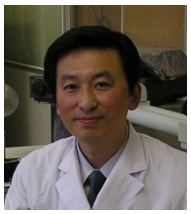
Present and Future for Research of Organ Regeneration

Eiji Kobayashi, MD.,Ph.D.
Keio University School of Medicine

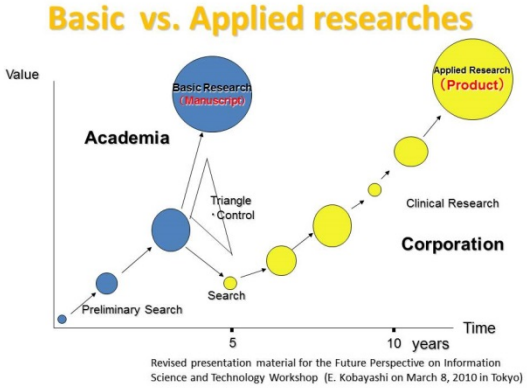


Introduction

I have been specializing in organ transplantation as a surgeon since the early days of my medical career. I am citing here the ancient painting of St. Cosmas and St. Damian
in order to emphasize the fact that there exists basically a difference in time spectrum between the basic research and the applied one (Figure 1).

The painting shows us that these famous two saints replaced the leg of the judge in Rome. It is told that the leg as a donor organ was the one from the Ethiopian condemned criminal. What I'd like to convey through this painting is that since the ancient days there have been attempts to subsidize the lost or missing parts of human body for the organ transplantation, and over the centuries these attempts have become real. I have done liver transplantations as my specialty in organ transplantation. Nevertheless, I have been in doubt how long we still should rely on the donors' organs for transplantation even in the future such as in 50 years, in 100 years. Lots of different approaches have been developed so far for creating organs. Based on these trends, I'd further refer to the subject as below. The terminology "regeneration" is commonly used in this research field. In my course of study I have concluded in using "Organ Fabrication" which fits better for the purpose of fabricating the organ again. In my knowledge acquired over the years I would give you the updated trends divided into three research fields.

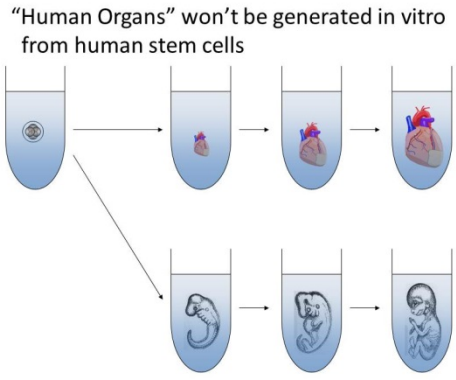
What the slide shows is something like teaching a fish to swim (Figure 2).



So to speak "Basic research" is the

research which has been progressing in high speed with revolutionary findings. But sometimes the outcomes tend to be hidden underground. There are cases that we can't check the updates in magazines nor at conferences, furthermore, on internet nor at MEDLINE. I regard them affirmative because what they do is the applied research for the purpose of fixing as a medical care through product planning. What I intend to explain is that there exists time spectrum between the basic research and the applied one. It is very important to balance the Triangle control: considering the situation, disclosing to the public and protecting the intellectual property. Also it is extremely important to see the Triangle from ethical point of view.

The most important point I have found when I started organ fabrication research is that we will never create organs in vitro (Figure 3).



Some findings have been studied why it is not viable but still it is under the process of pointing out the crucial reasons. It might be a repetition but it is clearer than the sky that whatever we command the stem cells in vitro to induce differentiation for the development or growth to organs, it does not turn out. For example, we can't fabricate islet in pancreas by using the receptor which reacts on sugar. This phenomenon never takes place in vitro. It is proven that we can create cells which secrete insulin. On the contrary, it does not come with the vital function which releases insulin reacted on glucose. If we could create this kind of function, we would fabricate human body in vitro. I have been doing intensive researches for what kind of element is lacking (Figure 4). Stem cells are indispensable for generating organs. In the course of generation, stem cells themselves create scaffold. As stem cells multiply themselves and also turn out to be other supporting cells by increasing and decreasing innumerable growth factors and in the end they create human organs perfectly. I have been always thinking how we can make this vital process possible through organ fabrication.

