Yamaton P Project: The Development of verification model of human derivative insulin-secreting cells for the treatment of diabetes

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Preface

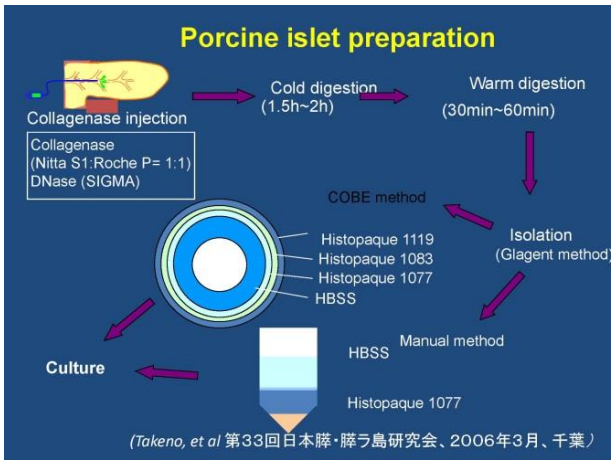
The medical products are segmented into two; pharmaceutical products and medical devices. The former is mainly dealt by top-notch corporations with 17,000 products totaling 800 billion yen in sales, the latter is dominated 80% by Small and Medium Size Enterprises; (SMEs) with 300,000 products totaling 220 billion yen in sales. This market structure is characteristic unique for Japanese medical industry (Data surveyed in 2008). Furthermore, most of medical devices used in Japan are from overseas. On the other hand, products for regenerative medicines in general act not only as pharmaceutical cells for treatment but medical devices for cell infusion. In November 2014 the newly implemented regulations for the safety of regenerative medicine have been put in place. The development of human derivative insulin-secreting cells for the treatment of diabetes is merely desired field of research.

I have been researching for the development of medical product for the treatment of diabetes transplantable to human under the name of ñ**Yamaton P**ò. **Yamato** represents ancient naming for Japan ñYamatoò, **Ton** derives from pig in Japanese language and **P** is the acronym for Pancreas. I will explain about our basic strategic plan after outlining the background.

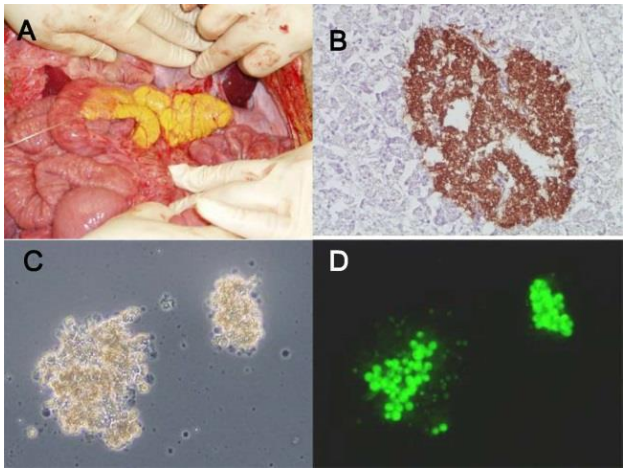
How to source cells for Yamaton P project

(1) The research for islet segregation from pancreas of pig

I had been segregating the islets of pigs when we enucleated pancreas of sacrificed pigs after the experiments 10 years before having launched ñPig projectò at Jichi Medical University. There was a necessity to organize the information about experimental pig cases due to the fact that the reports from overseas on the segregation of islets from livestock pigs dominated the trends. Therefore, at the pig center of Jichi Medical University, we have segregated islets of sacrificed mini pigs (Mexican hairless, Crown and Chinese) according to the protocol in the following table and surveyed the recovery rate and so on.



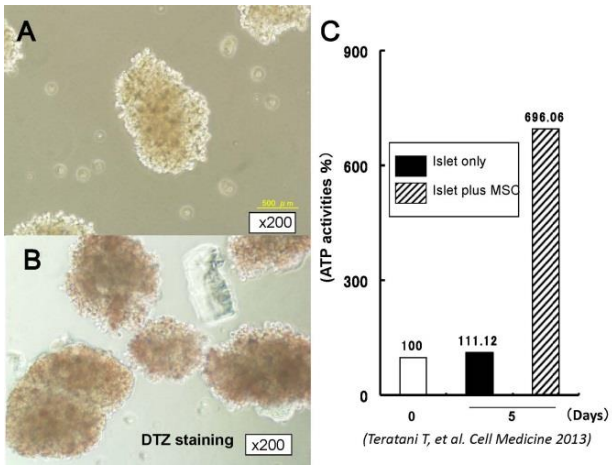
As a result, there was no difference in terms of the recovery and survival rates among mini pigs of Mexican hairless (8 cases), Crown (8) and Chinese (4). Based on the data I have done co-research with Dr. Naoya Kobayahi (Okayama University) and looked into the possibilities of shipping them to the domestic co-researchers. It took over 20 hours to transfer the islet of mini pigs by refrigerated delivery service but we found that the deteriorated islet in transfer recovered its function in cultivation together with fibroblast derived from human(*Miki A, et al. Cell Transplant 2006*). Also I segregated the islet of GRP-induced Kinka-pig for the research of imaging in time for islet transplantation (*Kawarazaki A, et al. J Biomed Opt 2009*).



