Study on the Reproductive Function in Transgenic Pig Harboring Human Erythropoietin (hEPO) Gene

Hyun-Gi Lee[1,3], Hwi-Cheul Lee[1], Hak-Jae Chung[2], In-Sul Hwang[1], Myoung-Seob Choi[1], Sung-June Byun[1], Seunghoon Lee[1], Min-Ji Kim[1], Jae-Seok Woo[1], Won-Kyong Chang[1], Poongyeon Lee[3], Hoon-Taek Lee[4,†] and Jin-Ki Park[1,†]

[1]Animal Biotechnology Division, National Institute of Animal Science, RDA, Suwon 441-706, Korea

[2]Hanwoo Experimental Station, National Institute of Animal Science, RDA, Pyongchang 232-952, Korea

[3]Animal Genetic Resources Station, National Institute of Animal Science, RDA, Namwon 590-832, Korea

[4]Department of Animal Science, KonKuk University, Seoul 143-701, Korea

ABSTRACT

Our previous study showed that transgenic (TG) pigs harboring human EPO (hEPO) gene have been shown to have reproductive disorders, including low pregnancy rates, irregular estrus cycle and low little size. To investigate these reasons, we assessed estrus behavior (standing response) and plasma 17β-estradiol (E_2) level, which partly reflect reproductive function, during the estrus cycles after synchronization and superovulation by hormone treatments. Then, we analysed blood composition and expression of hEPO gene in TG pigs. Pigs were injected with PG600. After 10 days, pigs were fed with Regumate porcine for 6 days. Blood samples were collected from jugular vein. Analysis of blood composition and E_2 level were measured by Hemavet 950 and E_2 ELISA kit, respectively. And, the expression of hEPO gene in reproductive organs was quantitated by real-time RT-PCR. The percentage of estrus behavior in TG was significantly decreased. Hematocrit (HCT), hemoglobin (Hb) concentration and red blood cell (RBC) number were significantly higher in TG than wild type (WT). On the other hand, high expression of hEPO gene in TG was observed in the mammary gland as well as in the uterus. Moreover, plasma E_2 level was significantly higher in TG than WT. These results suggest that nonspecific expression of hEPO gene in the other organs of TG may affect blood composition and plasma E_2 level, thereby causing reproductive disorders.

(Key words : hEPO, Transgenic pig, E_2 level, Hematocrit, ELISA)

INTRODUCTION

Erythropoietin (EPO), an approximately 30-kDa glycoprotein composed of 165 amino acids, has 3 N-glycosylation sites and 1 O-glycosylation site (Fisher, 1997). The liver is the primary site of production of EPO in the fetus, and there is a gradual shift from the liver to the kidney shortly after birth and is stimulated by tissue oxygenation, hypoxia, or anemia (Sasaki et al., 1987). The main role of EPO is regulation of erythrocyte production (Horiguchi et al., 2005) by feedback mechanism: increased oxygen level causes decreased EPO production, which results in reduction of erythrocytes that in turn reduces the oxygen level (Macmillan et al., 2001). EPO plays a role in the number, proliferation, apoptosis, function, and phenotypical differentiation of immature endothelial cells from early development through adulthood. This role indicates a common regulatory pathway for vasculogenesis and hematopoiesis (Muller-Ehmsen et al., 2006). Although angiogenesis actively occurs during embryo development, it is notably quiescent in normal adult mammal, except during adult female reproductive cycles (Hanahan and Folkman, 1996). Particularly, the cyclic development of the uterine endometrium is closely related to angiogenesis and is regulated by 17β-estradiol (E_2) secreted from the ovarian follicle (Yasuda et al., 1998). Recombinant EPO has a proliferative effect on the cultures of human umbilical vein endothelial cells and bovine adrenal capillary endothelial cells (Anagnostou et al., 1994), and it stimulates angiogenesis in vitro (Carlini et al., 1995; Ribatti et al., 1999; Ashley et al., 2002; Heeschen et al., 2003). Thus, EPO plays a role in angiogenesis, which may play a major role in the cyclic development of the uterine endometrium. A previous

study reported that high E_2 level in the culture medium for mouse uterus tissue culture resulted in increased EPO level (Yasuda *et al.*, 1998); thus, E_2 and EPO may be closely related in the uterus.

