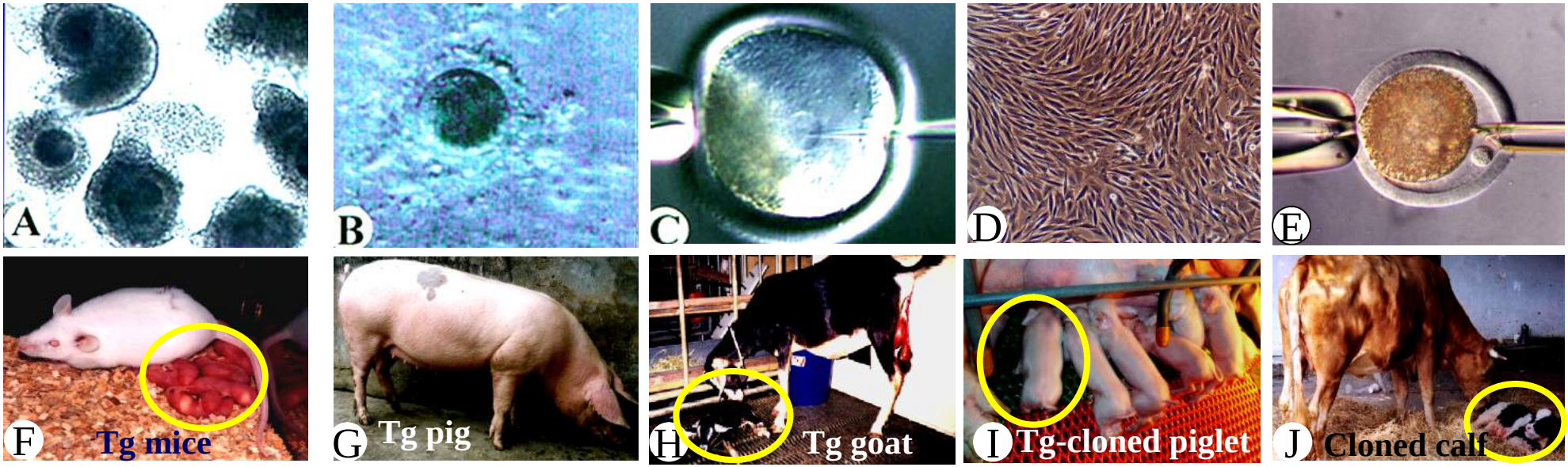
Recent Advancement of Researches Related to Generation of Transgenic and Cloned Animals



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What are transgenic animals?

- Transgenic animals might be defined as that animals whose genetic make-up has been modified by addition or deletion of a specific DNA sequence.
- An animal resulting after the transgenic manipulation is termed as a founder animal.
- The altered chromosomal DNA appeared to be germ-line transmitted into future generations.

Why transgenic Animals?

To make the Tg farm animals fitting to following purposes:

- **Improving the efficiency and quality of animal production**
  - Production performance (meat, milk wool, uterilization of nutrients, etc.)
  - Reproduction performance(pregnancy rate, ratio of new-born to surviving animals)
  - Health (resistance and susceptibility to disease, immune response)
  - Quality of animal products (taste, biological value, etc.)
  - Processing of animal products (storage, expiring, structure, etc.)
- **Enzymes**
  - Nutrients (baby food, diet, geriatric diets)
- **Gene farming**
  - Pharmaceuticals for human and veterinary medicine (vaccines, growth factors, blood coagulating factors, antibodies, etc.)
  - Raw materials (proteins for further industrial processing)
- **Animal models and organs**
  - Models of human diseases (high blood pressure, atherosclerosis, cancer, etc.)
  - Organ donors for xenotransplantation

Brem and Muller, 1994

The different systems producing recombinant proteins

	Amount	Extraction	Post-translational modifications
Bacteria	+ + + +	+ +	+
Yeast	+ + + +	+ + +	+ +
Fungi	+ + + +	+ + +	+ +
Transgenic plants	+ + + +	+ + ?	+ +
Baculovirus	+ + + +	+ + +	+ + +
Mammalian cells	+	+ + + +	+ + + +
Transgenic animals	**+ + + +**	**+ + + +**	**+ + + +**

Houdebine (1994)

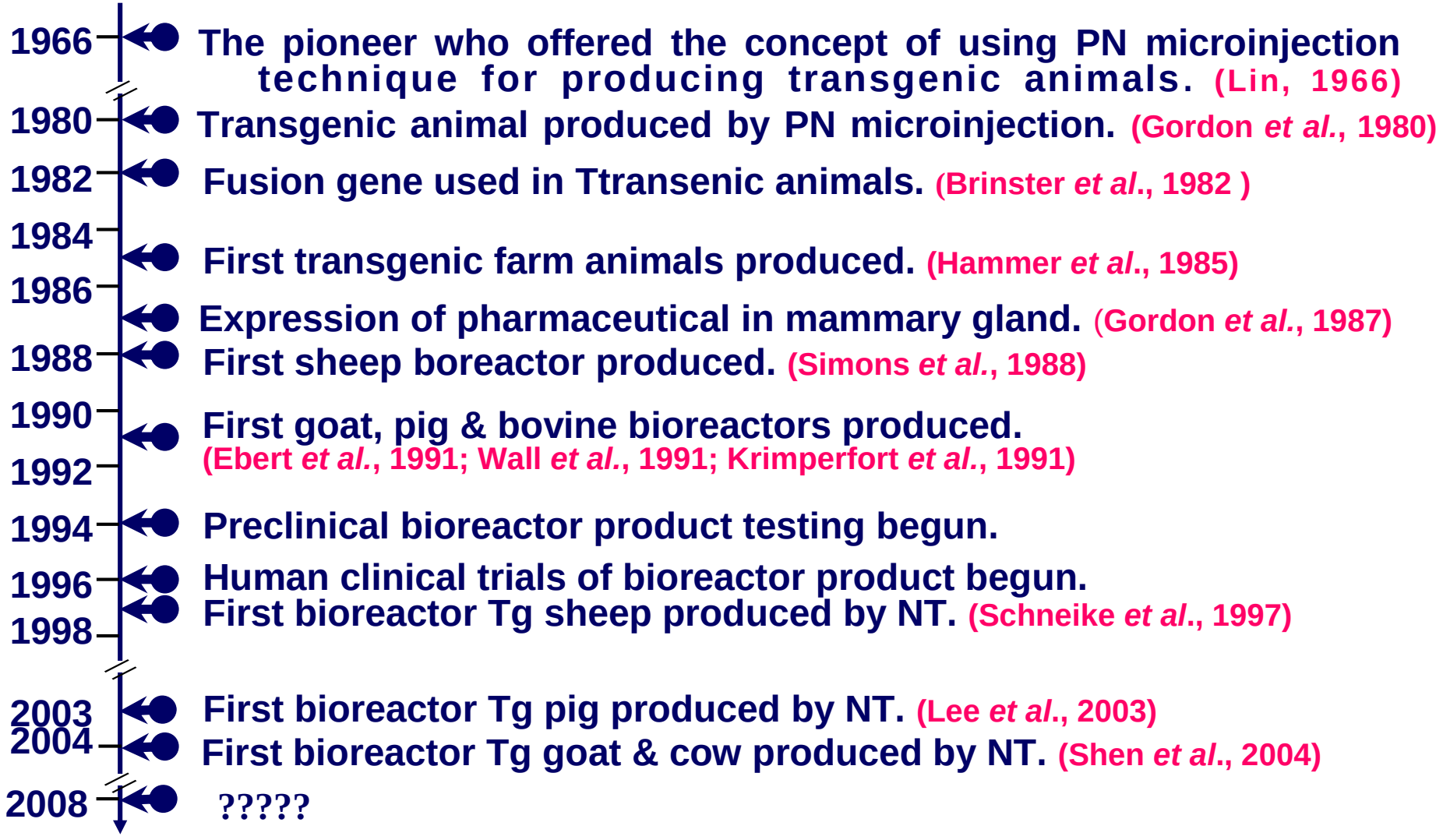
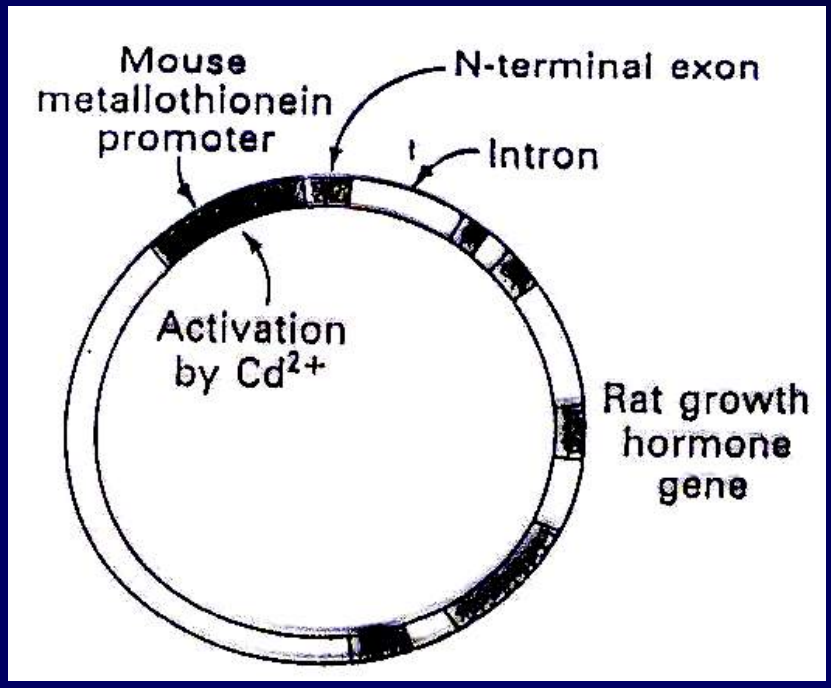


Fig._. Transgenic Animal Milestones

Pioneer studies related to generation giant Tg animals



(Palmiter *et al.*, 1983)

(Pursel *et al*., 1987)

Growth performance of Tg pigs harboring the *MT-bGH* gene

Group of animal tested	Ave. daily gain (gm)	Ave. feed efficiency (kg feed/ kg B.W.)	Remarks
G0			
Control	743 ± 36	3.12 ± 0.15	1. B.W.=30~60 Kg
Tg pigs	690 ± 65	2.62 ± 0.12	2. CP16%
	p = 0.480	p = 0.026	
G2			
Control	760 ± 24	2.99 ± 0.12	1. B.W.=30~90 Kg
Tg pigs	874 ± 30	2.46 ± 0.162	2. CP18% + 0.25% Lysin
	p = 0.016	p = 0.026	
G3			
Control	867 ± 21		1. B.W.=30~90 Kg
Tg pigs	933 ± 31	ND	2. CP18% + 0.25% Lysin
	p = 0.098		
G4			
Control	869 ± 44		1. B.W.=30~90 Kg
Tg pigs	988 ± 62	ND	2. CP18% + 0.25% Lysin
	p = 0.015		

Problems associated with Tg animals expressed rGH, hGH, bGH, or pGH gene(s)

1. Gastric ulceration (30%)
2. Severe synovitis (over 90%)
3. Pereicaridtis & endocarditis
4. Cardiomegaly
5. Nephritis
6. Pneumonia (over 80%)
7. Gilts were anoestrus &boars no libido → Infertile
8. Hyperglycemia (Glucoseuria)
9. High motality →
   - 6% Still born,
   - 20% Die before weaning,
   - 30% Do not survive to market.
10. Decrease appetite & feed consumption.

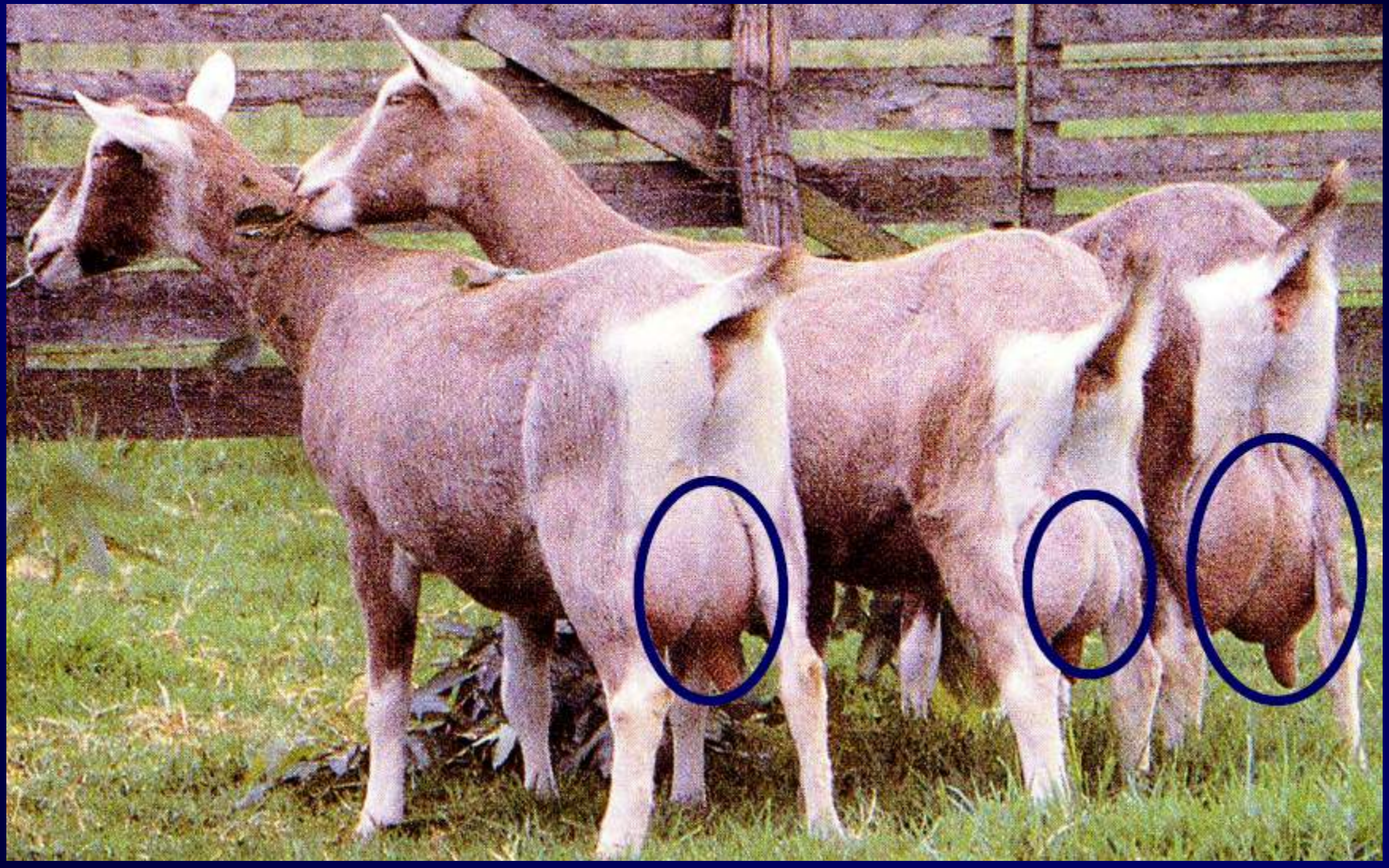
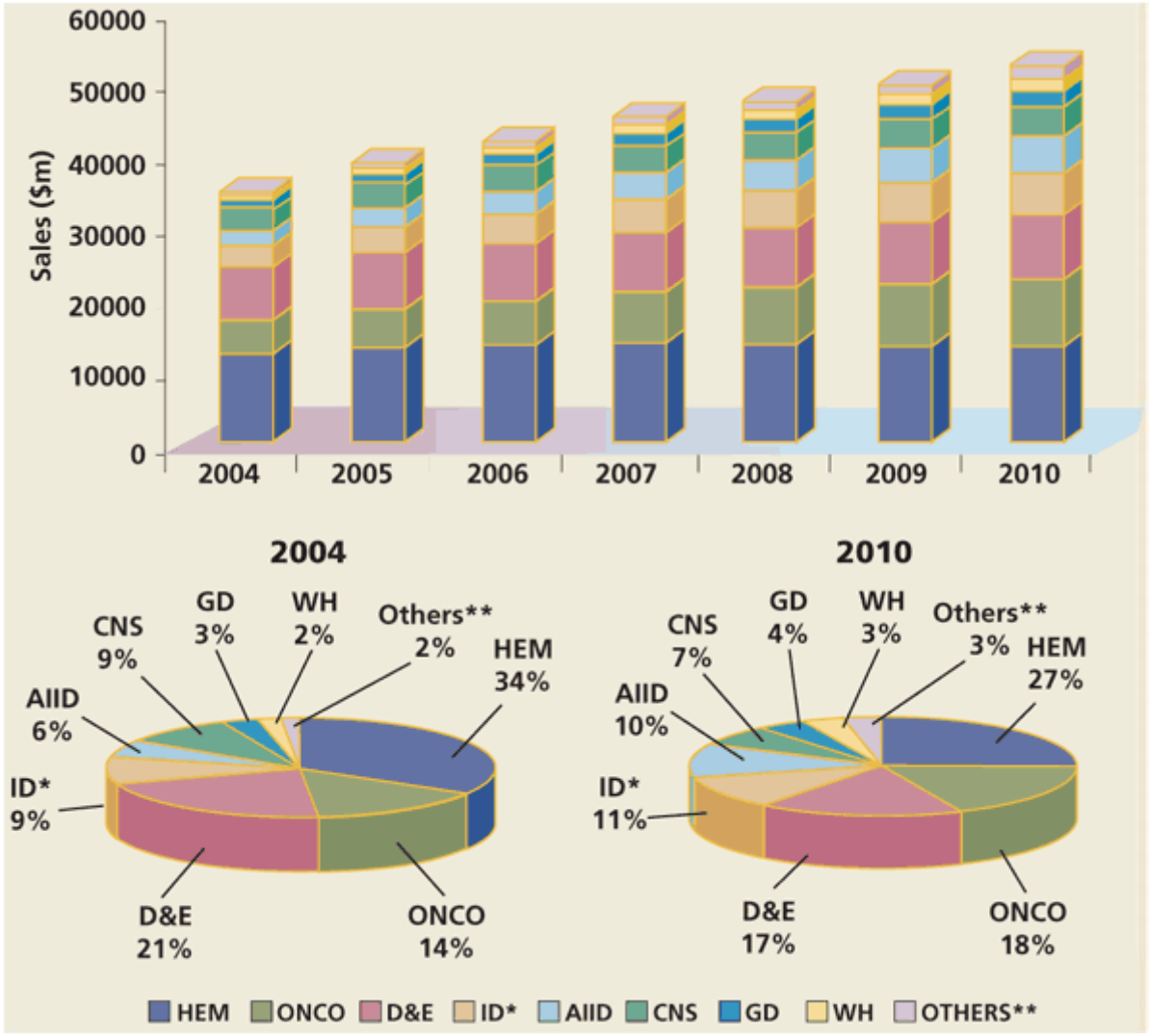


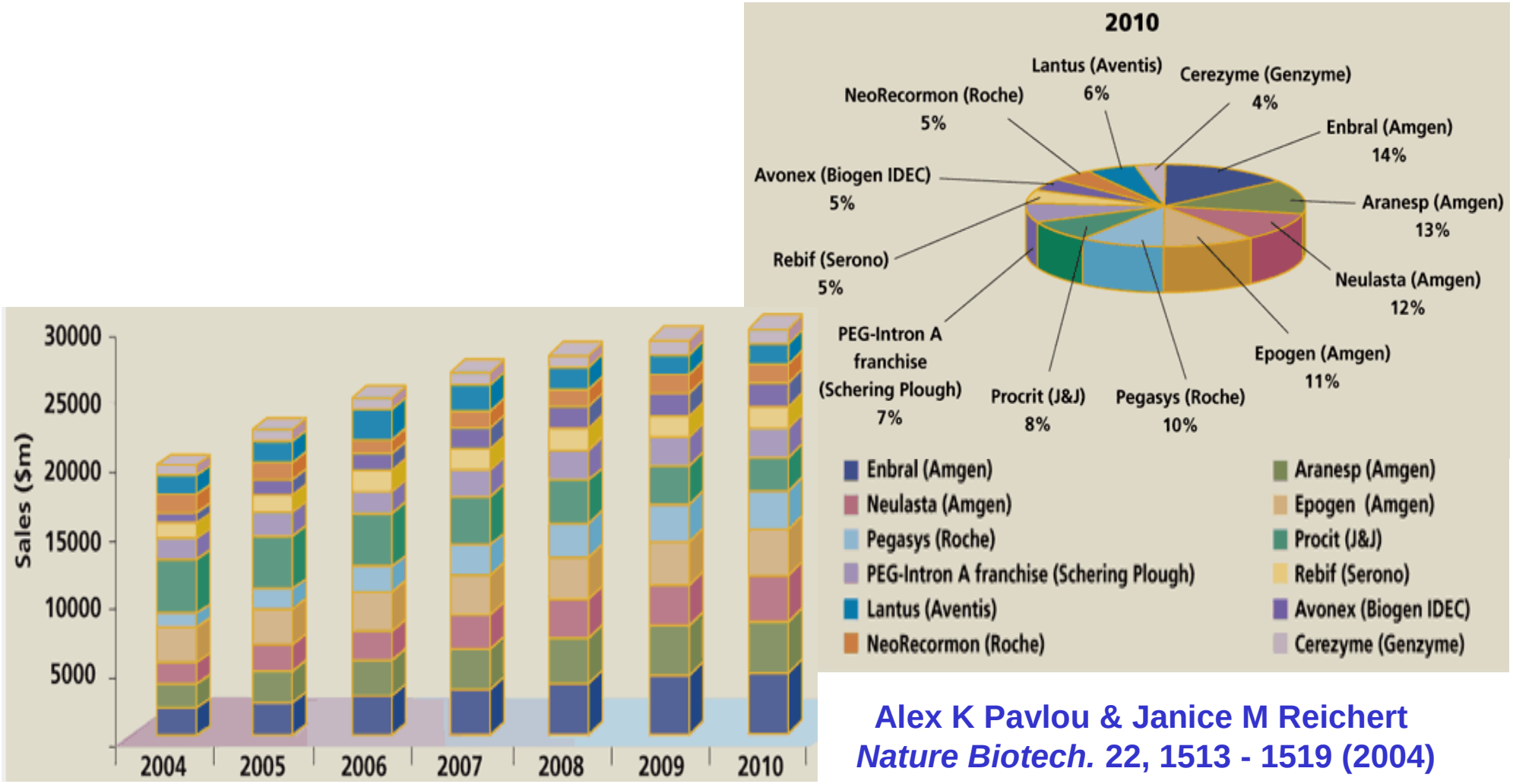
Fig. _. To use the mammary glands as a bioreactor



AIID: Arthritis, inflammation and immune disorders;
CNS: Central neural system; D&E, diabetes and endocrinology;
GD: Genetic disorders;
HEM: Hematology;
ID*: Infectious diseases including HIV;
ONCO: Oncology;
Others:** Respiratory plus cardiovascular;
WH: Women's health.

Alex K Pavlou & Janice M Reichert
Nature Biotech. 22, 1513 - 1519 (2004)

Fig._. Market growth from 2004 to 2010.



Alex K Pavlou & Janice M Reichert
Nature Biotech. 22, 1513 - 1519 (2004)

Fig._. Distribution of market subsegments generated by the industry's leading recombinant protein brands within the 57% total market share in 2010.

