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Cloning of Homozygous α1,3-Galactosyltransferase Gene Knock-Out Pigs by Somatic Cell Nuclear Transfer

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1. Introduction

To overcome donor shortage in organ transplantation, xenotransplantation using organs of genetically modified pigs has been actively investigated (Ekser and Cooper, 2010). Thus far, the development of genetically modified pigs has mainly focused on overcoming the immune rejection of the xenograft. The creation of knock-out pigs lacking the xenoantigen α1,3-galactosyltransferase (GalT-KO) (Dai et al., 2002; Kolber-Simonds et al., 2004; Lai et al., 2002; Phelps et al., 2003) and transgenic pigs expressing complement regulatory factors (Fodor et al., 1994; Takahagi et al., 2005) has been successfully achieved.

We have produced genetically modified pigs by the heterozygous knock-out of α1,3-galactosyltransferase gene and the co-expression of two transgenes, human decay-accelerating factor (hDAF) and N-acetylglucosaminyltransferase III (GnT-III) (Takahagi et al., 2005). Natural breeding of the heterozygous GalT-KO pigs could produce live homozygous GalT-KO pigs (Fujimura et al., 2008a; Fujimura et al., 2008b; Takahagi et al., 2007) with the two transgenes. The three genetic modifications made to these pigs, namely, the GalT-KO plus expression of GnT-III and hDAF, should reduce rejection when their organs are used for discordant transplantation. More specifically, Galα1,3-Gal epitopes, which cause hyper-acute rejection, have been removed from the pig cells, thereby

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reducing the antigenicity of the cells to human natural antibodies and suppressing the complement cascade reaction in the host. However, for the clinical application of xenotransplantation, it will be necessary to perform further genetic modifications in these pigs to overcome the various obstacles that exist in xenotransplantation (Ekser and Cooper, 2010; Klymiuk et al., 2010).

One strategy for achieving a more advanced genetic modification of the pigs is the use of somatic cell nuclear transfer (SCNT). This approach should make it possible to create pigs with multiple genetic modifications based on the existing GalT-KO/transgenic pig. Using the SCNT technology shown in Fig. 1, it should be feasible to introduce novel genetic modifications through sequential cloning. This approach might be used for removal of non-Gal antigens, controlling delayed xenograft rejection or cell-mediated immunity. In order to pursue such an approach, it is essential to know the reliability with which existing genetically modified pigs can be reproduced through somatic cell cloning technology. Here, we examined the feasibility of this strategy using three lines of GalT-KO pigs that had been created previously. We also describe the current efficiency of somatic cell cloning for generating genetically modified pigs for xenotransplantation research and discuss the prospects of applying this technology to achieve more sophisticated genetic modification of the pigs.

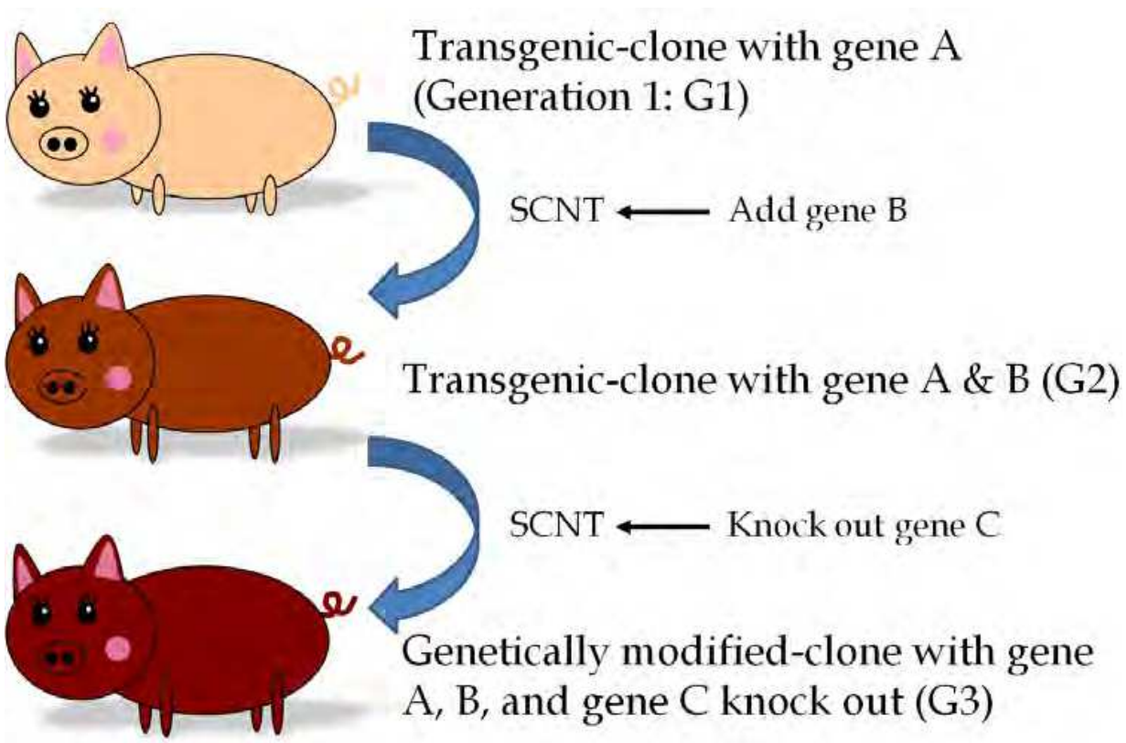


Fig. 1. Multiple genetic modifications of pigs using sequential somatic cell cloning

2. Reproducing GalT-KO pigs by cloning

In this study, we used somatic cell cloning to reproduce three types of GalT-KO pigs (DK3-1 male, DK3-2 female, DK3-9 female). These three types of pigs are all homozygous for the GalT-KO and were produced by intercrossing siblings derived from a mating of a heterozygous GalT-KO+hDAF+GnT-III pig to a wild-type pig (Table 1). DK3-1 and DK3-2 express two transgenes, namely, hDAF and GnT-III (Takahagi et al., 2005). However, the integration patterns of the two transgenes (i.e., heterozygous or homozygous integration) were not examined. By contrast, DK3-9 is a homozygous GalT-KO pig with no integrated transgenes.

