

## Production of transgenic piglets using ICSI–sperm-mediated gene transfer in combination with recombinase RecA

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### Abstract

Sperm-mediated gene transfer (SMGT) is a method for the production of transgenic animals based on the intrinsic ability of sperm cells to bind and internalize exogenous DNA molecules and to transfer them into the oocyte at fertilization. Recombinase-A (RecA) protein-coated exogenous DNA has been used previously in pronuclear injection systems increasing integration into goat and pig genomes. However, there are no data regarding transgene expression after ICSI. Here, we set out to investigate whether the expression of transgenic DNA in porcine embryos is improved by recombinase-mediated DNA transfer and if it is possible to generate transgenic animals using this methodology. Different factors which could affect the performance of this transgenic methodology were analyzed by studying 1) the effect of the presence of exogenous DNA and RecA protein on boar sperm functionality; 2) the effect of recombinase RecA on *in vitro* enhanced green fluorescent protein (EGFP)-expressing embryos produced by ICSI or IVF; and 3) the efficiency of generation of transgenic piglets by RecA-mediated ICSI. Our results suggested that 1) the presence of exogenous DNA and RecA–DNA complexes at 5 µg/ml did not affect sperm functionality in terms of motility, viability, membrane lipid disorder, or reactive oxygen species generation; 2) EGFP-expressing embryos were obtained with a high efficiency using the SMGT–ICSI technique in combination with recombinase; however, the use of IVF system did not result in any fluorescent embryos; and 3) transgenic piglets were produced by this methodology. To our knowledge, this is the first time that transgenic pigs have been produced by ICSI–SMGT and a recombinase.

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### Introduction

Transgenesis is a powerful biotechnological tool for the generation of genetically modified animals with applications in such different areas as veterinary medicine, biomedicine, and agriculture. DNA pronuclear microinjection has been the most popular system to generate transgenic animals. Transgenic mice, sheep, pigs, and cattle have been successfully produced using this technique. However, besides being expensive, this technique is still inefficient when it is used to generate transgenic farm animals (0.5–4%; Niemann & Kues 2000, Nakanishi *et al.* 2002, Wall 2002). Different methodologies have been developed to improve the efficiency of generation of transgenic pigs over the last decade and a half (reviewed by Gadea & Garcia-Vazquez (2010a)). These include infection with retroviral vector (Whitelaw *et al.* 2004), nuclear transfer with modified somatic cells (Lai *et al.* 2002), and sperm-mediated gene transfer (SMGT; Lavitrano *et al.* 2002).

The first report showing that exogenous DNA could be introduced into sperm was provided by Brackett's group in rabbits (Brackett *et al.* 1971). Lavitrano *et al.* (1989) described a new method for transgenic animal production: SMGT. This method is based on the ability of sperm to bind, internalize, and transport exogenous DNA into an oocyte during fertilization (Francolini *et al.* 1993, Zani *et al.* 1995, Lavitrano *et al.* 1997). SMGT has been used more or less successfully in the production of transgenic embryos and animals in a large number of species (reviewed in Smith & Spadafora (2005)).

Although transgenic animals have been obtained using SMGT, its efficiency is still low, mainly due to the spermatozoon's low uptake of exogenous DNA, thereby reducing the number of fertilized oocytes with transfected spermatozoa (Anzar & Buhr 2006). In addition, inter- and intra-species success variability is still an unsolved problem associated with this technology. Other studies have used 'augmentation' techniques, such as electroporation or liposomes, to 'force' sperm to capture

transgenes (Lai *et al.* 2001). More recent studies have introduced transgenes directly into the reproductive tract of male animals in what is known as testis-mediated gene transfer (Celebi *et al.* 2003, Coward *et al.* 2007).

Another innovation in SMGT has been the use of ICSI to deliver transgene-containing sperm cells directly into the egg, a process known as 'ICSI-mediated transgenesis' (ICSI-Tr) which was reported for the first time in mice (Perry *et al.* 1999) and later developed in pigs (Lai *et al.* 2001, Pereyra-Bonnet *et al.* 2008, García-Vázquez *et al.* 2009, Wu *et al.* 2009). These techniques (pronuclear injection and ICSI) are considered as passive methods for transgenesis (Shinohara *et al.* 2007), which rely on the repair mechanisms of the host for transgene insertion. In 'active transgenesis', recombinases or transposases are injected into oocytes to increase the efficiency of transgene integration into the genome (Shinohara *et al.* 2007).

In the mouse, both the bacterial recombinase protein recombinase-A (RecA; Kaneko *et al.* 2005, Moreira *et al.* 2007) and a mutated hyperactive Tn5 transposase protein (\*Tn5p; Suganuma *et al.* 2005) increase transgenesis several fold as compared with conventional methods such as pronuclear microinjection (Nakanishi *et al.* 2002) and traditional ICSI-Tr (Perry *et al.* 1999). The RecA protein from *Escherichia coli* is one of the best characterized of the recombinases, and plays an important role in homologous recombination and DNA repair in *E. coli* (Kowalczykowski & Eggleston 1994, Shinohara & Ogawa 1995). It initiates pairing by binding to single-stranded DNA (ssDNA) and by forming a helical nucleoprotein presynaptic filament. RecA was shown to protect ssDNA from degradation by creating a protective coating through its binding to the latter (Chow *et al.* 1986). Such a protective mechanism has also been suggested to influence ssDNA stability during pronuclear microinjection-mediated transgenesis with RecA:ssDNA complexes during pig and goat transgenesis attempts (Maga 2001, Maga *et al.* 2003). This RecA coating of ssDNA resulted in higher embryo survival and transgene integration frequencies. Moreira *et al.* (2007) have used RecA in mouse SMGT-ICSI system, and they observed that RecA-complexed and non-complexed enhanced green fluorescent protein (EGFP) DNA attaches differently to fresh sperm cells, and resulted in a higher level of embryo mosaicism.

The objective of this study was to investigate whether SMGT is improved by recombinase-mediated DNA transfer. Different factors which could affect the performance were analyzed by studying 1) the effect of the presence of exogenous DNA and RecA protein on boar sperm functionality; 2) the effect of RecA on *in vitro* EGFP-expressing embryo production by IVF and ICSI; and finally, 3) the evaluation of transgenic piglet production by ICSI-SMGT technique and RecA combination by embryo transfer.

## Results

### ***Effect of the presence of exogenous DNA and RecA protein on boar sperm functionality***

In our first experiment, we evaluated the effect of fresh sperm cells co-incubated with ssDNA complexes with *E. coli* recombinase RecA on boar sperm functionality. For this evaluation, eight replicates were performed. Spermatozoa were incubated with linearized plasmid EGFP (DNA) or RecA-DNA complexes (40:1 or 40:3 protein:DNA w/w) at concentrations of  $10^8$  cells/ml and 5–15 µg DNA/ml. Sperm viability, membrane lipid disorder, reactive oxygen species (ROS) generation, and motion parameters were evaluated by flow cytometry and computer-assisted sperm analysis (CASA) for control (no DNA), dsDNA (5 µg), RecA 5 (5 µg), and RecA 15 (15 µg) after 2 h of co-incubation.