A: Complete view of GFPT pig pancreas **B**: Islet tissue

C: Islet right after segregation (Visible light) **D**: Same as C (Under exiting light)

In addition from 2009, I started the experiments for the improvement of deterioration of preserved islet through the usage of MSC.



A: Islet of pig segregated by Dr. Gotoh team (Tohoku University)

B: With DTZ dyeing

C: Recovery of ATP activation with co-cultivation of deteriorated islet of pig together with MSC.

In any case, the tunica covering islet of pigôs pancreas is extremely weak and is subject to deterioration by the collagenase processing compared to that of human. The segregation of pig islet is technically difficult and high in cost. I have come to a conclusion that it would be created in cultivation in the future.

(2) The challenge to fabricate transplantable cultured islet by utilizing experimental pigs

Dr. Yoko Mullan (The City of Hope, USA) has reported first time in the world that islet cells enucleated from pig fetus pancreas starts functioning through cultivation (*Tsunosa T, et al. Transplant Proc1989*). In the consequence, it has been reported that cultured pig islet acquired through this method is applicable for the transplantation to human (*Mullan Y. Xenotransplantation1995*).

In 2008 under direct supervision of Dr. Mullan in collaboration with Prof. Nagashima (Meiji University) we tried to establish the method.

A: Livestock pig 65 days after birth (Kadoi Farm, Meiji University)

B: Enucleated fetus pig

C: Direct supervision of Dr. Mullan (at Jichi Medical University, Laboratory for Organ Regenerative Medicine on 22nd of October 2008)

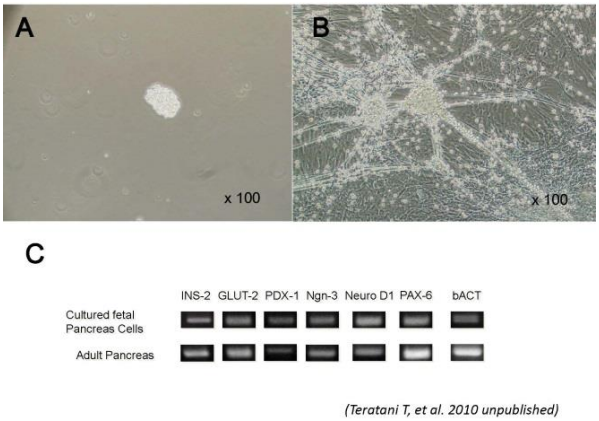
A: Loosen the enucleated pancreas by a pair of tweezers

B: Collect with 10-cc tube and wash

C: Add collagenase

D: Humidify and vibrate it in 37° Celsius water bath for 5 minutes

Hereafter, we washed it twice to three times and started cultivation in 10cm dishes while confirming of cell density. From approximately 25 days after the cultivation we confirmed of the growth and assembly of cells. And we have repeated the same experiments over and over.

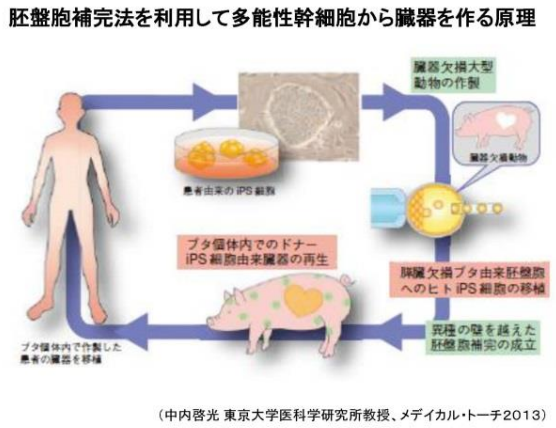


(Teratani T, et al. 2010 unpublished)

A: Resected endocrine cells from 56-day embryo livestock pig (1st day of cultivation)
B: 24th day of cultivation
C: Gene emersion of the same cell (by Dr. Takumi Teratani in August 2010)