We produced transgenic (TG) pig that secrete recombinant human EPO (hEPO) in its milk under transcriptional regulation of the mouse whey acidic protein (mWAP) gene promoter and have been breeding these pigs for 4 generations (Park *et al.*, 2006b). On the other hand, we have been observed various physiological anomalies such as low reproduction rate, low sperm motility (Park *et al.*, 2006a), irregular estrus cycle, and defective reproductive behavior in the hEPO TG pig. Among the reproductive disorders, irregular estrus cycle is especially a major problem in our research since it affects reproductive performance and production of milk.

In the present study, to investigate why reproductive disorders, especially irregular estrus cycle have been observed in hEPO TG pig. We assessed estrus behavior, blood composition, expression of hEPO in kidney, where EPO is produced in adult pig, mammary gland, which is transgenic target, and plasma E_2 levels.

MATERIAL AND METHODS

Induction and Detection of Estrus

For estrus induction, wild type (WT, n=7) and transgenic (TG, n=6) sows were treated with PG600 (pregnant mare's serum gonadotropin (PMSG) 400 IU, human chorionic gonadotropin (hCG) 200 IU; Intervet, Unterschleissheim, Germany) and examined for estrus at 12-h intervals for 3 to 7 days. Estrus detection was conducted by standard protocols of observation of pudendal swelling, standing to be mounted by the boar (Horsley *et al.*, 2005).

Estrus Synchronization and Measurement of Plasma E_2 Levels

To induce estrus synchronization, TG (n=3) and WT (n=2) sows were fed 5 ml of Regumate Porcine (Intervet) per gilt for 6 days to suppress estrus. On the last day of Regumate Porcine feeding, we transferred 10 ml of blood from the jugular vein to a heparin-treated tube and isolated plasma by centrifugation at 3,000 rpm for 10 min. Plasma samples were maintained in a deep freezer at $-70°C$ and used for the measurement of E_2 by using the Swine Estradiol ELISA test kit (Endocrine Technologies, CA, USA) according to the manufacturer's protocol. Briefly, 25 µl of plasma was reacted with anti-rabbit E_2 antibody at 37°C for 2 h, and the absorbance was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

Blood Analysis

Approximately 20 µl of blood from the jugular vein of TG (n=3) and WT (n=3) sows with induced estrus was used for blood composition analysis. Within 1 h of bleeding, white blood cell (WBC), red blood cell (RBC), hemoglobin (Hb) and hematocrit (HCT) were measured using Hemavet 950 (Drew Scientific Inc., Dallas, TX, USA).

RNA Extraction and cDNA Synthesis

To assess hEPO mRNA expression in TG pig tissues, the tissues were homogenized using a mortar after freezing in liquid nitrogen. RNA samples were isolated from the homogenates by using the RNeasy Mini kit (Qiagen, Valencia, CA, USA), and RNA concentration was measured by using Nanodrop ND-1000 spectrophotometer. Approximately 800 ng of RNA samples were used for the synthesis of cDNA using the First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany).

Quantitative Real-Time Polymerase Chain Reaction

We used custom-designed hEPO specific Hybprobes (Tib-Molbiol, Berlin, Germany) for quantitative real-time polymerase chain reaction (PCR) for cDNAs from each tissue by using the LightCycler DNA master hybprobe (Roche) contained in the LightCycler kit (Roche). Nucleotide sequences for Hybprobes were deduced from EPO (NM_000799) and GAPDH (AF 017079) sequences as follows: EPO (forward primer, 5'-CGAGAATATCA-CGACGGGCT-3'; reverse primer, 5'-GAAGAGTTTGCG-AAAGTGT-3'; probe FL, 5'-AGCTGCATGTGGATAAA-GCCGTCAGTG-FL-3'; LC, 5'-640-CCTTCGCAGCCTCA-CCACTCTGCT-p-3') and GAPDH (forward primer, 5'-ATTGCCCTCAACGACCACT-3'; reverse primer, 5'-GG-CCTCTCTCCTCCTCGC-3'; probe FL, 5'-GCCTCCAAG-GAGTAAGAGCCCCT-FL-3'; LC, 5'-640-ACCACCAAC-CCCAGCAAGAGCAC-p-3'). The reaction mixture contained 2 µl of cDNA as template, 4 mM $MgCl_2$, 1 µM primer mix, and 0.2 µM probe mix. Thermal profile was as follows: preincubation at 95°C for 10 min, 45 cycles of amplification at 95°C for 5 sec, 63°C for 20 sec, and 72°C for 14 sec. Melting was conducted from 45°C to 95°C at increment of 0.5 C/s, and cooling was conducted at 40°C for 30 sec.