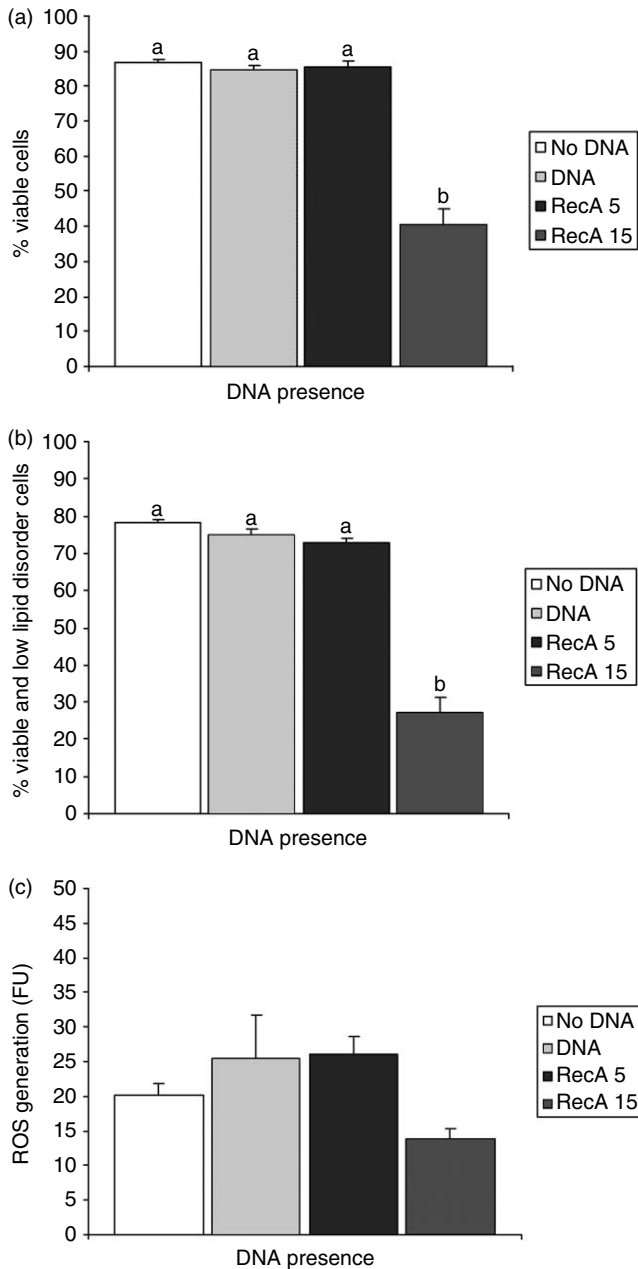
Sperm viability was measured by propidium iodide (PI) staining, which was significantly reduced by the presence of RecA-DNA complexes at a high DNA concentration of 15 µg/ml (Fig. 1a,  $P < 0.01$ ), but the presence of 5 µg/ml of DNA or RecA-DNA complexes did not significantly decrease the viability compared with the control. Exactly the same effect was observed when lipid membrane disorder and viability were simultaneously measured (stained by merocyanine 540 and Yo-Pro-1). The percentage of live cells with low lipid membrane disorder was significantly reduced in the presence of RecA-DNA 15 µg/ml complexes (Fig. 1b,  $P < 0.01$ ). However, ROS generation (measured by 2',7'-dichlorofluorescein diacetate) was not different in the different experimental groups (fluorescent units; Fig. 1c,  $P = 0.36$ ).

The motion parameters measured by CASA were affected by the presence of DNA and RecA-DNA complexes. There was a significant reduction in the percentage of progressive motile cells and the other motion parameters in the group RecA 15 compared with the other experimental groups (Table 1;  $P < 0.01$ ). Progressive motility was not reduced in the DNA and RecA 5 group compared with the control. Indeed, sperm velocities (curvilinear velocity (VCL), straight line velocity (VSL), and average path velocity (VAP)) were increased in these groups (Table 1;  $P < 0.05$ ) with the same pattern that was observed in the ROS generation data (Fig. 1c).

Once it was observed that 15 µg of DNA had a detrimental effect, this condition was not used in further experiments.

### ***In vitro production of EGFP-expressing embryos by ICSI using spermatozoa incubated with DNA and RecA recombinase***

Spermatozoa were incubated with dsDNA or RecA:ssDNA complexes, and later injected into the *in vitro* matured (IVM) oocytes (a total of 248 oocytes



**Figure 1** Effect of DNA incubation on (a) sperm viability ( $P < 0.01$ ), (b) membrane lipid disorder ( $P < 0.01$ ), and (c) ROS generation ( $P = 0.36$ ). The spermatozoa were divided into different groups: no DNA group: no dsDNA incubation; DNA group: dsDNA incubation; RecA 5 and 15 groups: sperm incubation with ssDNA:RecA complex using 5 and 15  $\mu\text{g}$  of DNA respectively. <sup>a,b</sup>Different letters indicate significant differences ( $P < 0.01$ ).

were used in nine replicate experiments). Cleavage rate, blastocyst formation rate, number of cells per blastocyst, and rate of EGFP-expressing embryos were evaluated. Injection of fresh sperm with RecA:ssDNA complexes into MII oocytes resulted in fertilization and developmental rates comparable with those obtained by ICSI performed in the control group using fresh sperm incubated with dsDNA ( $P > 0.05$ , Table 2). This result

suggests that neither naked ssDNA nor RecA:ssDNA was deleterious to embryo survival at the concentrations used. However, the use of RecA:ssDNA complexes clearly increased the percentage of EGFP-expressing embryos in relation to the control group ( $P < 0.01$ ). EGFP fluorescence was detected in 87.88% of embryos in the RecA group (in seven of the nine replicates, all the analyzed embryos expressed the protein) and 51.56% of embryos in the control group. This difference in EGFP expression was greater when only blastocysts were analyzed (28.6% for control versus 83.3% for RecA group; Table 2,  $P < 0.01$ ). Embryos-expressing EGFP are shown in Fig. 2.

### Evaluation of in vitro production of EGFP-expressing embryos by IVF using spermatozoa incubated with RecA recombinase

A total of 584 *in vitro*-matured porcine oocytes were divided into three experimental groups (spermatozoa not incubated with DNA or incubated with dsDNA or RecA:ssDNA complexes) were used for this experiment. Co-incubation of the spermatozoa with dsDNA only induced a lower cleavage rate than in the RecA:ssDNA complexes group (41.2 vs 25.1%,  $P < 0.01$ , Table 3), and the control group (sperm not incubated with DNA) remained in a middle ranking position (38.9%, Table 3). The production of blastocysts and the mean number of cells per blastocyst were similar in all experimental groups ( $P = 0.59$  and  $P = 0.27$  respectively, Table 3). Finally, no EGFP-expressing embryos were obtained.

### Production of transgenic piglets

#### Pregnancy rates

A total of 798 oocytes were injected with spermatozoa incubated with RecA:ssDNA complexes and immediately transferred into the oviducts of seven prepuberal crossbred sows. The results of the embryo transfer produced by ICSI-SMGT-RecA are reflected in Table 4. The sows D, E, and F were diagnosed as pregnant 25 days after the embryo transfer, a rate of pregnancy success of 42.8%. A total of 15 piglets were born (five of them stillborn). The average weight of the litters was 1.1 kg, and the pigs that were born alive did not show anatomical or physiological alterations, and had a normal growth.

#### Transgene presence and expression

Transgene presence was analyzed by means of PCR. The transgene was detected in seven of 15 animals (46.6%), four of them were live animals (F1–F4) and three of them were dead animals (D6, D7, and E3; Table 4 and Fig. 3).

In four transgenic animals that were born alive, EGFP expression was analyzed in different tissues (liver, spleen, greater omentum, kidney, blood, adipose tissue, tail, and ear), as detailed in Table 5 and Fig. 4.

**Table 1** Motility parameters measured by computer-assisted sperm analysis. Boar spermatozoa incubated with dsDNA (DNA) or single-stranded DNA (ssDNA):recombinase-A (RecA) using 5 and 15 µg of DNA respectively.

