Effects of Endogenous Rat Growth Hormone Gene Expression Suppressed by an Antisense RNA Transgene on Reproductive Functions in Transgenic Rats

Kazuya Matsumoto[1, 2], Naomi Nakagata[3], Susumu Ueda[4], Yutaka Toyoda[5], Eimei Sato[6], Kazuhiro Saeki[1, 2], Yoshihiko Hosoi[1, 2], and Akira Iritani[1, 2]

ABSTRACT

In homozygous transgenic rats harboring an antisense RNA transgene targeted to the rat growth hormone (GH) gene, the suppression of rat GH gene expression lowers the plasma GH concentration and retards growth. We determined the effects of suppressed endogenous rat GH gene expression on the reproductive ability of homozygous transgenic rats. Compared with nontransgenic females, the number of young at the first parturition from homozygous transgenic females significantly decreased lower ($p < 0.01$) despite the genetic background of mated males. We assessed ovarian physiology by means of sequential studies of ovulation efficiency, follicular growth and the fertilizing ability of oocytes in homozygous transgenic females. Natural ovulation efficiency was significantly reduced by 48% ($p < 0.01$) in homozygous transgenic females compared with those of nontransgenic females. The ovaries of homozygous transgenic rats responded to exogenous gonadotropin in a similar manner to those of nontransgenic rats. The number of eggs in homozygous transgenic females, injected with 7.5 and 15 IU pregnant mare's serum gonadotropin (PMSG) followed 55 hr later with 15 IU human chronic gonadotropin (hCG) and in controls, did not significantly differ. In addition, there was no difference between the fertilizing ability of oocytes from homozygous transgenic and nontransgenic female rats. These results indicated that rat ovarian physiology, especially follicular development and/or ovulation, is mediated by GH. We also demonstrated that GH action on the ovarian function might not be directly related to those of gonadotropins. Therefore, the dwarf transgenic rat is a useful animal model with which to study the biological relevance of GH in ovarian physiology.

INTRODUCTION

The potential involvement of GH in regulating reproductive functions has been demonstrated in rabbits (1) , pigs (2-4), bovine (5-7), and humans (8-10). Much information is available about the biological influence of GH on the rat ovary, because the rat is a popular model for endocrinological analyses. In vitro studies have located GH receptor mRNA in the rat ovary (11). In rat granulosa cells, GH induces progesterone and plasminogen activator synthesis (12). Furthermore, GH accelerates the in vitro maturation of follicle- and cumulus-enclosed rat oocytes obtained from PMSG-treated rats (13). Ovarian IGF-I and IGF-I receptors are distributed in rat granulosa cells (14). These finding indicate the involvement of GH on ovarian physiology, although it remains to be clarified whether the effects are di rect and/or mediated by IGF-I generated by GH exposure (15).

To date, several groups have described the in vivo influence of GH on reproductive functions by manipulating the GH level. Supranormal levels of plasma GH generated by injection of exogenous rat GH have little consequence on rat sexual maturation (16). Ovarian morphology has been studies in transgenic mice overexpressing bovine or human GH (17-19). Overexpressed GH suppressed fertility in females at degrees ranging from mild to severe (17, 19). However, these approaches could not entirely mimic in vivo GH influence

1. Department of Genetic Engineering, Kinki University, Wakayama 649-6493, Japan
2. Institute of Biology-Oriented Science and Technology, Kinki University, Wakayama 642-0017, Japan
3. Center for Animal Resources & Development, Kumamoto University, Kumamoto 862-0976, Japan
4. Nippon Institute of Biological Science, Tokyo 198-0024, Japan.
5. The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan.
6. Laboratory of Animal Reproduction, Graduate School of Agriculture Science, Tohoku University, Sendai 981-8555, Japan

on ovarian physiology, because human GH in mice has lactogenic activity (18) and luteal failure in transgenic mice expressing bovine GH is secondary to insufficient luteal progesterone secretion (20, 21). In contrast, substantial evidence also indicates that the reproductive function can be influenced by a low level of GH (22-24). Gruaz et al. (25) have found that a GH deficiency induced by passive immunization against rat GH-releasing factor (GRF) does not significantly delay sexual maturation. They demonstrated that normal GH and/or IGF-I secretion was not required for sexual maturation. However, ovarian functions were not mentioned in that study.

