Ovarian TGF-β1 Regulates Yolk Formation Which Involve in Egg Weight of Korean Native Ogol Chicken

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ABSTRACT : Proliferation and differentiation of ovarian cells are regulated by gonadotrophins and various intraovarian factors, with many of their actions dependent on growth factors. Transforming growth factor-β (TGF-β) has been reportedly involved in the regulation of ovarian follicular development. The overall objectives of the present study were to examine the influence of TGF-β1 expression in ovarian follicular development or yolk formation and to investigate the association of egg weight with ovarian TGF-β1 expression at 60 wk. Egg weights of 70 Korean Native Ogol Chicken (KNOC) were recorded from 20 to 60 wk. Ovaries were taken at 60 wk, and TGF-β1 was measured with ELISA, respectively. Based on egg weight up to 60 wk and TGF-β1 expression in ovary, the chickens were divided into high and low groups. Egg weights and follicle weight in the high TGF-β1 group were higher than those in the low groups. Also, TGF-β1 expression and follicle weight in high egg weight group were higher than those in the low groups. Taken together, the results indicate that TGF-β1 is associated with egg weight in KNOC. This association of TGF-β1 with egg weight in KNOC supports the report that TGF-β is mainly involved in the development and differentiation of follicles in the poultry. Further studies about other endocrine factors related to yolk formation are required to fully understand the endocrine mechanism of egg weight in Korean Native Ogol Chickens. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 11 : 1546-1552)***

Key Words : TGF-β1, Egg Weight, Ovary, KNOC

INTRODUCTION

A complex array of externally and locally derived regulatory agents is required for the maintenance of ovarian function (Hsueh et al., 1984). The somatic cell types in the ovarian follicle, thecal and granulosa cells, are the target cells for many of these regulatory agents. Cellular interactions between thecal cells and granulosa cells are postulated to have an integral role in the control of oocyte development. Ovarian follicular development, from initial recruitment to the eventual release of the oocyte, is a spectacular process involving many complex interactions both between follicles during recruitment and selection and between cell types within the developing follicle (Law et al., 1995). It is increasingly apparent that the coordination of events within the developing follicle is dependent upon many local growth factors that modulate the more general systemic gonadotrophin signal from the pituitary. One of these growth factors implicated in this control system is the transforming growth factor-β (TGF-β). TGF-β is a polypeptide that has effects on both cellular proliferation and differentiation. TGF-β generally inhibits growth of epithelial cells and stimulates mesenchymal cell growth (Moses et al., 1985). This highly conserved protein is produced by many cell types and acts via unique cell surface receptors (Derynck et al., 1985).

In mammals, mRNAs for TGF-β1 and β2 are expressed in the ovary and can be regulated by gonadotrophins and steroids (Mulheron et al., 1992). In the adult rat, Teerds and Dorrington (1992) have demonstrated that immunoreactive TGF-β1 levels peak in thecal cells around the time of antrum formation. Also, the similarities between mammalian and avian TGF-β gene expression are remarkable, especially in the light of the distinctive patterns of avian follicular development and the differing steroidogenic capacities of ovarian cell types of the two classes of vertebrates (Law et al., 1995). However, there are few reports regarding ovarian TGF-β in poultry. In adult rats, TGF-β1 and -β2 activity was found in granulosa cells only during the preovulatory period (Teerds and Dorrington, 1992), suggesting that the TGF-βs may be paracrine or autocrine regulators of ovarian follicular development.

The majority of plasma estradiol (E_2) is derived from matured follicles (Robinson et al., 1986). E_2 in conjunction with progesterone (P_4) primes the release of LH, which plays multiple roles throughout follicular development (Wilson and Sharp, 1976; Wu et al., 2000). E_2 was reportedly related to egg productivity (Su and Silversides, 1996) and associated with the regulation of yolk formation and egg weight (Palmer and Bahr, 1992; Whitehead et al., 1993), which were in turn closely related to vitellogenesis in hen. In the process of vitellogenesis, yolk formation occurs in the liver and is regulated by gonadotropin and steroid hormones. The yolk protein precursor, vitelloginin, is transported via blood to the ovary, where it is cleaved into the two yolk proteins, lipovitelline and phosvitin (Williams and Sharp, 1978). Similarly, growth factors, such as IGF-I,

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were mainly originated in the liver, as well. In addition, IGF-I may be synthesized by tubal glands and incorporated into the albumen during egg formation (Cox and Guillette, 1993). Bosche et al. (1995) reported that IGFs regulate the expression and secretion of TGF-βs as part of a coordinated series of changes in the growth factor gene expression that occur during cell proliferation and the initiation of differentiation. These data suggest the possibility of regulation of vitellogenesis by growth factors. However, there were few reports regarding interaction between growth factors and vitellogenesis in poultry until present.