Statistical Analysis

To determine the statistical significance between treatments, all data were subjected to one-way ANOVA with data values, and Duncan's multiple range tests were used for post-verification. Differences among the treatment effects were considered significant at $p<0.05$.

RESULTS

Estrus Behavior and Blood Composition

Fig. 1 shows the percentage of estrus behavior in TG and WT pig. Five in seven WT pigs (71.4%) showed estrus behavior, however, it was significantly decreased in TG pigs (16.7%, n=1/6). The RBC, Hb and HCT values was significantly higher in TG pigs, while there was no difference in WBC levels between the TG and WT pigs (Fig. 2).
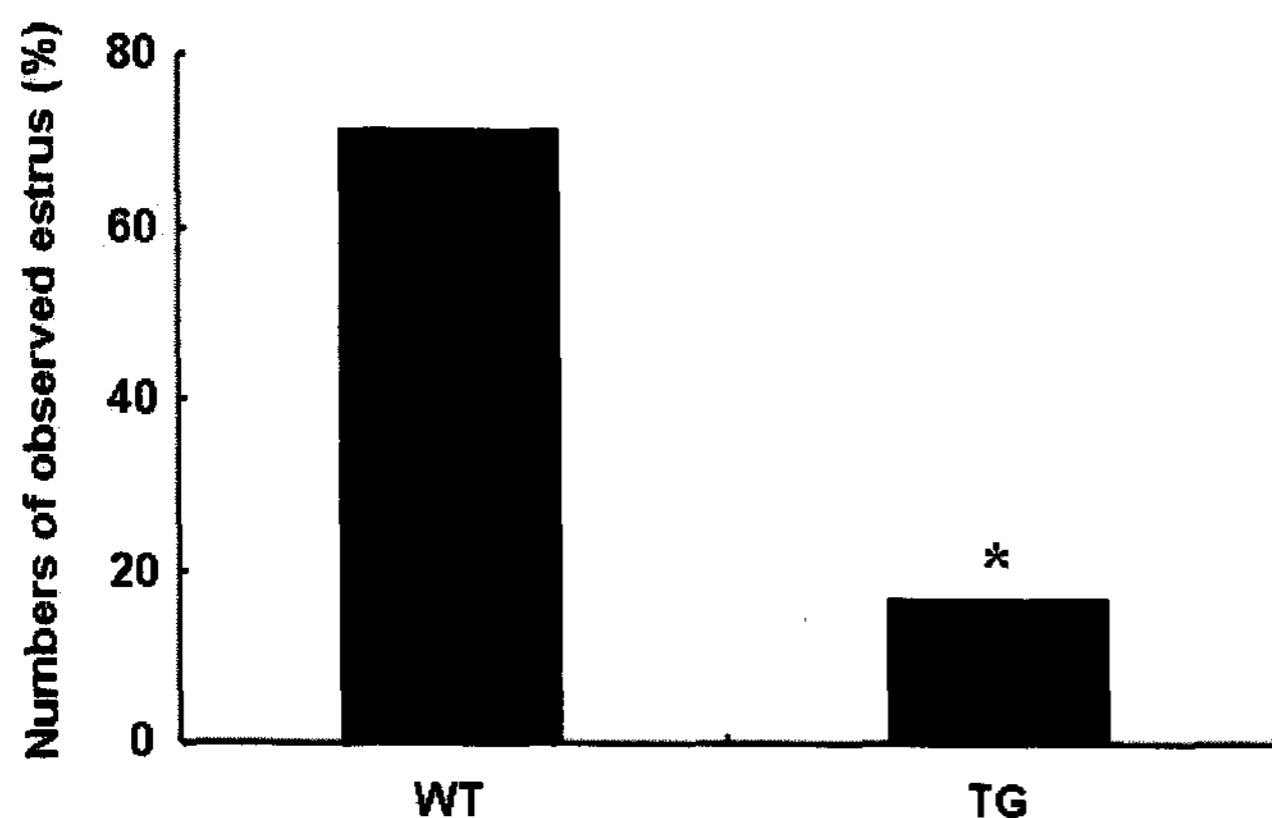