We demonstrated the inhibition of ~35-40% in endogenous rat GH mRNA levels in homozygous transgenic rats for an antisense RNA transgene targeted to the rat GH gene. Their growth rates and plasma GH concentrations were reduced by ~51-70% and ~59-71% compared with those of nontransgenic littermates, respectively (26, 27). Histochemical and hematological analyses have not revealed any remarkable pathological alterations besides dwarfism in these homozygous transgenic rats. No histological abnormalities were evident in any tissues involving pituitary, ovary, and testis (unpublished observations). However, the littersize in a homozygous transgenic rat line was reduced, although transgenic males and females were fertile. In this study, we found that life-long suppression of endogenous GH suppressed reproductive ability in the female rat. We therefore investigated the biological role of GH on ovarian function, considering the effects of normal and excessive levels.

MATERIALS AND METHODS

Animals

In this study, we selected the offspring of the transgenic rat line, TRE4 910329-32 carrying about 10-20 copies of transgene, from two lines of dwarf homozygous transgenic rats (26). All transgenic animals were from brother-sister mating from each transgenic generation and maintained in light-controlled (light from 3 : 00 to 17 : 00 hr) and air-conditioned rooms. To determine whether a rat GH reduction affects the reproductive ability of transgenic males or females, homozygous transgenic and nontransgenic female rats were mated with homozygous transgenic and nontransgenic males. The number of offspring in each crossing was recorded at first parturition.

Analysis of DNA

Genomic DNA was isolated from rat tail tissues and genotyped by Southern blotting as described (26, 27). Essentially, to identify homozygous transgenic rats, integrated transgene copy numbers were determined by analyzing Southern blots with a Bio Image (Millipore Co., Ann Arbor, MI). Homozygosity was confirmed by outcrossing with nontransgenic rats (Wistar).

Analysis of Ovulation Efficiency

Mature transgenic and nontransgenic females at 7 weeks of age with a 4-day estrus cycle, were sacrificed at estrus. The number of naturally ovulated oocytes enclosed inside intact cumulus cells, in the ampulla of the oviduct was counted. Immature homozygous transgenic and nontransgenic female rats at 4-5 weeks of age were injected with 7.5, 15, and 30 IU PMSG to stimulate follicles, followed 55 hr later with 15 IU hCG to induce superovulation. Females were sacrificed 16 hr after hCG injection, and the number of cumulus-enclosed oocytes in the oviduct was recorded

In Vitro Fertilization

The procedures for in vitro fertilization were essentially as reported by Toyoda et al. (28). Spermatozoa were collected from the cauda epididymides of mature Wistar male rats purchased from Clea Japan, Inc. (Tokyo, Japan) and incubated in 0.4-ml drops of HTF medium (29) under liquid paraffin (Squibb, E.R. Squibb & Sons, Inc., Princeton, NJ). The sperm suspension (5000-6000 sperm/μl) was incubated for 1 hr to allow for capacitation at 37℃ under 5% CO_2 in air. Sequentially, 20 μl of this sperm suspension was added to 0.4-ml drops of equilibrated HTF medium and incubated for 4 hr to allow for capacitation under the same conditions. Oocytes were collected from the excised oviducts of homozygous transgenic and nontransgenic female rats, that were superovulated as described above. Ten

to twelve cumulus masses were recovered in the HTF medium containing sperm incubated 16 hr after the injection of hCG. Morphological normal fertilized oocytes were collected from the sperm suspension 5 hr after insemination. After washing three times in HTF medium, the fertilized eggs were incubated in the same medium at 37℃ under 5 % CO_2 in air. Finally, the number of fertilized oocytes was recorded 12 hr after insemination.

Statistical Analysis

Experiments were independently performed 2 - 3 times. The numbers of ovulated and in vitro fertilized oocytes are presented as the means + SD for n animals. The differences between each group were compared by Student's t-test, and the differences with $p < 0.05$ were estimated to be significant.

RESULTS

The littersize in homozygous rats of the transgenic TRE4 910329-32 line was reduced as shown in Fig. 1. However, no histological and pathological abnormalities were detectable in any tissues of the transgenic rats and the relative weights of the ovary and testis to the body weight were similar to those of the control (data not shown). We therefore investigated whether GH suppression affects the reproductive ability of homozygous transgenic male or female rats. The numbers of pups at first parturition of homozygous transgenic female rats were reduced by about 66% compared with those of nontransgenic females, whether they were mated with homozygous transgenic or nontransgenic males ($p < 0.01$) (Fig. 2).

