World Rabbit Sci. 2007, 15: 161 - 166

EFFECTIVE GENERATION OF GENETICALLY MODIFIED RABBITS BY SPERM MEDIATED GENE TRANSFER.

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Abstract: Many reports in the past decade showing successful sperm mediated gene transfer (SMGT) of foreign DNA into both non-mammalian and mammalian animals. Fresh collected, extender washed rabbit sperm cells were co-cultured with linear 14.3 kb gene construct and used for AI. Transgene integration in rabbit genome was verified by PCR analysis. The PCR results show that transgenic founder rabbits transmitted transgene to F1 generation. Our preliminary results suggest that SMGT could be an effective method for production of genetically modified rabbits.

Key words: Transgenic rabbit, sperm mediated gene transfer (SMGT), human Factor VIII

INTRODUCTION

The rabbit is a source of meat but transgenic rabbits may also serve to generate models for biologicial and medical studies. Recently, the rabbit has attracted much attention from the biotechnology community. Several features make it an attractive model for transgenics and cloning, including: the rapid onset of sexual maturity, a short gestation period, a relatively larger number of offspring per litter, year-round reproductive capacity, and an average life-span of 9 years. Also noteworthy is that the rabbit genome is estimated to be 3 billion base pairs long, almost equal to the size of the human genome (Fadiel *et al*., 2003).

Genetically modified organisms (GMOs) now offer the oportunity to improve both the production and charactristics of conventional strains of animals and plants currently exploited in agriculture. As a potential tool for genetically manipulating animals, sperm mediated gene transfer (SMGT) has the advantages of simplicity and cost-effectiveness, in contrast with more established methods of transgenesis such as pronuclear microinjection (Lavitrano *et al.*, 2006).

Brackett *et al.* (1971) first described that rabbit sperm could take up exogenous DNA and transfer it to ova through fertilization. Eighteen years after this original description of sperm-mediated DNA transfer by Brackett and coworkers Lavitrano *et al.* (1989) first published an efficient way to create transgenic mice by introducing foreign DNA into an egg via the sperm.

Recently, Wang *et al*. (2003) and Shen *et al*. (2006) showing successful sperm mediated gene transfer of foreign DNA in rabbit. The successful *in vitro* uptake of DNA constructs by animal sperm cells has been documented in many species (for review see Smith and Spadafora, 2005).

We want to demonstrate here that simply incubation of washed sperm cells with DNA followed artificial insemination (AI) could be used for production of genetically modified rabbits.

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Received *December 2006* - Accepted *February 2007*.

MATERIALS AND METHODS

Gene construct

The construct used for SMGT consisted of a 2.5 kb murine whey acidic protein promoter (mWAP), 7.2 kb cDNA of the human coagulation factor VIII (hFVIII), and 4.6 kb of 3' flanking sequences of the mWAP gene (Figure 1) (Paleyanda *et al.*, 1997). The plasmid pPolyIII-D was digested with *Not* I to release the 14.3 kb insert and gel purified in a QIAEX II (Qiagene, Hilden, Germany). DNA concentration was determined spectrophotometricaly.

Animals

Does of M91 and P91 lines (derived from NZW) were artificially inseminated. Forty-eight hours before AI 25 I.U of PMSG per doe (Sergon, Bioveta, Czech Republic) was administrated. After AI the females were immediately injected with 2.5 µg synthetic GnRH (Supergestran, Ferring-Léciva, Czech Republic) (Parkanyi *et al.*, 2005).

Sexually maturated PCR positive males were mated and females inseminated with wild-type counterparts and F1 generation was analysed for transgene integration. All newborn kits were analysed by PCR.

Preparation of sperm

After evaluation, semen from the selected rabbit donors was collected using an artificial vagina (Carluccio *et al*., 2004). Sperm cells were twice washed in Diluent mixture for rabbit sperm (DMRS) with antibiotic (Art. Nr: 13540/0001, MINITÜB, Tiefenbach-Germany) to completely remove seminal plasma. After this treatment, the sperm/diluent was kept at room temperature. Sperm cells were resuspended and counted by using a hemocytometric chamber.

Sperm DNA uptake and artificial insemination

Washed sperm cells were diluted in DMRS at concentration 96×10^6/ml. Gel purified *Not* I fragment of gene construct was added at 35 ng/10^6 sperm cells. The tube was continualy inverted to prevent sedimentation of sperm. After incubation 60 minutes at room temperature DNA treated sperm cells suspension was used (0.5 ml per female) for AI (Carluccio *et al*., 2004).