I have divided today's topics into 3 sections (Table 1).

Table 1 **Classification of approach to produce the transplantable organs**

Category	Proof-of-concept rodent model		Pig model	
Whole animal (in vivo organogenesis)	pancreas	(Kobayashi T et al. Cell 2010)	pancreas	(Matsunari H et al. PNAS 2013)
	liver	(Hata T et al. Ann Surg 2013)	liver	(Fisher JE et al. Liver Transplant 2013)
	kidney	(Usui J et al. Am J Pathol 2012)	SCID pig	(Suzuki S et al. Cell Stem Cells 2012)
Donor of embryonic primordium	kidney	(Matsumoto K et al. Stem Cells 2012)	pancreas	(Hammerman M. Organogenesis 2012)
	liver	(Takebe T et al. Nature 2013)		
Donor of decellularized organ	heart	(Ott HC et al. Nature Med 2008)		
	kidney	(Ross EA et al. JASN 2009)	kidney	(Orlando G et al. Ann Surg 2012)
	liver	(Uygun BE et al. Nature Med 2010)	liver	(Yagi H et al. Cell Transplant 2013)
	Lung	(Ott HC et al. Nature Med 2010)		

*(**Table** modified from Hata T, et al. Organogenesis 2013)*

The first is the research field of animal embryo; the research trend to fabricate organs by using animal organs. The second is the study resembling heterologous transplantation; the fabrication of organs by transplanting animal fetus-like. The last is what I have been involved in; the research for organ fabrication. The study to create organs by imitating the vital reactions to the extreme which would never be possible in vitro. The underlined in red is the outcome of my research paper. I'd like to introduce it step by step.

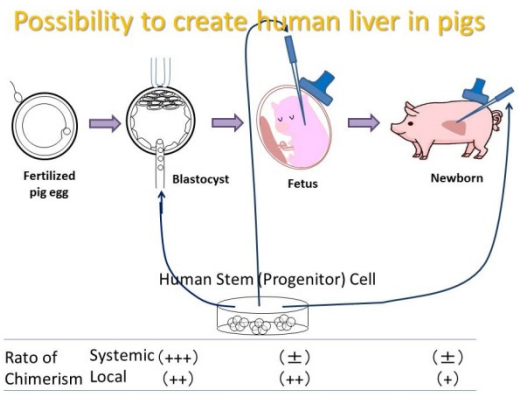
I explain in due course, but I would focus on the point that the basic research and the applied research, there exists a sort of time spectrum in between. It is commonly told that it is important to evidence the proof of concept for the basic research using mice and rats. In case the size of laboratory animals changes to the size of human body, the story would be totally different. Rat, say, 20-day rat means in 3 weeks it gives a birth after the pregnancy. The span of life cycle is very short. On the contrary, in case of using pigs, as the pregnancy period is 114 days, the time spectrum would be the one that the development of the applied research slows down. I explain the fusion technique for the alveolus in placenta. To fabricate human organs in pig body is one of the researches of Dr. Onishi. It has been long time since the research started to create human organs in the body of immune-deficient pig. Nevertheless, it is just recent that the birth of immune-deficient pig took place. Also further research on using animal organ prototype and creation of human organ prototype artificially have emerged. Furthermore, the research for using processed pig organ for the above purpose.

(1) In vivo bioreactor, Animal

Industry

By the way, now I'd refer to the most disputable point for the trial research "in vivo bioreactor". The issue has been well discussed at conferences. The point is that how far extent it is allowed the scientific technology for fabricating human organs in pigs by inserting human stem cells in the pig fertilized egg while preventing the emersion of pig organs. In the attempt of fabricating human organs in pigs, I'd point out how epoch-making the technology involved would be. As the trial surgery has never been done yet between human and pig, I'd explain the upcoming progress with facts and assumptions.

This slide shows the assumption flow how and what stage the human stem cells and precursor cells are injected in the process of pig's fertilized egg growth to fetus and to the birth of baby pig (Figure4).



