differentiation from pluripotent stem cells into hepatocytes was performed by mimicking the hepatocyte differentiation during embryonic development. During embryonic development, hepatocytes are differentiated from the definitive endoderm and the definitive endoderm is generated from the mesendoderm, which is induced under the influence of Nodal, a transforming growth factor beta (TGF-beta) family gene product (Zaret, 2000; Zaret & Grompe, 2008). The hepatocyte differentiation from the definitive endoderm or mesendoderm is induced by FGF signalling from the cardiac mesoderm and BMP-4 signalling from the septum transversum mesenchyme (Duncan, 2003; Si-Tayeb et al., 2010; Zaret, 2001). Thus, the procedure for induction of hepatocyte differentiation from

pluripotent stem cells consists of two steps: the first is definitive endoderm induction by activin, which acts in a similar way to Nodal, the second is hepatocyte induction by FGF and BMP-4. However, because the cells induced with this procedure are not fully maturated, we further induce maturation with serum-free Lanford medium, which is developed for maintaining human hepatocytes in vitro (Sasaki et al., 2009; Yoshie et al., 2010).

2.1.1 Induction of hepatocyte differentiation from mouse ESCs

Although, in most studies, the differentiation-inducing experiments were performed under serum-containing conditions (e.g. Ishii et al., 2005), the hepatocytes induced using these conditions have inevitable risks, such as viral infection advising against use in possible future clinical applications. Therefore, we propose a simple two-step induction method under serum-free conditions as below (Yoshie et al., 2010).

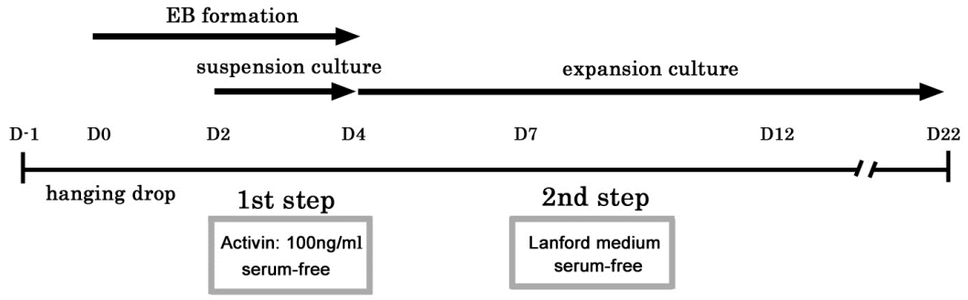

Fig. 1. Experimental design for the induction of hepatocytes from mouse ESCs.

As a first step, the mesendoderm was induced by activin as shown in Fig. 1. The expression of the mesendodermal marker goosecoid and the primary endoderm marker Foxa2 were increased approximately twofold under serum-free conditions than under serum-containing conditions at 100 ng/ml of activin which mimics the role of Nodal signals (Kubo et al., 2004), suggesting that unknown serum factors inhibited hepatic differentiation.

Next, as a second step, hepatocytes were induced from the mesendoderm with serum-free Lanford medium, which has been developed for maintaining human hepatocytes (Lanford et al., 1989). Once immersed in the Lanford medium, the embryoid bodies (EBs) began to show typical hepatic features by day 17, including the expression of hepatic lineage markers albumin (ALB), alpha fetoprotein (AFP), transthyretin (TTR) and α1-anti-trypsin (AAT) detected by RT-PCR, and ALB, AFP and cytokeratin-18 (CK18) detected by immunostaining. On day 22, these cells seemed to have become mature, functional hepatocytes characterized by the expression of metabolizing enzymes, including DPPIV/UDP-glucuronosyl transferase (Ugt1a1), Slcola4, cyp3a11, cyp2b10 and cyp7a1 detected by real-time PCR, a 50-fold greater cyp3A11 response than the control with 100uM dexamethasone stimulation. These results indicate that this simple two-step induction method under serum-free conditions induces high quality hepatic lineage cells directly from mouse embryonic stem (ES) cell-derived mesendoderm.

2.1.2 Induction of hepatocyte differentiation from human ESCs or iPSCs

From embryonic events we learn how to differentiate hepatocytes from pluripotent stem cells. Put simply, FGF produced from cardiac mesoderm triggers hepatic buds from the endoderm-originated tube, BMP-4 from septum transversum promotes differentiation and

proliferation, cytokine, such as OSM, leads them to the final stage (Duncan, 2003). Many methods to differentiate have been developed and practiced (Cai et al., 2007; Hu et al., 2003; Ishii et al., 2008; Pei et al., 2009; Soto-Gutierrez et al., 2006; Teratani et al., 2005). Curiously, however, they deleted BMP-4 produced from septum transversum, which is an indispensible factor for hepatic development. So we have developed a new protocol to differentiate EB prepared from H1,khES1,2,3 ES lines into hepatocytes (Sasaki et al., 2009): 25 ng/ml acidic fibroblast growth factor (aFGF) and 25ng/ml basic fibroblast growth factor (bFGF for the first 3 days; 20 ng/ml BMP-4and 20 ng/ml hepatocyte growth factor (HGF) for the next 3 days, and 20 ng/ml oncostatin M (OSM) and 20 ng/ml vascular endothelial