Milk production capacity of various species of animals

Item	Transgenic animal species						
	Mice	Rat	Rabbit	Pig	Sheep	Goat	Cattle
Length of lactation, day	21	21	35	28	250	250	305
Milk protein concentration, %	1.5	8.9	14	4.8	5.5	2.9	3.5
Milk volume per days at peak lactation	1.6 mL	40 mL	262 mL	12.8 L	2.4 L	4.7 L	35 L
Harvestable volume per lactation	9.0 mL	80 mL	1.4 L	260 L	306 L	600 L	9,000 L
Max. transgene protein produced	22 g/L	11 g/L	1 g/L	1 g/L	11(35)^{g}g/L	3 g/L	NDA
Transgene protein production capacity	198 mg	880 mg	1.4 g	260 g	3.4 kg	1.8 kg	?
Transgene protein production efficiency, %	147	12.4	.7	2.1	20.0	10.3	?

Wall, 1996

Estimated annual worldwide requirements and potential value of recombinant proteins from Tg animals

	Pharmaceutical						
Item	F-Ⅷ	FⅨ	GC	Protein C	AT Ⅲ	Fibrin	Albumin
Estimated quantity needed, kg	0.3	4	10	10	21	150	315×10^3
Current cost, US$/gram	2.9×10^6	40,000	100,000	100,000	7,000	1,000	3.56
Annual market, US$ $\times$ 10^6	0.87	160	1,000	100	150	150	1,120

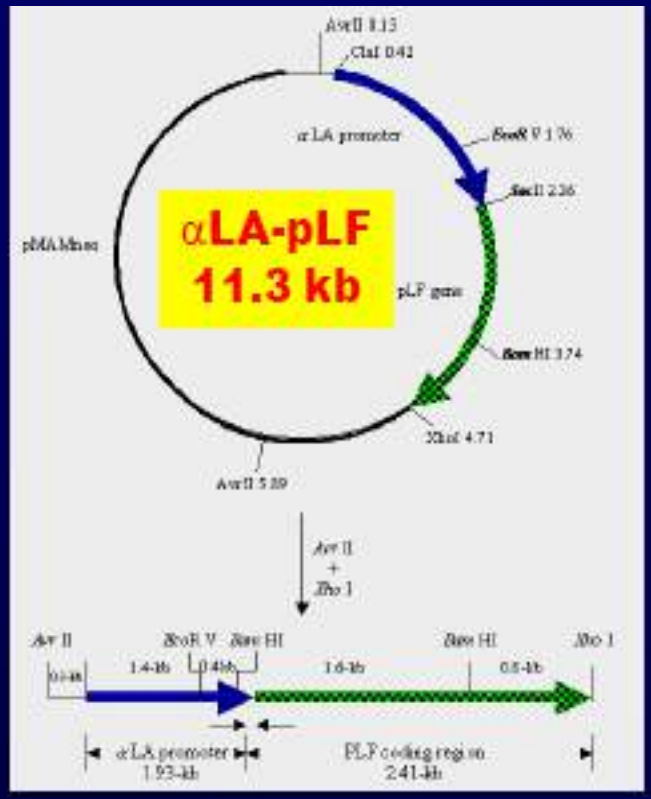
Wall, 1996

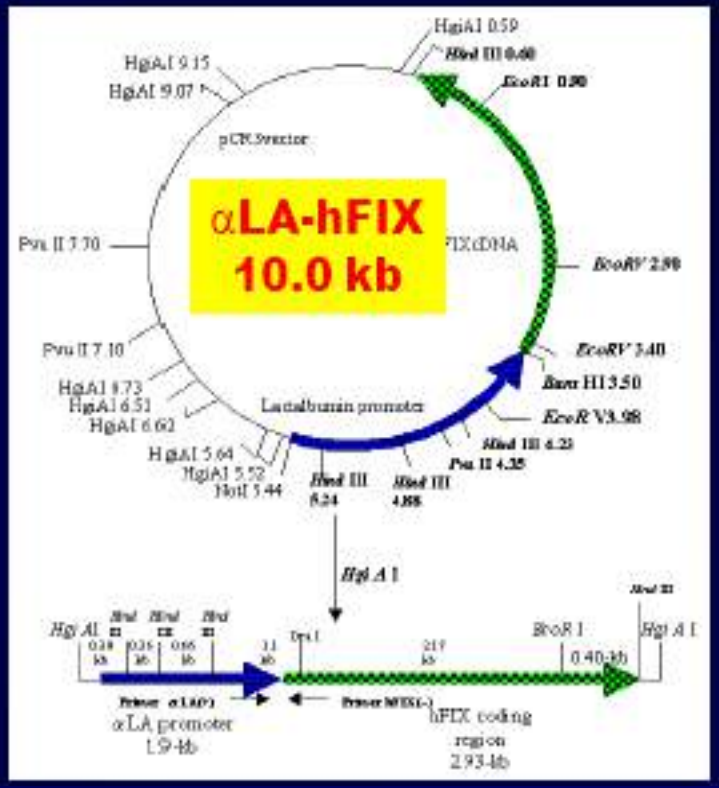
Methods for transfer of foreign gene(s) into farm animals

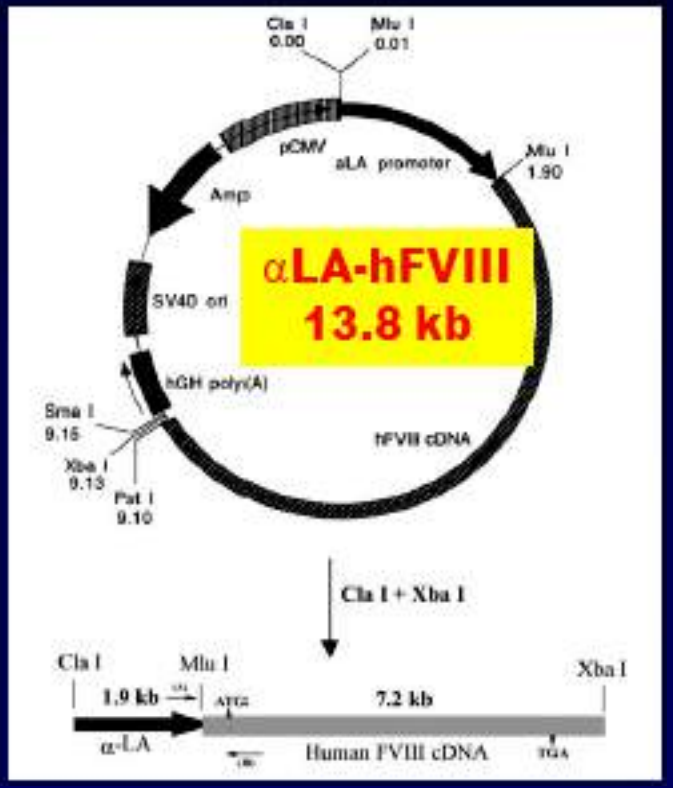
- DNA microinjection
- Embryonic stem cells
- Retroviral vectors
- Lentivirus
- Sperm vector
- Nuclear transfer of somatic cells

Purposes of Generation Transgenic Animals in Taiwan

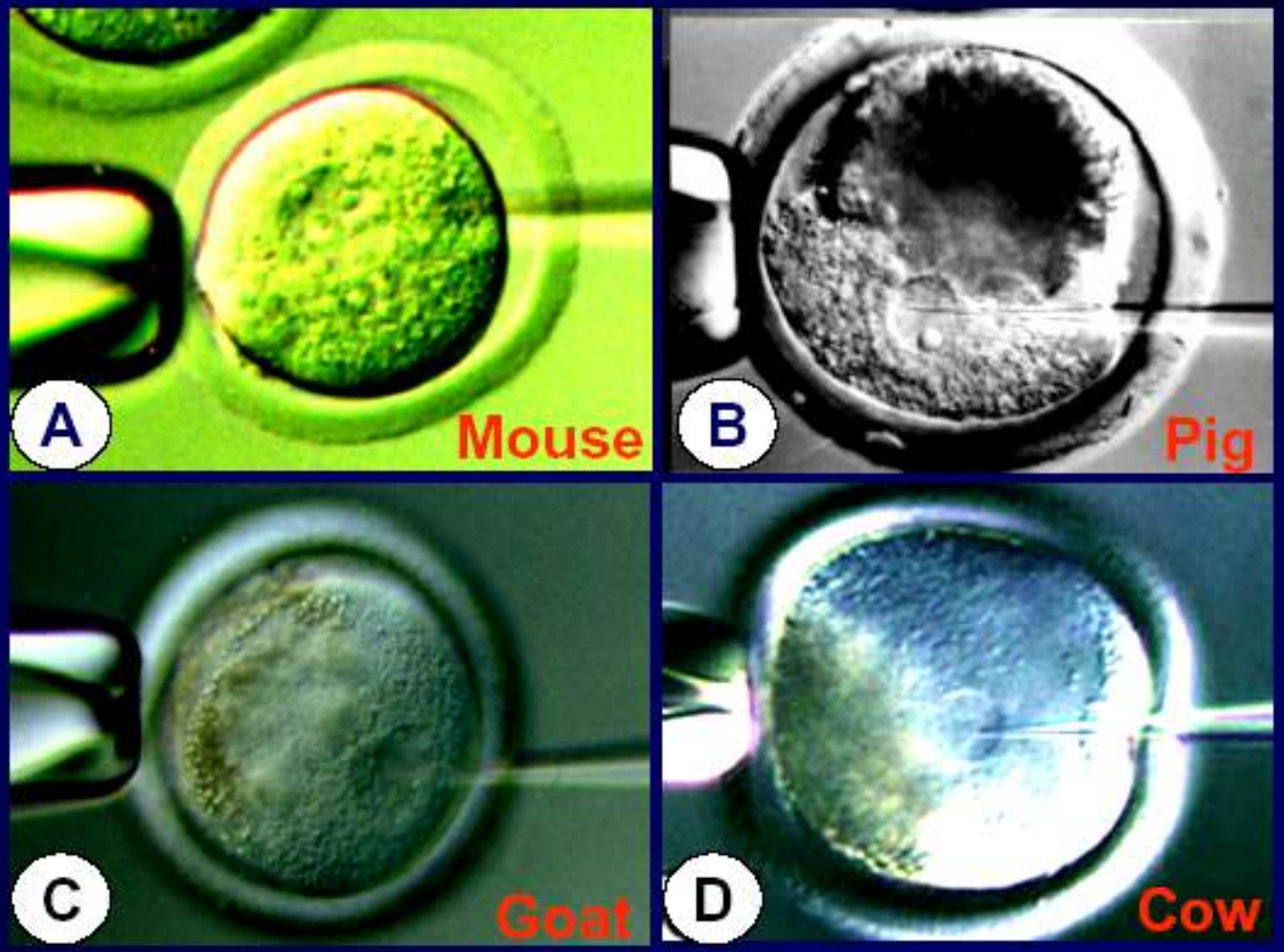
1. To improve resistance of piglets suffering from diarrhea and anemia by increase of lactoferrin concentration contained in the sow milk.
2. To enhance digestion abilities of pig on cellulose and phytate phosphorus contained in the hog diet.
3. To establish molecular pharming for mass production of pharmaceutics including human clotting factors VIII, IX and allergic proteins such as Derp(s).
4. To generate transgenic-cloned animals fitting to purposes of gene therapy and regenerative medicine requested.







Transgene(s) Driven by Tissue-Specific Promoter within the Mammary Gland



Introduce of foreign gene(s) into the pronucleus of newly fertilized eggs

New born Tg mice harboring the α*LA-hFIX* gene

(Wu *et al.*, 1997)

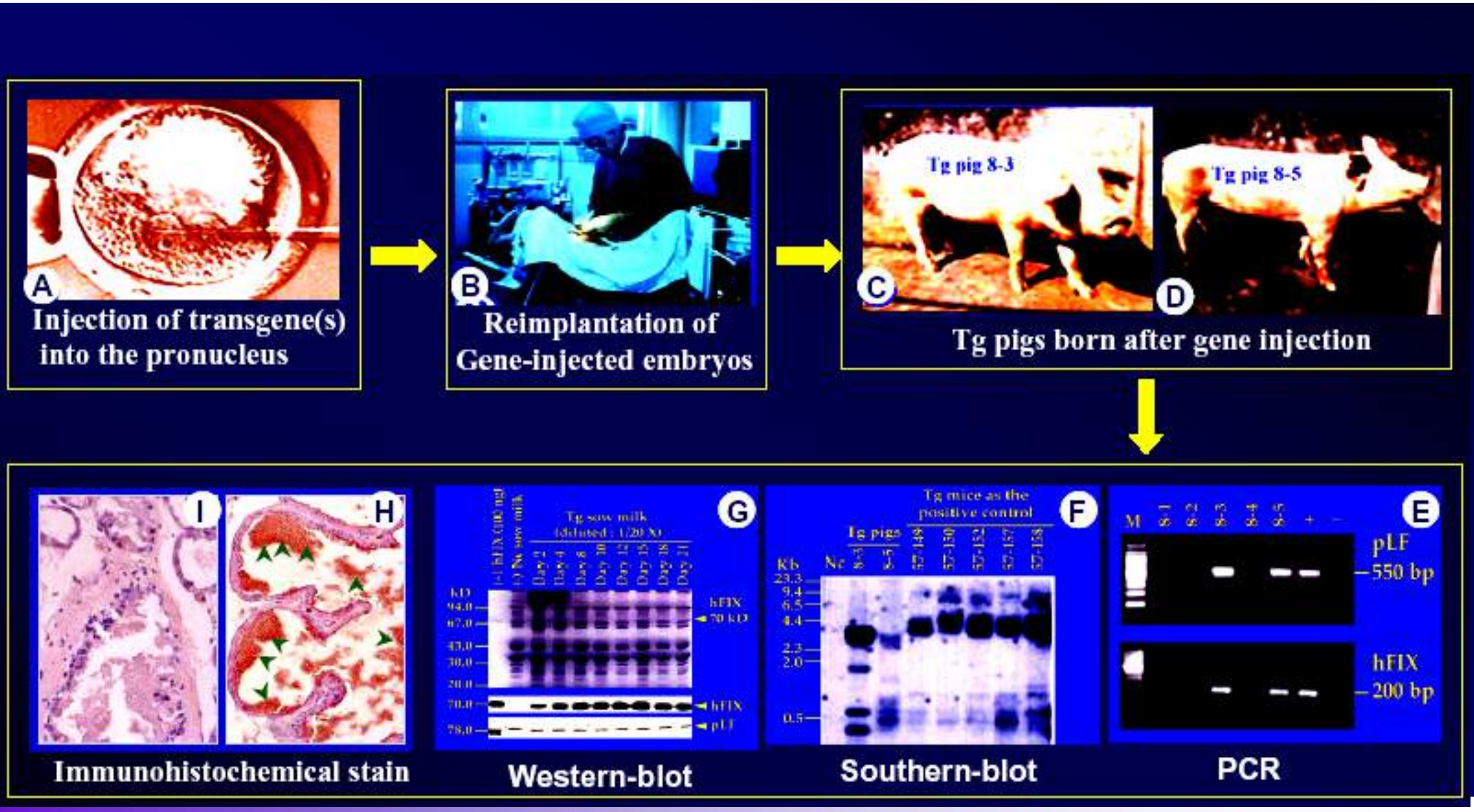


Fig. Transgenic pigs harboring both of *αLA-hFIX* and *αLA-pLF* transgenes

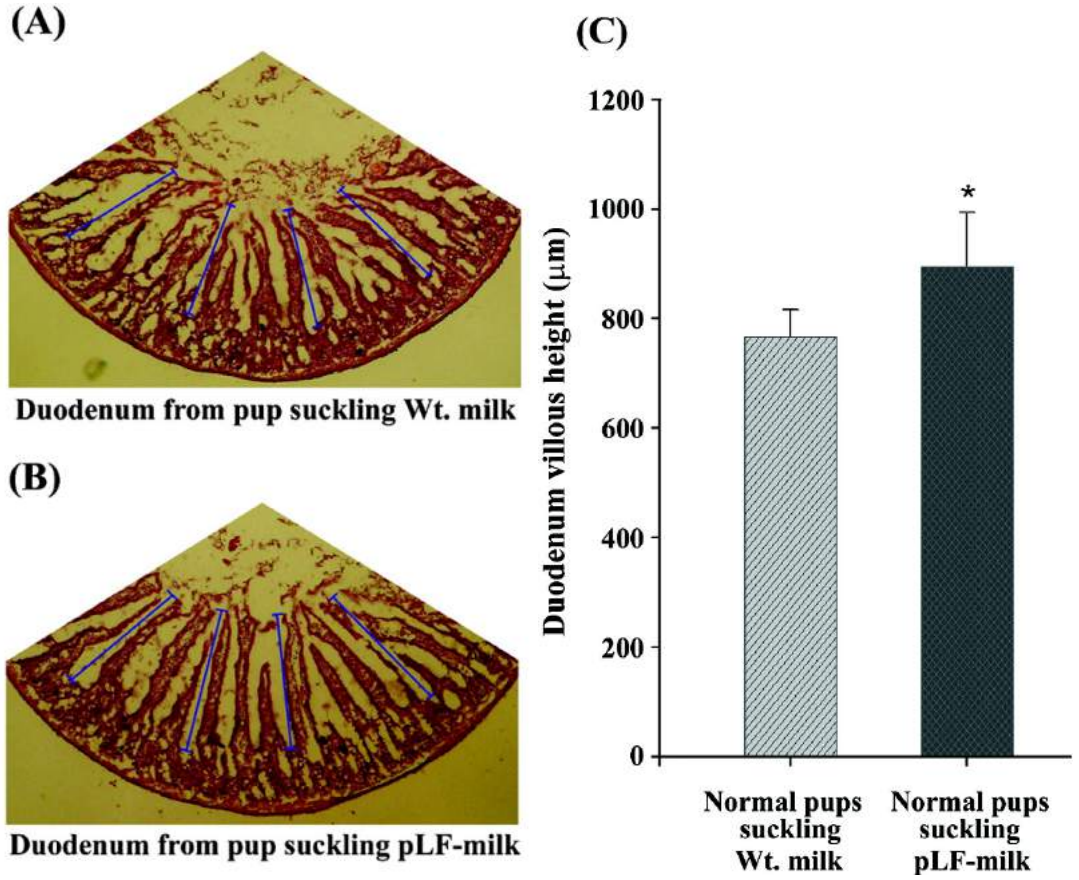


Fig._. Duodenum histology and villous length measurement of mouse pups suckling wild-type (Wt.) normal milks (**A**) compared with those suckling pLF-enriched milks derived from transgenic mice (**B**). For each intestinal section, duodenum villous height was estimated for at least 10 individual villi as shown by the blue lines. (**C**) The statistical data of duodenum villous height (μ m) between control and treated groups were presented as mean SD. *, $p < 0.05$.

Wu *et al*. (2007)

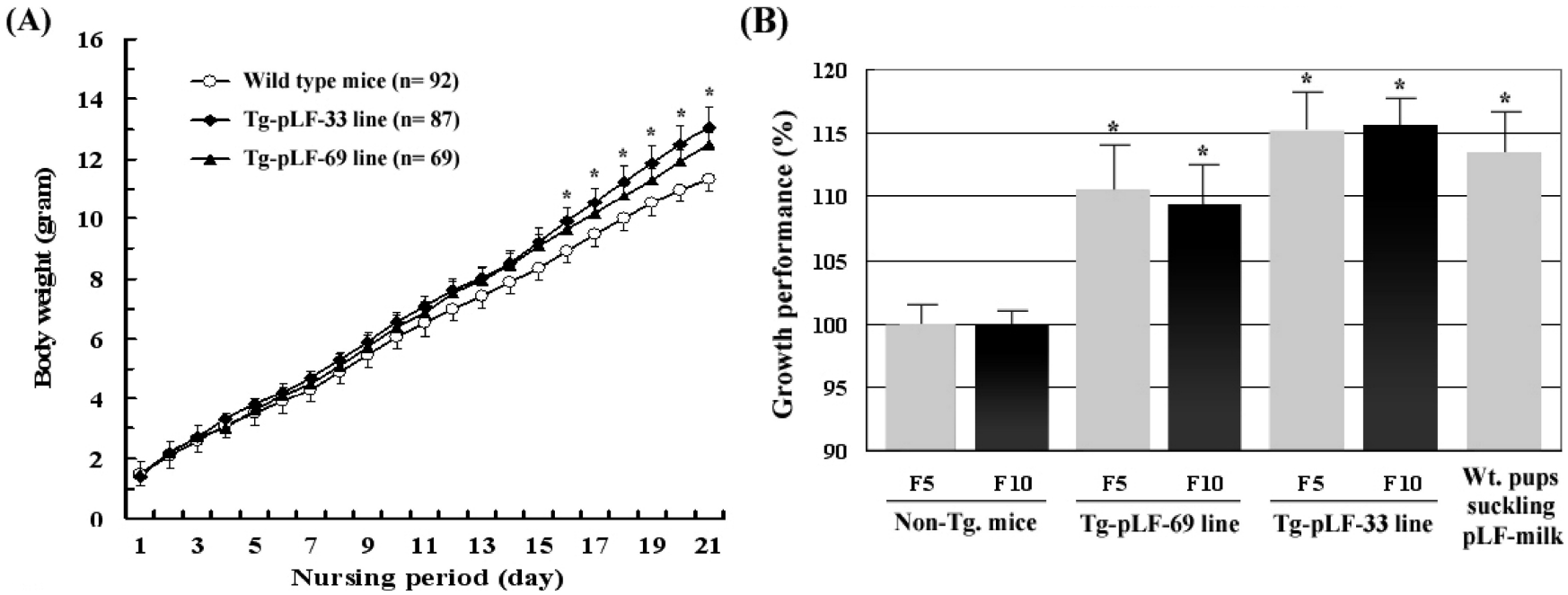
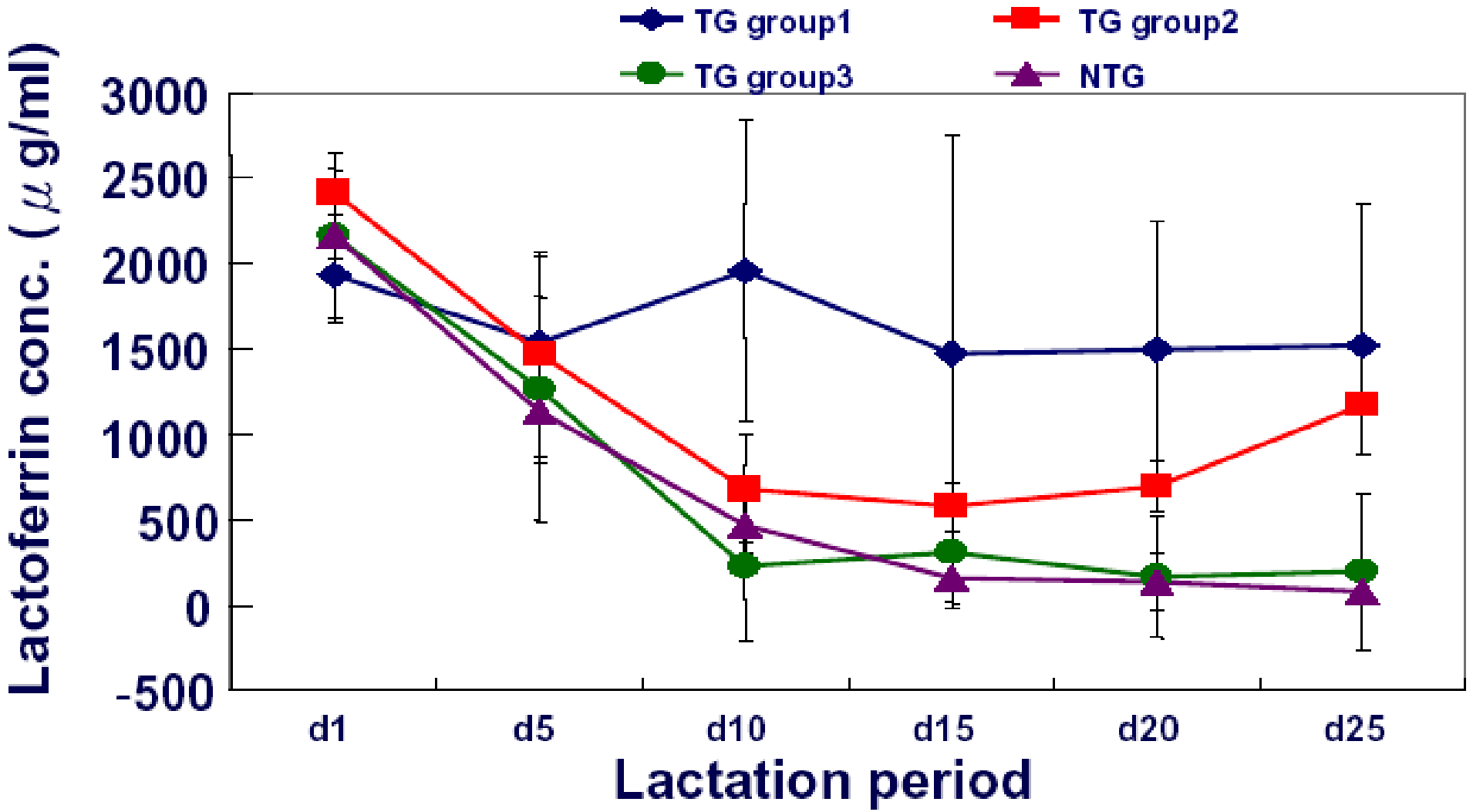


Fig._. Effects of pLF-enriched protein in the milk of transgenic mice on body weight gain during lactation period.

