Ⅰ. Major Research Results

1. Biotechnology Research

【Bio-medicine research】

(1) G-CSF transgenic animal production

We have developed wildtype G-CSF transgenic pig by microinjection on one-cell stage of in vivo fertilized oocytes. Protein medicine like EPO, G-CSF and GM-CSF would be a new choice of biosimilar to reduce cost of cancer treatment. One-cell stage embryos were prepared by superovulation treatment and natural mating was used to fertilized ovulated oocytes. After the surgical procedures of oocyte recovery, one-cell stage embryos were collected for microinjection. Approximately 25 eggs were implanted into 21 surrogate mothers (Table 1-1). G-CSF production vector containing the insulator, the mouse WAP promoter and bovine growth hormone poly A-containing sequences was used for transgenic microinjection.

Table 1-1. Transgenic pig production and Embryo transfer

Pig Numbers of superovulation	Total ovulated oocytes (mean)	Oocyte Recovery (%)	Microinjedted PN stage embryo (%)	Number of surrogate mother	Pregnancy/Delivery (No. of TG)
56	1,353 (24)	802 (59.3%)	526 (65.6%)	21	4 / 3 (1)

A total of 526 injected embryos were transferred to 21 surrogate mothers. Three pigs from 4 pregnants were delivered to full terms and gave birth to piglets and a G-CSF wild type transgenic pig was confirmed by PCR analysis (Fig. 1-1).

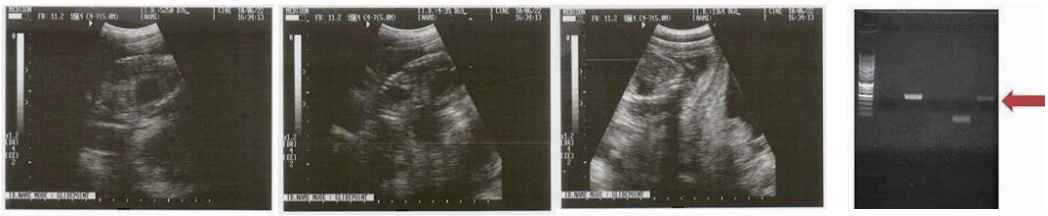


Fig. 1-1. Pregnancy test of transferred embryos and PCR results

(2) Transgenic line establishment and enhancement

We have developed four species of trangenic pigs (EPO, Factor VIII, vWF and tPA) which were confirmed by their productivity of biosimilars in pig milk. For strict industrial validation of biopharmaceuticals, the characteristics of exact transgene loci on genome, copy number of transgene were considered as important data for commercialization

The results of genomic location of transgene confirmed by FISH method were quoted from the data of '09. The copy number estimations of the transgene were analysed by qPCR. Each result of qPCR of transgenic pigs was compared to the results of amplification of Zar 1 gene of same samples.

The copy numbers of EPO transgene were about 3-4 with small variation and those of Factor VIII showed variations between 25 and 45 copies. The pigs of higher copy numbers should be tested their expression ability of productivity of biosimilars, because they were born from transgenic parents.As an orphan medicine, vWF transgenic pigs were classified three different lines. However, lower copy line (vWF-1) showed the highest expression level of biosimilar.

Tabel 1-2. The results of genetic loci and estimation of copy numbers

Species	Line	Locus in genome	estimation of copy numbers
Anemia (EPO)	EPO-1	5p11.2	3~4copy
Hemophilia (Factor VIII)	FVII-1	2p11.2	3~25copy, 45copy
Rare Hemophilia (vWF)	vWF-1	11p11.2	4~7 copy
	vWF-2	15q13.1	3~12 copy
	vWF-3	4p13	10~21 copy
Thrombosis (tPA)	tPA-1	-	-
	tPA-2	1p11	-

(3) Biosimilar protein purification from transgenic milk

The G-CSF trangenic pig was produced by microinjection technique and mutant G-CSF(F140N) was prepared for the next transgenic animals. This mutant G-CSF contained glycosylation site showed higher activity on the cell proliferations. To develop anti G-CSF antibody that could be a key molecule of protein purification, where subjected to 16 different cell lines for antibody screening tests.

As shown in Fig.1-2, cells produced in anti-G-CSF antibody isoforms. The mixture of anti G-CSF antibody cell line needed further isolation procedures.

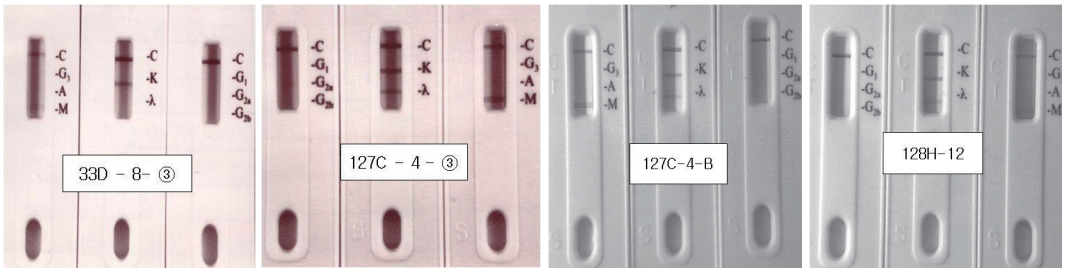


Fig. 1-2. Monoclonal antibody isotype tests

CSF-dependent cell line named M-NFS-60 cells was used for the estimation of G-CSF mutant activity (F140N). The survival of cells and proliferations showed the estimated activity of the wild type and mutant. In the figure 3, the activity of mutant G-CSF showed possibility of candidate medicine as a promoting agent of WBC cells.