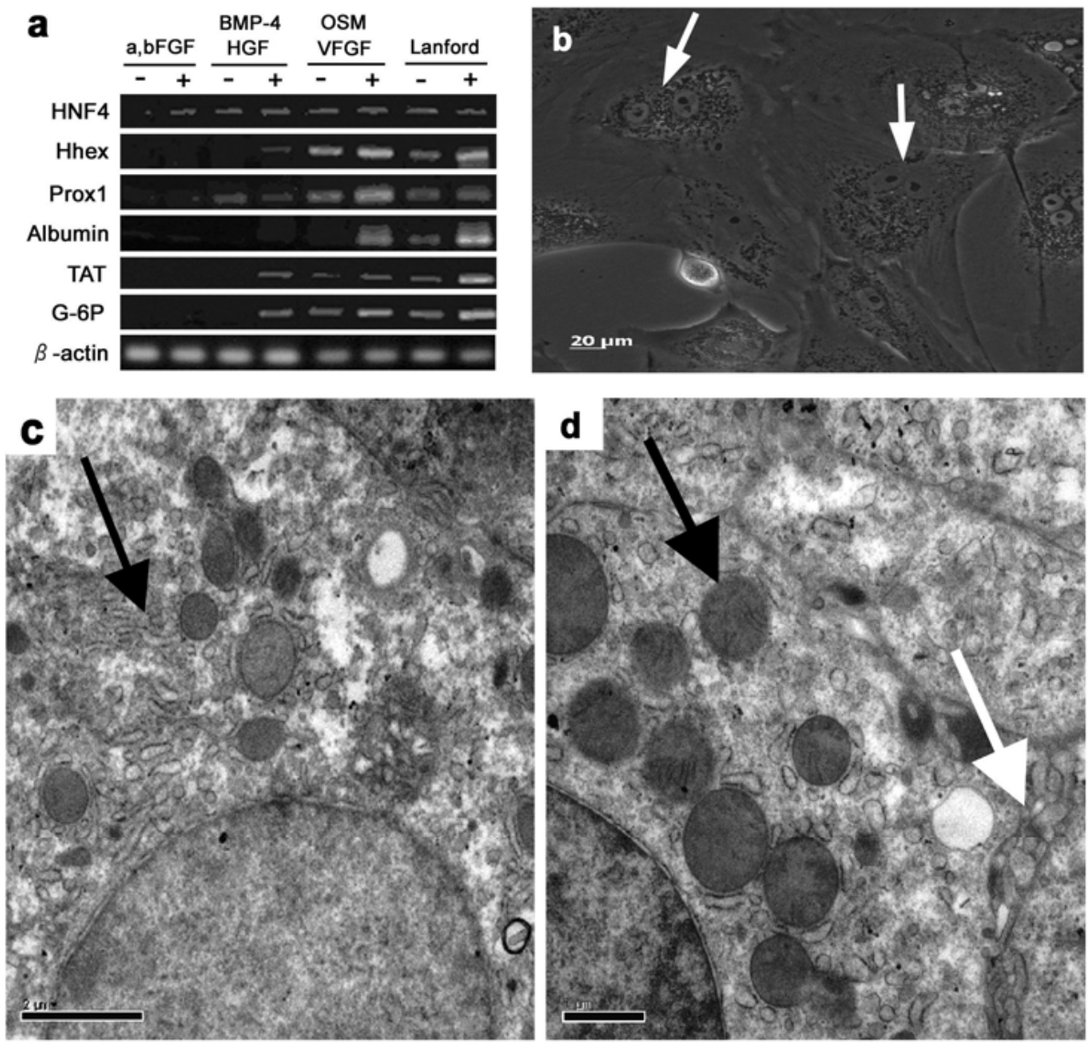

Fig. 2. Induction of hepatocyte differentiation from human ESCs . a. RT-PCR analysis of gene expressions associated with hepatic differentiation. Prox1 is definitely recognized in the OSM stage. Albumin is definite in the Lanford stage. G6P appears from the BMP-4 stage. b. The cluster of large cells with binucleate (arrows). c. Fine structures of the cytoplasm of differentiated hepatocytes. rER are layered (arrow). d. The junctional areas show bile canaliculi with microvilli (white arrow). Round mitochondria are rich in the cytoplasm (black arrow).

growth factor (VEGF) for the third set of 3 days; the modified Lanford medium for an additional 2 weeks. RT-PCR showed switch-on to hepatocyte maturation in the BMP-4 stage (Fig.2a), but it was quickly recognized that Lanford medium was a powerful promoter for cellular maturation. Therefore, Lanford medium was developed to maintain primary hepatocytes without serum. The contents contain growth factors and hormone, including EGF, LCGF, prolactin insulin and glucagon (Lanford et al., 1989). It remains unknown which factors are more effective, but it is true that some of them, or all, progress maturation powerfully (Sasaki et al., 2009; Yoshie et al., 2010). Drastic morphological changes had not been recognized during the periods from FGF stage to OMS, though gene expression showed signs of hepatocyte differentiation. However, several days later in the Lanford medium, the cells enlarged, took polyhedral forms and formed hepatocyte-like colonies with two-nuclei and large cytoplasm (Fig.2b). As described in our paper, they showed many gene expressions seen in mature hepatocytes, ICG uptake and albumin production. However, it was the TEM images that made us confirm that they were hepatocytes. They contained all the features with which morphologists determine that they are hepatocytes (Fig.2c, d). But this method, as well as other methods, has fatal defects. It is complicated, has no ability to produce a large number of hepatocytes at the final differentiation stage and requires high cost to use many growth factors. For clinical application, a simpler method has to be developed.

2.2 Hepatocyte reactivation or transdifferentiation from somatic cells

Although the liver is well known to have remarkable regenerative potential, the pathological liver has a limited ability to regenerate and the hepatocytes isolated from the living liver do not proliferate with ease in vitro (Clayton et al., 1983). However, by using gene transfer techniques similar to that used in the production of iPSC, reactivation of the hepatocytes or reprogramming of the somatic cells to hepatocytes is possible. It has been reported that rat primary hepatocytes, immortalized with oncogenic simian virus 40 T antigen (SV40Tag) could grow in vitro and maintain differentiated hepatic phenotype (Cai et al., 2000, 2002; Schumacher et al., 1996; Tada et al., 1998). Additionally, following transplantation into host rodents with liver failure, these immortalized hepatocytes function as well as primary hepatocytes and improve survival. However, although the SV40 T antigen can be excised by genetic engineering (e.g. Cre/lox recombination), the reprogramming to proliferative state by SV40Tag is thought not to be physiological as SV40 T antigen is a virus gene. We found some endogenous genes which when expressed in the proliferative hepatic cells could stimulate the growth of rat primary hepatocytes, following gene transfer, in vitro (unpublished data), so we expect that hepatocytes can be reactivated physiologically by these methods. However, to prevent immune rejection in cell therapy, these hepatocyte reactivation strategies have to utilize primary hepatocytes from the patient with end-stage liver pathologies or transfer the gene(s) into the patient's liver. Recently, it was reported that functional hepatocyte-like (iHep) cells could be induced directly from mouse tail-tip fibroblasts by gene transfer of Gata4, Hnf1a and Foxa3 (Huang et al., 2011). The transplanted iHep cells repopulated the livers of mice deficient in fumarylacetoacetate hydrolase (Fah) gene, restored liver functions and rescued almost half of recipients. Moreover, recently, we showed gene transfer unmediated transdifferentiation between hepatocytes and pancreatic cells (Kanoh et al., 2011). Thus, in addition to the induction from pluripotent stem cells, the strategy of direct induction from somatic cells will provide promising methods to prepare functional hepatocytes for regenerative medicine and toxicological tests.

