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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

The State of Health and the Reproductive Potential of Transgenic Mice Secreting Recombinant Human Lactoferrin in Milk

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Maintenance of transgenic animals' health and preservation of their reproduction is of primary importance for their long-term use as bioreactors of human medicinal proteins. It is known that many human bioactive proteins may have similar physiological effects in animals. To obtain transgenic animals secreting human medicinal proteins with milk, tissue-specific promoters are used in genetic engineering. The reproductive ability of transgenic animals is disturbed only if integration of a foreign genetic material into the animal genome results in injuries (insertions) in the DNA regions that are associated with the reproductive function. Insertions may also change any other important genomic regions of transgenic animals. To be used in practice, transgenic animals should ensure economically significant production of proteins of interest with milk. These issues were studied in a model experiment in mice transgenic for the human lactoferrin gene.

Mouse strains were obtained by breeding the primary transgenic animals transmitting the transgene with a high level of production of this protein with milk. The initial strains are characterized by a normal reproductive ability, correspond to the physiological norm characteristic for this animal species, and retain these characteristics (including the level of human lactoferrin production in milk) in a series of generations.

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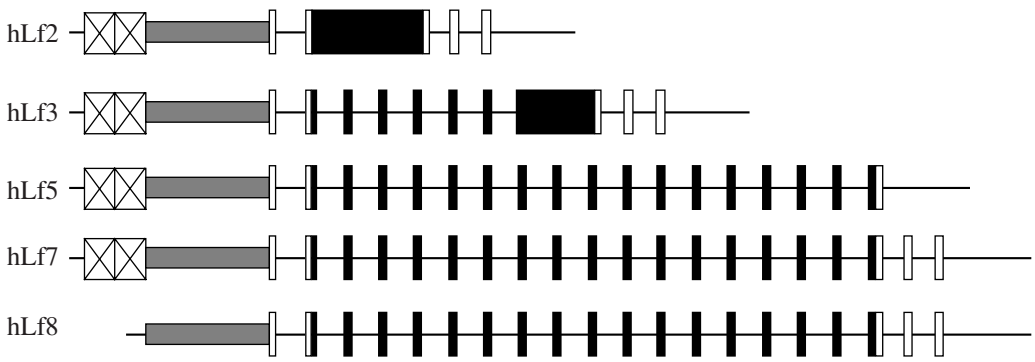
Lactoferrin is a human breast milk protein that protects a newborn from intestinal infections until it develops an own mechanism of immunological defense [1–3]. Obtaining this human protein with milk farm animals being used as bioreactors will make it possible to create an adequate nutrition for artificially fed infants. The bactericidal properties of human lactoferrin are promising for creating a wide spectrum of highly efficient and biologically safe drugs of a new generation [4–6].

OBTAINING AND BREEDING OF PRIMARY MICE TRANSGENIC FOR THE HUMAN LACTOFERRIN GENE

We used five genetic constructs created at the Institute of Gene Biology, Russian Academy of Science (figure), which contained the lactoferrin gene and ensured the production of this protein by transgenic animals with milk. The constructs were created on the basis of the pBC1 vector from the pBC1 Milk Expression Vector kit (Invitrogen, United States). The hLf2 construct, representing the 2100-bp lactoferrin cDNA, was cloned into the pBC1 vector at the *Xho*I restriction site. The hLf3 construct, a hybrid construct containing the lactoferrin gene, the first part of which was the genomic copy comprising exons 1–7 (14 479 bp long, from the ATG codon to the *Sma*I site) and the second part was cDNA (1331 bp, from the *Sma*I site to the stop codon), was cloned into the pBC1 vector at the *Xho*I site. These two parts were linked at the *Sma*I site located at exon 7. The hLf5 construct, the genomic lactoferrin sequence 35 013 bp long starting from the ATG codon, was cloned into the pBC1 vector at sites *Xho*I and *Not*I. The hLf7 construct, the genomic lactoferrin sequence 28 672 bp long starting from the ATG codon, was cloned into the pBC1 vector at the *Xho*I site. The hLf8 construct was derived from hLf7, from which



Structure of expression constructs carrying the human lactoferrin gene. Two copies of the insulator are indicated with crosses; the β-casein promoter is shown in gray; the coding and untranslated regions of the lactoferrin gene are shown in black and white, respectively.

the insulators upstream of the β-casein promoter were deleted.

