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REVIEW

Future of liver transplantation: Non-human primates for patient-specific organs from induced pluripotent stem cells

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Abstract

Strategies to fill the huge gap in supply *versus* demand of human organs include bioartificial organs, growing humanized organs in animals, cell therapy, and implantable bioengineered constructs. Reproducing the complex relations between different cell types, generation of adequate vasculature, and immunological complications are road blocks in generation of bioengineered organs, while immunological complications limit the use of humanized organs produced in animals. Recent developments in induced pluripotent stem cell (iPSC) biology offer a possibility of generating human, patient-specific organs in non-human primates (NHP) using patient-derived iPSC and NHP-derived iPSC lacking the critical developmental genes for the organ of interest complementing a NHP tetraploid embryo. The organ derived in this way will have the same human leukocyte antigen (HLA) profile as the patient. This approach can be curative in genetic disorders as this offers the possibility of gene manipulation and correction of the patient's genome at the iPSC stage before tetraploid complementation. The process of generation of patient-specific organs such as the liver in this way has the great advantage of making use of the natural signaling cascades in the natural milieu probably resulting in organs of great quality for transplantation. However, the inexorable scientific developments in this direction involve several social issues and hence we need to educate and prepare society in advance to accept the revolutionary consequences, good, bad and ugly.

Key words: Induced pluripotent stem cells; Hepatocytes; Tetraploid; Non-human primates; Anencephaly; Chimpanzee; Fumaryl acetoacetate hydrolase deficient; Hhex

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INTRODUCTION

There is a huge gap in supply *versus* demand of human organs for transplantation. Currently 108 614 patients in United States are waiting for an organ transplant according to United Network for Organ Sharing (UNOS)[1] against 7136 donors. There is a need to bridge this gap. Either we have to motivate more people to allow organ donation, or rely on alternative methods such as improved artificial organ support systems (dialysis machines, bioartificial liver, etc) or search for better ways to circumvent the problems, mainly immunological, with xenografts.

This includes improved methods for suppressing host immunity and growing "humanized organs" in animals. Recent developments with induced pluripotent stem cells (iPSC) have yielded a new option-growing organs from pluripotent stem cells derived from the patient's own tissues. Attempts have been made to grow

organs *in vitro* with mixtures of different stem cells and biocompatible scaffolds, but the development of an organ cannot be replicated *in vitro* due to its complexity. This is a major obstacle in the generation of organs, including those attempts to make organs derived from a patient's iPSCs, the ultimate goal in regenerative medicine. The straightforward method is to generate a fetus from iPSC in a surrogate mother for the sole purpose of organ harvesting, but obviously a host of ethical issues precludes this line of thought. Here, I will make an attempt to review the latest developments and discuss their prospects, taking the liver as a model organ.

Currently, more than 17 000 people in the United States are waiting for liver transplants. According to UNOS, about 5300 liver transplantations were performed in the United States in 2002.

BIOARTIFICIAL ORGANS-THE BIOARTIFICIAL LIVER: A WRONG ROAD?

The bio-artificial liver (BAL) is still in its infancy. BAL as a replacement for the normal liver is very unlikely. At most it is currently of use in bridging the gap between organ failure and transplantation or liver regeneration (as the liver has exceptional capacity to regenerate). BALs are largely unsuccessful because: (1) most of the liver cell lines are not functionally efficient and human iPSC-derived "hepatocytes" are not functional enough and difficult to obtain in sufficient quantities; (2) the special arrangement of hepatocytes into chords in sinusoidal spaces is important for their function; (3) the relationship and communication between hepatocytes themselves and between biliary epithelial cells, sinusoidal endothelial cells, etc, are quite important for functions of hepatocytes, such as active and passive transport of metabolites in the right direction and optimal gene regulation; (4) currently there is no source of functional hepatocytes in large quantities for bioreactors except from animals, which always pose a problem of infection, immune system activation, and functional incompatibility of essential proteins secreted by or possibly interacting with hepatocytes; (5) hepatocytes have a low life span under culture conditions, and it is difficult to maintain conditions close to that of the human microenvironment; and (6) difficulties in developing complex membranes which allow highly selective exchange of biologically important molecules.

The efficiency of bioartificial liver devices such as the "extracorporeal liver assist device" (ELAD) or non-biological devices such as the "molecular adsorbent recycling system" (MARS) and other models are not very different from dialysis alone. The current data show that only the MARS system reduces mortality in acute liver failure and in acute exacerbations of chronic liver failure, although this reduction is non-significant[2].