3. Applicative aspects of the prepared hepatocytes

3.1 Research for realization of the cell replacement therapy

The major problems concerning realization of the hepatic cell therapy are: (i) existence of undifferentiated cells in the induced hepatocyte sample and (ii) difficulty in the establishment and functioning of transplanted hepatocytes. To solve the first problem, we are trying to establish two selection techniques: 1) isolation of immature hepatocytes with a cell surface marker Liv2 antigen and 2) purification of differentiated hepatocytes with indocyanine green (ICG). The immature hepatocytes isolated with Liv2 antigen retain proliferative capacity and therefore the collection of immature hepatocytes can be amplified in this step. Although the maturation to functional hepatocyte is usually difficult, the flow cytometry with laser beam for ICG could specifically purify functional hepatocytes, because uptake of ICG is hepatocyte-specific function and ICG is incorporated by only functional hepatocytes. The second problem could be partly solved by improving the transplant method. Because the liver with cirrhosis cannot accept transplanted cells, we are examining the transplant method that plants hepatocytes into the submucosa of the duodenum as mentioned in the last section of this chapter. In this way, the bile produced by the hepatocyte may be flowed into the lumen of the duodenum appropriately.

3.1.2 Isolation of immature hepatocytes with Liv2 antibody

Although various cytoplasmic markers, such as ALB, AFP and glucose-6-phosphatase (G6P), have been reported as useful for identifying immature and mature hepatocytes, these are not surface markers, so it is impossible to isolate only living hepatocytes using the fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) method. An isolating method using several surface antigens of hematopoietic markers to select hepatocytes has been reported (Suzuki et al., 2000), but the method is insufficient due to inaccurate cell counts and contamination of other types of cells in addition to hematopoietic cells and hepatocytes. Liv2, which was first reported by Watanabe et al. (Watanabe et al., 2002) is selectively detected in the cell surface of fetal mouse liver cells of embryonic day E9.5 to E12, so it can be used as a surface marker to isolate immature hepatocytes.

We have performed immunocytochemical analysis to verify the presence of Liv2 in immature hepatocytes derived from ES cells (Takashimizu et al., 2009) and we found clusters of Liv2-positive cells in EB outgrowth with the same patterns as the immunoreactivity seen in the E9.5 immature hepatocytes. In addition, we analyzed microlocalization of the Liv2 antigen by immuno-TEM (ImmunoGold) and confirmed definitely that Liv2 is a surface antigen. Next, we analyzed the property of Liv2-positive cells isolated by MACS. Cell counting and MTT assay revealed that Liv2-positive cells isolated by MACS from the primary hepatocytes obtained from the fetal livers of E12 mouse embryos have moderate proliferative potential (Figure 3).

Double-Immunostaining of the isolated cells of 1 day after Liv2-MACS showed that all of Liv2-positive cells analyzed express Prox1, AFP and ALB (Figure 4). Both Prox1 and AFP are markers of immature hepatocytes and not express in the adult liver cells. Thus, it was demonstrated that in the cells obtained from E12 fetal livers, only immature hepatocytes were positive for Liv2.

Further cultivation of the isolated cells confirmed that these Liv2-positive cells were progenitors of mature hepatocytes and could differentiate into mature hepatic cells. Immunocytochemical analysis showed that although population of Liv2-positive cells was decreased 5 days after culture, positive cells for AFP and ALB increased (Figure 4).

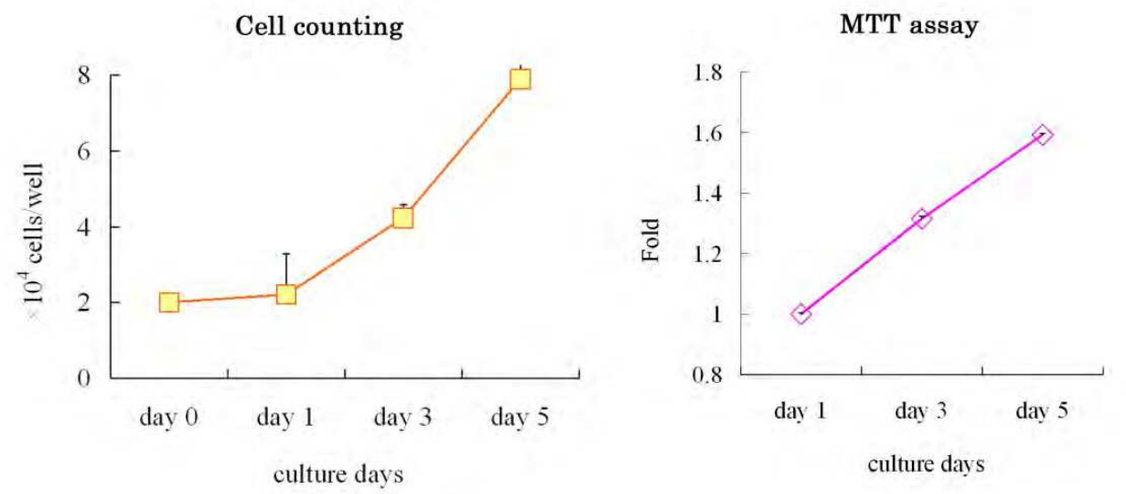

Fig. 3. Proliferation of Liv2-positive cells isolated by MACS. a: cell counting. b: MTT assay.

