TITLE: Some endocrine traits of transgenic rabbits. II. Changes in hormone secretion and response of isolated ovarian tissue to FSH and ghrelin

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SHORT TITLE: hormones in ovaries of transgenic rabbits

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SUMMARY

In the present in-vitro experiments we examined FSH- and ghrelin-induced changes in ovarian hormone secretion by transgenic rabbits. Fragments of ovaries isolated from adult transgenic (carrying mammary gland-specific mWAP-hFVIII gene) and non-transgenic rabbits from the same litter were cultured with and without FSH or ghrelin (both at 0, 1, 10 or 100 ng/ml medium). The secretion of progesterone (P_4), estradiol (E_2) and insulin-like growth facor I (IGF-I) was assessed by RIA. It was observed that ovaries isolated from transgenic rabbits secreted much less P_4, E_2 and IGF-I, than the ovaries of non-transgenic animals. In control animals FSH reduced E_2 (at doses 1-100 ng/ml medium) and IGF-I (at 1-100 ng/ml), but not P_4 secretion, and ghrelin promoted P_4 (at 1 ng/ml) and IGF-I (at 100 ng/ml), but not E_2 output. In transgenic animals, the effects were reversed: FSH had a stimulatory effect on E_2 (at 100 ng/ml) and ghrelin had an inhibitory effect on P_4(at 10 ng/ml). No differences in the pattern of influence of FSH on P_4 and IGF-I and of ghrelin on E_2 and IGF-I were found between control and transgenic animals. The present observations suggest (1) that both FSH and ghrelin are involved in rabbit ovarian hormone secretion, (2) that transgenesis in rabbits is associated with a reduction in ovarian secretory activity, and (3) that transgenesis can affect the response of ovarian cells to hormonal regulators.

INTRODUCTION

It was previously demonstrated (Sirotkin et al., 2007) that transgenesis (introduction of the mammary gland-specific mWAP-hFVIII gene) in rabbits can be associated with substantial changes in plasma and milk concentrations of corticosterone, progesterone (P_4), testosterone, estradiol (E_2) and insulin-like growth factor I (IGF-I). These hormonal changes can be associated with an increased incidence of pathological changes in organs, increases in concentration (Suvegova et al., 2004) and aneuploidy (Parkanyi et al., 2004) of leucocytes in blood, the appearance of new types of bone tissue, increased aneuploidy in bone marrow (Martiniakova et al., 2005) and changes in sperm quality (Chrenek et al., 2006). Nevertheless, it remaines unknown whether these changes are mediated by central regulators (hypothalamo-hypophysial system), or whether transgenesis could target peripheral endocrine glands and their response to upstream hormonal regulators.

Gonadotropins (FSH, LH and related hormones) are the best known regulators of gonads. They can stimulate or inhibit ovarian steroid hormone secretion, either directly or through local IGF-I (Spies et al., 1997, Makarevich et al., 2000; Hillier,2001; Fair, 2003; Mazerbourg et al., 2003). In contrast to gonadotropins, the role of the newly detected hormone ghrelin, which is produced in stomach and other tissues (van der Lely et al., 2004), in the control of the ovarian functions is poorly examined. There are two reports describing the ability of ghrelin to inhibit testosterone secretion and the expression of key steroidogenic enzymes in rat testis (Tena-Sempere, 2005) and of its ability to stimulate the secretion of P_4, E_2, arginine-vasotocin and IGF-I in cultured chicken ovarian fragments (Sirotkin et al., 2006, Sirotkin and Grossmann, 2006, 2007). The effect of ghrelin on hormone secretion by mammalian ovarian cells has not yet been studied.

The aim of present studies was to examine (1) whether both FSH and ghrelin are involved in the control of hormone secretion by rabbit ovarian cells, (2) whether transgenesis (introduction of mWAP-hFVIII gene) in rabbits is associated with changes in the secretory activity of ovarian cells, and (3) whether transgenesis is associated with changes in the response of ovarian cells to FSH and ghrelin. The secretion of P_4, E_2 and IGF-I was measured.