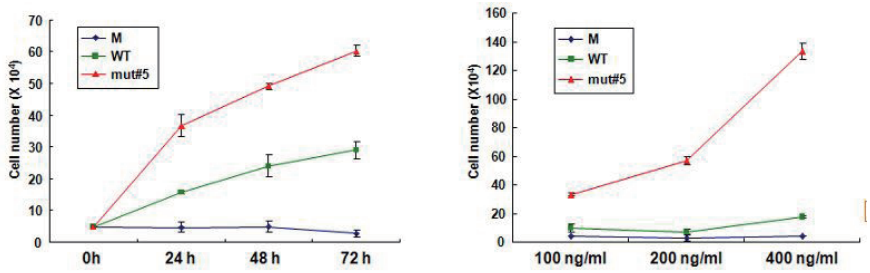


Fig. 1-3. The effect of G-GSF wild type and mutants on cell numbers

(4) Development of Transgenic cattle producing functional proteins

4-2. Study of transformation efficiency using stem cells

Stem cells were isolated from in vivo blastocysts and the stem cell culture conditions were established to the kind of feeder cell, and the efficiency was analyzed towards the development. A total of 85 embryos were collected from 14 cows (6.1 embryos/herd), in these 38 embryos were outgrowth cultured. From the blastocyst stage in vitro or in vivo experiment were performed to establish ES cell using MEF or STO cell which wasremoved zona pellucida, or a hatched embryos were cultured. The results showed no signiticance in between MEF and STO cells were cultured butthe embryos were attached to a feeder cell represents the form of a flat based morphology. To date, stem cells cultured in cattle have not been clearly established and further investigations will be needed to establish the culture conditions in the feeder cells.

Table 1-3. Construction of cattle in vivo embryos for stem cells by feeder cell conditions

Outgrowth embryo	Feeder cell	Attached to feeder cells	Established ES-like cell line	Final number of passages
1	STO	×	×	-
2	STO	×	×	-
3	STO	○	○	2~
4	STO	○	○	3~
5	MEF	×	×	-
6	MEF	×	×	-
7	MEF	○	×	-
8	MEF	○	○	3~

Table 1-4. In vivo and in vitro embryonic stem-like cells derived from blastocysts in cows

Source of Blastocyst	Number of Bl seeded	Number of Bl attached	Established ES-like cell line	Final number of passages
in vivo	6	3	2	〉4
in vitro	10	4	0	〉2

To establish in vitro and in vivo ES cells from the blastocyst stage, 6 and 10 embryos were cultured on the feeder cells and found 4 and 3 were attached respectively. The blastocyst stage comparison in between ES-like cell culture in in vitro and in vivo was found to be unsuccessive in in vitro and successive differentiation in in vivo of the embryonic stem cell respectively which needs further investigation to understand the mechanistic key function that behinds the differentiation.

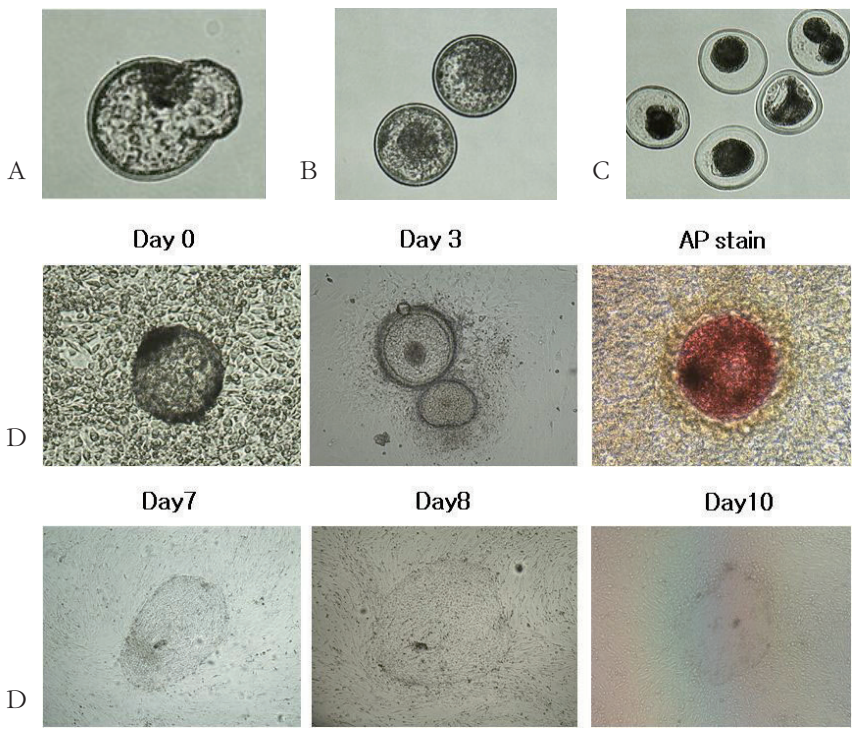


Fig. 1-4. Bovine embryonic stem-like cells of blastocyst outgrowth
(A, B: in vivo blastocyst from cattle. C: under grade unfertilized and fertilized embryo, D: Embryos of the outgrowth culture. E: outgrowth of embryos cultured day 7-10)