Fig. 1. The percentage of estrus behavior in WT and TG pig. WT, wild-type pig (n = 7); TG, transgenic pig (n = 6). *, Significant difference ($p<0.05$) compared with the WT group.
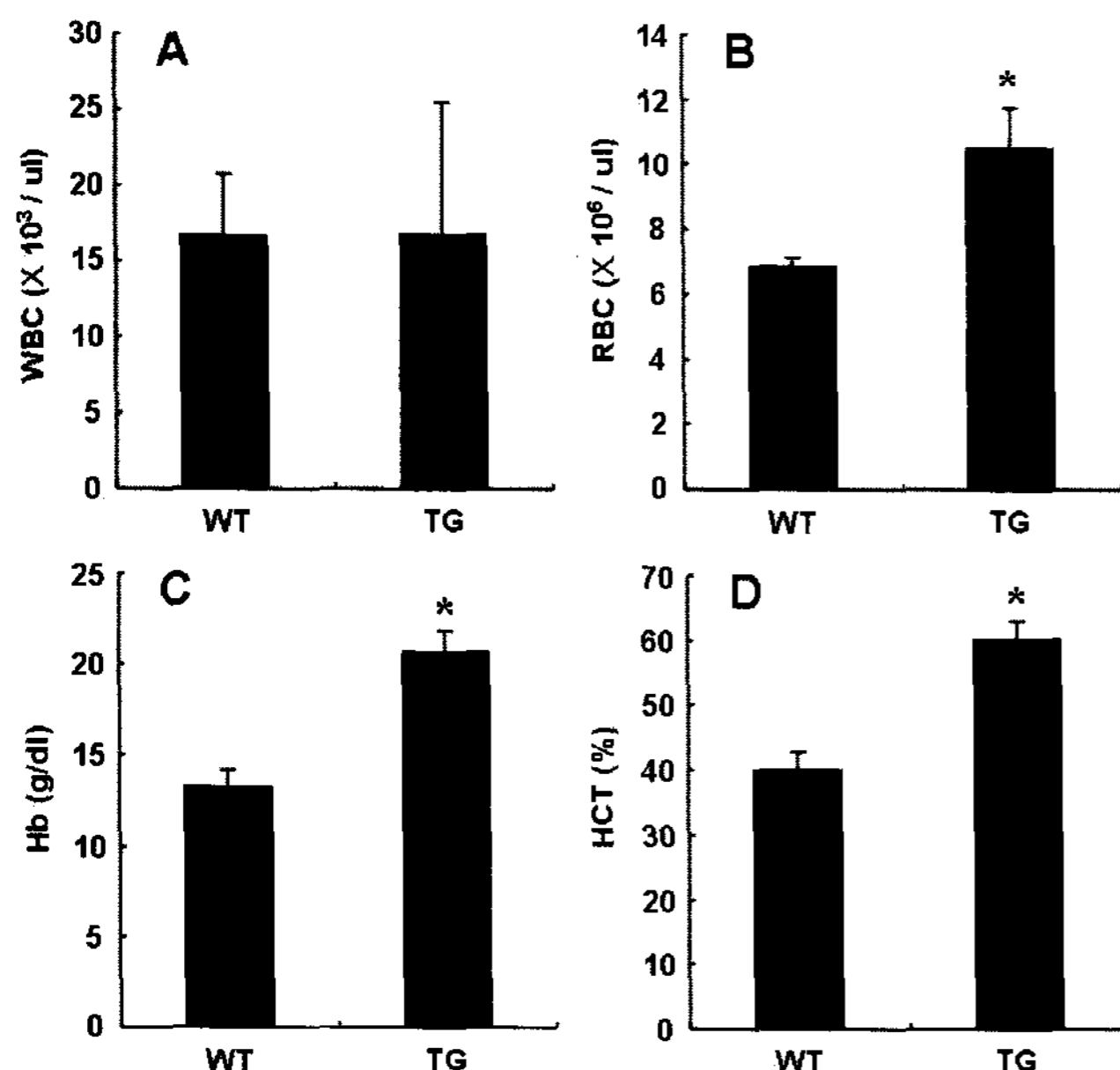

