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ABSTRACT

At present, the techniques available for the generation of transgenic domestic species are pronuclear injection, and more recently, transformation of fetal fibroblasts followed by nuclear transfer (CIBELLI, 1998). The pronuclear injection technique leads to a random integration of the introduced DNA with unpredictable results and to a high cost associated with the generation of transgenic animals. In cattle and sheep, the technology of nuclear transfer with somatic cell nuclei offers exciting possibilities for reducing the cost of transgenic cattle and seep. In swine and rabbit, unfortunately, it has not been possible to utilise nuclear transfer using somatic cells for generation of a live offspring. Isolation, maintenance, and genetic manipulation of pluripotent embryo-derived embryonic stem (ES cells) could be a promising alternative (MARTIN 1981; EVANS 1981).
Embryonic stem cells enable the engineering of precise modification to the mouse genome by gene targeting (JOYNER 1991). Although, there are reports of cultured cell contribution to chimeras in golden hamster, rat, pig and rabbit, definitive ES cell lines that contribute to the germline, have not been demonstrated in any species but mouse.
We aimed to establish rabbit ES cell line that may contribute to the germ line as well. A strategy originally used for creating ES cell lines from non permissive mouse strains (MC WHIR 1996) was adapted for rabbits. OctNeo transgenic mice was created and transgenic embryos of this strain was exposed to a medium containing G418 antibiotics to establish an ES cell-line with an adaptive selection advantage, because the availability of a transgenic line avoid the need of pronucleus- zygote microinyection..
In our experiments the OctNeo vector was coinjected with the Green Fluorescent Protein encoding EGFP vector (OKABE 1997). Since a joint insertion of the two vectors is expected, the presence of the EGFP also marks the insertion of the OctNeo vector. More than 900 microinjected rabbit embryos have been transferred to the recipient mothers. After the PCR analysis of DNA of the 106 progenies born so far, and two OctNeo/EGFP transgenic rabbit were found. Transgenic founders were mated to non transgenic rabbits, the transmittance of the transgene in the progenies is currently examined.

INTRODUCTION

Mouse embryonic stem cell lines (ES cell lines) are undifferentiated, permanently dividing cells (MARTIN 1981; EVANS 1981). These cells are originally derived from the proliferating inner cell mass of a preimplantation mouse embryo. ES cells are able to contribute to any tissue of developing chimera embryo if they are introduced into an embryonic environment by injection into a host blastocyst or by aggregation of two eight-cell stage host embryos. Most importantly, they can form gametes. In the last few years, mouse ES cells have become a leading means of introducing genetic alterations into the mouse

genome (JOYNER 1991). Although there are reports of cultured ES cell contributions to chimeras in golden hamster (DOETSCHMAN 1988), rat (IANNACCONE 1994), rabbit (GRAVES 1993) and pig (PIEDRAHITA 1990), definitive ES cell lines that contribute to the germline have not been demonstrated in any species, but mouse (NAGY 1990, 1993).

McWhir and his colleagues at the Roslin Institute have recently developed a novel drug selection strategy for the isolation of mouse ES cells (McWHIR 1996). In these studies, a construct was built in which the gene for resistance to the drug G418 (neo) was controlled by a promoter Oct3/4 that is normally active only in the undifferentiated cells of the epiblast. OctNeo transgenic mice were generated by pronuclear injection of the construct into embryos from a strain that normally does not permit isolation of ES cells. Embryos from these transgenic mice were cultured in the presence of G418. Within the embryos cultured in the presence of G418, cells that differentiated were immediately removed because their resistance to the drug was lost. This allowed the epiblast to expand and give rise to permanent ES lines. We intend to generate transgenic rabbit line by pronuclear injection of the OctNeo construct into rabbit embryos.

The OctNeo vector was coinjected with the EGFP vector (OKABE 1997). Green fluorescent protein (GFP) is a useful marker gene found in *Aequorea victoria*. GFP is nontoxic and shows green fluorescence without exogenous substrates or co-factors. The fluorescence of Enhanced Green Fluorescent Protein (EGFP) is 35 times stronger than the GFP (TAKADA 1997). The expression of EGFP can easily be observed by fluorescent microscopy. The co-injection of a marker gene with the DNA construct of the gene of interest in most cases results in the co-integration and co-expression of the foreign DNA (MC FAHRLEN 1996, PRUNKARD 1998).

