Co-expression of Human Proteins (IL-10, TPO and/or Lactoferrin) into Milk of Cross-Breed Transgenic Mouse

Zhen-Yu Zheng[1], Hyo-Sang Lee[2], Keon Bong Oh[2], Deog-Bon Koo[2], Yong-Mahn Han[3] and Kyung-Kwang Lee[2,†]

[1]*The College of Animal Husbandry and Veterinary, Henan Agricultural University, 95 Wenhua Rd., Zhengzhou, China*
[2]*Center for Regenerative Medicine, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, Korea*
[3]*Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 305-701, Korea*

ABSTRACT

We have previously produced transgenic (TG) mice expressing the human lactoferrin (hLF), interleukin-10 (hIL-10), and thrombopoietin (hTPO) proteins in the milk. In this study, we examined whether simple crossbreeding between two kinds of a single transgenic mouse can produce double transgenics co-expressing two human proteins.. The *hLF* male, and the *hIL-10* male were crossbred with the *hIL-10* and *hTPO* females, and the *hTPO* female, respectively. PCR analysis for genotyping showed 32%, 23%, and 24% double transgenic rates for *hLF/hIL-10*, *hLF/hTPO*, and *hIL-10/hTPO* transgenes, respectively. We analyzed the expression levels of the human proteins from double transgenic mice and compared those with their single transgenic siblings. All double transgenic co-expressed two human proteins at comparable levels to singles', unless hTPO was not co-expressed: for hLF, 1.1 mg/ml in hLF/hIL-10, whereas 0.5 mg/ml in hLF/hTPO; for hIL-10, 4.1 mg/ml in hIL-10/hLF, whereas 1.4 mg/ml in hIL-10/hTPO. The downregulation of hTPO to half level of singles' was observed in double transgenic mice. The possible reason why hTPO co-expressed might lead to down-regulation of another human protein was discussed. These results suggested that double transgenic generated by crossbreeding between two singles' could be useful system for bioreactor.

(Key words : Human lactoferrin, Human TPO, Human IL-10, Double transgenic mouse)

INTRODUCTION

The transgenic animal technology transferring foreign genes into the embryos by microinjection of the recombinant DNA have been rapidly developed for mice, rats, rabbits, swine, sheep, and cattle. Eventually, the transgenic animals has been readily used to study functions of unknown genes, and to generate a susceptible model to a chemical compound for testing in biomedical research and a genetic model for research in effect of a gene on human disease. In addition the transgenic animal system have been used to express human therapeutic proteins, such as alpha-1 antitrypsin (Wright *et al.*, 1991), antithrombin (Edmunds *et al.*, 1998), human lactoferrin (Platenburg *et al.*, 1994), human erythropoietin (Massoud *et al.*, 1996), human thrombopoietin (Sohn *et al.*, 1999) and monoclonal antibodies (Pollock *et al.*, 1999).

We have isolated and characterized milk protein promoters to find available regulatory elements that control mammary gland- and lactating-specific gene expression, from various animals, including mouse (Lee *et al.*, 1998), rat (Lee *et al.*, 1996), cow (Kim *et al.*, 1999; Oh *et al.*, 1999), and goat (Lee *et al.*, 2000). Among those, the isolated promoters from bovine and caprine β-casein genes have been extensively employed to generate transgenic mice and led to high level expression of various therapeutic proteins, lysozyme (Lee *et al.*, 1998), hLF (Kim *et al.*, 1997; Kim *et al.*, 1999; Han *et al.*, 2005), hTPO (Sohn *et al.*, 1999), growth hormone (Lee *et al.*, 1996; Choi *et al.*, 1998; Oh *et al.*, 1999), IL-10 (Sohn *et al.*, 2003), and granulocyte colony stimulating factor (Ko *et al.*, 2000) into their milk. Eventually, we have generated transgenic livestock animals, Korean native goat (Ko *et al.*, 2000; Lee *et al.*, 2000), and cow (Han *et al.*, 2005).

Nevertheless successful generation of transgenic animal producing human proteins, disadvantage has emerged that transgenic systems essentially required high-cost to be available. Additionally, to safely manage generated transgenic animals, many kinds of costs would be re-

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† Corresponding author : Phone: +82-42-860-4420, E-mail: leekk@kribb.re.kr

Table 1. PCR primers used to identify transgenic mice

Transgene	Primer sequences	Annealing temperature
hLF	5′- cgc tag gtt ata ttg ctg -3′ 5′- tgg caa ccc act tca gta -3′	52℃
hIL-10	5′- ata gcc cct aga gtt cta -3′ 5′- tgg caa ccc act tca gta -3′	55℃
hTPO	5′- gga gct gac tga att gct cct cgt -3′ 5′- cct gac gca gag ggt gga ccc tcc -3′	65℃

quired directly and indirectly. Therefore, we hypothesized that in context of management, the cost could be decreased if simple crossbreeding between two kinds of a single transgenic mouse produces double transgenic animals, and if aberrant effects of co-expressed active transgenes do not occur. To address this issue, we chose three transgenic mice expressing, *hLF, hTPO,* and *hIL-10,* respectively, and examined those expression levels.

