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# Increased of the Red Blood Cell in Peripheral Plasma of Transgenic Pigs Harboring hEPO Gene

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## ABSTRACT

The present study were performed to analysis the hematocrit and the red blood cells content into the blood plasma of the transgenic pigs harboring recombinent human erythropoietin gene (rhEPO). Mouse whey acidic protein (mWAP) linked to rhEPO gene was microinjected into pronuclei of porcine one-cell zygotes. After delivered of offspring, PCR analyses identified one mWAP-rhEPO transgenic founder offspring($F_0$). The first generation of transgenic pig ($F_0$) harboring mWAP-hEPO appeared to be a male, and the second generation ($F_1$) pigs were made by natural mating of $F_0$ with domestic swine, and male and female transgenic pigs ($F_1$) were identified by PCR. The blood samples from transgenic and normal pigs were collected for 50 days during lactation and were counted the red blood cell (RBC) numbers and Hematocrit (HCT) content into the blood. The transgenic pigs expressing rhEPO in their blood gave rise to higher RBC numbers and HCT contents than control animals. rhEPO was secreted both in the blood and milk of genetically engineered pigs harboring rhEPO gene. Therefore, this study provides a model regarding the production of transgenic pig carrying hEPO transgene for biomedical research.

(Key words : Transgenic pig, Hematocrit, Red blood cell, Erythropoietin)

## Ⅰ. INTRODUCTION

The domestic pig is attractive as a laboratory animal in a wide range of medical and pharmacological research field, because of having similar anatomical and physiological characteristics to human (Bustad and McClellan, 1996; Douglas, 1972; Swindle et al., 1994). It has been used for drug testing and a disease model (Mount and Ingram, 1971; Swindle and Smith, 1998). Transgenic technology in domestic pig has already been recognized as a possible bioreactor producing therapeutic proteins (Cameron et al., 1994; Janne et al., 1994; Wagner et al., 1995; Wei, 1997) and recently has received attention as organ donors in xenotransplantation (Cozzi and White, 1995).

Microinjection of foreign DNA into the pronucleus of a fertilized egg has been the most widely used and most successful method for pro-

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ducing transgenic mice and livestock (Brinster et al., 1981; Costantini F and Lacy, 1981; Gordon et al., 1980; Wagner et al., 1981a; Wagner et al., 1981b).

The discovery of human erythropoietin (hEPO), its purification, the cloning (Jacobs et al., 1985) of the gene for hEPO, and the availability of purified recombinant EPO for the treatment of anemia have been major advances in medicine. Bonsdorff and Jalavisto (1948) gave this humoral substance the name EPO, which was known today to be the hormone that controls red blood cell production. Erythropoietin is a glycoprotein with a molecular mass of approximately 30 kDa, which circulate in plasma of the human with 165 amino acids with three N-linked and one O-linked acidic oligosaccharide side chains in the molecule. Most of these advances have occurred over the past 15 to 20 years. Carnot and DeFlandre (1906) demonstrated an increase in peripheral red blood cell counts in normal rabbits injected with plasma from rabbits made anemic by bleeding. Carnot and colleagues reported that they have observed that regeneration of blood after blood letting is under the influence of a humoral process and stated the substance as the generic hemopoietin.

The reports (Hjort, 1936; Krumdieck, 1943; Erslev, 1953) that large volumes of plasma or serum from donor rabbits following a bleeding stimulus, when injected into normal recipient rabbits, produced a brisk increase in peripheral blood reticulocytes were very important in confirming the existence of a humoral factor that controls erythropoiesis. The expression of recombinant protein genes in the milk of transgenic animals is a recent approach toward the production of human therapeutic proteins at costs lower than those for cell culture. Milk protein genes are activated in a strict temporal pattern. WDNM1 and $\beta$-casein are already highly expressed early in pregnancy. The whey acidic protein (WAP) and $\alpha$-lactalbumin are highly expressed only at the end of gestation, and especially WAP seems to play a critical role in the control of these actions (Robertson et al., 1995). The upstream region of the rabbit whey acidic protein (rWAP) gene was reported to direct high level expression of foreign proteins into transgenic mouse milk (Devinoy et al., 1994; Thepot et al., 1995; Christa et al., 2000). Milk gene expression increases markedly after the second half of gestation, and in mice the peak is reached during lactation (Gabrowski et al., 1991; Puissant et al., 1994).

Many genetic modifications in swine were one of our interests in agriculture and human medicine. The transgenic animal introducing hEPO gene were produced in mouse and rabbit by Zbikowska et al. (2002) and Aguirre et al. (1998). Recently, our group was success to produce the transgenic pig harboring rhEPO gene(Lee et al., 2002). In this study, WAP promoter and rhEPO genes were used to produce the transgenic pigs. The purpose of the present studies were to characterize the transgenic pigs secreting recombinant human erythropoietin (rhEPO) under the control of promoter.