Treatment	% Progressive motility	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
No DNA	46.7±1.2 <sup>a</sup>	97.5±1.8 <sup>a</sup>	35.3±0.8 <sup>a</sup>	59.9±1.4 <sup>a</sup>	35.9±0.5 <sup>a</sup>	58.9±0.5 <sup>a</sup>	60.7±0.7 <sup>a</sup>	3.9±0.1 <sup>a</sup>	6.7±0.1 <sup>a</sup>
DNA	43.9±1.4 <sup>a</sup>	106.4±2.1 <sup>b</sup>	38.9±1.0 <sup>b</sup>	65.3±1.6 <sup>b</sup>	35.8±0.6 <sup>a</sup>	58.6±0.8 <sup>a</sup>	59.9±0.8 <sup>a</sup>	4.2±0.1 <sup>a</sup>	6.7±0.1 <sup>a</sup>
RecA 5	46.8±1.7 <sup>a</sup>	109.7±1.9 <sup>b</sup>	39.0±1.3 <sup>b</sup>	66.3±1.7 <sup>b</sup>	35.4±0.8 <sup>a</sup>	58.4±0.9 <sup>a</sup>	60.2±0.8 <sup>a</sup>	4.2±0.1 <sup>a</sup>	6.3±0.2 <sup>a</sup>
RecA 15	20.3±3.1 <sup>b</sup>	58.1±7.8 <sup>c</sup>	17.3±2.6 <sup>c</sup>	31.7±4.7 <sup>c</sup>	22.9±3.2 <sup>b</sup>	42.7±5.6 <sup>b</sup>	41.7±5.7 <sup>b</sup>	3.0±0.5 <sup>b</sup>	4.1±0.5 <sup>b</sup>
P value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity of the curvilinear trajectory; STR, straightness; WOB, wobble (VAP/VCL); ALH, amplitude of lateral head displacement; BCF, beat cross frequency. <sup>a,b,c</sup>Different superscripts in columns indicate significant differences ( $P<0.05$ ).

The presence of EGFP expression was confirmed in all tissues analyzed by western blot (Fig. 4). However, when we analyzed the tissues from the three stillborn animals generated, we observed differential EGFP expression (Table 6). From the animal E3, only one tissue sample analyzed (cartilage) was detected as expressing EGFP (expression in 8.3% of the tissues analyzed). On the other hand, the tissues from the piglet D6 (Table 6) showed the expression of EGFP in more than half of the samples (expression in 63.6% of the analyzed tissues), and in D7, EGFP expression was detected in 54.5% of tissues analyzed.

Using the same amount of sample in all the western blot analysis (15 µg of protein per sample), the intensity of expression signals from the animal F1 was consistently weaker than from other animals (F2, F3, and F4), and the transgene signal distribution was less intensive in blood, adipose, tail, and ear than in other tissues (Table 5). Levels of EGFP expression in stillborn animals were the greatest in samples from liver, lung, and heart (Table 6).

Finally, we confirmed these results with an immunohistochemistry study of different tissue samples (Fig. 5).

## Discussion

In recent years, transgenic pigs have been indispensable in the field of biomedicine, with applications in several research areas (Petters & Sommer 2000, Niemann *et al.* 2003, Prather *et al.* 2003, Gadea & Garcia-Vazquez 2010b). Nevertheless, the pronuclear microinjection approach is not an efficient method for the production of transgenic pigs. However, in the mouse, ICSI-SMGT has been shown to be a valuable tool for the production

of transgenic animals (Perry *et al.* 1999, Moreira *et al.* 2004, 2007). In pigs, ICSI efficiency is limited (García-Roselló *et al.* 2009), and the application of this transgenic technique has only been reported in a small number of studies (Smith & Spadafora 2005). Recently, a new transgenic model has been developed, in which ICSI-SMGT is combined with recombinase and transposase to increase the efficiency of the transmission of the exogenous gene. This technique has been denominated 'active transgenesis' (Shinohara *et al.* 2007). We investigated the possibility of exploiting the endogenous properties of recombinase proteins that allow them to associate with, bind, and recombine DNA, and their compatibility with IVF and ICSI-SMGT techniques, in order to generate transgenic pigs.

### Effect of the presence of exogenous DNA and RecA protein on boar sperm functionality

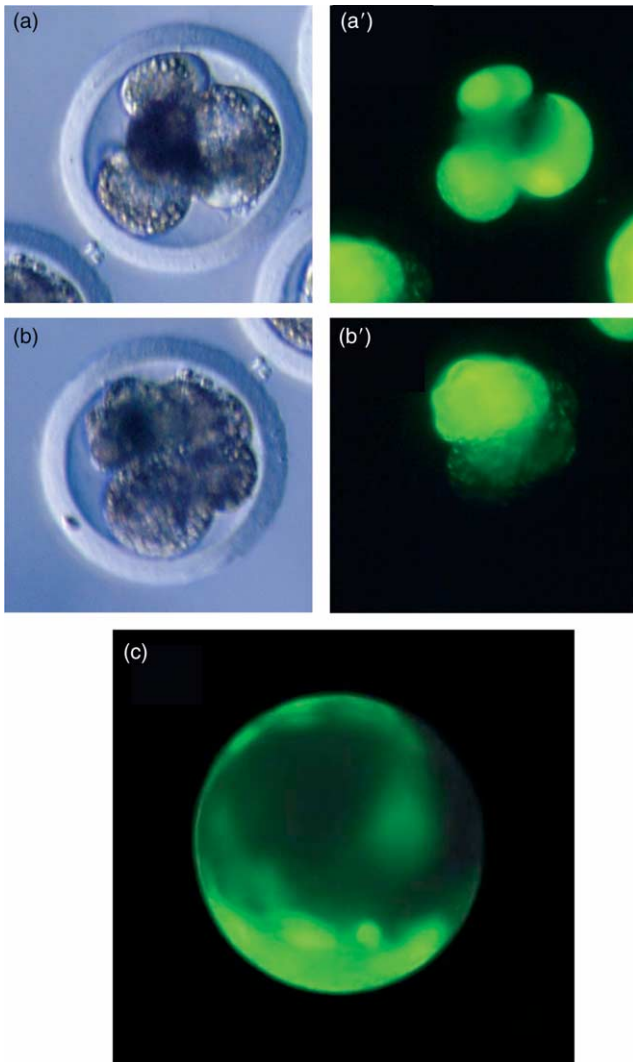
Exogenous DNA concentration has been shown to affect transgenic efficiency by SMGT-ICSI in mice, rats, and pigs (Hirabayashi *et al.* 2005, Moreira *et al.* 2007, Wu *et al.* 2009). Although high concentrations of exogenous DNA increased the possibility of DNA uptake by spermatozoa, it also decreased sperm cell motility, viability, and fertilizing capacity (Lavitrano *et al.* 1989). Maione *et al.* (1997) also observed that high DNA concentrations induced strong nuclease activity in sperm cells, yielding not only cleavage of the foreign DNA, but also the degradation of sperm chromosomal DNA. Thus, a process resembling apoptosis is triggered in spermatozoa when increased concentrations of exogenous DNA are used for SMGT (Lanes *et al.* 2009).

**Table 2** *In vitro* embryonic development of oocytes injected with recombinase-A (RecA) treated or fresh spermatozoa incubated with DNA and enhanced green fluorescent protein (EGFP)-expressing embryos.

Sperm treatment	Complex	No. of oocytes (replicates)	Cleavage rate (%)	Blastocyst rate* (%)	No. of cells/blastocyst	Total % EGFP expression*	% EGFP-expressing blastocysts	Mosaicism rate (%)
ICSI + fresh sperm	dsDNA	117 (9)	54.7	10.9	43.3±9.9	51.6 <sup>a</sup>	28.6 <sup>a</sup>	48.1
ICSI + fresh sperm	ssDNA + RecA	131 (9)	50.4	18.2	37.6±5.7	87.9 <sup>b</sup>	83.3 <sup>b</sup>	39.6
P value			0.50	0.25	0.60	<0.01	<0.01	0.47

<sup>a,b</sup>Different superscripts in columns indicate significant differences ( $P<0.05$ ).