We investigated the possible influence of a GH reduction on ovarian physiology of the rat, including follicular growth, ovulation, and fertilizing ability of ovulated oocytes. The number of naturally ovulated oocytes was 48% lower ($p < 0.01$) in homozygous transgenic, than in nontransgenic females as shown in Fig. 3. However, the ovarian response to exogenous gonadotropin in homozygous transgenic and nontransgenic rats was the same (Table 1). As shown in Fig. 4, the number of eggs in homozygous transgenic females superovulated with 7.5 and 15 IU PMSG followed 55 hr later with 15 IU hCG, was not significantly different from that of the control. However, in 30 IU of PMSG, many follicular cysts were found in all ovaries from the group given (data not shown). There was no difference in fertilizing ability of oocytes from homozygous transgenic and nontransgenic females, although that of oocytes from homozygous transgenic rats given 30 IU PMSG was much lower than that from nontransgenic animals (Fig. 5).

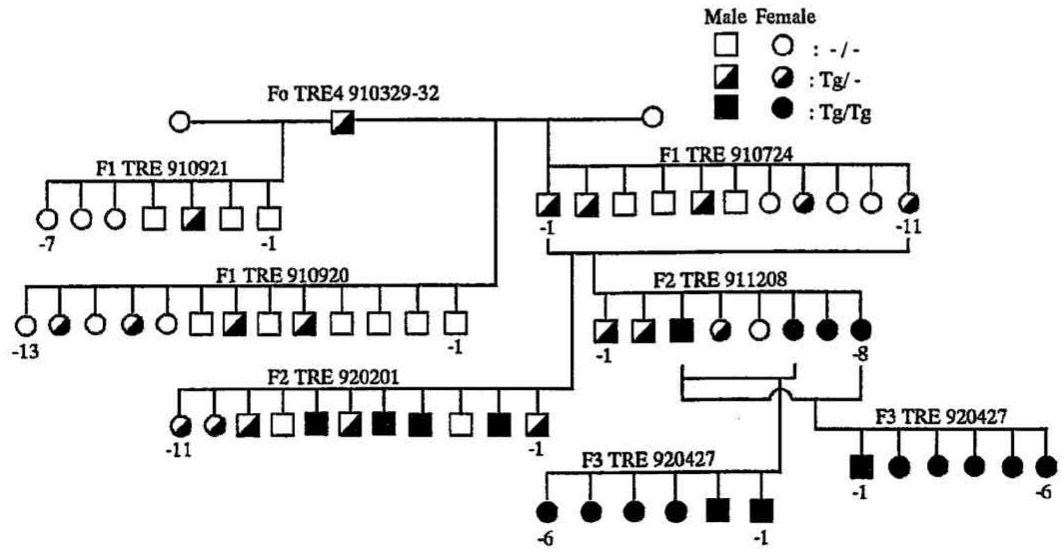

Fig. 1. Pedigree of the transgenic rat line designated Fo TRE4 910329-32. Nontransgenic, heterozygous, and homozygous transgenic rats are shown as -/-, Tg/-, Tg/Tg, respectively. Squares indicate males and circles indicate females.