## Ⅱ. MATERIALS and METHODS

### 1. rhEPO Gene to Used the Transgenic Pig Production

The 2.6 kb mouse WAP (mWAP) promoter region was prepared from the plasmid by digesting with restriction enzymes, EcoR Ⅰ and Kpn Ⅰ. The human EPO (hEPO) genomic DNA (2.6 kb) was prepared by PCR adding BstE Ⅱ and Bgl Ⅱ at 5'-end and 3'-end respectively. The 2.6 kb SV40 polyA fragment was amplified by PCR and cloned into PCR Ⅱ-TOPO vector (pSV40). mWAP promoter was cloned into pSV40 in EcoRI, Kpn Ⅰ site and human EPO cDNA was joined into the vector at

BstE Ⅱ, Bgl Ⅱ sites. The mWAP-hEPO were digested with Bam H I and EcoR I to obtain the fragment containing 7.8 kb (mWAP-hEPO) and 3.9 kb pCRII-TOPO vector region.

**2. Generation of Transgenic Pigs**

Semen from transgenic pig was collected by hand pressure method. Collected semen was diluted with BTS medium (GmbH and Co., Germany). Diluted transgenic pig semen (1 to 4 ratio for semen to medium) was inseminated to swine on natural estrus cycle. Piglets were produced from pregnant females. Extracted genomic DNA from the tail of piglets were subjected to detection of transgene by PCR analysis. Transgenic male and female F1 pigs after PCR confirmation were chosen, and bred each other by natural mating after their sexual maturation.

**3. Analysis of Blood Samples**

Blood samples were obtained from control and two transgenic pigs (named 0~5, 0~81). Red blood cell (RBC) content measurement was performed by Celltac MEK-5108k (Nihon Kohden, Japan) procedure. Blood samples collected from a ear vein during, 200㎕ of samples were added into 1.5ml centrifuge tube and subjected to the analysis of RBC content. After mixed 1:1 with blood and dilution solution, 50㎕ of blood samples were injected into the instrument. Diluted samples were mixed with buffer (Celltac) on 200 magnification at first, and this dilution were additionally diluted to 1: 40000 in buffer with stepwise washing five times. Hemolynact (eruption regent) were droped into the two hundred times diluted sample, and for each samples RBC were counted by Celltac MEK-5108k.

**4. Detection of hEPO Protein from Transgenic Pig Blood**

For the detection hEPO in blood, 25 ㎕ of sample dilution buffer were added to each well of microtiter plate then 25 ㎕ of milk were added to each well and incubate the plate for 1 hr at 37℃ in a moist chamber. After incubation, the plate was washed three times with 300 ㎕ washing buffer per well. The microtiter plate was incubated for 1 min after each washing procedure. After washing and tapping the microtiter plate on filter paper, 50 ㎕ of the diluted conjugate were added to each well. After incubation of the microtiter plate for 1 hour at 37℃ in a moist chamber, the plate was washed four times with 300 ㎕ washing buffer per well. After another washing and tapping, 50 ㎕ of substrate solution were added to each well, and the plate was incubated at 37℃ in a moist chamber. The enzymatic reaction was regularly stopped after 30 minutes incubation by adding 50 ㎕ of 3 N sodium hydroxide per well. All of the procedures including analysis were carried out using the EPO-ELISA medac (Diagnostika, Germany) procedure.

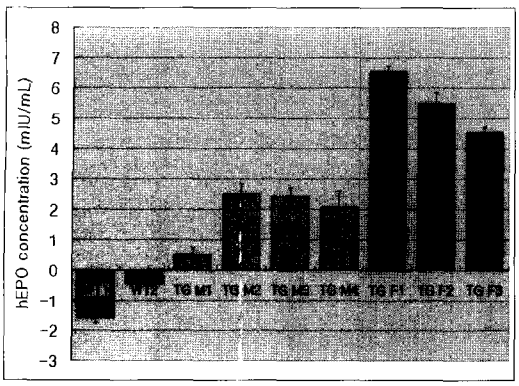
## Ⅲ. RESULTS

**1. Expression of hEPO Transgene in the Blood of Transgenic Pigs**

The concentration of the rhEPO in transgenic pigs was assessed by ELISA. Blood samples were collected from 4 male and 3 female transgenic pigs and 2 control pigs with the same age pigs and tested pigs were consist of 2 control pigs (no transgenic pig). Significant difference was observed in concentration of the rhEPO between male and female transgenic lines tested. As showed in the Fig. 1 and 2, a high level of rhEPO expression was observed in transgenic pigs. In particular, transgenic females expressed great amount of rhEPO in their blood.