Pig code number	Gal-T KO*	Transgenes**	sex
DK3-1	homozygous	hDAF and GnT-III	male
DK3-2	homozygous	hDAF and GnT-III	female
DK3-9	homozygous	NIL	female

*α1,3-galactosyltransferase gene knock out
** hDAF: decay-accelerating factor ; GnT-III: N-acetylglucosaminyltransferase III

Table 1. Original Gal-T KO pigs cloned by somatic cell nuclear transfer

3. Somatic cell cloning technology for reproduction of GalT-KO pigs

3.1 Nuclear donor cells for somatic cell cloning: Preparation of preadipocytes and cell cycle synchronization

Various types of primary cultured cells, such as fetal fibroblast cells, salivary gland progenitor cells and mesenchymal stem cells, have been used as the nuclear donor for somatic cell cloning (Faast et al., 2006; Kurome et al., 2008b; Matsunari et al., 2008b). For the cloning of GalT-KO pigs in this study, we used preadipocytes (Tomii et al., 2005; Tomii et al., 2009), which have consistently produced good results in somatic cell cloning in our laboratory.

Primary cultures of preadipocytes were established as reported previously (Tomii et al., 2005; Tomii et al., 2009) from the subcutaneous fat tissue of three homozygous Gal T-KO pigs at 6 months of age (Fig.2a, b). Subcutaneous fat tissue was collected by abdominal incision under general anesthesia and then washed with Dulbecco's phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 75 μg/ml penicillin G and 50 μg/ml streptomycin. Connective tissue surrounding the fat was removed using sterile scissors (Fig. 2b). Small pieces of fat tissue were then excised and incubated in Dulbecco's Modified Eagle Medium (DMEM) with 2% (w/v) bovine serum albumin (BSA, Fraction V) and 0.1% (w/v) collagenase (Wako Pure Chemical, Osaka, Japan) for 1 h at 37˚C (Fig. 2c). Dissociated cells were collected by filtration through 100-μm nylon mesh (Falcon 352360, Becton Dickinson, Franklin Lakes, NJ, USA). Following centrifugation at 150 *g* for 5 min, only mature adipocytes that remained in suspension near the surface of the supernatant were collected (Fig. 2d), and these adipocytes were washed several times. The mature adipocytes (1×10^5) were then incubated in a T12.5 cell culture flask (growth area, 12.5 cm^2; Falcon 353018, Becton Dickinson) filled to the brim with DMEM containing 20% (v/v) fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA) and tightly capped. The

flask was inverted and incubated in a humidified atmosphere of 5% CO_2 in air at 37˚C to encourage the adipocytes to attach to the inner ceiling of the flask (Fig. 2e). Following incubation for 7 days, morphological transition to a fibroblast-like shape, accompanied by a gradual decrease in the amount of cytoplasmic lipid droplets, was confirmed in cells attached to the flask's inner ceiling surface. Following confirmation of firm cell attachment, the flask was again inverted, and routine culture methods for adherent cells were implemented (Fig. 2f). The medium was changed every three days, and after two passages, preadipocytes were resuspended in fresh media supplemented with 10% (v/v) dimethyl sulfoxide (CELLBANKER® 1, Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) and were then frozen in liquid nitrogen.

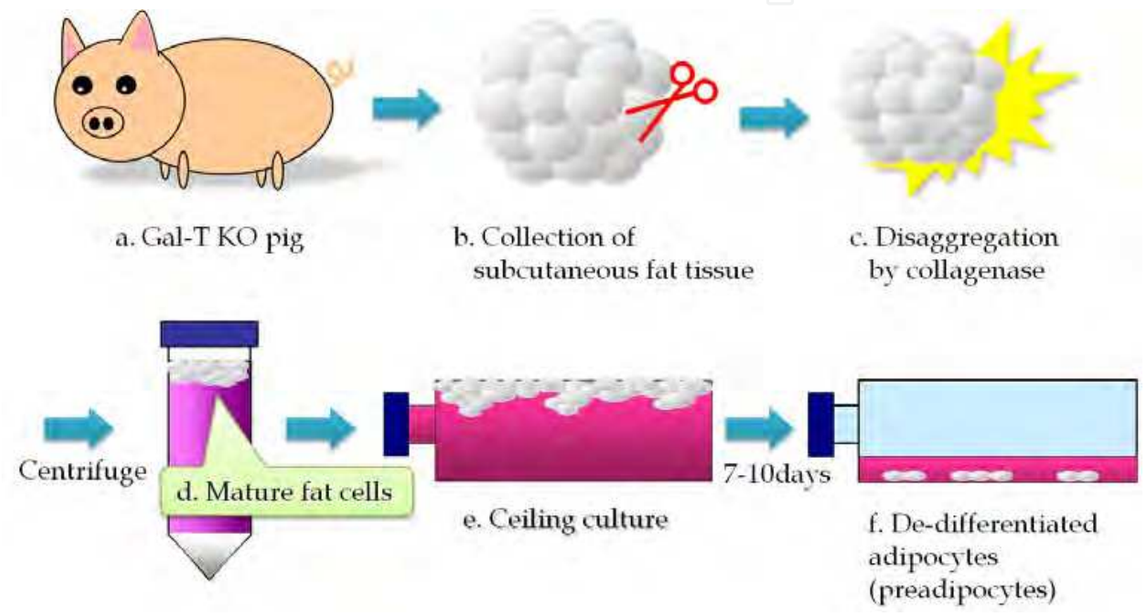
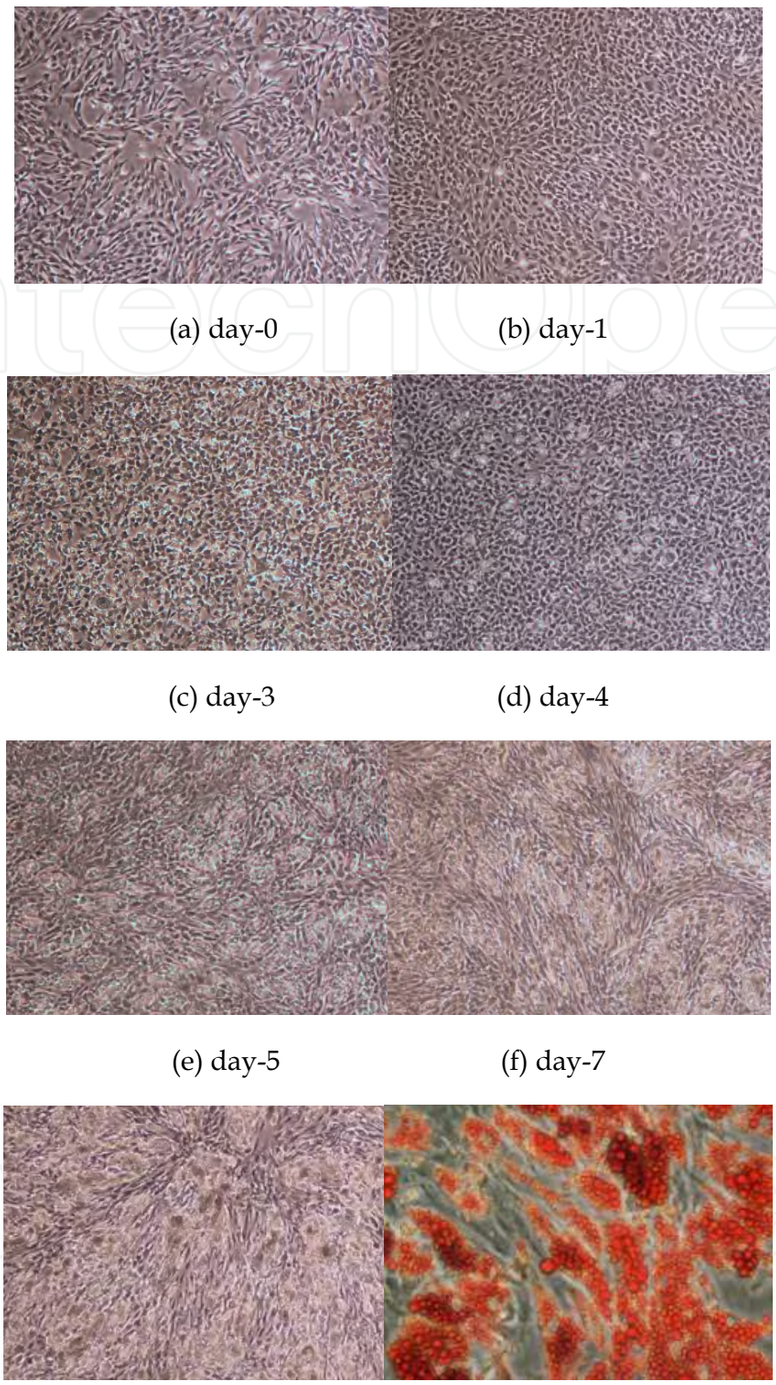