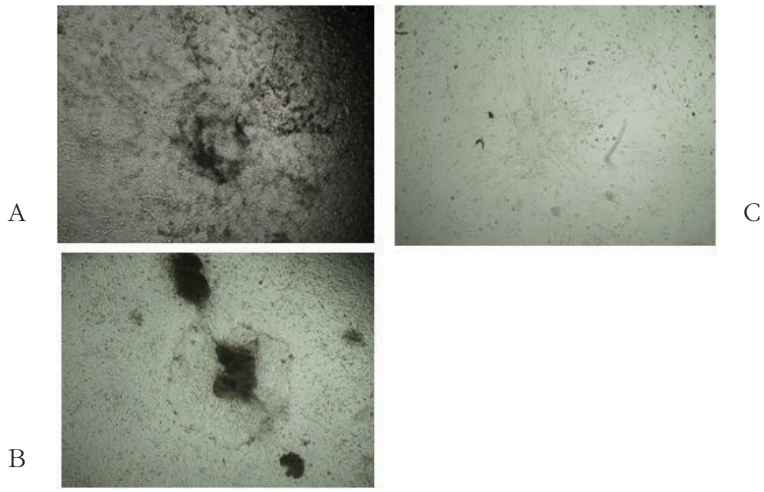


Fig. 1-5. Bovine embryonic stem-like cells of blastocyst outgrowth culture
(A, B: outgrowth culture of embryos. C: differentiation of outgrowth embryos)

The production of induced pluripotent stem cell (iPS) was induced in the somatic cell by non-viral vector where introduced by electroporation and AMAXA transfection, which was resulted in higher transfection efficiency (69:8 cells/flasks) in electroporation method.

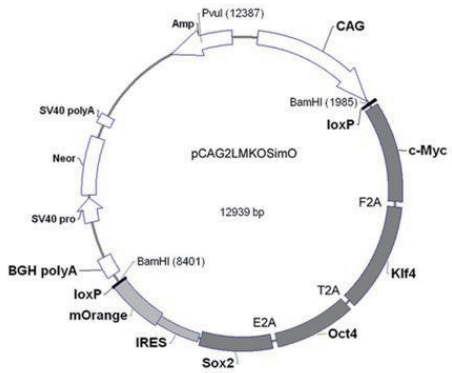


Fig. 1-6. iPS stem cell used to build a non viral vector (pCAG2LMKOSimO vector).
The four reprogramming factors are translated from a single mRNA encoding c-Myc, Klf4, Oct4 and Sox2 linked with three different 2A sequences, F2A, T2A and E2A. The sequences of the 2A peptides and their 'skip' sites by. c-Myc, Klf4, Oct4 and Sox2 coding regions linked by three different 2A peptide sequences of F2A, T2A and E2A (reprogramming cassette) are transcribed from the CAG enhancer/promoter. The reprogramming cassette is followed by IRES mOrange. The reprogramming cassette and ires mOrange are flanked by loxP sites. pCAG2LMKOSimO is linearized with PvuI before transfection

Table 1-5. Transfectrion conditions and number of GFP-positive cells

Treat	Passage number	DNA (ug)	Cells per flask (75cm2)	% of GFP positive cell(-100)	GFP positive cells per flask
Electroporation	6.5	2	2,000,000	0.48 (±0.17)	19.5 (±3.5)
Amaxa	6.5	2	2,000,000	0.22 (±0.07)	9 (±2)

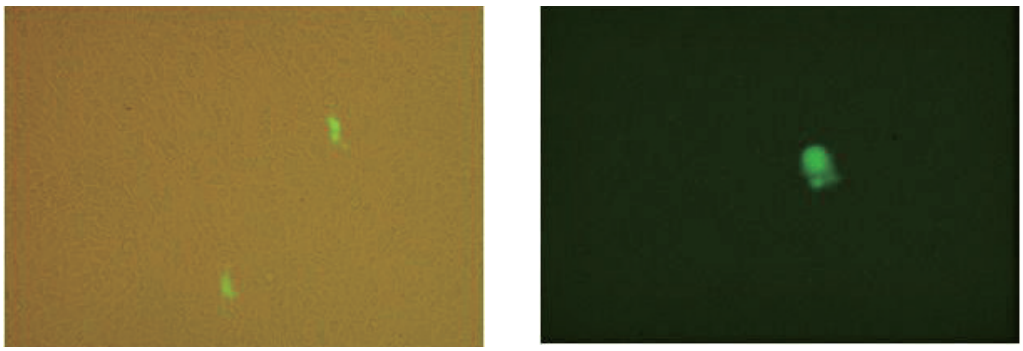
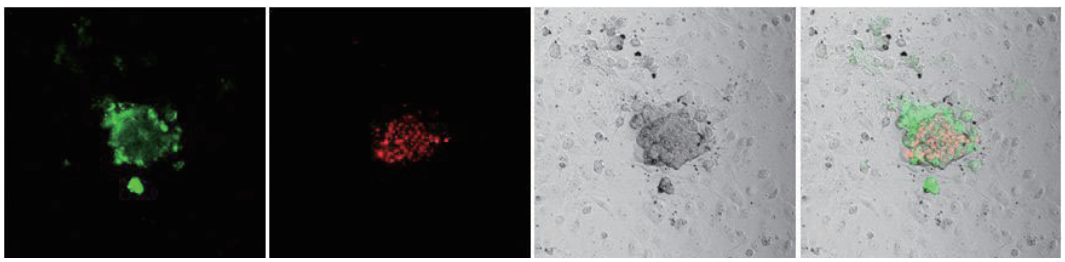


Fig. 1-7. pCAG2LMKOSimO vector for GFP expression after transfection(48hr)



A B C D

Fig. 1-8. Expression of pluripotency markers in pig iPS cells detected byimmunostaining. (A : Immunostaining of iPS cells were positive for NANOG, B : Nuclear staining, C: morphology of iPS colony, D: mergy image

(5) Development of Transgenic animal bioreactor producing therapeutic proteins

5-1. Oviduct-specific EGFP expression in transgenic chickens

We demonstrated the ability of the 2-kb promoter fragment of the chicken ovalbumin gene to drive tissue-specific expression of a foreign EGFP gene in chickens. Recombinant lentiviruses containing the EGFP gene were injected into the subgerminal cavity of 539 freshly laid embryos (stage-X). Subsequently, the embryos were incubated to hatch using phases II and III of the surrogate shell ex vivo culture system. The twenty-four chicks (G0) were hatched and screened for the EGFP with PCR. Two chicks were identified as transgenic birds (G1), and these founders were mated with wild-type chickens to generate transgenic progeny. In the generated transgenic hens (G2), EGFP was expressed specifically in the tubular gland of the oviduct. These results show the potential of the chicken ovalbumin promoter for the production of biologically active proteins in egg white.

