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Morphological restoration of gonadotrope population by thymulin gene therapy in nude mice

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Summary. The integrity of the thymus during the first week of life is necessary for a proper maturation of the pituitary-gonadal axis as revealed by the significantly reduced levels of circulating gonadotropins in congenitally athymic (nude) mice. In the present work we studied the impact of athymia and the effect of neonatal thymulin gene therapy on the pituitaries of adult nude mice. Also circulating thymulin and gonadotropin levels were evaluated. We used an adenoviral vector expressing a synthetic gene for the thymic peptide thymulin (metFTS) termed RAd-FTS. On postnatal day 1, each experimental heterozygous (nu/+) and homozygous (nu/nu) pup of both sexes received a single bilateral i.m. injection of RAd-FTS or RAd-GFP/TK, a control vector expressing green fluorescent protein. On postnatal days 51-52, mice were bled and sacrificed, their pituitaries were immediately dissected, fixed and immunostained. Morphometry was performed by means of an image analysis system. The following parameters were calculated: volume density (VD: cell area/reference area), cell density (CD: number of cells/reference area), and cell size (expressed in μm^2). Serum thymulin levels were measured by a bioassay and gonadotropin levels were assayed by RIA. It was observed that neonatal thymulin gene therapy in the athymic mice restored their serum thymulin levels and prevented the reduction in circulating gonadotropin levels. The histometrical analysis revealed that the treatment prevented the reduction in gonadotrope CD and the VD in athymic mice. Our data suggest that thymulin gene therapy may be an effective strategy to approach reproductive deficits associated with endocrine thymus dysfunction.

Key words: Pituitary-gonadal axis, Thymulin gene therapy, Nude mice, Hupophysiotropic activity, Rad-FTS

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Introduction

The integrity of the thymus during perinatal life is necessary for a proper maturation of the pituitary-gonadal axis as revealed by the endocrine alterations caused by neonatal thymectomy (Michael et al., 1980) or congenital absence of the thymus in mice (Rebar et al., 1980). Congenitally athymic (nude) female and male mice show significantly reduced levels of circulating gonadotropins (Rebar et al., 1981, 1982). In homozygous (nu/nu) females the time of the first ovulation is delayed (Besedovsky and Sorkin, 1974), fertility is reduced (Rebar et al., 1981) and follicular atresia is increased so that premature ovarian failure results (Lintern-Moore and Pantelouris, 1975). Similar abnormalities result from neonatal thymectomy in normal female mice (Michael et al., 1980; Nishizuka and Sakakura, 1971). In homozygous adult nude CD-1 male mice, gonadotropin responses to immobilization stress are reduced as also are serum basal levels of the same hormones as compared to the heterozygous counterparts (Goya et al., 2001). Additionally, athymic male mice display reduced secretion of gonadotropins but these hormonal abnormalities were not associated with reproductive deficiencies (Rebar et al., 1982).

Thymulin, which is exclusively produced by the thymic epithelial cells (TEC), consists of a nonapeptide component (facteur thymique sérique or FTS) coupled in an equimolecular ratio to the ion zinc (Dardenne and Bach, 1975; Dardenne et al., 1982), which confers biological activity to this molecule (Dardenne et al.,

1974; Bach, 1983).

It has been recently hypothesized that thymulin plays a relevant role in the hypothalamo-pituitary ovarian axis in rodents (García et al., 2000, 2005; Hinojosa et al., 2004).

There is documented evidence that thymulin possesses gonadotropin-releasing activity. Thus, thymulin has been shown to stimulate luteinizing hormone (LH) release from perifused rat pituitaries (Zaidi et al., 1988). Thymulin stimulated gonadotropin release from dispersed rat pituitary cells in a dose-related manner, an effect that declined with the age of the pituitary cell donors (Brown et al., 2000). We reported that thymulin immunoneutralization during early life in normal mice induces a significant decrease in cell density (CD) of somatotrope, lactotrope and corticotrope populations in peripubertal mice (Camihort et al., 2006). This and other studies suggest that thymulin may play an important role in thymus-pituitary communication, particularly during early life.

Recently, a synthetic DNA sequence coding for thymulin has been constructed and used to develop a recombinant adenoviral vector termed RAd-FTS. When intramuscularly (i.m.) administered to adult thymectomized (Tx) mice and rats (whose circulating levels of thymulin are nondetectable), RAd-FTS induced sustained supraphysiological serum levels of biologically active thymulin which remained high for at least 112 days in mice and for over 320 days in rats (Reggiani et al., 2006; Morel et al., 2008).