\*Related to cleaved embryos.



**Figure 2** Porcine embryos expressing EGFP produced by injecting spermatozoa that had been coincubated with RecA:ssDNA. (a) and (b) four-cell embryos under bright light. (a') and (b') the same embryos shown under blue light. (a') Total EGFP expression. (b') Mosaic EGFP expression. (c) Blastocyst under blue light (original magnification  $\times 200$ ).

In our experiments, sperm motility and viability were not affected by the presence of foreign DNA at a concentration of 5  $\mu\text{g/ml}$  RecA-coated or -uncoated DNA. However, when 15  $\mu\text{g/ml}$  of DNA were used in a RecA complex, the viability and motility significantly

decreased. These results are in accordance with previous reports that confirmed that bovine and porcine spermatozoa can bind to exogenous DNA and maintain their motility (Rieth *et al.* 2000, Alderson *et al.* 2006, Kang *et al.* 2008). Interestingly, one previous report (Chan 2000) described how transfected sperm had even higher percentages of total motility and progression when compared with controls. However, some controversial aspects exist in relation to sperm motility, so in another report, transfected spermatozoa were reported to be immotile (Anzar & Buhr 2006). The low viability and the absence of motility in spermatozoa bound with exogenous DNA could be associated with endonuclease activation (Maione *et al.* 1997, Spadafora 1998), which causes apoptosis-associated DNA cleavage and subsequent cell death (Anzar & Buhr 2006).

In this study, no significant differences were found in ROS production in spermatozoa among experimental groups. ROS are known mostly for their detrimental effects on sperm functions (Twigg *et al.* 1998). Szczygiel *et al.* (2003) demonstrated that incubation of spermatozoa with exogenous DNA leads to breaks in paternal chromosomes, and it is well known that spermatozoa deficiency in nuclear DNA repair systems results in their increased susceptibility to oxidative stress and DNA fragmentation (De Lamirande *et al.* 1997).

**In vitro production of EGFP-expressing embryos by ICSI using spermatozoa treated with RecA recombinase**

Efficient ICSI-mediated gene transfer requires use of sperm with plasma membranes damaged by freezing and thawing or by a surfactant (Perry *et al.* 1999). Damage to the sperm plasma membranes causes DNA to associate with important consequences for the transfer of DNA into oocytes (Perry *et al.* 1999). However, sperm pretreatments that markedly disrupt the plasma membrane cause DNA fragmentation (Szczygiel *et al.* 2003), or can damage the sperm nucleus and lead to lower efficiency of embryogenesis following ICSI (Kurome *et al.* 2006, García-Vázquez *et al.* 2009). It is also commonly found that transgenes integrated by passive transgenesis commonly form long concatameric arrays (Suganuma *et al.* 2005). Such transgene loci are not desirable,

**Table 3** *In vitro* embryonic development of oocytes IVF with recombinase-A (RecA) treated or fresh spermatozoa incubated with or without DNA and enhanced green fluorescent protein (EGFP)-expressing embryos.

Sperm treatment	Complex	No. of oocytes (replicates)	Cleavage rate (%)	Blastocyst rate* (%)	No. of cells/blastocyst	% EGFP expression*
IVF + fresh sperm	–	59 (4)	38.9 <sup>a,b</sup>	21.7	36.40 $\pm$ 9.2	–
IVF + fresh sperm	dsDNA	382 (4)	25.1 <sup>a</sup>	21.8	37.26 $\pm$ 3.3	0
IVF + fresh sperm	ssDNA + RecA	143 (4)	41.2 <sup>b</sup>	15.2	28.45 $\pm$ 3.3	0
<i>P</i> value			<0.01	0.59	0.27	

<sup>a,b</sup>In the same column indicate significant differences ( $P < 0.05$ ).

\*Related to cleaved embryos.

**Table 4** Production of piglets derived from ICSI-sperm-mediated gene transfer produced zygotes.

Recipient	Embryo transferred	Pregnancy	Piglets	Status at delivery	Gender	Weight at delivery (kg)	PCR			
A	102	—	—	—	—	—	—			
B	102	—	—	—	—	—	—			
C	95	—	—	—	—	—	—			
D	113	+	#1	Live	Female	1.5	—			
			#2	Live	Female	1.1	—			
			#3	Live	Female	1.3	—			
			#4	Live	Male	0.8	—			
			#5	Live	Female	1.1	—			
			#6	Stillborn	Male	1.2	+			
			#7	Stillborn	Male	1.0	+			
			#8	Stillborn	Female	0.4	—			
						Total live: 5	Average: 1.05			
E	119	+	#1	Live	Female	0.7	—			
			#2	Stillborn	Male	0.6	—			
			#3	Stillborn	Female	1.2	+			
									Total live: 1	Average: 0.8
			#1	Live	Male	1.4	+			
F	125	+	#2	Live	Female	1.8	+			
			#3	Live	Female	1.4	+			
			#4	Live	Male	1.5	+			
									Total live: 4	Average: 1.5
G	142	—	—	—	—	—	—			
Total				Total live: 10	Female: 7 Male: 3	Average: 1.1	Total: 7			

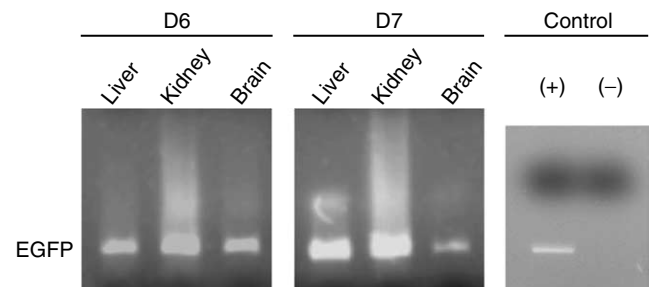
because they often have the potential to generate aberrant RNAs that can cause gene silencing (Garrick *et al.* 1998).

Thus, in our second experiment, the aim was to see whether the use of RecA exerts a protective effect on the chains of ssDNA, translating into an improvement in embryonic development and in the rate of production of EGFP-expressing embryos through ICSI, when the sperm was incubated with RecA:ssDNA complexes. The results confirmed that embryo development produced by ICSI was not affected by the use of RecA, but also that the use of RecA represented an improvement in the percentage of EGFP-expressing embryos produced (87.9 vs 51.6%), confirming previous studies that reported that the use of recombinase RecA increased the efficiency of production of transgenic embryos using pronuclear microinjection in farm animals (Maga 2001, Maga *et al.* 2003) and ICSI in mice (Kaneko *et al.* 2005). The role of RecA in transgenesis could be the protection of ssDNA from degradation, as previously suggested for pronuclear injection of RecA:ssDNA complexes (Maga *et al.* 2003). This mechanism of protection has also been suggested to influence the stability of ssDNA during the transgenesis process using pronuclear microinjection with RecA:ssDNA complexes in pig and goat (Maga 2001, Maga *et al.* 2003). When ICSI-Tr is performed with live sperm cells, RecA may facilitate transgene integration, first by protecting the DNA from nucleases present in the sperm solution before sperm injection, and second by protecting it from the intracellular oocyte degradation that usually occurs in its absence (Moreira *et al.* 2007).

The EGFP fluorescence of embryos injected with RecA:ssDNA complexes demonstrates transgene stability, suggesting that RecA plays a different, possibly enzymatic, role in transgene integration (Kaneko *et al.* 2005).