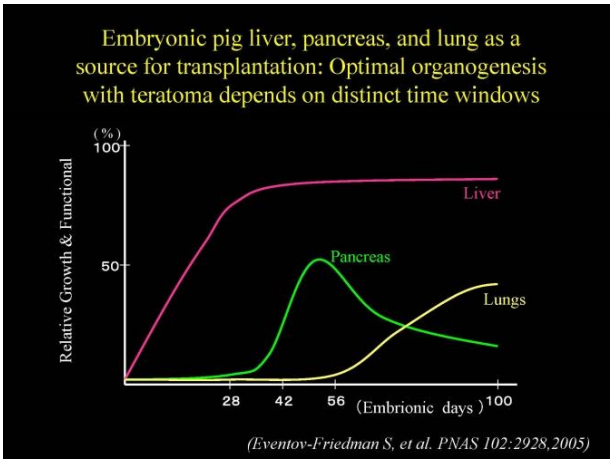
This method has led to the feasibility of generating islet in large quantities by enucleating precursor of pancreas endocrine cells through the improvements in cultivation conditions.

Recently, Professor Nakauchi (Tokyo University) has shown the possibility of generating human pancreas based on the theory of blastocyst supplement method (*Kobayashi T, et al. Cell 2010*). In the consequence, they succeeded in generating a pig lacking in pancreas which possibly turns out to be the base for the experiments in collaboration with Dr. Nagashima (Meiji University) (*Matsunari H, et al. PNAS 2013*).



Nevertheless, we need to look into the feasibility of blastocyst supplement method between pig and human besides the ethical issue of generating animal embryonic cell. I have been proposing the methodology of isolating fetus pancreatic primordia of pig for

cultivation to solve the above challenge (*MEXT: Ministry of Education, Culture, Sports, Science & Technology in Japan, March 2014*).



It represents the protocol to cultivate the enucleated pancreas from fetus embryo of sacrificed pig between 8 and 9 weeks without acquiring human-pig chimeric organ even if it emerges. When pancreas is generated, the endocrine and exocrine cells increase simultaneously. However, as exocrine cells cannot be cultivated with a conventional method, it is expected that the specified endocrine cells related to insulin secretion are generated.

The risk of xenogeneic transplantation should be always considered on the emersion of cells from this method regardless the generative types such as insulin secreting cells cultivated from pig embryo or human-derived one induced in pig body.

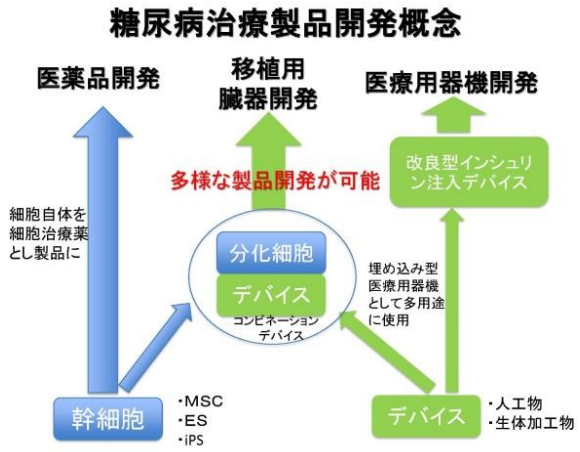
(3) Induction of in-vitro insulin secreting cells

Professor Osafune team (Kyoto University) in pursuit of the regenerative medicine for diabetes has been fabricating transplantable pancreatic cells derived from iPS cells. They have partially succeeded in improving the blood glucose level of model mice with diabetes through the methodological development of generating pancreatic precursor cells for transplantation (*Unpublished*).

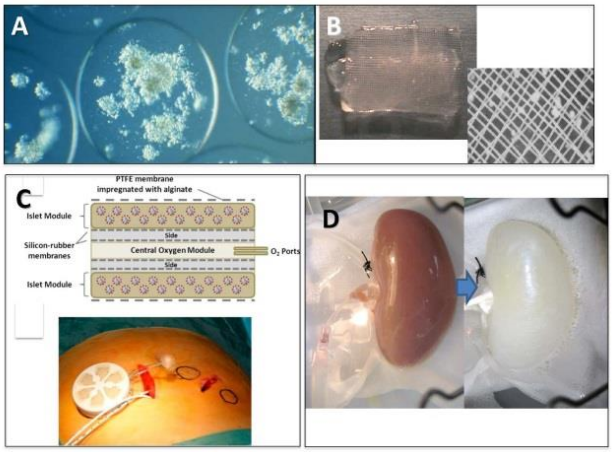
Currently insulin-secreting cell induced from human stem cells is not functioning enough. On the other hand, it is expected that it gradually grows once transplanted to in vivo. It means that the project **Yamaton P** will be focused on the researches for inducing differentiation of human-derived islet and transplantation methodology.