MATERIALS AND METHODS

Generation of F-11 Double-Transgenic Mice

Previously, we reported separately generations of transgenic mice expressing *hLF* (Kim *et al.*, 1999), *hIL-10* (Sohn *et al.*, 2003), and *hTPO* (Sohn *et al.*, 1999) into milk. Briefly, the transgenic mice were produced by microinjecting an *hLF, hIL-10,* and *hTPO* reconstruct gene into fertilized zygotes derived from C57BL/CBA F1 background strain, respectively. 10 generations (F10) of three transgenic strains, *hLF, hIL-10,* and *hTPO* were crossbred by following schemes shown in Table 2. The PCR technique was used to detect *hLF, hIL-10* and/or *hTPO* transgenes from F11 progenies using each transgene specific primer (Table 1).

Analysis of Expression Level of Transgene by ELISA

The transgenic mice of F11 identified as single and/or double's were crossbred with a wild type male, and then allowed to nurse all progenies after parturition. Milk was collected from transgenic females of 10-day lactation. The expression level of protein in milk was determined by ELISA. The process for collecting and sampling of milk in detail were described in previous studies (Kim *et al.*, 1999; Sohn *et al.*, 1999; Sohn *et al.*, 2003). The concentrations of hLF, hIL-10 and hTPO were separately measured using an ELISA kit following instructions of manufactures (Calbiochem, USA; R & D system; PharMingen, respectively).

RESULTS

Generation of Double Transgenic Mice

To generate double transgenic mice was demonstrated in Table 2. The lineages chosen from three different transgenic mice were as followed: for *hLF,* a lineage 28 carrying seven copies and expressing at 0.6 mg/ml of level in founder (Kim *et al.*, 1999); for *hTPO,* a lineage 15 carrying a single copy and expressing at 1.5 mg/ml of level in founder (Sohn *et al.*, 1999); for *hIL-10,* a lineage 6 carrying a single copy and expressing 1.6 mg/ml of level in founder (Sohn *et al.*, 2003). The transgenic males for *hLF* and *hIL-10* were crossbred with *hIL-10* and *hTPO* transgenic females, respectively. The genotyping for their progenies was performed using primer sets for three transgenes (Table 1). The transmission rate, which was for the inheritance at least one transgene between two transgenes, was shown to be followed Mendelian pattern of inheritance (Table 2). Although the *hLF* transgene appeared to be transmitted less than 50%, the most was shown to be 50% of transmission rate. Notably, we observed that the rate to be double's carrying two transgenes was not deviated from expectation of principal of Mendel, showing around 25% of transmission, independently of transgenic genotypes of mother and/or father.

Co-Expression in Double Transgenic Mice Milk

To examine whether two human proteins could be expressed simultaneously in milk of double transgenic mice, and similar to levels of singles, we collected milk from 10-day lactating single and double transgenic mice.

Table 2. Transmission rate of double transgenic mice cross breeding of different transgene single transgenic mouse

Transgenic mice (No)	No. of offsprings born	No. of offsprings analyzed	No. (%) of transmission rate			
			hLF	hTPO	hIL-10	Double transgenic
hLF♂× hIL-10 ♀(4)	28	28	14 (50.0)	-	14 (50.0)	9 (32.1)
hLF♂× hTPO ♀ (3)	26	26	11 (42.3)	15 (57.7)	-	6 (23.1)
hIL-10♂× hTPO♀ (3)	29	29	-	15 (51.7)	14 (48.3)	7 (24.1)

Table 3. Concentration of human protein in milk of F11 genenrations of transgenic females (mg/ ml)

Protein \ Transgene	Single transgenic			Double transgenic		
	hLF	hTPO	hIL-10	hLF/ hIL-10	hLF/ hTPO	hIL-10/ hTPO
hLF	1.1±0.4	-	-	1.1±0.4	0.5±0.1	-
hIL-10	-	-	3.9±1.0	4.1±0.7	-	1.4±0.2
hTPO	-	0.9±0.3	-	-	0.5±0.1	0.5±0.1

ELISA showed that from single transgenic mice expression levels of hLF, hIL-10, and hTPO were 1.1±0.4 mg/ ml, 3.9±1.0 mg/ml, and 0.9±0.3 mg/ml (Table 3), respectively. In double transgenic mice, co-expressed hLF/hIL-10, hLF/hTPO and hIL-10/hTPO protein was shown to comparable levels to their singles, 1.1±0.4 (hLF)/4.1±0.7 mg/ml (hIL-10), 0.5±0.1 (hLF)/0.5±0.1 mg/ml (hTPO) and 1.4±0.2 (hIL-10)/0.5±0.1 mg/ml (hTPO) respectively.

