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A Study on *In Vitro* Model for Mammary-Specific Gene Expression

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유선 특정의 유전자 발현을 위한 세포 배양 모델에 대한 연구

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요 약

형질 전환동물의 유선을 이용한 단백질의 생산이 보편화되고 있지만 원하는 단백질이 만들어지기 까지 많은 시간과 노력이 필요하며 기술적인 어려움이 항시 따른다. 그래서 보다 쉽게 재조합된 유전자의 발현 정도를 시험하는 *in vitro*에서의 시험방법의 개발은 중요한 의미가 있다고 하겠다. 따라서 본 연구에서는 인간의 유방암을 가진 환자의 유선에서 유래된 MCF_7 cell line을 이용하여 유선 특정의 유전자 발현을 위한 세포 배양 모델을 개발하고자 하였다. 우선 소의 카제인의 cDNA를 MMTV-LTR의 통제하에 clone하였으며, 이것을 $CaPO_4$ 의 침전법으로 MCF_7 cell에 transfection 시킨 다음, HAT 배양액으로 선발하였으며, dexamethasone으로 유도시키고, 발현되는 정도를 면역 항체를 이용하여 분석하였다. 선발된 세포는 dexamethasone에 의하여 MMTV promoter가 유도되는 것을 확인할 수 있었다. 따라서 MCF_7 cell과 같이 다양한 steroid receptor를 가지고 있는 세포는 유선 특정의 유전자 발현을 위한 세포 배양 모델에 유용하게 사용이 될 수 있을 것이다.

(Key words : *In vitro* model, Mammary-specific gene, Transgenic animal, MCF_7 cell line)

I. INTRODUCTION

Since the first transgenic animals were produced (Gorden et al., 1980; Palmiter and Brinster, 1986), this technology has been applied to various fields (Jaenisch, 1988; Westphal, 1989; First, 1991; Ebert and Selgrath, 1991; Kim et al., 1993). One of such applications is production of foreign proteins secreted into milk of lactating animals (Simons et al., 1987; Bayna and Rosen, 1990; Velander et al., 1997). Many foreign proteins including human blood proteins were produced to a various degree by this transgenic technology and some proteins are already commercially available (Clark et al., 1987). The expression levels of the foreign proteins seem to come from the nature of all segments of the DNAs microinjected (Vonderhaar and Ziska, 1989; Yom and Bremel, 1993; Yom et al., 1993). Many foreign genes that were expressed into milk were linked to the promoters from milk protein genes. However, their expression level and specificity in mammary glands were often unpredictable (Yom and Bremel, 1993). Therefore, in this study a development of *in vitro* model for mammary-specific gene expression was attempted using MCF_7 cell line.

The MCF_7 cell line is a stable epithiliod cell line obtained from pleural effusion of a female patient with metastatic breast cancer, which responded to a hormone theraphy through estro-

Table 1. Steroid receptor concentrations and their Kd in MCF7 cell line

Steroids	[R] (fm/mg protein)	Kd
DHT	40	2.8×10^{-10}
Estradiol	100	0.6×10^{-10}
Progesterone	300	1.2×10^{-9}
Glucocorticoid	800	8.0×10^{-9}

gen receptors. They affect the number of progesterone receptors which is modulated by estrogen (Horwitz and McGuire, 1978). The MCF_7 cell line is a human breast cancer cell line with many steroid hormone receptors such as estrogen, androgen, progesterone, and glucocorticoid (Cato et al., 1986). The receptor concentrations and their Kds are shown in Table 1. Among the steroid receptors in MCF_7 cells, glucocorticoid receptors have the highest concentration having the Kd at 8.0×10^{-9}. There was no cross competition for estrogen receptors (Glover and Darbre, 1989), but progesterone competed for androgen and glucocorticoid binding. Androgen, but not glucocorticoid, competed for progesterone binding to their receptors. The MCF_7 cells have been used for studying the hormonal interrelationship involved tumor response to endocrine therapy. It is known that progesterone, androgenes, estrogens, glucocorticoids affect the function of mammary glands.

Mouse mammary tumor virus (MMTV) is a murine retrovirus that is mostly expressed in lactating mammary glands of some strains of mice (Henrard and Ross, 1988). Glucocorticoid reponse element (GRE) is responsible for MMTV RNA and protein production in cell culture exposed to glucocorticoids (Cato et al., 1987). Glucocorticoids, progestines and androgens all induce the transcription of MMTV upon binding of their respective receptors to GRE. This element is located between −202 and −59 base of 5′ upstream of the start of transcription on MMTV long terminal repeat (LTR) region (Cato et al., 1988). Previously we reported that in transgenic mice MMTV promoter can be lactation-specific and inducible by dexamethasone (Yom et al., 1993). Prolactin is involved in regulation of MMTV expression in normal mouse mammary epithelium (Munoz and Bolander, 1989).