HUMANIZED LIVER IN ANIMALS

Pigs are the preferred animal for humanized organs, although primates like chimpanzees or gibbons would the ideal for the generation of "humanized" organs. We have made remarkable progress in the last 10 years in the field of xeno-immunology of pig-to-nonhuman primate transplantation, and we are expecting clinical trials in the near future. A common school of thought is for engineered animals lacking certain antigens so that their organs can be used for transplantation in human patients with a reduced chance of immune rejection[3]. Pigs can be genetically modified for xenotransplantation by alteration of immunologically important genes such as human decay-accelerating factor (hDAF), and CD46 (membrane cofactor protein), α-galactosyl transferase knockout (GT-KO), CD55 or CD46, CD59 transgenics, as well as human leukocyte antigen (HLA)-Ⅱ transgenics, including DP, DQ, and DR[3,4]. HLA-DR15+ transgenic pig skin pieces were grafted onto severe congenital immunodeficiency (SCID) mice reconstituted intraperitoneally with HLA-DR15+ human peripheral blood mononuclear cells. The dermal graft survived and was integrated[4]. Using GT-KO pigs and novel immunosuppressant agents, 2 to 6 months' survival of heterotopic heart xenotransplants has been achieved. The issue of hyper-acute rejection is more or less solved with hDAF and GT-KO pigs, but acute humoral xenograft rejection, injury to the endothelium leading to thrombotic microangiopathy and coagulation dysregulation, remains unsolved for a meaningful survival rate to be achieved. Baboons died following massive internal bleeding and profound thrombocytopenia post-transplantation of livers from GT-KO pigs transgenic for CD46[5,6].

STEM CELLS, IPSC AND SOPHISTICATED SCAFFOLDS MEET THE ANGIOGENESIS OBSTACLE FOR GENERATING ORGANS

The genesis of organs is a very complex process. Organs such as the brain, liver and kidney have extremely complicated architecture and contain several cell types. The relationship between cells, their specific orientation, and physical and chemical characteristics are of crucial functional importance. Thus, even if we generate genetically intact and fully functional hepatocytes, biliary epithelial cells, angiogenic precursor cells, sinusoidal endothelial cells, kupffer cells and so on, we are unlikely to regenerate (or generate *de novo*) a liver through co-culture of these cells, injecting these cells in a defined proportion into a damaged liver, or populating an appropriate scaffold or matrix. A highly sophisticated scaffold or matrix with spatial and temporal cues-chemical, mechanical, ionic, electric charge or surface properties-for homing of different cell types is unlikely to be successful in the near future, considering the complexity of the micro architecture of organs required for normal physiological function. One of the major barriers to successful generation of organs *in vitro* is our inability to generate the vascular architecture necessary for growth, development

and maintenance of any organ. Recently, attempts have been made to use natural scaffolds by decellularization of an entire organ, the liver in this example, and preserve its vascular network. Preliminary studies showed the possibility of being able to efficiently re-cellularize the bioscaffold using perfusion cell seeding with primary human fetal liver progenitor cells and endothelial cells in a bioreactor[7]. However, as noted above, numerous difficult technical issues remain to be addressed to efficiently deliver primary human liver progenitor cells to generate functional hepatic tissue. Availability of decellularized human liver scaffolds would be another problem.

Although iPSC technology offers wonderful possibilities for generating practically every cell type from adult somatic cells through a pluripotent stem cell intermediate, currently this has limited applications in, for example, regeneration of tissues of lesser complexity such as bone marrow and adipose tissue with a genetic modification [example: C-C chemokine receptor type 5 (CCR5) in the bone marrow stem cell gene therapy of acquired immunodeficiency syndrome (AIDS) or adipocyte gene therapy in inherited forms of diabetes or lipodystrophy][8] or without a genetic modification (as in the management of leukemia or degenerative disease, old age), or drug testing. For example, iPSC-derived hepatocyte-like cells, and proximal or distal renal tubular epithelium for hepatic or renal toxicity testing, respectively, are useful in new drug development or assessment of drug response to different human genotypes, a step towards personalized medicine.

Small organs or tissues can be engineered successfully using scaffolds, for example, blood vessels or urinary bladder. By culturing cells on a biodegradable scaffold such as polyglycolic acid, and later passing media in a pulsatile fashion under optimum pressure, was found useful in generating functional small-caliber arteries[9]. The pulsatile flow triggers collagen deposition and alignment of the fibers and this is critical for attaining mechanical maturity to withstand pressure met under natural conditions[10].