(A) Growth curve of pups during the lactation period among three different groups, one control group of wild-type mice and two test groups of transgenic mice.

(B) Enhanced growth performance of preweaning mice from different generations (F5 and F10) and different pLF-expressed transgenic mouse lines (Tg-pLF-69 and Tg-pLF-33) compared with the non-transgenic (Non-Tg) mouse lines. Wild-type mouse (Wt.) pups (*n* = 29) feeding with the milks from the transgenic mice (Tg-pLF-33 line) were also applied to evaluate their growth performance.

Wu *et al*. (2007)



Changes of lactoferrin concentrations contained in milk of Tg sow's during the lactation period

(Wu *et al.*, 1998)

Reproductive performance of Tg sows harboring the *αLA-pLF* gene

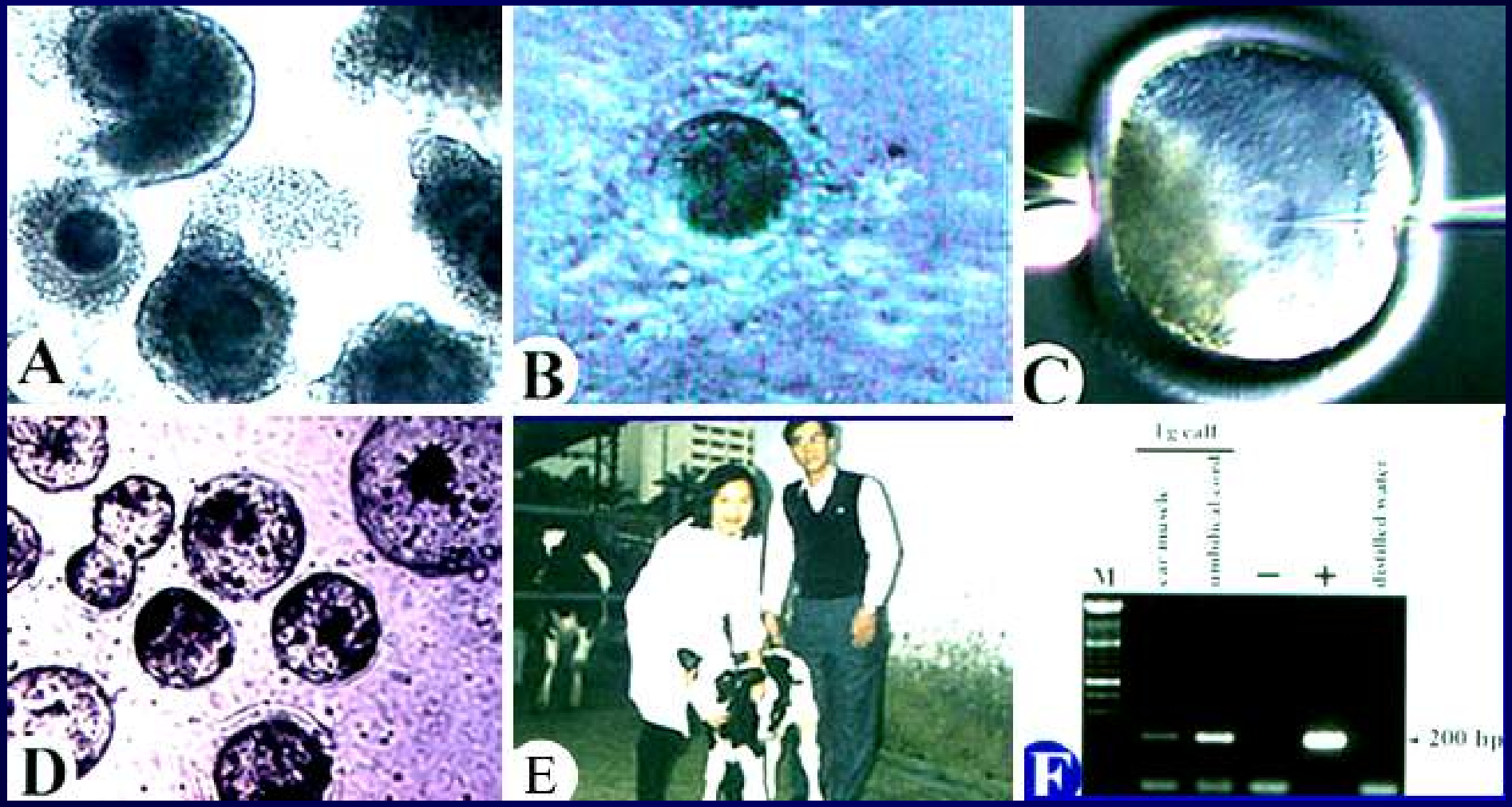
Ear no. of Tg sows	No. of litters farrowed down	Total piglets born alive	% of transgene to be germ-line transmitted	% of piglets survived after weaning
Y064-11	4	35	11.4	100
Y078-11	4	33	63.6	87.9
Y076-12	4	40	55.0	100
Y111-14	4	41	48.8	100
Y111-16	2	12	66.7	100
Y064-10	3	29	55.2	100
Y090-15	4	41	68.3	100
Y144-11	3	28	60.7	100
Overall or average	28	269/28 9.6/litter	136/269 50.6	265/269 98.5

(Wu *et al.*, 1999)

Concentration and clotting activity of hFⅨ contained in the milk of transgenic mice and sows

Source of rhFⅨ	n	Conc. of rhFIX in milk (μg/ml, mean ± SD)	Clotting activity (%, mean ± SD)
Tg mice milk No.61)	4	45.64 ±16.32	9.15 ± 0.21
Control mice milk	3	0	0
Tg sow milk(No.8-3)	16	408.34 ± 55.0	14.18 ± 2.45
Control sow milk	3	0	0

(Wu *et al.*, 1999)



A Tg calf harbouring the *a-LAhFIX* gene born after transfer of cow embryos generated by techniques of IVM, IVF, gene injection, and subsequently cultured *in vitro*

(Sung *et al*., 1999)

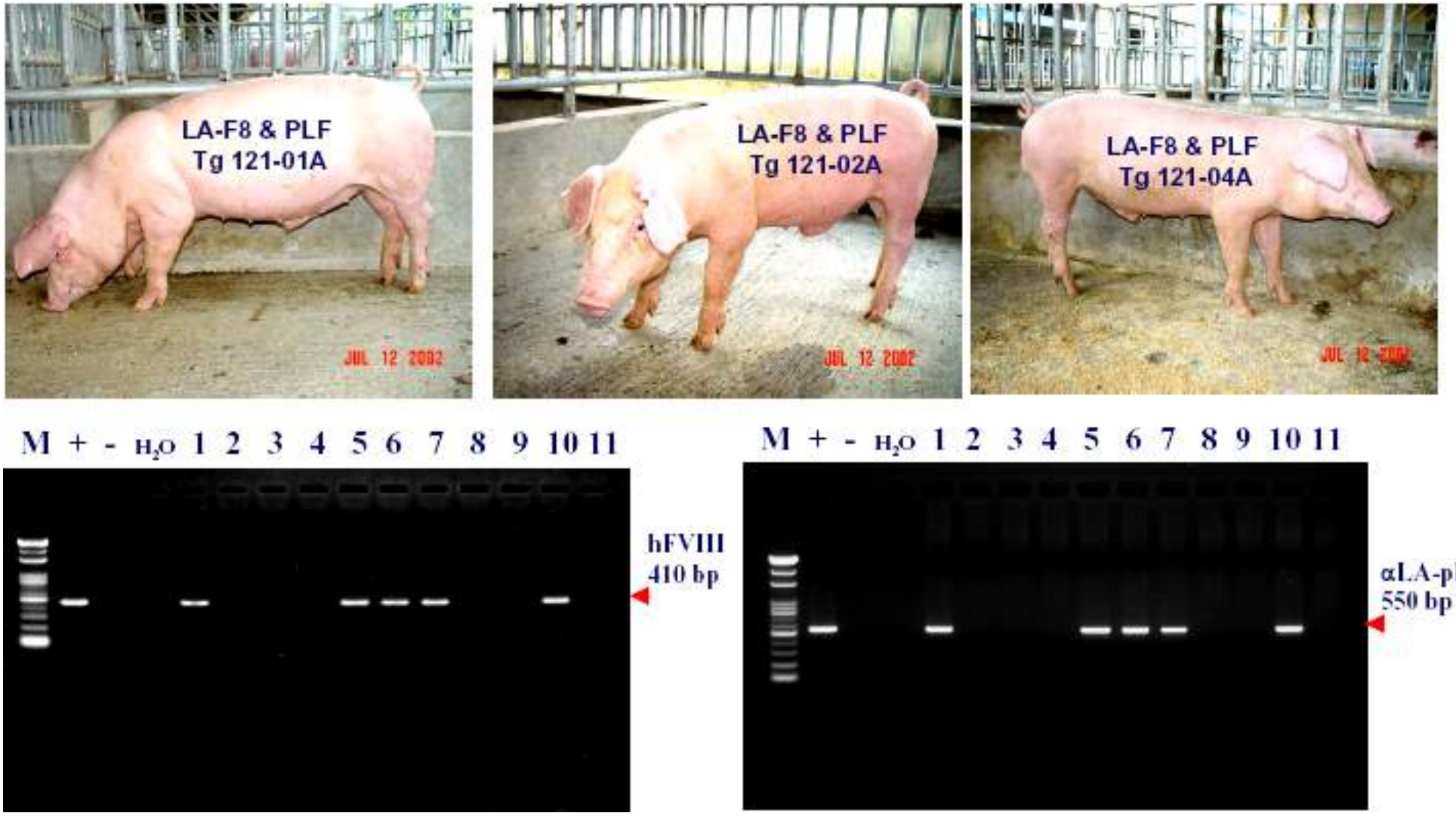
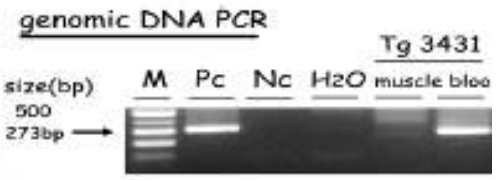
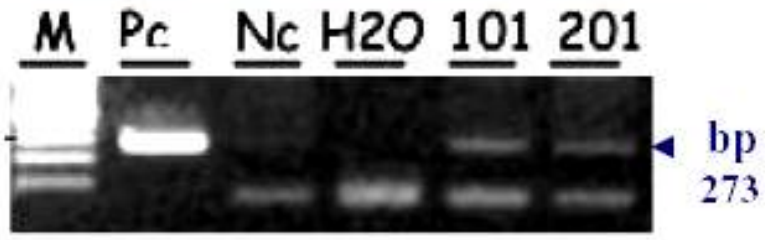
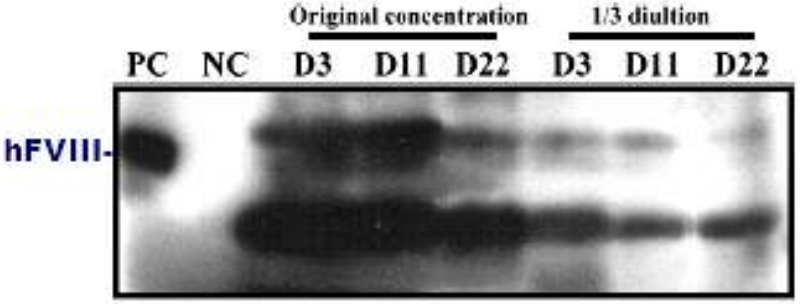


Fig. Transgenic pigs harboring both of *αLA-pLF* and *αLA-hFVIII* transgenes

(Wu *et al.*, 2003)







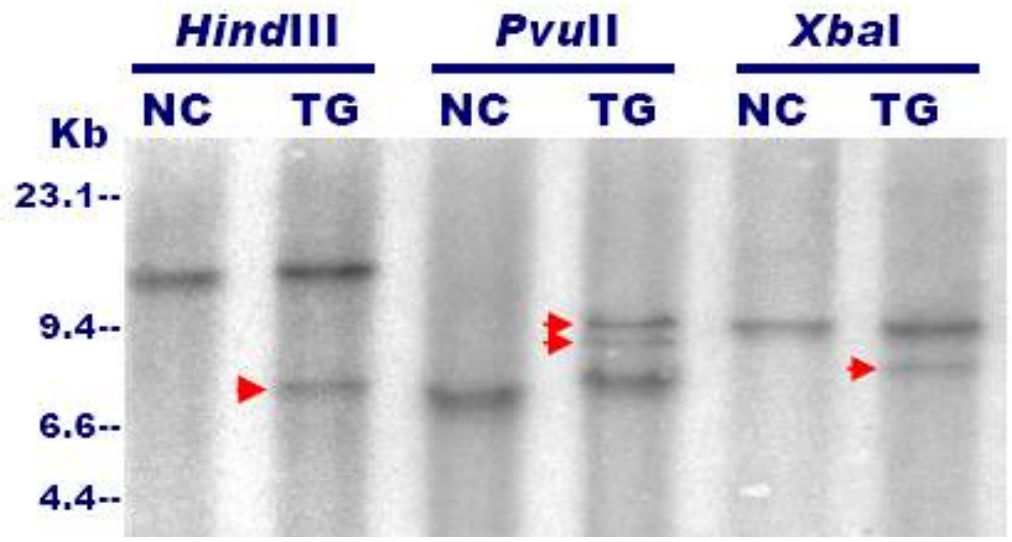


Fig. Tg-3431 dairy goat harboring *αLACN –FVIII AC*

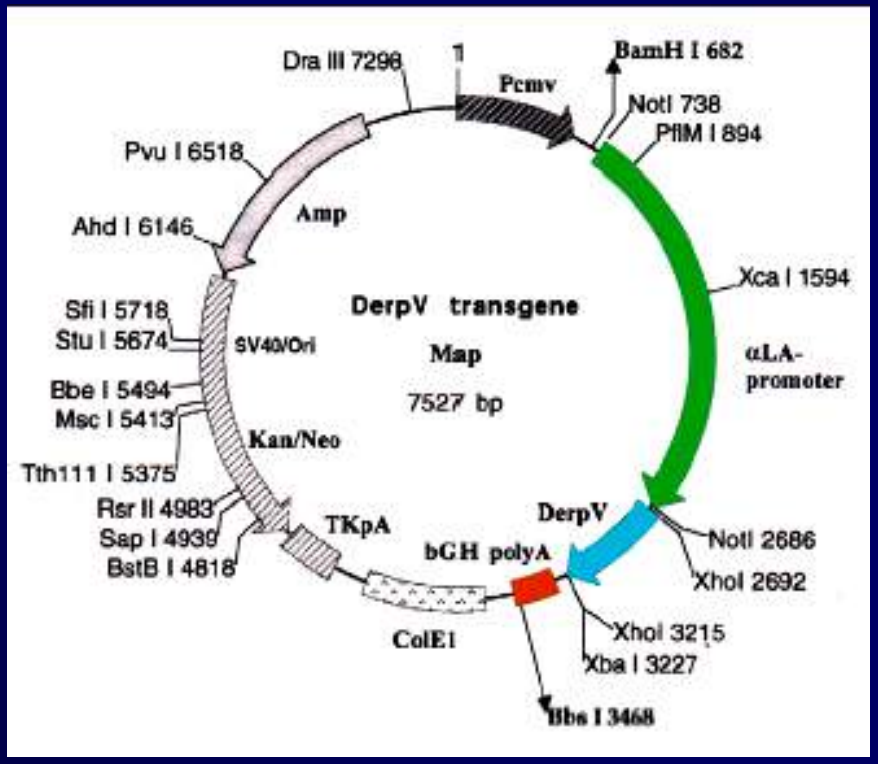
(Lin *et al.*, 2002)

Recombinant hFVIII concentration and clotting activity in the transgenic milk

Source of hFVIII	n	Conc. of hFVIII in milk (μg/ml, Mean ± SD)	Clotting activity assay (U*/ml, Mean ± SD)
Tg milk (No. 15)	1	19.87 ± 0.96	3.60 ± 0.59
Tg milk (No. 25)	3	15.08 ± 4.84	1.56 ± 0.24
Tg milk (No. 36)	4	40.84 ± 4.91	9.46 ± 2.04
Control milk	4	0	0

* One unit of rFVIII was defined as equivalent to the amount of human FVIII normally present in 1 ml of plasma, approximately 200 ng.
Results presented are the average of two independent assays.

Chen *et al*. 2003



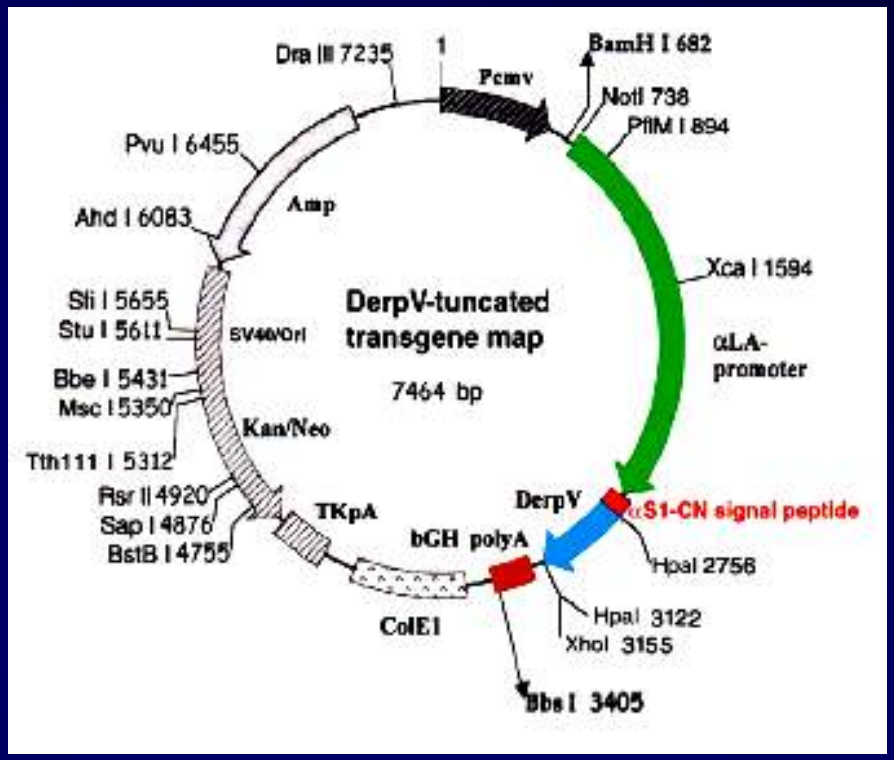
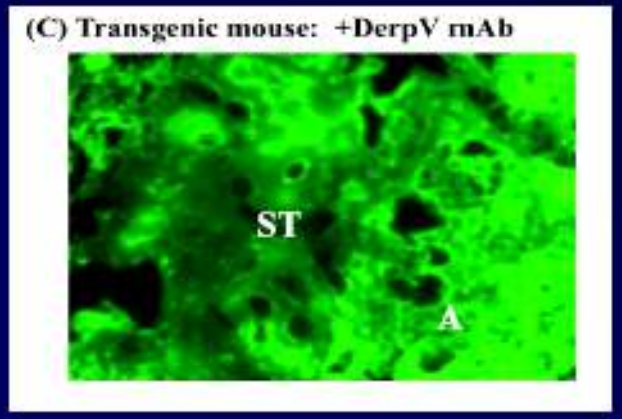
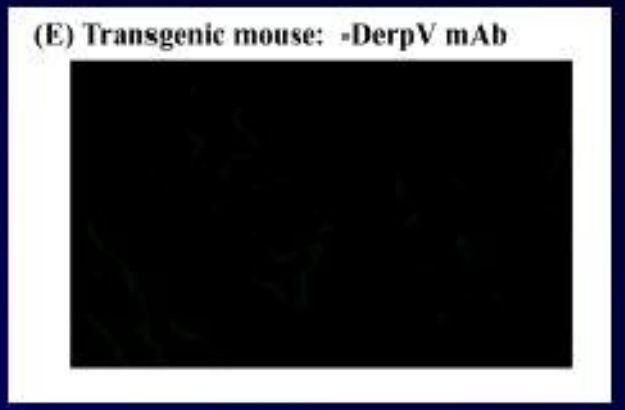


Fig. Plasmid DNA map of constructed *aLA-CN-derp5i* (left) and *aLA-CN-derp5t* (right) transgene(s).

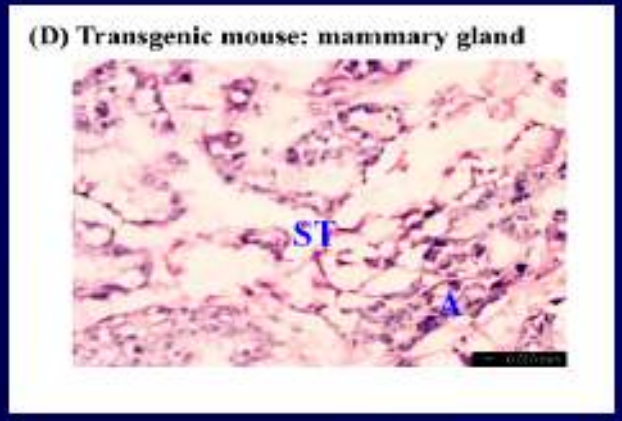
(Chen *et al.*, 1999)

FITC-immunofluorescent stain :





Hematoxylin & Eosin stain :



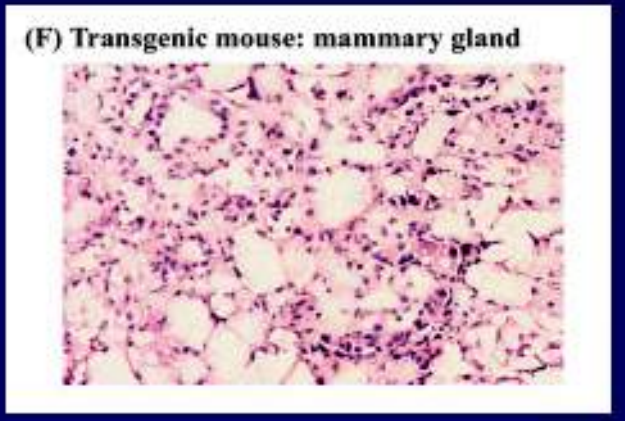


Fig. Immunofluorescent staining assay against to mammary gland tissue of the *aLA-derp5t* Tg mice at day-12 during the lactation period.

