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Role of thymulin on the somatotropic axis *in vivo*

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ABSTRACT

Aims: There is clear evidence for the existence of a bi-directional thymus–somatotropic axis and several studies suggest that the thymic peptide thymulin may be involved in this communication. We undertook to assess the impact of serum thymulin immunoneutralization in C57BL/6 mice and that of neonatal thymulin gene therapy (NTGT) in nude mice on body weight (BW) gain and on the histomorphometric profile of the somatotrope population.
Main methods: Immunoneutralization of thymulin was done from postnatal day 1 to 35 by i.p. injections of rabbit anti-thymulin serum (α-FTS) and normal rabbit serum (NRS) in controls. NTGT was implemented in nudes using an adenoviral vector expressing a synthetic gene for thymulin (RAd-FTS). On postnatal day 1, heterozygous (nu/+) and homozygous (nu/nu) pups received a single bilateral i.m. injection either RAd-FTS or RAd-GFP (a control vector expressing green fluorescent protein). BW gain was recorded and at the end of the study the pituitaries were immunostained for growth hormone (GH). Serum GH and thymulin were determined by radioimmunoassay and bioassay, respectively.
Key findings: Thymulin immunoneutralization induced a significant decrease in BW gain, serum GH and somatotrope cell density as well as an increase in somatotrope cell size. NTGT markedly increased BW gain, serum thymulin ($P<0.01$) and somatotrope cell and volume density in nu/nu mice.
Significance: Our results suggest that thymulin plays a relevant physiological role on the thymus–somatotropic axis in mice.

Introduction

Thymulin is a thymic metallopeptide involved in several aspects of intra- and extrathymic T-cell differentiation (Bach, 1983). Thymulin which is exclusively produced by the thymic epithelial cells (TEC) (Dardenne et al., 1974), consists of a biologically inactive nonapeptide component termed FTS (an acronym for serum thymus factor in French), coupled in an equimolecular ratio to the ion zinc (Gastinel et al., 1984), which confers biological activity to the molecule (Dardenne et al., 1982). Thymulin production and secretion is influenced directly or indirectly by the neuroendocrine system (Savino and Dardenne, 2000). A particularly relevant pituitary hormone is growth hormone (GH) which can influence thymulin synthesis and secretion. *In vitro*, human GH can stimulate thymulin release from TEC lines (Timsit et al., 1992) which are known to possess specific receptors for GH (Ban et al., 1991). Animal studies have shown that treatment of aged dogs with bovine GH partially restored their low thymulin serum levels (Goff et al., 1987). In old mice, treatment with ovine GH increased their low circulating thymulin levels and enhanced the concanavalin A (Con A)-dependent proliferative response of their thymocytes, as well as interleukin-6 production (Goya et al., 1992). In old rats, combined treatment with GH and thyroxine was also able to restore partially their reduced thymulin levels (Goya et al., 1993).

Conversely, there is evidence for a thymus–somatotropic axis. Thus, in homozygous adult nude CD-1 male mice, GH responses to immobilization and cold stress are reduced as also are serum basal levels of this hormone and thyrotropin as compared to the heterozygous counterparts (Goya et al., 1995). Furthermore, thymulin has been found to stimulate GH release in dispersed rat pituitary cells at doses from 10^{-8} to 10^{-3} M (Brown et al., 1998), whereas others have reported that thymulin doses of 10^{-11} M have no effect on GH secretion in incubated rat pituitary fragments (Hadley et al., 1997). Immunoneutralization studies have strengthened the hypothesis that thymulin is a physiologic mediator of the perinatal influence of the thymus on neuroendocrine maturation. Thus, neonatal immunoneutralization of circulating thymulin in otherwise normal C57BL/6 mice induced significant morphologic alterations in the gonadotropic and thyrotrophic