Korean Native Ogol Chicken (KNOC), which is a protected species under the Korean government (Protected Species Act No. 265), is a dual purpose (egg and meat) chicken with low egg productivity. In the past, the economic traits of KNOC were analyzed by statistical and genetical approaches (Han, 1996). Recently, Kang et al. (2000) has shown that IGF-I is negatively related to the egg weight in KNOC. In addition, studies on the relationship between steroid hormones and egg productivity of the KNOC have revealed that E_2 is positively associated with egg weight (Kang et al., 2001). However, there are no reports on the role or even the presence of TGF-βs in the developing ovarian follicles of KNOC.

Therefore, the overall objectives of the present study were to analyze the relation for the ovarian TGF-β1 at 60 wk to both endocrine factors (IGF-I, E_2, and P_4) and weights of ovary and follicles and to investigate the association of ovarian TGF-β1 expression with egg weight.

MATERIALS AND METHODS

Animal

Seventy-unselected female Korean Native Ogol Chicken (KNOC) (Protected Species Act No. 265) were purchased from KNOC Breeding Farms (Yeonsan, Korea). They were raised at the Korea University Animal Breeding Center with a diet (16% crude protein, 3% crude fat, 7% crude fiber, 15% crude ash, 3% Ca, 0.45% P, 0.58% methionine+cystine, 2.75 Mcal/kg metabolizable energy) ad libitum. All chicks were housed in individual cages and received 15 h of light/d for 17 wk followed by 15 min increment in light/wk up to 17 h of light/day (Ohh, 1988). Egg weights were recorded daily from the onset of laying until the age of 60 wk. The average egg weight was calculated every 5 wk until 60 wk old.

Blood collection and serum preparation

Blood (3-5 ml) was taken from the wing vein every 10 wk from 20 wk until 60 wk. Also, blood collection was conducted from 11:00 to 15:00. In order to obtain serum, blood samples were stood at room temperature for 2 h and centrifuged for 20 min at 1,000×g, and the supernatant was stored at -70°C until use.

Isolation for ovary and follicles

To study any possible association among ovarian TGF-β1 expression, weight of the ovary and follicles, ovary and follicles (F1-F5) were isolated from 11:00 to 15:00 at 60 wk. Ovaries were washed in PBS, their weights measured, and follicles (F1-F5) identified. Small yellow follicles (SYF) from the ovary were also counted.

Protein extraction

The ovaries were washed 3 times in an ice-cold Ringer solution (125 mM NaCl, 1 mM $CaCl_2{\times}2H_20$, 5 mM KCl; pH 7.5) to remove any adhering yolk. These samples were homogenized in pre-chilled RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 50 mM Tris; pH 8.0) containing protease inhibitors (100 mM PMSF, 1.0 mg/ml Aprotinin) and incubated at 4°C for 30 min. The samples were centrifuged at 10,000 ×g in a pre-chilled centrifuge at 4°C for 15 min. The supernatant from ovary lysate was collected and stored at -70°C until being analyzed for TGF-β1.

Radioimmunoassay (RIA)

The recombinant chicken IGF-I (GroPep. Pty., Ltd., Australia) was iodinated by the chloramine T method (Lee and Henricks, 1990). Iodinated IGF-I was purified on a Sephadex G-50 column and aliquots were stored -20°C until used.

Serum IGF-I concentration was determined after removing serum IGF binding proteins were removed using an acid-ethanol method (Daughaday et al., 1980). Briefly, each sample was acidified with acid-ethanol (87.5% ethanol, 12.5% HCl) and stood for 30 min at room temperature. Then samples were centrifuged at 1,800×g for 30 min, they were neutralized with 0.2 ml of 0.855 M Tris-base. IGFBPs-removed supernatant was mixed 0.1 ml of RIA buffer (30 mM sodium phosphate, 0.02% protamine sulfate, 10 mM EDTA, 0.05% Tween-20, 0.02% sodium azide; pH 7.5), and incubated with rabbit anti-human IGF-I polyclonal antiserum (GroPep. Pty. Ltd.; final dilution of 1:10,000) and 15,000 cpm [^{125}I] IGF-I in RIA buffer for 16-18 h at 4°C. Then, 0.1 ml of goat anti-rabbit IgG antibody (GroPep Pty., Ltd.) was added and the mixture was incubated for 1 h followed by an additional 1 h incubation with 0.1 ml of normal rabbit serum at 4°C. After addition of 1 ml RIA buffer, the tubes were centrifuged for 10 min at 3,000×g at 4°C. The supernatant was aspirated and the pellet was counted in a gammer-counter. The IGF-I amount was determined by logit-log plots and the intra-assay coefficient of variation was 7.8%.