MATERIALS AND METHODS

Animals, DNA microinjection

Mice: (C57BlxCBA) F1 females and males were used for generation of embryos. The DNA microinjection procedure was generally carried out according to the protocol of Hogan et al. (HOGAN 1994). The fertilised one-cell embryos were collected from the oviduct of superovulated (C57BlxCBA) F1 females that were mated to (C57BlxCBA) F1 males. The male pronucleus of the mouse zygote was microinjected with solution of linearized DNA in 2 ng/µl concentration in M2 media. The injected embryos were cultured in a microdrop of M16 medium (SIGMA) in a humidified atmosphere of 5% CO_2 and 95% air at 37^oC until transfer, or until they developed into blastocysts.

Rabbits: Animals used in this study were sexually mature New Zealand White females which were superovulated by intramuscular administration of follicle stimulating hormone to enhance the yield of ova (20 I.U. PMSG/kg body weight), followed 3 days later by intravenous delivery of chorionic gonadotropin (180 I.U HCG/ animal). The animals were mated to fertile males or by artificial insemination. The typical yield were 20-30 zygotes flushed from the oviducts 20 hours later. The efficiency of embryo yield were increased by keeping the females for one week in long day length /18 hours of light/ before embryo collection, previous to a long period in short day length /8hours of light/. The large male pronucleus of the rabbit zygote was microinjected with solution of linearized DNA in 2 ng/µl concentration in PBS+20% FCS media. 12-12 injected zygotes were implanted into each oviduct of pseudopregnant recipient females, that had been hormonally induced a day before (120 I.U. HCG per recipients) (URBAN 1998).

Cell culture

Rabbit one-cell stage embryos were flushed from the oviducts of naturally mated females. Embryos were cultured in B2 medium. On Day 5, blastocysts were placed individually onto mitomycin C-arrested mouse embryonic fibroblasts in ES cell culture medium in four-well culture dishes kept at 38.5^{o}C with 5% CO_2 in air. ES cell culture medium consists of high-glucose DMEM (SIGMA) supplemented with 15% FBS (GIBCO), 0.5% nonessential amino acids (SIGMA), penicillin (SIGMA), streptomycin (SIGMA), 0.1mM 2-mercaptoethanol (GIBCO) and 4 mM glutamine (SIGMA). Human LIF (1000 U/ml, Sigma) or recombinant murine LIF (1000 U/ml, Gibco) were added to culture medium. Embryos generally attached to the layer of fibroblasts within 48 hours. On Day 7 in culture, the inner cell mass was mechanically isolated, trypsinised and replated onto four-well dishes. ES-like colonies were subsequently passed onto four-well dishes in seven-day intervals when individual colonies were subcloned.

Analysis of transgenic embryos, PCR method

Construction of transgenes:
EGFP vector: the plasmid (pCX-EGFP) contains the CMV IE enhancer, the chicken ß-actin promoter, the EGFP reporter gene and the rabbit ß-globin 3' flanking sequence (SOE 1997).
OctNeo vector: the pOctneo plasmid was constructed by ligation of two fragments: the
1,95 kb fragment of the mouse Oct3/4 promoter (OKAZAWA 1991), and the 1,7 kb modified bacterial neo gene linked to the 0,65 kb human growth hormone 3' poly-A signal (SELFRIDGE 1992) in a pUC8 vector.

Genomic DNA preparation:
Genomic DNA of the pups was prepared from the tips of tails (at the mice), and from the ears and blood (at the rabbits). According to the standard protocol (HOGAN 1994). The pups were screened for transgnenesis by PCR.

PCR amplification:
PCR amplification was carried out in a mixture of 1xPCR buffer, containing 200μM dNTP, 1,25 mM $MgCl_2$ and 0,1 μM primers and 1.5 units of Taq DNA polimerase (Promega). The following PCR amplification were performed in the thermocycler:
EGFP vector: 94°C 30 sec, 52°C 1 min, and 72°C 1 min (40 times).
OctNeo vector: 94°C 45 sec, 60°C 1 min, and 72°C 1 min (30 times).

Primers:
EGFP1: 5'-TTCGGCTTCTGGCGTGTGAC-3'
EGFP2: 5'-CCTTCAGCTCGATGCGGTTC-3'
Octneo1:5'- TGCTCTGATGCCGCCGTGTTCCGGCTG-3'
Octneo2:5'- ATCGCCATGGGTCACGACGAGATCC-3'

RESULTS AND DISCUSSION

Non transgenic ES cell lines establishment

We have derived two independent types of cell lines from rabbit embryos. Five independent putative ES cell lines and four trophoblast cell lines have been established to date. One type displays morphology identical to primary outgrowths of trophectoderm. The trophoblast-like colony grew rapidly. There were about 100 colonies in each well. The second cell type represents presumptive pluripotent ES cells derived from the inner cell mass, grew as colonies of small cells with a high nucleus: cytoplasm ratio. ES-like cells grew very slow in colonies of small cells. Generally, 50 cells in one colony, and 2 or 4 colonies in one well were observed. There was not significant difference between hatching rates when human or murine LIF was added to the culture medium. However, the rate of ES-like cell line differentiation

was significantly higher in the non-treated group and mouse LIF treated group. However, after few passages, these ES like cells have been differentiated even in the presence of human LIF and we could not establish a well-dividing ES cell line by this traditional method.