Fig. 2. Preparation of nuclear donor cells for somatic cell cloning from a GalT-KO pig: establishment of preadipocytes

After thawing by routine procedures preadipocytes were used as nuclear donors following cell cycle synchronization by serum starvation according to the method described previously (Tomii et al., 2005; Tomii et al., 2009); that is, preadipocytes were cultured in DMEM supplemented with 20% FBS to sub-confluence followed by culture for 2 days in DMEM with 0.5% FBS.

The ability of the obtained preadipocytes to re-differentiate into mature adipocytes was confirmed by the induction of differentiation *in vitro* (Tomii et al., 2005; Yagi et al., 2004). Sub-confluent preadipocytes were cultured for 4 days in DMEM supplemented with 20% (v/v) FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 5 µg/mL insulin and 0.25 µM dexamethasone to induce adipogenic differentiation. After replacing the medium with DMEM containing 20% FBS, the cells were further cultured for 6 days. To examine the lipid droplet accumulation, cells cultured for 10days after differentiation induction were fixed in 10% (v/v) formalin and stained with Oil Red O for 20min.



(a) day-0 (b) day-1

(c) day-3 (d) day-4

(e) day-5 (f) day-7

(g) day-10 (h) Oil Red O stained

Fig. 3. Re-differentiation of preadipocytes established from the GalT-KO pig (DK3-2)

Preadipocytes in sub-confluence before the induction of re-differentiation (a) and 1 to 10 days (b-g) after re-differentiation induction (x40). Re-differentiated cells on day 10 after fixation and staining (x200).

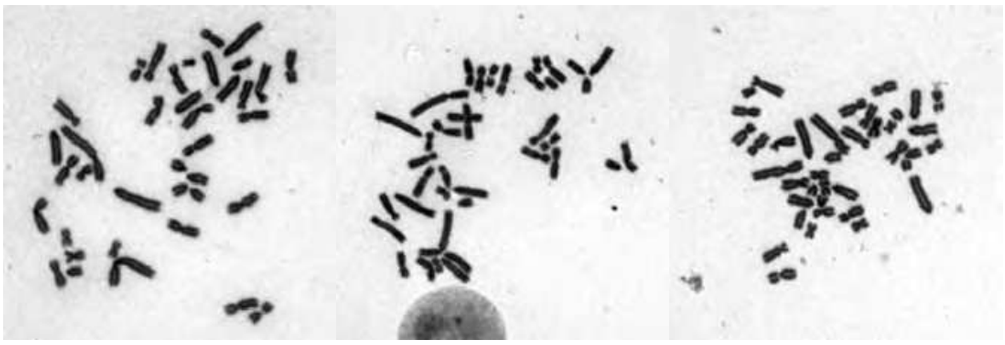
3.2 Karyotyping of nuclear donor cells

It is essential that the nuclei of the donor cells have a normal chromosomal constitution when creating somatic cell cloned animals. An examination of the cells showed that the normal chromosome number was present in 74% to 88% of the cells of the primary culture cell lines used in this study (Table 2, Fig. 4).