The primary transgenic mice were obtained by the conventional procedure of DNA microinjections into the zygote pronucleus. The results are summarized in Table 1.

Out of 54 primary transgenic mice carrying the human lactoferrin gene (27♀ and 27♂), 11 animals gave no progeny and 43 primary mice proved to be able to breed; however, 9 of them did not transmit the transgene (6♀ and 3♂). One primary transgenic female and its transgenic progeny did not produce human lactoferrin with milk. Thus, a considerable proportion of primary transgenic animals (37%) were discarded for different reasons. The content of human lactoferrin in the milk of primary transgenic mice varied depending on the genetic construct used as well as in different individuals (Table 2).

Several strains of transgenic mice (to *F*8) producing human lactoferrin with milk at a concentration of 10–15 g/l were then obtained from the primary transgenic mice that had a high level of expression of human lactoferrin in milk.

TISSUE SPECIFICITY OF RECOMBINANT HUMAN LACTOFERRIN EXPRESSION AND ITS QUALITY

The production of recombinant human lactoferrin with milk of transgenic mice was tissue-specific. Trace amounts of human lactoferrin were only detected in the brain and muscle tissue of the animals.

In a special study we showed that the recombinant human lactoferrin secreted in milk of transgenic mice is identical in physicochemical characteristics and biological activity to the lactoferrin obtained from breast milk [7].

PHYSIOLOGICAL AND PATHOMORPHOLOGICAL STUDIES OF THE TRANSGENIC MICE

In total, population studies were performed with approximately 2 500 mice. The comparison with respect to the number of progeny, individual development, and survival of juveniles did not reveal any significant differences between the experimental and control animals. The transition of the transgene to the progeny in *F*1 was somewhat lower than the calculated level; however, starting from *F*2, the average value of

Table 1. Results of experiments on obtaining primary mice transgenic for the human lactoferrin gene

Construct	Quantity of microinjected zygotes	Quantity of recipients	Quantity of embryos transplanted to one recipient	Quantity of born pups (% of transplanted zygotes)	Quantity of transgenic animals (% of transplanted zygotes)	Transgenic animals, percent of born pups
hLf2	400	19	21.05	13 (3.25)	5 (1.25)	38.46
hLf3	610	38	16.05	92 (15.08)	18 (2.95)	19.57
hLf5	380	24	15.83	36 (9.47)	10 (2.63)	27.78
hLf7	358	21	17.05	121 (33.80)	16 (4.47)	13.22
hLf8	257	16	16.06	92 (35.80)	5 (1.95)	5.43
Total	2005	118	16.99	354 (17.66)	54 (2.69)	15.25

Table 2. Human lactoferrin content in milk of 18 primary transgenic female mice producing this protein

Genetic construct	Number of primary transgenic mice	Production of human lactoferrin with milk, g/l
hLf2	221	4.00
hLf3	277	6.10
	1118	8.70
	385	14.00
	263	10.20
	230	0.87
hLf5	112	1.50
	115	4.80
	116	33.00
	146	40.00
hLf7	705	4.20
	787	5.60
	790	8.09
	793	3.70
	809	7.40
hLf8	1252	3.30
	1238	0.22
	1174	1.50

the transgene transition did not drop below 40%. The proportion of males (♂) and females (♀) in the transgenic progeny was approximately the same (Table 3).

For assessment of the health status of experimental animals in the Laboratory of Biological Tests of the Shemyakin–Ovchinnikov Institute of Organic Chemistry (Pushchino Branch) and at the Department of Histology, Cytology, and Embryology of Sechenov Moscow Medical Academy, we performed pathomorphological and histological analyses of four (2♂ and 2♀) transgenic mice carrying the LTF5 construct, which ensures the highest level of human lactoferrin production in milk in comparison with intact (control) four (2♂ and 2♀) mice. We examined mature animals (*F*1–*F*8) receiving the standard diet.