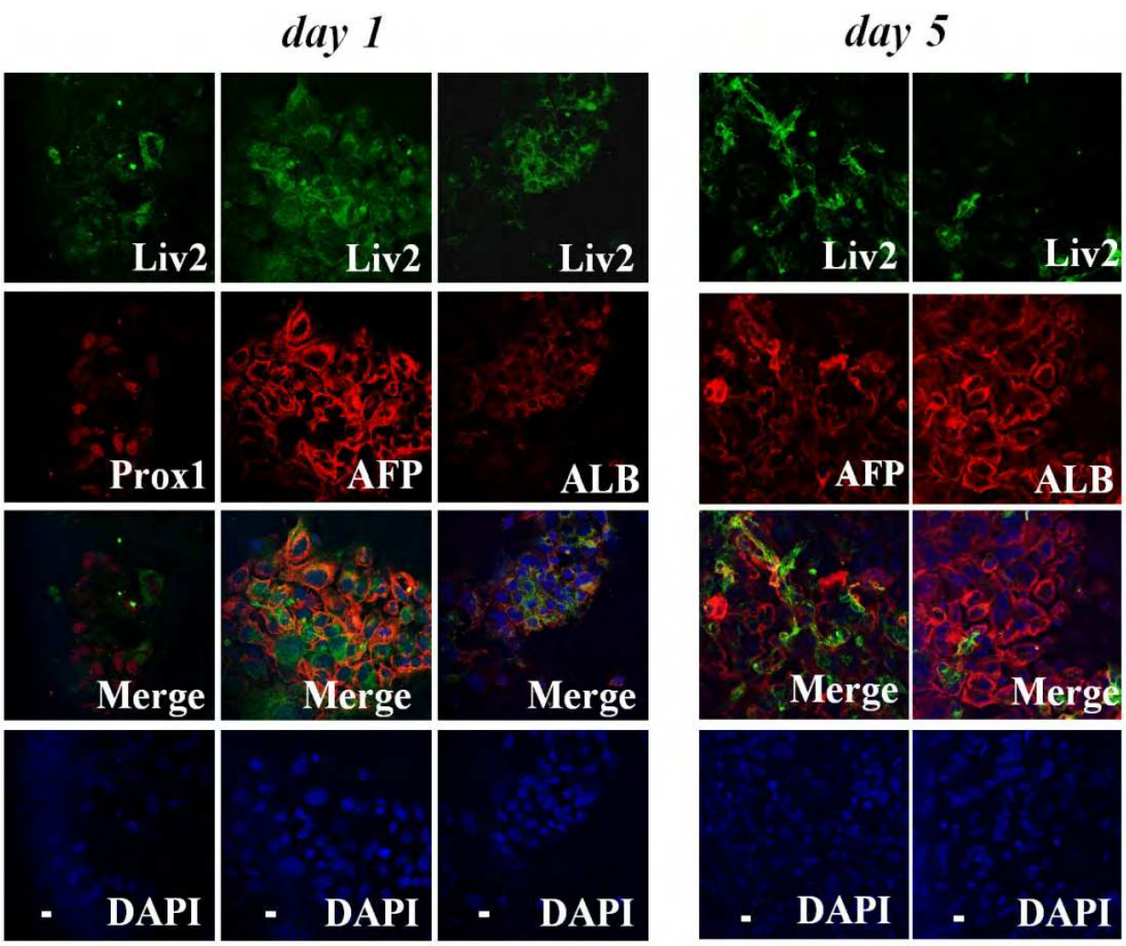

Fig. 4. Immunocytochemical analysis of cultured cells isolated by MACS with Liv2 antibody.

RT-PCR analysis revealed that the expression of mature hepatocyte markers CYP7A1, G6P, TAT and TTR was initially very low or absent and increased gradually afterward (Figure 5A). Moreover, the expression of CK7, a maker of bile duct cells, was extremely low on the first day and drastically up-regulated on day 5 (Figure 5b), suggesting that the Liv2-positive cells were bipotential progenitors capable of producing both hepatocytes and bile duct cells.

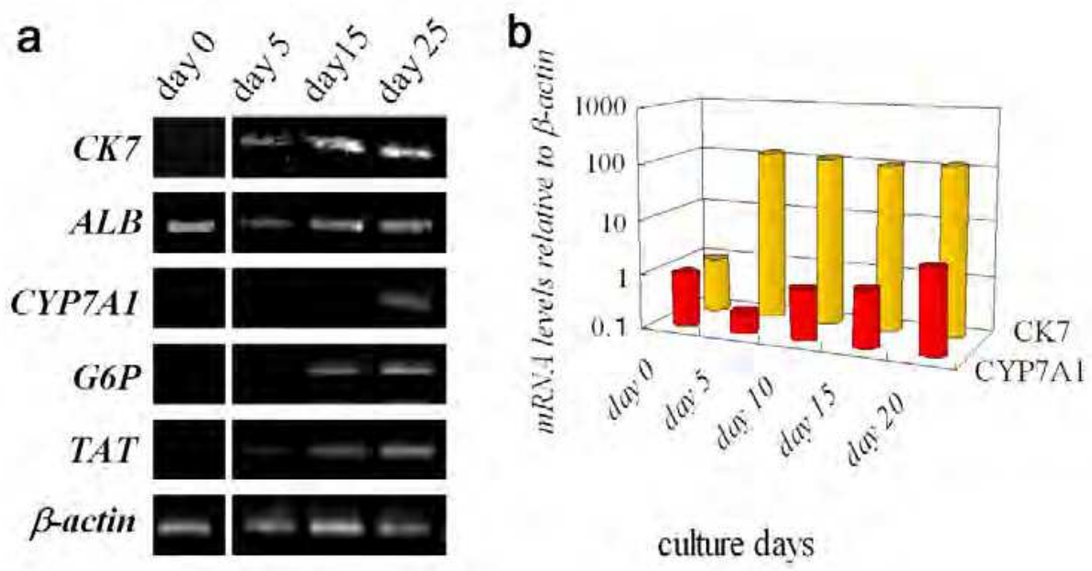

Fig. 5. Analysis of gene expression of mature hepatocyte makers in Liv2 positive cells cultured with Lanford medium. a; RT-PCR，b; Real-Time RT-PCR.

Taken together, these data strongly suggest that Liv2-positive cells are immature hepatocytes normally contributing to embryonic development and that anti-Liv2 antibody can be used to isolate immature hepatocytes derived from ESCs.