Concentration of ovarian TGF-β1

Ovarian TGF-β1 was determined by TGF-β1 Emax Immuno Assay System (Promega Co., USA). To measure the active form of TGF-β1, extracted protein was diluted with DPBS. Then, 1 μl of HCl was added to 50 μl of diluted sample and the mixture was incubated for 15 min at room temperature. After incubation, 1 μl of NaOH was added and the neutralized mixture was used for the assay. Flat-bottom 96 well plates were coated with TGF-β1 coat monoclonal antibody and a second specific polyclonal antibody bound the captured TGF-β1. The amount of specifically bound polyclonal antibody was detected using a species-specific antibody conjugated to horseradish peroxidase. The color development was stopped and the intensity of the colors was measured at 450 nm.

Selection for experimental groups

Chicks were divided into high and low groups based on ovarian TGF-β1 concentration at 60 wk and egg weight profiles up to 60 wk. Selection criteria for high and low groups were 20% in upper and lower classes.

Statistical analysis

Levels of TGF-β1 and IGF-I, egg weight, and ovary and follicle weight from high and low groups were statistically analyzed using the Duncan's One Way ANOVA procedure of Statistical Analysis System (SAS Institute, 1995).

RESULTS

Ovarian TGF-β1 and egg weights of unselected KNOC

Ovarian TGF-β1 expression at 60 wks of age and changes in egg weights from 20 to 60 wks of age of the unselected group were examined (Table 1). Average ovarian TGF-β1 concentration was 1.83±1.28 ng/mg at 60 wks of age. Egg weight gradually increased until 50 wks of age (50.54 g) and then declined.

Comparison of egg weight between high and low ovarian TGF-β1 groups

Unselected KNOCs were divided into two groups depending on their ovarian TGF-β1 expression at 60 wk: a high TGF-β1 group with higher than 3.66 ng/mg and a low TGF-β1 group with less than 0.44 ng/mg, comparising the upper and low 20% of the unselected group, respectively. Egg weights were compared between the two groups (Figure 1). Although significant differences were not detected between the two groups, egg weight in the high ovarian TGF-β1 group tended to be higher than that in the low group from 45 to 60 wk.

Comparison of serum IGF-I and E_2 concentration between high and low ovarian TGF-β1 groups

Both groups were compared for serum IGF-I and E_2 concentration (Figures 2 and 3). In the comparison of IGF-I pattern, a significant difference ($p<0.05$) was detected at 50 and 60 wk (Figure 2). Also, the average E_2 concentration was significantly different between high and low groups only at 40 wk (198.0±80.3 and 106.0±43.1 pg/ml; $p<0.05$). At another week of age, a large variation among individuals were observed.

Comparison of TGF-β1 concentration between high and low egg weight groups

Similar to KNOC grouping by ovarian TGF-β1, the high egg weight group with higher than average 49.5 g and the low egg weight group with less than average 46.2 g were selected and their TGF-β1 concentrations were compared (Table 2). Large individual variation was detected, which led to no significant difference between the two groups.

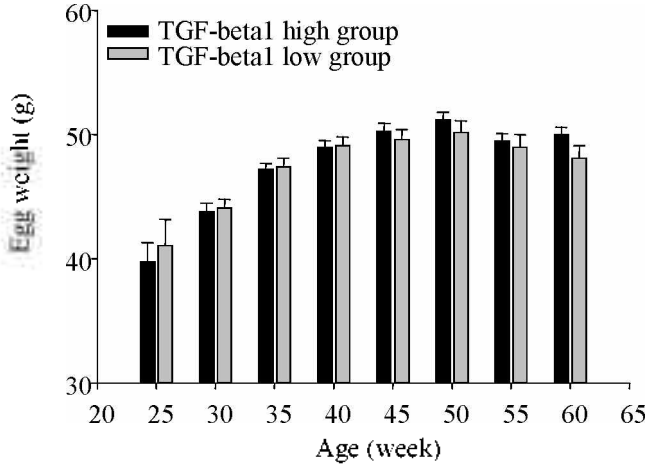

Figure 1. Comparison of egg weights between high and low ovarian TGF-β1 expression groups. Values are the mean±SE.

