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Production of Transgenic Chimeric Rabbits and Transmission of the Transgene Through the Germline

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ABSTRACT Here we report that improved reproductive technologies combined with an efficient microinjection method and in vitro cultivation medium enabled us to create germ line chimeric rabbits. To follow the fate of the chimeric embryo a blastomere marked with the human blood coagulation factor VIII (hFVIII) transgene was microinjected into a morula stage wild type embryo. The degree of chimerism in different tissues was estimated by real-time PCR and was found to be in the range of 0.1–42%. Among the four chimeric animals, one was identified as a chromosomal intersex and two were germline chimeras. *Mol. Reprod. Dev. 68: 435–440, 2004.*

Key Words: reproductive technologies; developmental biology; germline chimeric rabbit; real-time PCR

INTRODUCTION

The rabbit has several advantages over other laboratory animals and transgenic rabbits are used both as large animal models of genetic and acquired diseases and as bioreactors for producing recombinant proteins (reviewed by Bösze et al., 2003). Present utilization of transgenic rabbits is limited by the low efficiency of microinjection and the absence of proven embryonic stem cell (ES) lines. Cloning of genetically modified donor cells would be an attractive alternative to targeted gene modification. It is promising that the first cloned rabbits from adult somatic cells did not show any obvious morphological abnormalities in the offspring (Chesne et al., 2002). Nevertheless somatic cell cloning is still a very demanding method with a number of unpredictable perturbations in gene expression (Wilmut et al., 2002). Therefore a rabbit ES cell line, which would allow precise genetic modification and could colonize the germ line would be very useful. It was a decade ago when pluripotency of cultured rabbit inner cell mass (ICM) cells were demonstrated by analyzing chimeric fetuses (Giles et al., 1993) but despite years of effort, reliable rabbit ES cell lines have not been obtained (reviewed by Houdebine, 2002). ES-like cells have been isolated and used to create chimeric rabbits (Graves and Moreadith, 1993; Schoonjans et al., 1996) but germ-line transmission of chimerism or transgenes from these animals was not reported. To evaluate the degree of chimerism and the extent of germline transmission we took advantage of a recently-developed transgenic rabbit line (Hiripi et al., 2003), homozygous for the presence of the human blood coagulation factor VIII (hFVIII). Using improved ES cell generation methods (Gallagher et al., 2003; Schoonjans et al., 2003), we previously compared treatments and culture conditions for rabbit ES cells (Gócza et al., 2002). Our ultimate aim is to establish an ES cell line from rabbits which will enable us to perform targeted gene modifications. Towards that aim an indispensable first step was to develop an efficient method to create rabbit chimeras from preimplantation stage embryos.

MATERIALS AND METHODS

Micromanipulation, In Vitro Culture, and Transfer of Embryos

All animals used were sexually mature New Zealand White rabbits. Superovulation and embryo recovery from the donor does oviducts was performed as published (Hiripi et al., 2003). Eight cell embryos were obtained 44 hr after insemination. From the flushed precompacted transgenic embryos the mucin coat and zona pellucida were removed by incubation in 0.5% pronase (Sigma P8811) solution for 10 min. The blastomeres were separated individually by pipetting the zona free embryos up and down in a glass capillary tube several times. The recipient 16-cell stage embryos were obtained from wild type fertilized does. The holding pipettes for the recipient embryos during the cell

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injection were prepared from borosilicate glass capillaries by use of a microcapillary puller (Backhoffer, Type 462) and a microforge (Narishige, MF-9). The tip of the pulled capillary was broken at 80–100 μm inner diameter. Contrary to the traditional method of making holding capillaries the mouth of the pipette was not fire polished, in order to keep the rabbit embryo covered by the mucin coat in a stable position during micromanipulation. For the injection pipette the tip of the pulled glass capillary was broken with the glass bulb of the microforge at 40 μm inner diameter.

After that the capillary was ground at 45° with a microgrinder (Narishige, PB-7) and a small spike was melted to the tip of the pipette. Only one blastomere was injected into the recipient embryo by a microcontroller-regulated syringe. Following micromanipulation, the chimeric construct embryos were cultured in vitro until compaction in RDH medium (Jin et al., 2000) in a CO_2 incubator at 38.5°C, 5% CO_2 and 98% relative humidity in air. The RDH medium was prepared by mixing Ham's F10, RPMI, and DMEM medium at the concentration of 1:1:1, supplemented with 5 mM taurine (Sigma, T8691) 0.3% BSA (Sigma, A3311). Recipient does received 84 μg GnRH analog intramuscularly (Receptal®, Intervet International B.V. Boxmeer, Holland) in 11–12 hr asynchrony with the donor rabbits. Four to 12 compacted morula stage embryos were transferred to each oviduct of the recipient does by laparoscopy (Besenfelder et al., 1998).