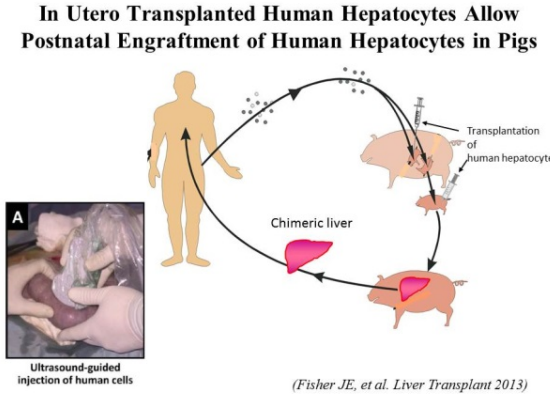
It is obvious that the chimera animal would be born if we inject human stem cells into the blastocyst of pig. It is assumed that the chimera animal consisting of various organs human and pig-orgin mixed would be born. The absolutely ideal method that Dr. Nakauchi of Tokyo University has invented is that the pig-related gene is terminated in the organ emerging process. The theory won't be applied to any kind of organs but we can find a very promising gene candidate. Once terminated the gene for pig organs, the gene for human organs start working and with high probability the human organs are born with very high chimeric rate. It means that a single organ with high human gene is born to the focal extent.

In the latter half of the 19th century, there took place lots of experimental researches with Dr. WE Beschorner as a chief person to use pig as a reactor for generating human organs. Basically the concept is resembling to mine in terms of creating human-gene cells and chimera in pig body. This method is translated as "in vivo bioreactor"; animal factory, the research for developing human organs in the live pig. If injected in the newborn baby pig, we can slow down the rejection ratio of accepting human organ in its body or even exterminate the rejection completely. Recently there are cases that the precursor human cells are injected in the very early stage of pig infancy. The purpose of using small animals is that we inject human stem cells in the newborn stage of pigs, which leads to the differentiation in shape of chimera animal by using the "in vivo bioreactor" method when we inject it in pig's fertilized egg or

embryo or newborn baby stages. It is natural to think that chimera ratio would go up if we inject it at the earliest period of pig's cell formation.

What I want to inform you is that there have been tries and errors up to now in regard to injecting human stem cells in the latter stage of embryo or newborn baby pig. Dr. Beschrorner to whom I have referred has been a driving force to push the research forward. Unfortunately he passed away 5 years ago on account of the malignant tumor. The venture capital he inaugurated has been with no one to succeed since then. You can check the name of the company on the internet as "Chimerix". It is clear that the venture capital existed with the purpose of doing researches to fabricate human organs in the body of pig.

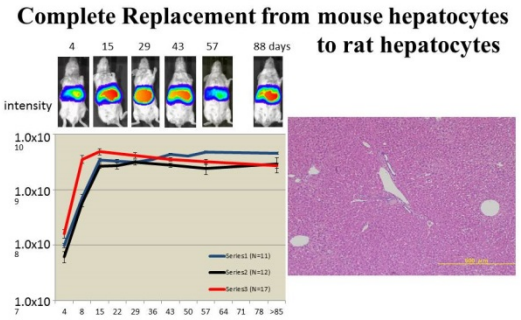
The same kind of research has been reported by Dr. Fisher (Figure 5).



His method is to inject human stem cells in embryo of pig once and once again when it is born as a newborn pig. He has reported that maximum 50% of pig liver can be converted to human's one. The slide shows that Dr. Fisher is aiming to inject at the liver of pig by way of pig uterus. The important point here is that even up to now only 50% of human stem cell have been converted. It means that all of the vascular systems stay with pig origin.

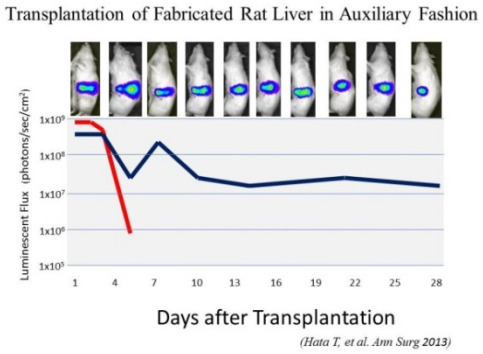
We have tried further development of the above method by creating a special mechanism as a pig gene which destroys the liver part inside the pig. We create a specialized pig which comes with the pig gene auto-collapse-liver function when the pig is given a special medication. Then we inject the human stem cells in the embryo or newborn pig liver and replace the collapsing pig liver for the human one. We believe that a sort of systemic chimera animal which is not pig nor human will never born because we do not use the fusion technique for the alveolus in placenta. The purpose of the research is to evaluate the advantage of the method compared to the direct pig organ transplantation as a heterogeneous organ transplantation. The target animals are 20-gram mouse as if it were pig versus 200-gram rat as human. The purpose is to grow rat liver in mouse. The mouse has uPA gene which destroys its liver by itself. Furthermore, the mouse has been modified with the immune deficiency SCID. Therefore, it is designed to accept human cells. Using this kind of mouse,