**In vitro production of EGFP-expressing embryos by IVF using spermatozoa treated with RecA recombinase**

Porcine IVF with spermatozoa coincubated with exogenous RecA-coated and -uncoated DNA did not produce any EGFP-expressing embryos in our experimental conditions. Several researchers have investigated the use of porcine IVF-SMGT for transgenic embryo production, but none were successful in producing protein-expressing embryos (Horan *et al.* 1992, Lai *et al.* 2001, Bolling *et al.* 2003).



**Figure 3** PCR analysis of genomic DNA (liver, kidney, and brain) from D6 and D7 piglets. Positive control (+) (genomic DNA from EGFP-transgenic mouse); negative control (genomic DNA from nontransgenic pig).

**Table 5** Enhanced green fluorescent protein-expressing tissues in F1, F2, F3, and F4 piglets.

No. of piglet	Li	Sp	Om	Ki	Bl	Ad	Ta	Ea
F1	+	+	+	+	+	+	++	+
F2	+++	++	++	++	+	+	+	+
F3	+++	++	++	++	+	+	+	+
F4	++	+	+++	+++	+	+	+	+++

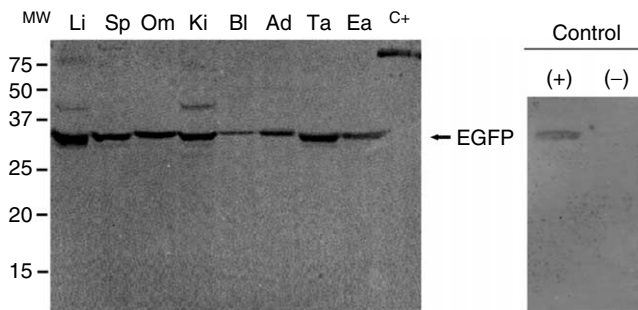
Li, liver; Sp, spleen; Om, greater omentum; Ki, kidney; Bl, blood; Ad, adipose; Ta, tail; Ea, ear. The evaluation was done using the following criteria: +, weakly positive; ++, medium positive; +++, strongly positive. The results are reported as lines intensity.

Chan *et al.* (2000a, 2000b) were not able to obtain any transgenic embryos using primate spermatozoa by IVF but were successful in obtaining transgenic embryos by ICSI. Nevertheless, limited success has been reported with bovine IVF-SMGT (Gagné *et al.* 1991, Sperandio *et al.* 1996, Rieth *et al.* 2000).

It has been previously reported that there is a window of opportunity, in which the exogenous DNA binds to spermatozoa, and it coincides with the early stage of capacitation (Lavitrano *et al.* 2002). It is possible that under our experimental conditions, sperm capacitation occurs very quickly, so that DNA binding can occur, but perhaps the acrosome reaction takes place too early and thus the spermatozoa die. In this case, changes in the sperm membrane functionality might modulate the time of DNA binding; the populations of viable sperm attached to the DNA may determine success or failure in the production of transgenic animals using this method. Under our experimental conditions, we have confirmed that the cells with the most DNA bound are the dead cells or the cells with severe membrane alterations in boar spermatozoa (García-Vázquez *et al.* 2009).

On the other hand, it is possible that the DNA binding induces the alteration and death of the cell by endonuclease activation in an apoptotic-like process (Anzar & Buhr 2006). It has been suggested that mature spermatozoa contain nucleases that when induced by internalization of foreign DNA might cause degradation of both sperm chromosomal DNA and added exogenous DNA (Maione *et al.* 1997, Spadafora 1998, Szczygiel

*et al.* 2003). Such DNA degradation might decrease the possibility of a sperm carrying exogenous DNA and participating in fertilization (Kang *et al.* 2008). In line with this, the medium developed by Lavitrano *et al.* (2002) containing EDTA and free of Ca<sup>2+</sup> allows the capacitation process to occur slowly. With this medium free of Ca<sup>2+</sup>, the probability that the endogenous endonucleases act on foreign DNA is reduced. However, calcium and bicarbonate ions are necessary for the sperm capacitation process in swine (Harrison 1997), and when the Ca<sup>2+</sup> concentration in the media is increased, this has been shown to lead to an increase in penetration rate and in the mean number of sperm per penetrated oocyte (Abeydeera & Day 1997). The IVF medium used in our experiments is TALP medium that contains high levels of Ca<sup>2+</sup>, which could induce the action of endonucleases on foreign DNA. Moreover, the capacitated sperm that can move freely within the cumulus matrix may bind to the surface membrane but not cross it (Myles & Primakoff 1997). Moreover, Chan *et al.* (2000a, 2000b) showed that the signal of rhodamine-labeled plasmid-bound spermatozoa is lost at the oocyte surface during sperm interaction with the zona pellucida (ZP), but this is not the case with the ICSI technique. In IVF approaches, immediately after sperm penetration, the contents of cortical granules are secreted and the enzymes could modify the ZP in a process to prevent sperm penetration (Stein *et al.* 2004). These data suggest that both mechanical and enzymatic actions must be considered.



**Figure 4** Expression analysis of EGFP genetic modified piglet by SDS-PAGE and western blot. Different tissues were studied (Li, liver; Sp, spleen; Om, greater omentum; Ki, kidney; Bl, blood; Ad, adipose tissue; Ta, tail; Ea, ear; MW, molecular weight; C(-), wild-type control; C(+), mouse ZP3-GFP or EGFP-transgenic mouse) from F2 piglet generated with RecA:ssDNA. Sample from liver showed high levels of expression.

### Production of transgenic piglets by embryo transfer

Using ICSI-Tr in mice, the transfection efficiency is on average, 2.5% of oocytes injected (OI) or 20% of animals born (AB), with very low rates of mosaicism (Perry *et al.* 1999). Recently, a more efficient version of this method was reported where the efficiencies of OI and AB were 4.6 and 45% respectively (Moreira *et al.* 2007). In pigs, previous reports have obtained transgenic piglets using SMGT-ICSI. Yong *et al.* (2006) transferred embryos produced by ICSI and spermatozoa from a transgenic boar (created by Cabot *et al.* (2001)) to six recipient females and obtained a transgenic pig, and Naruse *et al.* (2005) obtained a single transgenic pig after three embryo transfers.

**Table 6** Enhanced green fluorescent protein-expressing tissues in D6, D7, and E3 piglets.

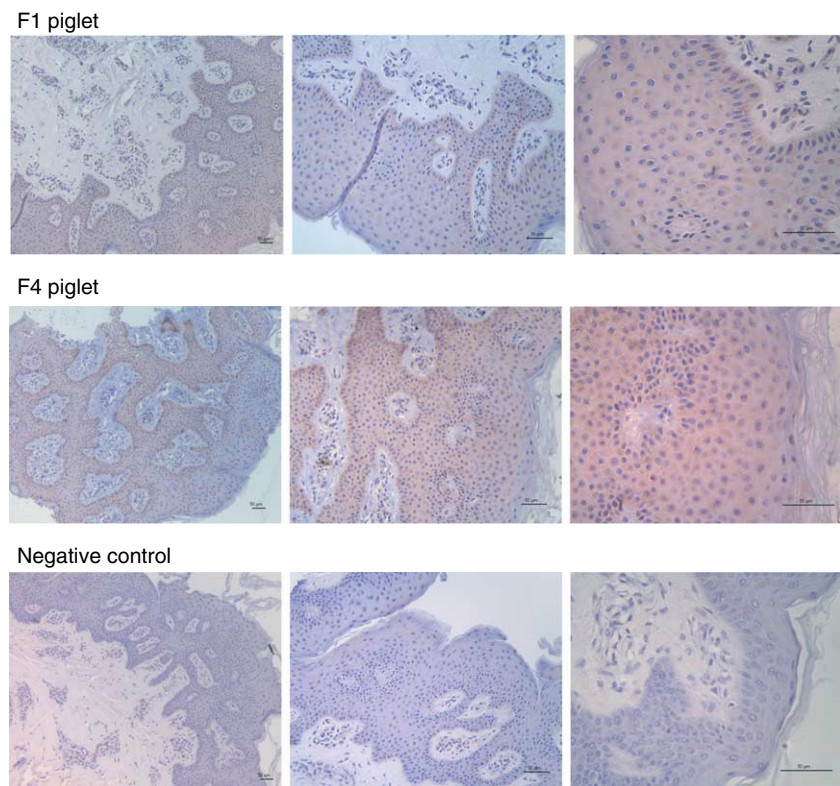
No. of piglet	Li	Ki	Br	Sk	Sm	Lu	He	Te/Ov	Sp	Di	Bv	Ca
D6	+	ND	++	+	++	+++	+++	–	–	+	–	–
D7	+++	++	–	++	++	–	–	ND	–	++	++	–
E3	–	–	–	–	–	–	–	–	–	–	–	+