Chromosome Analysis

Chromosome analysis was performed from peripheral blood lymphocyte cultures (Moorhead et al., 1960). Complete mitoses were analyzed for the number of the smallest chromosomes. Metaphases containing nine of the smallest chromosomes (pairs of 18, 19, 20, 21, and Y) were evaluated as male, those containing eight as female cells.

Analysis of Tissues to Detect the hFVIII Transgene

DNA was extracted from ear biopsies of liveborn and stillborn rabbits using the conventional salting-out method (Laird et al., 1991). The presence of the hFVIII transgene was analyzed by PCR using the primers: 5′GCC TCT CAG AGT CAC CAC TTC CTC TGT TGT3′ 5′AAG ACG CTG GGT TGG TCC GAT ACT ATT TAC3′ which define a 1,020 bp region of the hFVIII cDNA as described earlier (Hiripi et al., 2000a). Chimeric animals were sacrificed under anesthesia and the following tissues were dissected and their DNA PCR-tested for the presence of hFVIII: brain, liver, lung, skin, spleen, kidney, muscle, heart, and genital organs of both sexes.

Real-Time PCR Procedure

Real-time PCR is a quantitative and precise method to determine the copy number and zygosity of transgenes in transgenic animals (Tesson et al., 2002). The degree of chimerism in different hFVIII positive tissues was determined by real-time PCR using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The endogenous κ-*casein* gene is represented by two copies in both the normal and the hFVIII transgenic cells, therefore, it was used to determine the total cell quantity.

The fate of the hFVIII transgenic blastomere was followed by the quantification of the *hFVIII* gene in the different tissues of chimeric rabbits. As the donor blastomere was hemizygous for hFVIII, the absolute quantity of the transgene is equal to the amount of hFVIII transgenic cells in a particular tissue. The quantity of normal cells (non-transgenic) was determined using the following equation:

$$n = (k - 2 * \mathrm{hFVIII})/2$$

where n is the quantity of normal cells, k is the quantity of *casein* gene and hFVIII is the quantity of transgenic cells.

Absolute quantification of both genes were based on standard curves generated from serial dilutions of plasmids containing either the κ-*casein* gene (Hiripi et al., 2000b) or hFVIII cDNA (Halter et al., 1993). The two target genes were amplified separately in tubes using 40 ng template DNA in the TaqMan Universal PCR Master Mix (Applied Biosystems, cat no. 4324018). Thermal cycler parameters were 95°C, 10 min; (95°C, 10 s; 60°C, 1 min) ×45.

Detection was performed using the fluorogenic 5′ nuclease technology (TaqMan, Lee et al., 1993). Primer and probe oligonucleotide sequences were designed with the Primer Express v2.0 program (Applied Biosystems) using default parameters. Reaction mix consisted of 0.25 μM from each primer and 0.2 μM probe. Primer and probe sequences for the rabbit κ-*casein* gene were as follows: Primers: 5′AGC CAG TGG TGA GCA CTG AAG T3′ 5′GCA GTT GCT TCC GTG GTA GTC3′ Probe 5′VIC-CAG AGG CTT CCC CAG AGC TCA TCA TCA-TAMRA3′.

For the detection of human clotting factor VIII, the pre-optimized Assays-on-Demand primer/probe mix (Applied Biosystems, cat no. Hs_00240767_m1) was used according to the manufacturer's instructions. Samples were run in duplicate. Moreover tissues representing above or below average degrees of chimerism or showing the worst repeatability were selected and repeated in duplicate again.

RESULTS

Embryo Survival and Pregnancy Rates of Chimeric Construct Embryos Created by Microinjection

Transgenic embryos were produced by mating superovulated wild type donor females with a hFVIII transgenic homozygous buck (Hiripi et al., 2003). A single blastomere marked with the hFVIII transgene was microinjected into a 16-cell stage wild type embryo. The chimeric embryos survived the micromanipulation at a high rate as monitored at the end of the in vitro

TABLE 1. Efficiency of Embryo Transfer for Chimeric Construct and Control Embryos and the Ratio of Chimeric Offspring

	Number of transferred embryos	Number of pups (% of transferred embryos)	Number of live-born pups (% of transferred embryos)	Number of chimeras (% of pups)
Micromanipulated embryos	87	30 (34.5)	28 (32.2)	4 (13)
Control embryos	52	22 (43.2)	18 (34.6)	—

culture period. Manipulating at this stage has the advantage over inner cell-mass injection that morulae are less differentiated than blastocysts. Moreover, Moens et al. (1996) detected chimerism only in those fetuses, which were microinjected with fetal gonadal cells at 8- to 16-cells stage.

