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Short Note

Production of Recombinant Human Protein C in the Milk of Transgenic Rabbits from the F3 Generation

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The stability of transgene transmission, milk production and milk content of recombinant human protein C (rhPC) in transgenic rabbits of the F3 generation was determined. Transgenic rabbits carrying the 4.2kb mouse whey acid protein promoter and 9.4kb genomic human protein C were produced after mating transgenic (F2 generation) and non-transgenic rabbits. PCR analysis of F3 samples showed that the transgene was transmitted. Milk production, obtained at the 10^{th}, 15^{th}, 20^{th} and 30^{th} day of the first lactation by the weight-suckle-weight method showed no significant difference between transgenic and non-transgenic does. Concerning rhPC secretion, Western blotting detected a light chain (21 kDa) of rhPC in the milk and an ELISA test confirmed rhPC at the level of 0.109-0.301 μg/ml. A milk sample from a non-transgenic rabbit was hPC negative. In conclusion, the stability of hPC transgene transmission and production of rhPC protein C was confirmed in generation F3 of transgenic rabbits, with similar efficiency as in F1 and F2 generations, without reduction of milk production.

Key words: rhPC, transgenic, rabbit, milk production.

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The selection criterion for choosing the most suitable species for gene farming can be based on the quantity of protein needed per year. Compared with the larger farm animals, rabbits have unique features. The protein content of rabbit milk is 2.5 times higher than sheep milk, 4.8 times higher than goat milk and about 5.5 times higher than cow´s milk. A lactating rabbit female can produce 170-220 g of milk per day and yield up to 10 kg of milk per year under semiautomatic hygienic milking conditions (DUBY *et al*., 1993).

Another criterion which influences the choice of species for transgenesis are reproductive capabilities focused on reproductive interval and number of offspring per litter (production of new generations with reasonable efficiency), with stable transgene transmission in the offspring's genome. ZINOVIEVA *et al*. (1998) reported that the IGF-1 transgene was stably transmitted and expressed in six generations, CHRENEK *et al*. (2004) showed stable hFVIII transgene trasmission in three generations of transgenic rabbits produced using different microinjection techniques.

Housing of transgenic animals and production operations seem to be less expensive than that of fermentation and cell culture facilities. In general, cell culture production is 2-3 times more expensive than transgenic animal technology (HODGSON 1992). Thus, in terms of several economical, reproductive and hygienic (sanitary) aspects, the rabbit could be a suitable species for gene farming.

This study demonstrates the stability of hPC (human protein C) transgene transmission, content of recombinant human protein C in the milk of transgenic rabbits derived from the F3 generation and compares milk production between transgenic and non-transgenic females lactating on the first lactation.

Material and Methods

Biological material

A line of transgenic rabbits was generated by microinjecting a WAP-hPC gene construct into the pronucleus of a fertilized egg (CHRENEK *et al*. 1999). F3 generation rabbit offspring were gained after mating transgenic (F2 generation) and non-transgenic rabbits. The PCR technique was used to detect WAP-hPC gene construct integration in the transgenic rabbit from generation F3 as reported previously (CHRENEK *et al*. 2002).

Milk collection

Milk production of transgenic and non-transgenic does was estimated on the 10th, 15th, 20th and 30th day of lactation by the weight-suckle-weight method. Milk samples were taken from lactating females on the 15th, 20th and 30th day of first lactation. In order to stimulate milk letdown, intramuscular injection of 5IU of oxytocin (Leciva, Czech Republic) was applied 10 min before milk collection. The milk samples were either analyzed immediately or stored at –80°C before further processing.

Detection of hPC gene expression

The presence of rhPC (recombinant human protein C) in transgenic and non-transgenic rabbit milk samples was detected by Western blotting as described previously (CHRENEK *et al*. 2002). Shortly, defatted milk samples were electrophoresed by SDS-PAGE under non-reducing conditions. Proteins were then transferred by semi-dry trans-blot onto a ECL Hybond membrane (Amersham Pharmacia Biotech, Sweden). The membrane was blocked in 3% H_2O_2 for 15 min and in 5% BSA in TTBS (Tris buffer Tween 20) for 1 hour, and probed with sheep anti-human protein C antibody (Dako A/S, Denmark). Specific bands were visualized by incubation with peroxidase-conjugated rabbit anti-sheep IgG antibody (Dako A/S, Denmark).

Secretion of rhPC into transgenic milk was determined quantitatively by ELISA-kit (Assechrom protein C:Ag, Diagnostica Stago, France), according to the manufacturer's manual. Milk samples were diluted in the range of 1:10-1:20.

Results and Discussion

Transgenic animal production

Integration of the WAP-hPC gene in ear samples of two rabbit females (no.116 and no.117) from the F3 generation was detected by PCR (data not shown). The transgenic rabbits were apparently normal and crossing with non-transgenic males yielded a litter of normal size without any disturbance during lactation. Stable transgene integration in transgenic rabbit using a different gene construct (hFVIII) in the F2 generation was also detected in the same laboratory (CHRENEK *et al*. 2004). ZINOVIEVA *et al*. (1998), obtained six generations of transgenic rabbits with stable integration and production of biologically active IGF-1 without any negative effect on the physiological or reproductive performance. Phenotypic and genotypic stability of hPC gene expression has been reported in several multiple lines of transgenic animals including mouse and pig (CHEN *et al*. 2002, VAN COTT *et al*. 1997). Our results correspond to data of earlier studies.