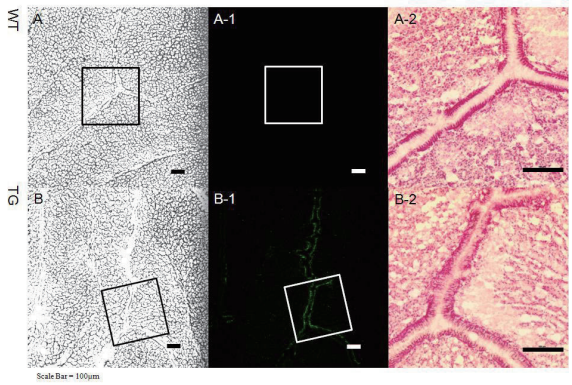


Fig. 1-9. Tissue-restricted expression of EGFP.

【Bio-organ research】

(1) Production of transgenic cloned pigs for xenotransplantation

1-1. Characterization of donor cells added with follicular fluid

The cell cycle stage of the donor cells cultured with 10% of porcne follicular fluid (pFF) for 72 hr were analyzed by FACS (Fig. 1-10). The population of G0/G1 stage was significantly higher in pFF group (90.34%) compared to FBS (81.84%) ($P<0.05$).

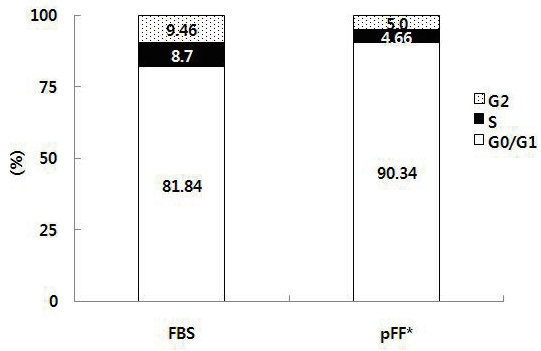


Fig. 1-10. Effects of follicular fluid on cell cycle stages
FBS: DMEM + 10% FBS ; pFF: control + 10% pFF
* p〈0.05

1-2. Effects of pFF on protein expression in conditioned medium

Two dimentional electrophoresis (2DE) was used to select differentially expressed proteins in both FBS and pFF conditioned medium (Fig. 1-11).

According to 2DE results, 790 spots were selected in the medium containing 10% of pFF. The spots identified were expressed at least 2 times. Based on the 2DE results, it can be postulated that porcine follicular fluid was found to many unidentified proteins

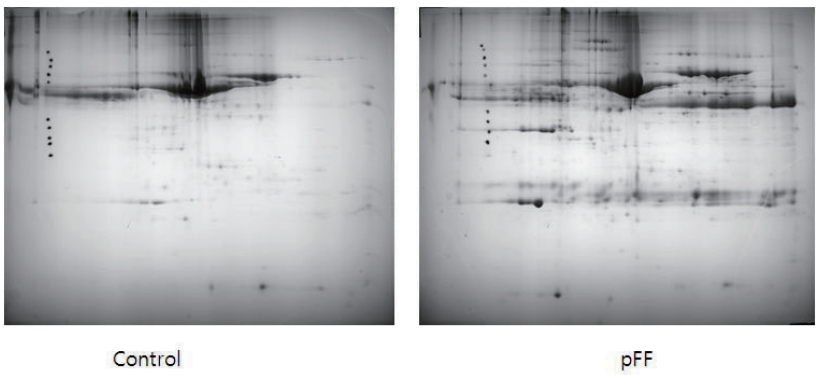


Fig. 1-11. Different expression patterns of total proteins using 2DE with 790 differentially expressed proteins in pFF treated group.

1-3. Peptide mass fingerprint(PMF) analysis

Peptide mass fingerprint (PMF) was performed to analyze the strongly expressed spots (Table 1-6). According to PMF analysis, 12 spots were identified as 17β-hydroxysteroid dehydrogenase type 1, properdin, adiponectin, transferrin and albumin.

Table 1-6. Peptide mass fingerprint(PMF) analysis of the differentially expressed spots

File.No	GI	Description	Access
103	161779844	17beta-hydroxysteroid dehydrogenase type 1 [Sus scrofa]	ABX79407
105	126352512	thiopurine S-methyltransferase [Equus caballus]	NP_001075374
106	148724910	B-factor, properdin [Sus scrofa]	CAN87698
3105	114158576	Adiponectin	Q3Y5Z3
3106	76445989	serum albumin [Bos indicus]	ABA42866
4306	30794280	serum albumin precursor [Bos taurus]	NP_851335
6111	125947	Ig lambda chain C region	P01846
7104	4505753	phosphoglyceratemutase 1 [Homo sapiens]	NP_002620
7108	52353352	serum albumin precursor [Sus scrofa]	NP_001005208
7202	833800	transferrin [Sus scrofa]	CAA30943
7204	164318	albumin [Sus scrofa]	AAA30988

1-4. Functional analysis

Table 1-7 showed the biological process, expression cite, and molecular function of the identified spots. 17β-hydroxysteroid dehydrogenase type 1 has functions of estrogen biosynthetic process in biological process, cytoplasm in expression cite, and catalytic activity in molecular function. Transferrin has functions of cellular iron ion and homeostasis in biological process, endosome in expression cite, and binding to ferric ion in molecular function.