Fig. 2. Comparison of blood composition in WT and TG pigs using Hemavet 950. WT, wild-type pig (n = 3); TG, transgenic pig (n = 3). Panel A, WBC, white blood cell; panel B, RBC, red blood cell; panel C, Hb, hemoglobin; panel D, HCT, hematocrit. *, Significant difference ($p<0.05$) compared with the WT group. Data are expressed as mean values of blood composition with error bars indicating standard deviation, SD.
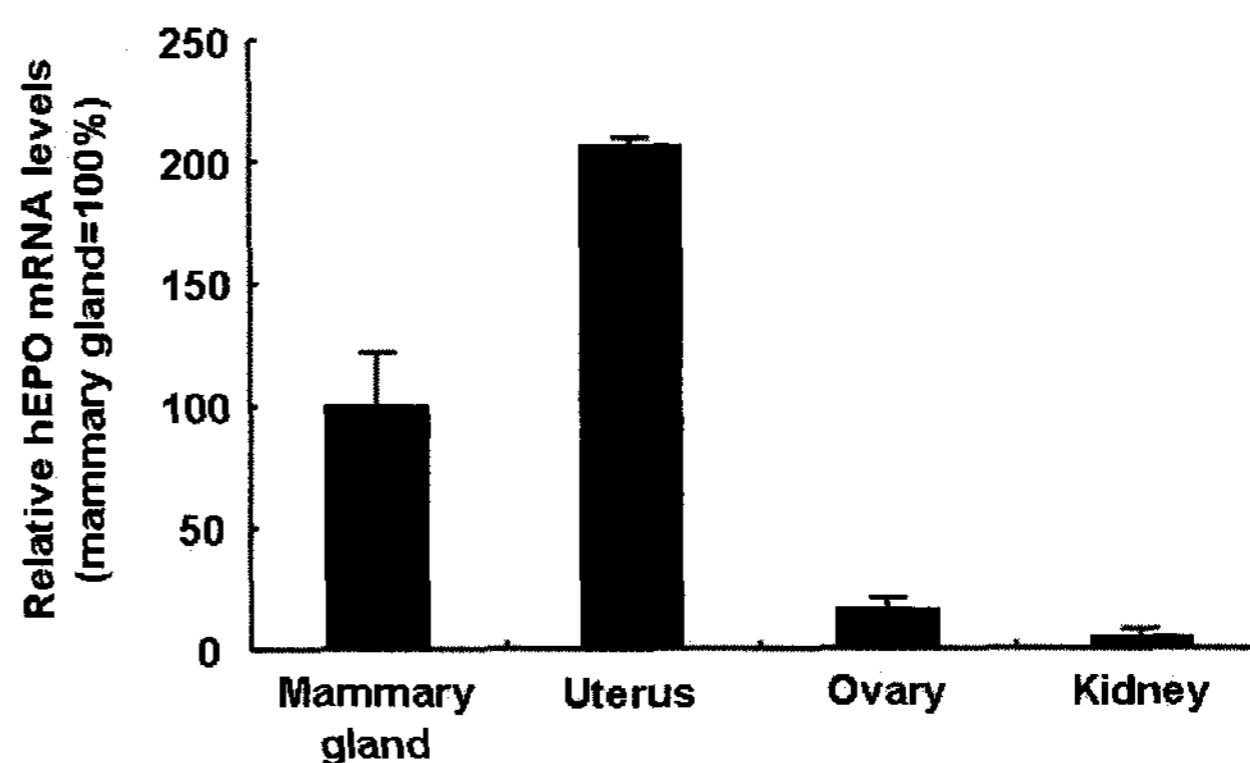