Milk production

Transgenic females, no.116 and no.117, showed a gradual increase of milk production until day 20, afterwards it decreased during the 10 day period until the end of the experiment (Table 1). No significant differences were obtained between transgenic and non-transgenic females at the first lactation.

Milk production of transgenic females was comparable with those of non-transgenic does. SCHRANNER (1993) reported similar results (day 10 – 0.18kg, day 20 – 0.21kg, day 30 – 0.17kg), and this observation is in agreement with previously published reports of other authors (CHRASTINOVA *et al*., 1997, DRAGIN *et al*., 2004).

Detection of hPC gene expression

In milk samples from both lactating transgenic females from the F3 generation, Western-blotting analysis revealed a 21kDa specific band, which

Table 1

Milk production of transgenic and non-transgenic does at first lactation (kg)

Female	Days of lactation			
	10th	15th	20th	30th
transgenic				
no. 116	0.16	0.18	0.19	0.14
no.117	0.18	0.22	0.32	0.20
non-transgenic				
no. 12	0.18	0.21	0.24	0.17

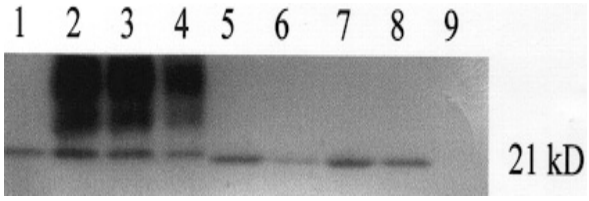

Fig. 1. Western blott analysis of transgenic and non-transgenic rabbit milk samples. Lanes: 1 – blood plasma (1:40); lane 2, 3 and 4 – hPC (2.5μg, 1.25μg and 0.62μg of hPC); lane 5 – milk from transgenic female no.117 (1:10); lane 6 – milk from transgenic female no. 117 (1:20); lane 7 – milk from transgenic female 116 (1:10); lane 8 – milk from transgenic female 116 (1:20); lane 9 – milk from non-transgenic female.

corresponds to a light chain of rhPC. This band was not seen in the milk from a non-transgenic female, but was detected in human plasma samples and in recombinant hPC preparations used as positive controls (Fig. 1).

The concentrations of rhPC produced in the mammary gland of both transgenic females from the F3 generation, determined by ELISA, ranged within 0.109 to 0.351 μg/ml depending on lactation day (Table 2). No signal was detected in the milk of the non-transgenic female.

However, the concentration of rhPC in the mammary gland of transgenic rabbit females was low, similar to F1 and F2 generations (Table 3) of transgenic rabbits (CHRENEK *et al*. 2002). The concentration of recombinant hPC changes concomitantly with a change in milk yield during lactation, with peak on the 20th day of lactation.

The concentration of rhPC in milk among different generations and even among animals within the same generation was highly variable (for instance, F2 generation 0.030-0.375 μg/ml). This means that transgene expression is an individual parameter of each female. It is possible that such variability is due to a different copy number of the transgene or localization of the transgene on the chromosome. Otherwise, it may be affected by different milk yield, eventually different milk composition (content of fat, proteins and others), which can interfere with the proper measurement of rhPC.

VAN COTT *et al*. (2001), a using different hPC gene construct, obtained rhPC secretion in transgenic pig milk, in a range of 40 to 1200 μg/ml. These authors also suggested that pigs with rhPC expression levels less than 500 μg/ml had no sig-

Table 2

Concentration of rhPC in milk of transgenic and non-transgenic females (μg/ml)

Female	Days of lactation			
	15th	20th	30th	Average
transgenic				
no. 116	0.209	0.351	0.190	0.250
no.117	0.109	0.236	0.127	0.157
non-transgenic				
no.12	0.0	0.0	0.0	0.0

Table 3

Concentration of rhPC in milk of three generations of transgenic females (μg/ml)

Female	Days of lactation				
	15th	20th	30th	Average	Author
F1 generation					CHRENEK *et al*. 2002
no. 8 (I-lactation)	0.102	0.136	0.110	0.116	
no.8 (II-lactation)	0.155	0.300	0.186	0.213	
F2 generation					CHRENEK *et al*. 2002
no.105	0.235	0.560	0.330	0.375	
no. 110	0.023	0.048	0.019	0.030	
F3 generation					present study
no. 116	0.209	0.351	0.190	0.250	
no. 117	0.109	0.236	0.127	0.157	

nificant differences in milk protein composition compared to non-transgenic pigs. The same authors concluded that transgenesis and rhPC secretion in milk was not related to any abnormality of milk production such as mastitis or other mammary gland disorders.

In conclusion, the present study confirms the stability of hPC transgene transmission and proves that rhPC can be steadily secreted over multiple generations with no interference of milk production.

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