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Research article

Effective generation of transgenic pigs and mice by linker based sperm-mediated gene transfer.

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Abstract

Background: Transgenic animals have become valuable tools for both research and applied purposes. The current method of gene transfer, microinjection, which is widely used in transgenic mouse production, has only had limited success in producing transgenic animals of larger or higher species. Here, we report a linker based sperm-mediated gene transfer method (LB-SMGT) that greatly improves the production efficiency of large transgenic animals.

Results: The linker protein, a monoclonal antibody (mAb C), is reactive to a surface antigen on sperm of all tested species including pig, mouse, chicken, cow, goat, sheep, and human. mAb C is a basic protein that binds to DNA through ionic interaction allowing exogenous DNA to be linked specifically to sperm. After fertilization of the egg, the DNA is shown to be successfully integrated into the genome of viable pig and mouse offspring with germ-line transfer to the F1 generation at a highly efficient rate: 37.5% of pigs and 33% of mice. The integration is demonstrated again by FISH analysis and F2 transmission in pigs. Furthermore, expression of the transgene is demonstrated in 61% (35/57) of transgenic pigs (F0 generation).

Conclusions: Our data suggests that LB-SMGT could be used to generate transgenic animals efficiently in many different species.

Background

The introduction of foreign genes into animals forms the basis of a powerful approach for studying gene regulation and the genetic basis of development. Microinjection is the preferred method for introduction of a foreign gene into the mouse, a reliable technique developed by Gordon and his colleagues in 1980 [1]. Attempts to utilize this technology to produce transgenic livestock such as pigs, goats, sheep, and cattle have been made with only limited success due to low efficiency. Only 10–17% of transferred microinjected zygotes were born alive and less than 1% of them were transgenic animals (F0 generation) [2]. There are many reasons for this decrease in efficiency: low transgene integration rates, low embryo viability, and high skills requirement. Efficiency is critical because of the labor-intensive techniques and the high cost of animals. Other available gene transfer strategies for generating transgenic livestock include nuclear transfer and retroviral-mediated gene transfer. Unfortunately, all of these techniques have found limited applications. The present methods for nuclear transfer have low overall efficiency (typically between 0 and 3%) and are error prone as summarized by Wilmut [3]. High technical skills and intensive labor are also required. The problems associated with retroviral vectors are species specificity, transgene size limitation and inactivation, low titers, and public acceptance [4,5].

Sperm-mediated gene transfer was suggested by Brackett *et al.* as early as 1971 [6]. In 1989, Lavitrano *et al.* reported utilizing spermatozoa coated with exogenous DNA as vectors for *in vitro* fertilization to generate transgenic mice [7]. The report sparked wide spread excitement in the scientific community and a revolution in gene transfer technology was anticipated [8,9]. Since then, numerous efforts to duplicate these experiments have failed [10,11]. On the other hand, dozens of reports have been made in the past decade showing successful sperm-mediated transfer of foreign DNA into both non-mammalian and mammalian animals with or without modifications such as fusion with liposomes or electroporation (for recent reviews [12–14]). However, still lacking are the convincing and reproducible data for the exogenous DNA integration pattern, gene expression, and germ-line transmission.

In 1999 Perry *et al* generated transgenic mice with SMGT by utilizing detergent or a freeze/thaw process to disrupt the mouse sperm membrane, causing enhanced DNA binding and presumably entry of the foreign DNA into the sperm [15]. Nevertheless, the technique still required an efficiency limiting microinjection step [16]; i.e, the manual injection of the DNA coated sperm into the oocyte.

If DNA binding to sperm could be increased without interfering with fertilization, SMGT might become an efficient and simpler method of transgenesis. Receptor-mediated gene transfer was first demonstrated by Wu et al. [17] using polycation-conjugated asialoglycoprotein. The positive charges allowed binding to DNA's, large polyanionic molecules. This strategy had been successfully applied to many receptors and cells *in vitro* and *in vivo* using antibodies, transferrin, asialofetuin, galactose, folate, and other proteins (peptides) or carbohydrates (for recent reviews [18–20]). DNA coupled with antibodies or antibody-fragments offer the ability to target the selected cells and facilitate internalization of the complexes *via* receptor-mediated endocytosis. If a sperm reactive antibody with a basic region could be identified, it may possibly serve as a benign biological cross-linker between DNA and sperm.

We report here the production of a monoclonal antibody (mAb C) that can be used as a cross linker to facilitate the binding of exogenous DNA to sperm. Our data suggest that LB-SMGT can efficiently generate transgenic animals in all tested species.

Results

Generation of a monoclonal antibody capable of binding to the sperm of different species

We developed a monoclonal antibody by over-immunizing 6-week-old Balb/c mice with washed sperm from 12-week-old FVB/N mice collected by epididymal dissection. A hybridoma (mAb C) that did not interfere with *in vitro* fertilization in mice and was capable of binding to the mouse sperm cell surface was identified. The purified monoclonal antibody (mAb C) obtained from the ascites is a basic protein.

In order to demonstrate that mAb C could bind to a variety of sperm from different species, flow-cytometric analysis was performed. The histograms in Fig. 1 show the FITC fluorescent signal of mAb C treated sperm from all tested species shifted to the right compared to sperm with only secondary antibody and two other non-related mAbs from the same immunoglobulin class (data not shown). This suggests that mAb C can specifically bind to the surface of mammalian (pig, mouse, cow, goat, sheep, human) and avian (chicken) sperm. Interestingly, mAb C seems to bind to two to three populations of sperm cells in goat and sheep (Fig. 1e,1f). Since sperm has been reported to be a heterogeneous population [21–23], it is possible that different amounts of the antigenic surface protein are expressed. Besides human, we also noticed that a portion of the chicken and sheep sperm population did not interact with mAb C as can be seen by the FITC fluorescent signal at the background level of the 2° Ab negative control (shadowed areas under the solid lines which do not shift to the right). It is likely that some sperm do