There is only one report adopting a similar strategy by purifying hepatic progenitor cells rather than mature hepatic cells with surface antigens (Li et al., 2010). They successfully isolated hepatic progenitors by FACS with c-Kit and EpCAM antibody, and demonstrated that these c-Kit-negative, EpCAM-positive cells could undergo long-term expansion with sustained hepatoblast-like characteristics. Moreover, they cloned the hepatoblast-like cells and showed these clones repopulated in the host livers without inducing tumorigenesis. Although EpCAM expressed in hepatic progenitor cells rather than adult hepatocytes, EpCAM also expressed in many other cells such as undifferentiated ESCs and definitive endoderm cells. Therefore, they complemented EpCAM in positive selection with c-Kit in negative selection since c-Kit is only expressed on ESCs, definitive endoderm cells and some mesoderm cells, but not hepatic cells. However, there may be other cells with c-Kit-negative and EpCAM-positive other than hepatic progenitor and the success of their strategy is thought to depend on their efficient induction of definitive endoderm from mouse ESCs. On the other hand, to isolate immature hepatocytes, we use single antibody (Liv2) highly specific to immature hepatocytes. Although anti-Liv2 antibody specifically recognizes rodent Liv2 antigen, but not human and gene encoding, its Liv2 antigen is still unclear, however, a human counterpart must exist considering the evolutionary relationship between rodent and human. Developing tools for biochemical analysis, such as mass

spectrometry, are expected to reveal the Liv2 gene. Thus, we expect the development of an isolating system for human immature hepatocytes with anti-Liv2 antibody recognizing human Liv2 in the future.

3.1.3 Isolation of mature hepatocytes with ICG

There are a lot of protocols for generating hepatocytes from ESCs or iPSCs in vitro, but purifying mature hepatocytes is still difficult and many different cell types remain. Several studies have used ESC lines transfected with the green fluorescent protein reporter gene controlled by promoters of hepatic genes, such as Foxa2 (Gouon-Evans et al., 2006), Afp (Yin et al., 2002), ALB (Heo et al., 2006; Soto-Gutierrez et al., 2006) and a1-antitrypsin (Aat) (Duan et al., 2007). However, these strategies need genetic manipulation that is not suited to the future therapeutic applications and GFP is known to have slight toxicity to cells. Moreover, mature hepatocytes rather than immature hepatocytes are known to be unstable in vitro. Thus, a particularly safe method is required for selecting mature hepatocytes derived from ESCs or iPSCs.

Indocyanine green (ICG) is a nontoxic organic anion that is eliminated exclusively by hepatocytes and is clinically used as a test substance to evaluate liver function (Berk & Stremmel, 1986). The uptake and release of ICG are frequently used to identify and/or evaluate ESC-derived hepatocytes (Agarwal et al., 2008; Farzaneh et al., 2010; Yamada et al., 2002). The fluorescence of ICG can be observed at 800-900 nm with appropriate excitation of near 780 nm. Therefore, we developed a flow cytometer equipped with an excitation laser of 785 nm to detect ICG-positive cells (Yoshie et al., manuscript in preparation). We determined optimal concentration and incubation time for detection of ICG uptake and showed that rat primary hepatocytes and ESC-derived hepatocytes selectively took up ICG. Although irradiation of ICG with a laser at 100 J/cm^2 was reported to generate singlet oxygen ($^1O_2$), which is damaging to cells (Hirano et al., 2007), the power of our laser (FISHMAN-R) was 5 mJ/cm^2 and much less likely to generate singlet oxygen. In fact, in analysis with trypan blue exclusion, the analyzed cells were not damaged by FISHMAN-R laser flow cytometry.

Similar sorting methods of hepatocytes or hepatocyte-like cells by use of antibodies are frequently reported, e.g. isolation of mature hepatocyte-like cells by FACS with sialoglycoprotein-receptor (Basma et al., 2009). However, because the purification of mature hepatocytes is thought to be a final step and the mature hepatocytes have limited proliferative potential, this purification step must be massive i.e. sorting a lot of hepatocytes. Therefore, use of antibodies in this step is inadequate in the cost performance, as the antibodies are expensive. Our detection system which relies on the selective uptake by hepatocytes of ICG, a stable and inexpensive fluorescent chemical compound, overcomes this problem and will lead to the development of an effective system for purifying hepatocytes derived from ESCs, iPSCs or somatic cells.

3.2 Use of hepatocytes for toxicology and drug development

The liver is centre of metabolism and the hepatocytes are responsible for metabolizing most compounds in vivo. Therefore, the cultured hepatocyte can be used to predict how drugs are metabolized and to what extent they may be toxic. These tests include use of primary rodent hepatocytes and human immortalised hepatocyte cell lines, such as HepG2 (Rudzok et al., 2010). However, the rodent hepatocytes have species-specific differences in metabolizing function from human (Lake, 2009) and immortalised hepatocytes have poor

hepatic function and sometimes have an abnormal karyotype (Wong et al., 2000). Thus, human primary hepatocytes have become a standard tool for evaluating hepatic drug metabolism in vitro. However, to do so demands a lot of functional hepatocytes and the utility of the human primary hepatocyte is seriously limited. Our induction and selection methods will be helpful to meet the demand of these examination systems as an alternative to primary human hepatocytes. Particularly, dual selection strategies mentioned above are suited for scalable production of human hepatocytes. Immature hepatocytes isolated with Liv2 were suggested to have unlimited proliferative potential (Figure 3) and could serve the scalable production. Although expanded immature hepatocytes have to differentiate into mature hepatocytes, our protocol could efficiently induce maturation with Lanford medium and the functional, mature hepatocytes that must be in large amounts could be purified inexpensively by FACS with ICG.