MATERIAL AND METHODS

Collection and culture of ovarian tissue

The origin, housing, breeding and detection of transgenic broiler rabbits carrying the mammary gland specific WAP-hFVIII gene construct was describe previously (Sirotkin et al., 2007). Ovarian tissue isolated from both transgenic and non-transgenic offspring from the same litters from generation F_3 (adult sexually mature females, 9 months of age) was used. Ovarian function was activated by i.m. injection of pregnant mare serum gonadotropin (PMSG, Werfaser, Alvetra und WERFFT, Vienna, Austria, 20 IU/kg body mass). One hour after injection, rabbits were killed at a local slaughterhouse in accordance with EU and local ethical and hygiene requirements. Ovaries were collected and transported to the laboratory at the ambient temperature in a glass container within 0.5 h of slaughter. Thereafter ovaries were washed in Dulbecco's PBS (D'PBS) with 1% antibiotic-antimycotic solution (Sigma, St.Louis, USA), placed in 100 mm diameter culture dishes (Gama, České Budejovice, Czech Republic) and dissected using a blade knife to 8 approximately equal parts (weight 4,8-5,6 mg). These ovarian fragments were washed again 3 times in D'PBS with antibiotic-antimicotic and cultured in 1 ml of BioWhittaker ^{TM} DME/F12 1:1 mixture supplemented with 10% BioWhittaker ^{TM} fetal calf serum (both from Cambrex BioScience, Verviers, Belgium) and 1% antibiotic-antimycotic solution (Sigma) in Falcon ^{TM} 24-well plates (Becton Dickinson, Lincoln Park, USA) at 37^0C and 5% CO_2 in humidified air.

Control groups contained either no cells (blank control) or cells but no exogenous hormones. Experimental groups (originating from the ovaries of either transfected or non-transfected animals) received 1, 10 or 100 ng/ml of immunological grade porcine FSH (pFSH-I-1, APF10640B) kindly provided by Dr. A.P.F. Parlow, National Hormone and Pituitary Program, Torrance, CA, USA) or research grade octanoylated recombinant human ghrelin 1-18 (PGH-3625 PI; Peptides International, Inc., Louisville, Kentucky, USA) prepared by solid phase peptide synthesis using an Fmoc/t-butyl strategy and purified on a reverse phase HPLC column. The hormones were dissolved in the culture medium immediately prior to the experiment. After 24 h culture the medium from plate wells was gently aspirated and frozen at -18^0C to await RIA.

Validation of ovarian cells viability

To determine the viability of ovarian cells after culture, all ovarian fragments were carefully dissected using a multiblade knife in Petri dishes with culture medium described above. The resulting suspension of granulosa cells was passed through a steel sieve to separate large pieces and cellular fragments, washed three times by centrifugation (200 xg) and resuspension in sterile incubation medium as above. 200 ul aliquots of cell suspension were incubated in Lab-Tek chamber-slides (Nunc, Inc., Naperville, USA) at 37^0C and 5% CO_2 in humidified air during 5 days. Microscopic inspection after such culture showed the formation of an approx. 70% confluent cell monolayer. Chamber-slides with monolayers were washed 3 times in ice-cold D'PBS, fixed for 20 min in 4% paraformaldehyde in D'PBS, washed in PBS (2 x 5 min) and subjected to immunocytochemical analysis by using DAB-reagent for visualization of PCNA (marker of proliferation) and bax (a marker of apoptosis), as described previously (Makarevich et al., 2000; Sirotkin et al., 2006).

Hormone analysis

Concentrations of P_4, E_2 and IGF-I contained in ovarian fragments-conditioned incubation medium weree determined in 20-50 μl of medium using RIA/IRMA kits from DSL (Webster, TX, USA) according to the manufacturer's instructions. The characteristics of the assay were described previously (Sirotkin et al., 1998; Makarevich et al., 2000; 2004).

Statistics

Each experimental group was represented by 6 culture wells, each containing one ovarian fragment. The data shown are the values obtained in two different series of experiments: one to examine the effect of FSH and the other to detect the effect of ghrelin. The experiments were performed on separate days, using separate pools of cells, each obtained from 3 control and 3 transgenic animals. After RIA, the values of the blank control (cell-free medium) were subtracted from the value determined in cell-conditioned medium to exclude any non-specific background. The rates of substance secretion were calculated per mg ovarian tissue/day. Significant differences between the experiments were evaluated using two-way ANOVA. When effects of treatments were revealed, data from the experimental and control groups were compared by Duncan's multiple range test. Differences from the control at $P<0.05$ were considered as significant.

RESULTS

The cultured ovarian fragments secreted substantial amounts of P_4, E_2 and IGF-I. Cells isolated from ovarian fragments after 1 day culture were able to attach to the substrate and form a monolayer. Immunocytochemical analysis of these cells showed the presence of substances associated with proliferation (PCNA, localised mainly in nuclei) and apoptosis (bax, localised both in nuclei and cytoplasma; Fig.1).