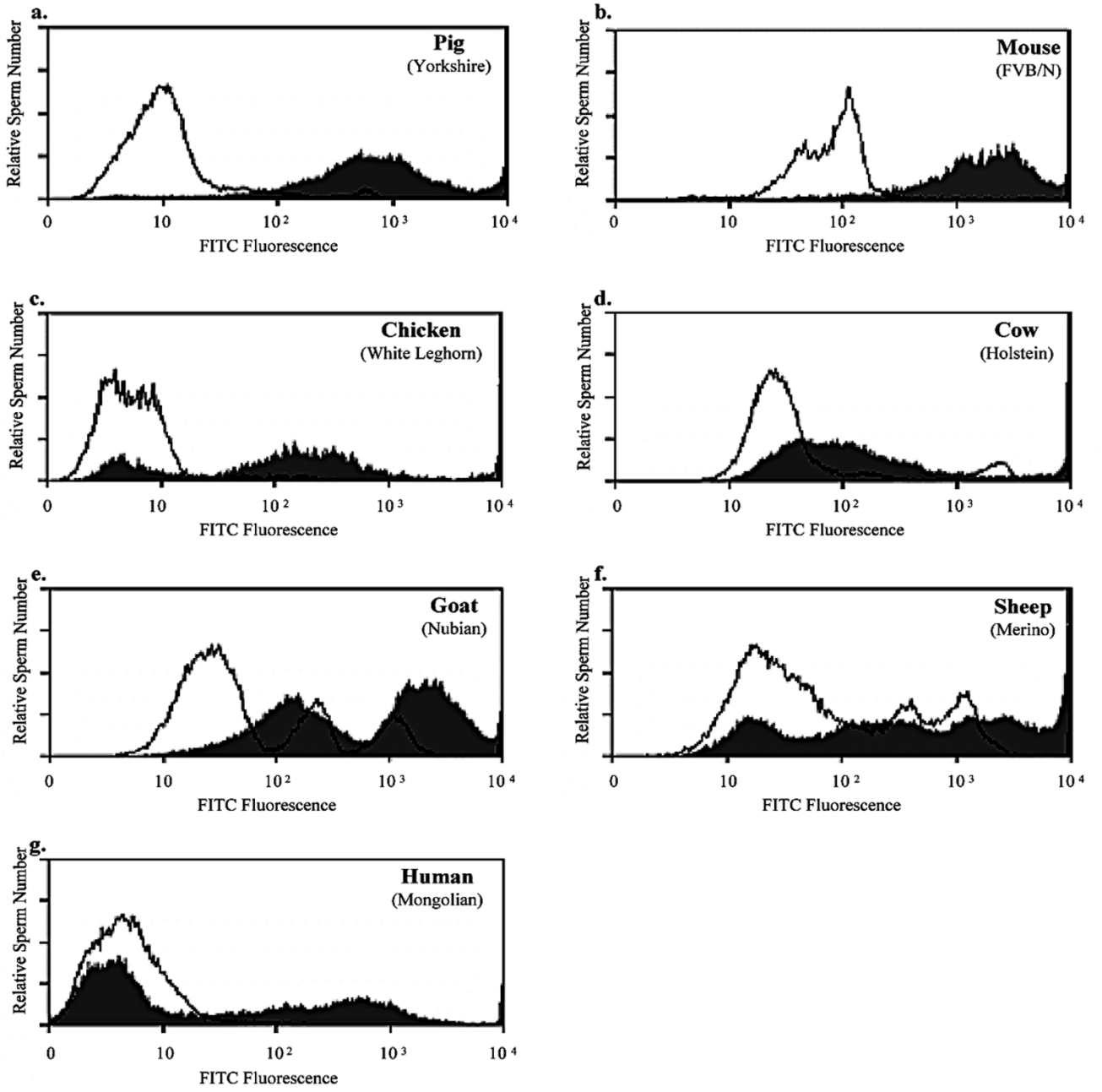

Figure 1

Flow cytometric analyses of mAb C binding to sperm from different species. Panels **a-g**, show results from flow-cytometric analyses of mAb C binding to sperm from pig (Yorkshire), mouse (FVB/N), chicken (White Leghorn), cow (Holstein), goat (Nubian), sheep (Merino) and human (Mongolian) respectively. Areas under solid lines represent FITC-conjugated goat anti-mouse immunoglobulin secondary antibody (2° Ab) (ICN) serving as a negative control in all sperm analyses. Shadowed areas represent mAb C and 2° Ab binding to sperm from different species. The experiments were performed at various times.

not express the surface protein in certain developmental stages and therefore cannot interact with mAb C.

Interaction between mAb C (linker protein) and DNA

mAb C is a positively charged, basic protein and all DNA molecules are negatively charged. To study mAb C's capability to interact with DNA, we added an increasing

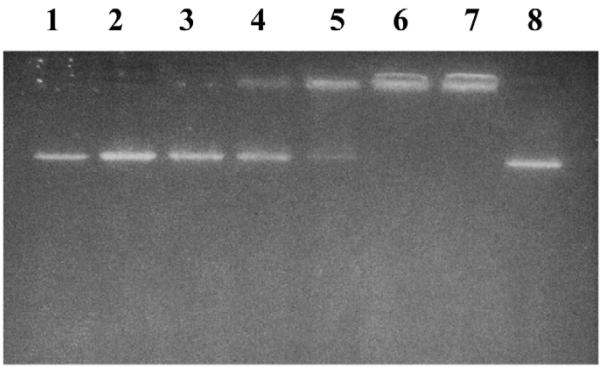

Figure 2
Ionic interaction analysis of mAb C and DNA. 0.3 μg of Sal I-linearized pSEAP-2 control DNA was mixed with water, medium or mAb C for 40 minutes at room temperature with occasional mixing and then subjected to 1% agarose gel electrophoresis. Lane 1: DNA in dH_2O; lane 2 and 8: DNA in Modified Tyrode's medium without BSA (MTM); lane 3: DNA plus 0.1 μg mAb C in MTM; lane 4: DNA plus 0.3 μg mAb C in MTM; lane 5: DNA plus 1 μg mAb C in MTM; lane 6: DNA plus 3 μg mAb C in MTM; and lane 7: DNA plus 10 μg mAb C in MTM.

amount of mAb C to a constant amount of Sal I-linearized pSEAP-2 control DNA encoding for secreted human alkaline phosphatase under the control of the SV40 early promoter and the SV40 enhancer. This resulted in its partial neutralization and the formation of an antibody-DNA complex with various electrical charges as can be seen when the mixtures are separated by agarose gel electrophoresis (Fig. 2). DNA smears caused by diverse rates of migration in the gel when compared with the controls can be observed in lanes 4 and 5. When the amount of added mAb C reached 3 μg or more in lane 6 and 7, the resulting complexes had a net electrical charge of zero and no longer responded to the electric field in the gel, remaining in the original sample loading point. This suggests that the DNA interacts with mAb C via ionic interaction.