In the present study, we describe the effects of neonatal thymulin gene therapy on the morphology and function of pituitary gonadotropes in nude mice.

Materials and methods

Animals and experimental procedures

Gene therapy experiments

The offspring of NIH homozygous (nu/nu) nude male and heterozygous (nu/+) female mice were used. The parent mice were purchased from the Animal Core Facility of the National University of La Plata, Argentina. All mice were maintained on a γ-irradiated chow diet and sterilized water. Animals had free access to food and water and were kept at 22°C with a light/dark cycle of 12/12 h. All experiments on nude animals were done following the Animal Welfare Guidelines of the NIH.

On postnatal day 1 or 2, each experimental heterozygous (nu/+) and homozygous (nu/nu) pup of both sexes received a single bilateral i.m. (hindlegs) injection of 10^8 plaque forming units (pfu) with RAd-FTS or RAd-GFP/TK used as a control vector, in 10 μl vehicle (5 μl per side). On postnatal days 51-52, mice were bled and immediately sacrificed by cervical dislocation, their pituitaries were immediately dissected, fixed and immunostained. The experimental groups analyzed were: nu/nu mice injected with RAd-GFP/TK or RAd-FTS and nu/+ mice injected with RAd-GFP/TK, both females and males. Thus six groups of mice were studied.

Adenoviral vectors used

RAd-FTS

A DNA sequence coding for the biologically active thymulin analog, termed methionine-FTS or metFTS, was constructed. A recombinant adenoviral (RAd) vector harboring the synthetic gene for metFTS was constructed by a variant of the two plasmid method (Hitt et al., 1998) employing the AdMax® plasmid kit (Microbix, Canada). This procedure has been described in detail elsewhere (Reggiani et al., 2006).

Briefly, this kit uses a shuttle plasmid (pDC515) and the genomic plasmid pBHGfrt(del)E1,3 FLP. The thymulin synthetic gene (metFTS) was inserted into the shuttle and both plasmids were cotransfected into HEK293 cells. In cotransfected HEK293 cells enzyme-directed recombination generated the genome of the desired recombinant adenoviral vector, RAd-FTS. The newly generated RAd was rescued from HEK293 cell lysates and plaque purified. It was further purified by ultracentrifugation in CsCl gradient and titulated by a serial dilution plaque assay.

RAd-(GFP/TK)$_{fus}$

An adenoviral vector termed RAd-(GFP/TK)*fus*, was constructed in our laboratory following the above general procedures and was used as a control vector in the gene therapy studies. The vector harbors a hybrid gene encoding the *Aequorea victoria* enhanced green fluorescent protein fused to herpes simplex virus type 1 thymidine kinase (GFP/TK)*fus* (a kind gift from Dr. Jacques Galipeau, McGill University, Montreal, Canada). The vector was expanded in HEK293 cells and purified and titrated as indicated above.

Thymulin Bioassay

Biologically active thymulin was measured in serum by a rosette bioassay described in detail elsewhere (Dardenne and Bach, 1975). This method is based on the ability of thymulin to restore the inhibitory effect of azathioprine (Az) on rosette formation in spleen cells from thymectomized mice. The inhibitory activity of samples was compared with that of a standard curve using synthetic thymulin. Serum values were expressed as fg/ml bioactive thymulin.

Pituitary hormone assays

Serum levels of luteinizing hormone (LH) and

follicle-stimulating hormone (FSH) were measured by radioimmunoassay using the mouse materials provided by Dr. A. F. Parlow, Pituitary Hormones and Antisera Center, UCLA Med. Center, U.S.A. Serum concentrations (ng/ml) of LH and FSH were expressed in terms of mouse LH RP-2 and rat FSH RP-2, respectively.