(Chen *et al.*, 2001)

Flow chat of animal test studies:

for evaluation the immunosuppressive effect of derp5 on improvement of the lung function

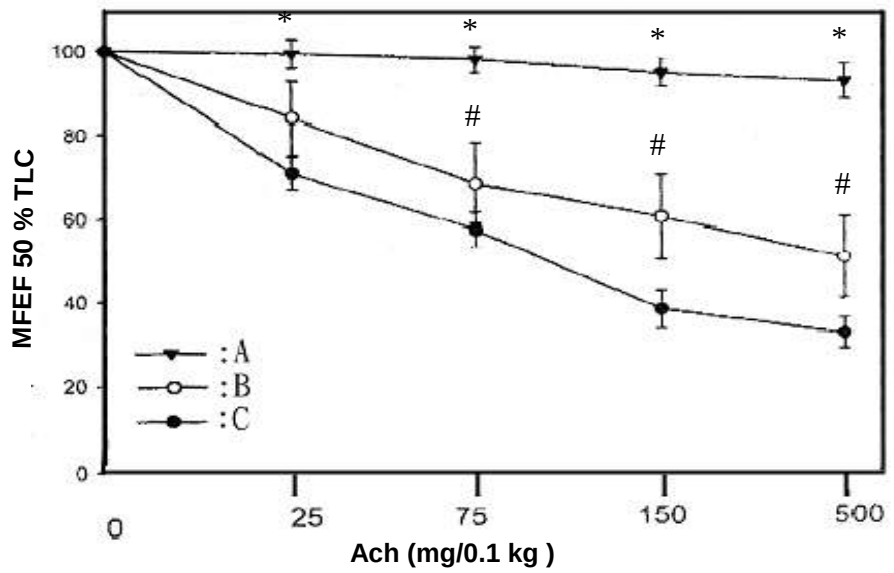
New born mice nursing for 21 days → (1 month later) Sensitization → (14 days) Boost → (21days)

Inhalation challenge → (18 hrs) Measurement of lung function

	Treatment	Sensitization	Boost	Challenge
Group A (n=5)	**Derp5 transgenic milk**	**0.1 ml $Al(OH)_3$/mouse**	**0.1 ml $Al(OH)_3$/mouse**	1.6 mg derp5 /mouse
Group B (n=5)	**Derp5 transgenic milk**	**10 μg Derp5 + 4mg $Al(OH)_3$/mouse**	**10μg Derp5 + 4 mg $Al(OH)_3$/mouse**	1.6 mg derp5 /mouse
Group C (n=5)	**General mouse milk**	**10 μg Derp5 + 4mg $Al(OH)_3$/mouse**	**10μg Derp5 + 4 mg $Al(OH)_3$/mouse**	1.6 mg derp5 /mouse

(Chen *et al.*, 2001)

(A)



(B)

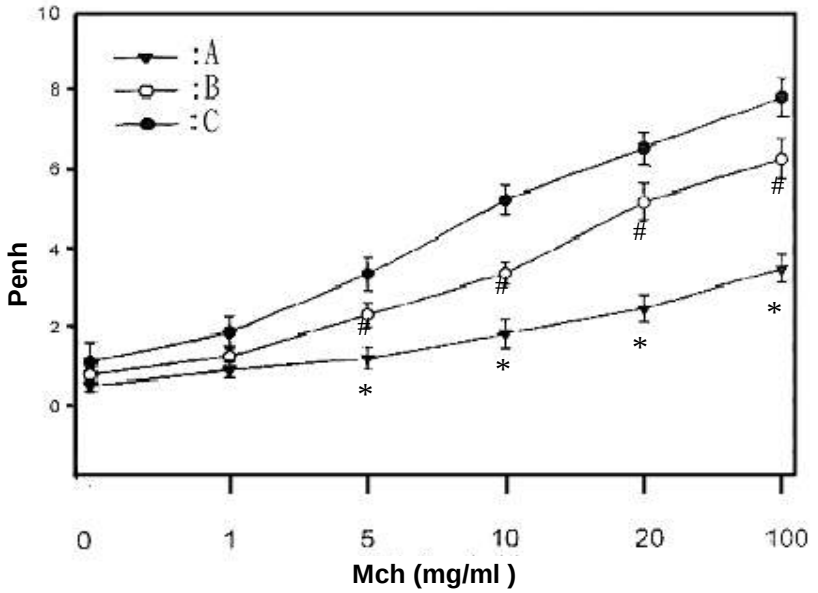


Fig. Effect of oral intake the transgenic milk contained derp5 on suppression of airway hyperreactivity expressed by measurement of (A) 50% total lung capacity and (B) increases in enhanced pause (Penh), against to various dosages of acetylcholine (Ach) and methacholine (Mch) treatment, respectively.

(Chen *et al.*, 2001)

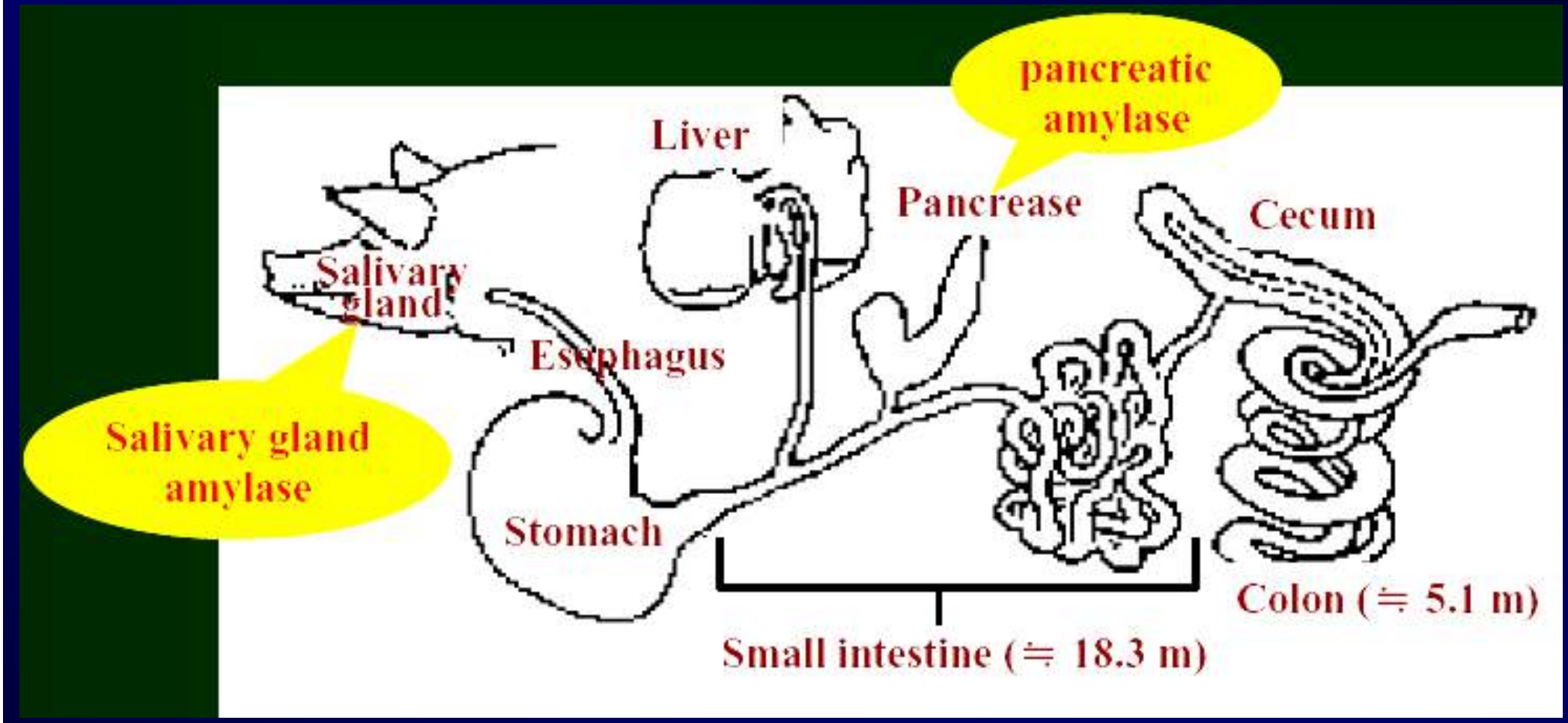


Fig. The principal digestive track of pig.

(Lin *et al.*, 2004)

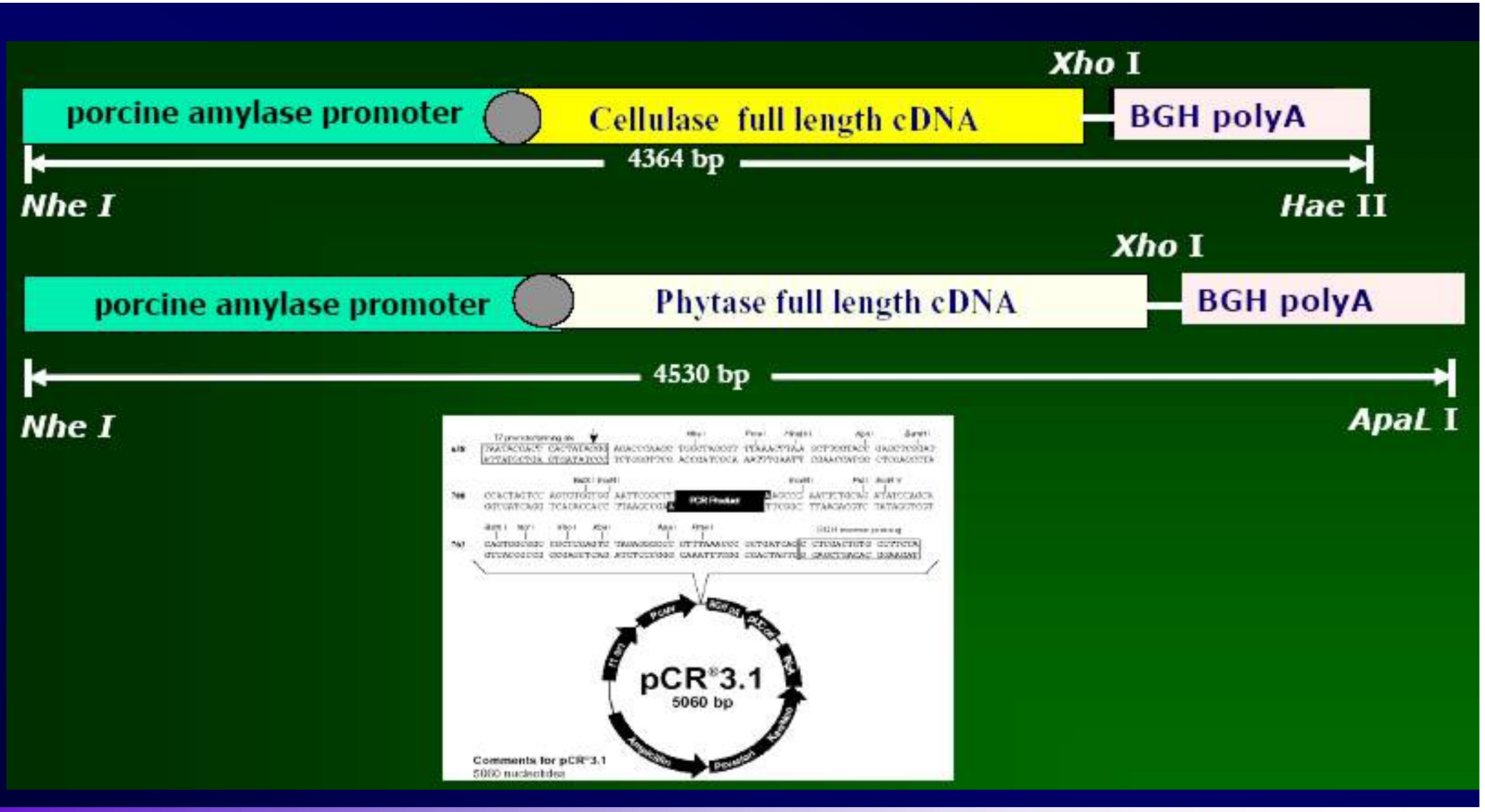


Fig. Transgene(s) Driven by Pancreas-Specific Promoter for ensuring the Tg protein(s) to be secreted into small intestine

(Lin *et al.*, 2004)

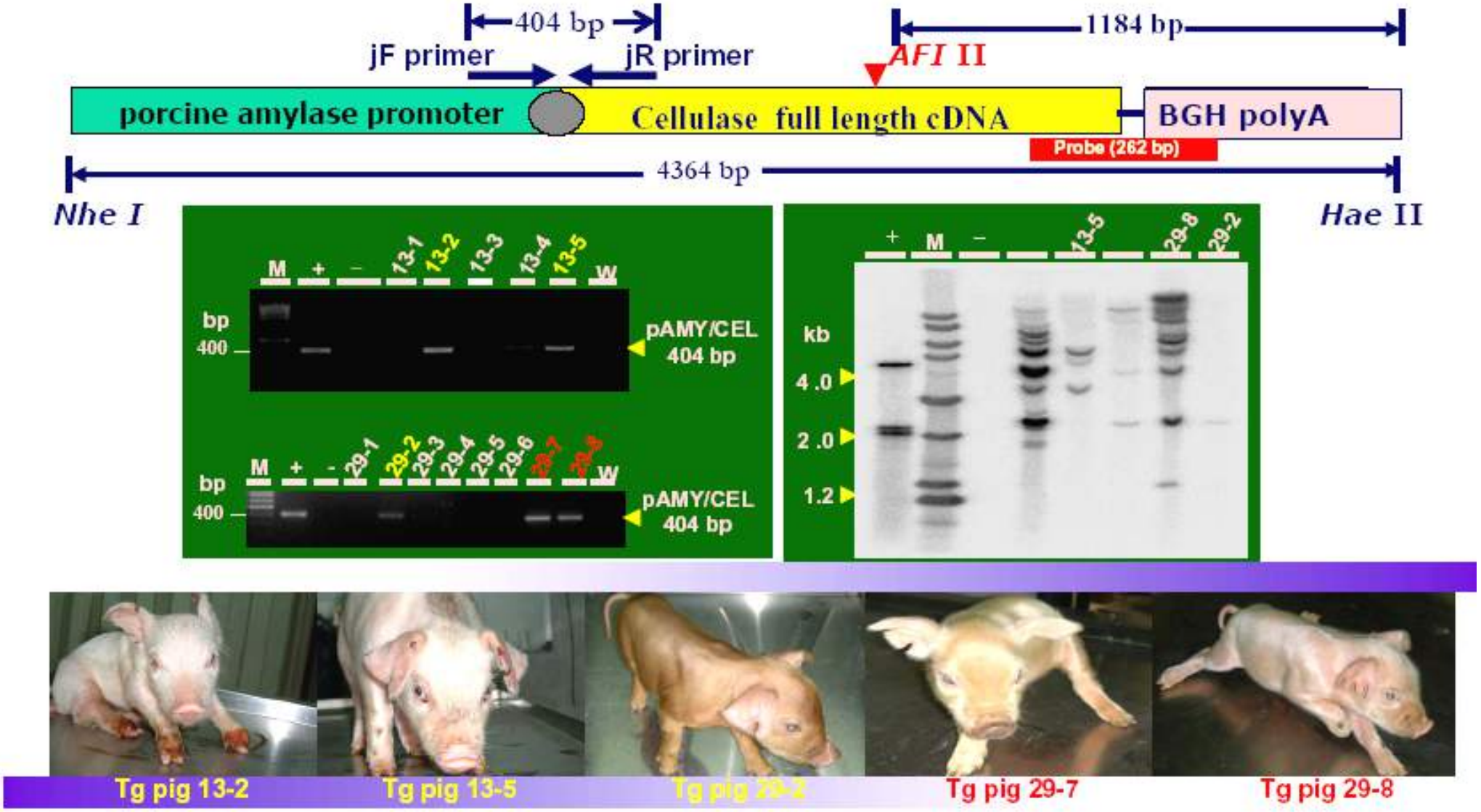


Fig. Transgenic piglets harboring the *pAMY-CEL* transgene

(Lin *et al.*, 2004)

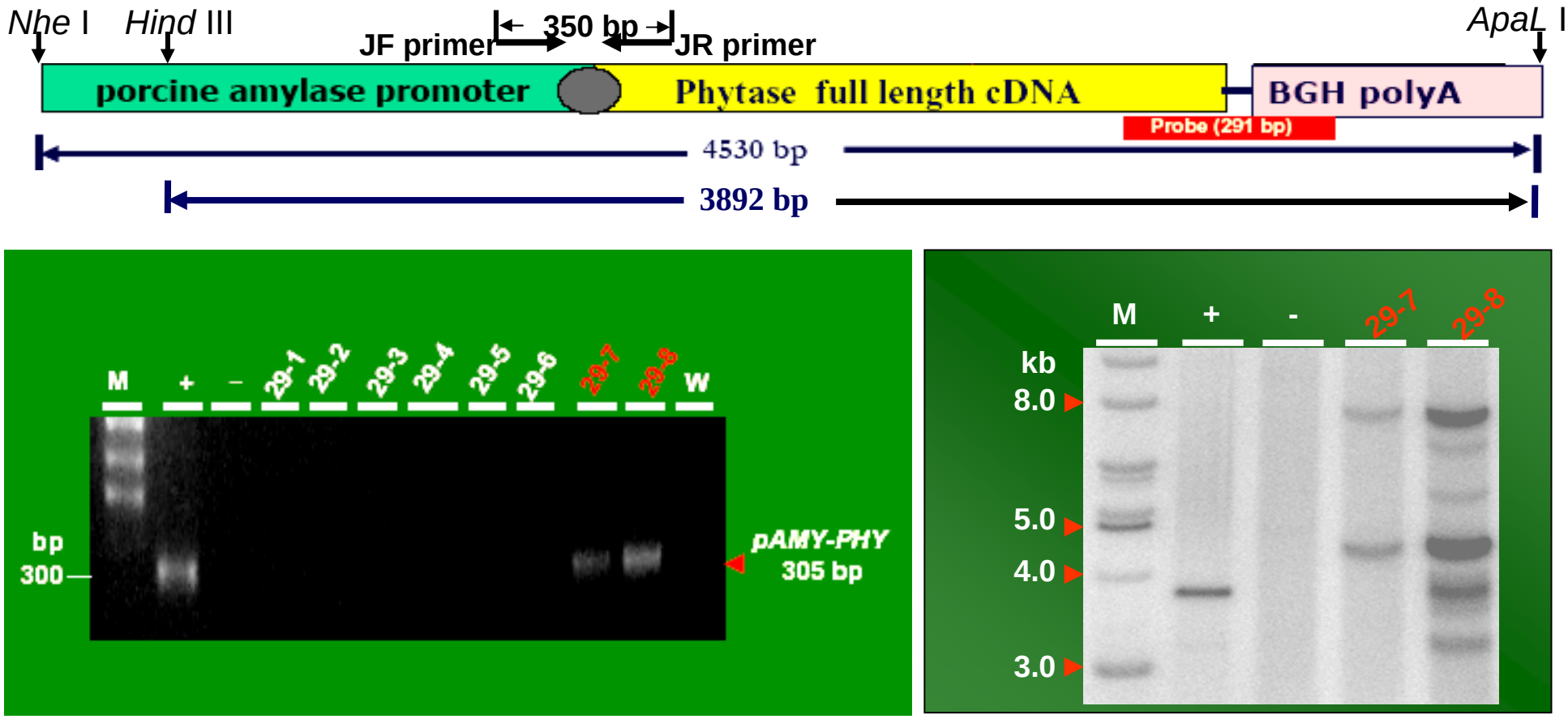


Fig. Transgenic piglets harboring the *pAMY-PHY* transgene

(Lin *et al.*, 2004)

Table_. Formulation of experimental diets for transgenic and conventional pigs

Ingredients (%, w/w)	Low phosphate diet	common diet
Corn	43.40	43.40
Soybean meal (44%CP)	15.00	15.00
Barley	30.00	30.00
Casein protein	2.00	2.00
Corn starch	8.00	8.00
Mono Calcium Phosphate	0.00	0.45
Limestone ($CaCO_3$)	0.55	0.55
NaCl	0.20	0.20
ZnO	0.004	0.004
Vitamin premix[1]	0.40	0.40
Total (100 kg)	100	100
	Calculated nutritive values	
Ca	0.38	0.46
Total P	0.35	0.44
Available P	0.09	0.14
CP	15.03	15.03
Ca/total P ratio	1.09	1.05
ME, Kcal/kg	3230	3230

[1]Vitamin premix provided per kilogram of diet: 1400 IU of vitamin A, 160 IU of vitamin D_3, 6 IU of vitamin E, 0.5 mg of vitamin K, 0.8 mg of riboflavin, 0.8 mg of thiamin, 6 mg of vitamin B_{12}.

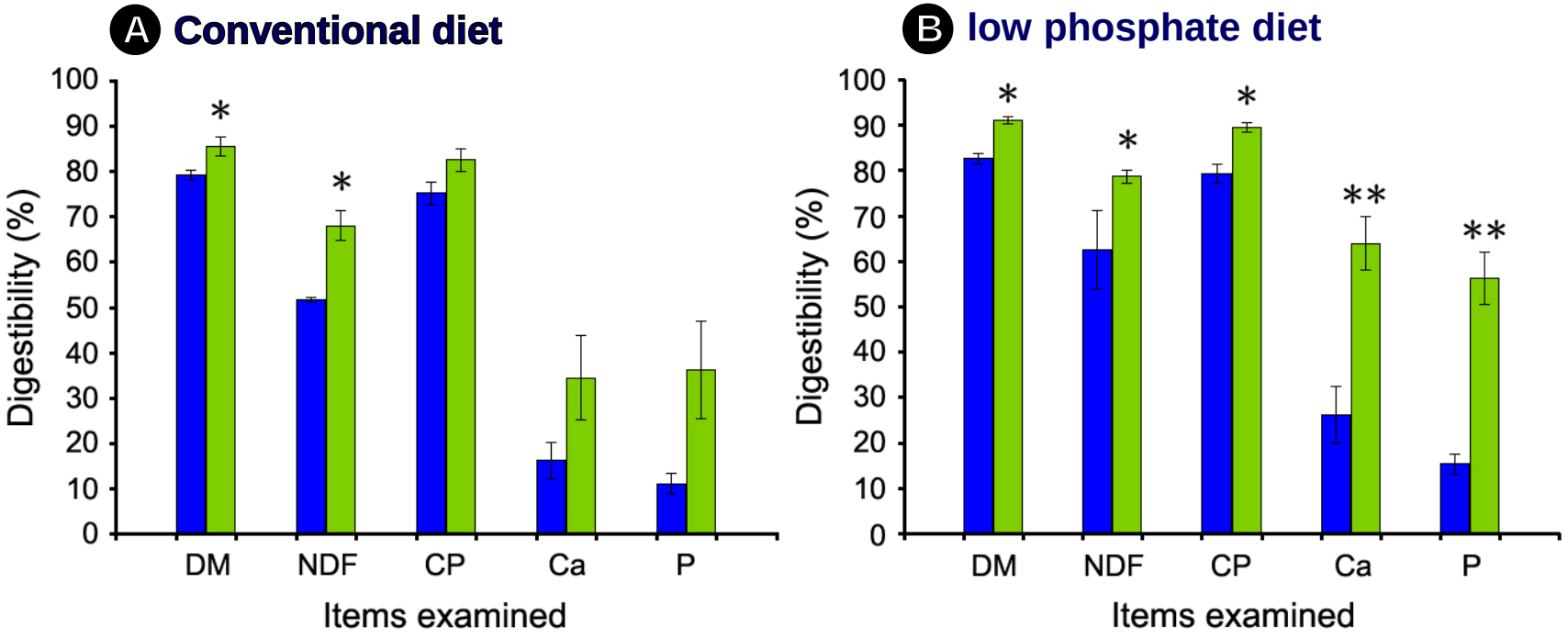


Fig._. Comparisons on the digestibility between non-transgenic pigs (■) and transgenic pigs (■) that were fed with the conventional diet (A) and/or with the low phosphate diet (B). Estimations of digestibility were conducted according to the indicatory methods based on each of items examined, including dry matter (DM), neutral detergent fiber (NDF), crud protein (CP), calcium (Ca), and phosphorus (P) etc. found in the feces against to each of that in-tacked from the diet.

