Liver-bud Transplantation: A Bud That May Bloom into the Future of Regenerative Medicine

The development of the human liver starts in the 3rd to 4th week of gestation with the outgrowth of a bud of proliferating endodermal cells from the ventral foregut. These endodermal cells differentiate into hepatoblasts in response to stimulatory signals from the adjacent cardiac mesenchyme and the mesenchyme of the septum transversum, in the form of fibroblast growth factor (FGF) and bone morphogenic protein (BMP) respectively. Without these signals, this bud would develop into the pancreas. The endodermal cells intermix with the adjacent mesenchyme of the septum transversum in the form of cords. Starting at the 6th to 7th week of gestation, a subpopulation of hepatoblasts starts to express biliary specific antigens and develop into a solid ring of biliary cells. Differentiation into biliary cells is regulated by hepatocyte nuclear factor (HNF)-6 and Notch pathway. Fibroblasts and stellate cells are derived from the mesenchyme and Kupffer cells presumably originate from the yolk sac, as they antedate bone marrow development. The developing liver incorporates the vitelline vein which runs from the yolk sac to the heart. The inferior part of the vitelline vein forms the portal vein and the superior part forms the common hepatic vein with the midsection becoming capillarized. Thus, the development of the liver is a complex process, requiring the presence of hepatic differentiated endodermal, mesenchymal and endothelial cells to form the vasculature, and involving complex signaling processes and interactions between these components. Importantly it has to be realized that the mesenchymal cells not only provide the scaffolding but are vital for the differentiation, maturation and integration of the various cell types into a functioning organ. Because of the complex and imperfectly understood nature of organogenesis, it was believed that it was almost impossible to recapitulate the steps necessary for organogenesis *in vitro*. The article by Takebe et al¹ in *Nature* sets out to disprove this belief by generating, for the first time, a functional three-dimensional human liver model *in vitro*. The authors hypothesized that co-culturing all three cell lineages in the presence of appropriate growth media and signaling molecules would induce development similar to that seen during embryonal hepatogenesis.

Undifferentiated human induced pluripotent stem cells (iPSCs) were first stimulated to differentiate into endodermal cells and then into hepatocytic lineage by appropriate culture media, differentiation factors and growth

factors including human BMP4, human fibroblast growth factor (hFGF), recombinant human activin A, recombinant human hepatocyte growth factor (hHGF) and recombinant human epidermal growth factor (hEGF). To generate human liver buds *in vitro*, 1×10^6 human iPSC derived hepatic endoderm cells (iPSC-HEs), $0.8\text{--}1 \times 10^6$ human umbilical vein endothelial cells (HUVECs) and 2×10^5 human mesenchymal stem cells (MSCs) were mixed, resuspended in a mixture of essential growth medium and hepatocyte culture medium and plated on solid medium in a 24-well plate. Forty-eight hours after seeding, these two-dimensional cell clusters self-organized into macroscopically visible three-dimensional cell clusters representing human iPSC derived liver buds (iPSC-LBs). There was a formation of endothelial network with homogenously distributed human iPSC-HEs as seen by fluorescent protein-labeled cells. Compared with iPSC-HEs alone, cells in human iPSC-LB had significantly increased expression of early hepatic marker genes such as alpha-fetoprotein (AFP), retinol binding protein 4 (RBP4), transthyretin (TTR) and albumin.

To test whether these iPSC-LBs could be vascularized and grow into completely functional mature liver, self-organized human iPSC-LBs were detached after 4–6 days of culture, collected and transplanted into a preformed cranial window of an immunodeficient mouse. Human blood vessels in the iPSC-LBs connected with host vasculature within 48 h compared to iPSC-HEs transplanted without endothelial cells which failed to vascularize and engraft. Also, the vascular networks were similar in density and morphology to those of adult livers in iPSC-LBs transplants, compared to the much less dense vasculature in only HUVECs with human MSC transplants. Hepatic cells in human iPSC-LBs proliferated rigorously, particularly until 2 months after transplantation.

Histologically the human iPSC-LB transplants were similar to actual human liver buds, consisting of hepatic cord-like structures and became characteristic of adult liver after 60 days with similar molecular markers. Majority of the cells matured to become AFP negative while remaining albumin positive, characteristic of adult liver cells. Albumin production was higher and gene expression profiling suggested more mature hepatocytes in this model compared to transplanted iPSC derived mature hepatic cells alone. This suggests the importance of a three-dimensional vascularized tissue for successful engraftment, maturation and function. Furthermore, challenging the transplanted mice with drugs having hepatic metabolism produced human liver specific metabolites. Also,

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metabolome analysis of the iPSC-LBs was done to characterize the metabolic products of sugar, amino acid and nucleotides. Metabolic profile was similar to that of the adult human liver rather than the original iPSC-HEs. The iPSC-LBs were also transplanted into the mesentery in some mice, a more realistic and practical site for human studies, and good engraftment was achieved with improved survival after experimentally induced liver failure of the native mice livers.

The difference between previous stem cell based transplantation approaches and the present study is in the use of all the three principal cell types that are present during normal organogenesis, viz. endodermal cells, endothelial cells and mesenchymal cells, along with the requisite growth and differentiation factors. Compared with culture of iPSC-HEs alone, expression of markers of early hepatogenesis was much more pronounced in this model. Stem cell based therapies in liver failure have failed mainly because of short lived improvements in liver function. This is presumably because of poor engraftment of the stem cells when injected either in systemic or portal venous circulation. Allowing for development of a solid three-dimensional liver bud prior to transplantation, combined with the presence of all three cell types, allowed for successful engraftment, which was also dependent upon vascularization of the graft by the recipient vessels. Because of this, the graft continued to mature and function for a longer duration. Vascularization was better in the iPSC-LBs compared to HUVECs and MSC transplants. Not only synthetic functions but also the metabolic and detoxification functions resembled adult livers after 60 days.

This study is a first of its kind proof-of-concept that early organogenesis can be recreated *in-vitro* and that the developed organ-bud can subsequently be transplanted into a recipient to generate a functional three-dimensional, vascularized organ. The immediate utility is in developing models of the human liver, and other organs, in animals to study the human metabolism of various

drugs and toxins. In the long-term there is enormous therapeutic potential of using organ-bud transplantation for treating organ failure in the face of acute shortage of organs for transplantation and the generally disappointing results of stem cell transplantation.

Several bench-to-bedside issues remain to be addressed before this approach can even be considered practicable. Although, iPSCs are a particularly attractive option as there are no ethical concerns associated with embryonal PSCs, several controversies about iPSCs have remained unresolved since they were first produced in 2006. Although, iPSCs can be derived from the patient's own somatic cells, it is not clear whether they would be immunogenic and whether immunosuppression would be needed, as these cells express many antigens not found in normal adult cells. Also, there is theoretical risk of activation of oncogenes or insertion of retroviral genes leading to mutations during induction of somatic cells into PSC. Teratoma formation is considered a major obstacle to stem cell based regenerative medicine by the FDA. The efficiency of the process of induction is quite low with only a small fraction of cells attaining pluripotency. Also, whether these bud grafts will be able to grow sufficiently to be suitable for implantation and anastomosis and whether they will be able to meet the recipient's need after explantation or failure of the native organ, remains to be seen.

REFERENCE

1. Takebe T, Sekine K, Enomura M, et al. Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature*. 2013;499:481–484.

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