Table 1-7. Functional analysis of the differentially expressed spots

File.No	GO(BP)	GO(CC)	GO(MF)
103	estrogen biosynthetic process	cytoplasm	catalytic activity
105	metabolic process	cytoplasm	methyltransferase activity
106	proteolysis	-	catalytic activity
3105	positive regulation of glucose import	extracellular region	eukaryotic cell surface binding
3106	transport	extracellular space	-
4306	transport	extracellular space	metal ion binding
6111	-	-	antigen binding
7104	regulation of glycolysis	cytosol	bisphosphoglyceratemutase activity
7108	maintenance of mitochondrion location	extracellular space	toxin binding
7202	cellular iron ion Homeostasis	endosome	ferric iron binding
7204	maintenance of mitochondrion location	extracellular space	toxin binding

1-5. Effect of transferred embryos on pregnancy

The effect of the number of transgenic NT embryos transferred on pregnaney shown in Table 1-8. The pregnancy rate was higher in 〉 129 group (13/20, 65%) than that of 〈 90 (1/6, 16.7%).

Table 1-8. Effect of the number of NT embryos on pregnancy

ET*	Pregnancy(%)	Remarks
〈 90	1/6(16.7)	X1: still birth
〉 129	13/20(65.0)	X5: delivery X4: maintenance X4: still birth

* NT embryos transferred

1-6. Effect of medium on pregnancy

In table 1-9, effect of culture medium type on pregnancy rate was evaluated. The

pregnancy rate was higher in the serum reduced medium containing 2% FBS (8/12, 66.7%) when compared to control supplemented with 10% FBS (3/7, 42.9%).

Table 1-9. Effects of donor cell culture medium on pregnancy

Medium	Pregnancy(%)	Remarks
DMEM (10% FBS)	3/7(42.9)	X3: maintenance
Serum reduced medium (2% FBS)	8/12(66.7)	X5: delivery X1: maintenance X2: still birth

* Containing 500nM Roscovitin

1-7. Effect of donor cell type on pregnancy rate

Normally donor cells were cultured for 3 days. At that time, the cells were cultured more than 95% of culture dish. However, we used the donor cells immediately after thawing. The pregnancy rate was higher in frozen-thawed group (10/17, 58.8%) than that of control group (9/29, 31%) (Table 1-10). Because the status of donor cells was G0/G1 stage immediately before freezing, it can be possible to use the cells for NT.

Table 1-10. Pregnancy rate according to donor cell conditions

Cell type	Pregnancy(%)	Remarks
Cultured	9/29(31.0)	X2: delivery
Frozen-thawed*	10/17(58.8)	X5: delivery X3: maintenance

* cells without additional in vitro culture after thawing

1-8. Production of transgenic cloned pigs for xenotransplantation

We successfully produced the transgenic cloned pigs for xenotransplantation (Fig. 1-12).

Fig. 1-12. Production of transgenic cloned minipigs for xenotransplantation

(2) Establishment of mass production system on transgenic pigs

2-1. Production of GalT KO F1 pigs

Using artificial insemination, we produced 60 F1 pigs. Among them, 24 F1 pigs were evaluated to GalT KO. Unfortunately, two of 24 GalT KO F1 pigs died immediately after the birth (Table 1-11).

Table 1-11. Production of GalT KO transgenic pigs

No. of			Sex of litters	
Dams	Litters	GT-KO	♂	♀
1	4	2	1	1
2	5	2	2	-
3	10	3	1	2
4	13	6 (1)	3	3
5	14	8	2	6 (1)
7	9	2 (1)	-	2 (1)
8	5	1	1	-
계	60	24 (2)	10	14 (2)

( ): No. of pups died

2-2. Analysis of hormone levels

Testosterone level in GalT KO F1 pigs : The level of testosterone was lower in GalT KO pigs than that of normal pigs at 2~3 months. During their growing periods, the testosterone level was not stable, however, the level was increased in male at 9 months after birth (Fig. 1-13).

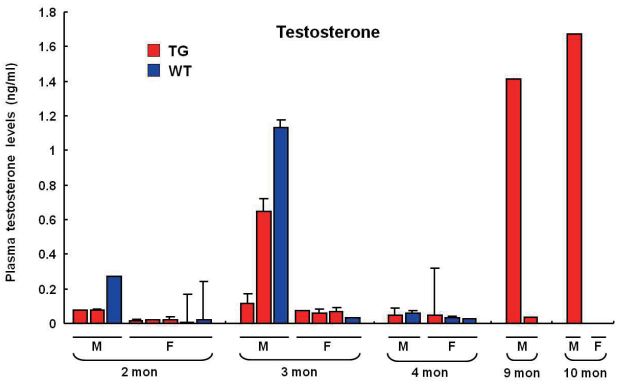


Fig. 1-13. Testosterone level of GalT KO pigs

The LH level was not significantly different in both GalT KO female pigs and normal pigs at 2 month after birth. The bottom level was maintained during 3 to 4 months after birth. However, the LH level was increased after sexual maturity in female GalT KO pigs (Fig. 1-14).

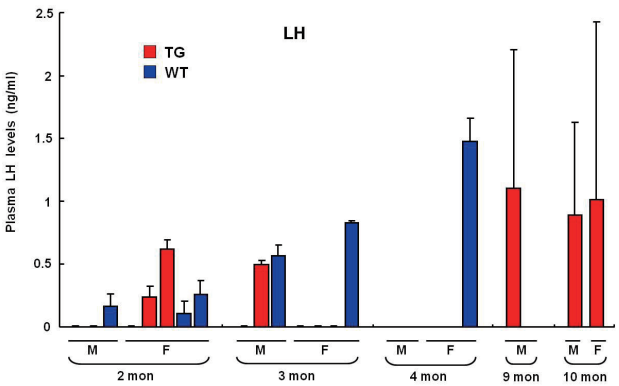


Fig. 1-14. LH level of GalT KO pigs

2-3. Analysis of blood and SLA genotyping in miniature pigs

Blood genotyping : By PCR amplification, all the NIH MHC Inbred miniature pigs showed OO blood genotypes (Fig. 1-15).