4. Further problems

4.1 In vitro tissue formation using induced hepatocytes

Besides immature hepatocytes being acceptable in some cases of hepatocyte transplantation due to hepatic maturation after transplantation, immature hepatocytes are absolutely unacceptable for use in evaluating hepatic drug metabolism in vitro. The mature functional hepatocytes are required in drug tests. However, in vitro induction of hepatic maturation or terminal differentiation has not been achieved enough. Realization of fully functional hepatocytes may require tissue organization similar to that of the liver in the living body. There is a dilemma in this problem: although the purification of hepatocytes is necessary for the improvement of the quality of induced hepatocytes, fully functional liver tissue requires various types of cells other than hepatic parenchymal cells. To solve this problem, we have three options: (i) selecting several types of cells and organizing them into tissue structure, (ii) selecting the precursor cells for histogenesis of liver, inducing differentiation and prompting self-organization and (iii) constructing artificial culture microenvironments mimicking native environments of liver. The culture microenvironments, such as microwell architecture (Mori et al., 2008), are well known to improve the function of cells, including hepatocytes (Sharma et al., 2010).

Previously, we showed the induction of retinal cells by co-culturing with ESC-derived RPE cells (Yue et al., 2010). Now, we try to apply a combination of these co-culture and microwell methods to induce mature function of hepatocytes.

4.2 Hepatocytes transplantation: Where or how are hepatocytes transplanted?

Purified pluripotent stem cell-derived hepatocytes are transplanted into patients with fatal hepatic disease, in whom hepatic tissue structures are newly developed and function. Thus, a new therapy for incurable hepatic disease substituting liver transplantation is established, which is a final goal of regenerative medicine.

The problems to overcome for successful regenerative medicine are to determine the appropriate sites for effective cellular survival and function, and the effective way, i.e. which should be selected for transplantation, cell type or tissue type. The best way still remains unknown. Most experimental studies show injection of pluripotent stem cell-derived hepatocytes through the vein or directly into the liver as cell type. Mouse ESC-derived hepatocytes were injected into the liver through the portal vein (Yamada et al., 2002). HGF-treated bone marrow mesenchymal cells were transplanted through the caudal

vein into the liver of CCl4-injured rats and this was effective against liver damage (Oyagi et al., 2006). After multipotent progenitor cells derived from human umbilical cord blood were transplanted into the left lateral lobe of the liver of the CCl4-injured rats or through the portal vein, they differentiated into the hepatocyte-like cells and were useful in treating the injury (Moon et al., 2009).

But there is extreme pessimism about these ideas, methods and practices because fatally damaged livers provide poor and severe environments, such as extreme fibrosis in cirrhosis, rapid cellular necrosis in liver failure etc. for transplanted fresh hepatocytes. It is more reasonable to transplant hepatocytes into good conditions with a rich blood supply ectopically. In fact, the first transplanted site performed in a human patient with cirrhosis was the spleen (Mito et al., 1992). According to their report, a part of the cirrhotic liver was resected, from which intact hepatocytes were isolated and were injected into the spleen; afterwards, the survival was confirmed. Their experimental reports using rats indicated that 6 months later splenic tissues were substituted by the hepatic tissues consisting of hepatic cords and hepatic sinus (Kusano & Mito, 1982; Mito et al., 1979). Except the spleen, fat tissues are good sites for breeding hepatocytes and their survival has been shown in fat pads (Jirtle & Chalopoulos, 1965). One of the authors transplanted rat primary hepatocytes into the rat mesenteric fat tissues and confirmed their survival and tissue structure (Sasaki et al., 1983) (Figure 6a). But ectopic transplantation has a significant problem: it has no way and route to discharge bile juice. Any definite answers have not been given yet, but we have some hints that might help to resolve the problem. For example, glands e.g. duodenal glands or pancreatic tissues are found in the submucosa of the intestine (Fig.6b, c). Clinically ectopic tissues e.g. pancreatic or stomach tissues are recognized. In evolution, primitive hepatic tissues are incorporated into the midintestine in the earth worm as chloragocytes with varied functions, including a storing function of endogenous substances, such as glycogen and lipids, and metals (Ireland & Richards, 1977) and intensive DAB reactivity (Fischer & Horvath,

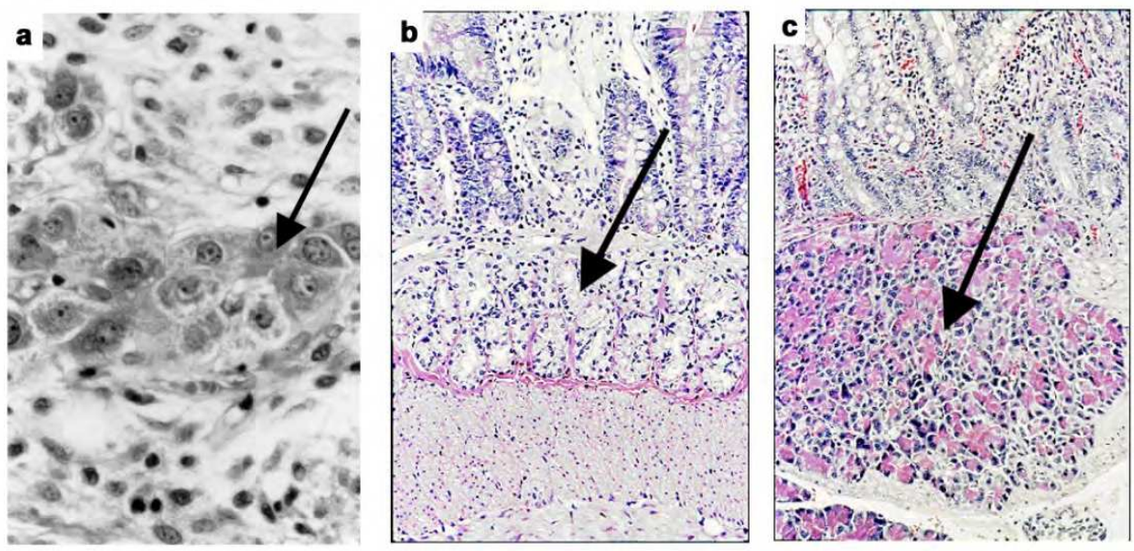

Fig. 6. Strategy of ectopic transplantation. a. The survival hepatocyte cluster in the rat intra-mesenterium (arrow). b. The duodenal gland in the submucosa of the rat duodenum (arrow). c. The pancreatic tissue in the submucosa of the small intestine (arrow).

1978). The next function in shipworms is midgut glands consisting of large cells with binucleate (Strunk, 1959) and enhanced hepatic function in crabs and lobsters as hepatopancreas (James, 1989). In mammalian embryonic development, it is well-known that hepatic buds grow from the duodenum described above. Therefore, our idea is to transplant hepatocytes into the submucosa and return them into the mother site, the intestine.