when the rat liver cells are injected in the newborn mouse, about 98% of the mouse liver is replaced by the rat one (Figure 6).



(Hata T, et al, Ann Surg 2013)

The most important outcome from the research is whether the replaced liver can really stand the organ transplantation process or not. This image shows that the rat liver with gene marker as firefly luminescent gene inserted should work properly if the transplanted liver functions properly (Figure 7).



(Hata T, et al. Ann Surg 2013)

In general, the extremely strong immunological rejection occurs if we transplant rat liver in the mouse because it is a heterologous transplantation. But the result of this experiment shows that the mouse chimera liver with rat hepatocytes grown in the recipient rat has become sustainable with the amount of immunosuppressive agent which exceeds the acceptance limit in regular case. The above experiment leads to the assumption that the pig celled mixed with human-like cells might suffer from less immunological rejection rather than heterologous transplantation.

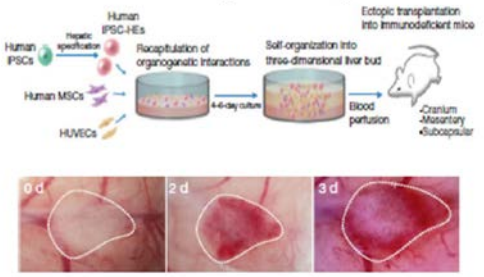
By the way, my personal view on the scientific and ethical issue of the fusion technique for the alveolus in placenta will be given in the last part. The issue I would pick up here is whether we can continue killing pigs in order to catch up with the demands for alternative organs. This is the fundamental question. One of our challenges for our future research is how we can overcome the reliance on animals for organ transplantation: the heterologous transplantation. At the moment, the problem of heterologous transplantation jeopardizes the clinical application. Even we enhance the chimeric rate, the heterologous transplantation problem stays. It means that we can never fabricate 100% pure organ free from animal origin even we generate human organs in pigs because it is chimeric. Therefore, the challenge how to overcome the criteria of heterologous transplantation should be discussed fully as one of the most important issues.

(2) Organ Bud Transplantation

In regard to another field of research, it has become a global topic that we use “Organ Bud”, so to speak the organ

prototype for transplantation. The idea is to fabricate organs through the usage of organ buds. This splendid research has been originated by Dr. Taniguchi of Yokohama Municipal University whose article was published by "Nature" magazine (Figure 8).

Vascularized and functional human liver from an iPSC-derived organ bud transplant

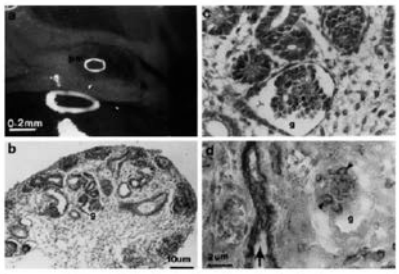


(Takebe T, et al. Nature 2013)

The project is aimed at fabricating organ bud by inducing differentiation to the human iPS stem cells while culturing vascular endothelial cell and scaffold simultaneously. And this organ bud is inserted in the brain surface where extremely abundant vascular plexuses are centered. It turns out to be liver bud through the formulation of vessel structures inside itself.

Let me explain the updates of organ bud transplantation. As it is not ethically possible to use human fetus organs, there have been various researches to transplant embryo organs of pigs into human bodies. The rationale for these endeavors is that the antigenicity of the pig embryo organs is considered extremely low. Also the research goes based on the fact that the sterilization of pig embryo is deemed possible. Dr. Marc Hammerman of Washington University is mainly involved in this research. He has been well known for numerous experiments for kidney and pancreas (Figure 9).