Table 1. The ovarian TGF-β1 expression at 60 wk and egg weight of unselected Korean Native Ogol Chicken

Traits	No. of Chicks	Weeks							
		25	30	35	40	45	50	55	60
TGF-β1[1] (ng/mg)	70	-	-	-	-	-	-	-	1.83±1.28
AEW[2] (g)	70	40.51±2.66	43.69±3.95	47.33±2.53	48.57±2.81	49.67±3.18	50.54±2.72	48.84±3.59	48.80±3.56

[1] All values are expressed as mean±SD.

[2] Average egg weight.

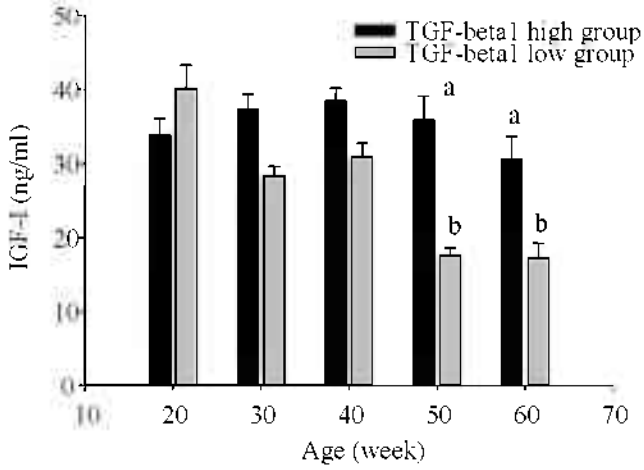

Figure 2. Comparison of serum IGF-I expression between high and low TGF-β1 groups. Values, expressed as ng per ml, are the mean±SE. Means with different superscript differ significantly ($p<0.05$).

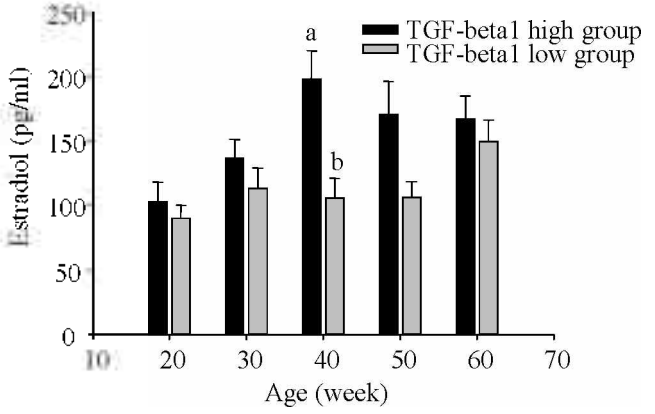

Figure 3. Comparison of E_2 expression between high and low ovarian TGFβ1 expression groups. Values are the mean±SE. Means with different superscripts differ significantly ($p<0.05$).

Table 2. Comparison of ovarian TGF-β1 concentration between high and low egg weight group at 60 wk[1]

Groups	No. of Chicks	TGF-β1 (ng/mg)
EW high (>49.5 g)	15	1.88±1.37
EW low (<46.2 g)	15	1.65±1.29

[1] All values are expressed as mean±SD.

Comparison of IGF-I and E_2 concentration between high and low egg weight groups

Both groups, the high and low egg weight groups, were compared for IGF-I and E_2 levels (Figures 4 and 5). In the comparison of IGF-I levels, the low egg weight group showed a higher amount than the high group from 20 to 40 wks and then the pattern was reversed (Figure 4). Conversely, E_2 concentration in the high group was higher than that in the low group during all periods examined (Figure 5). In particular, E_2 concentration was significantly

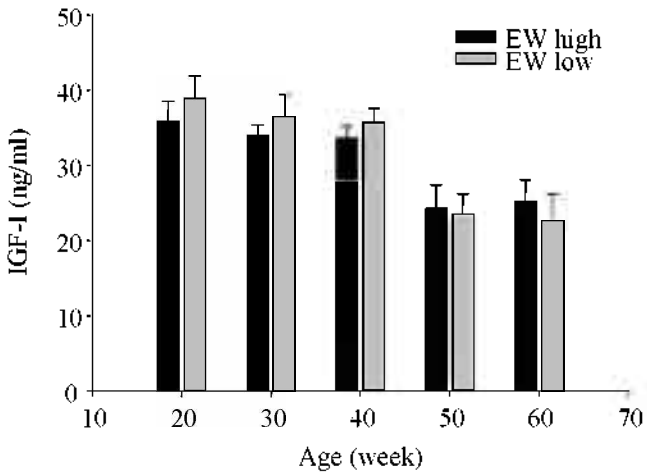

Figure 4. Comparison of serum IGF-I concentration between high and low egg weight groups. Values are expressed as the mean±SE.