During the external examination of mice bodies and visual assessment of the state of organs of the abdominal and thoracic cavities, cervical organs, as well as the brain and spinal cord, no pathological deviations characterizing each group of examined animals was detected. This allowed us to conclude that the state of the animals was satisfactory.

When performing histological studies of tissues and organs of experimental and control animals, we studied the following internal organs and tissues of mice: the brain, the spinal cord, the spleen, mesenterial lymph nodes, the thymus, lungs, the heart, kidneys, the uterus and ovaries (in lactating females), appendages, the prostate and testicles (in males), skin, skin with the mammary gland, the biceps muscle of thigh, the thyroid gland, the bladder, the pancreas, the liver, the stomach, and intestines (duodenum, nestis, ileum, caecum, colon, and rectum).

The analysis of histological preparations of the brain and spinal cord as well as the intramural ganglia and nerves, revealed no pathological changes in the experimental and control animals. Similar normal morphological patterns were observed in the experimental and control animals in gonads (testicles and ovaries), skin derivatives (hair and glands), adrenal glands, organs of the cardiovascular system, lungs, and organs of the excretion system. The morphological pattern of the skeletal muscle tissue of the biceps muscle of the thigh, skeletal muscle tissue, skin and spinal cord was similar and unchanged both in the experimental and control groups. In the biceps muscle of the thigh of one

Table 3. Transmission of transgenes hLf3, hLf5, hLf7 during breeding of some primary transgenic female and male mice

Genetic construct	Number of primary transgenes	Quantity of studied progenies	F1			Quantity of studied progenies	F2		
			Quantity of transgenes, %				Quantity of transgenes, %		
			total	♂	♀		total	♂	♀
Breeding of primary transgenic females									
hLf3	263, 277, 385, 1118	93	29 (31)	13 (45)	16 (55)	43	9 (21)	4 (44)	5 (56)
hLf5	112, 116, 146,	39	27 (69)	13 (48)	14 (52)	131	66 (50)	32 (48)	34 (52)
hLf7	643, 787, 790, 793, 809	93	21 (23)	15 (71)	6 (29)	82	37 (45)	14 (38)	23 (62)
	Total	225	77 (34)	41 (53)	36 (47)	256	112 (44)	50 (45)	62 (55)
Breeding of primary transgenic males									
hLf3	281, 373, 382, 398, 1112	154	52 (31)	20 (38)	32 (62)	158	74 (47)	35 (48)	39 (52)
hLf5	113, 138, 145, 172	162	40 (28)	19 (45)	21 (55)	82	27 (38)	15 (53)	12 (47)
hLf7	516, 699, 704, 803, 825, 864	248	78 (31)	34 (44)	44 (56)	125	46 (47)	24 (57)	22 (43)
	Total	564	170 (30)	73 (43)	97 (57)	365	147 (40)	74 (50)	73 (50)

animal, we distinguished a zone in which partial lysis of several muscle fibers and simultaneous well-expressed formation of new young fibers (myotubule stage) were observed. The discovered zone may correspond to posttraumatic regeneration of the skeletal muscle fiber. The study of digestive glands revealed similar morphological patterns in animals of both groups. We also detected small local zones in the exocrine sections of the pancreas of one experimental and one control animals that contained cells with vacuolized cytoplasm. The hematopoietic and immunogenic organs had similar morphological parameters in the experimental and control animals. Note a relative increase in the germ centers of lymphatic nodes of both groups of animals.

The lungs of experimental and control animals had a similar morphology of airways and respiratory section, except for the zone of the subpleural atelectasis of an unclear etiology, observed in one of the control animals.

The results of the population studies allowed us to make a general conclusion that the mice transgenic for the human lactoferrin gene correspond to the physiological norm for this species, because single changes detected on the histological preparations were equally characteristic of experimental and control animals. Importantly, the reproductive function and the high level of lactoferrin production with milk of transgenic animals were retained.

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