Our first trial was to confirm whether human ES cell-derived embryo bodies, which were treated with the previous cocktail (Takashimizu et al., 2009) and containing ICG-positive cells (Fig.7a), survived in the submucosa or in the intestine. Injection was insecure in that it was difficult to confirm where it was injected or whether hepatocytes or EBs remained there. Then, we have established a new technique for transplantation. The peritoneum and muscle layer of the nude rat was cut by an electronic scalpel and separated (Fig.7b), in whose shallow furrow EBs were arranged in order (Fig.7c). After the wound was closed (Fig.7d), one or two weeks later, the transplanted site was prepared for histological investigation. In the submucosa, unique tissues including duct like structures were found (fig.7e). The vascular

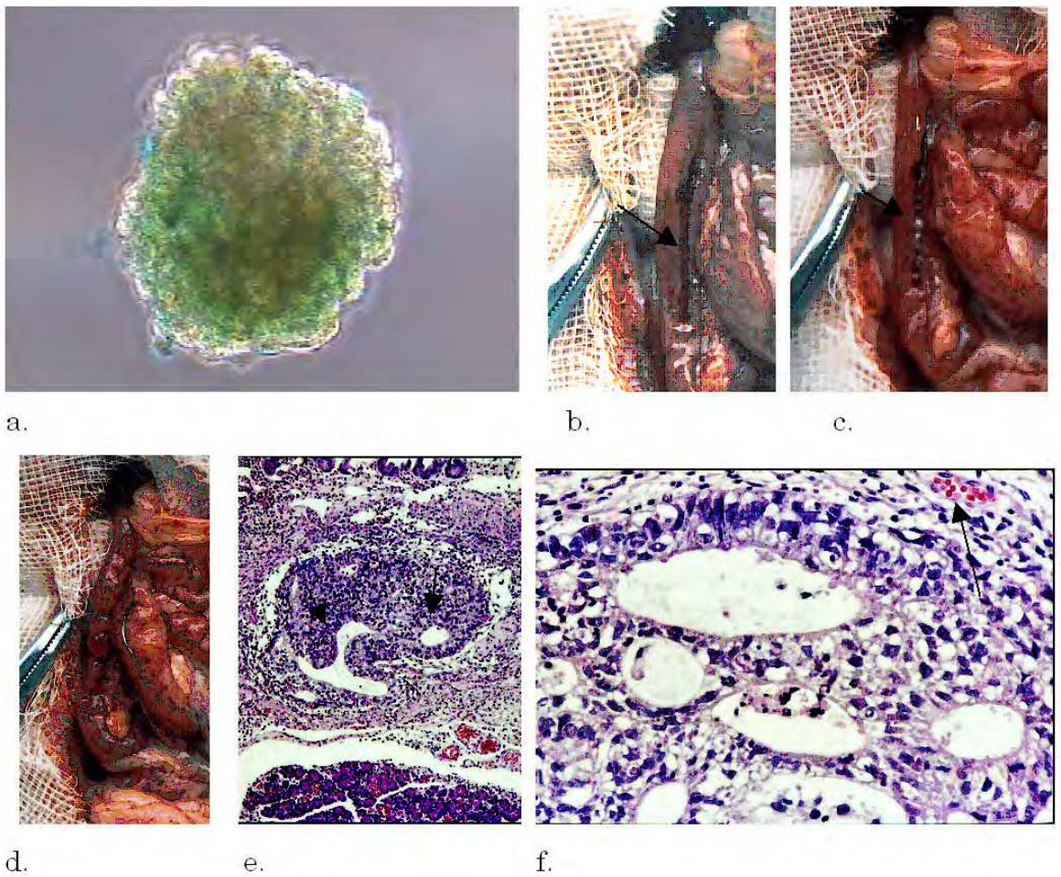

Fig. 7. Transplantation of human ESC-derived hepatocyte-like cells into a rat duodenum. a. Human EB containing ICG positive cells (green). b. The linear cut line performed on the surface of the nude rat duodenum (arrow). c. EBs (arrow) are arranged in the shallow furrow. d. The wound was closed with 7-0 nylon. e. Histology of the treated duodenum. EB survives in the submucosa along with duct formation (arrows). f. Vascular supply is confirmed (arrow), but is not sinusoid-like.

supply did not penetrate the cell cluster, but rounded the periphery (Fig.7f). Hepatic sinus did not appear to be differentiated yet. We recognized transplanted cell clusters survived in this method, but did not confirm that they differentiated into functional hepatocytes from EBs containing ICG-positive cells. At that time, we did not have the method to purify pluripotent stem cell-derived hepatocytes from mixed varied cell types yet. As the ICG-selection method has been established, as described above, purified hepatocytes will be transplanted and be confirmed to function and discharge bile juice into the intestine.

Further, for clinical trials, we provide a new technique, fibre scope transplantation. To cut the muscular layer is to subject patients to opening the abdomen, whereas if hepatocytes were injected into the submucosa, patients' discomfort would significantly decrease. But it remains unknown how hepatocytes are injected into the submucosa with the fibre scope, as fibre scope has not yet been developed for rats. The scope with the smallest diameter for use on a dog was tried through the stomach to attain the duodenum (Fig.8a). It was very difficult to inject into the mucosa on the same side, but was successful in swelling the mucosa on the opposite side (Fig.8b). Fibre scope transplantation will be an effective tool for endoderm-derived differentiated cells in addition to hepatocytes.