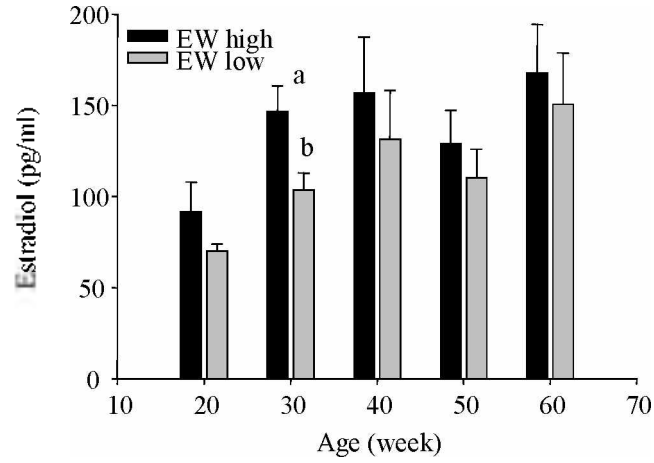

Figure 5. Comparison of serum E_2 concentration between high and low egg weight groups. Mean±SE (n=15) with different superscripts differ significantly ($p<0.05$).

different at 30 wk (146.9±41.5 pg/ml and 103.6±24.8 pg/ml; $p<0.05$).

Comparison of follicle weight and ovary weight between groups selected by ovarian TGF-β1 expression or egg weight

Weight profiles of follicle and ovary of overall unselected groups and of the selected groups by TGF-β1 and egg weight are summarized in Table 3. Although the groups did not show any statistical difference in follicle and ovary, the high TGF-β1 expression group tended to show heavier follicle weight but not ovary weight. When follicle weight and ovary weight were compared between high and low groups by egg weight, no significance was shown, which is similar to the comparison result based on ovarian TGF-β1 expression.

Table 3. Comparison of follicle weight, ovary weight, among TGF-β1 and EW selection groups[1)]

Selection traits	Groups	No. of Chick	Follicle Weight (g)					Ovary weight[2)] (g)
			F1	F2	F3	F4	F5	
TGF-β1	High (>3.66 ng/mg)	15	12.9±4.6	9.4±2.6	4.7±2.1	1.5±1.5	0.4±0.5	27.1±16.6
	Low (<0.44 ng/mg)	15	12.3±3.9	7.9±3.4	3.9±2.4	1.5±1.1	0.4±0.2	31.4±10.9
EW[3)]	High (>49.5 g)	15	12.8±5.4	8.9±4.4	4.6±3.0	1.5±1.5	0.5±0.5	27.4±15.1
	Low (<46.2 g)	15	10.8±4.9	7.2±3.3	3.5±2.1	1.2±1.0	0.3±0.2	25.9±12.7
Overall (unselected)		70	11.9±4.5	8.4±3.5	4.3±2.5	1.5±1.2	0.4±0.4	28.6±13.4

[1)] All values are expressed as mean±SD.
[2)] F1 to F5 follicle weight plus SYF weight.
[3)] Egg weight.

DISCUSSION

KNOC expresses hybrid characteristics of both broiler and layer, with low egg productivity. The annual egg production and average egg weight of KNOC are approximately 120 and 50 g (Nahm, 1997), which are far less than that of White Leghorn (>300 and >60 g) (Su and Silversides, 1996). There are not many comparative studies on the characteristics of these two strains, but it is not difficult to suspect that this may be due to different endocrine mechanisms for egg production.