Long-term in vitro culture of rabbit embryos results in no implantation after transfer to the uterus, due to the lack of a mucin layer (Adams, 1970). Rabbit oviducts secrete mucin at about 20 hr post coitum, and this substance is known to be essential for successful implantation in vivo. Deposition of mucin and adequate cell number in cultured rabbit embryos may aid development after transfer to recipients. Therefore in our experimental regime the chimeric construct embryos were transferred either on the day of manipulation or after 12 hr in vitro cultivation into the oviduct of the recipient does, which were synchronized 11–12 hr behind the donors. Data obtained from the transfer of 87 micromanipulated embryos show that rabbit embryos prepared in this way gave rise to chimeric rabbits at 13% efficiency (Table 1). The animals were in good physical condition and revealed no gross pathology.

Chromosome Analysis

The diploid number of chromosomes (2N = 44) for the domestic rabbit was first established by Painter in 1926 using amnion cells from 14-day embryos (Chan et al., 1977). Identification of unbanded X- and Y-chromosomes is problematic in this species as none of them are surely distinguishable from autosomes (Frankenhuis et al., 1990). Sexing by counting the smallest chromosomes in complete mitoses was successfully carried out in all four animals.

The two chimeric does were 44,XX and one of the bucks 44,XY. As a young adult the chimeric animal no. 375 was identified as hypogonadic, but later on it sired 27 offsprings. The hypogonadic male proved to be an XX/XY chromosomal intersex with 54 XX and 14 XY cells (Table 2).

Detection of Chimerism

PCR analysis of tissues, germ cells, and offspring. The rabbits born from embryo transfer and the offsprings of the chimeric rabbits were analyzed for the presence of the hFVIII transgene from DNA samples obtained from ear biopsies (Fig. 1). Chimerism was detected from the tissue samples of all four chimeric animals by PCR analysis when they were sacrificed. Figure 1 shows that in case of the hypogonadic buck no. 375 and doe no. 16 out of the nine analyzed tissues three (33%) and four (44%) were chimeric, respectively. The female no. 16 died at the 30th days of its first pregnancy from a bacterial infection, which were not related to the experiment. Therefore, only tissue samples from its five fetuses were analyzed for the presence of the hFVIII transgene. It is interesting to note, that the ovarian tissues of female no. 16 like no. 374 were chimeric by PCR analysis. Significantly higher degree of chimerism, 80 and 91%, respectively was detected in the tissue samples of doe no. 374 and buck no. 334. The semen of buck no. 334 was found to be hFVIII transgene positive in each samplings, and it was used to inseminate normal New Zealand White does. Notably, among the 10 offsprings of doe no. 374 one hFVIII transgenic pub was identified with PCR from ear biopsy.

Real-Time PCR Analysis

Real-time PCR analysis was restricted to the hFVIII transgenic tissue samples of the two chimeric rabbits, which exhibited high levels of chimerism by PCR analysis. The analysis was based on the absolute quantification of the hFVIII and κ-*casein* gene's copy numbers (Fig. 2). The real-time PCR results presented in Table 3

TABLE 2. Results of Chromosome Analysis From the Peripheral Blood of Chimeric Rabbits

Chimeric rabbit no.	Phenotypic sex	Ratio of male metaphases %	Ratio of female metaphases %
16	Doe	0	100
374	Doe	0	100
334	Buck	100	0
375	Hypogonadic buck	20.5	79.5

Complete mitoses were analyzed for the number of the smallest chromosomes.
Metaphases containing nine of the smallest chromosomes (pairs of 18, 19, 20, 21, and Y) were evaluated as male, those containing eight as female cells.