To further demonstrate the ability of DNA to specifically bind to sperm *via* mAb C, we treated the sperm of pig, mouse, chicken, cow, goat, sheep, and human with mAb C or control antibodies. In the presence of mAb C, sperm from all tested species significantly bound 25~56% more ^{32}P-labeled DNA compared with control reactions without antibody ($p < 0.001$) or with a non-related mAb ($p < 0.0001$) (Table 1). This increase in specific binding might be critical to the success of LB-SMGT. It was observed that less DNA bound to chicken sperm, but this is not unexpected since the physical size of chicken sperm is less than one third that of mammalian sperm. A sizeable count was also observed in the sperm without mAb C treatment. It has been reported that a certain level of non-specific ionic interaction exists between DNA molecules and various sperm surface proteins of non-mammals and mammals [13,14]. However, our data indicates that mAb C may critically enhance DNA binding to sperm through a specific sperm surface antigen.

Generation of transgenic pigs by surgical oviduct insemination

For this study, Duroc, Yorkshire, and Landrace female pigs (gilts) between 10 and 14 months old were selected for LB-SMGT experiments using surgical oviduct insemination (summary in Table 2). Forty-three offspring (#1–43) from 7 gilts (total 27 gilts were inseminated with a 26% pregnancy rate) were obtained in an initial experiment. To ensure detection of all transgenic samples, including those with a single copy of the transgene, we have examined genomic DNA from the tail region of piglets with Bgl I digestion, which has two internal restriction sites in the pSEAP-2 control DNA. Fig. 3a shows that ten out of seventy-five (13%) F0 generation piglets: 5 (not shown), 17, 26, 36, 40, 42, 43, 44, 48 and 64, had a 1.3 kb positive hybridization band. Animals 44–75 were obtained from a separate experiment (32 offspring from 8 gilts, total 30 gilts inseminated with a 28% pregnancy rate) to demonstrate reproducibility. Furthermore, analysis of sperm genomic DNA from a limited number of 8-month-old F0 generation animals (boars) showed that two out of eleven (18%), numbers 25 and 30, had a hybridization signal (data not shown).

As an alternative method to detect transgenic pigs, we have also looked for the expression of heat stable SEAP in the sera collected from the 70-day-old F0 generation animals. The results are summarized in Fig. 4. In the control study, the chemiluminescent signals range from 4×10^6 to 2×10^7 RLU when assayed without the heat deactivation step as displayed in the top panel of Fig. 4. There was no obvious difference in the level of total alkaline phosphatase activity between the genetically modified F0 generation animals and nontransgenic controls, nor was any difference observed in the controls of the different breeds. The bottom panel of Fig. 4 shows the results of an assay after heat inactivation of the endogenous alkaline phosphatase activity. A reading of more than 2×10^4 RLU of SEAP activity was set as positive in the genetically modified pigs, which is significantly higher than in the nontransgenic controls ($6.9 \times 10^3 \pm 3.7 \times 10^3$ RLU). Thus, twenty-one out of thirty-six (58%) group one piglets (F0 generation) were shown to express the thermostable human SEAP enzyme. The sera from the second group of F0 generation animals (#44–75) were assayed and showed similar results: fourteen out of twenty-two (64%) pigs showed comparable levels of expressed SEAP ($>2 \times 10^4$ RLU) (data not shown). In both experiments, due to the