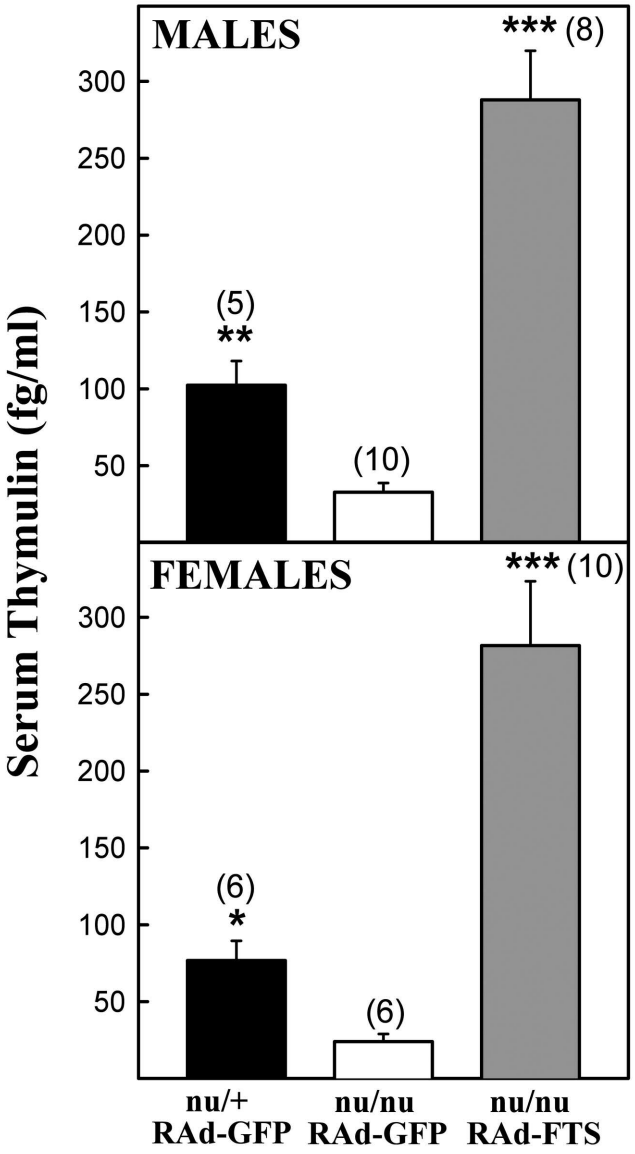

Fig. 1. Effect of neonatal thymulin gene therapy on serum levels of thymulin in female and male nude mice. Vectors were injected i.m. on the day of birth or on the day after. On postnatal day 51-52 the animals were bled and thymulin assayed. Serum thymulin values (fg/ml) are expressed as mean ± SEM; n values are shown in parentheses. Asterisks indicate the level of significance of differences respect to homozygous nude injected with RAd-GFP/TK. *: P<0.05; **: P<0.01; ***: P<0.001.

Immunohistochemistry

Stated in brief, pituitary tissues from 5 animals of each group were fixed in Bouin's fluid and embedded in paraffin. Serial sections of 4 μm were obtained at different levels of the blocks following a ventral-to-dorsal sequence. The sections were immunostained, and then incubated for 1 h at room temperature with the primary antibody, anti-FSH and anti-LH (Dako, CA, USA), diluted 1:100. Thoroughly washed sections were then treated for 30 min with a ready-to-use EnVision reaction system (Dako, CA, USA). The peroxide-sensitive chromogen was diaminobenzidine (Cónsole et al., 2001). In all instances, the specificity of the primary antiserum was monitored either by observing its ability to block the immunocytochemical reaction after its preabsorption with an excess of the related antigen or by its replacement with normal rabbit serum or phosphate-buffered saline.