Fig. 3. Relative mRNA levels of hEPO in the reproductive organs. Each value is normalized using porcine GAPDH.
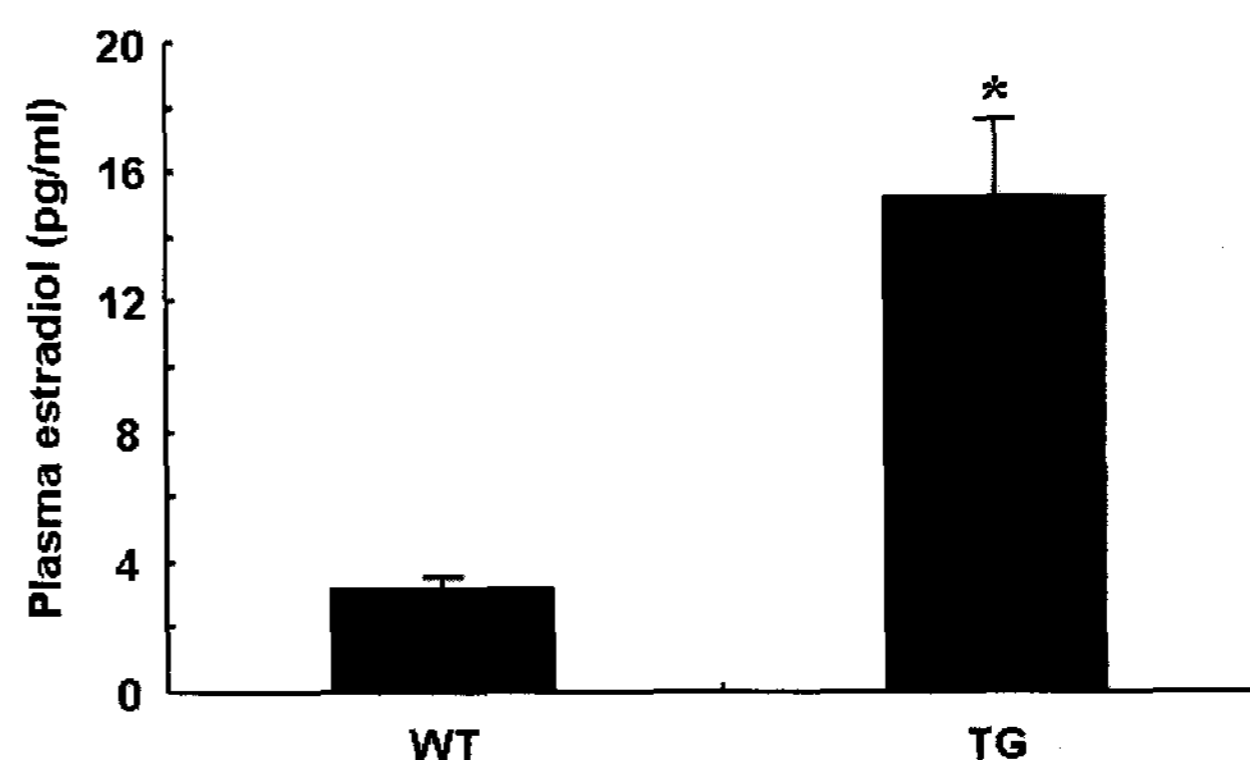

Fig. 4. Comparison of plasma E_2 levels in TG and WT pigs. WT, wild-type pig (n = 2); TG, transgenic pig (n = 3). *, Significant difference ($p<0.05$) compared with the WT group. Data are expressed as mean values of the plasma E_2 levels with error bars indicating standard deviation, SD.

Expressions of hEPO mRNA and Plasma E_2 Levels

The hEPO gene is not expressed in WT pigs. As shown in Fig. 3, hEPO mRNA expression in TG pig was higher in mammary gland (transgenic target) compared with ovary and kidney. Also, high level of hEPO mRNA expression was observed in the uterus. Expression of this gene in uterus (not transgenic target) was approximately 2-fold higher than that of the mammary gland. Plasma, which is collected from estrus synchronized pigs by Regumate Pocine, E_2 level was significantly higher in TG pig (TG vs. WT, 15.21 pg/ml vs. 3.16 pg/ml) (Fig. 4).