	DK3-1	DK3-2	DK3-9
Proportion of cells with normal chromosome number (2n=38)	74% (37/50)*	86% (43/50)*	88% (44/50)*

*Number of metaphase chromosome plates examined

Table 2. Chromosome composition of the nuclear donor cells used for somatic cell cloning of the Gal-T KO pigs



(a) DK3-1 (b) DK3-2 (c) DK3-9

Fig. 4. Normal chromosome numbers of the GalT-KO cells used for somatic cell cloning

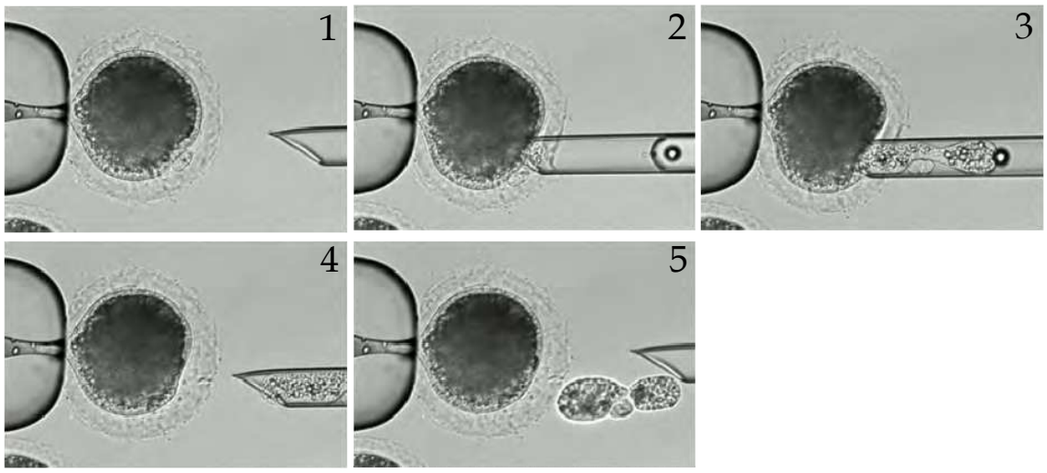
3.3 Preparation of recipient oocytes for somatic cell cloning

Ovaries were recovered from gilts at a local slaughterhouse and transported to the laboratory in PBS containing 0.1% (w/v) polyvinyl alcohol (PVA), 75 μg/ml penicillin G, and 50 μg/ml streptomycin. Follicular fluid and cumulus-oocyte complexes (COCs) were aspirated from the follicles (diameter, 3 to 6 mm) using a 20-gauge needle attached to a 10-ml disposable syringe. COCs with several layers of compact cumulus cells were selected and cultured in NCSU23 medium (Petters and Wells, 1993) supplemented with 0.6 mM cysteine, 10 ng/ml EGF, 10 IU/ml eCG (ASKA Pharmaceutical, Tokyo, Japan) and hCG (ASKA Pharmaceutical), and 10% (v/v) porcine follicular fluid. Oocytes were cultured for 22 h with eCG and hCG in a humidified atmosphere of 5% CO_2 in air at 38.5°C and then for 18 to 20 h without these hormones in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C.

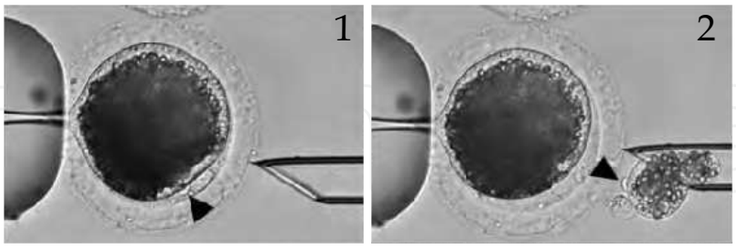
3.4 Somatic Cell Nuclear Transfer (SCNT)

Nuclear transfer of preadipocytes was performed as described previously (Tomii et al., 2005; Tomii et al., 2009). *In vitro*-matured oocytes with expanded cumulus cells were

treated with 1 mg/ml hyaluronidase and denuded of cumulus cells by pipetting. Enucleation was performed using a chemically assisted method developed by Yin et al. (Yin et al., 2002). Following culture in NCSU23 medium supplemented with 0.1 μg/ml demecolcine, 0.05 M sucrose (Nacalai Tesque, Kyoto, Japan), and 4 mg/ml BSA for 0.5 to 1 h, mature oocytes that had the first polar body were enucleated by aspirating the polar body and adjacent cytoplasm using a beveled pipette (diameter, 30 μm) in 10 mM HEPES-buffered Tyrode lactose medium containing 0.3% (w/v) polyvinylpyrrolidone (PVP), 0.1 μg/ml demecolcine, 5 μg/ml cytochalasin B (CB), and 10% FBS (Fig. 5a). When a protrusion was observed on the surface of an oocyte, it was also removed with the polar body (Fig. 5b). Enucleation was confirmed by staining the cytoplasts with 5 μg/ml bisbenzimide (Hoechst 33342).



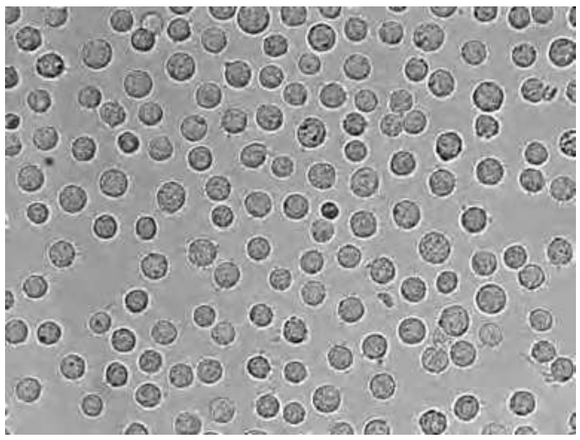
(a) Enucleation



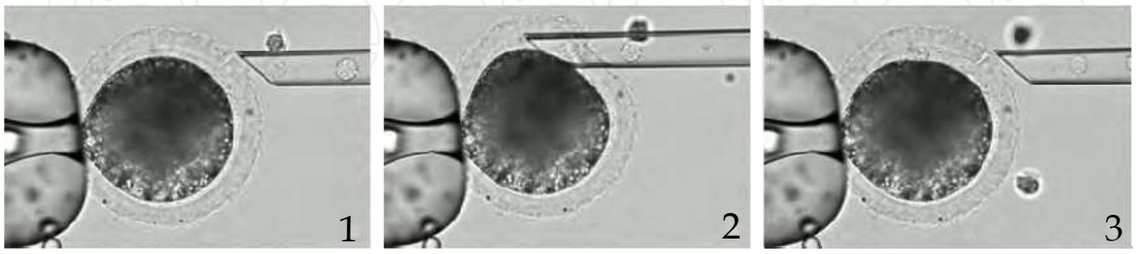
(b) Protrusion

Fig. 5. Enucleation of a porcine *in vitro*-matured oocyte: preparation of the recipient cytoplast for somatic cell cloning
(a) Removal of the first polar body and adjacent cytoplasm by aspirating with a beveled pipette (1-5)
(b) A protrusion (arrow head) visible on the surface of an oocyte was removed with the polar body (1-2)