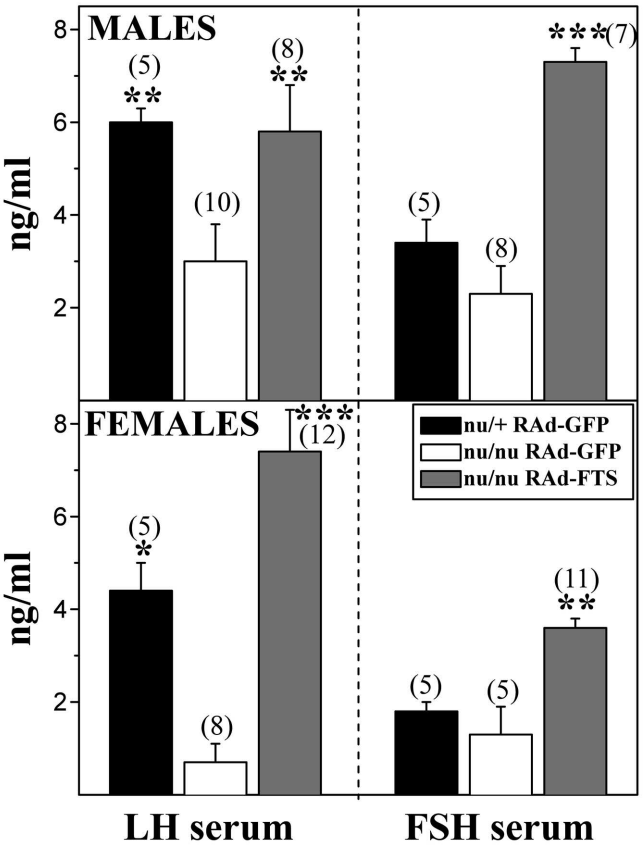

Fig. 2. Effect of neonatal thymulin gene therapy on serum levels of gonadotropins in female and male nude mice at 51-52 days.- Asterisks indicate the level of significance of differences respect to homozygous nude injected with RAd-GFP/TK. *: P<0.05; **: P<0.01; ***: P<0.001; n values are shown in parentheses.