DISCUSSION

We previously reported that transgenic boar sperm harboring hEPO gene has low sperm viability than WT boar (Park et al., 2006a). This observation may result in the decreased fertilization capability and then caused by small number of liter size. Among the our hEPO

transgenic pig, several female have been shown to have reproductive disorders, including low pregnancy rates, irregular estrus cycle and low little size. Here, we focused on the irregular estrus cycle, which may causes decreased rates of pregnancy by directly and/or indirectly decreasing reproductive performance in male pigs, in hEPO transgenic pig. On the other hand, sexual behavior in female pigs is characterized by both proceptive and receptive behavior. Standing response is regarded as most important signal for mounting male during mating (Signoret, 1971). In the present study, estrus behavior (standing response) in hEPO TG pig was significantly decreased. This result suggests the possibility of defective hormonal regulation in the h-EPO TG pig.

Next, we determined the blood compositions of TG and WT pigs to compare reproductive disorders such as low pregnancy rates and irregular estrus cycle. As reported previously (Kim et al., 2007), TG pigs showed high RBC, Hb and HCT values than WT pigs, however, no significant difference was observed in WBC levels between TG and WT pigs. In previous studies, TG animals showed foreign EPO gene expression in the salivary gland, uterus, and ovary, with symptoms of erythrocyte polycythemia despite using the mammary gland-specific promoter; our TG pigs also showed similar phenotypes. Additionally, the tissue-specific mWAP promoter was reported to have leakage of expression (in the salivary gland and kidney of mouse) (Paleyanda et al., 1994). In the present study, to identify non-specific hEPO expression in TG pigs, we designed a hEPO-specific probe to perform quantitative real-time PCR. Non-specific expression of hEPO was observed in the reproductive organs of TG pigs, i.e., in the ovary and uterus. The uterus, in particular, showed higher expression level than the mammary gland, which is the target organ of the transgene. Although the WAP gene is expressed mainly in the mammary gland, WAP RNA were found in the pituitary gland, pancreas, adrenal gland, tongue, liver, thymus and heart atria (Pittius et al., 1998). The mechanism of non-specific expression in our study is not known but is probably due to shorter promoter fragments. Paleyanda et al. (1994) previously showed that a 4.1 kb mWAP promoter fragment directs expression of recombinant human protein C specifically to the mammary gland. However, we used a 2.6 kb mWAP promoter, thereby may lead to non-specific expression. Taken together, non-specific expression of the foreign EPO transgene appears to affect hematopoiesis in TG pigs. These observations also suggest the possibility of "other" leakages in different tissues of TG pigs. Also, there is a strong possibility that this non-specific hEPO expression can affect the physiology of pig uterus by angiogenesis. Angiogenesis in the uterus is closely related not only to the estrus cycle but also to EPO (Yasuda et

al., 1998). Thus, this non-specific hEPO expression in the uterus might result in the irregular estrus cycle of hEPO TG pigs. Furthermore, over-expression of hEPO in the uterus might influence angiogenesis in the uterine endometrium, resulting in irregular estrus cycle.

On the other hand, since E_2 concentration is closely related to the estrus cycle, in order to investigate the irregular estrus cycle of TG pigs, we compared E_2 concentrations of TG and WT pigs after estrus synchronization by Regumate Porcine treatment for 6 days. Sasaki reported that EPO induces angiogenesis along with VEGF (vascular endothelial growth factor) controlled by E_2 and is required for cyclic angiogenesis in the uterus (Sasaki, 2003). Thus, non-specific expression of hEPO in the uterus of TG pigs might affect the porcine estrus cycle. Based on these results, we propose that the difference in the secretion levels of E_2 during the preovulatory periods is one of the possible causes of usually low pregnancy rates in TG pigs. Studies on changes of other hormones in TG pigs, particularly estrus or pregnancy related hormones, should be conducted to elucidate the problems associated with reproduction in hEPO TG animals or other blood factor TG animals. Based upon the results of estrus behavior and blood composition, as well as h-EPO mRNA expression and plasma E_2 level, these results suggest that nonspecific expression of hEPO gene in the other organs of TG may affect blood composition and plasma E_2 level, thereby causing reproductive disorders. Further studies are needed to assess the hypothalamic hEPO gene expression in hEPO TG pig, because endocrine regulations such as reproductive behavior, estrus cycle, and so on are under the control of hypothalamus.