Li, liver; Ki, kidney; Br, brain; Sk, skin; Sm, skeletal muscle; Lu, lung; He, hearth; Te/Ov, testicle/ovary; Sp, spleen; Di, digestive aparatus; Bv, blood vessels; Ca, cartilage. The evaluation was done using the following criteria: +, weakly positive; ++, medium positive; +++, strongly positive; ND, not determined. The results are reported as lines intensity.

In our studies, after embryo transfer to seven receipt females, we obtained three pregnancies. Just a few research groups have been able to obtain piglets by ICSI-derived embryos, and the success using ICSI with *in vitro*-matured oocytes is even more limited (reviewed by García-Roselló *et al.* (2009)). From 15 pigs born, seven were transgenic (46.6%), with four of them (live piglets) with protein expression in all the tissues (57.1%). In line with this, Kaneko *et al.* (2005) observed that all transgenic mice produced by RecA-ICSI were fully transgenic with all of them passing the trait to the next generation. Sukanuma *et al.* (2005) observed similar facilitated transgene insertion during a transposase enzyme-mediated insertion of a transposon during ICSI microinjections as a complex named transposome (TN:ICSI). However, Moreira *et al.* (2007) found that although the transgenesis efficiency of the common ICSI-based transgenesis procedure with frozen-thawed sperm

cells is similar to that of ICSI with fresh sperm cells and RecA-DNA complexes, the traditional ICSI-Tr procedure with frozen-thawed spermatozoa is much more efficient for maintaining a low frequency of founder animal mosaicism.

Three of the animals generated by this RecA:ssDNA complex approach had mosaic expression, suggesting a delayed integration of the transgene in the host genome after the first cell division (Kaneko *et al.* 2005, Moreira *et al.* 2007). Thus, Shinohara *et al.* (2007) affirmed that one of the problems of active transgenesis in mice is the high level of mosaicism. According to Chan *et al.* (1999), the process of integration in the embryos that express GFP in 100% of the blastomeres probably occurred in the first cellular cycle, the pronuclear state before the replication of the DNA. In the embryos that have 50% of the cells expressing GFP, there are two possibilities: 1) the integration of transgenes occurred after the



**Figure 5** Immunohistochemistry study of sections of skin from transgenic piglets (F1 and F4) using an anti-EGFP antibody and comparable tissue sections from a nontransgenic control piglet. Scale bars, 50 µm.



replication of the DNA in the first cellular cycle; or 2) the integration occurred before the replication of the DNA in the second cellular cycle, at the two-cell stage. Similarly, an embryo that expresses GFP in 75% cells was probably derived from a combination of numerous integration events.

In the current study, five of the piglets were stillborn. In the ICSI technique, the manual selection of a spermatozoon and injection of the whole cell, complete with all acrosome and plasma membranes intact, result in abnormalities of sperm decondensation, retention of the acrosomal structures and proteins, and exclusion of the paternal nuclear centriole from the mitotic apparatus (Hewitson *et al.* 1999, Terada *et al.* 2000). These concerns have led to the predictions of chromosomal abnormalities and developmental defects that could cause the deaths of the animals. Techniques for the *in vitro* production of embryo are associated with a higher incidence of embryonic and fetal loss (Hyttel *et al.* 2000). In addition, three of these stillborn piglets were transgenic. Some authors have inferred that the presence of a great number of integrated copies of the transgene (Kaneko *et al.* 2005, Naruse *et al.* 2005) may affect the viability or functionality of some of the vital organs, for example, D6 in heart and lung, and D7 for liver and kidney, although the number of integrated transgene copies has not been analyzed in this study.

In the transgenic piglets born in our study, it was not possible to detect any direct fluorescence. Direct EGFP fluorescence detection is not the most sensitive indicator of EGFP expression. Undetectable direct EGFP fluorescence may be due to protein missfolding or to partial translocation of the peptide containing the recognized epitope (Chan *et al.* 2000a). Alternatively, the lack of visible fluorescence could also be due to low protein expression. The amount of the protein in the different tissues might not be enough to produce direct fluorescence. In other studies in which embryo fluorescence was undetectable by direct GFP imaging, when the embryo was labeled with an anti-GFP antibody, a fluorescence signal was detected under a microscope (Chan *et al.* 2000b).

The exact mechanism by which RecA may facilitate random transgene integration is not clear. The RecA protein may also act by protecting the DNA from degradation by cellular enzymes, thereby increasing the half-life of the DNA probes resulting in increased associations with the genome for a greater chance of producing a transgenic animal. The protection offered by RecA may also increase the probability that the transgene integrates after the one-cell stage. Another possibility is that the transgene DNA is directed into the genome through the use of a microhomology search (Kaneko *et al.* 2005). Such a mechanism has been described in the case of integration of *Agrobacterium tumefaciens* T-DNA into the host plant genome (Valentine 2003). The use of a RecA mutant that retains

its ssDNA-binding properties but is deficient in strand exchange activity (Cazaux *et al.* 1998) may shed light on the role of RecA in the transgenesis process (Kaneko *et al.* 2005).

In summary, the use of a system of ICSI–SMGT using IVM oocytes and spermatozoa incubated with RecA:ssDNA complexes is a viable methodology to successfully produce transgenic piglets, and opens the way for the production of new transgenic pig models.

## Materials and Methods

### Ethics

This study was developed following institutional approval from the University of Murcia, and it was performed in accordance with the Animal Welfare regulations of that institution.

### Material

#### Media and chemicals

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma–Aldrich Química S.A. The oocyte maturation medium was NCSU-37 supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 µg/ml insulin, 50 µM β-mercaptoethanol, 10 IU/ml equine chorionic gonadotropin (eCG; Folligon, Intervet International B.V., Boxmeer, Holland), 10 IU/ml human chorionic gonadotropin (hCG; VeterinCorion, Divarsa, Barcelona, Spain), and 10% porcine follicular fluid (v/v).

After microinjection, oocytes were cultured until embryo transfer in TALP medium (Rath *et al.* 1999) consisting of 114.1 mM NaCl, 3.2 mM KCl, 8 mM Ca-lactate·5H<sub>2</sub>O, 0.5 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.35 mM NaH<sub>2</sub>PO<sub>4</sub>, 25.1 mM NaHCO<sub>3</sub>, 10 ml/l Na-lactate, 1.1 mM Na-pyruvate, 5 mM glucose, 2 mM caffeine, 3 mg/ml BSA (A-9647), 1 mg/ml polyvinyl alcohol (PVA), and 0.17 mM kanamycin sulfate. The medium used for embryo micromanipulation was Dulbecco's PBS (DPBS) supplemented with 10% FCS.

The embryo culture medium was NCSU-23 containing 0.4% BSA (A-8022), 75 µg/ml potassium penicillin G, and 50 µg/ml streptomycin sulfate (Macháty *et al.* 1998).