In this report, to develop an *in vitro* model for mammary-specific gene expression, MCF_7 cells were transfected with MMTV-α_{S1} casein cDNA, vector pMSG α_{S1} CN, by $CaPO_4$ precipitation. Transfectants were selected in HAT media and induced with dexamethasone, an analog of glucocorticoid. The cells were fixed, incubated with chicken anti-casein and then FITC-labeled rabbit anti-chicken antibodies, and analyzed by a microscope. The expressions with or without dexamethasone were compared. Yom et al. (1993) reported that MMTV-α_{S1} casein cDNA was expressed in the transgenic mouse milk. The same construct but in a mammalian vector was used in this study and MCF_7 cells were evaluated as an *in vitro* model for the study of mammary-specific gene expression.

Ⅱ. MATERIALS AND METHODS

1. Cloning of expression vector

The cloning strategy was described previously (Yom et al., 1993; Yom, 1997). Briefly, the 5′ end of the bovine cDNA was ligated to the 3′ end of MMTV promoter. The pC_{184}, a pBR_{322} clone with bovine α_{S1} casein cDNA, was provided by Dr. Mackinlay at University of South Wales (Stewart et al., 1984). The pBluescript with KS polylinker (pBS_{KS}), a derivative of pUC_{19}, was obtained from Stratagene (La Jolla, CA). The pMSG, a mammalian vector with MMTV LTR; and the pUC-4K, a pUC derivative with kanamycin resistance sequence; were

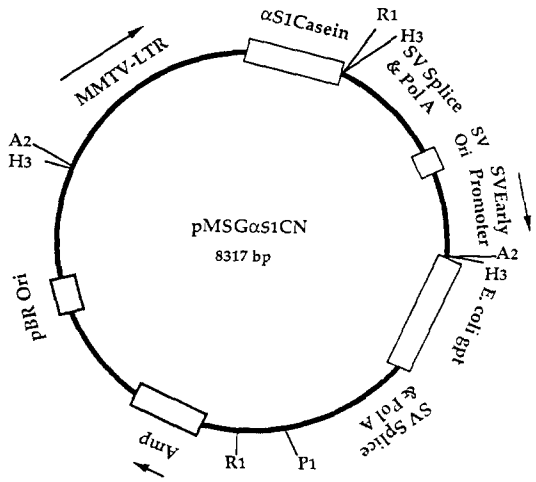

Fig. 1. Map of a mammalian vector pMSG α_{s1} CN. The bovine cDNA is under the control of MMTV-LTR. The gpt protein from *E. coli* is under the SV40 early promoter. Thus this vector can be selected in HAT medium for gpt expression under the control of SV40 early promoter. The arrows indicate transcription directions.

purchased from Pharmacia Inc. (Piscataway, NJ). The pMSG αs_1 CN as shown in Fig. 1 was constructed by ligation of a fragment from Spe I and Xho I digestion of pBS αs_1 CN to a fragment from Nhe I and Xho I digestion of pMSGK. Enzymes were from Promega Co. Gene clean kit was obtained from Bio 101 Inc. (La Jolla, CA).

2. Culture media

For cell growth media, MEM with non-essential amino acid (Sigma Chem. St. Louis, MO) was supplemented with 5% FCS (fetal calf serum, Sigma Chem.). The HAT selection media (Miller and Buttimore, 1986) were made as follows. MEM, 5% FCS, xanthine (250μg/ml), mycophenolic acid (25μg/ml), hypoxanthine

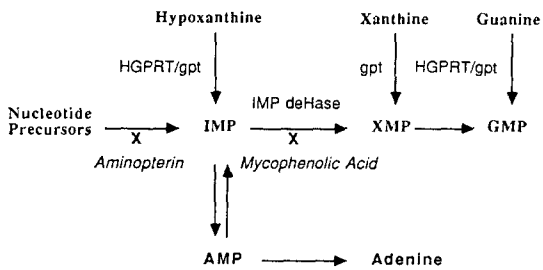

Fig. 2. Mechanism of gpt selection in HAT medium. As indicated, aminopterin blocks the conversion of nucleotide precursors into IMP and mycophenolic acid blocks the conversion of IMP into XMP.

(15μg/ml), aminopterin (2μg/ml), and thymidine (10μg/ml). The materials for HAT media were from Sigma Chemical Co. The mechanism of HAT selection is shown in Fig. 2.