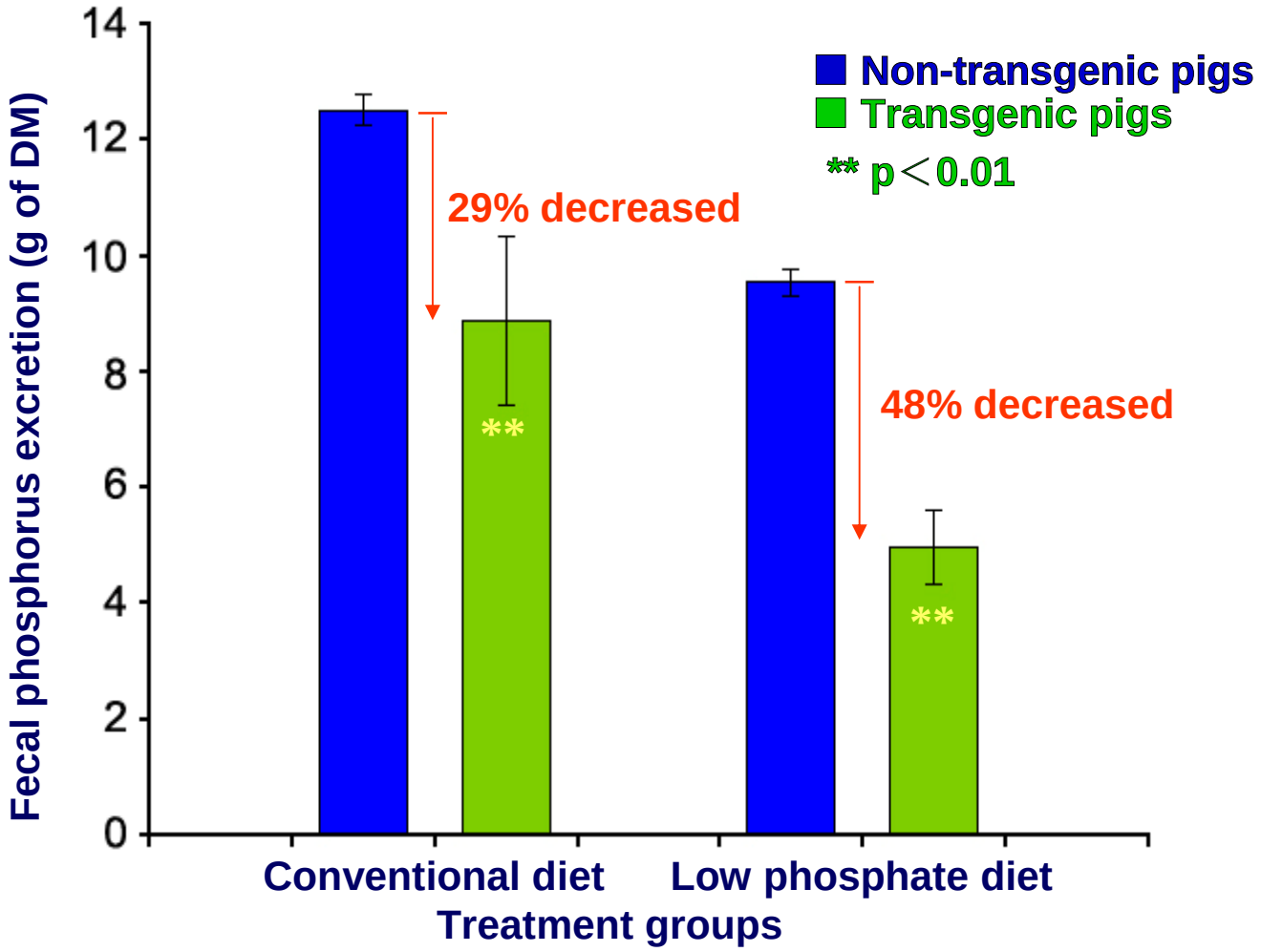


Fig._. Transgenic pigs resulted in significant reduces of fecal phosphorus output when comparisons were made to those from that of non-transgenic pigs.

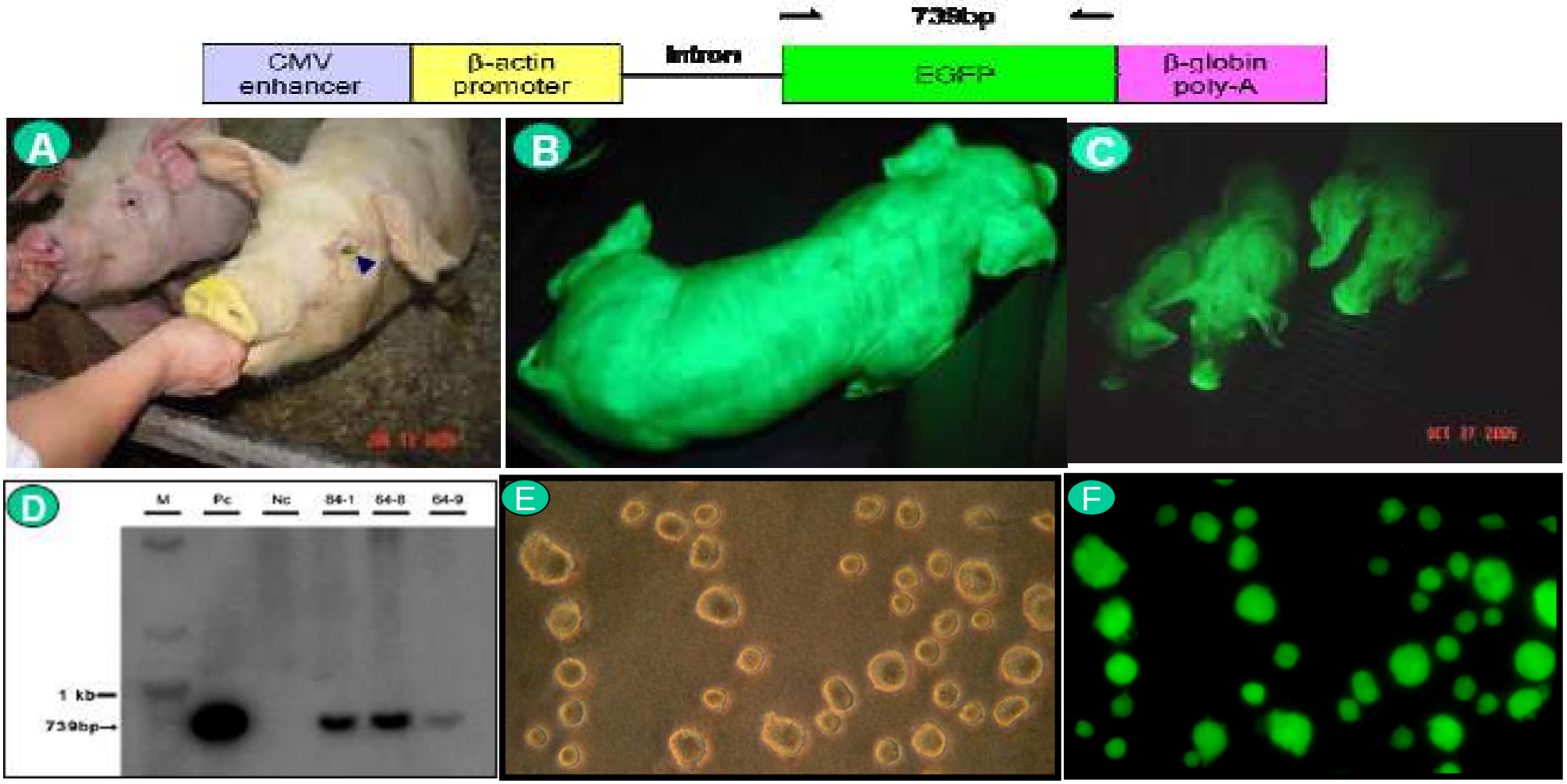


Fig. Southern blot assay of *ECOR* I digested genomic DNA for EGFP gene in transgenic pigs generated by pronuclear microinjection. Based on the fluorescence-activated cell sorter analysis against to the *in vitro* cultured msenchymal stem cells isolated from femur bone marrow of these Tg pigs, over 99.9% of the cultured cells appeared to express the EGFP.

(Wu *et al*., 2006)

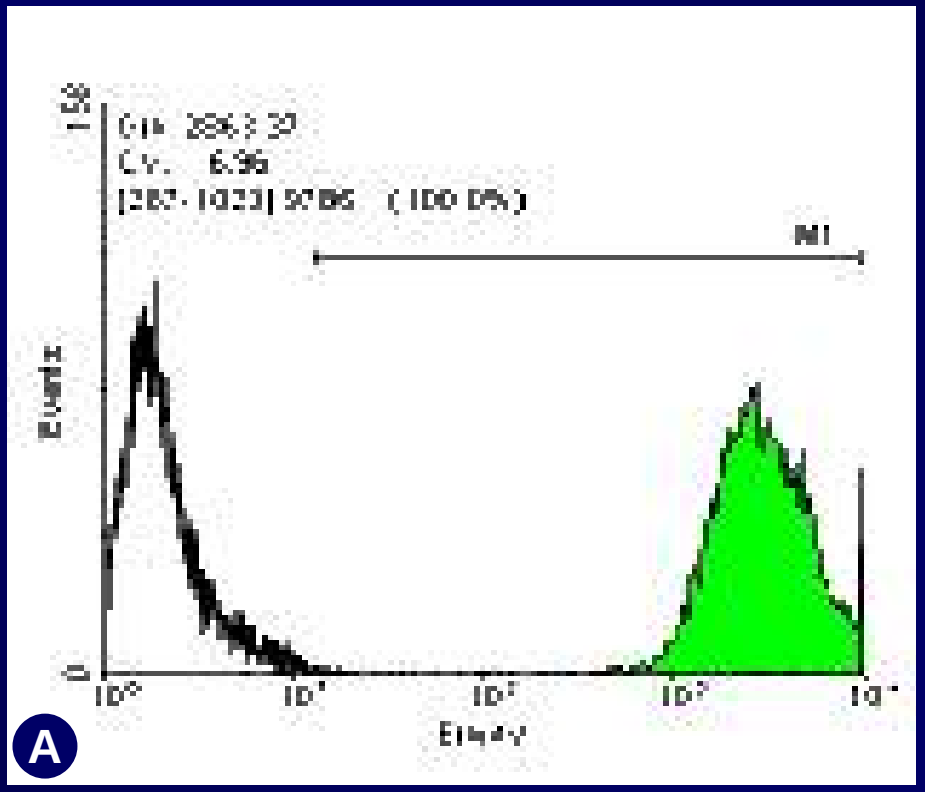
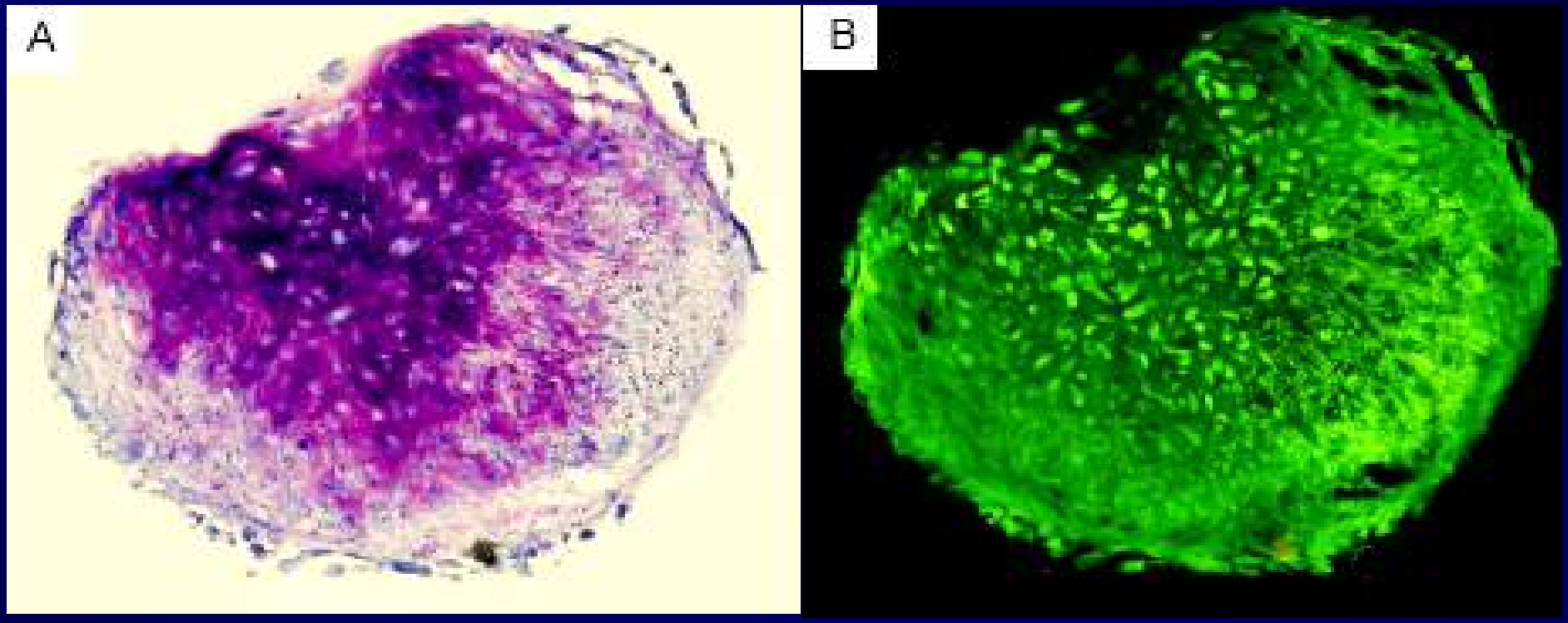


Fig._. EGFP expression profile of pMSCs examined by (A) flow cytometric and (B) western blot analyses.

Hsiao et al. (2006)



(200 X)

Fig._. Chondrogenic differentiation of BM-MSCs from EGFP transgenic pig.

(A) Proteoglycan were examined by toluidine blue staining.

(B) BM-MSCs derived chondrogenic lineage express high level of EGFP.

Hsiao et al. (2006)

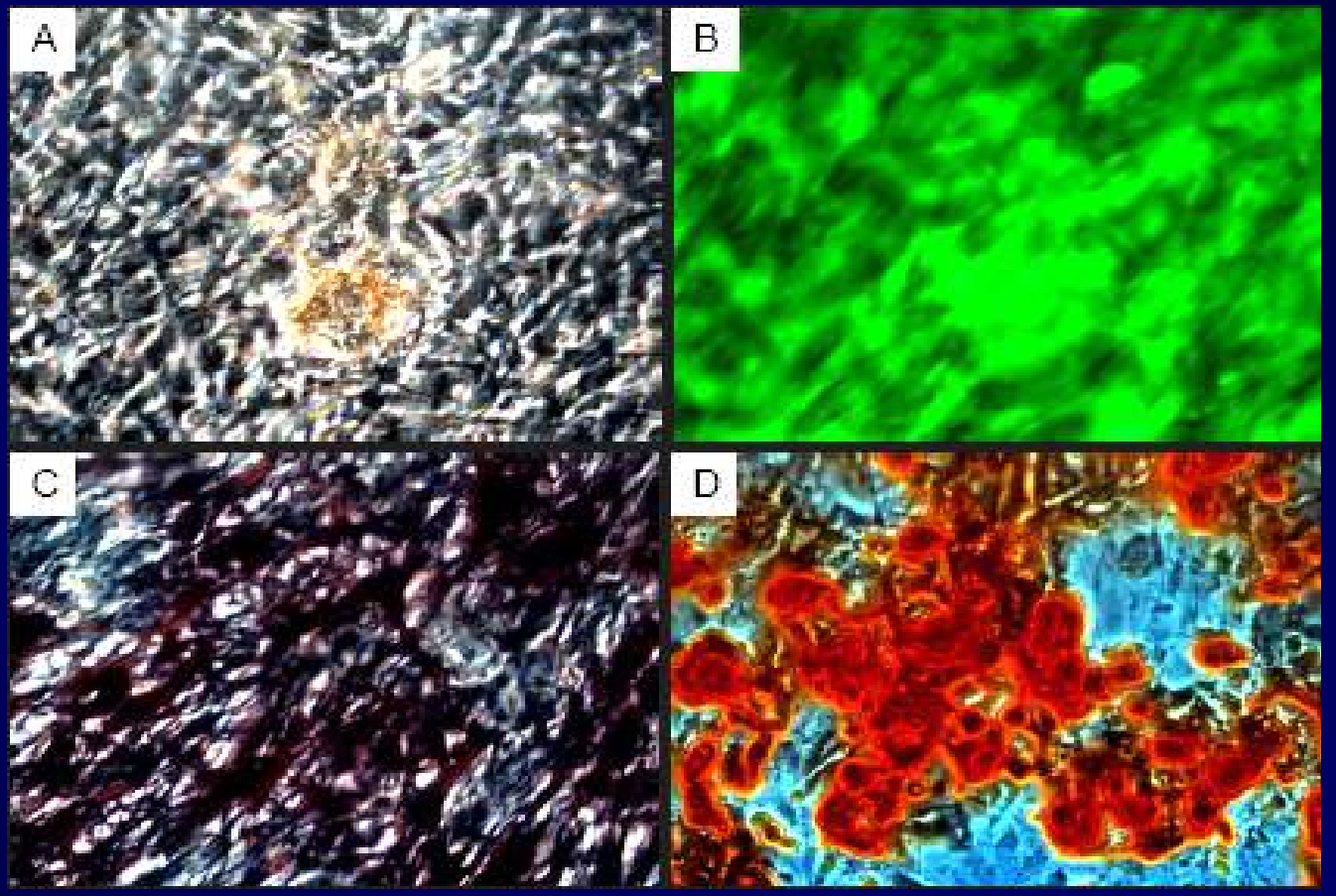


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Fig._. Osteogenic differentiation of BM-MSCs from EGFP transgenic pig.

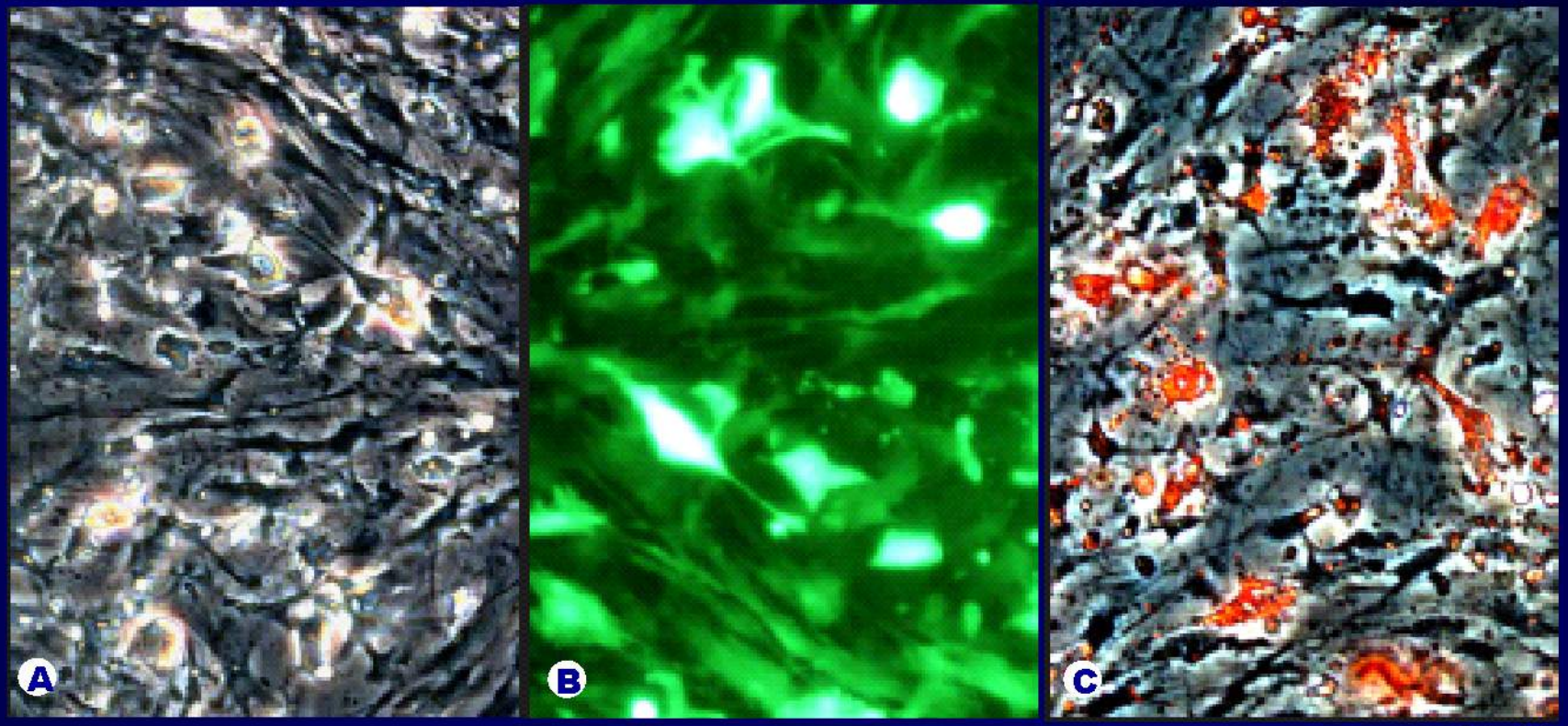
(A) BM-MSCs derived osteogenic lineage shown that both of **multilateral phenotype and deposited calcium** were easily observed.

(B) **EGFP** was **highly expressed throughout** in those **osteogenic lineage cells** differentiated form BM-MSCs of the EGP Tg-pig.

(C) **Alkaline phosphatase activity** were examined by **BCIP/NBT substrate.**

(D) **Calcium deposition** were examined by **alizarin red staining.**

Hsiao et al. (2006)



(200 X)

Fig._. Adipogeneic differentiation of BM-MSCs from EGFP transgenic pig.

(A) BM-MSCs derived adipogenic lineage showed a lipid-filled phentype.

(B) High level of EGFP was expressed after adipogenic differentiation.

(C) Lipid droplet were examined by Oil red O staining.