First step toward establishing the OctNeo/EGFP transgenic ES cell line

In the second series of experiments the strategy of McWhir was used (McWHIR 1996). Transgenic rabbits carrying OctNeo construct were produced. In our experiments, the OctNeo vector was coinjected with the EGFP vector into the male pronuclei of one-cell stage mouse or rabbit embryos. Since a joint insertion of the two vectors was expected, it is hoped that the presence of the EGFP also indicates the insertion of the OctNeo vector after injection.

In vitro experiments

We analysed the mouse and rabbits embryos after pronuclear injection first in vitro. Mouse embryos were placed in M16 medium (HOGAN 1994) and the rabbit embryos in B2 medium (GRAVES 1993). The EGFP transgenic embryos the expression of the EGFP protein and the OctNeo transgenic embryos could be detected with G418 selection. Six (8.9%) EGFP positive green mouse embryos and seven (4.0%) green rabbit embryos were found. Three (4.5%) G418 resistant mouse embryos and four (2.3%) rabbit embryos were found. No OctNeo-EGFP transgenic mouse embryo, but one (0.57%) OctNeo-EGFP transgenic rabbit embryo was found (*Table 1*).

Table 1.

IN VITRO	mouse	%	rabbit	%
No. of Zygotes injected	67	-	174	-
No. (%) OctNeo positive G418 resistant embryos	3	4.5	4	2.3
No. (%) EGFP positive green embryos	6	8.9	7	4.0
No. (%) OctNeo/EGFP positive embryos	0	0	1	0.6

In vivo experiments

255 microinjected mouse and 918 rabbit zygotes have been transferred to the recipient mothers. 55 (21.6%) mouse and 106 (11.5%) rabbit progenies have been born. After the PCR analysis of DNA of the new-borns, two (3.6%) OctNeo PCR positive, and three (5.5%) GFP expressing mice, six (5.7%) OctNeo PCR positive and three (2.8%) EGFP PCR positive rabbits were found. We got two (1.9%) OctNeo/EGFP transgenic rabbit, but could not detect OctNeo/EGFP transgenic mice (*Table 2)*. In the following period the co-injection of mouse and rabbit embryos with EGFP and OctNeo vectors will be continued. We try to find the reason why no double transgenic mice, and less double transgenic rabbit were born than expected based on earlier results (McFAHRLEN 1996, PRUNKARD 1998).

While six OctNeo-transgenic rabbits were found following the PCR-analysis of DNA of tail samples, no transgenic animal were detected in the PCR analysis of blood DNA-samples (*Table 3)*. From these, and from other results of in vitro experiments, it can be concluded that in OctNeo positive rabbits a mosaic insertion of the transgene was observed (WILKIE 1986). In rabbit and mouse embryos expressing EGFP mostly showed mosaicism and the transgene insertion could not be confirmed. Testing of PCR positive rabbits is currently under way.

Table 2.

IN VIVO	Mouse	%	Rabbit	%
No. Zygotes injected	382	-	1529	-
No. Zygotes transferred	255		918	
No. (%) Newborns	55	21.6	106	11.5
No. (%) Survived newborns	55	21.6	67	7.3
No. (%) OctNeo PCR positive newborns	2	3.6	6	8.9
No. (%) EGFP PCR positive newborns	3	5.5	3	4.5
No. (%) OctNeo/EGFP PCR positive newborns	0	0	2	3.0

Table 3.

Transgenic founder rabbits	**SEX**	**OctNeo PCR**	**OctNeo PCR**	**EGFP PCR**
		Ear	Blood	Blood
44	F	+	-	+
85	F	+	-	+
93	F	+	-	-
97	M	+	-	-
99	-	+	-	-
100	F	+	-	-
331	F	-	-	+

There are several steps to be done to obtain an OctNeo/EGFP transgenic rabbit ES cell-line. First, the transgenic rabbit lines should be established, then embryos derived from these rabbits can be used to develop a method to establish ES cell-line. Successful adaptation of OctNeo transgenic mouse ES cell line derivation method, rabbit ES cells may help to create more appropriate transgenic models for human diseases such as arthreriosclerosis, and HIV-1 infections disease than mouse.

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