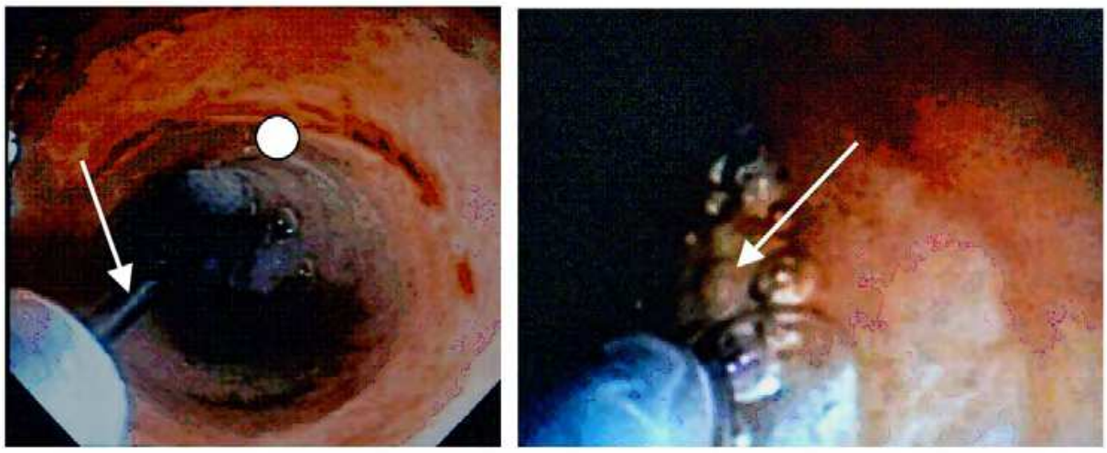

Fig. 8. Fibre scope transplantation. a. The interior of the nude rat duodenum. The red mucosa and the needle for injection of the fibre scope (arrow) are recognized. White circle is the mark for the needle to insert. b. The needle is inserted into the mucosa. The mucosa swells after fluid injection (arrow).

On the other hand, another problem is whether cell transplantation or tissue transplantation is better, more effective or reasonable. It will take a long time to determine this, or each method may be used according to varied conditions, such as kinds of disease, its progress and permitted transplanted site. But in conclusion we prefer tissue transplantation, because effective function is due to cell-to-cell interaction and tissue organization. Kikuchi et al. have developed a unique in site cell micropatterning system (Kikuchi et al., 2009). A photoresponsive cell culture surface was geometrically processed in situ with the UV irradiation to increase the cell adhesiveness of HepG2. After confirmation of HepG2 adhesion to the first dot pattern, fibroblast, which adhered easily to the plate without the UV irradiation were introduced to fill gaps among the HepG2 dot pattern. This simple

geometric pattern caused a 50-fold increase of CYP3A than conventionally cultured HepG2. Cell transplantation breaks once-established cell-to-cell interaction and requires reorganization in vivo, which creates double the work.

But three methods to complete tissue transplantation have to be established, the in vitro method of tissue organization, which geometric patterning described above helps, the technique to transport in vitro established tissue into in vivo without destruction and the method to acquire rapid blood supply after transplantation.

Scaffold-engineering may be essential to tissue transplantation, because it is easily transported in intact conditions and is processed for drug delivery systems e.g. slow releasing angiogenic factors (Hou et al., 2011). Collagen or modified collagen sponges were conventionally used as cell carriers in many labs, as well as in ours (Imamura et al., 2004; Kanematsu et al., 2004; Takimoto et al., 2003). The above collagen-based scaffold is not complete, because cells did not expand rapidly within the sponge in vitro. After transplantation, the carrier was bounded on the periphery by the non-penetrating host tissue. Moreover, function, such as urea synthetic rate, decreased below the control level (Hasirci et al., 2001). In addition to collagen-based materials, many other materials and methods are proposed (Kano et al., 2008; Katsuda et al., 2010; Torok et al., 2011), which will steadily improve tissue transplantation techniques. In the future it is expected that simpler, easier, more effective and more practical transplantation techniques will be developed.

5. Conclusion

In this chapter, we described the production and selection of hepatocytes and show a strategy for regenerative medicine using pluripotent-derived hepatocytes. There are two sources to obtain hepatocytes: (i) somatic cells, such as mature hepatocytes themselves and (ii) multi- or pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). We have reported the methods for induction of differentiation from pluripotent stem cells. Our protocols for induction of differentiation from pluripotent stem cells mimic the hepatocellular differentiation during embryonic development.

One important problem of the differentiation-mediated preparation of hepatocytes is how to exclude undifferentiated cells, while a lot of hepatocytes are produced. Additionally, it is impossible to improve the efficiency of hepatocellular differentiation to 100% and the remaining undifferentiated cells have the potential to cause teratoma after being transplanted into the host. To isolate hepatocytes, we proposed a dual selection method. The first is isolation of immature hepatocytes by cell sorting with Liv2, which specifically detects immature hepatocytes. The second is purification of mature hepatocytes by low cost sorting with ICG, following massive expansion of immature hepatocytes and induction of differentiation into mature hepatocytes with Lanford medium. The flow of our method is as below:

i. Induction of immature hepatocytes from pluripotent stem cells
ii. Isolation of proliferative immature hepatocytes with anti-Liv2 antibody
iii. Large scale culture expanding the immature hepatocytes and induction of differentiation into mature hepatocytes
iv. Low cost and safe purification of functional mature hepatocytes by FACS with ICG

The next step is in vitro and in vivo tissue formation. The culture condition mimicking tissue organization improves the function of cells, including hepatocytes. Moreover, to

realize the therapy for patients with incurable hepatic disease, it is necessary to promote tissue organization artificially in vivo. We propose that for the artificial organization of hepatic tissue, heterotropic transplantation into submucosa of the duodenum is most suitable, since the liver with incurable disease does not accept the transplanted hepatocytes easily.

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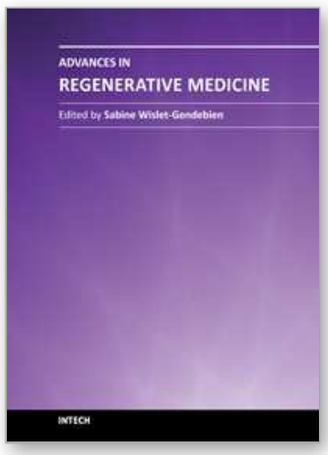

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Even if the origins of regenerative medicine can be found in Greek mythology, as attested by the story of Prometheus, the Greek god whose immortal liver was feasted on day after day by Zeus' eagle; many challenges persist in order to successfully regenerate lost cells, tissues or organs and rebuild all connections and functions. In this book, we will cover a few aspects of regenerative medicine highlighting major advances and remaining challenges in cellular therapy and tissue/organ engineering.

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