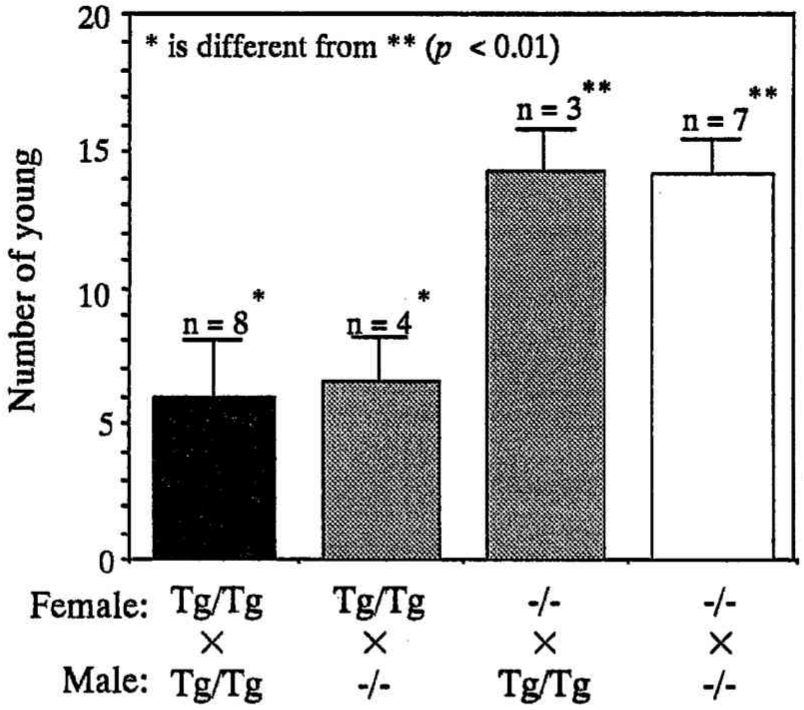

Fig. 2. Comparison of number of young at first parturition of homozygous transgenic (Tg/Tg) and nontransgenic (-/-) female rats reciprocally mated with homozygous transgenic and nontransgenic males from the TRE4 910329-32 line. The numbers of young are presented as the means + SD.

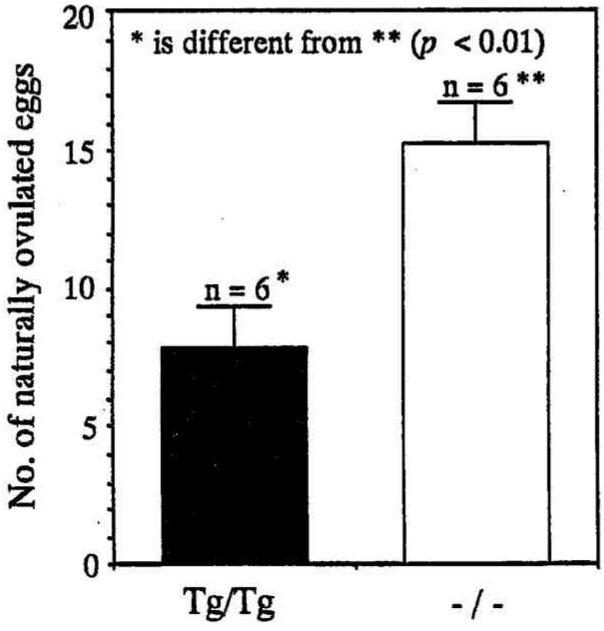

Fig. 3. Comparison of number of naturally ovulated oocytes in homozygous transgenic (Tg/Tg) and nontransgenic (-/-) female rats. Each result is the mean + SD.

Table 1 Response of homozygous transgenic (Tg/Tg) and nontransgenic (-/-) femalerats to induce superovulation with exgenous gonadotrophins

Rats	Dose of PMSG (IU)	Dose of hCG (IU)	No. of rats	
			Used	Ovulated (%)
Tg/Tg	7.5	15	10	8 (80)
	15	15	10	10 (100)
	30	15	10	6 (60)
-/-	7.5	15	5	2 (40)
	15	15	5	5 (100)
	30	15	5	3 (60)