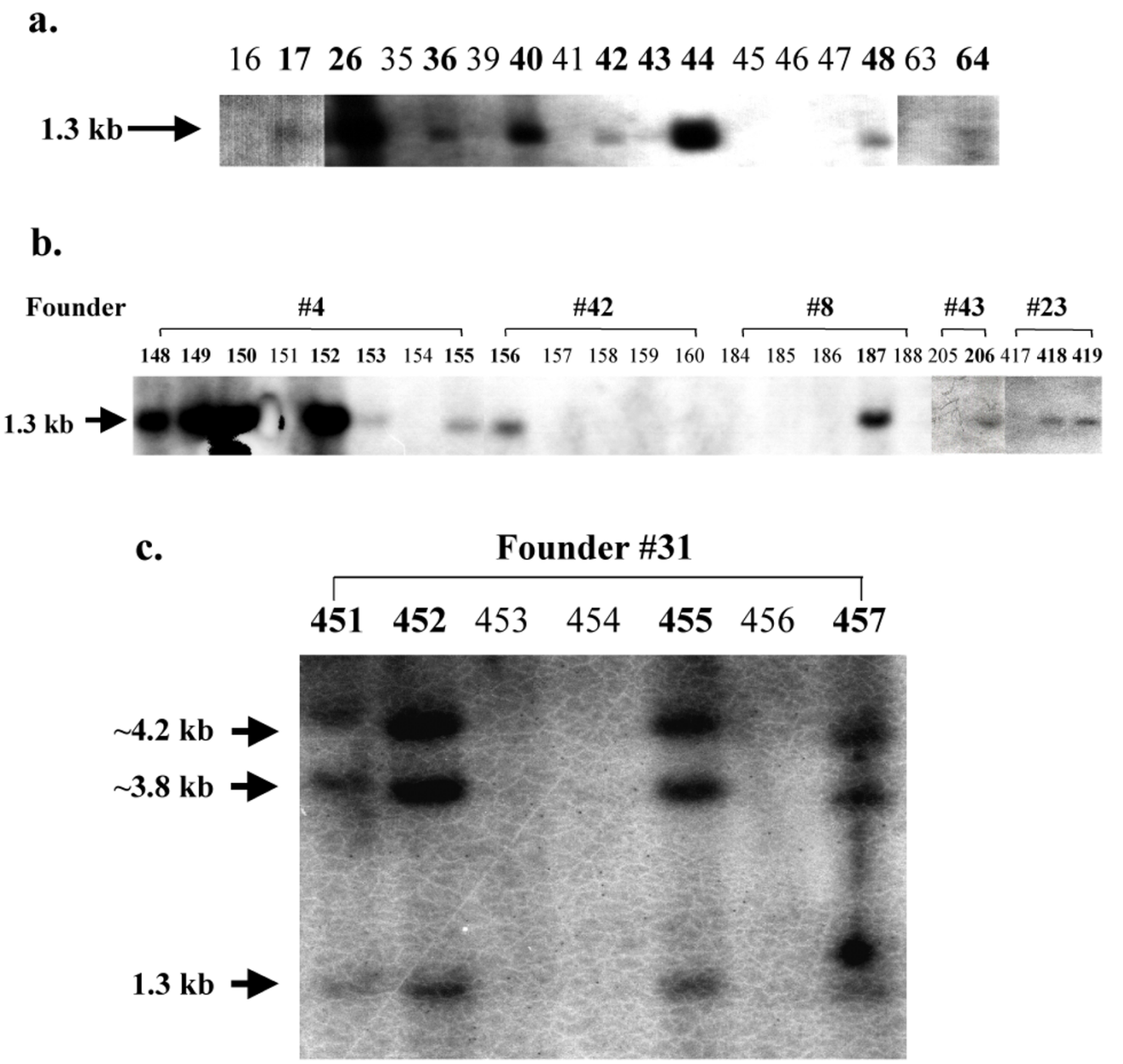

Figure 3
Southern blot analysis of transgenic pigs. a, Tail genomic DNA of group I and II F0 generation animals digested with Bgl I. **b**, and **c**, Ear genomic DNA from group I offspring (F1) digested with Bgl I. The numbers in bold indicate positive detection on the blot.

mosaic condition in many of the transgenic pigs, the number of F0 generation animals expressing human SEAP in their sera (average 60%), which might be secreted from different organs or cells, is greater than the number of animals found to be positive (13%) by Southern blot analysis of a small localized area (tail tip).

The amount of expressed human SEAP in the transgenic pigs' sera was estimated using a standard curve. We select-

Table 1: DNA binding assay.

Treatments	No mAb		Non-related mAb		mAb C	
Species	**(cpm)**	**Average**	**(cpm)**	**Average**	**(cpm)**	**Average**
Pig (Yorkshire)	12,971	13,342	10,531	12,285	16,269	16,806
	13,713		14,038		17,343	
Mouse (FVB/N)	12,471	14,143	11,541	12,597	18,139	18,727
	15,814		13,653		19,314	
Chicken (Leghorn)	5,830	6,107	N/D	N/D	7,294	8,580
	6,383		N/D		9,865	
Cow (Holstein)	12,766	13,582	15,351	14,635	20,417	19,530
	14,398		13,918		18,643	
Goat (Nubian)	17,749	17,162	15,374	15,686	20,385	19,964
	16,574		15,997		19,543	
Sheep (Merino)	15,367	14,313	14,018	13,426	19,368	18,903
	13,259		12,834		18,437	
Human (Mongolian)	10,518	11,225	9,865	11,111	16,439	17,353
	11,932		12,357		18,266	

Table 2: Summary of transgenic pig analysis.

		F0 Generation				F1 Generation		
Animals (F0)	**Strain**	**Sex**	**Southern* (Tail)**	**Southern* (Sperm)**	**SEAP Assay****	**Southern (Ratio)**	**Positive Pigs**	**Littermate**
1	D	M	-		+			
2	D	M	-		-			
3	D	M	-		+++			
4	D	M	-		-	7/16	148–150, 152, 153, 155, 474	148–155, 469–476
5	D	M	+	-	-			
6	D	F	-		+			
7	D	F	-		-			
8	Y	M	-	-	-	1/5	187	184–188
9	Y	M	-	-	-			
10	Y	M	-	-	+			
11	Y	M	-	-	+	0/7		138–144
12	Y	M	-	-	-			
13	Y	F	-		NA			
14	Y	F	-		+	0/12		460–468, 477–479
15	Y	F	-		+++			
16	Y	M	-		+++			
17	Y	M	+		+++			
18	Y	F	-		-	0/10		401–410
19	Y	M	-		+++			
20	Y	M	-	-	+			
21	Y	M	-	-	+			
22	Y	F	-		++	0/12		215–226
23	Y	F	-		+++	2/15	418, 419	411–425
24	Y	M	-		++			
25	Y	M	-	+	+++	0/3		198–200

Table 2: Summary of transgenic pig analysis. ***(Continued)***

26	Y	F	+		++	0/10		145, 430–438
27	Y	F	-		NA			
28	Y	F	-		NA	0/8		381–388
29	Y	F	-		-	0/2		166, 167
30	Y	M	-	+	-			
31	Y	M	-		++	7/30	441, 442, 448, 451, 452, 455, 457	439–459, 480–488
32	Y	F	-		NA			
33	Y	F	-		+	0/6		389–394
34	Y	M	-	-	-			
35	Y	M	-	-	+++			
36	L	F	+		NA			
37	L	F	-		NA			
38	L	M	-		-			
39	L	M	-		-			
40	L	M	+		NA			
41	L	F	-		-	0/9		175–182, 197
42	L	F	+		-	1/5	156	156–160
43	L	F	+		++	1/9	206	201–209
44	D	M	+		+			
45	D	M	-		-			
46	D	F	-		+			
47	D	F	-		NA			
48	D	F	+		+			
49	D	F	-		+++			
50	D	F	-		+++			
51	D	M	-		++			
52	D	M	-		++			
53	D	M	-		+++			
54	D	M	-		NA			
55	D	F	-		+			
56	D	F	-		+			
57	D	F	-		-			
58	D	F	-		++			
59	D	M	-		+++			
60	L	F	-		NA			
61	L	M	-		NA			
62	L	F	-		NA			
63	L	M	-		NA			
64	L	M	+		NA			
65	L	F	-		-			
66	L	F	-		NA			
67	L	F	-		NA			
68	L	M	-		-			
69	L	M	-		-			
70	L	M	-		++			
71	L	F	-		-			
72	L	M	-		NA			
73	L	F	-		-			
74	L	M	-		-			
75	L	M	-		+			

D: Duroc; Y: Yorkshire; L: Landrace. *: + or - indicated detectable or undetectable hybridization signal in Southern blot analysis. **: - represents SEAP enzyme activity less than 2×10^4 RLU; + represents SEAP enzyme activity between $2–4 \times 10^4$ RLU; ++ represents SEAP enzyme between $4–6 \times 10^4$ RLU; +++ represents SEAP activity more than 6×10^4 RLU.