Preadipocytes were used as nuclear donors following cell cycle synchronization by serum starvation for 2 days. A single donor cell was inserted into the perivitelline space of an enucleated oocyte (Fig. 6). Donor cell-oocyte complexes were placed in a solution of 280 mM mannitol (Nacalai Tesque) (pH 7.2) containing 0.15 mM $MgSO_4$, 0.01% (w/v) PVA, and 0.5 mM HEPES and were held between two electrode needles (Fig. 7). Membrane fusion was induced with a somatic hybridizer (SSH-1; Shimadzu, Kyoto, Japan) by applying a single direct current (DC) pulse (200 V/mm, 20 μsec) and a pre- and post-pulse alternating current (AC) field of 5 V, 1 MHz for 5 sec, respectively. The reconstructed embryos were cultured in NCSU23 medium supplemented with 4 mg/ml BSA for 1 to 1.5 h, followed by electrical activation. Reconstructed embryos were then washed twice in an activation solution containing 280 mM mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.01% (w/v) PVA and were aligned between two wire electrodes (1.0 mm apart) of a fusion chamber slide filled with the activation solution. A single DC pulse of 150 V/mm was applied for 100 μsec using an electrical pulsing machine (ET-3; Fujihira Industry, Tokyo, Japan). Activated oocytes were treated with 5 μg/ml CB for 3 h to suppress extrusion of the pseudo-second polar body.



(a) Nuclear donor cells



(b) Insertion of donor cell

Fig. 6. Insertion of nuclear donor cells into recipient cytoplast
(a) Nuclear donor cells derived from the homozygous GalT-KO pig (DK3-1)
(b) A single donor cell was inserted into the perivitelline space of an enucleated oocyte

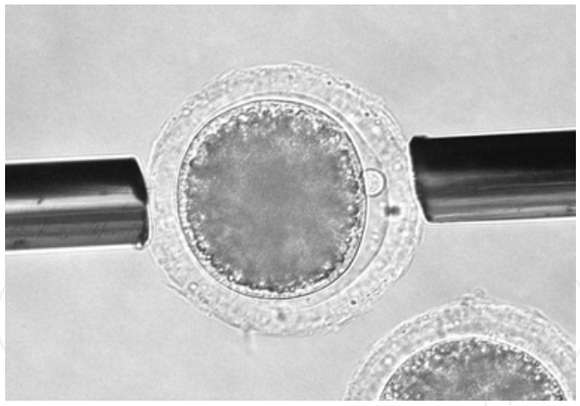


Fig. 7. Fusion of a nuclear donor cell and recipient cytoplast

An electric pulse was applied to a cell-oocyte complex using two electrodes (the chopstick method).

3.5 Culture of SCNT embryos

In vitro culture of SCNT embryos was performed in 20-μl droplets of PZM-5 (Functional Peptide Inc., Yamagata, Japan). The dish was maintained under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C. Beyond the morula stage, embryos were cultured in PZM-5 supplemented with 10% FBS. Cleavage and blastocyst formation of the reconstructed embryos were monitored during culture for 7 days. On Day 7, the number of cells in the blastocysts was counted after fixation and staining.

3.6 The effects of a histone deacetylase inhibitor on the development of SCNT embryos

Some of the SCNT embryos produced here were cultured in the presence of a histone deacetylase inhibitor (HDACi) Scriptaid, and their subsequent development was examined. The SCNT embryos, which had been undergone electrical activation and cytochalasin B treatment, were cultured in the presence of 500 nM Scriptaid in PZM5 for 15–20 hours and were then transferred to normal medium for further culture. The effects of HDACi were assessed by the rate of SCNT embryos that developed into blastocysts and the cell numbers in the blastocysts obtained.

3.7 Embryo transfer

Crossbred (Large White / Landrace × Duroc) prepubertal gilts weighing from 100 to 105 kg were used as recipients of the SCNT embryos. Gilts were treated with a single intramuscular injection of 1000 IU eCG (ASKA Pharmaceutical) to induce estrus. Ovulation was induced by an intramuscular injection of 1500 IU hCG (Kawasakimitaka Pharmaceutical, Kanagawa, Japan) given 68 h after the injection of eCG. Cloned embryos cultured for 1 or 2 days were surgically transferred into the oviducts of recipients approximately 51-54 h after hCG injection. Embryos cultured for 6 days were transferred at the blastocyst stage to the uterine horns of the recipients approximately 146 - 148 hr after hCG injection.

3.8 Analysis of cloned piglets

Homozygous GalT gene knock out in the cloned pigs was confirmed by PCR as described previously (Fujimura et al., 2008a; Fujimura et al., 2008b; Takahagi et al., 2005). Briefly, tail chips derived from the cloned pigs and a control non-transgenic pig were lysed overnight at 55°C in a lysis solution (10 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.5 % SDS, and 100 μg/mL Proteinase K). The lysates were extracted with phenol-chloroform followed by ethanol precipitation. The genomic DNA was resolved in TE buffer.

The primer sets used were the same as those described in previous reports (Fujimura et al., 2008a; Fujimura et al., 2008b; Takahagi et al., 2005). In the case of a targeted allele in which the GalT gene has been replaced with a marker gene (hygromycin-resistance gene), a DNA fragment of approximately 10 kb was amplified; however, in the case of the wild-type allele, a DNA fragment of approximately 7.6 kb was amplified. Amplification was performed on genomic DNA using the Expand Long Template PCR System (Roche; the forward primer was 5′-AGAGGTCGTGACCATAACCAGAT-3′, and the reverse primer was 5′-AGCCCATCGTGCTGAACATCAAGTC-3′). After an initial denaturation step of 94°C for 2 min, PCR amplification was performed for 30 cycles of 94°C for 15 sec, 65°C for 30 sec and 68°C for 10 min with a 20-sec extension per cycle, and this was followed by incubation at 68 C for 7 min. Amplification products were analyzed by 1 % agarose gel electrophoresis.