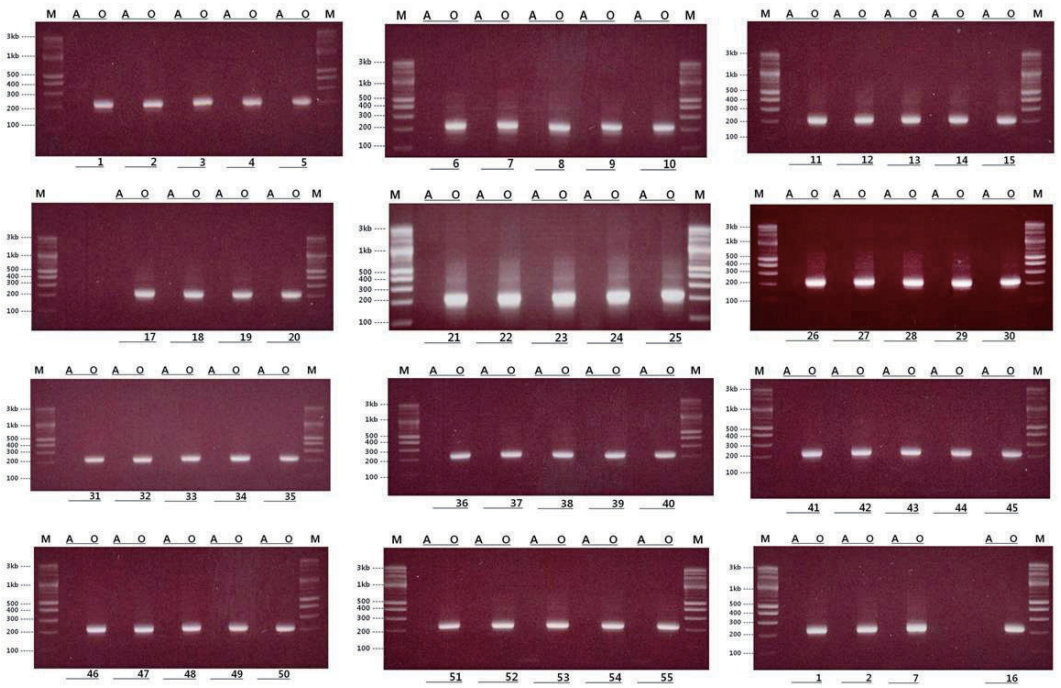


Fig. 1-15. Analysis of blood genotype in NIH MHC Inbred miniature pigs

Analysis of SLA class I and II in NIH MHC Inbred minipigs : Using allele specific primer, SLA class I and II type was analyzed by PCR (Table 1-12). Among 37 NIH MHC Inbred minipigs, 25 minipigs were evaluated to homozygote (68%) and 12 minipigs were determined as heterozygote (32%), respectively.

Table 1-12. Haplotype of SLA class I & II in NIH MHC inbred minipigs

No.	Sex	alive	Class I				Class II				Haplotype
			SLA1	SLA3	SLA2	Haplo-type	DRB1	DQB1	DQA	Haplo-type	
8001-1	F	0	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	02XX	02XX	Lr-0.2/0.2	Lr-2.2/2.2
8002-1	M	0	(02XX 07XX) 04XX	04XX	02XX	Lr-2.0/2.0	02XX	02XX 04XX	02XX	Lr-0.2/0.4	Lr-2.2/2.4
8003-2	M	X	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8004-2	F	X	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8004-3	F	X	(02XX 07XX) 04XX	04XX	02XX	Lr-2.0/2.0	02XX	02XX 04XX	02XX	Lr-0.2/0.4	Lr-2.2/2.4
8005-2	F	0	(02XX 07XX) 06XX	03XX 08XX 04XX	02XX 03XX	Lr-2.0/3.0	02XX 03XX	02XX 03XX	01XX 02XX	Lr-0.2/0.3	Lr-2.2/3.3
8007-6	F	X	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8007-7	F	X	04XX	04XX	04XX	Lr-4.0/4.0	02XX	02XX	02XX	Lr-0.2/0.2	Lr-4.2/4.2
8008-4	M	X	04XX	04XX	04XX	Lr-4.0/4.0	02XX	02XX	02XX	Lr-0.2/0.2	Lr-4.2/4.2
8009-1	F	0	(02XX 07XX) 04XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8010-1	F	0	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	02XX 04XX	02XX	Lr-0.2/0.4	Lr-2.2/2.4
8013-1	F	0	(02XX 07XX) 04XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8013-2	F	0	(02XX 07XX) 04XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8015-1	F	0	04XX	04XX	04XX	Lr-4.0/4.0	02XX	02XX	02XX	Lr-0.2/0.2	Lr-4.2/4.2
8016-3	F	0	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8018-1	F	0	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8019-1	F	0	04XX	04XX	04XX	Lr-4.0/4.0	02XX	02XX	02XX	Lr-0.2/0.2	Lr-4.2/4.2
8022-2	F	0	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	02XX 04XX	02XX	Lr-0.2/0.4	Lr-2.2/2.4