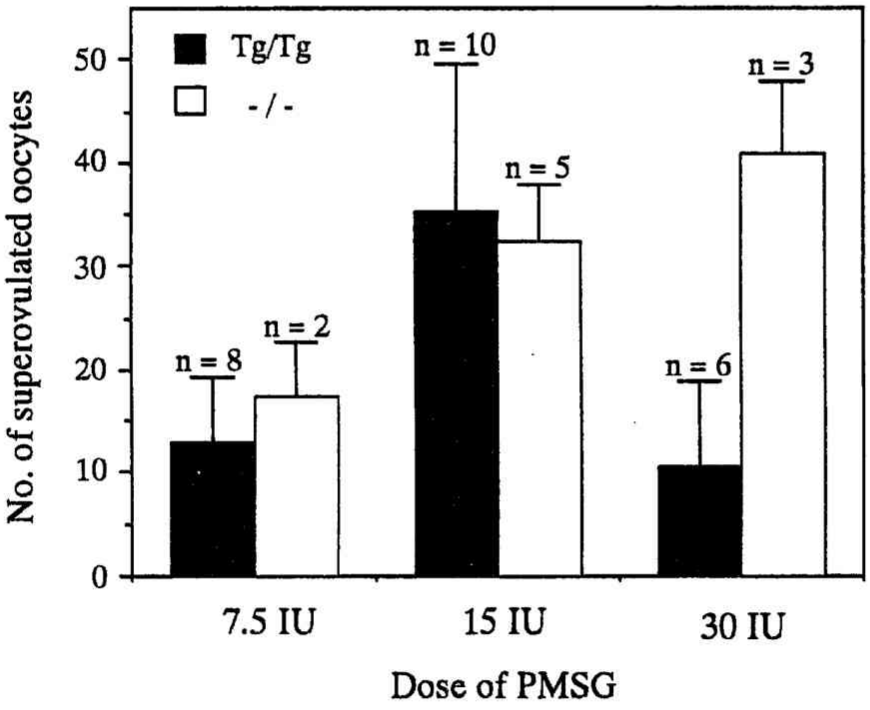

Fig. 4. Comparison of number of superovulated oocytes in homozygous transgenic (Tg/Tg) and nontransgenic (-/-) female rats injected with 7.5, 15, and 30 IU PMSG and 55 hr later with 15 IU hCG. Each result is the mean + SD of three individual experiments.

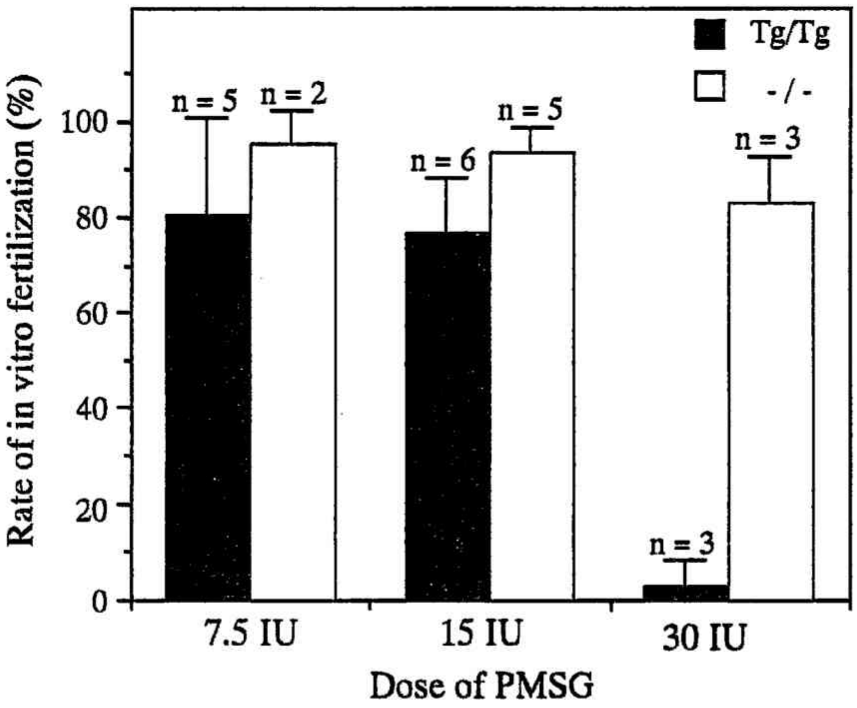

Fig. 5. In vitro fertilization of oocytes from homozygous transgenic (Tg/Tg) and nontransgenic (-/-) female rats superovulated by injections of 5, 15, and 30 IU PMSG, followed 55 hr later with 15 IU hCG. The fertilization rate in each group is plotted with the vertical bar representing SD.

DISCUSSION

This study confirmed that low circulating rat GH levels resulting from rat GH gene expression suppressed by the antisense RNA transgene were related to mild reproductive deficits in females. The smaller litter from these homozygous transgenic females was due to a decrease in the number of naturally ovulated eggs. These in vivo findings provided direct evidence that GH plays a role in several ovarian functions, especially follicular growth and /or ovulation. This notion is consistent with the results of a study in which GH deprivation was achieved by administering anti-GRF serum; that the number of pups was significantly reduced in these rats (25). The biological effects of GH on body weight are dose-dependent in vivo (26, 27). Additionally, the rate of suppression in the natural ovulation efficiency of homozygous transgenic females shown here was compatible with the repression rate of the plasma GH concentration as compared with that in nontransgenic rats (27). Thus, these results indicate that GH stimulates ovarian physiology in a dose-dependent manner.