Swine fertilization medium (SFM; Lavitrano *et al.* 2002) was used for processing the spermatozoa (SFM: 1 l contains glucose 11.25 g, Na citrate (2H<sub>2</sub>O) 10 g, EDTA (2H<sub>2</sub>O) 4.7 g, citric acid (H<sub>2</sub>O) 3.25 g, Trizma 6.5 g supplemented with 6 mg/ml BSA (SFM/BSA)).

### Methods

#### Transgene construction and PCR detection

The EGFP plasmid construct (pEGFPN1, 5.4 kb; Clontech Laboratories, Inc.) used for our experiments contained the human CMV immediate early promoter and the enhanced GFP gene. This construct was linearized with AflIII prior to use. The transgene was purified using an Elu-Quit DNA Purification Kit (Schleicher & Schuell, Dassel, Germany) following the manufacturer's instructions. DNA was resuspended in TE (10 mM Tris and 0.1 mM EDTA, pH 8).

Transgene presence was confirmed by EGFP PCR (Gutiérrez-Adán & Pintado 2000). EGFP PCR was performed using the primers GFP-1 (5'-TGA ACC GCA TCG AGC TGA AGG G-3') and GFP-2 (5'-TCC AGC AGG ACC ATG TGA TCG C-3'), specifically amplifying a 340-bp portion of GFP DNA. DNA was extracted from different tissues of the piglets to analyze for the presence of the transgenic DNA. Amplification was carried out in a total volume of 25  $\mu$ l (1 $\times$  of PCR mix containing 1U Taq polymerase, 2.5  $\mu$ l 10 $\times$  buffer, both from Promega, 100  $\mu$ M each dNTP, 0.1  $\mu$ M each primer, and 2.5 mM MgCl<sub>2</sub>). Samples were loaded directly from ice onto the heating block at 92 °C to minimize the time required to reach denaturation temperature. The PCR protocol was an initial step of 92 °C (2 min), followed by 32 cycles of 92 °C (30 s), 59 °C (30 s), and 72 °C (30 s) and a final extension cycle at 72 °C (10 min). PCR products were resolved on 1.5% Tris/borate/EDTA agarose gels, followed by staining with ethidium bromide and visualized using u.v. light.

#### *Semen collection and preparation of sperm for DNA uptake*

Fresh semen was routinely collected from mature fertile boars ('Lo Navarro' S.A., Murcia, Spain) using the manual method and a dummy (King & Macpherson 1973). The sperm-rich fraction was collected in a pre-warmed thermos flask, and the gel fraction was held on a gauze tissue covering the thermos opening.

Semen was prepared according to the method described by Lavitrano *et al.* (2002) and García-Vázquez *et al.* (2009). Spermatozoa (10<sup>8</sup> cells/ml) were incubated with transgene (pEGFP) or RecA:ssDNA linearized EGFP transgene complexes (5 or 15  $\mu$ g DNA/ml) in SFM with BSA at 16 °C.

#### *Preparation of RecA:ssDNA complexes*

RecA:ssDNA complexes were prepared as described previously (Kaneko *et al.* 2005, Moreira *et al.* 2007). Denatured ssDNA was quenched on ice, and the appropriate quantity of RecA solution was added in order to satisfy a 40:1 or 40:3 protein:DNA (weight:weight) ratio, ensuring coating of all ssDNA and a final DNA concentration of 10 or 30 ng/ $\mu$ l respectively. The mixture was incubated in ice for 1 h, and then mixed with fresh semen at 16 °C for 30 min for embryo production or 2 h for evaluation of sperm function.

#### *Sperm motility assessment using a computer-assisted analysis system*

Motion parameters were determined using a CASA system (Sperm Class Analyzer, Microptic, Barcelona, Spain). A 7- $\mu$ l drop of the sample was placed on a warmed (37 °C) slide and covered with a 24 mm<sup>2</sup> cover slip. The setting parameters were 25 frames, in which spermatozoa had to be present in at least 15 in order to be counted. Images were obtained at  $\times$ 200 magnification with a phase microscope. Spermatozoa with VAP <20  $\mu$ m/s were considered as immotile. A minimum of five fields per sample was evaluated, counting a minimum of 200 spermatozoa per subsample. The CASA-derived motility characteristics measured were VCL (mm/s), straight-line velocity (VSL, mm/s), VAP (mm/s), linearity of the curvilinear

trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), amplitude of lateral head displacement (ALH, mm), and beat cross frequency (BCF, Hz).

#### *Analysis of seminal parameters by flow cytometry*

Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter Inc., Miami, FL, USA). A 15-mW argon ion laser operating at 488 nm excited the fluorophores. Data from 10 000 events per sample were collected in list mode, and four measures per sample were recorded. Flow cytometric data were analyzed using the program Expo32ADC (Beckman Coulter Inc.) using a gate in forward and side scatter to exclude eventual remaining debris and aggregates from the analysis.

*Assessment of membrane lipid disorder.* To detect the increase in plasma membrane lipid packing disorder, sperm samples were stained with merocyanine 540 (M540) and Yo-Pro 1 (Harrison *et al.* 1996). Stock solutions of M-540 (1 mM) and Yo-Pro 1 (25  $\mu$ M, Invitrogen S.A.) in DMSO were prepared. For each 1-ml diluted semen sample (5–10 $\times$ 10<sup>6</sup>), 2.7- $\mu$ l M540 stock solution was added (final concentration of 2.7  $\mu$ M), and 1  $\mu$ l of Yo-Pro was added (25 nM final concentration). M 540 fluorescence was collected with a FL2 sensor using a 575-nm band-pass filter, and Yo-Pro 1 was collected with a FL-1 sensor using a 525-nm band-pass filter. Cells were classified as having low merocyanine fluorescence (viable and low disorder), high merocyanine fluorescence (viable and high disorder), or Yo-Pro-1 positive (dead).

*Plasma membrane integrity.* Seminal samples (1 ml of semen 5–10 $\times$ 10<sup>6</sup> spermatozoa) were incubated with 5  $\mu$ l of PI stock solution (500  $\mu$ g) final solution 2.5  $\mu$ g at room temperature for 10 min. PI fluorescence was collected with a FL2 sensor using a 575-nm band-pass filter.

*Generation of ROS.* Production of ROS was measured by incubating the diluted spermatozoa in TALP in the presence of 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) (0.5  $\mu$ M; Gadea *et al.* 2005). This fluorogenic probe is commonly used to detect cellular ROS production. H<sub>2</sub>DCFDA is a stable cell-permeable nonfluorescent dye that is de-esterified intracellularly, thus turning to highly fluorescent 2',7'-dichlorofluorescein upon oxidation. In total, 10 $\times$ 10<sup>6</sup> cells/ml were incubated for 60 min with 5  $\mu$ l from H<sub>2</sub>DCFDA stock solution (final concentration 0.5  $\mu$ M) before being measured by the flow cytometer. Green fluorescence was recorded with a FL1 sensor using a 525-nm band-pass filter. Measurements were expressed as the mean green intensity fluorescence units (mean channel in the FL1).

#### *Oocyte collection and in vitro maturation*

Within 30 min of slaughter, ovaries from prepuberal gilts were transported to the laboratory in saline (0.9% w/v NaCl) containing 100  $\mu$ g/ml kanamycin sulfate at 37 °C, and then washed once in 0.04% (w/v) cetrimide solution and twice in saline. Oocyte-cumulus cell complexes (COCs) were collected from nonatretic follicles (3–6 mm diameter), and washed twice in 35-mm plastic Petri dishes containing DPBS supplemented

with 4 mg/ml PVA. Groups of 50 COCs were cultured in 500- $\mu$ l maturation medium for 20–22 h at 38.5 °C under 5% CO<sub>2</sub> in air. Once cultured, the COCs were washed twice, transferred to fresh maturation medium without hormonal supplements or dibutyryl cAMP, and cultured for 20–22 h (Funahashi & Day 1993).