No.	Sex	alive	Class I				Class II				Haplotype
			SLA1	SLA3	SLA2	Haplo-type	DRB1	DQB1	DQA	Haplo-type	
8022-4	F	0	04XX	04XX	04XX	Lr-4.0/4.0	02XX	02XX	02XX	Lr-0.2/0.2	Lr-4.2/4.2
8024-1	F	0	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8024-2	F	X	04XX	04XX	04XX	Lr-4.0/4.0	02XX	02XX	02XX	Lr-0.2/0.2	Lr-4.2/4.2
8025-1	F	0	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	02XX 04XX	02XX	Lr-0.2/0.4	Lr-2.2/2.4
8026-2	F	0	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8026-3	F	X	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8026-6	M	0	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	02XX 04XX	02XX	Lr-0.2/0.4	Lr-2.2/2.4
8027-1	F	0	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8027-3	M	X	02XX 07XX	04XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8028-2	F	X	(02XX 07XX) 04XX	04XX	02XX	Lr-2.0/2.0	02XX	02XX	02XX	Lr-0.2/0.2	Lr-2.2/2.2
8030-2	F	0	02XX 07XX	01XX 03XX 04XX 08XX	02XX	Lr-2.0/2.0	02XX	02XX 04XX	02XX	Lr-0.2/0.4	Lr-2.2/2.4
8030-3	F	0	02XX 07XX	01XX 03XX 04XX 08XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4
8033-1	F	0	02XX 07XX	01XX 03XX 04XX 08XX	02XX	Lr-2.0/2.0	02XX	02XX	02XX	Lr-0.2/0.2	Lr-2.2/2.2
8033-2	F	0	(02XX 07XX) 04XX	01XX 03XX 04XX 08XX	02XX 04XX	Lr-2.0/4.0	02XX	02XX	02XX	Lr-0.2/0.2	Lr-2.2/4.2
8034-1	F	0	(02XX 07XX) 04XX	01XX 03XX 04XX 08XX	02XX 04XX	Lr-2.0/4.0	02XX	02XX 04XX	02XX	Lr-0.2/0.4	Lr-2.2/4.4

No.	Sex	alive	Class I				Class II				Haplotype
			SLA1	SLA3	SLA2	Haplo-type	DRB1	DQB1	DQA	Haplo-type	
8036-1	F	0	02XX 07XX	01XX 03XX 04XX 08XX	02XX	Lr-2.0/2.0	02XX	04XX	02XX	Lr-0.4/0.4	Lr-2.4/2.4

(3) Studies on the increase of implantation efficiency in cloned embryos

3-1. Effect of promoter type on pregnancy rate

The pregnancy rate was 40% (2/5) in non-TG cell, 25% (4/25) in CMV promotor, and 15% (3/20) in Transposase, respectively. The difference was not significant in both promotors (Table 1-13).

Table 1-13. Effect of promotor types on pregnancy

Transgenic methods	No. of surrogates	No. of pregnant surrogates (%)
Non-TG cell	5	2(40%)
CMV ptomotor	20	4(25%)
Transposase	20	3(15%)

3-2. Analysis of implantation rate on recipient's breeds

The pregnancy rate was 28.6% in Landrace, 25% in Landrace (L) & Duroc (D) F1 hybrid, 18.7% in L & Yorkshire (Y) F1 hybrid, and 14.3% in Duroc, respectively. However, no pregnancy was shown in Yorkshire(Table 1-14).

Table 1-14. Effect of recipient type on pregnancy

Breeds	No. of surrogates	No. of pregnant surrogates (%)
Landrace	7	2(28.6%)
Duroc	14	2(14.3%)
Yorkshirer	3	0(0%)
L x Y F1	16	3(18.7%)
L x D F1	4	1(25%)

3-3. Measurement of fetal growth using ultrasound during pregnancy

The body length of fetus was 14.4cm at the end of the pregnancy. And the diameter of fetal heart was 3.64cm at 15 weeks of pregnancy (Table 1-15).

Table 1-15. Analysis of fetal growth during pregnant period(㎝)

Week	4	5	6	7	8	9	10	11	12	13	14	15
G-sec	3.17	5.54	5.08	6.50	7.68	9.32	9.80	10.90	-	-	-	-
Fetus	1.80	2.13	3.73	5.35	5.97	7.22	8.19	9.09	12.43	13.19	13.97	14.40
Heart	-	-	-	1.10	1.40	1.65	1.77	1.95	2.47	2.83	3.06	3.64

3-4. Measurement of fetal body length using ultrasound

Using ultrasound, the body length without head of fetus was measured at 15.9±4㎝ immediately before parturition. After Caesarean section, the body length of litters was 16± 2cm (Table 1-16). It may be helpful to decide the timing of C-sec.

Table 1-16. Measurement of body length of fetal or litters

	Part	Length(cm)
Ultrasound	Body	15.9±4
Real	Head	9±1
	Body	16±2
	CRL	25±2

3-5. Measurement of fetal organ size during pregnancy

Comparison of fetal organ size in both normal and cloned miniature pigs was shown in Figure 1-16. Transgenic cloned pigs died within several days after birth as a result of developmental defects. We measured fetal organ sizes of both normal and minipig using ultrasound. The development of fetal organ in minipig was usually delayed for 2~3 days compared to that of normal pig.

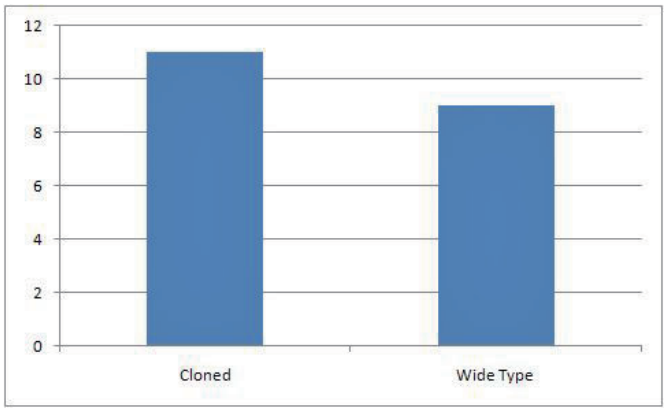


Fig. 1-16. Comparison of fetal organ size in both normal and cloned minipig

3-6. Decision of appropriate time to caesarean section (c-section)

To decide c-section point, we examined fetal count, body weight, and developmental status (Table 1-17). The important factors in successful c-section were the size of fetus (around 14cm) and the day (116~117 days after embryo transfer).