Growing larger organs is a major problem because oxygenation and metabolite exchange becomes difficult as the thickness increases. Self-assembly of cells, for example cardiomyocytes, can take place in thin sheets (< 80 μm), and increasing the thickness by sequential deposition of multiple cardiac sheets has to be slow enough to allow the host vasculature to sprout into and vascularize each layer before the next layer is deposited[11]. However this method is very impractical in humans because of the necessity of multiple surgeries. Furthermore, this approach is unlikely to be successful for more complicated organs like the liver, not only because the liver has different types of cells in a highly ordered manner, but it also has a complicated dual vasculature forming the sinusoids. Following a nature mimetic approach, a vascular tree should have a capillary network (10 μm-20 μm) which can be generated by induction of sprouting by cytokines and co-culture with related cells; the intermediate microvessels (50-500 μm) may be obtained by microfabrication-microfluidic techniques and finally the microvasculature (about 2 mm) is produced by a combination of tissue engineering methods[12]. Unfortunately, achieving vascularization in a tissue by assembling all these and finally generating a fully vascularized organ which is functional is a very complicated process making this approach undesirable.

MAKING GENETICALLY HUMAN ORGANS IN ANIMALS

It is an ingenious idea to generate genetically human organs in animals. With the recent advancement in iPSC technology, transgenic technology and embryo manipulation, it is possible to generate organs of one animal species in another one. The best achievement in this direction is reported by Kobayashi *et al*[13] in Cell 2010. Mouse wild-type iPSCs injected into Pdx1 -/- rat blastocysts (Pdx is a critical gene for genesis of the pancreas and hence Pdx1 -/- rats are pancreatogenesis-disabled) developmentally compensated for the vacancy of the pancreatic "developmental niche", generating almost an entirely iPSC-derived rat pancreas inside the mouse, and mouse iPSC-derived pancreas inside the Pdx-/- mouse. Similarly it could be possible to generate a human pancreas (and other organs) in animals, for example in monkey, pig or sheep, which are genetically modified to support implantation and development of an embryo containing cell clusters/organ of human origin.

Production of a chimeric embryo/fetus[14] was performed largely to study organogenesis, cell migration, cell lineages, cell destination, development and function of the immune system, rather than with the aim of generating live chimeric animals for organ/tissue harvesting. However, efforts to make live intergeneric chimeric animals (for example rat-mouse chimera) were unsuccessful because of incompatibility between the fetal parts of the placenta and the uterus[15,16]. The only exceptions we know are hybrids like geep (a sheep and a goat)[15-17]. Thus it is one of the major achievements of 2010 to produce a rat-mouse intergeneric chimera by injecting mouse pluripotent stem cells into rat blastocysts.

NEW WORLD MONKEYS COULD BE USED TO GENERATE GENETICALLY HUMAN ORGANS

Rats and mice belong to same family (Muridae) and subfamily (Murinae), but of a different genus, while human beings and chimpanzees belong to the same genus and there are seven species in the sub-family "Hominini" which contains man, chimpanzees, gorillas and orangutans. Man is closer to the chimpanzee than the rat is to the mouse (Table 1). Modern molecular studies have spectacularly confirmed this prediction and have refined the relationships, showing that the common chimpanzee

Table 1 Comparison between mouse and rat *versus* chimpanzee and human

	Mouse	Rat	Chimpanzee	Human
Size	20 g-40 g	250 g-520 g	35 kg-75 kg	45 kg-100 kg
Chromosomes	20 pairs	21 pairs	24 pairs	23 pairs
Genome similarity	96.50%		98%	
Gestation period	20 d	22 d	9 mon	9 mon
Birth weight	0.5 g-1.5 g	5 g-6 g	1.5 kg-2 kg	2 kg-4 kg
Liver	4-5 distinct lobes	4 distinct lobes	3 lobes which are not separate	3 lobes which are not separate
Gallbladder	Present	Absent	Present	Present

(*Pan troglodytes*) and bonobo (*Pan paniscus* or pygmy chimpanzee) are our closest living evolutionary relatives[18]. This opens an exciting possibility to generate and harvest human organs, genetically identical to the recipient, in new world monkeys. There is about 98% sequence similarity between human and chimpanzee genomes, and the global variation between humans at the single nucleotide level has been estimated at about 0.1%[19,20]. Chimpanzee body temperature, general blood biochemistry (glucose, sodium, potassium, calcium, phosphate, insulin, hemoglobin, urea, etc), red blood cell count, white cell count, platelet count, osmolarity, plasma protein composition, etc, falls within the range of human values[21]. It may be noted that rather than genetic differences, what makes humans unique are "aspects of human uniqueness which arose because of a primate evolutionary trend towards increasing and irreversible dependence on learned behaviors and culture"[20].