#### *ICSI with the construct (EGFP)*

The spermatozoa were incubated with transgene (pEGFP) or RecA:ssDNA complexes at 16 °C for 30 min, and then, the sperm samples were prewarmed at 38 °C for 5 min before ICSI. COCs cultured for 44 h in maturation medium were mechanically stripped of cumulus by gentle aspiration with a pipette. Denuded oocytes were washed two times in supplemented DPBS medium and transferred to ICSI drops. ICSI was performed as described by García-Roselló *et al.* (2006) and García-Vázquez *et al.* (2009).

#### *IVF and embryo culture*

COCs cultured for a total of 44 h in maturation medium were stripped of cumulus mechanically by gentle aspiration with a pipette. Oocytes were washed three times with TALP medium, and groups of 30–35 oocytes were transferred into each well of a four-well multidish containing 250  $\mu$ l of IVF medium previously equilibrated at 38.5 °C under 5% CO<sub>2</sub>. The sperm suspension (250  $\mu$ l) was added to fertilization wells to obtain a final concentration of 3000 cells/oocyte. At 2-h post insemination (hpi), oocytes were washed twice with fresh NCSU-23 by gentle aspiration through a glass pipette and allowed to continue in culture at 38.5 °C under 5% CO<sub>2</sub>.

After 2 h (in the case of IVF) and 20–24 h (in the case of ICSI), putative zygotes were washed three times in NCSU-23 previously equilibrated overnight, transferred into microdrops at a rate of 5  $\mu$ l of NCSU-23 media/embryo and covered with mineral oil (embryo tested) in a Petri dish, and incubated at 38.5 °C and 5% CO<sub>2</sub> in 100% humidified air for 7 days.

#### *Microscopic embryo development evaluation*

After culture, the embryos were evaluated under a stereomicroscope, and a sample was fixed in absolute ethanol for 24 h. After staining with Hoechst 33342 (10 mg/ml in PBS), cell nuclei were counted under an epifluorescence microscope.

#### *Analysis of EGFP expression in embryos*

Expression in embryos was examined 144 h post fertilization. The embryos were examined by epifluorescence microscopy (Diaphot 300, Nikon, Tokyo, Japan) with FITC filters (excitation range of 395–470 nm and emission spectrum of 509 nm). This enabled the clear identification of nonfluorescent (non-EGFP expressing) and fluorescent embryos (EGFP expressing), which were scored accordingly.

#### *Embryo transfer*

Estrus and superovulation in prepuberal crossbred recipient gilts were induced by i.m. administration 1250 IU of eCG (Folligon,

Intervet, Boxmeer, The Netherlands), followed after 72 h by 750 IU of hCG (Veterin Corion, Divisa Farmavic S.A., Barcelona, Spain). Preoperative anesthetic and analgesic induction of animals was carried out by a combination of ketamine (100 mg/ml) 10 mg/kg (Imalgene 1000, Merial Laboratorios S.A., Barcelona, Spain), medetomidine (1.0 mg/ml) 0.2 mg/kg (Domtor, Pfizer S.A., Madrid, Spain), midazolam (5 mg/5 ml) 0.2 mg/kg (Dormicum, Roche), and morphine hydrochloride (20 mg/ml) 0.2 mg/kg (Morphine Braun 2%, B. Braun Medical S.A., Barcelona, Spain) administered i.m. The anesthetic level was maintained using isoflurane (Isoflo, Laboratorios Dr Esteve S.A., Barcelona, Spain) vaporized in oxygen at 2–3%.

Oviducts from animals were exposed through a mid-ventral incision. Over 100 putative zygotes, produced by ICSI 2–3 h before surgery, were introduced into both oviducts (~50 putative zygotes in each one) by a Tom Cat Catheter (Kendall Co., Mansfield, MA, USA). The sows were kept under the usual farm conditions, and 25–28 days after transfer, pregnancy diagnosis was carried out by ultrasonography (100 FALCO-VET scan, Esaote España S.A., Barcelona, Spain).

#### *Analysis of EGFP expression in piglets*

*Direct observation.* EGFP expression was evaluated by direct visualization of piglets through blue light with a maximum excitation of 488 nm by a lamp that emits u.v. light.

*Western blotting.* Proteins were extracted from different tissues of the piglets. Samples were dissolved in sample buffer in reducing (5%  $\beta$ -mercaptoethanol (vol/vol)) conditions. After boiling for 5 min, samples were separated by 12% SDS/PAGE. In brief, 4% stacking and 12% separating gels were used and run using 25 mM Tris/0.2 M glycine buffers, pH 8.6, containing 0.1% SDS for 1.5 h at 150 V at room temperature. For western blots, the SDS-PAGE slab gels were equilibrated for 30 min in 25 mM Tris and 192 mM glycine in 20% (vol/vol) methanol, and proteins were electrotransferred to PVDF membrane at 100 V for 1 h.

The membrane was incubated in blocking buffer (TBST-BSA 5%) for 1 h, and later washed two times for 10 min with washing buffer (TBST) and rocked gently with primary antibody (rabbit polyclonal anti-GFP; Abcam, Cambridge, UK; 1:1000) for 1 h, followed by incubation with secondary antibody (goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:20 000) for 1 h. The detection procedure was carried out in a dark room using an ECL plus Kit (Amersham Pharmacia Biotech, Inc). The membranes were scanned using a Typhoon 9410 imager (Amersham Pharmacia Inc. Biotechnology, Bioscience) for detection of chemiluminescence.

To assess EGFP expression in tissues, SDS-PAGE and western blots were performed in putative transgenic animals as well as negative and positive control animals to verify further the EGFP expression. The positive control, for each one of the analyses, was made using EGFP transgenic mouse (29 kDa) or ZP3-GFP mouse (82 kDa). The EGFP from our analysis has a molecular weight of 29 kDa.

### Immunohistochemistry

Samples of different tissues were fixed at 4 °C in 4% paraformaldehyde overnight, dehydrated, and embedded in paraplast wax (McCormick, Richmond, IL, USA). Sections (5 µm) were placed on Superfrost Plus microscope slides (Menzel, Braunschweig, Germany). Slides were then deparaffined in xylene, rehydrated through a descending ethanol series, and washed in PBS.

The tissue sections were treated with proteinase K (10 µg/ml) for 3 min and washed twice with PBS containing 0.1% Tween 20 (PBT). Then, endogenous peroxidase was blocked with 0.3% hydrogen peroxide in PBS, pH 7.2. After that, the sections were blocked in PBS containing 1% BSA for 1 h at room temperature and incubated in a moist chamber with Rabbit Polyclonal anti-GFP antibody (Abcam) (1:500). After washing in PBS, the sections were incubated with goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotechnology Inc.; 1:4000). The staining was obtained using EnVision FLEX Mini Kit, High pH (Dako UK Ltd, Cambridge, UK). The sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted in DPX. Images were collected with a Leica DM 6000 microscope joined to a digital camera (Leica DFC 280, Wetzlar, Germany) and Software Leica Application Suite.

### Statistical analysis

Data are presented as means  $\pm$  s.e.m. and analyzed by ANOVA considering the specific sperm treatment as the main variable. When ANOVA revealed a significant effect, values were compared by the least significant difference pairwise multiple comparison *post hoc* test (Tukey). Differences were considered statistically significant at  $P < 0.05$ . Cleavage and blastocyst data (categorical data) were modeled according to the binomial model of parameters by arcsine transformation of the data, and were analyzed by ANOVA.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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