3. Cell culture

The MCF_7 cells were grown close to confluency in T75 flask with 12ml culture medium and washed with 1X Hanks balanced salt solution (HBSS), detached with 1ml trypsin/EDTA, split 1:10, replated in 10cm dishes with 10ml culture medium, grown for 1 d and fed with 9ml fresh medium 2 hours prior to transfection.

4. Purification of DNA

The plasmid pMSG αs_1 CN was isolated by alkaline lysate procedure, digested with RNAse, further purified with Gene Clean, run on 1% agarose gel to examine its purity, and precipitated with absolute ethanol. The 10μg of DNA pellet was air-dried in a sterile tissue culture hood and resuspended in 450μl of sterile H_2O and 50μl of 2.5M $CaCl_2$ was added.

5. Transfection by cacium phosphate precipitation

A 500μl of 2X Hepes-buffered saline was plac-

ed in a sterile 15ml conical tube and bubbled with a 2ml pipet attached to a mechanical pipettor. The DNA/$CaCl_2$ solution was added dropwise with a Pasteur pipet and immediately vortexed for 5sec. The mixture was allowed to precipitate for 20min at room temperature and evenly distributed with a Pasteur pipet over a 10cm dish of cells. The dish was gently agitated to mix the precipitate and medium, and incubated for 12h and the medium was removed. The cells were washed twice with 2X PBS, incubated in 10ml of culture medium for 2d, and changed to the selection medium.

6. Cloning of transfected cells

The transfected cells were selected in HAT medium for 3 weeks with every 3d change of fresh selection medium. The isolated colonies were identified by a microscope and the visible colonies were picked by sterile glass cloning cylinders. The isolated cells were detached by trypsin/EDTA and replated in a dish with culture media several times, and grown for 4 weeks.

7. Expression analysis by FITC-2nd Ab

The diluted cloned cells were grown on pieces of glass slide in culture media for 3d. A 0.1μM of dexamethasone in fresh culture media was incubated for 24h for induction of MMTV promoter in the transgene construct. The slides were washed with PBS, fixed in 50/50 of acetone/methanol mixture for 2 min and washed with PBS. The chicken anti-casein Ab was diluted 1/1,000, 1/10,000, and 1/100,000 in 0.04X MOPS buffered saline with 0.1% gelatin and 0.05% Tween 20, and each dilution was applied to the fixed cells and incubated for 1hr. The slides were washed 4X in PBS with 0.05% Tween 20. The FITC-conjugated rabbit anti-chicken antibody was diluted 1/50, 1/100, and 1/500 in the MOPS buffered saline, and each dilution was applied to all combination of 1st Ab-treated slides and incubated for 1hr. The slides were washed 8X in PBS with Tween 20 and preseved in petri dish with a filter saturated with 1X PBS until microscope analyis was performed.

Ⅲ. RESULTS AND DISCUSSION

To test whether the transgenes are inducible by dexamethasone and to examine whether bovine casein protein can be expressed under the control of MMTV promoter *in vitro*, MCF_7 cells were transfected with pMSG αs_1 CN, selected for gpt protein, and cloned as described as above. Fig. 1 shows the map of the mammalian vector pMSG αs_1 CN. The arrows indicate the directions of transcription. The bovine casein cDNA is under the control of MMTV-LTR (MMTV promoter), thus it will respond to dexamethasone induction. The gpt protein from *E. coli* is under the control of SV40 early promoter. Therefore, cells with this vector can be selected in HAT medium for gpt expression under the control of SV40 early promoter.

As shown in Fig. 2, the mechanism of gpt selection in HAT medium is summerized as described by Mulligan and Berg (1981). As indicated by X, aminopterin blocks the conversion of nucleotide precursors into IMP and mycophenolic acid bloks the conversion of IMP into XMP. Mammalian cells can not utilize xanthine, thus can not survive in HAT medium (Miller and Buttimore, 1986), whereas the cells with pMSG αs_1 CN will survive because it will express gpt protein that converts xanthine to XMP. Nonetheless, this process proceeded slowly, thus the selection took 2~3 weeks.