The direct involvement of plasminogen activator in the ovulation process has been demonstrated (30). That study using double-targeted mice lacking of both functional tissue-type plasminogen activators (PA) and urokinase-type PA, suggested that PAs together with other proteases produce the proteolytic activity required for degradation of the follicular wall during ovulation. GH induces tissue-type PA synthesis without any other intermediary protein in rat granulosa cells (12). Indeed, Northern blotting showed that GH receptor gene is expressed in the rat ovary, suggesting that GH directly affects that issue (11). Yoshimura et al. (31) have shown in the in vitro perfused rabbit ovary that GH is not directly essential for the ovulatory process, whereas GH enhances gonadotropin-induced ovulation. Therefore, the suppressed ovulation shown here is likely to be secondary to a decrease in plasminogen activator in granulosa cells induced by a low GH plasma concentration.

IGF-I is essential for several ovarian functions including steroidogenesis (32), follicular growth (33), and oocyte maturation (34). High levels of the IGF-I and IGF-I receptor genes

are expressed in the granulosa cells of actively growing rat follicles (14). A study of the in vitro perfused rabbit ovary has demonstrated that GH stimulates the ovarian production of IGF-I, which in turn mediates the induction of follicular growth and oocyte maturation (31). Therefore, GH may indirectly exert its effects on ovarian physiology via intra-ovarian IGF-I. This notion was confirmed by the in vitro finding that the GH effect on meiotic maturation in cumulus-enclosed oocytes can be inhibited by antibodies against IGF-I (35), suggesting that GH requires the intermediation of locally produced granulosa cell-derived IGF-I to fulfill its role on oocyte maturation. Furthermore, the data from this study of homozygous transgenic rats with low circulating levels of GH, support the view that GH is critical for follicular growth and oocyte maturation, although the IGF-I level remains to be determined in the ovary of homozygous transgenic females.

This study showed that there was no difference in ovulation efficiency between transgenic and nontransgenic females treated with 7.5 or 15 IU PMSG, indicating that a low GH level did not affect the superovulation induced with exogenous gonadotropins. These results suggest that there is no functional comprehensive mechanism among gonadotropins and GH on the ovarian function. However, GH synergistically enhances the effects of gonadotropins in rabbit follicular growth and ovulation (31). This discrepancy in the synergistic action of gonadotropins and GH remains to be explained, but it might be related to differences between hCG (31) and the PMSG used in this study. Therefore, GH may not only enhance the gonadotropin effects, but also have an independent influence upon ovarian physiology.

In conclusion, we demonstrated that the mild reproductive deficits in homozygous transgenic female rats characterized by repressed GH gene expression and a consequently low plasma GH level, are directly caused by a reduction in ovulation efficiency. We demonstrated that the in vivo stimulatory role of GH on follicular development and/or ovulation is dose-dependent. Furthermore, there is no functional comprehensive relationship between GH and gonadotropic actions upon ovarian function. However, it remains to be determined whether these actions of GH are direct or indirect via locally generated factors including IGF-I and PA. Therefore, the dwarf transgenic rat with a life-long depletion of endogenous GH seems to be a unique animal model from which to obtain information about the in vivo biological influences of GH upon ovarian function.

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要　約

成長ホルモン・アンチセンスDNA導入トランスジェニックラットにおける繁殖性の検討

松本和也、中潟直己、上田　進、豊田　裕、佐藤英明、佐伯和弘、細井美彦、入谷　明

成長ホルモン（GH）を標的にしてアンチセンスDNAをラットに導入したトランスジェニックラットでは、導入遺伝子由来のアンチセンスRNAにより内在性ラット成長ホルモン遺伝子の発現が特異的に抑制されていることが明らかになっている。そのため、このトランスジェニックラットでは、正常ラットに比較して血漿成長ホルモン量が30～40%減少し、体重も30～50%減少していた。また、妊性はあるものの、ホモ接合体のトランスジェニックラット同士の交配では、産子数が正常ラットに比較して低下していることが観察された。

本研究では、この原因を探るためにトランスジェニックラットの繁殖性について検討を加えた。正常ラットとトランスジェニックラットの相互交配によって、トランスジェニック雌ラットにその要因があることが認められた。さらに排卵卵子数、性腺刺激ホルモンに対する感受性、排卵卵子の正常性を検討したところ、産子数の低下は排卵卵子数に起因すること、また性腺刺激ホルモンに対する卵巣の感受性が低いことが明らかになった。このことは、成長ホルモンが卵巣機能とくに卵胞発育に対して何らかの関与をしていることを示しており、今後成長ホルモンが卵巣機能に及ぼす影響を調べる上で、このトランスジェニックラットは有効であることが示唆された。