There are multiple possible approaches to generate a human organ in a chimpanzee or a higher primate. One approach is to make a true chimera by populating the chimpanzee donor blastocyst with patient specific human iPSC, which is modified to have genes for development of the liver but deficient in genes for brain development. This ensures that under no circumstances will a human brain develop inside an animal or grow with cells of animal origin. The chimpanzee donor blastocyst should be deficient in the genes which are critical and specific for the development of the organ in question (Pdx1 in pancreas and probably Hhex in liver). However, for the human liver we have yet to identify the most suitable liver-specific gene which can be knocked out without affecting general development of the fetus. Foxa1, 2 and 3, Gata-6, HNF-4a, HNF-1a, Hhex, Sox-9 are among key genes involved in foregut-liver development[22-26]. Alternatively, the blastocyst may be deficient in a protein which is metabolically important, and whose deficiency would result in selection pressure, such that only the iPSC-derived cells would survive. Taking the liver as a model organ, fumaryl acetoacetate hydrolase (FAH)-deficient chimpanzee blastocysts would be a good example which would be populated with human patient derived iPSC with the normal (wild type) FAH gene. During the development of the fetus, human liver cells expressing FAH would have a survival advantage over chimpanzee liver cells lacking FAH. Fumarylacetoacetate, a toxic metabolite, will accumulate in FAH deficient liver cells and kill them[27]. This selection process can be controlled at will using NTBC to facilitate a smooth and optimum rate of cell replacement without affecting the liver architecture. The introduction of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) inhibits p-hydroxyphenylpyruvate dioxygenase (the second enzyme in tyrosine degradation) and stops the formation of the toxic metabolites[27]. Thus, giving animals NTBC and slowly weaning them off might lead to a liver which is exclusively composed of human liver cells inside a chimpanzee fetus. One major worry in creating human organs in animals is the formation of germ cells from human donor cells in the gonads of the recipient animal, although the possibility is remote. Using animal blastocysts as well as the surrogate mother animal where the critical genes for spermatogenesis/oogenesis are knocked out, the theoretical possibility of germline transmission of human genes can be ruled out. A practical approach would be to use a mixture of human patient-derived iPSC with nonhuman primate embryonic cells/iPSC, in which some specific genes for fertility (with no effect on implantation or the development of the embryo, e.g., an acrosomal protein for male infertility) are knocked out for introduction into a tetraploid embryo (tetraploid complementation technique)[28]. It may be noted that there are reports of efficient generation of iPSC from non human primates[29]. There exist several methods to generate genetically intact 'virus free' iPSC from adult primate cells[30-34].

The proposal is very attractive but we can anticipate the following problems: (1) a chimpanzee-human mosaic fetus may not survive (though unlikely) because: (a) growth factors and transcription factors and/or signaling pathways may be incompatible; (b) cell adhesion molecules or response to directional molecule gradients may be different; and (c) the developing fetus may abort due to unforeseen reasons (e.g., failure of the tetraploid complementation technique or implantation and development); (2) possible immune rejection on transplantation even after perfusion washes and immune cell depletion due to small quantities of antigens, for example glycoproteins, adhering to the vessel walls, interstitial spaces, growth of some animal blood vessels into the "human" organ, etc; (3) a possibility that iPSC-derived organs are more prone to tumors; and (4) ethical issues involved in making human-chimpanzee mosaic embryos which might survive to near full term, even if it is ensured that human brain (or certain types of human neurons important in cerebral cortex for human identity) will not be present in the fetus by using human iPSC knockout for genes specific for brain development.

Despite these problems success is very likely because the genetic difference between mouse and rat is greater than that between human and chimpanzee and it was proved by Kobayashi *et al* that it is possible to generate a rat pancreas in a mouse. The immunological rejection

is less likely to be a major problem at least in the case of the liver because: (1) liver is a very immune tolerant organ compared with several other organs such as the kidneys[35,36]; (2) the patients own cells will be used to generate the new liver, ensuring 100% HLA matching; (3) better and less toxic immune-suppressants and immune-modulators are currently available; and (4) one can perform immunodepletion on the liver prior to harvest, first by treating with an immunocyte-specific mitogen and then treating with cyclophosphamide; this will push the immunocytes into mitosis which would then be preferentially killed by cyclophosphamide. There are more than a few ways to overcome the obstacle presented by the immune system in this setting, including the induction of immunological tolerance in the host[37]. Novel methods such as inhibition of leukocyte costimulatory molecules may offer a way to suppresses T cell activation resulting in immune suppression[38]. Several studies have found increased abnormal epigenetic changes, mutations in coding regions, and copy number variations in induced pluripotent cells compared with normal in a small proportion of cells[39-42].