Hsiao et al. (2006)

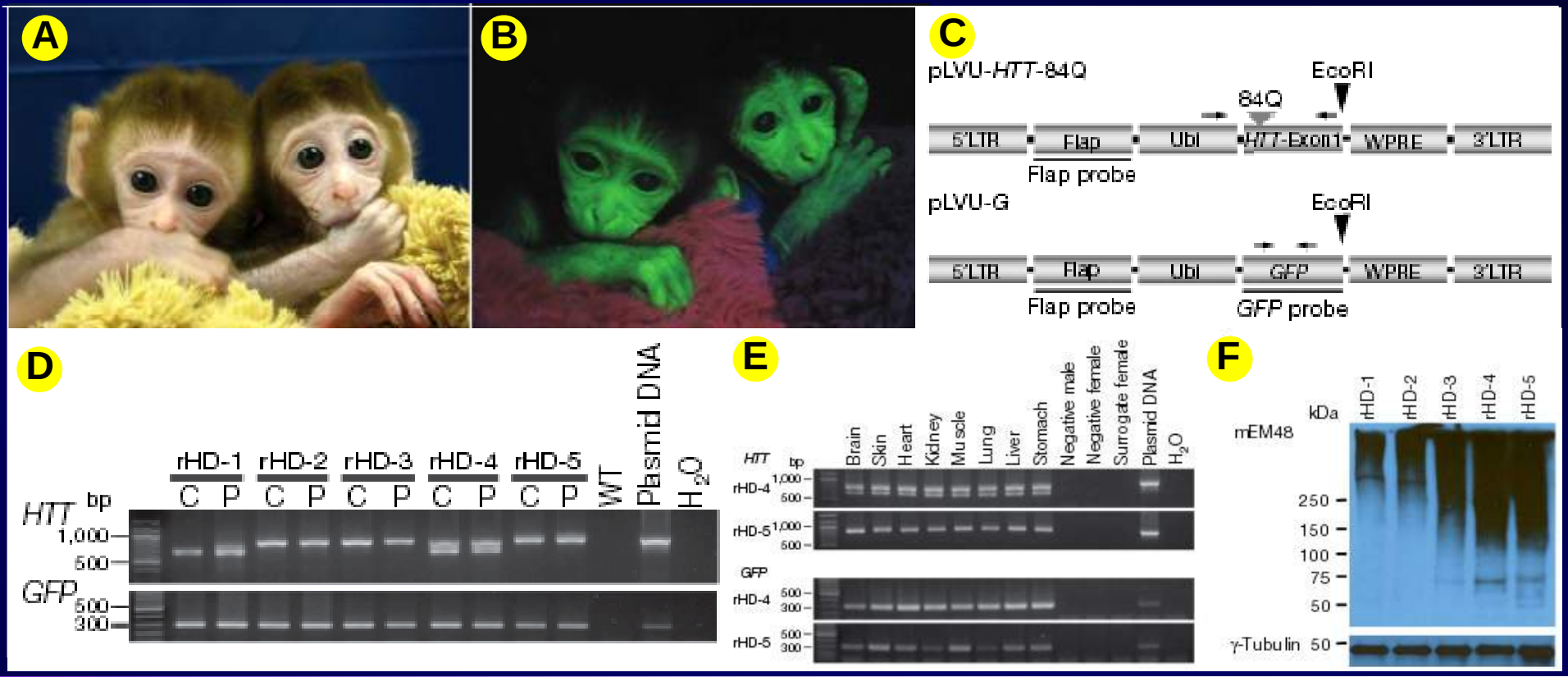


Fig._. Generation of a transgenic model in monkeys of Huntington's disease (HD).

The Tg HD monkeys rHD-1 (left) and rHD-2 (right) are shown. Transmission light image (A) and fluorescent image (B) showing GFP expression inHDmonkeys. C, Top panel: lentiviral vector that carries exon 1 of the HTT gene with 84 CAG repeats (pLVU-HTT-84Q). Bottom panel:lentiviral vector that carries the GFP gene (pLVU-G). Arrows indicate the positions of PCR primers; arrowheads denote restriction digest sites. Flap, HIV-flap sequence; GFP, green-fluorescent-protein gene; HTT, huntingtin gene; LTR, long terminal repeat; Ubi, ubiquitin promoter; WPRE, woodchuck post-transcriptional regulatory element. D, E, The presence of transgenes inHDmonkeys was confirmed by PCR analysis using primer sets specifically for mutant HTT (top panels) and for the GFP gene (bottom panels). PCR of the cord (C) and placental (P) tissues of all HD monkeys (D), and PCR of different tissues collected from rHD-4 and rHD-5 (E). Expression of the transgenic mutant HTT was confirmed by western blot analysis (F) using the placental tissues. Immunostaining was performed using mouse-monoclonal-mEM48 antibody (top panel) and an antibody against c-tubulin (bottom panel).

Yang *et al.* (2008)

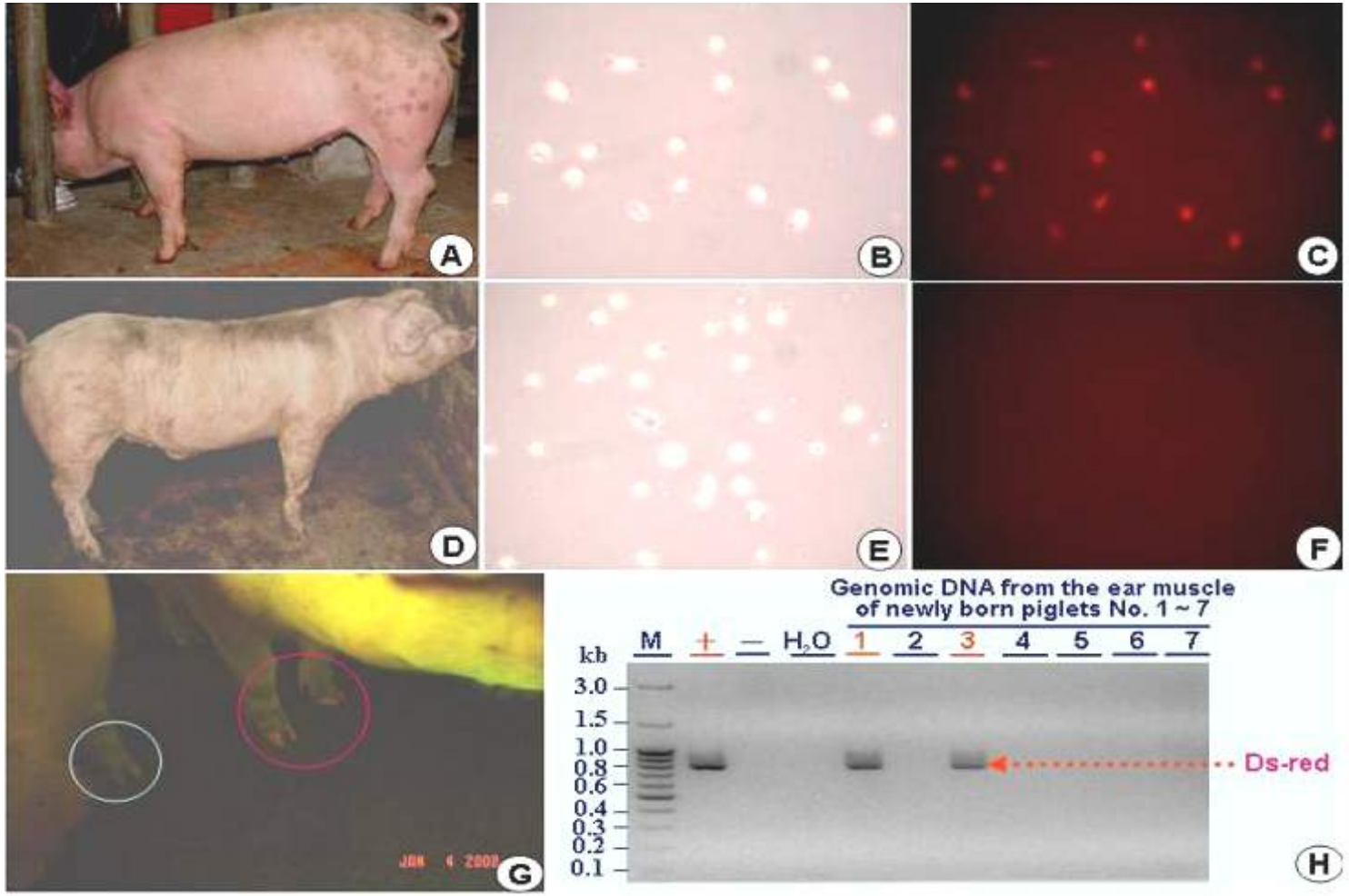


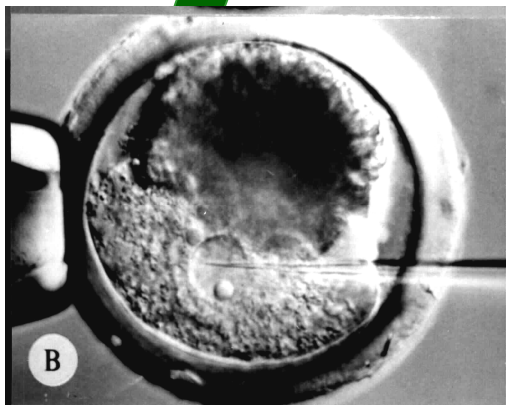
Fig._. Tg pigs harboring the DsRed gene successfully generated by method of pronuclear microinjection. The expression of red fluorescent protein gene was shown in the skin and white blood cells of the Tg pig (A, B and C) when compared with those of the control pig (D, E and F). The red fluorescent protein also expressed on the hoofs of the Tg pig (G). The transgene was detected by PCR amplification (H).

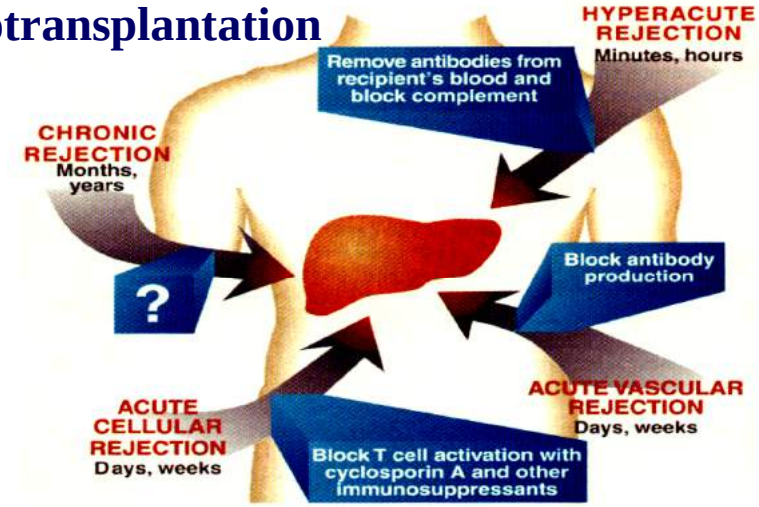
(Wu *et al*., 2007)

Generation of Tg pigs for Xenotransplantation

Generation of Tg pigs harboring gene(s) related to immuno-rejection

To meet Requirement of xenotransplantation





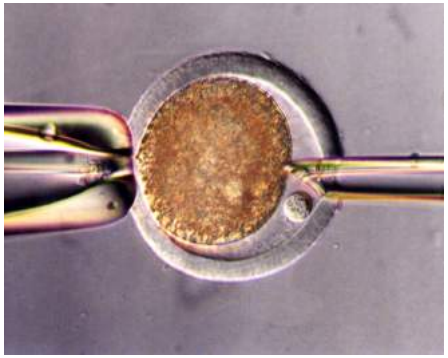
Transgenic pigs harboring transgene(s) for xenotransplanation purpose

Transgene(s)	Tg founder(s) generated	Date of parturition	Gender	Germ-line transmitted status of transgene(s)
Human decay accelerating factor				
CMV-hDAF	Y116-12A	4/15/99	♀	Germ-line transmitted (F1)
	Y167-10A	5/28/99	♀	Germ-line transmitted (F1)
Human leucocyte antigen class II				
HAL-DP A1+B1	Y113-7&-8	10/18/96	♂ / ♀	Germ-line transmitted (F3)
HAL-DQ A1+B1	Y122-02A	4/21/99	♂	Germ-line transmitted (F1)
HAL-DQ A1	Y215-10A	6/30/00	♀	to be verified
HAL-DR B1	L218-12A	7/8/99	♀	Germ-line transmitted (F1)
HAL-DR A1+B1	D146-13A	4/30/00	♀	Pregnancy in progression

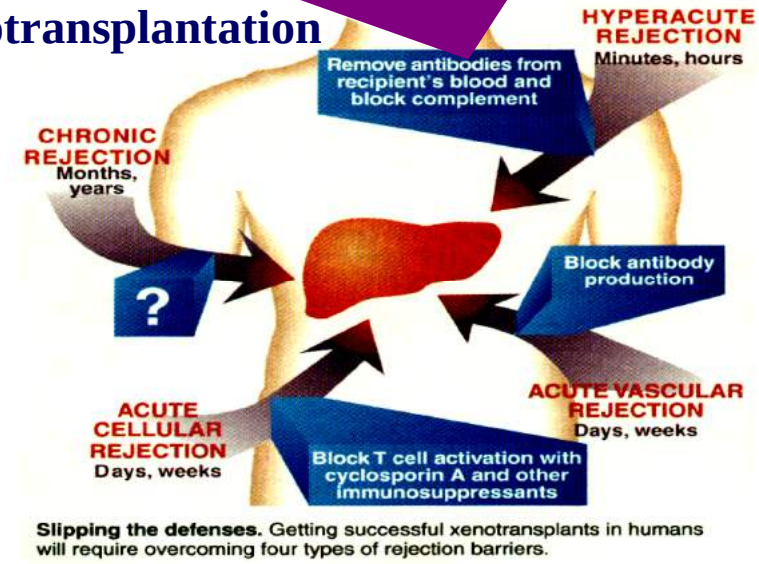
(Dr. Chin-fu Tu, ATIT)

We do anticipate the cloning of human embryos should be allowed for fitting to purposes of Cell-, tissue- and organ-engineering and meet the requirement of xenotransplantation

Generation of Cloned-Tg pigs harboring gene(s) related to immuno-rejection



Meet to the requirement of xenotransplantation



Slipping the defenses. Getting successful xenotransplants in humans will require overcoming four types of rejection barriers.

Generation of Tg pigs harboring knock-in gene(s) related to immune rejection

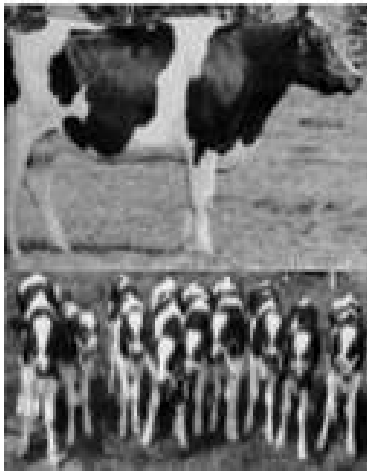
Milestone in Studies Related to Animal Cloning

Authors	Years	Big breakthrough
Spemann	1938	The pioneer offered the concept of animal cloning by nuclear transfer techniques.
Briggs & King	1952	Created the first cloned tadpoles were obtained after the NT-based tech, using donor nucleus from embryonic cells.
Gordon; McKinnell	1962	First cloned frogs developed to adult, using donor nucleus from embryonic cells.
Gordon & Lasky	1970	First cloned frogs developed to adult, using donor nucleus from adult cells.
Willadsen *et al.*	1984	First cloned sheep born after embryo-splitting and some other species including rabbit, cattle, pig, goat, and monkey were also cloned thereafter.
Wilmut *et al.*	1997	Dolly was born after NT using DN from mammary epithelia cells of an adult (6-yrs) ewe.
Wilmut *et al.*; Wakayama *et al.*; Cibelli *et al.*; Baguisi *et al.*	1998~	Cloned animals including mouse, cattle, goat, pig, cat, dog and monkey were achieved, using donor nucleus from adult cells.

(Wilmut *et al*., 1997)

(Wakayama *et al*., 1998)

(Baguisi *et al*., 1999)



(Wells *et al*.,1999)

(Kubota *et al*., 2000)

(Chan *et al*., 2000)

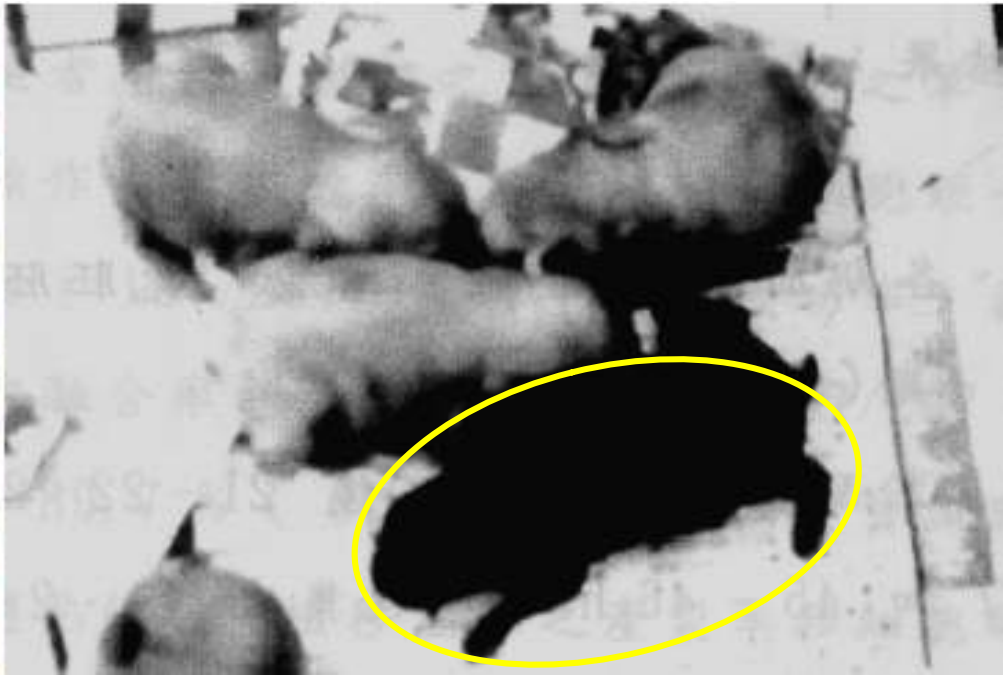
(Shin *et al*. , 2002)

(Vanderwall *et al*., 2003)

(Lee *et al*., 2005)

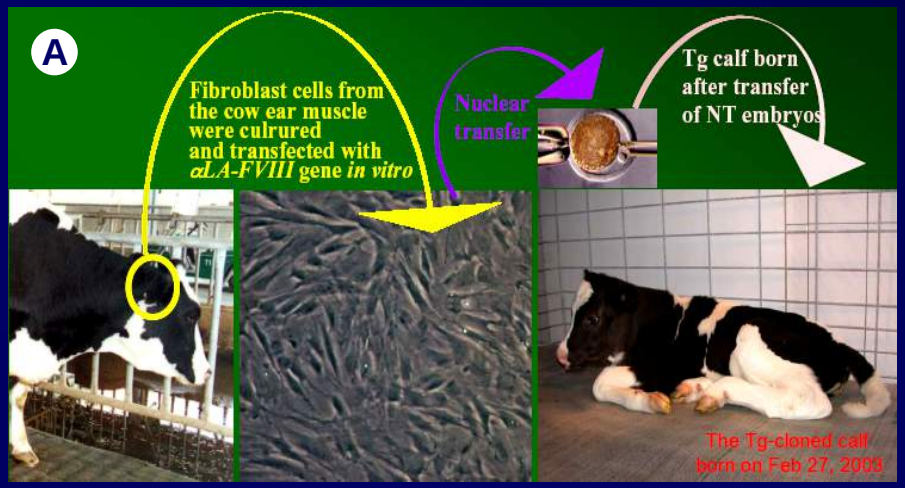
Fig. Several species of mammalian animals have been successfully cloned since Dolly was cloned in 1997.

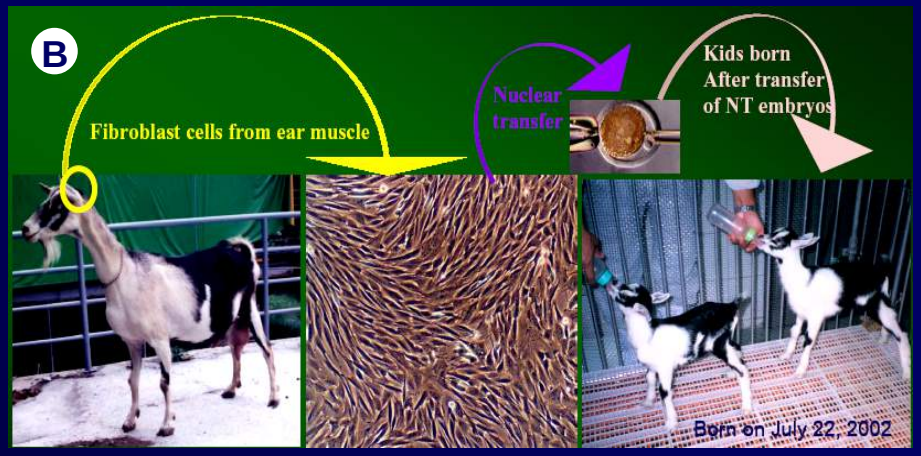
(Chen and Wu, 1993)

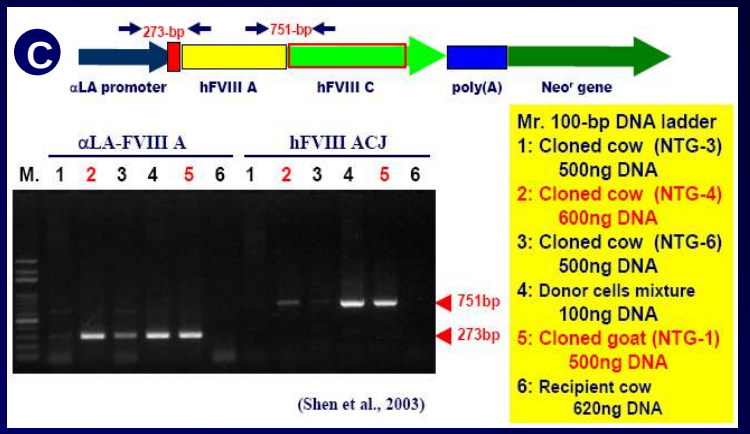


(Shen *et al.*, 1997)

Fig. Cloned pigs and rabbit (black hair) generated after the nuclear transfer by using blastomere from the 4- and 8-cell embryos, respectively.







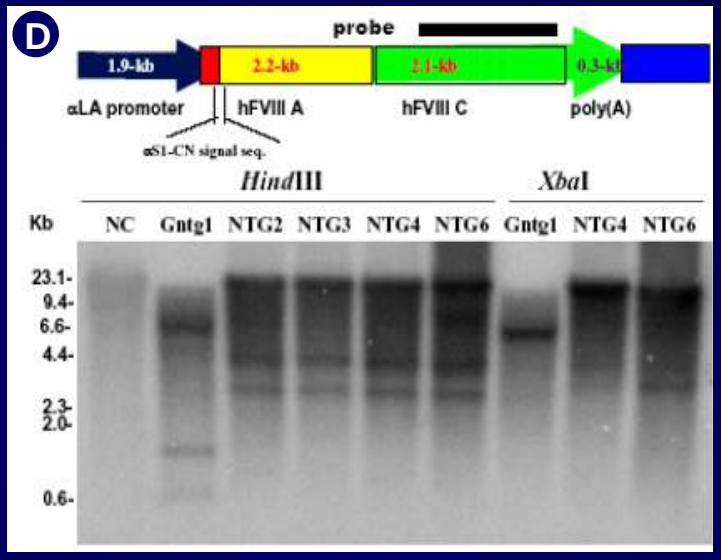


Fig. Cloned-Tg calf and kids harboring *αLA-FVIII* gene born after transfer of NT embryos generated by using ear-muscle fibroblast cells, after gene transfection, from of dairy cow and goat as the donor cells, respectively.