The diversity of treatment products for diabetes

Formally I have referred that regenerative medicine products have both factors such as treatment cells as pharmaceutical products and medical devices to inject them;



The background of our research for insulin-secreting cells transplantable to human has been already stated. The below are the devices to inject these cells;



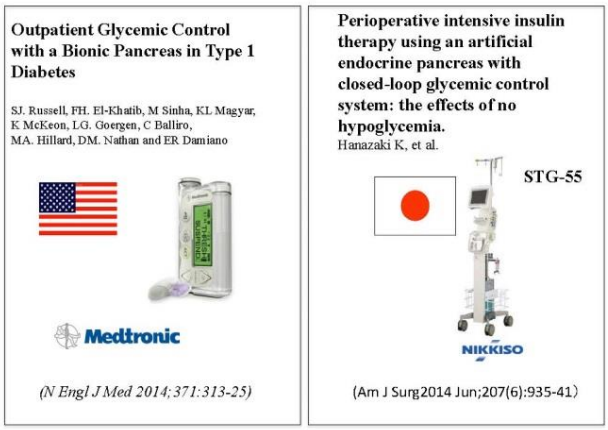
A: Micro-capsulated products: The clinical research is about to start at Dia Trans Inc., a joint venture between Living Cell Technology Inc. in New Zealand and Otsuka Pharmaceutical Factory Inc. to manufacture pig fetus islet. The islet is not isolated because it is solely for abdominal cavity transplantation.

B: Macro-capsulated products: Dr. Sumi team (Kyoto University) has been researching for the feasibility of macro capsule with mesh built-in reinforced by PVA. It has an advantage to be picked up in case of emergency to avoid dispersion of pig islet after transplantation.

C: Chamber-shaped products: the materials consist of less foreign matter reaction in-vivo such as mesh-shaped and silicon ones. The image is the device to avoid lack of oxygen to the transplanted islet by transferring oxygen to the chamber reported by Dr. Ludwing B (PNAS 2013)

D: Decellularized products: The decellularized pig organ (in this case, kidney) acts as a base for refilling insulin-secreting cells which is currently under our research. Apart from A to C products, it has no immune-segregating characteristics.

The development of devices for transplantation is subject to change in accordance with the progress in researches for human-derived cells. The chambers developed so far has been targeting immune segregation taking xenogeneic transplantation such as pig islet into consideration. Therefore, in order to verify the effectiveness and safety for preclinical,monkey model with diabetes representing primates has been indispensable. However, it has been questioned whether this monkey model with diabetes is necessary for verifying the effectiveness and safety for human islet cells induced artificially.



The effectiveness of these devices have been verified with pig models with physical and insulin-secreting amount similarities to human. I strongly have a belief that prompt screening for finding best matches between cells for transplantation and devices to inject them for further improvements leads to so-to-speak **Double One** (Only One, the best One) products.

MMP with total pancreatectomy for the purpose of drastic evaluation of new products

As mentioned in the previous paragraph, the monkey models with diabetes have been utilized as large experimental animals when we pay attention to the history of islet transplantation development. It is extremely important for preclinical trials to ensure the

effectiveness and safety to apply pig islet to human including controls over xenogeneic immune responses. Nevertheless, how effective it would be if we apply it to verify human-derived insulin secreting cells? It will require lots of human labor and become costly to manage experimental animals. On the other hand, pig model with diabetes has been more versatile in overseas as a tool to verify insulin-injection devices thanks to insulin similarity between pig and human.

In the field of developing therapeutic medicine for diabetes, pig model is highly promising for the purpose of verifying the effectiveness of human-derived insulin secreting cells. In fact, in Europe mini pigs with pancreatectomy have been standardized in this research field.

Goettingen Minipigs (GMP): Comparison of Two Different Models for Inducing Diabetes

A Strauss,V Moskalenko, C Tiurbe, I Chodnevskaja, S Timm, V Wiegering,CTGermer and K Ulrichs

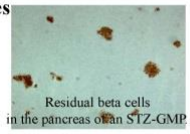


Table : Persons, qualifications and time needed to induce the two diabetes models in the Goettingen minipig and care for the diabetic animals prior to grafting the isolated islets of Langerhans.