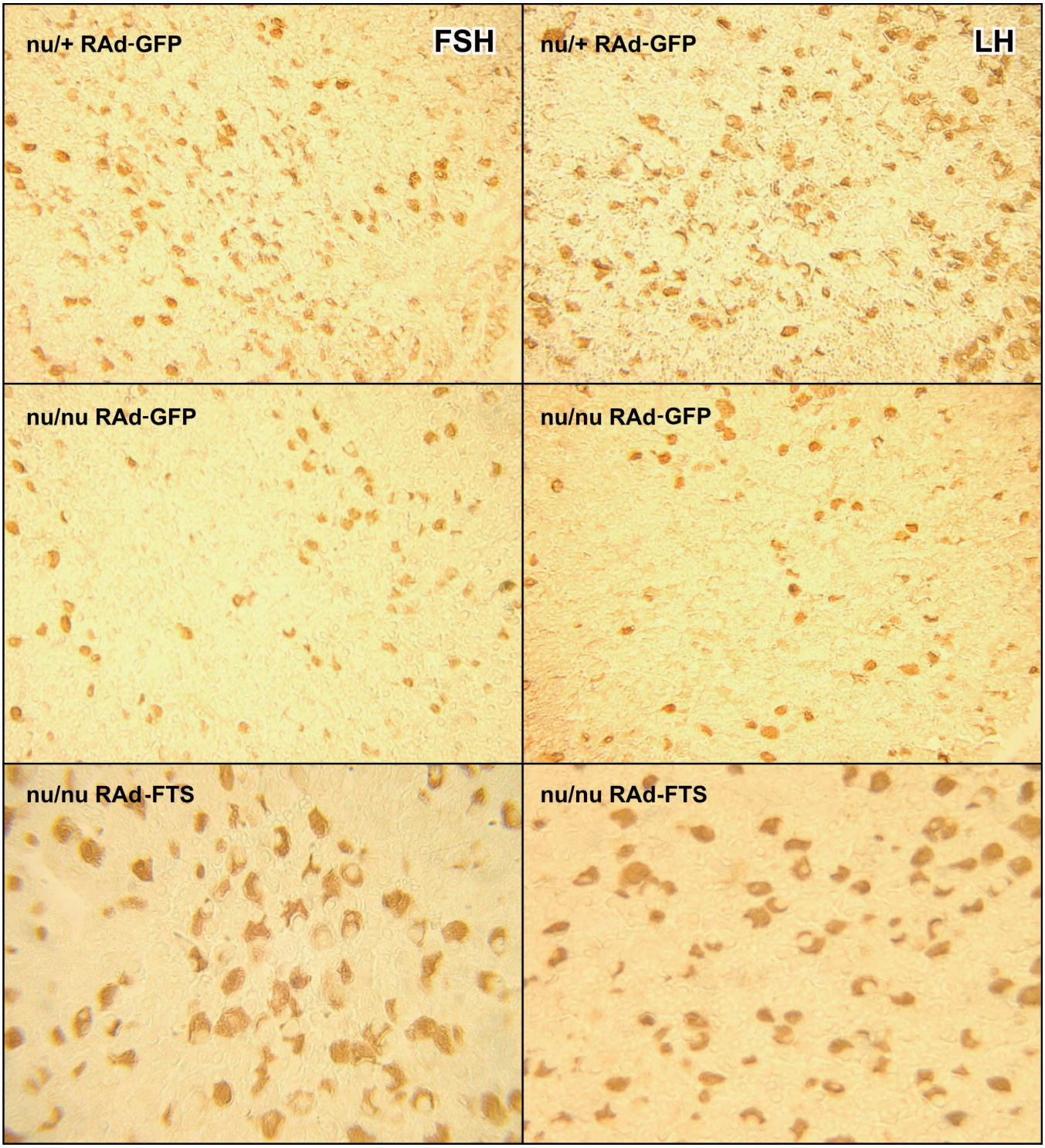

Fig. 3. Representative fields of specifically immunostained gonadotrope cells in the three experimental groups: nu/+ RAd-GFP/TK, nu/nu RAd-GFP/TK and nu/nu RAd-FTS. EnVision system peroxidase. Bar: 25 μm.

Morphometry

Morphometry was performed as reported in detail previously (Cónsole et al., 2002). Measurements of immunostained pituitary cells were made by means of an image-analysis system (Imaging Technology, Optimas 5.2). The cells and reference area (RA) were analyzed in each field on an average of ten micrographs taken from two levels (e.g. a and b). These measurements were recorded and processed automatically and the following parameters subsequently calculated: volume density (VD= Σ cell area/RA), cell density (CD = number of cells/RA), and cell size (CS, expressed in μm^2). RA represents the total area throughout which the cells were scored. Thus, this area divided into the sum (Σ) of the individual cell areas (A) yielded VD, a parameter that represents an estimate of cell mass according to generally accepted criteria. The number of cells (CD) was calculated by dividing the immunostained area of the pituitary tissue by the mean individual cell area. For this parameter, 100 cells were recorded in each field.

Statistical analysis

Data are expressed as mean ± SEM, unless otherwise indicated. Statistical comparisons among experimental groups were performed by the Student's *t*-test or by ANOVA followed by the Tukey test when the ANOVA was significant.

Results

Hormone assays

At 51-52 days of age, control nu/nu mice showed low levels of serum thymulin as compared to control nu/+ counterparts. A single neonatal i.m. injection of RAd-FTS markedly increased the circulating levels of biologically active thymulin in athymic female and male nude mice (Fig. 1).

Serum LH and FSH levels decreased in athymic female and male mice as compared to nu/+ control counterparts. Neonatal thymulin gene therapy increased gonadotropin levels in nu/nu mice of both sexes tested at 51-52 days of age (Fig. 2).

Immunohistochemical and histometric studies

Immunostained FSH- and LH-cells stood out in sharp relief, exhibiting an ochre definite granular cytoplasmic pattern, with some vacuolisation. The immunolabeled gonadotrope cells corresponding to representative fields of the histometry performed in the three experimental groups are shown in Fig. 3.

The analysis revealed changes induced by the injection of RAd-FTS on the gonadotrope population of the *pars distalis*. The FSH and LH cell population showed a significant ($p<0.01$) decrease in cell density (CD) and volume density (VD) in athymic nude mice of both sexes as compared to heterozygous counterparts. Neonatal thymulin gene therapy significantly increased the CD, cell size (CS) and volume density (VD) of the gonadotrope population in nu/nu mice of both sexes tested (Fig. 4).