Ovarian follicular development and ovulation is the culmination of proliferative and differentiative activities of both granulosa and theca cells. One important aspect of the ovarian function is the need for a rapid stimulation and inhibition of follicular growth. The majority of developing follicles, however, undergo atresia, in which proliferation is stopped and the follicle degenerates. Therefore, the control of granulosa cell growth appears to be complex and requires factors that both stimulate and inhibit growth. TGF-β has been implicated as an important intrafollicular regulator of follicle development in the mammalian ovary (May et al., 1996). In several porcine reports, TGF-β may play a physiologically significant role in controlling differentiation of immature and moderately mature porcine granulosa cells via an autocrine/paracrine mechanism (Kubota et al., 1994). In addition, ovarian thecal cells in culture were found to synthesize and secrete TGF-β (Skinner et al., 1987). Therefore, these indicate that ovarian thecal cells produce TGF-β, which can regulate granulosa cell growth and differentiation and suggest that TGF-β is a local regulator of follicular function (Engelhardt et al., 1992). It is certain that there are differences in follicular developmental mechanism present between mammals and birds. However, Law et al. (1995) has reported the first demonstration of TGF-β gene expression in the ovary of a nonmammalian species. In this report, similarities between mammalian and avian TGF-β gene expression were remarkable, especially in light of the distinctive patterns of avian follicular development and the differing steroidogenic capacities of ovarian cell types of the two classes of vertebrates.

In chickens, the decrease in egg production with age is in part caused by a reduction in the number of follicles reaching the phase of rapid growth. Conversely, egg weight increased with age and is maintained without a decrease. Thus, the fewer follicles are present the greater quantity of yolk they receive proportionally, resulting in larger sized eggs (Williams and Sharp, 1978). Since, TGF-β is an inhibitor for granulosa cells, it is said that TGF-β inhibits growth of granulosa cells and suppresses a production of steroid hormones in process of egg production with age (Michael et al., 1987). As a result, the opportunity of ovulation and recruitment of follicles in turn decrease. Consequently, a certain portion for yolk formation can be used to enlarge the size of eggs. Therefore, it is likely that TGF-β1 plays a positive role in influencing factors for egg weight after all. In this study, the egg weight of high TGF-β1 group was higher than that of low group from 45 to 60 wk (Figure 1). Although high and low groups did not show any statistical difference, this result suggests that tissue- and time-specific differences in ovarian TGF-β1 function during increasing egg weight.

The steroid hormone E_2 is known to play important roles in ovarian physiology. The majority of plasma E_2 is derived from follicles (Robinson et al., 1986). In addition, E_2 in conjunction with P_4 primes the release of LH (Wilson and Sharp, 1976), which plays multiple roles throughout follicular development (Wu et al., 2000). A high preovulatory E_2 level is related to the activity of immature follicles and the maturation and growth of F1. Thus E_2 is a stimulator in vitellogenesis (yolk formation and maturation). Hormones, such as steroids and gonadotropins, are reportedly known to stimulate the expression of paracrine growth factors (Hsueh et al., 1984). Furthermore, actions of hormones to stimulate follicle development and growth are mediated in part through altering these local cell-cell interactions (Johnson, 1990). Thus, the locally produced paracrine factors that mediate cell-cell interactions are involved in primordial follicle development and the progression of follicle development during folliculogenesis is starting to be elucidated (Nilson and Skinner, 2001). Consistent with the above, the comparison of E_2 expression at 40 wk between high and low TGF-β1 groups showed 198 pg/ml and 106 pg/ml, respectively (Figure 3). Moreover,

the high egg weight group showed a tendency of higher E_2 expression than the low egg weight group during all periods (Figure 5), which is a similar result by Su and Silversides (1996). Also, the report by Whitehead et al. (1993) showing that E_2 is associated with controlling egg weight supported our results.

In the comparison of IGF-I between selection groups, high TGF-β1 group had higher IGF-I amount than low group. In contrast, the IGF-I concentration of the high egg weight group was not significantly different from that of the low egg weight group at 50 and 60 wk (Figure 2 and 4). These indicate that serum IGF-I is positively related to ovarian TGF-β1, but not to egg weight, and support a previous report for other poultry (Su and Silversides, 1996). In KNOC, serum IGF-I has a negative association with egg weight until 40 wk (Kang et al., 2000), which is contrary to the results from the present study. Since this study investigated any association between growth factors and egg weight until 60 wk, it is supposed that IGF-I may stimulate TGF-β1 expression for yolk formation at late egg laying period. Taken together, the results suggest the positive influence of growth factors (TGF-β1 and IGF-I) in the regulation of yolk formation and egg weight.

In conclusion, although significant differences in egg weight between the high and low TGF-β1 groups were not detected, our results suggest the involvement of TGF-β1 in ovarian follicular development and yolk formation by vitellogenesis. Studies regarding ovarian expression of endocrine factors related to egg weight are required to further understand the endocrine mechanism of yolk formation or ovarian follicular development in KNOC.

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