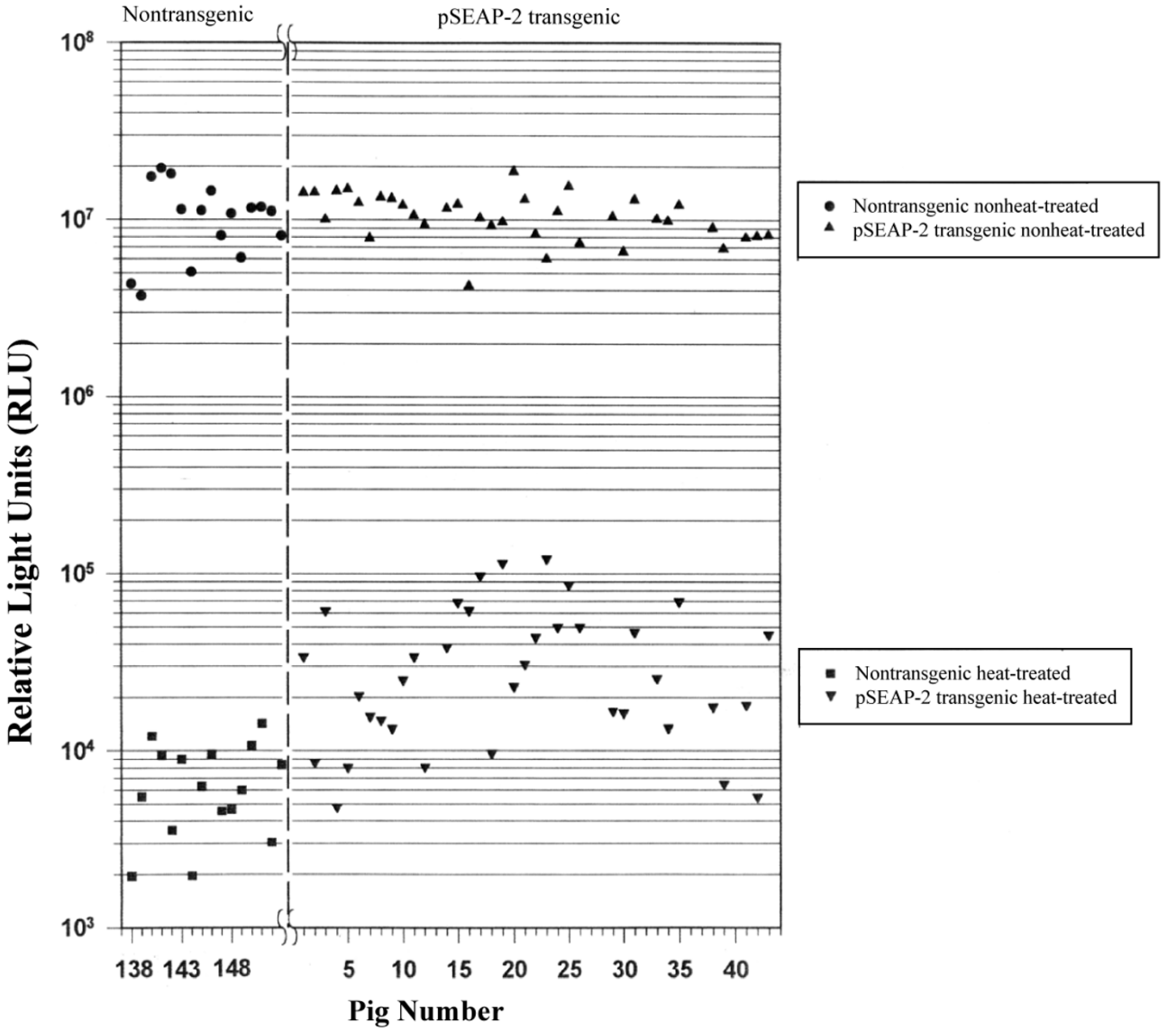

Figure 4
Expression of secreted human alkaline phosphatase (SEAP) from the transgenic pigs. Sera from 70-day-old piglets from nontransgenic controls and group I F0 generation animals were analyzed by using Great Escape SEAP Chemiluminescence Detection kit (Clontech Laboratories Inc). The average reading for controls is $6.9 \times 10^3 \pm 3.7 \times 10^3$ RLU. (●) represents nontransgenic nonheat-treated sera. (▲) represents transgenic nonheat-treated sera. (■) represents nontransgenic heat-treated sera. (▼) represents transgenic heat-treated sera. The breed, number, and sex of nontransgenic controls are: Duroc, 144(F), 145(F), 146(M), 153(M), 161(F), 162(M) and 163(M); Yorkshire, 141(M), 142(M), 143(M), 150(M), 151(F) and 152(F); Landrace, 138(M), 139(M), 140(M), 147(M), 148(M), 149(M), 156(F), 157(F), 158(F), 159(F) and 160(F).

ed founders with a human SEAP level between 2×10^4 RLU to 1.2×10^5 RLU as samples. Therefore, 18~112 ng of expressed SEAP was detected in 0.25 ml of serum sample from the 70-day-old pigs. The average weight of the 70-day-old pigs was 27.2 kg. If we assume that pig weight (kg) to pig blood volume (liter) is 13 : 1 and the ratio of blood volume to serum volume is 2 : 1, we can estimate that the transgenic pigs expressed 75.3 – 468.6 μg of human SEAP per pig. Moreover, since the animals were mo-