Transplantation of embryonic kidneys



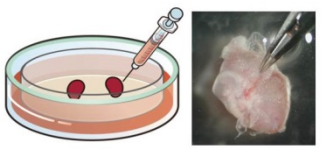
Photograph (a) and histological sections (b–d) of pig metanephroi 14 days post transplantation into mice

(Marc R. HAMMERMAN, Clinical Science 2002)

We have been doing similar kind of experiments for approximately 10 years. Our method is to use pig kidney bud inserted in human stem cells before the transplantation for the purpose of its chimera (Figure 10).

Generation of human-chimera organ with human stem cell insertion to pig kidney primordium

Patented Technology : Method of generating artificial kidney precursor

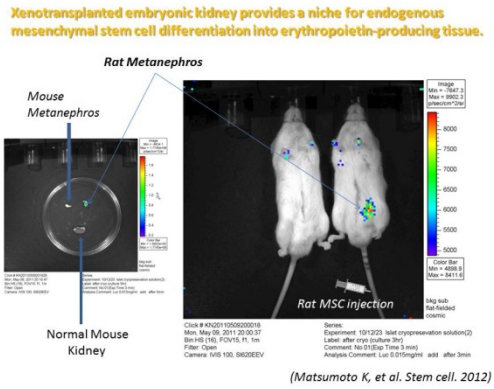


Chimera ratio is enhanced with human stem cell insertion

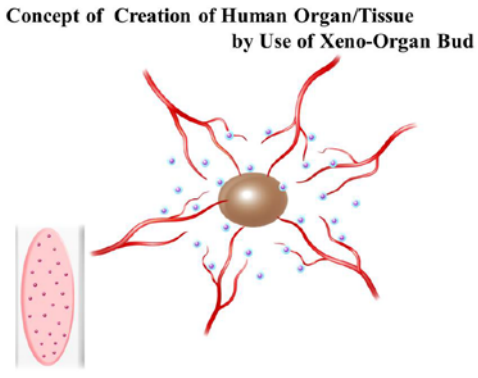
(June 5 2012 New Zealand Registered number :590122)

We have been continuing our research in order to prove how the inserted pig kidney bud turns out to be in human body. This is the same step that we have taken in the previous experiment between mice and rats which have been

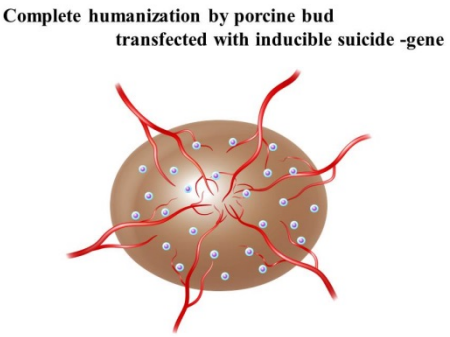
regarded as 20-gram mouse as if it were pig versus 200-gram rat as human (Figure 11).



The summary of the experiment is as follows: Transplanting pig kidney or organ bud to the patient, the aggressive transfer of blood vessels to the bud occur from the patient and the stem cells in the peripheral vessels are engrafted to the organ bud. As it induces differentiation, temporarily the immunosuppressive agent is required to initiate the growth of the organ bud (Figure 12).

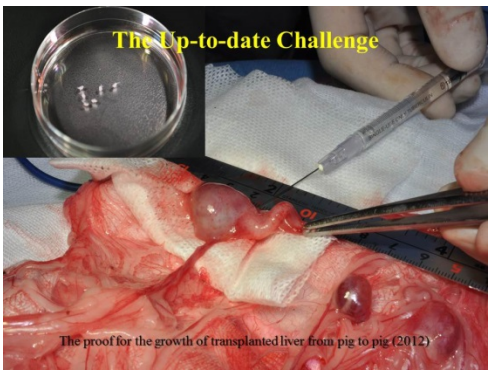


To a certain extent the differentiation induction progresses, we let the pig organ bud naturally disappear (Figure 13).



This method would deliver a total humanization if we could create a pig with the gene to kill itself through the application of medication.