Polymerase chain reaction analysis

Kits were sampled at day of born. DNA was isolated from ear tissue of rabbits. About 100 mg of DNA was subjected after first denaturation step (94°C 2 min) to 35 cycles of PCR amplification (94°C 20 s, 64°C 30 s, and 72°C 30 s, final 72°C 10 min) using 1 unit of Platinum Taq DNA polymerase (Invitrogen) and primers for the hFVIII cDNA #3693 and #3694 (Table 1) wich define a 601 bp region of hFVIII cDNA (Figure 2). Ten microliters of PCR products was analysed by electrophoresis in 1.5% agarose gel stained with ethidium bromide. The genomic DNA of the positive samples with primers #3693 and #3694 were further analysed by PCR with transgene specific primers WAP-5'f and #3694 and HF Ex26f and WAP-3'r (Table 1) using Phusion Hot Start HF DNA polymerase (Finnzymes) to check integrity of incorporated DNA. As a positive control for PCR amplification was used about 1 ng of *Not* I fragment of 14.3 kb mouseWAP-hFVIII cDNA construct.

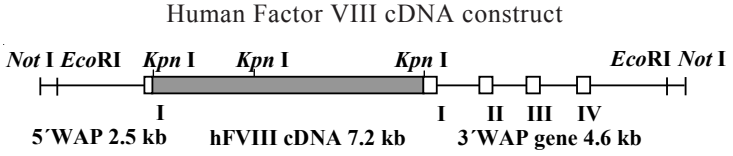

Figure 1: Map of hFVIII cDNA construct used for SMGT - 7.2 kb *Kpn* I fragment of human Factor VIII cDNA inserted into first exon of mouse WAP gene in vector pPolyIII-D (Paleyanda *et al.*, 1997) (Not drawn in scale).

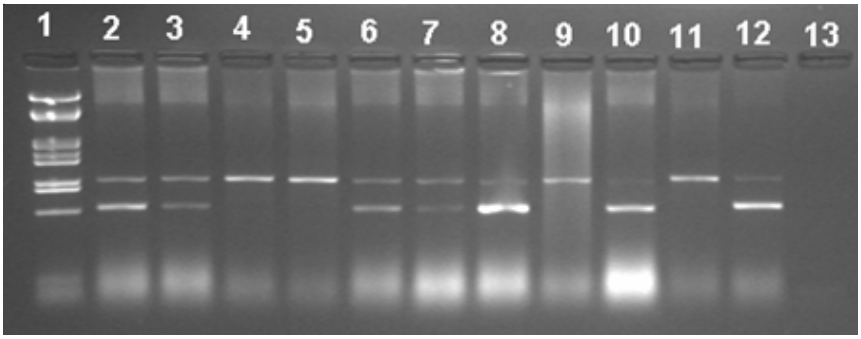

Figure 2: PCR analysis using primers for the hFVIII cDNA #3693: 5'-GTA GAC AGC TGT CCA GAG GAA-3' and #3694: 5'-GAT CTG ATT TAG TTG GCC CAT C-3' (Table 1) with a PCR product of 601 bp (Paleyanda *et al.*, 1997). Lane 1 – MW marker lambda DNA/EcoRI + HindIII (Promega), lanes 2, 3, 6, 7, 8, 10, 12 – positive animals, lanes 4, 5, 9, 11 – negative animals, lane 13 – negative control for PCR.

The conditions for Phusion Hot Start HF DNA polymerase were as follows: 100 ng of genomic DNA; 500 nM of each primer, 0.25 mM of each dNTP, 1 × GC buffer; 1 unit enzyme; total volume 25µl.

The cycling conditions for primers WAP-5'f and #3694 were as follows: first denaturation step 98°C 30 s; 98°C 10 s, 69°C 30 s, and 72°C 45 s 35 cycles; final extension 72°C 10 min; PCR product: 1788 bp.

The cycling conditions for primers HF Ex26f and WAP-3'r were as follows: first denaturation step 98°C 30 s; 98°C 10 s, 72°C 2 min 30 s 35 cycles; final extension 72°C 10 min; PCR product: 3.72 kb

Ten microliters of PCR products was analysed by electrophoresis in 0.7% agarose gel stained with ethidium bromide (Figures 3 and 4). The nucleotide-nucleotide BLAST was used to check the specificity of primers for PCR. The goal was to make sure that the primers will give a unique product from the target transgene.

RESULTS AND DISCUSSION

All kits appeared normal at birth, and there was no obvious difference in appearance between the transgenic and nontransgenic littermates. Summary of insemination results of SMGT experiments are in Table 2. Litter size of does inseminated with DNA treated sperm was in normal range (9.1±1.5). In total 86 live born and 5 dead born animals were PCR screened for transgene. Number of genetically modified kits after SMGT varied from 12.5% to 55.5%.

In Figure 2, PCR analysis of DNA samples from the ears of twelve kits showed that seven kits (lanes no. 2, 3, 6-8, 10 and 12) were positive for the hFVIII transgene whereas the others (lane no. 4, 5, 9 and 11) were negative. PCR using primers #3693 and #3694 was in positive animals amplified specific hFVIII cDNA fragment 601 bp. In all rabbit samples was also amplified 950 bp fragment which is

Table 1: Primers used for the mWAP-hFVIIIcDNA transgene analysis.