Table 1-17. Factors on appropriate Caesarean section time

Factors	Days after embryo trasnfer			
	113	117	124	125
No. of surrogate	3	2	1	1
Type of surrogate	L x Y	L x Y	L x Y	L x Y
Delivery	C-sec	C-sec	C-sec	Parturition
Mean No. of fetus	1	2	2	4
Body weight	430	694	478	868
No. of stillbirth	1	2	2	1
Health status	Poor	Good	-	Good
Amniotic fluid status	Good	Leak	Dry	Leak

(4) Expression pattern and function of genes associated with pregnancy

4-1. Effects of epigenetic regulation on cloned embryo development and pregnant efficiency

Expression of antioxidant genes such as catalase, Gpx1, and Mgst1 were low in level day-7 embryo, however, the genes were expressed highly in day-14 and -25 embryos especially in conceptus, endometrium, and extra-embryonic tissue (Fig. 1-17).

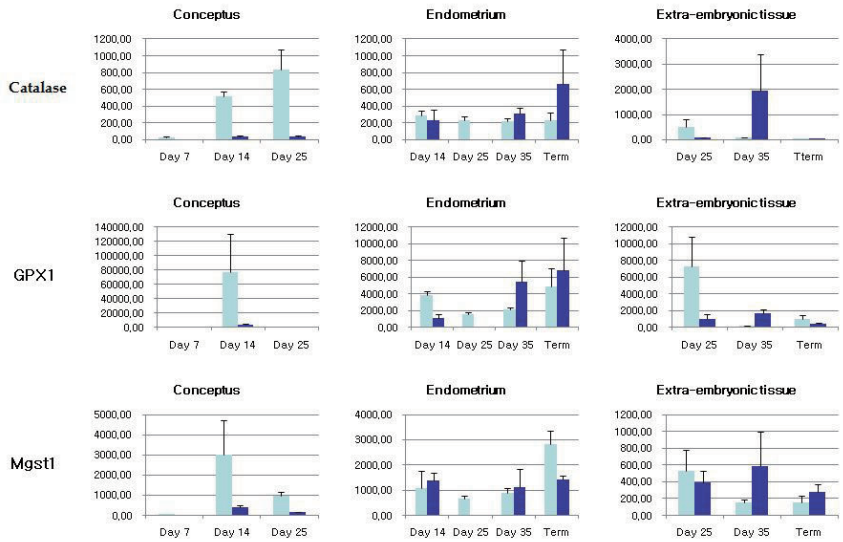


Fig. 1-17. Expression of antioxidant genes reproductive tissues

3-2. Effect of histone deacethylase inhibitor treatment on cloned embryo development

The blastocyst development was affected by the treatment of scriptaid (33.3%) and valproic acid (42.9%), respectively. However, control group was 17.6% of blastocyst development (Table 1-18).

Table 1-18. Effects of different supplementations on cloned embryo development

Treatment	No. of embryos developed to		
	Cultured	= 2-cell	Blastocyst(%*)
Control	155	85	15(17.6)
Scriptaid (500nM)	220	120	40(33.3)
Valproic acid (3mM)	72	35	15(42.9)

* blastocysts / cleaved embryos

Effects of different supplementations on apoptosis in cloned blastocysts were shown in Table 1-19. The difference was not statistically significant in total cell number in both control and HDAC group, however, apoptosis in cloned blastocysts was significantly lower in Scriptaid (15.2%) and Valproic acid (16.5%) treatment groups compared to that of control (22.1%) ($p<0.05$).

Table 1-19. Effects of different supplementations on apoptosis in cloned blastocysts

Treatment	No. of cells	
	Total	Apoptotic(%)
Control	39.2±3.3	22.1±1.7a
Scriptaid (500nM)	37.7±2.6	15.2±1.0b
Valproic acid (3mM)	42.5±3.1	16.5±1.5ab

Different superscripts were statistically significant ($p<0.05$).

4-3. Effects of histone deacethylase inhibitor on histone H4 acetylation in cloned embryos

To induce epigenetic modification of cloned embryos, Scriptaid or valproic acid were treated. After 48 hr of HDAC treatment, the embryos were performed to evaluate histone H4 acetylation using immuno- cytochemistry and confocal microscopy (Fig. 1-18). The intensity of acetyl H3K9 antibody was stronger in Scriptaid and Valproic acid than that of control.

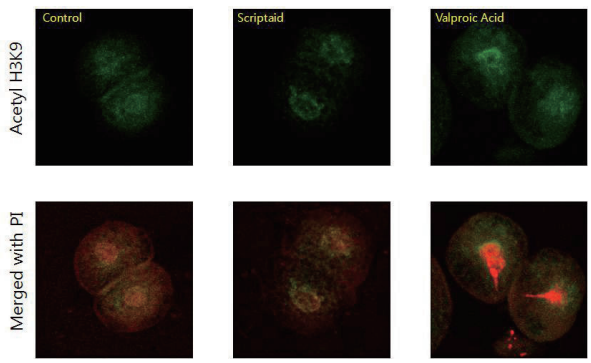


Fig. 1-18. Effects of Scriptaid and Valproic acid on histone H4 acetylation in cloned embryos