(Shen *et al.*, 2003)

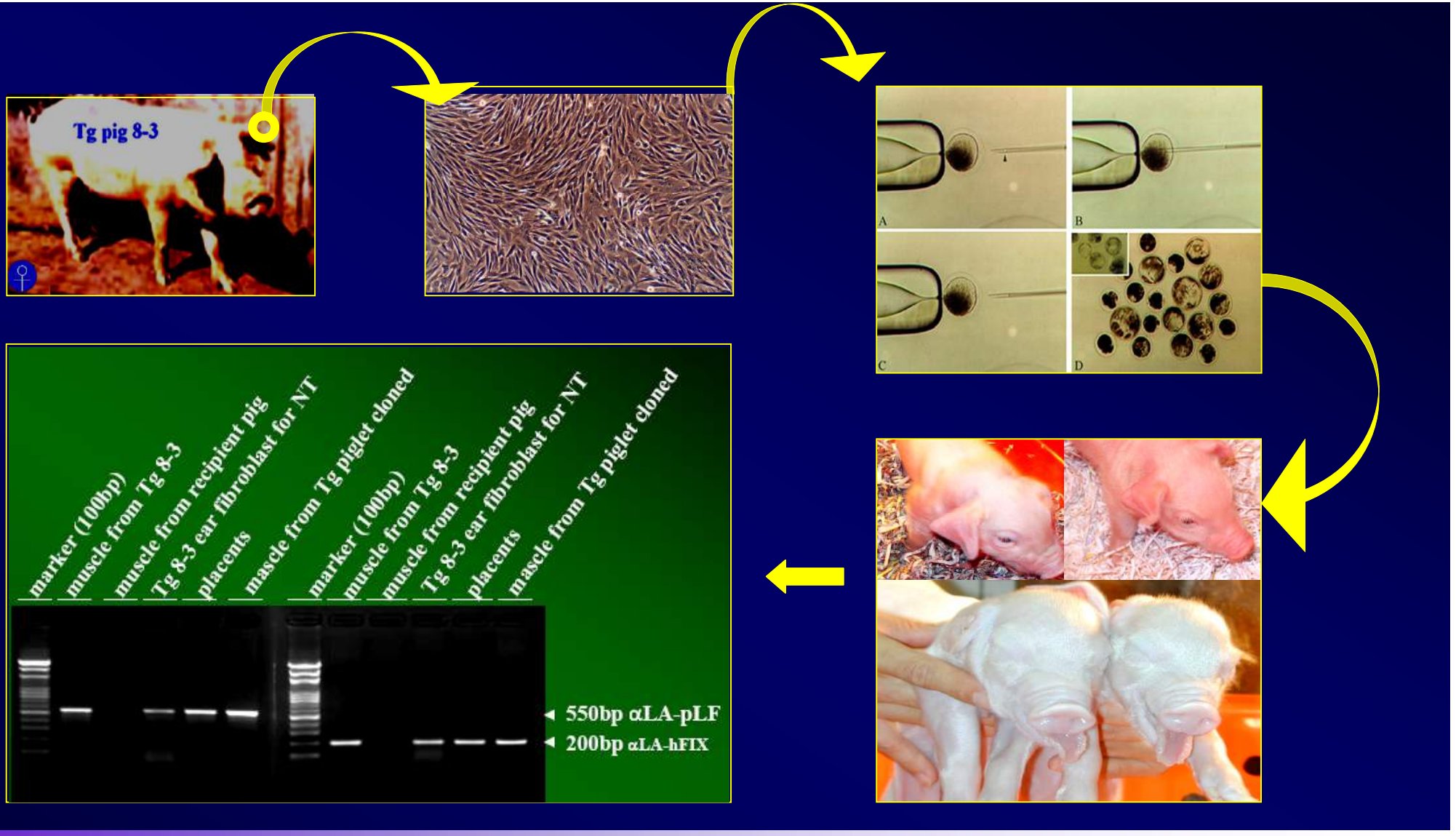


Fig. Cloned piglets harboring both of *αLA-pLF* 及 *αLA-hFIX* trnsgenes born after NT using ear muscle fibroblast cells from Tg 8-3 as the nucleus donor.

(Wu *et al.*, 2002)

Hindrances related to Tg-cloned animals

1. Transgenic and cloned animals can evoke apprehension and engender uncertainty related to issues including biosafety, food safety, ethical and social implications. While certain levels of oversight are appropriate, it is important to create new oversight mechanisms or regulatory burdens only when there are compelling reasons for doing so.

2. High incidence of abortion, fetal abnormalities, and postnatal losses have been observed.

3. More knowledge are required for further elucidation events related to the incomplete nuclear reprogramming by the somatic cloning processes.

Efficiency of cloning piglets using skin muscle fibroblast from the sow harboring double transgenes (α*LA-pLF* and α*LA-hFIX*) as the nuclear donor for nuclear transfer

cell passage No.	No. of donors	No. of collected oocytes	No. of enucleated oocytes	No. of NT embryos	No. of activated oocytes	No. of cultured embryos	No. of ET embryos	ID # of Recipient	remarks
P3	5	56	55	47	52	47	42	43-12	Aborted one fetus
P3	5	176	72	66	66	56	43	22-11	One cloned piglet born
P3	5	54	52	50	50	33	30	52-14	An aborted fetus
P4	5	108	56	50	50	47	46	24-11	
P5	6	145	130	90	90	69	67	54-11	Sick
P5	6	176	160	147	133	133	130	54-15	One cloned piglet born
P5	6	134	101	85	77	77	77	74-11	Aborted 4 fetuses
p6	6	170	115	96	96	96	91	70-11	
p6	6	108	104	100	97	97	96	75-12	
p7	6	122	95	50	44	44	43	83-10	Forty oocytes lost (vibr.)
p7	6	182	178	160	132	132	130	85-11	Two cloned piglets born
Total	62	1429	1118	941	887	831	795	11	Four cloned piglets and Six aborted fetuses

(Wu *et al.*, 2002)

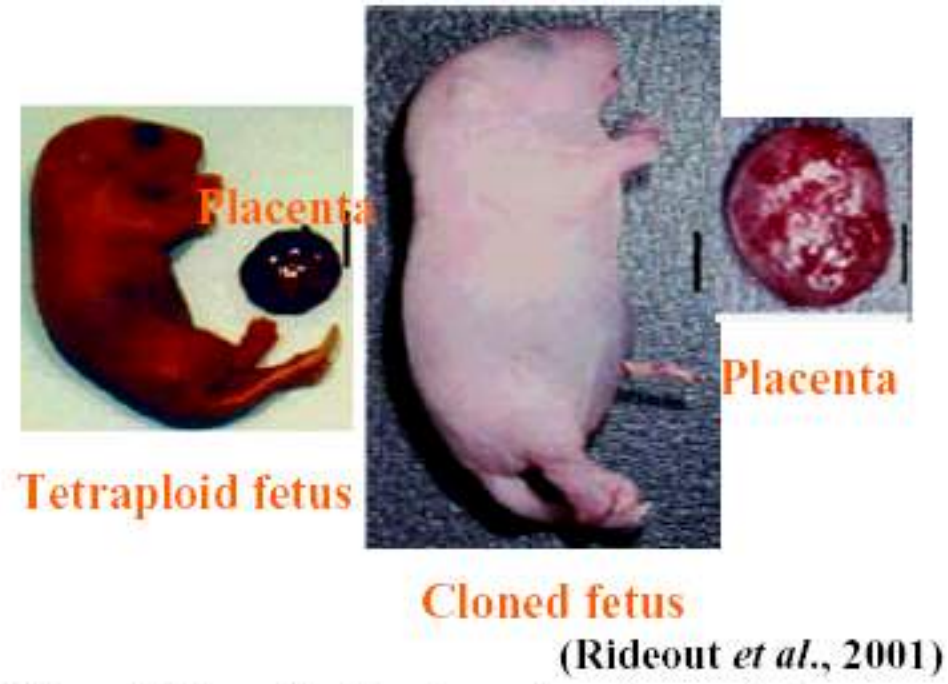
The health profile of cloned animals

Table 1. Literature survey of developmental problems in cloned animals

Species	Percentage healthy animals (healthy/total born)	Problems (% of reported problem cases) after birth	Follow-up period	Reference [a] Unpublished data
Cattle	100 (10/10)	None	4 weeks	1
	100 (2/2)	None	2 months	2
	100 (1/1)	None	7 months	3
	100 (1/1)	Diabetes (100). This animal survived into adulthood	8 months	4
	100 (5/5)	None	8–15 months	5
	80 (24/30)	Pulmonary hypertension, dilated cardiomyopathy (17)	1–4 years	6
	75 (3/4)	Internal hemorrhage umbilical artery (100)	NA	7
	66 (4/6)	Viral infection (50), dystocia (50)	10–12 months	8
	54 (13/24)	Dystocia (15), bacterial infection (8), kidney problems (42)	2–12 months	9
	50 (1/1)	Oversized, leg malformation (100)	NA	10
	50 (4/8)	Pneumonia (25), drawing in amniotic fluid (50), dystocia (25)	2–4 months	11
	44 (11/25)	Heart defects (57), liver fibrosis (29), pneumonia (7), osteoporosis (21), joint defects (14), anemia (42)	4 weeks	12
	40 (4/10)	None described	1 year	13
	25 (1/4)	Viral infection (66)	1 month	14
	0 (0/1)	Thymic atrophy, lymphoid hypoplasia (100)	NA	15
Sheep	100 (1/1)	None	6 years	16, 17 (K. Campbell)[a]
	100 (1/1)	None	3 weeks	18
	83 (5/8)	None described	3 years	19 (K. Campbell)[a]
	21 (3/14)	Kidney, liver, and brain defects	6 months	20
	0 (0/1)	Kidney and liver defects	NA	21
Goats	100 (3/3)	None	3 years	22 (E. Behboodi)[a]
	100 (5/5)	None	1 year	23
	50 (3/6)	Bacterial infection in the lungs (100)	1 year	24
Pigs	100 (1/1)	None	7 weeks	25
	100 (4/4)	None	1 week	26
	100 (2/2)	None	2 months	27
	100 (5/5)	None	9 months	28 (I. Colman)[a]
Mice	100 (8/8)	None	>3 months	29
	100 (4/4)	Obesity (100). This was not a lethal disorder	6 months	30
	100 (5/5)	Enlarged placenta (20)	6 months	31
	100 (6/6)	None	>2 months	32
	100 (3/3)	None	2 months	33
	99 (79/80)	None described	>3 months	34
	93 (15/16)	Umbilical hernia (100)	>3 months	35
	86 (19/22)	None described	>1 year	36
	40 (2/5)	Respiratory failure/umbilical hernia (40), failure to foster (20)	>3 months	37
	33 (1/3)	Respiratory failure (100)	>3 months	38
Total	77 (259/335)			

(Jose B. Cibelli *et al.*) Nature Biotechnology 20, 13 - 14 (2002)

Problems associated with the animal cloning techniques



(Rideout *et al.*, 2001)

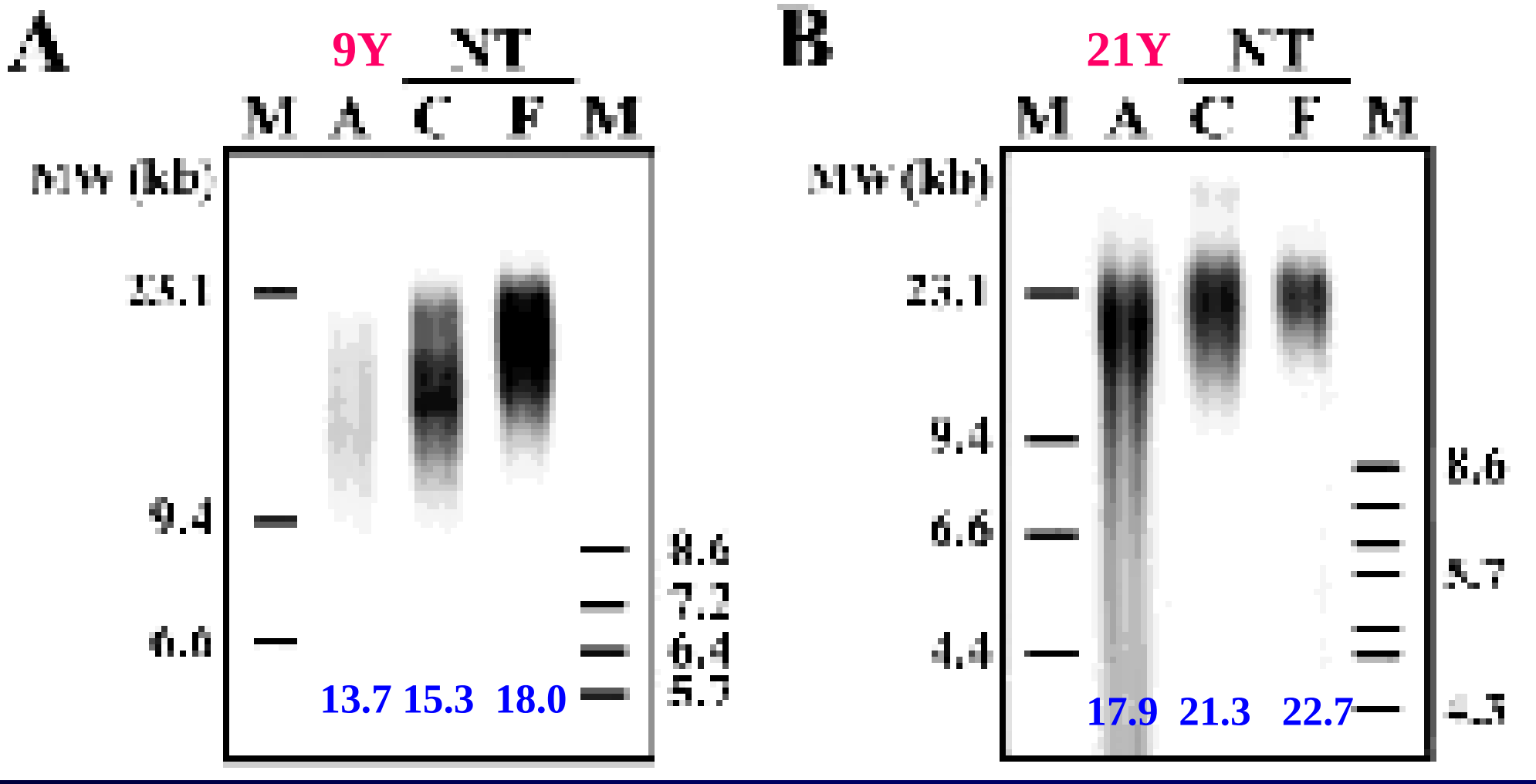
The ES cell-derived mice at term

Abnormalities observed including:

- ☆ Giant fetus
- ☆ Giant placenta
- ☆ Hypoplasia in blood circulation system
- ☆ Hypoplasia in respiratory system
- ☆ High abortion rate
- ☆ High postnatal loses

Fig. The length of telomeres and the activity of telomerase are not good criteria for the ageing

(Yamamoto et al., 1999)



Rebuilding of telomere length in cloned cattle.

Betts *et al.*, 2001

Interspecies Implantation and Mitochondria Fate of Panda-Rabbit Cloned Embryos

(Chen DY et al.) Biology of Reproduction 67, 637-642 (2002)

Somatic cell nuclei of giant pandas can dedifferentiate in enucleated rabbit ooplasm, and the reconstructed eggs can develop to blastocysts. In order to observe whether these interspecies cloned embryos can implant in the uterus of an animal other than the panda, we transferred approximately 2300 panda-rabbit cloned embryos into 100 synchronized rabbit recipients, and none became pregnant. In another approach, we cotransferred both panda-rabbit and cat-rabbit interspecies cloned embryos into the oviducts of 21 cat recipients. Fourteen recipients exhibited estrus within 35 days; five recipients exhibited estrus 43–48 days after embryo transfer; and the other two recipients died of pneumonia, one of which was found to be pregnant with six early fetuses when an autopsy was performed. Microsatellite DNA analysis of these early fetuses confirmed that two were from giant panda-rabbit cloned embryos. The results demonstrated that panda-rabbit cloned embryos can implant in the uterus of a third species, the domestic cat. By using mitochondrial-specific probes of panda and rabbit, we found that **mitochondria from both panda somatic cells and rabbit ooplasm coexisted in early blastocysts, but mitochondria from rabbit ooplasm decreased, and those from panda donor cells dominated in early fetuses after implantation.** Our results reveal that **mitochondria from donor cells may substitute those from recipient oocytes in postimplanted, interspecies clonedembryos.**

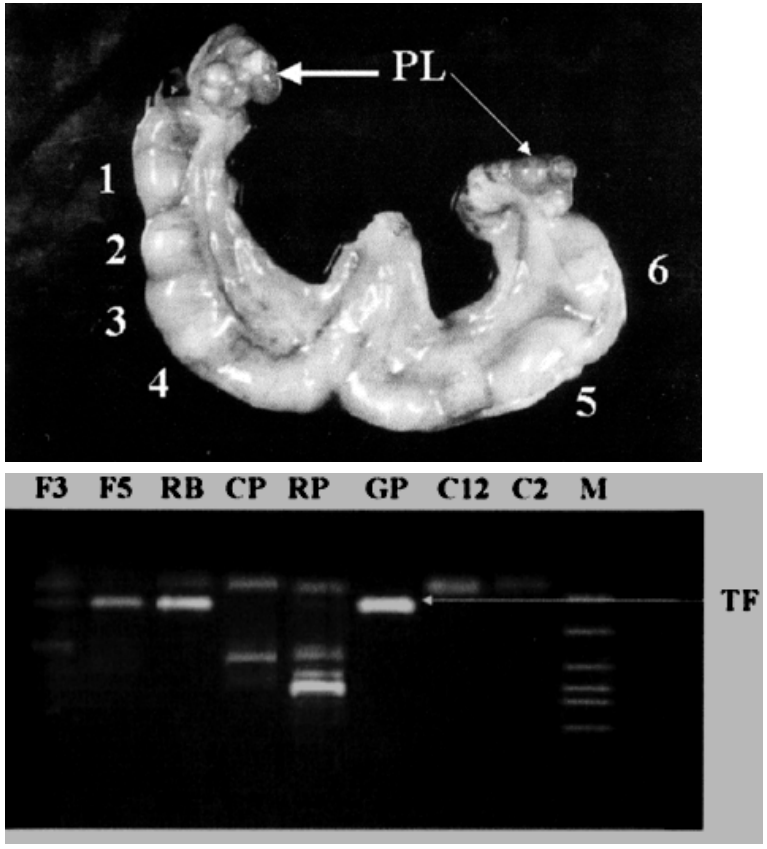
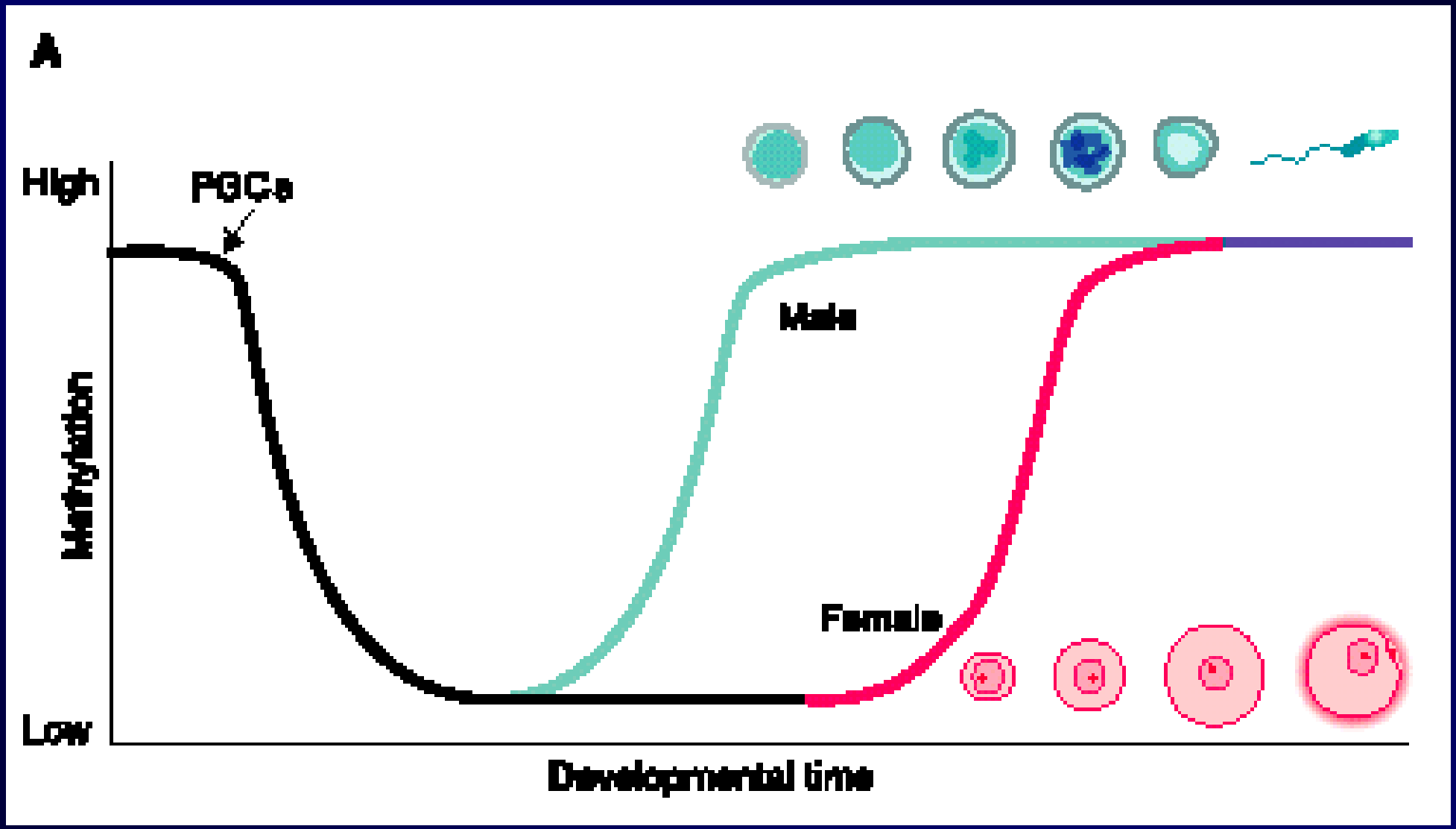
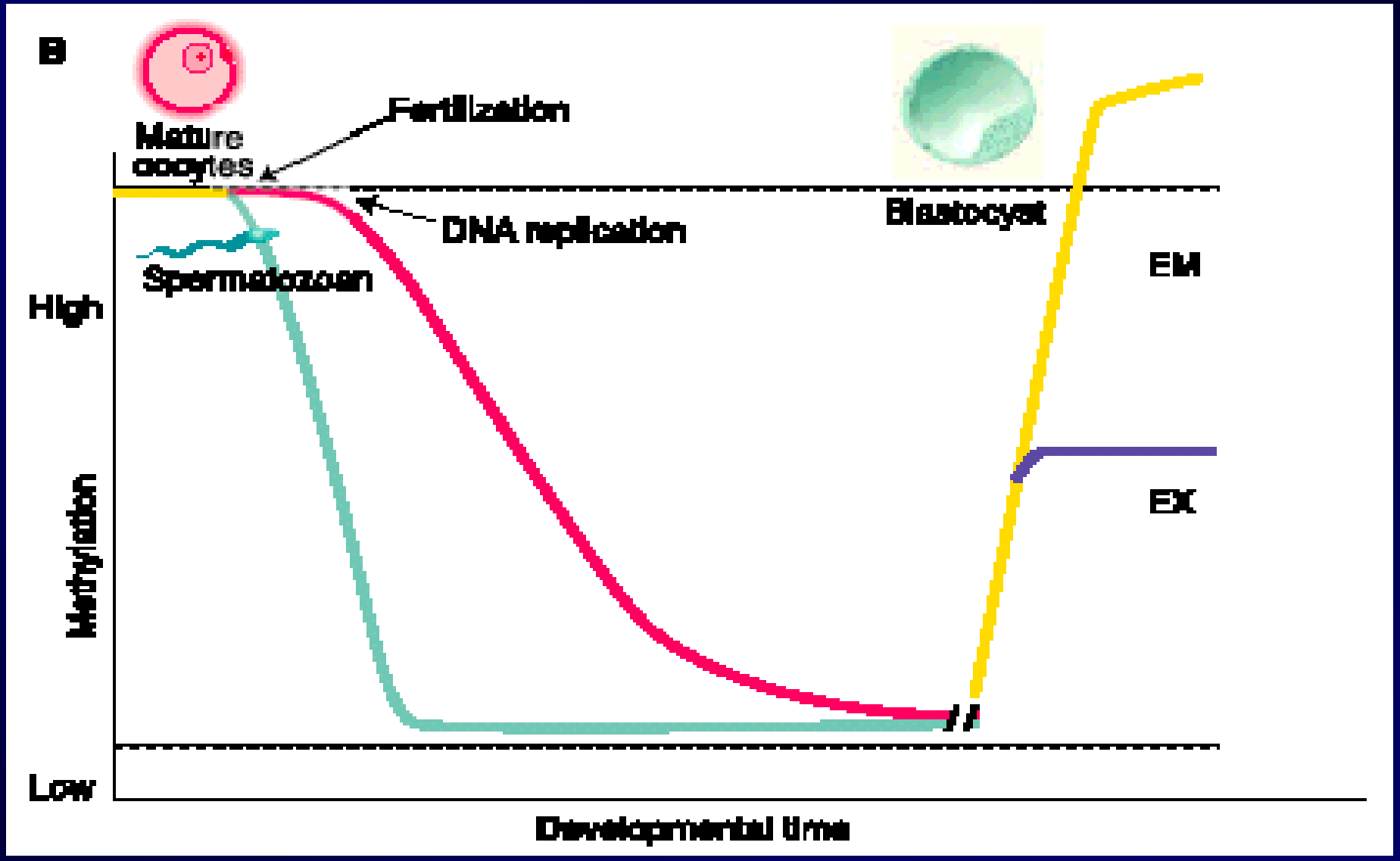