Currently this research is being done simultaneously. Nevertheless, there exist a limit that either liver or kidney, once inserted the bud, starts accumulating lots of secretion. The liver gathers bile and the kidney, urine. If the biliary duct and the urinary tract lack in respective organs, fluid retention occurs and hydronephrosis emerges in the kidney (Figure 14).

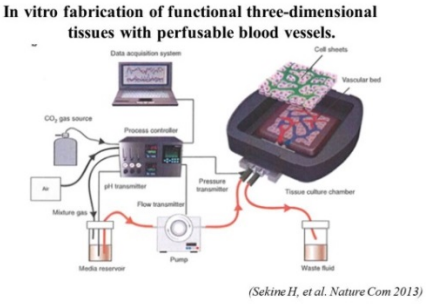


The biggest challenge we confront with is how we can provide urinary tract simultaneously. We have been trying to find solutions to the issue intensively together with Dr. Yokoo of Jikei University. Organ bud transplantation

involves short of scientific verification. It means that small organ bud does not grow after we transplant it to matured human body (patient). If there are no urine tract nor biliary duct, no way out for secretion, and a sort of blister emerges. The wonderful outcome researched by Dr. Taniguchi of Yokohama Municipal University is that the humanization of organ bud becomes possible. Our experiment, as we use xenogeneic organ bud, there always accompanies the problem of heterologous graft.

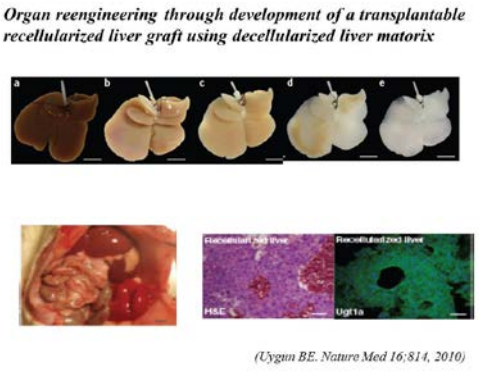
(3) Organ/Tissue Fabrication

The last theme of our research is organ fabrication technology. The word "Fabrication" means remaking. The early stage study has been done together with Dr. Okano of Tokyo Women's Medical University. In-vitro fabrication of a certain thickness of vascular organ with multiplying sheets on it if we can prepare for the vascular plexus outside the vitro (Figure 15).



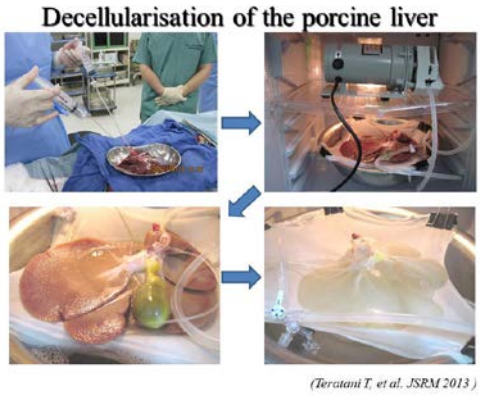
The limit is that further technical development is indispensable because we need to multiply the sheets in 3-dimentional and based on how it is formed, the oxygen and nutrition rates flown inside the vascular plexus depends totally on the fabricated blood vessels. It is still a big challenge whether we can accumulate thin and delicate blood vessel organs in multiple layers.

To further apply for the above technology, decellularization method has been developed. The slide shows the experiments on rats have been often done since 2010 in the United States (Figure 16).

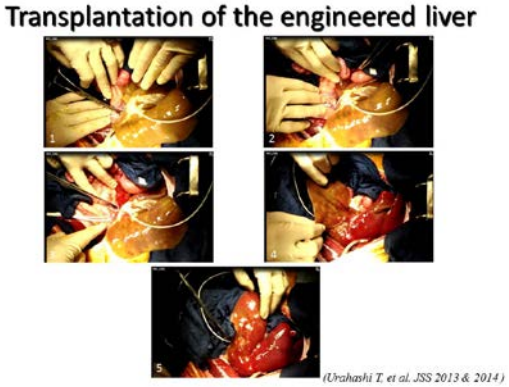


Both human liver from the cadaver and pig liver will do for decellularization taking the application to human into consideration. By using a kind of detergent we wash all of the cells from organs and leave room for organ fabrication. Then we fabricate organs on the scaffold which is filled up with cells again. We have been experimenting it on the small animals already. The biggest barrier stays with the shift from small animals to big ones to see whether the same methods are applicable or not. We have concluded that it would not be achieved so easily. To summarize our