Significant differences were found between the basal secretory activities of ovarian cells originating from transgenic and non-transgenic animals. In the first series of experiments the cells of transgenic rabbits secreted much less P_4 (Fig.2a) and E_2 (Fig.2b), but not IGF-I (Fig.2c) than those of non-transgenic animals. In the second series of experiments, ovarian

cells from transgenic rabbits secreted significantly less P_4 (Fig.3a), E_2 (Fig.3b) and IGF-I (Fig.3c) compared with non-transgenic ones.

FSH as able to affect the secretion of some hormones by cultured ovarian fragments, whilst its effect depended on the origin of ovarian tissue. It reduced the secretion of E_2 (Fig.2b) and IGF-I (Fig.2c), but not P_4 secretion by control ovarian tissue. The response of ovarian tissue from transgenic animals on FSH was similar in the case of P_4 (Fig.2a) and IGF-I (Fig.2c). However, in transgenic rabbits addition of FSH resulted not in inhibition but in stimulation of E_2 secretion (Fig.2b).

An influence of ghrelin on the secretory activity of ovarian tissue of both groups was demonstrated. With control cells it stimulated P_4 (Fig.3a) and IGF-I (Fig.3c), but not E_2 (Fig.3b) secretion. With transgenic cells, ghrelin inhibited P_4 secretion (Fig.3a). No significant differences were found between the groups in the pattern of influence of ghrelin on E_2 (Fig.3b) and IGF-I (Fig.3c).

DISCUSSION

Our observations confirm previous reports (Makarevich et al., 2000) of the ability of isolated and cultured rabbit ovarian cells to survive, to form monolayer, to express markers of proliferation and to secrete steroid hormones. Our observations confirm previous reports (Spies et al., 1997; Hillier, 2001) of production of steroid hormones by rabbit ovarian cells. This is probably the first demonstration of the presence of the apoptosis-associated substance bax and of the secretion of IGF-I by rabbit ovaries.

Results presented in Fig.2 confirm previous data (Spies et al., 1997, Hillier, 2001) on the involvement of FSH in the control of rabbit ovarian steroidogenesis. The lack of influence of FSH on P_4 and the inhibitory action of FSH on E_2 observed in our experiments could be due to FSH-induced luteinisation of rabbit ovarian tissue, which in rodents is associated with a reduction in E_2 production, but not with the formation of a P_4-producing corpus luteum (Hillier, 2001). This effect of FSH on steroidogenesis could also be explained by the development of negative feedbach mechanisms induced by PMSG injection before collection of the ovaries. This is the first report of the ability of FSH to control rabbit IGF-I. As in other mammals (Makarevich et al., 2000; Fair, 2003; Mazerbourg et al., 2003; Oksbjerg a kol, 2004), this could regulate basic ovarian functions and mediate FSH action.

Figure 3 shows a direct influence of ghrelin on mammalian ovarian function. Previously, only an indirect influence of ghrelin on reproduction, mediated by metabolism or hypothalamo-hypophysial system, had been suggested (Van der Lelly et al., 2004; Tena-Sempere et al., 2005). The influence of ghrelin on both P_4 and IGF-I observed in our experiments is the first demonstration of involvement of ghrelin in direct control of secretion of both peptide and steroid ovarian hormones. Furthemore, it indicates potential involvement of ghrelin in regulation of ovarian functions controlled by these hormones in mammals. This finding is in line with our recent observation that ghrelin can regulate the secretory activity, proliferation and apoptosis of cultured avian ovarian cells (Sirotkin et al., 2006, Sirotkin and Grissmann, 2006, 2007).

The results presented in Figs. 2 and 3 demonstrate that transgenesis may be associated with substantial changes in ovarian secretory activity: ovarian cells isolated from transgenic rabbits had a reduced ability to secrete both steroid and peptide hormones. This observation is in

agreement with the results of our in-vivo experiments (Sirotkin et al., 2007) demonstrating that transgenic rabbits had altered (mainly reduced) concentrations of peptide and steroid hormones in blood. Some discrepancy between Fig.2 and Fig.3 in effect of transgenesis on basal IGF-I release could be due to some random differences in material used in different experiments, but in the majority of cases ovarian cells isolated from transgenic rabbits secreted significantly lover amounts of hormones, than the cells of control animals. The reduced secretory activity of ovarian cells isolated from transgenic rabbits demonstrates that transgenesis may affect the endocrine system not (or not only) at the hypothalamo-hypophysial level, but directly at peripheral endocrine glands (ovaries).