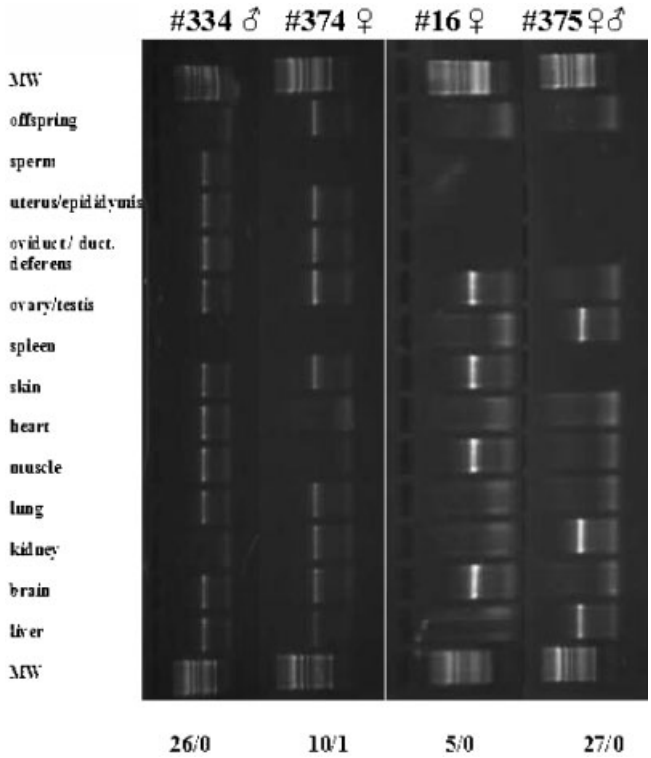

Fig. 1. Agarose-gel analysis of the hFVIII specific PCR products to demonstrate chimerism in tissue samples from rabbits: no. 334, 374, 16, and 375 and from ear biopsy of the offspring from 374 female. The expected size of the PCR band is 1,020 bp. The DNA size standard (MW) was the 1 kb ladder (Invitrogen, 10511-012). The number: 36/1; 20/1; 5/0; and 27/1 correspond to the total number of offspring analysed/number found to be positive by hFVIII-specific PCR.

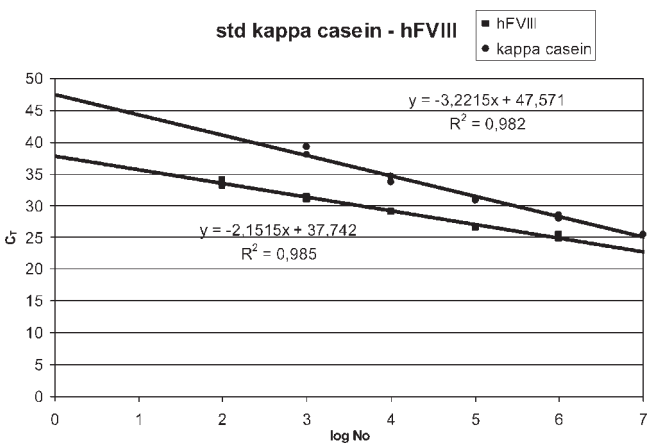

Fig. 2. Standard curve for the absolute quantitation of κ-*casein* and *hFVIII* gene copy numbers. Absolute quantification of both genes were based on this standard curve generated from serial dilutions of plasmids containing either the κ-*casein* gene (Hiripi et al., 2000b) or hFVIII cDNA (Halter et al., 1993). The amount of target sequence (N0) is inversely proportional to CT. CT corresponds to the number of PCR cycles at which the fluorescence intensity of the sample rises above threshold.

TABLE 3. Quantitative Analysis of the Degree of Chimerism in the Tissue Samples of Chimeric Rabbits by Real-Time PCR

Chimeric rabbit		Chimerism[a] (% ± SD)
No. 374	Brain[b]	16.02 ± 1.22
	Liver	7.17
	Lung	25.06
	Skin	1.82
	Kidney[b]	42.60 ± 1.44
	Uterus[b]	3.89 ± 0.66
	Oviduct	4.22
	Ovary	3.62
No. 334	Brain	1.45
	Liver	0.10
	Lung	30.20
	Skin	1.82
	Muscle[b]	0.37 ± 0.07
	Heart[b]	0.14 ± 0.02
	Epididymis[b]	13.47 ± 0.93
	Vas deferens	0.71
	Testis	0.37
	Semen[b]	1.11 ± 0.06

Tissue samples which were identified as positive for the presence of the hFVIII transgene by PCR were chosen for analysis.
[a]The degree of chimerism was expressed as the ratio of transgenic (hFVIII) to normal cells, as described in "Materials and Methods."
[b]Tissues were analyzed twice to test the reproducibility of the method.

along with the data shown in Figure 1 demonstrate that the microinjected blastomere is able to participate in all tissue types analyzed including the germline. Nevertheless its contribution to the different embryonic tissues varies from one animal to the other (compare brain, liver or kidney in nos. 374 and 334). It is worth noting that the degree of chimerism in the tissues which were found to be hFVIII transgenic by PCR varied randomly over a wide range, from 0.1% till 42%. The level of chimerism was low in the semen samples of the no. 334 buck (1.1%), which could explain the fact that among its 26 offspring no hFVIII transgenic were detected.