DISCUSSION

The transgenic animal technology as a bioreactor has been used as a powerful tool to produce human proteins into milk, which could offer a renewable source of commercially important proteins (Rudolph 1999). We performed extensively to generate transgenic animal, including mouse, Korean native goat, and cow, producing various pharmaceutical proteins into milk (elsewhere). Among those, we reported successful transgenic mice expressing commercially feasible human proteins in milk, such as hLF, hIL-10 and hTPO. To confirm whether double transgenic mice could be expression of two human proteins, we produced to double transgenic mice by cross breeding system in single hLF, hIL-10 and hTPO protein expression transgenic mouse. hLF which constitutes an important component of the innate immune system and may represent a novel therapeutic with broad spectrum potential is found in the secondary granules of polymorphonuclear leukocytes and in mucosal secretions such as milk (Ward *et al.*, 2002). Also hLF has antimicrobial due to its capable of chelate iron, which is essential for microbial growth, antifungal, antiviral, antitumor, anti-inflammatory and immunoregulatory activity (Velliyagounder *et al.*, 2003). hIL-10 which is regulates immune-mediated inflammation is produced by type 2 T cells that inhibits the production of IL-2 and interferon-γ (IFN-γ) by type 1T cells, IL-10 also profoundly inhibits a many macrophage functions, including monokine synthesis, nitric oxide (NO) production, and expression of costimulatory molecules (Chhabra *et al.*, 2008). TPO is the primary physiological regulator of platelet production and plays a pivotal role in promoting the proliferation and maturation of megakaryocytic progenitor cells and megakaryocytes (Bartley *et al.*, 1994; de Sauvage *et al.*, 1994). We hypothesized that it might be very useful if one transgenic animal could produce two independent human proteins into milk. To address this issue, we crossbred *hLF, hIL-10* and/or *hTPO* transgenic mice to generate double transgenic mice. Since transgenic animals are required for stable transmission and expression of transgene to long generation, we test transgenic rates to be double and expression levels of them. In many research groups the stability of trasngene transmission has been reported (Zinovieva *et al.*, 1998; Aigner *et al.*, 1999; Chrenek *et al.*, 2004). Notably, we reported that independent transgenic *hLF, hIL-10* and *hTPO* transgenic mice transmitted their transgenes to high numbers of generation in this volume.

In this study, the transmission rate of double transgenic line was summarized in Table 2. The transmission rates of single and double gene in F11 progeny was observed about 42 to 57% and about 23 to 32% respectively. Theses results that concerning the integration of transgene in the F11 of transgenic mice, in the milk, can expression of hLF, hIL-10 and hTPO protein showed that Mendelian pattern of inheritance in agreement with a study, which reported that the multiple lines of transgenic pigs are transmitted to offspring in the Mendelian fashion (Van Cott *et al.*, 1997). The concentration of human protein in the milk among lactation of F11 double transgenic females shown to Table 3. The F11 single transgene mice milk in the hLF, hIL-10 and hTPO protein was observed 1.1, 0.9 and 3.9 mg/ml respectively. Also, stable expression of the human protein in their milk of double transgenic female was stable expressed range from 0.5 to 4.4 mg/ml compared with single transgene female. As mentioned above, those human proteins are very active (Sohn *et al.*, 1999; Kim *et al.*, 1999 ; Sohn *et al.*, 2003), thus essentially required for accurate controlling of expression site and time. In original studies, we demonstrated that mammary gland-specific expression of hLF, hTPO, and hIL-10, while having a biological activity. In regard of expression level, it is likely that hTPO lead to down-regulation of co-expressed hLF and hIL-10, as well as hTPO itself. TPO was known to induce phosphorylation of STATs (Miyakawa *et al.*, 1996), including STAT3 which is a transcription factor mediating proliferation of hematopoietic cells (Majka *et*

al., 2002), whereas controlling cell death and tissue remodeling in the mouse mammary gland during involution (Zhao *et al.*, 2004). Interestingly, a kind of cytokine EPO, which is also involved in STAT signaling (Murray, 2007), was well known to be expressed at very low level in milk of transgenic animal (Massoud *et al.*, 1996; Rodriguez *et al.*, 1995; Mikus *et al.*, 2004). When preparing this manuscript, we already examined inheritance of hIL-10, hLF and hTPO expression up to 20 generations, and found that hTPO level was relatively constant at about 1.1 mg/ml between siblings and generations, whereas hLF and hIL-10 levels were shown to be highly variable (Zheng *et al.*, submitted). It is likely that hTPO exogenously expressed into milk might weakly induce repression of milk proteins gene expression, through autocrine signaling to STAT3. Moreover, co-expressed with other cytokine hLF and hIL-10, hTPO might induce to synergistic effect on it, consequently leading to down-regulation of human proteins. However, since all of double transgenic mice expressing active hLF and hIL-10, hLF and hTPO, and hIL-10 and hTPO were shown to normal breeding, and nursing for at least 10 days of lactation, the more reasonable explanation for why down-regulated by co-expression with hTPO still remain elusive.

In conclusion, our data indicated that the stable transmission of *hLF, hIL-10* and *hTPO* gene in the double transgenic mice form F11 generation that can be secretion of the human protein in their milk.

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