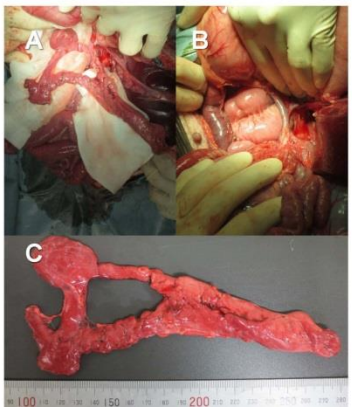
Type of work	Number of persons and qualification	Work time
diabetes induction with STZ	1 scientist or surgeon 1 animal caretaker	3 hours
diabetes induction with PE	2 surgeons 1 medical assistant 2 anaesthesiologists	6 hours
management of the STZ diabetes	1 animal caretaker	2 × 30 minutes/day
management of the PE diabetes (early phase)	1 surgeon	Up to 4 hours/day
management of the PE diabetes (late phase)	1 animal caretaker	2 × 30 minutes/day

Abbreviations: STZ: streptozotocin; PE: pancreas explantation in the animal operation theatre

(Diabetology & Metabolic Syndrome 2012, 4:7)

Despite the fact that the size of mini pig is smaller than regular pig but larger than dogs and monkeys for the verification purpose of human cell therapeutics. Consequently, it requires larger number of human cells for experiments and larger amount of immunosuppressive agents to be used after the xenogeneic transplantation between human and pig that ends up with costly trials. We have established a management system based on the creation of pancreatectomy model by Micro Mini Pig (MMP); the smallest experimental pig in the world.

The pancreatectomy is a relatively easy operation and applicable for verifying various medical products. In order to establish it as a reproducible and stable model, we have standardized it as SOP.

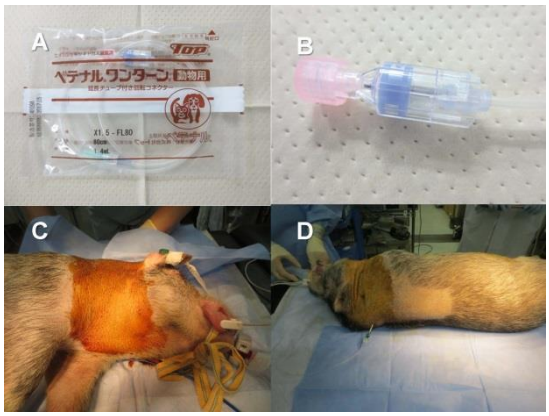


A: The image during operation; we separated by electric scalpel the tail and head part of pancreas respectively and isolated it by binding.

B: The operated field after resection; the blood flow is well kept at duodenum.

C: Resected pancreas

Postoperative management is quite important. Free-moving transfusion line is utilized for blood collection when necessary,



A: Tube for free-moving (Petenal One-turn)

B: Rotating part of IV tube

C: IV tube to be inserted into cervical vein

D: Tube to be picked up from the backside of cervical part

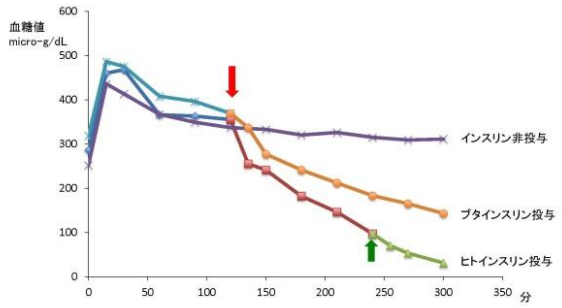
E: IV system after pancreatectomy

F: Free-moving image

G: Free to be fed

H: Blood collection through free-moving tube

We have done the following trial by utilizing the above mentioned procedures:



Intravenous Glucose Tolerance Test (IVGTT) was executed with rapid intravenous injection of 0.5g/kg at 0 second (approximately 18ml of 40% glucose liquid). The pancreatectomy model has kept the blood glucose level over 300 after the test. In addition, as a test for reactivity, another IV of 1 unit/kg (Humulin R immediate effect type) and pig insulin (Sigma I5523) were injected respectively 120 minutes after the glucose IV. Both worked for lowering the blood glucose level, the former (Humulin R) has shown better result. The result apparently shows the difference between reagent and medicine; it is considered that the latter is optimized for generating utmost efficiency. One of the test results shows that another Humilin R injection at 240 minutes (Green arrow) brought hypoglycemia symptom and immediate 5% glucose physiological saline solution IV was needed.

Thanks to the evaluation system developed by Drs Enosawa & Kyo (National Center for Child Health and Development, Tokyo,Japan), we are able to ideally judge the efficiency of fabricated human insulin-secreting cells and cell-injection devices on the integrated non-clinical-clinical evaluation scheme.