4. *In vitro* development of cloned embryos derived from the GalT-KO pigs: An index for successful pig cloning

In vitro culture can provide an approximate estimate of the competence of SCNT embryos to develop into live cloned offspring. Since transcription from the embryonic genome starts around the 4-cell stage in porcine embryos, the early cleavage stages may be able to progress regardless of the existence of genomic aberrations. We found that the cleavage rate to the 2- to 8-cell stage in cloned embryos reconstructed from cells with numerical chromosome anomalies was similar to that of cloned embryos derived from normal cells (unpublished data). Although *in vitro* development into blastocysts does not guarantee the normalcy of the SCNT embryos, the ability to reach this developmental stage is nevertheless a reasonable indicator of whether cloned offspring can be obtained from the SCNT embryos. This raises the question of what rate of development of SCNT embryos into blastocysts is necessary to ensure that cloned pigs can be obtained. This question is difficult to answer as the culture conditions for embryos differ among laboratories. However, in our experience, when the developmental rate of cloned embryos produced using a given type of nuclear donor cell was over 30%, cloned pigs could almost always be obtained. With our porcine somatic cell cloning procedures, the *in vitro* developmental rate of the SCNT embryos into blastocysts exceeds 50%, and in such cases, the pregnancy rate of the recipients after transfer of the cloned embryo is almost 100%.

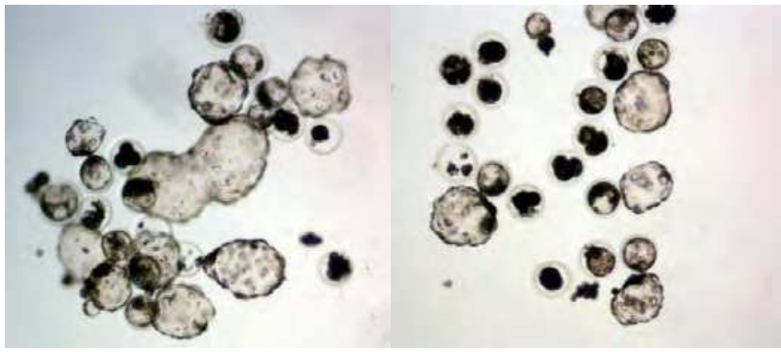
The *in vitro* development of the SCNT embryos is significantly improved by culture in the presence of a histone deacetylase inhibitor (HDACi), e.g., trichostatin A (TSA) and Scriptaid (Zhao et al., 2010). This improvement can be explained by the activation of transcription of genes involved in development, which results from histone hyperacetylation in the

chromosomes of the cloned embryos. Table 3 summarizes the *in vitro* development of SCNT embryos created from GalT-KO cells. The blastocyst formation rate in the SCNT embryos derived from GalT-KO pig cells (DK3-1) was significantly improved (51.4 vs. 77.1%, $P<0.05$) by Scriptaid treatment. As shown in Fig. 8, the morphology of the cloned blastocysts treated with HDACi was better than that of untreated blastocysts, and the number of constituent cells was high.

Scriptaid treatment	No. of NT embryos cultured	Embryonic development (%)		Average cell number in blastocysts (mean ±SEM)
		Cleaved	Blastocysts	
+	35	27 (77.1)[a]	27 (77.1)[a]	70.9 ±6.5[a]
-	37	33 (89.2)[a]	19 (51.4)[b]	62.2 ±5.3[a]

[a, b]Values with different superscripts in the same column differ significantly

Table 3. *In vitro* development of porcine SCNT embryos treated with Scriptaid



(a) Scriptaid(+) (b) Scriptaid(-)

Fig. 8. Morphology of cloned porcine blastocysts treated with Scriptaid

5. Creation of cloned pigs from GalT-KO cells

The results of transfer experiments with the cloned embryos are presented in Table 4. Transfer of the 547 cloned embryos reconstructed with the donor cells from DK3-1 into 6 recipients gave rise to 6 pregnancies (100%). Although 2 miscarried at 30 and 21 days of gestation, 2 recipients farrowed 10 piglets, including 2 stillborn piglets. From 2 other recipients, a total of 7 live full-term piglets were obtained by Caesarian section. The overall production efficiency of the cloned piglets was 3.1% from the recipients that farrowed or received a Caesarian section (17/547). Cloning efficiency of GalT-KO pigs in these experiments was comparable to that of non-KO pigs that we had reported previously (Kurome et al., 2006; Matsunari et al., 2008b).

As shown in fig., the DK3-1 cloned piglets were apparently healthy, though they were all sacrificed for analysis within 72 h. The average body weight and crown-rump length of the cloned piglets were 822.9 +/- 47.3 g and 24.7 +/- 0.6 cm, respectively (Table 5). The birth weights of the cloned pigs were much less than those of non-transgenic pigs with a common genetic back ground in our experimental farm (1.0 - 1.5 kg).

Origin of donor cell	Scriptaid treatment	SCNT embryos transferred	Recipients	Full term / Pregnant	Piglets or full-term fetuses* obtained (stillborn)**	Production efficiency of cloned offspring
DK3-1	-	547	6	4 / 6	17 (2)	17/547 (3.1%)
DK3-1	+	635	5	5 / 5	13 (5)	13/635 (2.0%)
DK3-2	-	176	2	2 / 2	2 (2)	2/176 (1.1%)
DK3-9	+	377	4	1 / 1	7 (0)	7/377 (1.9%)
DK3-9***	-	214	2	2 / 2	1(1)	1/214 (0.5%)

*Obtained by Caesarian section
**Within the piglets or full-term fetuses obtained
***Cloned embryos were produced from donor cells that were transported internationally

Table 4. The effects of Scriptaid treatment on the *in vivo* development of porcine SCNT embryos derived from Gal-T KO cells

Fig. 9. Cloned offspring produced by SCNT of the homozygous GalT-KO pig cells co-expressing hDAF and GnT-III (DK3-1)

We also conducted an experiment in which cloned embryos, created using the cells from DK3-1, were treated with the HDACi Scriptaid and then transferred to recipients (Table 4). A total of 635 cloned embryos were transplanted into 5 recipients, all of which became pregnant (100%). By natural birth or full-term Caesarian section, 13 offspring were obtained from the recipients (5 were stillborn). The efficiency of producing offspring was 2.0% (13/635), indicating that HDACi treatment did not provide a marked improvement. However, it is notable that the six pregnant recipients did not have miscarriages in the experiment that used the HDACi treatment.