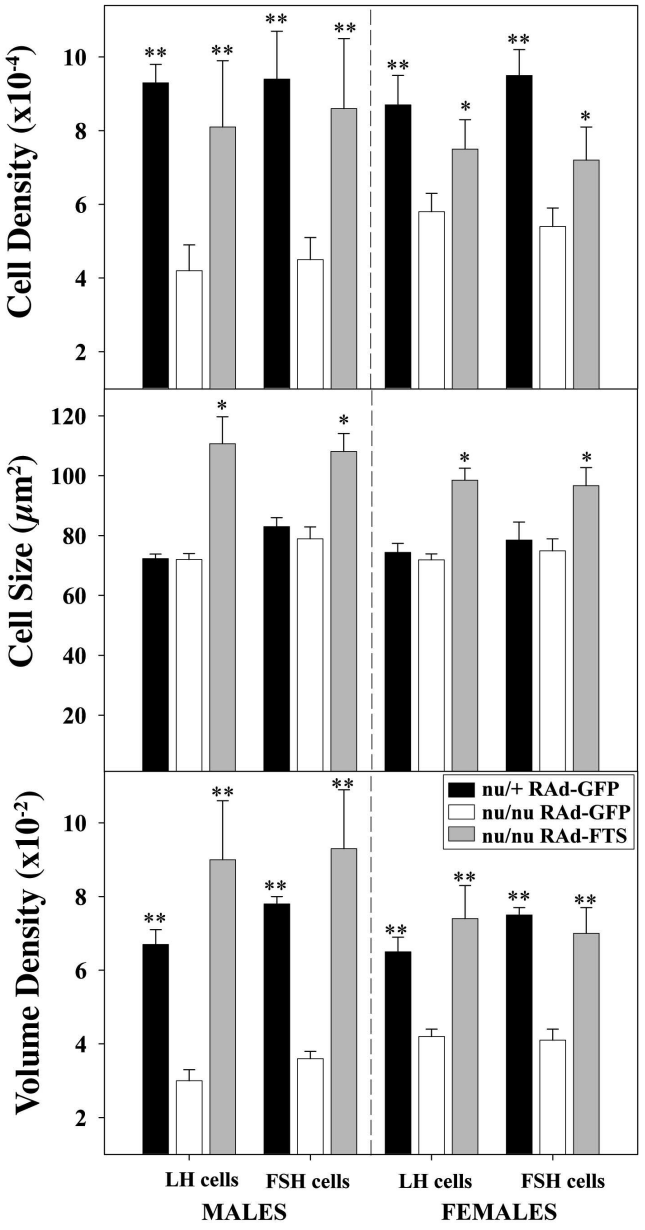

Fig. 4. Effect of neonatal thymulin gene therapy on histometry of LH and FSH cells in female and males nude mice.- Cell density, cell size and volume density of LH and FSH cell population in female and male pituitaries. Asterisks indicate the level of significance of differences respect to homozygous nude injected with RAd-GFP/TK. *: $P<0.05$; **: $P<0.01$; n=5 for all group.

Discussion

The most critical endocrine function of the thymus seems to be exerted in a very circumscribed period between the last days of pregnancy and the very first days of postnatal life in mice and rats (Pierpaoli and Besedovsky, 1975). Neonatal thymus grafting has been reported to prevent some of the endocrine alterations observed in neonatally thymectomized and athymic nude mice (Pierpaoli and Besedovsky, 1975; Pierpaoli et al., 1976). We have recently reported that neonatal serum thymulin imunoneutralization in normal mice depresses circulating levels of gonadotropins and increases gonadotrope cell size (Camihort et al., 2006). In the present study, we wanted to evaluate whether the thymic hormone thymulin is a major mediator of the effects of the thymus gland over the gonadotropic axis.

Considering the multi-hormone control exerted by the pituitary gland on thymulin secretion, it seems logical to expect that thymulin may in turn exert a specific effect on anterior pituitary cell populations. Since thymulin possesses hypophysiotropic activity *in vitro* (Zaidi et al., 1988; Brown et al., 1999, 2000; Goya et al., 2004) it could act directly on the adenohypophysis *in vivo* modulating the response of the gland to hypothalamic or other secretagogues or inhibitors. Alternatively, thymulin could also influence gonadotropin production by stimulating the release of cytokines or other neuroactive molecules from thymulin-responsive immune cells.

We have previously demonstrated that in thymectomized (Tx) rodents that RAd-FTS can induce supraphysiologic levels of serum thymulin for long periods (Reggiani et al., 2006; Morel et al., 2008). The present results are in line with the above reports.

Our results indicate that a single neonatal i.m. injection of RAd-FTS, but not RAd-GFP/TK (a control vector), increased the circulating levels of biologically active thymulin in homozygous nude mice of both sexes tested at 51-52 days of age. Athymic mice showed low but detectable levels of circulating thymulin, which is probably produced by a thymus rudiment which has been previously documented in nudes (Groscurth et al., 1975).

The reduced concentrations of gonadotropins seen in the athymic animals seem to be causally related to the absence of the thymus and support the idea that the thymus, directly or indirectly, is necessary for the development of the normal function of the hypothalamic-pituitary-gonadal axis in mice. In line with this, the finding that neonatal thymulin gene therapy prevents the deficit of circulating gonadotropins in adult nudes as well as the morphological abnormalities of gonadotrope cells observed, lends further support to the view that thymulin plays a major role as a mediator of the influence of the thymus in the maturation of neuroendocrine system during early life. Finally, our data suggest that thymulin gene therapy may be an effective strategy to approach reproductive deficits associated with thymus dysfunction.

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