Primer	Sequence of primer from 5' to 3'	nt position	GenBank Acc. No.
#3693	GTA GAC AGC TGT CCA GAG GAA	1204-1225	NM_000132
#3694	GAT CTG ATT TAG TTG GCC CAT C	1804-1782	NM_000132
WAP-5'f	CTA GAG CTG TGC CAG CCT CTT C	2309-2330	U38816
HF Ex26f	TGC CCT GAG GAT GGA GGT TCT G	7179-7200	NM_000132
WAP-3'r	GCC CAC TGT GGA TGG TGT CAT C	5745-5724	U38816

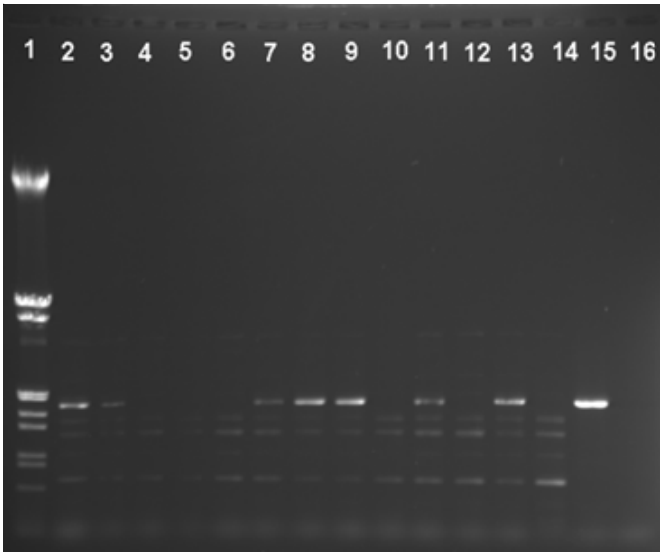

Figure 3: PCR analysis of F1 generation using primers WAP-5'f: 5'- CTA GAG CTG TGC CAG CCT CTT C -3' and #3694: 5'- GAT CTG ATT TAG TTG GCC CAT C -3' (Table 1) with a PCR product of 1788 bp. Lane 1 – MW marker lambda DNA/ EcoRI + HindIII (Promega), lanes 2, 3, 7, 8, 9, 11, 13 – positive animals, lanes 4, 5, 6, 10, 12 – negative animals, lane 14 – wild type negative animal, lane 15 – positive control, lane 16 – negative control for PCR.

probably amplified from rabbit coagulation factor VIII and served as internal control for functional PCR amplification. The exact sequence of this 950 bp fragment remain unclear, because BLAST search of primers sequences of the unfinished *Oryctolagus cuniculus* database showed no homology sequence.

After PCR screening with primers #3693 and #3694 positive DNA samples were diluted to 100 ng/µl in 10mM Tris-HCl (pH 8.5) and further analyzed to check the integrity of integrated DNA with primers from promoter region of transgene construct WAP-5'f and #3694 from exon 11 of human factor VIII cDNA for amplification of 1788 bp PCR product (Figure 3). The primers HF Ex26f from exon 26 of human factor VIII cDNA and WAP-3'r from 3' region of transgene construct for amplification of 3.72 kb PCR product (Figure 4).

From 86 live born kits 39 (45.3%) has been PCR positive with primers #3693 and #3694 and 31 kits (36%) has been PCR positive for all primers combination (Table 3) used for the mWAP-hFVIIIcDNA transgene analysis. Eight animals of F0 generation positive for 601 bp PCR product showed as a negative for amplification of 5' region and 3' region of transgene. Seven (22.5%) sexually maturated PCR positive animals, four males were mated and three females inseminated with wild-type counterparts and F1 generation was analysed for transgene integration. Each male shown normal libido and was mated with two wild-type does.

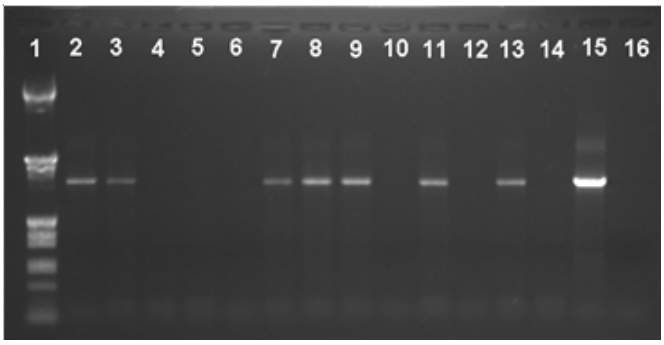

Figure 4: PCR analysis of F1 generation using primers HF Ex26f: 5'- TGC CCT GAG GAT GGA GGT TCT G -3' and WAP-3'r: 5'- GCC CAC TGT GGA TGG TGT CAT C -3' (Table 1) with a PCR product of 3.72 kb. Lane 1 – MW marker lambda DNA/EcoRI + HindIII (Promega), lanes 2, 3, 7, 8, 9, 11, 13 – positive animals, lanes 4, 5, 6, 10, 12 – negative animals, lane 14 – wild type negative animal, lane 15 – positive control, lane 16 – negative control for PCR.