**2. Generation of Transgenic Pigs**

Fig. 3 suggest that mWAP-hEPO gene was trans-



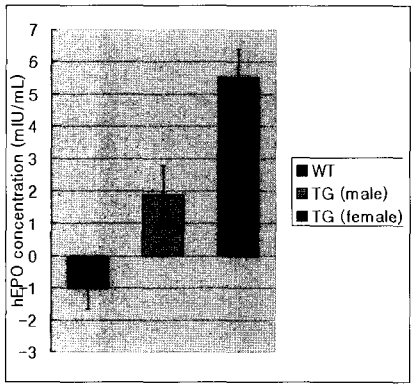
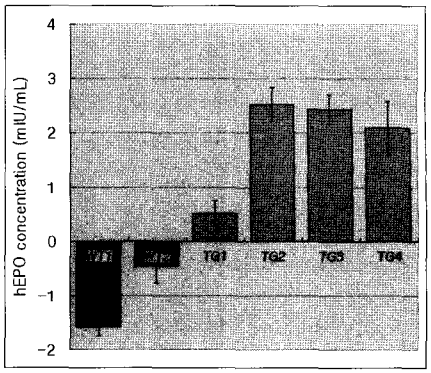




**Fig. 1. Comparison of rhEPO concentration in the blood of transgenic lines. WT: wild type pig (control), TG M: transgenic male, TGF: transgenic female. A graph in the left includes 2 WT, and 7 TG (4 male and 3 female). In the right, averages of hEPO concentration in the blood of WT and TG (male and female).**



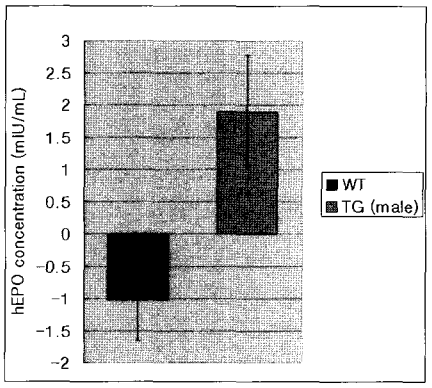




**Fig. 2. Comparison of rhEPO concentration In the transgenic and control pig's blood. WT: wild type pigs (control), TG : transgenic pigs. 2 WT and 4 TG pigs are shown in the left. A graph in the right represents averages of hEPO concentration in the blood of WT and TG.**

mitted into the sperm of transgenic founder ($F_0$) pig and two descendants ($F_1$). As a results, the extracted DNA from three different pigs were detected the transgene in three different sizes (304, 567 and 989 bp) after agarose gel electrophoresis.

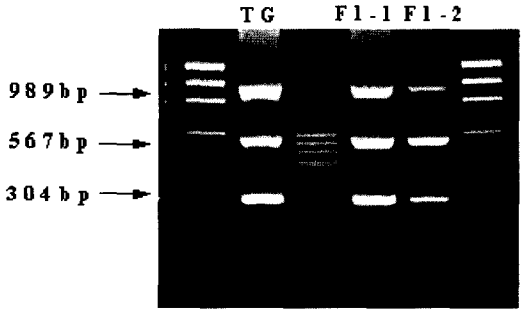
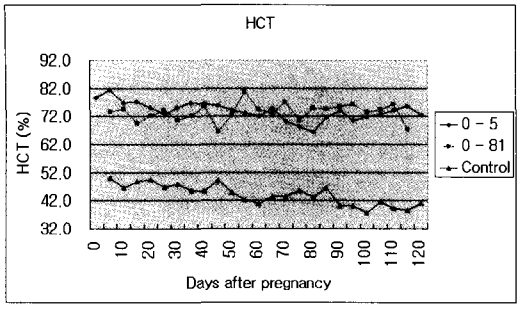



**Fig. 3. Detection of mWAP-hEPO gene transmitted into the sperm of transgenic pigs using PCR. Arrows indicate sizes of DNA bands in bp. TG: F0 transgenic founder pig; F1-1 and F1-2: F1 transgenic pig line 1 and 2.**

### 3. Variation in Red Blood Cell Number and HCT in Blood

To count of HCT values and RBC number in the blood, the blood samples collected from two transgenic and one control pigs during 120 days after