DISCUSSION

Since the first chimeric rabbits were reported (Gardner and Munro, 1974), successful attempts have used cells at stages of development nearly equivalent to the recipient embryo by the combination of two morulae (Yang and Foote, 1988); ICM cells injected into day-4 blastocysts (Babinet and Bordenave, 1980); or fetal gonadal cells injected at 8–16 cell stage embryos (Moens et al., 1996). Recently transgenic rabbit fetuses were produced as chimeras between embryos generated by somatic cell nuclear transfer and normal embryos. In future this method could be an alternative to microinjection for production of transgenic rabbits (Matsuda et al., 2002).

ES cells derived from mink morulae displayed also a high level of pluripotency (Sukoyan et al., 1993). Hence creating germ cell line chimeric rabbits using morulae

derived blastomeres is a useful step on the way of establishing a rabbit ES cell line. Jin et al. (2000) compared pregnancy rates while altering the synchronization time of recipients. Live born pups were obtained after 72 hr in vitro cultivation of embryos when recipient does were synchronized 1 day behind the donor. Cloned rabbit embryos were successfully implanted when transferred into recipients mated with vasectomized males 22 hr after the donor females (Chesne et al., 2002). The high rate of chimeric construct embryos born alive could be partly explained by the adaptation of the asynchronous timing for hormonal treatments of the recipient does. The chimeric rabbits including the chromosomal intersex male were fertile and did not show growth abnormalities. In the case of chimeric mice the relationship of chromosomal sex to functional germ cells has been long time analyzed in detail. It was first described by Tarkowski (1961) and confirmed by several authors that XX/XY chimeric mice constructed at an early embryonic stage develop as male (Mullen and Whitten, 1971; Gearhart and Oster-Granite, 1981; Yoshino et al., 1994; Tarkowski, 1998). Our data underline this observation in rabbits since the chromosomal intersex animal developed as a buck, albeit the ratio of male cells in its blood was only 20%. Babinet and Bordenave (1980) in their pioneering experiment reported germ line chimerism assessing it in the lymphoid organs with the help of allotypic immunoglobulin markers in the founders and their progeny. However, at that time no other markers were available to estimate the extent of chimerism. Since then a number of different laboratories have reported the formation of rabbit chimeras, but germline transmission in these animals was not confirmed (Yang and Foote, 1988; Giles et al., 1993; Moens et al., 1996; Schoonjans et al., 1996). Real-time PCR combined with transgenic technology enabled us to follow the fate of the hFVIII transgenic blastomere in the tissues and germ line of the chimeric animals, and the degree of chimerism was qualitatively as well as quantitatively determined.

The first report of manufactured mammalian chimeras (Tarkowski, 1961) introduced an entirely new method for analyzing early mammalian development. These chimeras have gained renewed importance, because this is a very informative approach for addressing questions about the mutant phenotype and the abnormal behavior of mutant cells (Rossant and Spence, 1998). Detailed GPI (glucose phosphate isomerase) analysis of diploid mouse ES cell chimeras showed that the ES cell contribution was widespread in tissues of chimeric animals. Certain tissues such as thymus and lungs had consistently low contributions. The same skewed distribution was also seen in control ICM aggregation chimeras of matched genotypes, indicating a strain-specific effect (Gócza and Merentes-Diaz, 1996).

In contrast our data show that in rabbit chimeras the cells derived from microinjected blastomeres were able to participate with equal probability in all tissue types including the germline. Moreover the contribution of the blastocyst derived cells to the different embryonic tissues and the degree of chimerism varied from one animal to the other. Further experiments are necessary to reveal the species specific differences in early embryonic determination.

CONCLUSIONS

Chimeric rabbits created earlier were poorly characterized and produced at low efficiency. In this study we created chimeric rabbits and followed the fate of the microinjected blastomere using the human factor VIII transgene as a marker. In our experiments chimeric construct embryos gave rise to chimeric rabbits at 13% efficiency. To estimate the level of chimerism of different tissues real-time PCR was used. The single microinjected blastomere was able to populate all examined tissue types, including the germ line.

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