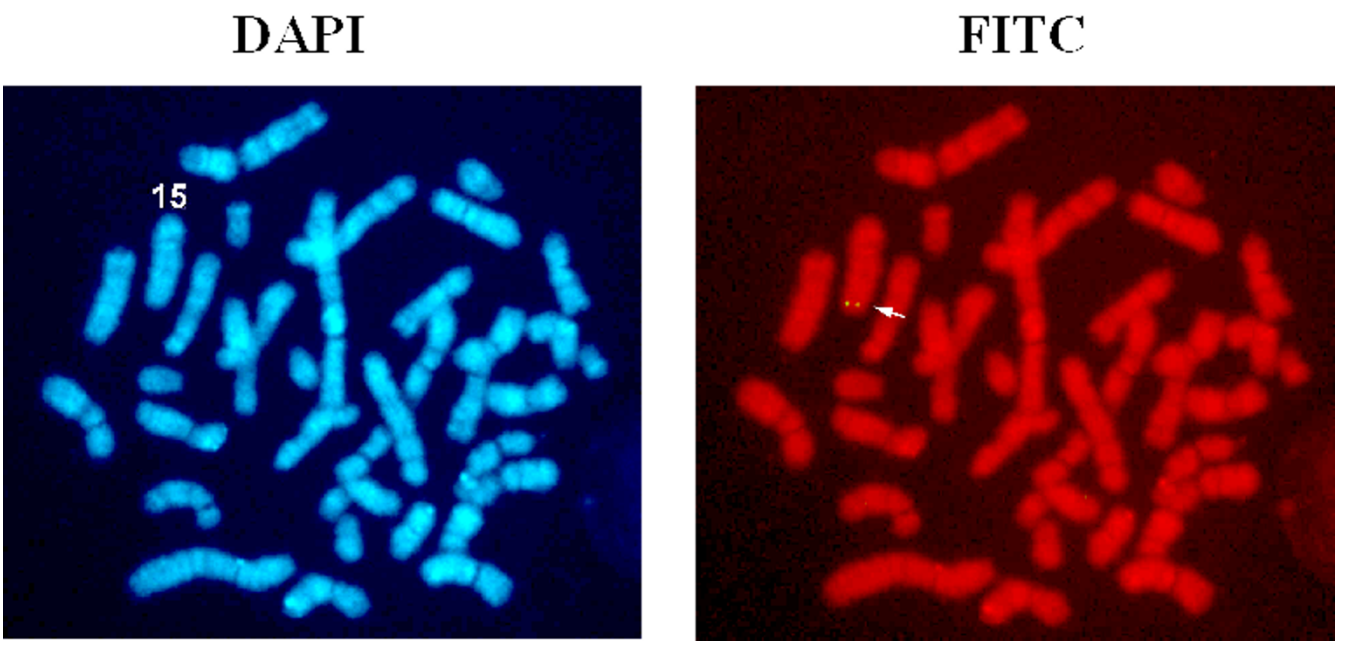

Figure 5
FISH analysis for transgene localization. The transgene was located to chromosome 15, region q25–q28, in TG pig #152 (F1 generation) by FISH. A 3.1 kb EcoR I/Sal I DNA fragment from the plasmid vector region of the pSEAP2-Control (# 2308–260) was used as a probe. The standard FISH experiment was performed by SeeDNA Biotech Inc. (Windsor, Ontario, Canada). The detailed localization was further determined based on the summary from ten photos. DAPI (4', 6'-Diamidino-2-phenylindole Dihydrochloride) is a DNA-specific fluorescent dye. FITC: fluorescein isothiocyanate.

saic, the potential transgene expression may be even higher.

In a subsequent study, sixteen F0 generation animals were randomly selected to mate with wild type pigs to estimate the transmission rate of the germ-line. The F1 generations from six (37.5%) animals from all three breeds were found to carry a 1.3 kb positive hybridization band (Fig. 3b) without gender selection (Table 2). Interestingly, the offspring of F0 #4 had a variety of hybridization intensities. This is indicative of multiple site transgene integration. We also observed extra high molecular weight bands in the offspring of F0 #31 (Fig 3c). Most likely, DNA rearrangement occurred in this case. Different intensities of hybridization signals on the same blot were also seen (Fig 3b). The estimated copy number in the F1 animals is 1~5, except for some offspring from F0 #4 (10~20 copies) (data not shown). This suggests that different copies of the transgene have integrated into the pig genome. As is common with the mouse microinjection method, we observed a high germ-line transmission rate, multiple site insertion, rearrangement, and multiple DNA-copy insertion using LB-SMGT in pigs. In addition, F0 #23, 42, and 43, but not #25, 26 and 33, which were scored positive by Southern blot or SEAP activity were able to transmit the transgene to some of their offspring (a limited number) as determined by Southern blot. Conversely, F0 #4 and 8, which didn't show any detectable hybridization signal against DNA from a small piece of tail, managed to pass the transgene to their offspring. This indicates that a number of transgenic pigs generated by LB-SMGT are mosaic and still have germ-line transmission capability. It is possible that the mosaicism we observed is due to transgene integration at various stages of early embryonic development.

To further demonstrate that the transgene was permanently integrated into pig genomic DNA, a FISH (fluorescence *in situ* hybridization) experiment was performed using a blood sample from a transgenic F1 pig (#152). As shown in Fig. 5, the transgene was located to chromosome 15, region q25–q28. Moreover, two out of nine progeny (F2) of this TG pig showed positive transgene hybridization bands by Southern blot analysis (Fig. 6). Therefore, we have demonstrated that the transgene can be stably transmitted to future generations in this line of pigs.

Generation of transgenic mice by in vitro fertilization

Generating transgenic mice by microinjection has been widely used in studies of gene function and regulation. To see if our LB-SMGT method can also be used in mice, FVB/N mouse sperm was collected by epididymal dissection and mixed with mAb C to form a sperm-mAb complex,

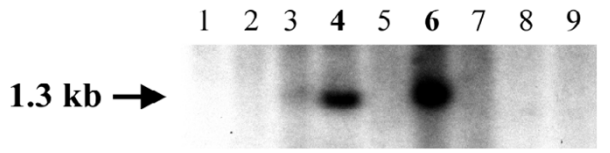

Figure 6
Southern blot analysis of F2 generation transgenic pigs. Genomic DNA from the ear of F2 offspring from TG pig #152 digested with Bgl I. The numbers in bold indicate positive detection on the blot.