FIG. 5. The results of PCR for mtDNA using panda specific D-loop primer. F, Fetuses from cat uterus; RB, panda-rabbit cloned blastocyst; CP, cat somatic cells; RP, rabbit somatic cells; GP, panda somatic cells; C12 and C2, negative controls; M, markers; TF, target fragment



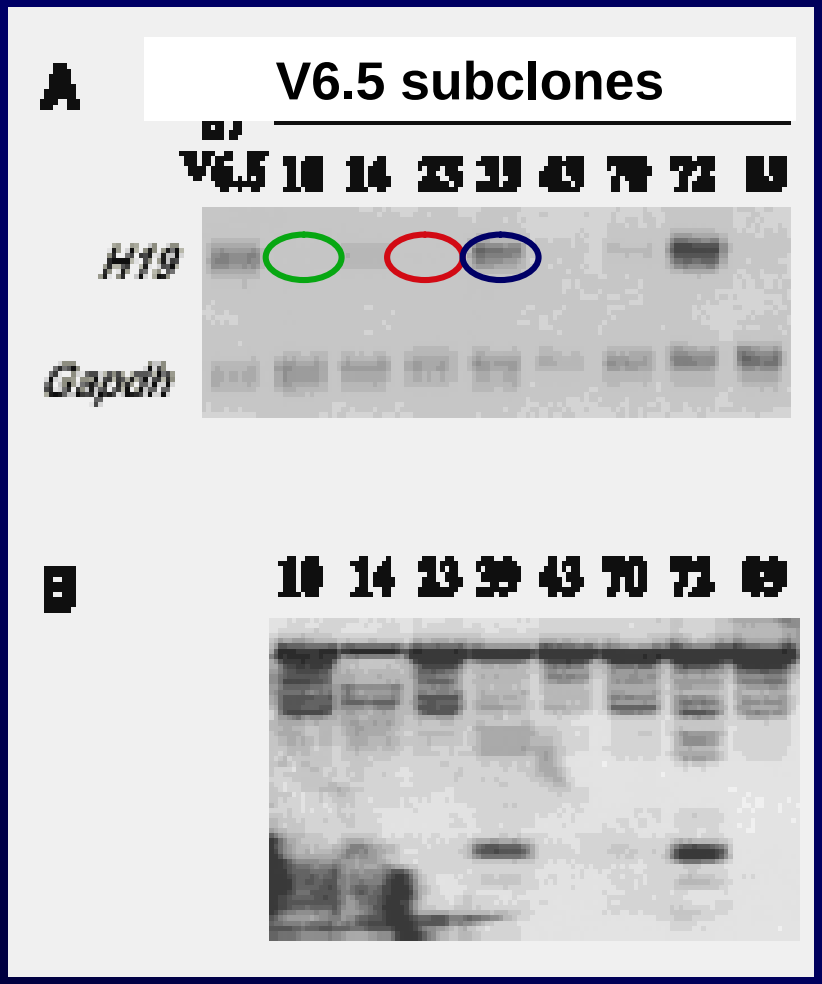
Methylation reprogrammimg in the germ line.

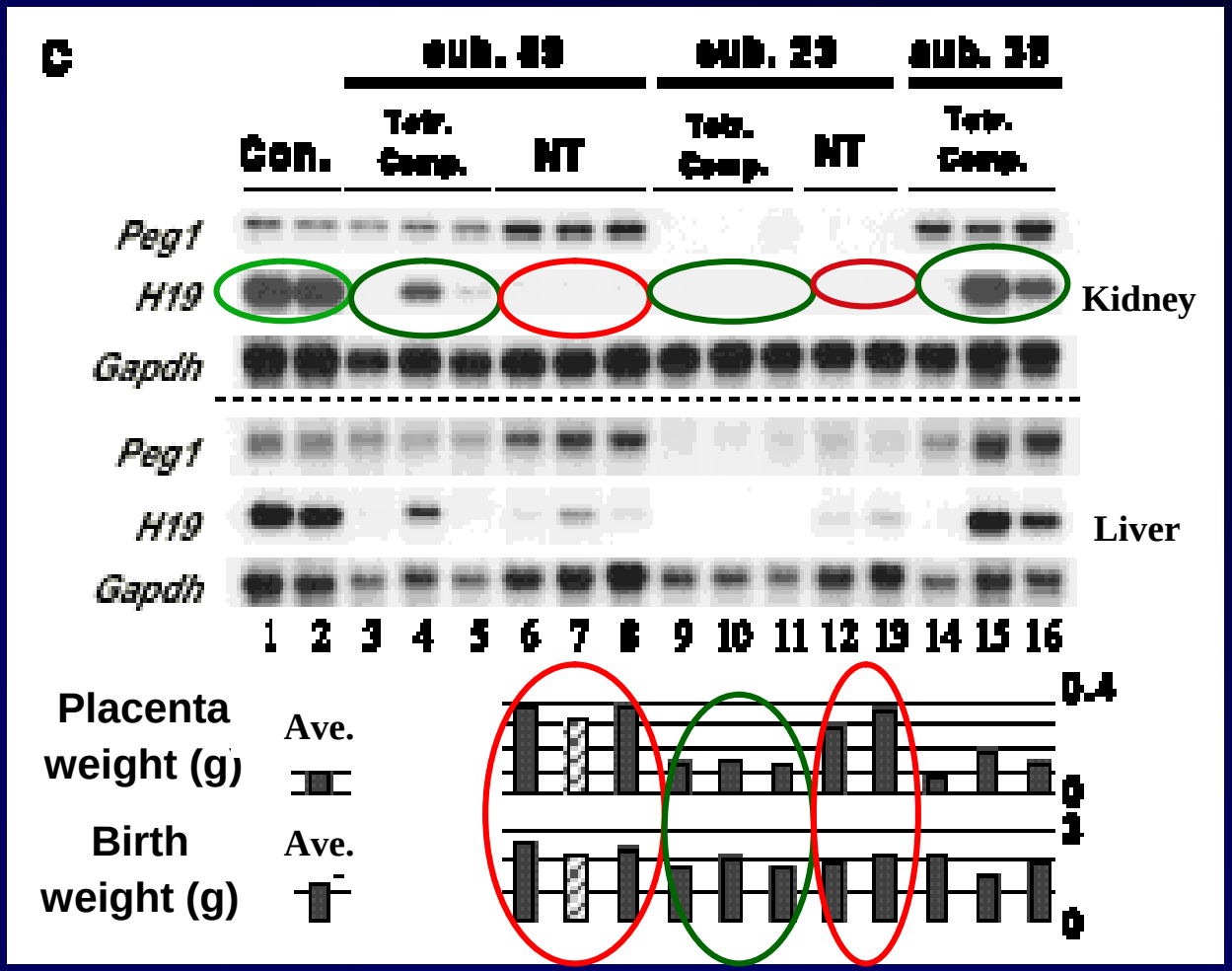
Reik *et al*., 2001



Methylation reprogrammimg in preimplantation embryos.

Reik *et al.*, 2001





H19 methylation and expression in subclones of the V6.5 ES cell line and expression in mice cloned from these subclones.

Humpherys *et al*., 2001

Double strain DNA size fractions from porcine embryos collected at various stages of development *in vivo*

Porcine embryos (≒ 200 blastomeres)

↓

Lysis

↓

Capturing mRNA by Dynal Oligo(dT) Beads

↓

CIP Treatment

↓

TAP Treatment

↓

RNA Oligo Ligation

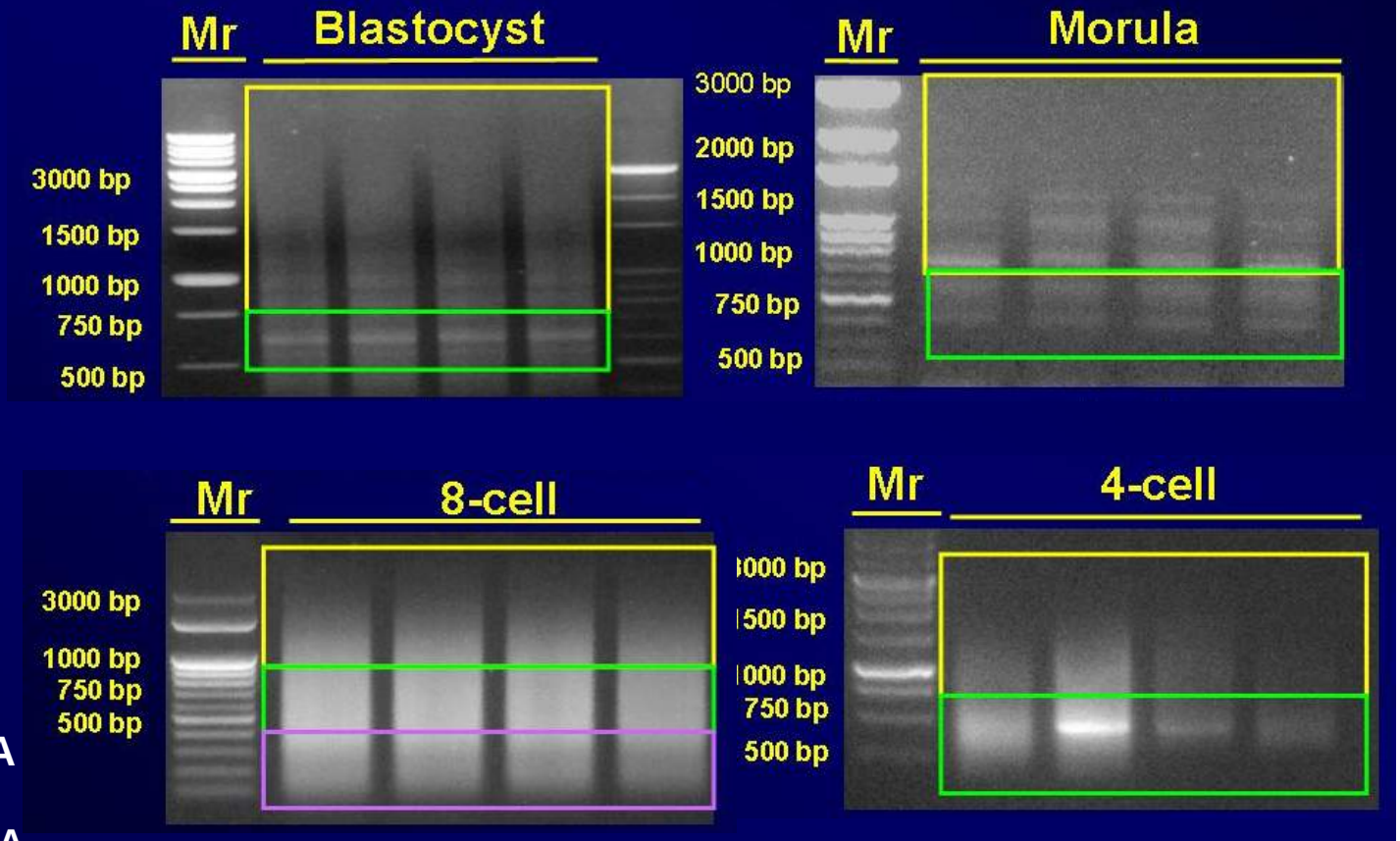
↓

1st strand cDNA Synthesis

↓

PCR to Amplify Full-length cDNA

4 PCR reactions were performed.
Each reaction used 2 ul of 1st cDNA
(about 1/10 of total 1st cDNA)

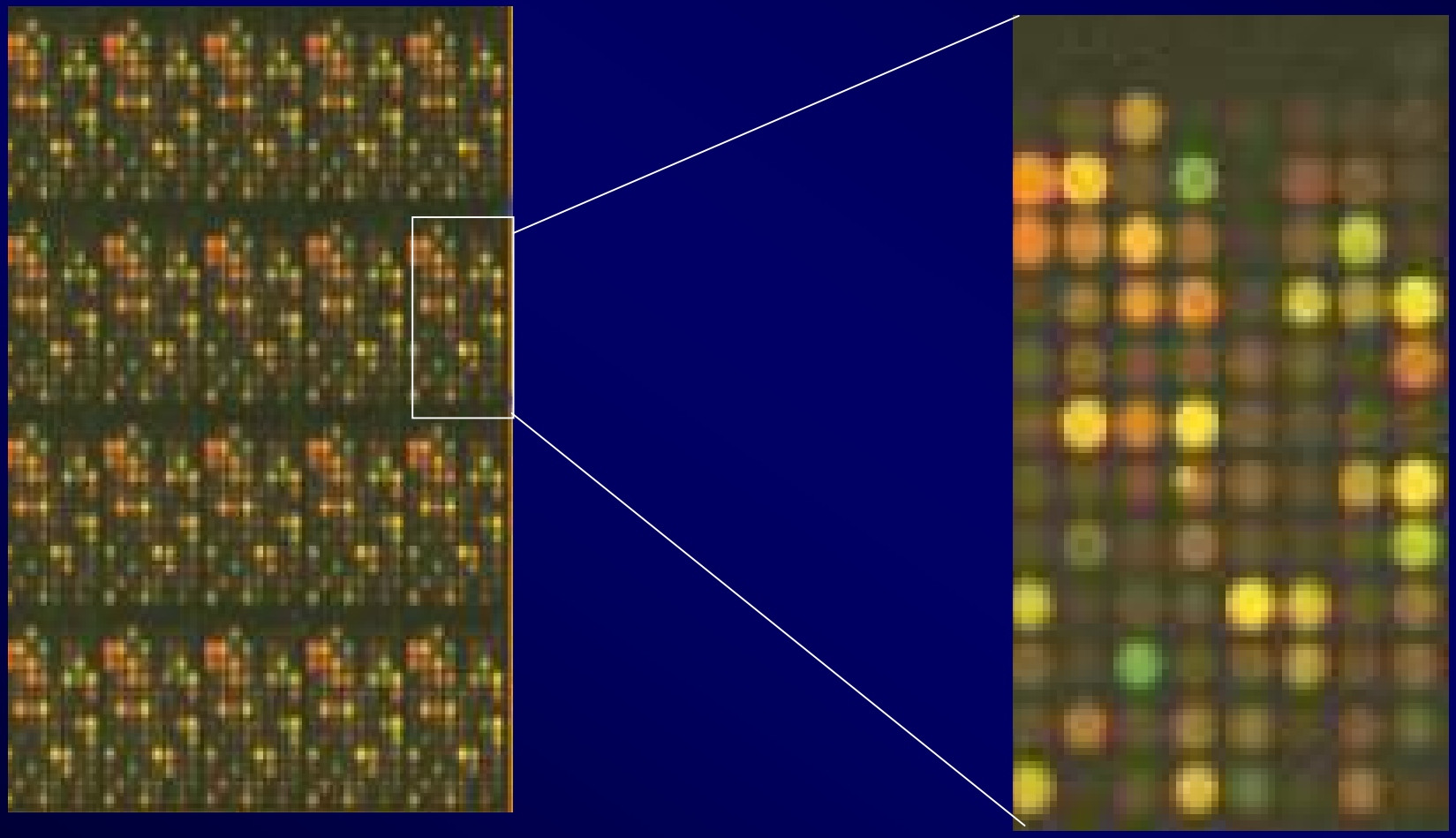


(Hsu *et al.*, 2005)

DNA sequencing on EST libraries constructed from various stages of porcine embryos

Stages of embryos for EST construct.	No. EST clones sequenced	No. of EST readed	Clusters	Singleton
Blastocyst	13,440	11,960		
Morula	18,816	15,581		
8-cell	14,976	6,433		
4-cell	18,432	8,422		
Oocyte	2,000	-		
Total	67,664	42,607	1,358	5,006

Development of gene chips based on full-length cDNA EST clones from porcine embryos



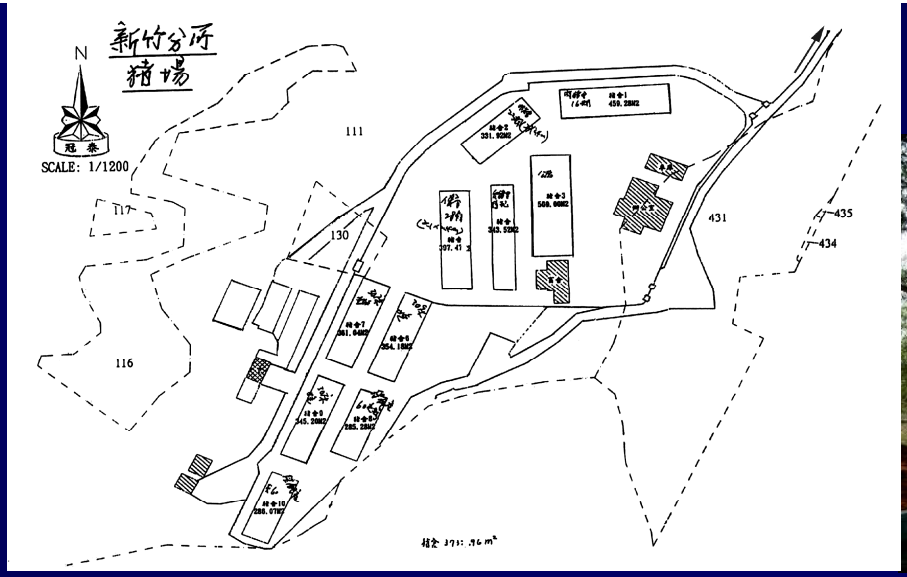
Future prospects:

To make various of large-animal models available for meeting to the purposes of biomedical research requested.

Establishment of large-animal models for biomedical research

1. **Pig models for research related to** liver-metabolic diseases
2. **Pig models for research related to** cardiovascular diseases
3. **Canine models for research related to** cancer diseases
4. **Pig models for research related to** drug screening
5. **Pig models for research related to** reproductive dysfunctions
6. **Pig models for research related to** regenerative medicine

Field-trait-core facilities for Bio-safety verification of Tg animals




場區入口


場區鳥瞰


SPF雞舍


豬舍參觀走道


分娩舍


公豬及待配舍

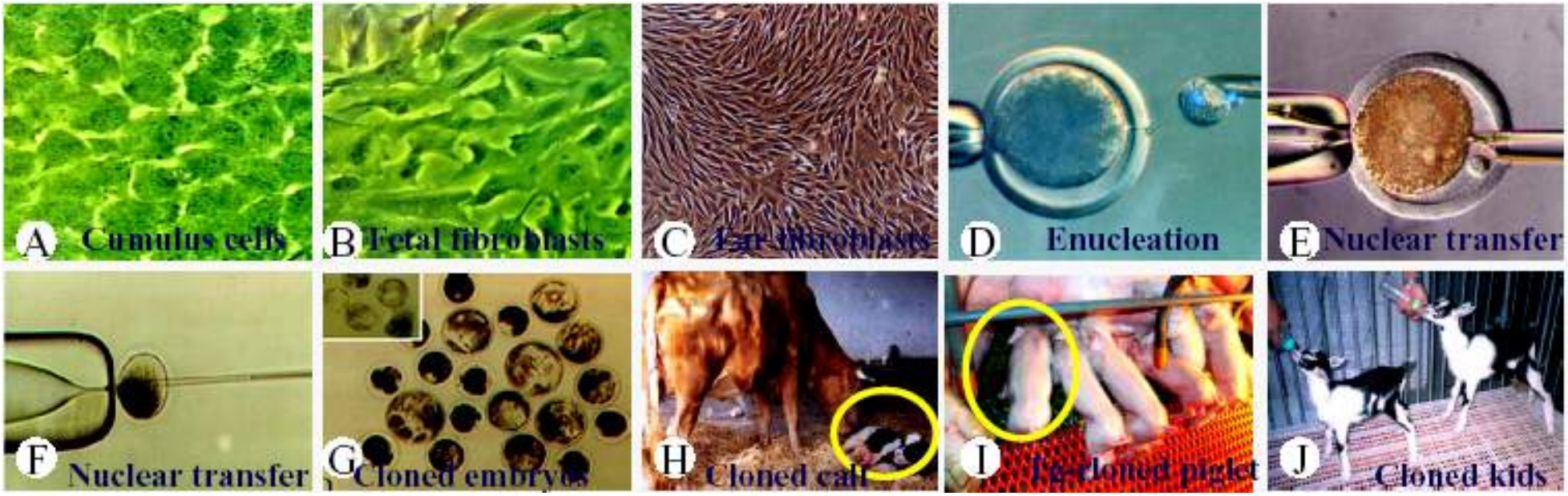

保育舍


基改豬、鷄之屠宰取樣場所

CONCLUSION

- Transgenic mice, pigs, dairy goats and/or dairy cow harboring transgene(s) of *αLA-pLF* , *αLA-hFIX* , *αLA-hFVIII, pAMY-CEL, pAMY-PHY* and/or allergic protein gene (Derp(s)) were successfully generated after microinjection of foreign gene(s) into their pronucleus and/or via the cloning strategy using ear muscle fibroblast cells after gene transfection.
- According to newborns obtained, an average transgenic efficiency around 20% is in common ; and the transgene(s) in Tg founders were confirmed to be highly germ line-transmitted.
- The bovine αLA promoter appeared to effectively direct the recombinant cDNA(s) at high levels of expression in mammary gland of the Tg mice, Tg pigs, and Tg dairy goats.
- The recombinant hFVIII and hFIX obtained from milk of Tg mice, Tg sows and Tg dairy goats all confirmed to possess coagulation activity equivalent to that obtained from human plasma.
- Studies related to examination the biological activities of recombinant cellulase and phytase expressed from Tg pigs harboring *pAMY-CEL*and *pAMY-PHY* transgene(s) are now in progression.
- Cloning by nuclear transfer in mammals using somatic cells has great potential applications in the coming future for fitting to purposes of cell-, tissue- and organ-engineering and gene therapy etc.

THANKS FOR YOUR ATTENTION



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