The cloned cells were fixed, incubated with chicken anti-casein and then FITC-labeled rabbit anti-chicken antibodies, and analyzed by a

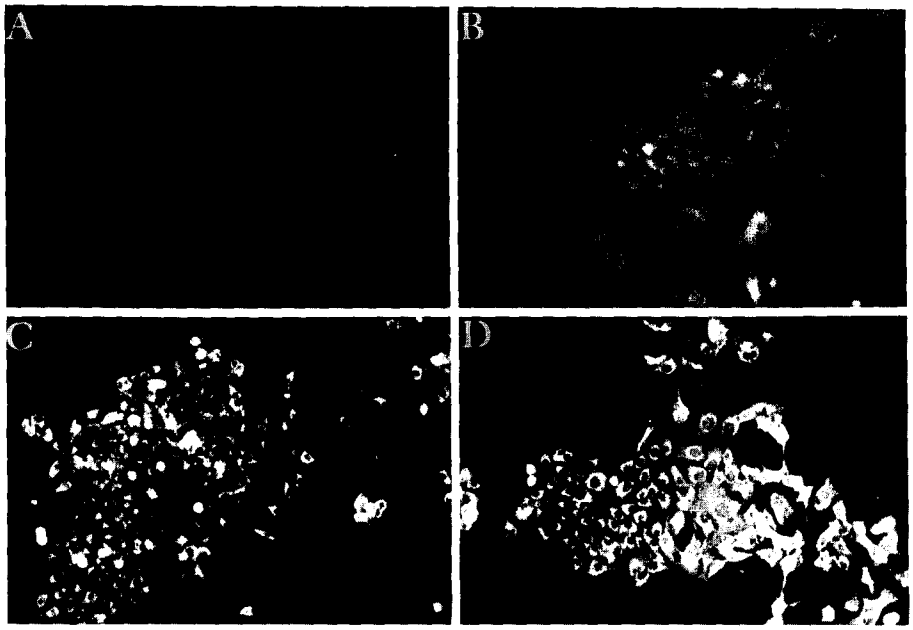

Fig. 3. Expression of bovine αs_1 casein in cloned MCF_7 cells using chicken anti-casein antibody from egg yolk and FITC-labelled rabbit anti-chicken-IgG antibody. The pictures were taken in the dark field fluorescence over UV. The samples were manually exposed for 120 sec. The combination of 1/10,000 dilution of chicken antibody and 1/100 dilution of rabbit 2nd antibody with FITC was optimal for the analysis.
A: Dexamethasone-induced, non-transfected cells.
B: Non-induced, but transfected cells.
C and D: Dexamethasone-induced, transfected cells.

microscope. Fig. 3 shows that the expression of bovine α_{s1} casein in stably cloned MCF_7 cells in the dark field fluorescence over UV. The samples were exposed for 120 sec. The combination of 1/10,000 dilution of chicken antibody and 1/100 dilution of rabbit 2nd antibody with FITC was optimal for the analysis. Fig. 3A was from dexamethasone-induced non-transfected cells, and B was from non-induced but transfected cells. C and D were dexamethasone-induced transfected cells. The cells in C and D were from independent clones. The analysis of the film scanning showed that dexamethasone induced 30~40 fold increase in the casein expression in transfected cells.

In this report, an *in vitro* model for mammary-specific gene expression was developed to evaluate the effectiveness of a transgene in transgenic animals. MCF_7 cells were transfected with the vector pMSG αs_1 CN by $CaPO_4$ precipitation. Transfectants were selected and induced with dexamethasone. The result showed that dexamethasone induced 30~40 fold increase in bovine α_{s1} casein expression. This result agrees with the report by Yom et al. (1993) where the same construct was expressed and further induced by dexamethasone in transgenic mouse milk. Therefore MCF_7 cells along with pMSG vector can be used as an *in vitro* model for the study of mammary-specific gene expression.

Ⅳ. SUMMARY

Recently the production of transgenic animals to express foreign proteins in mammary glands has been a routine procedure. However, it still takes a considerable time and effort, and is faced with various technical challenges until the protein of interest is successfully made. Thus, a development of an *in vitro* model for mammary-specific gene expression for recombinant genes was carried out in this study. To this end, bovine α_{S1} casein cDNA was inserted at the multiple cloning site of pMSG vector under the control of MMTV promoter. MCF_7 cells were transfected with pMSG α_{S1} CN by $CaPO_4$ precipitation. Transfectants were selected in HAT medium and induced with dexamethasone. The cells were analyzed with chicken anti-casein and FITC-labeled rabbit anti-chicken antibodies. The results showed that dexamethasone induced 30~40 fold increase in the MMTV- α_{S1} casein expression. Therefore MCF_7 cells, which have multiple steroid receptors, along with pMSG vector can be used as an *in vitro* model for the study of mammary-specific gene expression.