The body weight and length of the cloned piglets obtained from the 3 lines of GalT-KO cells are presented in Table 5.

DK3-1 Scriptaid (-)		DK3-1 Scriptaid (+)		DK3-2 Scriptaid (-)		DK3-9 Scriptaid (+)	
Weight (g)	Length (cm)	Weight (g)	Length (cm)	Weight (g)	Length (cm)	Weight (g)	Length (cm)
780	23	900	28	720*	23	1000	25
820	24	840*	25	480*	19	1000	26
820*	24	600*	23			1100	27
900	25	700*	25			900	25
1120	29	1500	27			700	23
580	23	1200	28			500	20
560	22	900	23			380	19
730	25	800	24				
1060*	27	900	25				
700	22	600	22.5				
700	23	600	24				
600	22	700*	26				
920	25	700	24				
1100	29						
900	25						
1120	29						
580	23						

*stillborn piglets

Groups	Average body weight (g) (mean± SEM)	Average body length (cm) (mean± SEM)
DK3-1 / Scriptaid (-)	822.9± 47.3	24.7± 0.6
DK3-1 / Scriptaid (+)	841.5± 72.0	25.0± 0.5
DK3-2 / Scriptaid (-)	600.0**	21**
DK3-9 / Scriptaid (+)	797.1± 104.2	23.6± 1.2

** SEM was not given due to limited sample numbers

Table 5. Body weight and length of the cloned piglets derived from the three types of the GalT-KO pigs

When 176 cloned embryos derived from DK3-2 were transferred into 2 recipients, both became pregnant, and 2 stillborn piglets were delivered (Table 4). The body weights and crown-rump lengths of these piglets were also much less than those of the normal piglets: 480 g compared with 720 g and 19 cm compared with 23 cm, respectively (Table 5). These stillborn piglets were delivered 2 days later than the expected date of farrowing. This delay may explain the weak DNA amplification signal obtained by PCR (Fig. 10).

Homozygous knock-out of the GalT gene was confirmed in all of the cloned pigs by PCR (Fujimura et al., 2008a; Fujimura et al., 2008b; Takahagi et al., 2005) (Fig. 10).

Transmission of the two transgenes, i.e., hDAF and GnT-III, was also confirmed in the cloned offspring. In the present study, we did not examine expression of these transgenes. However, faithful expression of transgenes in the cloned offspring produced by SCNT of a polytransgenic pig has been demonstrated in our previous studies (Fujimura et al., 2008a; Fujimura et al., 2008b; Takahagi et al., 2005). The influence of epigenetic modification on gene expression in cloned pigs needs to be investigated.

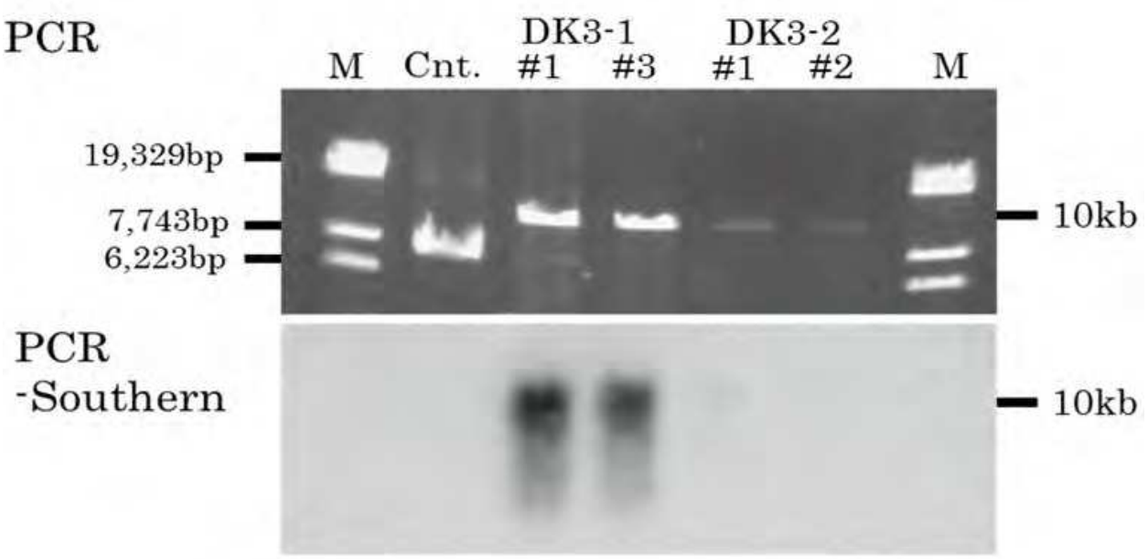


Fig. 10. PCR and PCR–Southern analysis of cloned pigs derived from homozygous GalT-KO pig somatic cells

Cloned pigs were produced from cells derived from DK3-9 (Table 4). DK3-9 is a GalT-KO homozygote and has no other genetic modification or transgene integration. The cloned embryos were treated with 500 nM Scriptaid for 15–20 hours. When 377 SCNT embryos were transplanted at the early cleavage stages (day 1 to 3), all 4 recipients became pregnant, and one sow farrowed seven live offspring. The average body weight and crown-rump length of the cloned piglets derived from the DK3-9 cells were 797.1+/- 104.2 g and 23.6 +/- 1.2 cm (Table 5). The other recipients had miscarriages. In the cloning experiments with DK3-9 cells, several miscarriages occurred, although the SCNT embryos had been treated with HDACi Scriptaid. This result is in contrast to the results of the cloning of DK3-1. It may be pertinent that the experiments with DK3-9 were performed during one of the hottest summers recorded in Japan, and this may have affected the recipients' pregnancies; further investigation will be necessary to identify the causes of the difference in experimental outcomes. Most of the cloned offspring with a normal range body weight were healthy and grew normally (Figs. 11, 12).