The present figures showed not only transgenesis-associated changes in basal ovarian secretory activity but also the principal changes in ovarian cell response to FSH and ghrelin treatments: transgenesis was associated with prevention and even reversal of the E_2 response to FSH and P_4 response to ghrelin treatment. Since FSH, P_4 and E_2 (Spies et al., 1997, Hillier,2001) and probably ghrelin (Tena-Sempere et al., 2005; Sirotkin et al., 2006) are important regulators of the ovary, it is possible that transgenesis influences reproduction through these hormones. Indeed, transgenesis with the mWAP-hFVIII gene construct results in changes in sperm quality (Chrenek et al., 2006) but has no effect on female fertity (Sirotkin et al., 2007).

The mechanisms through which transgenesis influences ovarian secretory ativity remain to be studied. Some changes may be primary and others secondary. For example, transgenesis-associated suppression of the P_4 and E_2 observed in our experiments could be due to decreased secretion of IGF-I, a known stimulator of ovarian steroidogenesis (Makarevich a kol, 2000; Fair, 2003; Mazerbourg et al., 2003; Oksbjerg a kol, 2004) or to a decreased response of ovarian cells to ghrelin, which in our experiments was potent stimulator of both IGF-I and P_4 secretion. Therefore, the trangenesis of rabbits with mWAP-hFVIII gene construct could affect endocrine and other systems probably not through hFVIII itself (Chrenek et al., 2005b; Sirotkin et al., 2007) but rather through destabilisation of the genotype, which in turn affects the production, secretion, reception or response to several important hormones. Our studies have demonstrated that transgenesis itself, independently of the introduced gene, could have unpredicted side-effects on the endocrine system, including suppression of ovarian hormone secretion and reversal of the response of ovarian cells to upstream hormonal regulators.

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LEGENDS TO FIGURES

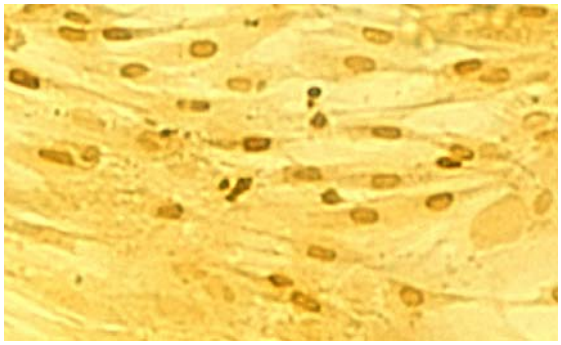
Fig. 1. Monolayers of ovarian cells isolated from cultured ovarian fragments of non-transgenic (control) rabbits and expressing markers of proliferation (PCNA, left) and apoptosis (bax, right; brown staining). Magnification x 200. .

Fig. 2. Effect of FSH on the secretion of P4 (a), E2 (b) and IGF-I (c) by cultured fragments of ovaries isolated from non-transgenic (control) and transgenic rabbits. Values are means ± S.E.M. * - significant ($p<0.05$) difference between control and transgenic animals.

Fig. 3. Effect of ghrelin on the secretion of P4 (a), E2 (b) and IGF-I (c) by cultured fragments of ovaries isolated from non-transgenic (control) and transgenic rabbits. Values are means ± S.E.M. * - significant ($p<0.05$) difference between control and transgenic animals.

Fig.1. Monolayer of ovarian cells containing markers of proliferation (left) and apoptosis (right)