A




B

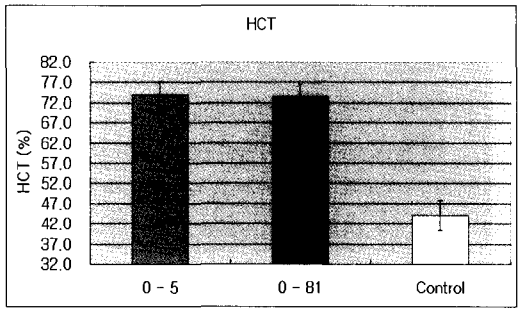
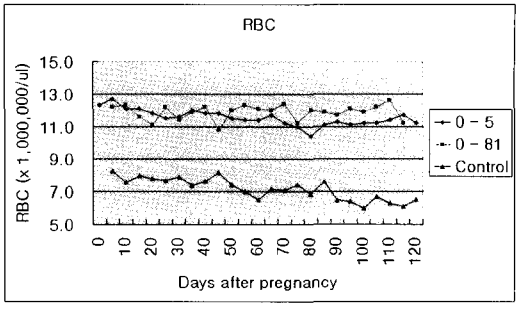



**Fig. 4. Variation in HCT values between transgenic and control pigs. 0~5 and 0~81: transgenic pigs; Control: rhEPO-negative pig. A: Variation of HCT content; B: Average of three different pigs.**

A




B

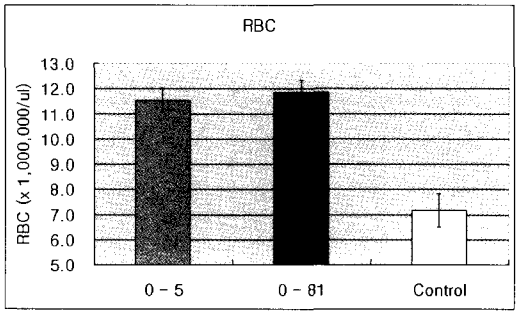



**Fig. 5. Variation in RBC numbers between transgenic and control pigs. 0~5 and 0~81: transgenic pigs; Control: rhEPO negative pig. A: Variation of RBC content; B: Average of three different pigs.**

gestation. As shown in Fig. 4 and 5, HCT and RBC number in blood samples of transgenic pigs (0~5 and 0~81) were approximately 40% higher than control and continuously maintained during entire pregnancy. This represents that mWAP promoter regulating the rhEPO expression was not restricted in mammary gland, but leaked into other organs, reflecting high rhEPO activity in blood of transgenic animals.

## Ⅳ. DISCUSSION

This study were chose EPO as a model protein for testing the feasibility of the mouse whey acidic protein (Valander et al., 1991) to target potential therapeutic proteins into milk. Moreover, hEPO is physiologically active at a very low concentration, so that the consequences of any unspecific expression can easily be evaluated. The results showed that human EPO was secreted into the milk(data not shown) and plasma of transgenic $F_1$ pigs. Several attempts have been undertaken to produce human EPO in the milk of transgenic mice and rabbits (Rodriguez et al., 1995; Suk et al., 1995; Massoud et al., 1996; Korhonen et al., 1997; Aguirre et al., 1998). Based on previously obtained results, hEPO was recognized as a very difficult growth hormone to be produced in transgenic animals. Transcriptional leakage and/or protein leakage from milk into general circulation caused symptoms similar to polycythemia in humans in most animals expressing human EPO (Suk et al., 1995; Massoud

et al., 1996; Korhonen et al., 1997). In addition to this, low EPO expression levels had been shown in the milk of transgenic mice (10 ng/ml) and rabbits (0.3 ng/ml) carrying the human EPO cDNA/rabbit WAP construct (Rodriguez et al., 1995). Transgenic mice and rabbits secreting up to 50 $\mu$g/ml of rhEPO in milk were also generated with a similar construct, the human EPO/cDNA associated with the rabbit WAP gene promoter (Massoud et al., 1996).

However, ectopic expression of the hormone has been reported as having deleterious effects on transgenic animals. The rabbits had an abnormally high amount of RBCs, as stated they could not reproduce and died prematurely. In EPO-transgenic mice developing EPO disease, the red blood cells ($P< 0.05$), hematocrit values ($P<0.01$) and blood hemoglobin levels ($P<0.02$) were almost double of control mice reported by Halina et al. (2002). Similar to above result, in this study, HCT and RBCs concentration into transgenic pig blood were also approximately higher 40% (Fig. 4 and 5) than those control pigs, and rhEPO protein was secreted in blood of mWAP-hEPO transgenic swine as a leaking. All the transgenic pig expressing rhEPO in milk, most probably as a result of massive overproduction and leakage of EPO into the bloodstream, as recognized as a major symptom of the disease shown in previous reports.

In conclusion, this study demonstrated that the regulatory region of mWAP gene directs production of protein into the milk of transgenic sow. Not only this model pig be beneficial for a commercial production of rhEPO, But also suggests that the mWAP promoter can be useful for expression of other important therapeutic proteins in milk. However, problems also exist. Due to leakage of rhEPO, only poor transgenic founders survived and could be evaluated. The best transgenic founders maybe died very early, or even during embryogenesis, because of the profound biological activity of the expressed protein.

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