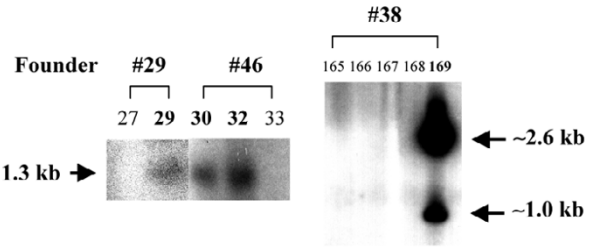

Figure 7
Genotype analysis of transgenic mice. Southern blot analysis of transgenic mice from F1 generation. The genomic DNA was isolated from 6-week-old mouse-tails and digested with Bgl I. The 1.3 kb Bgl I fragment of pSEAP-2 control DNA was used as a probe. The numbers in bold indicate positive detection on the blot.

then SalI linearized pSEAP-2 control DNA was added and in vitro fertilizations were performed. Initial screening by PCR showed 33% of the 10-day-old embryos carried the transgene (data not shown). Forty-seven F0 pups were born but no hybridization signal was detected in their tail samples by Southern blot. However, four (33%) transmitted transgenic mouse lines (F1) were found after twelve F0 generation animals were randomly selected to mate with wild type mice (Fig. 7). We also noticed that one out of five offspring from F0 #38 showed multiple hybridization bands (about 1 and 2.6 kb) indicating DNA rearrangement (Fig. 7). This may be a higher frequency of mosaicism than most published results in FVB/N mice produced by the microinjection method. In summary, our studies show that in the FVB/N inbred mice, LB-SMGT is a viable method for generating transgenic mice, although we observed a low fertilization rate (FVB/N is known as a difficult strain for IVF but is widely used for microinjection), few founders, and a high frequency of mosaicism. We may be able to improve the fertilization rate and simplify the method by using alternatives to in vitro fertilization such as oviduct, uterine, or artificial insemination. Since mAb C bound to all tested sperm from various species, LB-SMGT should be applicable with different strains of mice as well as with other rodents such as the rat.

Discussion

Our data show that transgenic animals such as pigs and mice can be generated efficiently, simply, and easily by LB-SMGT when compared to current techniques. Germline transmission to the F1 generation, 37.5% in pigs and 33% in mice, was highly efficient. Preliminary data using LB-SMGT through artificial insemination indicates the presence of the transgene in 49% (44/90) of chicken embryos by PCR (data not shown) and expression of the transgene, human interferon β, was detected in 53% (18/34) of chicks (F0 generation, data not shown). In addition, our flow cytometry and DNA binding data demonstrated that DNA could specifically bind to sperm *via* mAb C from all tested species, from birds to mammals including human. Therefore, our data suggests that LB-SMGT could be used to generate transgenic animals efficiently in most, if not all, species.

The standard protocol of artificial insemination for pigs requires a large volume of liquid (50–200 ml) and a large number of spermatozoa ($5–10 \times 10^9$ per insemination dose) due to the cervical folds, length and coiled nature of the uterine horns. Therefore, we chose surgical oviduct insemination in most of our pig experiments. However, we tried a limited number of sows using artificial insemination with about 10^8 spermatozoa in 10 ml with 10 μg linear DNA and 10 μg mAb C per insemination (less spermatozoa cause a low fertility, about 50%). Very encouraging data were obtained from the initial screening of F0 animals. The transgene was detected in a similar rate as using surgical oviduct insemination by Southern blot analysis. Recently, a deep intrauterine insemination technique, which dramatically reduces the number of sperm and volume required for conventional artificial insemination, has been reported [24]. It is our hope to adopt this new technique with our LB-SMGT in generating future transgenic pigs.

Surgical oviduct insemination, *in vitro* fertilization, and artificial insemination have all been recognized as simple and successful techniques for generating offspring in animals for many years. Here, we demonstrated the efficacy of using these techniques in combination with the LB-SMGT technology to generate transgenic animals such as pigs and mice. The LB-SMGT technology that we have described here has a universal application for producing transgenic animals in different breeds (Duroc, Yorkshire, and Landrace) of pigs and different species of animals (pig, mouse, and chicken) with less labor, skill requirement, and cost but with a high rate of success. Therefore,

a number of useful genes could be easily introduced into livestock to benefit modern agriculture and medicine.

Our data demonstrate that exogenous DNA is specifically bound to the sperm cell surface *via* the linker protein (mAb C) through ionic interaction. It has been suggested that polycationic reagents could spontaneously condense DNA into small particles known as polyplexes [25]. The antibody cross-linker may provide protection from DNAase activity designed to prevent foreign DNA from entering the egg [26,27]. It has been reported that part of the bound DNA is internalized into the sperm using SMGT [28]. However, it is yet unclear whether internalization into sperm is an essential process for transgene chromosomal integration and how mAb C affects this process. Exogenous DNA molecules may dissociate from the linker protein due to a change in pH, proteolysis, or other unknown mechanisms in the fertilized egg. This free DNA or possibly even linker-bound DNA might be integrated into the chromosome during early phases of embryonic development. This raises the interesting question of whether there is any similar linker existing in nature. If it exhibits a high gene transfer rate as observed in our experiments, such a linker might play a role in the proposed lateral gene transfer. The recently sequenced human genome contains as many as 223 bacterial genes that may have been laterally transferred into the human genome [29].