PCNA



Bax

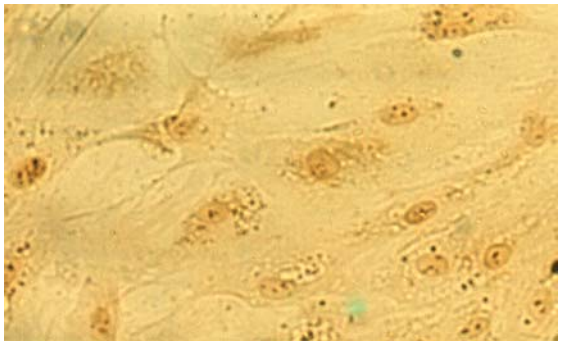
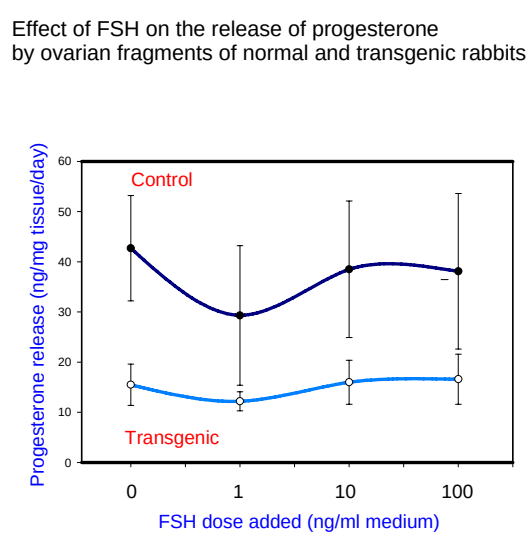
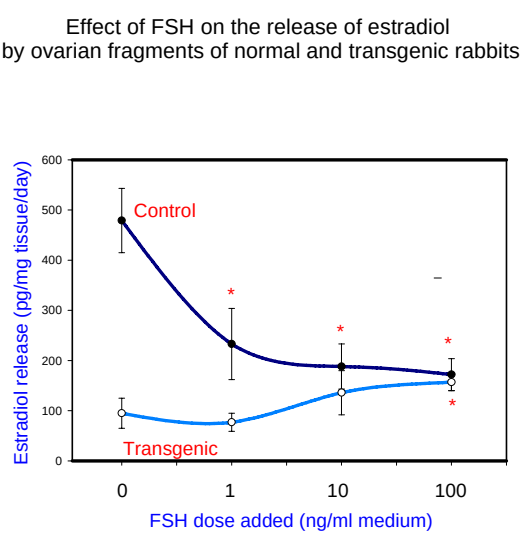


Fig. 2. Effects of FSH on the secretory activity of ovarian cells isolated from transgenic and non-transgenic rabbits

A.



B.



C.

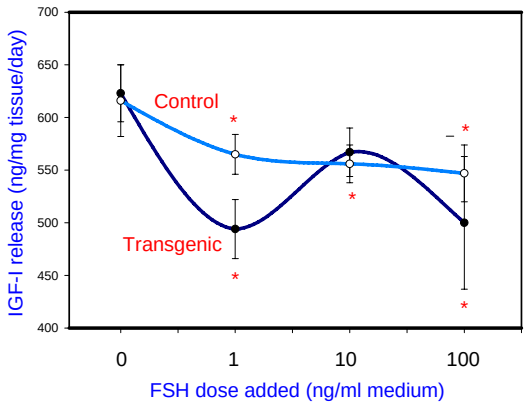
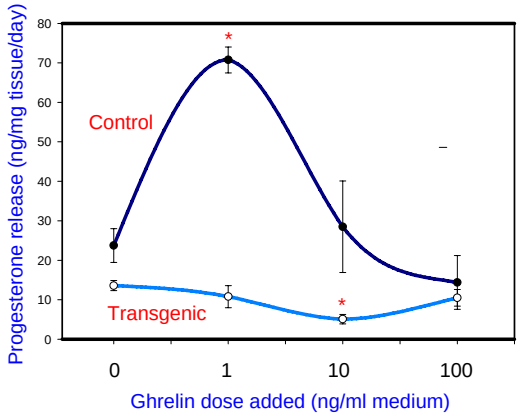


Fig. 3. Effects of ghrelin on the secretory activity of ovarian cells isolated from transgenic and non-transgenic rabbits

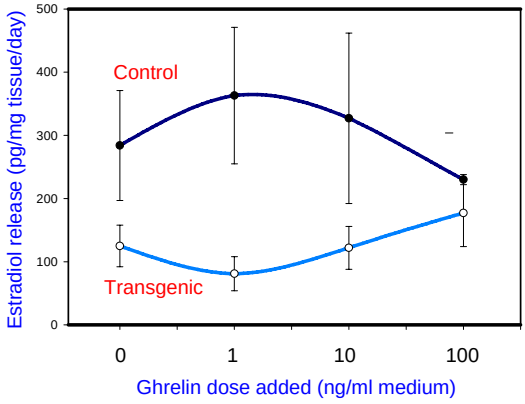
A.

Effect of ghrelin on the release of progesterone
by ovarian fragments of normal and transgenic rabbits



B.

Effect of ghrelin on the release of estradiol
by ovarian fragments of normal and transgenic rabbits



C.

Effect of ghrelin on the release of IGF-I
by ovarian fragments of normal and transgenic rabbits