Table 2: Summary of AI of sperm mediated gene transfer experiments.

Female no.	Live born kits	Dead born kits	No. of screened kits	No. of PCR positive[1] (%)	No. of live PCR positive[1] (%)
1	10	0	10	5 (50)	4 (40)
2	8	3	11	5 (45.4)	3 (27.2)
3	8	0	8	4 (50)	2 (25)
4	12	0	12	5 (41.6)	5 (41.6)
5	7	0	7	2 (28.6)	2 (28.6)
6	8	1	9	5 (55.5)	4 (44.4)
7	8	0	8	3 (37.5)	1 (12.5)
8	9	1	10	5 (50)	4 (40)
9	9	0	9	5 (55.5)	5 (55.5)
10	7	0	7	4 (57.1)	1 (14.3)

[1] PCR positive kits with all primers combination in Table 3.

Germline transmission to F1 offsprings are in Table 4. With exception of male no. 02 and female no. 11, all other founder rabbits transmit the transgene to F1 generation. The germline transmission of transgene to F1 generation varied from 0% to 25%. The germline transmission of transgene is the crucial point in assessing of a transgenic animal in this context. Sperm-mediated gene transfer is confirmed as a technique that permits a high rate of transgenic mammals.

It is experimentally documented that quality of semen is important factor in SMGT experiments (Lavitrano *et al.,* 2003). A guideline for the handling of rabbit bucks and semen can be used for selection of semen donors for SMGT experiments (IRRG, 2005). Wang *et al.*, 2003 used washed and lipofectin incubated sperm cells and showed that bovine serum albumin (BSA) incubation medium could effectively block exogenous DNA uptake in rabbit sperm cells. Therefore concentration of BSA in extender should be considered in SMGT experiments. For production of transgenic rabbits they use embryo transfer. Shen *et al*., 2006 employed dimethylsulfoxide (DMSO) as a medium to transfect testicular germ cells with exogenous DNA via repeated direct injection into animal testis. This technique requires surgical manipulation of anesthetised animals.

In our experiments we simply washed twice sperm cells from the selected rabbit donors in commercially available Diluent mixture for rabbit sperm, incubated with linear exogenous DNA and performed standard artificial insemination. No cost liposomes, or repeated animal testes injection or expensive equipment and laborious techniques such as embryo culture, embryo transfer and electroporation are required to obtain genetically modified rabbits.

Remarkable progress in gene mapping and genome sequencing endeavors in livestock will open a new set of possibilities for introduction of precise genetic modifications for agricultural applications. The host of possibilities includes progress in areas like milk production, growth rate, carcass composition, reproductive performance, and disease resistance. SMGT is the inexpensive methods for production of genetically modified livestock.

Table 3: Primers combination used for the mWAP-hFVIIIcDNA transgene analysis.

Forward primer	Reverse primer	PCR product
#3693	#3694	0.6 kb
WAP-5'f	#3694	1.788 kb
HF Ex26f	WAP-3'r	3.72 kb

Table 4: Germline transmission to F1 offsprings.

Rabbit		Live born kits	Dead born kits	No. of screened kits	No. of PCR positive[1] (%)	No. of live PCR positive[1] (%)
sex	no.					
M[2]	01	11	7	18	3 (16.6)	2 (11.1)
M	02	0	0	0	0 (0)	0 (0)
M	03	9	8	17	2 (11.7)	2 (11.7)
M	06	8	4	12	3 (25)	3 (25)
F	08	8	1	9	1 (11.1)	1 (11.1)
F	09	8	0	10	2 (20)	2 (20)
F	11	9	1	9	0 (0)	0 (0)

[1] PCR positive kits with all primers combination in Table 2. [2]M: male, F: female.

CONCLUSION

Rabbit sperm are able to carry foreign DNA into eggs at fertilization. SMGT method establishes a simple and straightforward technology of introducing DNA into rabbits, offering many advantages over other methods, which require considerable skill for micromanipulation. In the field may take advantage to transmit transgene to the progeny with high efficiency.

Acknowledgements: We would like to thank Mr. Jan Pecho and Dipl. Ing. Ivan Chlebec for taking care of the animals and for providing technical assistance. This work was financially supported by Ministry of Agriculture Slovak Republic and Slovak Research and Development Agency under the contract No. APVV-27-005505.

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