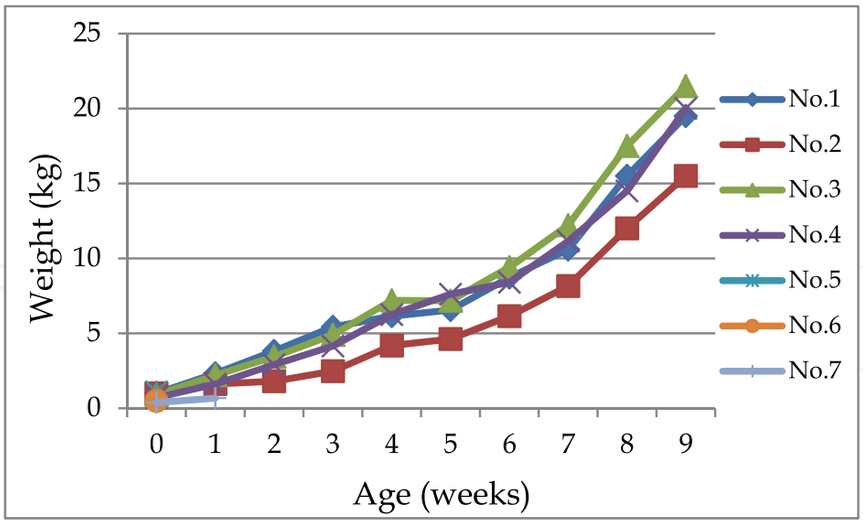


Fig. 11. Growth of the cloned pigs derived from the GalT-KO cells (DK3-9)

Fig. 12. Live offspring of a GalT-KO pig (DK3-9) produced by somatic cell cloning

The GalT-KO pig (DK3-9) cells that we created were transported by air from Japan to Germany and used to produce cloned pigs. As shown in Table 4, 214 cloned embryos were produced and transferred into 2 recipient sows. Both recipients became pregnant, and one cloned offspring was produced.

6. The prospect of the development of genetically modified pigs and the development of "rainbow pigs"

To bring xenotransplantation into clinical application, it is essential to overcome xenograft rejections, such as hyperacute rejection, delayed xenograft rejection, and cellular rejection

(Ekser and Cooper, 2010). Genetic modification of pigs by technologies based on SCNT would be the most promising approach to solve these issues. Furthermore, the advantage of SCNT is that additional genetic modification can be achieved based on the analysis of phenotypic expression of the resulting genetically modified pigs (Fig. 1). Genetically modified pigs so far developed as xenotransplantation donors including ours (Fujimura et al., 2008a; Fujimura et al., 2008b; Takahagi et al., 2005) will be further improved. With multiple gene modifications in pigs, the feasibility of serial somatic cell cloning (multi-generational cloning) would have important implications. In this regard, we have successfully produced the 4th generation of cloned pigs by serial cloning (Kurome et al., 2008a; Matsunari et al., 2008a). We have developed a process for multi-generational cloning of pigs, that is, a means of proceeding from the first clone generation (G1) to the second (G2) and then to the third generation (G3), and the efficiency of producing cloned pigs throughout this process was almost constant (Kurome et al., 2008a; Matsunari et al., 2008a). Notably, the cloned offspring did not show any shortening of their telomeres (Kurome et al., 2008a) and grew normally. Our results indicate that serial cloning is a feasible option for production of specifically designed pigs with multiple genetic modifications.

In this study, we investigated the feasibility of cloning the GalT-KO pigs by focusing on the introduction of multiple genetic modifications into pigs. The application of somatic cell cloning in xenotransplantation research is not limited to genetic modifications. Somatic cell cloning could also be used for reproducing large numbers of pigs with a preexisting genetic modification for use in organ transplantation experiments in primates and for other preclinical research (Kuwaki et al., 2005; Yamada et al., 2005). We have already started an organ transplantation experiment using the cloned GalT-KO pigs (unpublished). Additionally, as already discussed, while it is difficult to transport genetically modified pigs, it is a comparatively simple matter to transport cells from the pigs by air to overseas.

Development of genetically modified pigs has been attempted in several countries, including Australia, Britain, Germany, Japan, South Korea and the USA, with the aim of applying such animals in xenotransplantation. To realize their potential for clinical applications, it will be necessary to create pigs that incorporate all of the desired genetic modifications. We shall continue our pursuit of the ultimate goal of producing "rainbow" pigs—cloned pigs covering every possible genetic modification of medical interest.

7. Conclusion

In conclusion, the data presented in this chapter demonstrate that homozygous GalT-KO pigs can be efficiently reproduced by SCNT; hence, cloning technology is suggested to be a feasible option for proliferation of the genetically modified pigs. As part of the process of developing organ donors for xenotransplantation, somatic cell cloning provides an efficient and superior technology with high reliability and reproducibility for creating and reproducing pigs with multiple genetic modifications.

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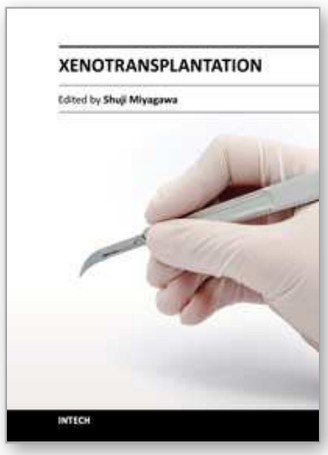

Xenotransplantation

Edited by Prof. Shuji Miyagawa

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Accompanied by the advent of animal cloning, the technique of nuclear transfer produced alpha1,3-galactosyltransferase-knockout (Gal-KO) pigs in many institutes, including the ones in Japan, at the beginning of 21st Century. In addition, the controversy of the risks of PERV has gradually minimized, because of the fact that there are no cases of PERV infections reported in humans. Furthermore, a large clinical wave for islet allotransplantation resumed the interest of xenotransplantation, especially porcine islet transplantation and some exceptions. Clinical trials were done in many countries so far, such as Sweden, China, Mexico, USA (Inventory of Human Xenotransplantation Practices - IXA and HUG in collaboration with WHO). In addition, a new clinical trial was approved by the government, and resumed the porcine islet transplantation research in New Zealand two years ago.

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