Materials and Methods

Flow cytometry analyses

The animal species used in this study were pig (Yorkshire), mouse (FVB/N), chicken (White Leghorn), cow (Holstein), goat (Nubian), sheep (Merino), and human (Mongolian). One million spermatozoa were distributed in 0.3 ml extender (dilution buffer) + 1.5 mg/ml BSA (extender formula varies per farm animal) and then mixed with 3 μg of purified mAb C primary antibody. The mixture was incubated for 40 min with occasional mixing. After one wash and centrifugation at 3000 rpm for 1.5 min to remove unbound mAb C, 0.3 ml of fluorescent (FITC) conjugated goat anti-mouse secondary antibody (2°Ab) (ICN Pharmaceuticals Inc., Costa Mesa, CA) diluted 1: 20 was added and incubated for 40 min with occasional mixing. After removal of unbound 2°Ab, samples were subjected to flow cytometry analysis with a MoFlo cytometer (Cytomation Inc., Fort Collins, CO). Sperm with two non-related immunoglobulins from the same class as mAb C and 2° Ab only were included in all experiments as negative controls.

DNA binding assay

3 μg of mAb C or a non-related control mAb were mixed with 2×10^6 sperm from different species in a reaction containing 300 μl of incubation buffer (extender + 1.5 mg/ml BSA) and incubated for 40 min at room temperature with occasional mixing. After washing twice with centrifugation at 3000 rpm for 1.5 min, the sperm pellet was resuspended in incubation buffer containing 500 ng of ^{32}P-labeled pSEAP-2 control DNA (Clontech Laboratories Inc., Palo Alto, CA; catalog #6052–1, vector map available at Clontech web site) made by T_4 DNA polymerase, and incubated for another 40 min. After washing the sperm twice with incubation buffer to remove unbound ^{32}P-labeled DNA, samples were measured by liquid scintillation counting. The data were statistically analyzed by paired t-test and a two-tailed p-value was drawn.

Surgical oviduct insemination of pigs

The standard protocol of pig surgical oviduct insemination was followed [30]. Briefly, on day 1 at 5:00 pm, every pig was injected with 1000 I.U. of PMSG. On day 4 at 5:00 pm (72 hr later), 1000 I.U. of hCG was given to every pig to stimulate ova production. On day 6 at 10:00 am, fresh semen was collected and mixed with extender (E Merck, Darmstadt, Germany) in a 1:1 ratio at 35°C. After cooling to room temperature, 1.5×10^7 sperm in 1 ml of incubation buffer (extender + 1.5 mg/ml BSA) were transferred into a 15 ml-Falcon tube and mixed with 5 μg of mAb C for 40 min with occasional gentle mixing. Then the tube was left undisturbed for 30 min. After most spermatozoa settled to the bottom to form a pellet-like precipitate, the supernatant containing unbound mAb C was removed. 0.9 ml of incubation buffer was added to the tube for an additional washing and steps were repeated as above. About 50% of the spermatozoa were lost during the washing process. Finally, the sperm precipitate was combined with 1.3 ml of incubation buffer containing 3 μg of Sal I-linearized pSEAP-2 control DNA and incubated for 40 min with occasional gentle mixing. Approximately 3 hours from the time of collection, 0.2 ml of the sperm-mAb C-DNA mixture (about 1.2×10^6 sperm) was injected into each side of the oviduct of an anesthetized ovulating pig. 7–10 pigs were surgically inseminated per day.

Southern blot analysis for transgenic pigs and mice

Genomic DNA was extracted from the tail or ear of animals using a standard phenol-chloroform extraction protocol. 10 μg of DNA was digested overnight with 25 units of Bgl I. The DNA samples were separated on a 0.8% agarose gel, and transferred onto a nylon membrane (Osmonics Inc., Minnetonka, MN) using a TurboBlot unit (Schleicher & Schuell Inc., Keene, NH). A 1.3 kb Bgl I DNA fragment (bp 3571–4839) corresponding to the plasmid region of pSEAP-2 control DNA was used as a template, to avoid any cross hybridization with endogenous phosphatase gene(s). The ^{32}P-dCTP Prime-it II kit (Stratagene, La Jolla, CA) was used for random priming. Hybridization was carried out at 42°C in UltraHyb hybridization buffer (Ambion Inc., Austin, TX) following the manufacturer's instructions.

In vitro fertilization of mice

The standard protocol of mouse *in vitro* fertilization was followed [31,32]. Briefly, on day 1 at 8:00 pm, 20 FVB/N female mice (9~12 weeks old) were each injected with 5 I.U. of PMS (Sigma, St. Louis, MO). After 48 hours, each mouse was given 5 I.U. of hCG (Sigma). On day 4 at 7:30 am, a FVB/N male mouse (9–20 weeks old) was sacrificed, the epididymis was dissected out, and sperm were collected. Approximately 0.3~1 million spermatozoa were incubated in 300 μl of Modified Tyrode's medium (pH 7~8) for 1 hour to capacitate them. Then, 3 μg of mAb C were added and incubation was carried out for another 40 min at 37°C with occasional gentle mixing followed by centrifugation at 3000 rpm for 1.5 min to remove supernatant. 300 μl of Modified Tyrode's medium was used to wash away any unbound mAb C. The sperm pellet was resuspended in 300 μl of Modified Tyrode's medium, 1 μg of Sal I-linearized pSEAP-2 control DNA was added, and the mixture was incubated for 40 min at 37°C with occasional gentle mixing. All females were sacrificed at 13.5 hours post-hCG treatment; their ovulated eggs were dissected out, combined with 300 μl of the sperm/mAb/DNA mix and incubated for 4 hours at 37°C to allow fertilization to occur. Fertilized eggs were washed with CZB medium three times and incubated in 300 μl of CZB medium for 20–22 hours. On day 5, 2-cell stage embryos were transferred to the oviduct of pseudopregnant CD-1 females.

Author contributions

KC carried out the pig farm operation and participated in its design and coordination. JQ carried out the molecular genetic studies, drafted the manuscript, and participated in its design. MSJ carried out the initial molecular genetic studies and participated in its initial design. YHL carried out the initial mouse studies and participated in its initial design. MCW carried out the surgical oviduct insemination of pigs. CDC, CKL, HLL, CTH, PYH, PYS, CWY, WJL, CHC, FYW, and YJL participated in the pig farm operation and its molecular genetic studies. LB and JB Jr. carried out the flow cytometry analysis. HIH and JX participated in the mouse and chicken studies. KW conceived of the study, drafted the initial manuscript, and participated in its design and coordination. KC, JQ, MSJ, YHL all contributed equally to this work. All authors read and approved the final manuscript.

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