REFERENCES

1. Anagnostou A, Liu Z, Steiner M, Chin K, Lee ES, Kessimian N, Noguchi CT (1994): Erythropoietin receptor mRNA expression in human endothelial cells. Proc Natl Acad Sci USA 91:3974-3978.
2. Ashley RA, Dubuque SH, Dvorak B, Woodward SS, Williams SK, Kling PJ (2002): Erythropoietin stimulates vasculogenesis in neonatal rat mesenteric microvascular endothelial cells. Pediatr Res 51:472-478.
3. Carlini RG, Reyes AA, Rothstein M (1995): Recombinant human erythropoietin stimulates angiogenesis in vitro. Kidney Int 47:740-745.
4. Fisher JW (1997): Erythropoietin: physiologic and pharmacologic aspects. Proc Soc Exp Biol Med 216: 358-369.
5. Hanahan D, Folkman J (1996): Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353-364.

6. Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, Mildner-Rihm C, Martin H, Zeiher AM, Dimmeler S (2003): Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood 102:1340-1346.

7. Horiguchi H, Oguma E, Kayama F (2005): The effects of iron deficiency on estradiol-induced suppression of erythropoietin induction in rats: Implications of pregnancy-related anemia. Blood 106:67-74.

8. Horsley BR, Estienne MJ, Harper AF, Purcell SH, Baitis HK, Beal WE, Knight JW (2005): Effect of PG 600 on the timing of ovulation in gilts treated with altrenogest. J Anim Sci 83:1690-1695.

9. Kim MO, Kim SH, Shin MJ, Lee DB, Kim TW, Kim KS, Ha JH, Lee S, Park YB, Kim SJ, Ryoo ZY (2007): Human erythropoietin induces lung failure and erythrocytosis in transgenic mice. Mol Cells 23:17-22.

10. Macmillan D, Bill RM, Sage KA, Fern D, Flitsch SL (2001): Selective *in vitro* glycosylation of recombinant proteins: semi-synthesis of novel homogeneous glycoforms of human erythropoietin. Chem Biol 8:133-145.

11. Muller-Ehmsen J, Schmidt A, Krausgrill B, Schwinger RH, Bloch W (2006): Role of erythropoietin for angiogenesis and vasculogenesis: From embryonic development through adulthood. Am J Physiol Heart Circ Physiol 290:H331-340.

12. Paleyanda RK, Zhang DW, Hennighausen L, McKnight RA, Lubon H (1994): Regulation of human protein C gene expression by the mouse WAP promoter. Transgenic Res 3:335-343.

13. Park CG, Kim S, Lee P, Han JH, Lee HG, Byun SJ, Yang BS, Lee CH, Lee HT, Chang WK, Park JK (2006a): Sperm Fertility of Transgenic Boar Harboring hEPO Gene is Decreased. Reprod Dev Biol 30:27-34.

14. Park JK, Lee YK, Lee P, Chung HJ, Kim S, Lee HG, Seo MK, Han JH, Park CG, Kim HT, Kim YK, Min KS, Kim JH, Lee HT, Chang WK (2006b): Recombinant human erythropoietin produced in milk of transgenic pigs. J Biotechnol 122:362-371.

15. Pittius CW, Hennighausen L, Lee E, Westphal H, Nicols E, Vitale J, Gordon K (1988): A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice. Proc Natl Acad Sci USA 85:5874-5878.

16. Ribatti D, Presta M, Vacca A, Ria R, Giuliani R, Dell'Era P, Nico B, Roncali L, Dammacco F (1999): Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization *in vivo*. Blood 93:2627-2636.

17. Sasaki H, Bothner B, Dell A, Fukuda M (1987): Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. J Biol Chem 262:12059-12076.

18. Sasaki R (2003): Pleiotropic functions of erythropoietin. Intern Med 42:142-149.

19. Signoret JP (1971): The mating behaviour of the sow. In: Cole, D.J.A.(eds), Pig production. Butterworths, UK, pp 295-313.

20. Yasuda Y, Masuda S, Chikuma M, Inoue K, Nagao M, Sasaki R (1998): Estrogen-dependent production of erythropoietin in uterus and its implication in uterine angiogenesis. J Biol Chem 273:25381-25387.