Ⅴ. REFERENCES

1. Bayna, E. M. and J. M. Rosen. 1990. Tissue-specific, high-level expression of the rat whey acidic protein gene in transgenic mice. Nucl. Acids Res., 18:2977-2985.
2. Cato, A. C. B., R. Miksicek, G. Schütz, J. Arnemann, and M. Beato. 1986. The hormone regulatory element of mouse mammary tumor virus mediates progesterone induction. EMBO J., 5:2237-2240.
3. Cato, A. C. B., D. Henderson, and H. Ponta. 1987. The hormone response element of the mouse mammary tumor virus DNA mediates the progestin and androgen induction of transcription in the proviral long terminal repeat region. EMBO J., 6:363-368.
4. Cato, A. C. B., P. Skroch, J. Weinmann, P. Butkeraitis and H. Ponta. 1988. DNA sequences outside the receptor-binding sites differentially modulate the responsiveness of the mouse mammary tumor virus promoter to various steroid hormones. EMBO J., 7:1403-1410.
5. Clark, A. J., P. Simons, I. Wilmut and R. Lathe. 1987. Pharmaceuticals from transgenic livestock. Trends in Biotechnology, 5:20-24
6. Ebert, K. M. and J. P. Selgrath. 1991. Changes in domestic livestock through genetic engineering. *In* Animal applications of research in mammalian development. Cold Spring Harbor Laboratory Press. pp. 233-266.
7. First, N. L. 1991. New advances in reproductive biology of gametes and embryos. *In* Animal applications of research in mammalian development. Cold Spring Harbor Laboratory Press. pp. 1-21.
8. Glover, J. F. and P. D. Garbre. 1989. Multihormone regulation of MMTV- LTR in transfected T-47-D human breast cancer cells. J. Steroid Biochem., 32:357-363.
9. Gorden, J. W., G. A. Scangos, D. J. Plotkin, J. A. Barbosa, and R. H. Ruddle. 1980. Genetic transformation of mouse embryos by microinjection of purified DNA. Proc. Natl. Acad. Sci., 77:7380-7384.
10. Henrard, D. and S. R. Ross. 1988. Endogenous mouse mammary tumor virus is expressed in several organs in addition to the lactating mammary gland. J. Virol., 62:3046-3049.
11. Horwitz, K. B. and W. L. McGuire. 1978. Estrogen control of progesterone receptor in

human breast cancer. J. Biol. Chem., 253: 2223-2228.

12. Jaenisch, R. 1988. Transgenic animals. Science, 240:1468-1474.
13. Kim, T., M. L. Leibfried-Rutledge and N. L. First. 1993. Gene transfer into bovine cells and embryos using replication-defective retroviral vectors encapsidated with xenotropic murine leukemia virus envelopes. Animal Biotechnology, 4(1):53-69.
14. Miller, A. D. and C. Buttimore. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol., 2895-2902.
15. Mulligan, R. C. and P. Berg. 1981. Selection of animal cells that express the *Escherichia coli* gene coding for xanthine-guanine-phosphoribosyl transferase. Proc. Natl. Acad. Sci. USA. 78:2072-2076.
16. Munoz, B. and F. F. Bolander, Jr. 1989. Prolactin regulation of MMTV expression in normal mouse mammary epithelium. Mol. Cell. Endo., 62:23-29.
17. Palmiter, R. D. and R. L. Brinster. 1986. Germ-line transformation of mice. Annu. Rev. Genet., 20:465-471.
18. Simons, J. P., M. McClenaghan, and A. J. Clark. 1987. Alteration of the quality of milk by expression of sheep β-lactoglobulin in transgenic mice. Nature, 328:530-532.
19. Stewart, A. F., I. M. Willis and A. G. Mackinlay. 1984. Nucleotide sequences of bovine α_{S1}-and k-casein cDNAs. Nucl. Acids Res., 12:3895-3907.
20. Velander, W. H., H. Lubon and W. N. Drohan. 1997. Transgenic livestock as drug factories. Sci. American, 276:1:54-58.
21. Vonderhaar, B. K. and S. E. Ziska. 1989. Hormonal regulation of milk protein gene expression. Annu. Rev. Physiol., 51:641-652.
22. Westphal, H. 1989. Molecular genetics of development studies in the transgenic mouse. Annu. Rev. Cell Biol., 5:181
23. Yom, H. C. 1997. A study on the transmission of a transgene in the offspring of transgenic mice. Korean J. Animal Reprod., 20:453-458.
24. Yom, H. C. and R. D. Bremel. 1993. Genetic engineering of milk composition:Modification of milk components in lactating transgenic animals. Am. J. Clin. Nutn., 58:299 S-306S.
25. Yom, H. C., R. D. Bremel and N. L. First. 1993. Mouse mammary tumor virus promoter directs high-level expression of bovine α_{S1} casein in the milk of transgenic heterozygous and homozygous mice. Animal Biotechnology, 4(1):89-107.

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