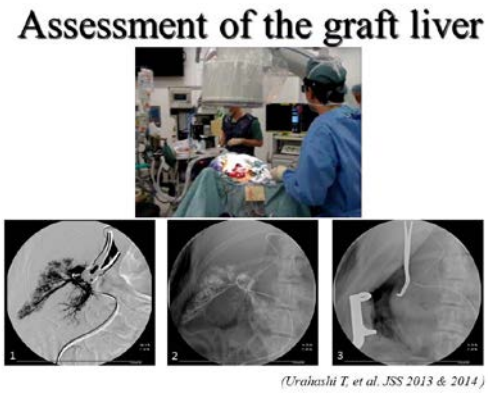
method, we freeze pig liver in a special manner and decellularize it (Figure 17).



The process takes only a minute and the massive liver turns out to be a jellyfish-like substance. Totally new liver is born by filling it up with the network of vascular endothelium and another liver cells (Figure 18).



There exist lots of challenges to overcome but we are seriously regard it as a new organ fabrication method. The slide shows one-week-after image of the newly born liver endowed with the fine vascular texture (Figure19).



This is the most recent study we have been focusing on.

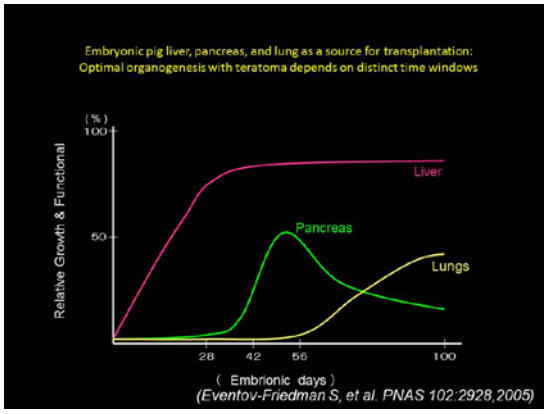
Still the issue emerges that we need to verify the method through the usage of bigger animals. It has not been fixed yet whether transplanting cells should be young and recently-fabricated ones or already-differentiated ones in order to best fit for human organs. Also there exist a big challenge how we can control strong thrombus by utilizing anticoagulant method when we are in need to use artificial blood vessels. We think it best from the applied science point of view to use the decellularized scaffold instead of heterologous transplantation. The vision to push forward this research is due to the fact that there are decellularized products available for pig cardiac valve and injuries.

Discussion

I'd like to refer to my personal views on the ethics of organ transplantation knowing that I should rely on the authorities. I am basically a surgeon. Also

I am a specialist in the applied research to save lives of patients in pursuit of organ fabrication in pigs. Therefore, I think it would be better for me to set the limit of rights and the range of responsibilities by myself. In regard to the ethical issues, it is indispensable to gather the views from the dedicated committee members. But it would be immature to count on them in terms of scientific rights and responsibilities.

The last slide shows my personal views on the supporting method for blastomeric vesicle (Figure 20).



There is no scientific evidence concerning the conglutination between pig and human. It naturally leads to the question how we can accept the ontogenetic development. We have already proven scientifically that kidney organ bud is applicable; we utilize organ bud of pig kidney extracted from 30-day pregnancy. In regard to organ bud of pancreas, 60-day one. The focus is how the chimera status would be when human and pig are ontogenetically unified. The following is my view backed up by scientific facts that there could be an order for pigs, both pregnant and unborn to sacrifice themselves as experimental animals if we can establish scientific guarantee and draw a line till when we are allowed to do so.

Acknowledgement

The above is the translation into English for the lecture in Japanese given (the recording of the lecture is already available on internet) at the Ministry of Education, Culture, Sports, Science and Technology where the workshop was held on March 20, 2014 on the treatment of animal-human chimeric embryo.

I thank my laboratory manager Mr. Kita for his translation skills into English and my secretary Ms. Takahashi for her putting my draft writing in order.