It may be noted that iPSC is a relatively new technology and it might take another decade for the technology to mature. Similarly, newer screening methods which would facilitate selection of genetically intact cells, such as faster methods for whole genome scanning for mutations and epigenetic abnormalities are expected to resolve these issues.

Any research involving implantation of human embryos into the uterus after *in vitro* manipulation at any stage of development in humans or primates is illegal. However using a non-human primates (NHP) embryo to develop a human organ inside a NHP fetus inside the uterus of a NHP may not be illegal in many countries. The National Academy of Sciences (United States) Guidelines, recommends that human-nonhuman chimeras will not be allowed to breed, but this recommendation is only voluntary[43-45].

MAKING HUMAN ORGANS IN PARTLY *IN VITRO* SYSTEMS-ETHICAL ISSUES

Many ideas which are quite logical cannot be put into practice because of ethical concerns. One great example is therapeutic cloning. Commoditization of human oocytes and human sperm or human embryos and human organs is considered unethical in many countries. Any *in vitro* or *in utero* culture of intact human embryos, regardless of the method of its derivation, beyond 14 d or formation of the primitive streak, whichever is earlier, is illegal. One of the main concerns is the identity of the embryo as a human. However, human identity is technically the development of the brain and the nervous system which defines and determines all emotions, pain, memories, self respect, ethics and self identification. Growing an embryo which is anencephalic (without brain) for organ harvest would be a solution. However carrying an anencephalic fetus may be emotionally devastating for the surrogate mother in some cases. This can be avoided in turn by having a "*in vitro*-uterus" /semi-artificial uterus system (or uterus with some of the supporting organs) to facilitate the growth of the anencephalic system containing the organ of interest. However, these futuristic concepts are well beyond the consideration of current society for ethical reasons and the unpredictable social and medical consequences.

SCANNING AND PRINTING AN ORGAN

Development of a fetus from a zygote is an example of directed self-assembly processes, in which, through chemical or physical gradients, or predetermined cell-cell and cell-extracellular matrix interactions, the developing organism gradually acquires its final shape. Thus it is logical to assume that if we could provide the appropriate gradients, position and neighbors, then cells will migrate, self-assemble, and establish the correct connections to form the organ. This is quite true for tissues or organs of low complexity such as cartilages, bone, skin, urinary bladder or heart valves, but is unlikely for complex organs such as the liver or brain. Thus the human cornea, urinary bladder, etc. may be ideal tissues/organs for bio-artificial/"engineered" organs rather than complex organs such as the liver.

Most organs are composed of several types of cells in a very specific order in 3-dimensional space which is critical for their function. The concept of inkjet printing opens up a solution to this problem because it allows precise delivery of multiple cell types and matrix components into pre-determined sites with high precision. Multiple cell types in suspension are placed, instead of ink, into different "ink" chambers of a sterilized cartridge and the printer is directed to arrange or "print" these cells in a specific order. It is also possible to use conventional 2-dimensional printing to generate cells of different phenotypes with differential coatings of cell adhesion molecules printed in a specific pattern on extremely thin films with differential cell adhesion properties, which would result in a final pattern formation through minimization of configuration energy, the driving force in cell rearrangement. If we could use a suitable matrix, a chemical gradient also can be printed[46,47].

There is concern that bioprinting would result in non-functional tissues. However, in an elegant experiment by Jacob *et al*[48], synchronous macroscopic beating was demonstrated throughout a sheet obtained by the fusion of chick cardiac cell spheroids through bioprinting.

Imagine a scanner that can scan in 3 dimensions in sub-nanometer resolution and store an enormous amount of data with spatial coordinates of each molecule in the scanned object! Similarly, imagine a 3-dimensional printer that can print at sub-nanometer resolution. If such a scanner and printer could exist, one could scan an entire organ no matter how complex it is, if not an entire human body and reconstruct (clone) it, perhaps so perfectly that it includes the memories! The printer

would be using all molecules which constitute the human body as its ink equivalent! This is science fiction today but tomorrow this may become a reality!

CONCLUSION

The development of iPSC technology has enabled us to generate cells which are very similar to pluripotent stem cells from adult cells. Improvements in this technology will have radical consequences in regenerative medicine, transplantation medicine, therapeutic cloning, and generation of patient-specific whole organs. Generation of iPSC-derived whole organs inside the uterus, making use of the natural developmental signals and environment may result in more natural and high quality organs for transplantation. In future, non-human primates or an "*in vitro*-uterus" may be useful for producing patient-specific organs such as the liver for transplantation. The society needs to be prepared in advance to accept the revolutionary consequences, good, bad and ugly, of these ongoing scientific developments.

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