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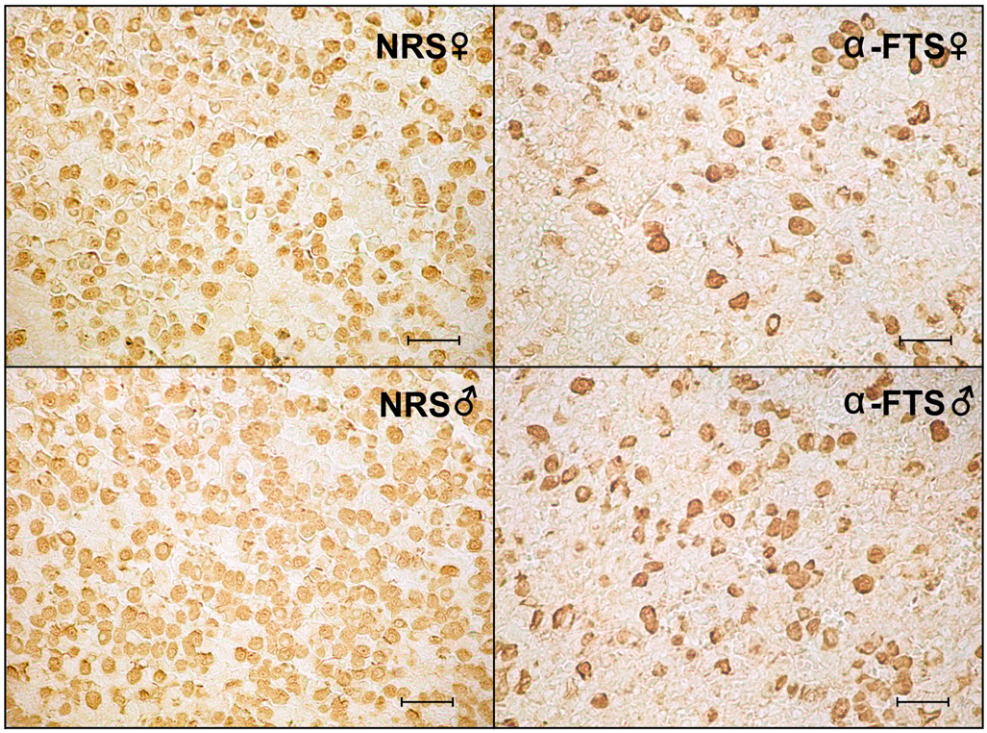

Fig. 1. Effect of thymulin quenching on the somatotrope population in C57BL/6 mice. The α-FTS group showed a decrease in cell density and an increment in cell size compared with the control group (NRS) both in female and male mice. EnVision system peroxidase. Bar: 35 μm.

anterior pituitary endocrine cell populations (Goya et al., 2007; Martínes et al., 2011).

We have constructed a synthetic DNA sequence coding for a biologically active thymulin analog called met-FTS and cloned it in a recombinant adenoviral (RAd) vector termed RAd-FTS. The design of the DNA sequence for met-FTS was optimized for expression in rat systems by choosing for each amino acid of the native peptide the codon more frequently used by rat cells (Reggiani et al., 2006).

In the present study we assessed the impact of immunoneutralization on the somatotrope population during early life of normal mice and the effect of neonatal thymulin gene therapy on the thymus–somatotropic axis in congenitally athymic nude mice.

Materials and methods

Adenoviral vectors used

RAd-FTS

Previously we constructed a DNA sequence 5′-ATGCAAGCCAAATCTC AAGGTGGATCC-AACTAGTAG-3′ that encodes a biologically active thymulin analog, met-FTS (met-QAKSQGGSN), here referred to as synthetic gene for thymulin. A recombinant adenoviral vector, termed RAd-FTS, harboring the synthetic gene for thymulin was constructed, by a variant of the two plasmid method employing the AdMax® plasmid kit (Microbix, Canada) as described elsewhere (Reggiani et al., 2006).

RAd-GFP (control)

An adenoviral vector termed RAd-GFP was constructed in our laboratory following the general procedures outlined above 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 (GFP) fused to herpes simplex virus type 1 thymidine kinase.

Animals and experimental design

Thymulin immunoneutralization experiments

Male and female C57BL/6 mice were used. On the first day of the study, 12 cages were used to place one adult male and female per cage. Beginning on the day of birth, pups received weekly i.p. injections of either normal rabbit serum (NRS group; 30 pups) or anti-thymulin rabbit serum (α-FTS group; 30 pups); the anti-thymulin serum was a kind gift from Dr. Jean-Marie Pléau. The first injection consisted of 20 μl serum per pup, whereas the subsequent injections consisted of doses of 8 μl serum per gram body weight (BW), until the end of the experiment on postnatal day 35. Animals were bled from the retro-orbital plexus and killed by cervical dislocation. The pituitaries were dissected, fixed and processed for histologic and immunohistochemical assessment as appropriate.

All experiments on animals were done following the Animal Welfare Guidelines of NIH (INIBIOLP's Animal Welfare Assurance No A5647-01).

Thymulin gene therapy experiments

The offspring of NIH homozygous (nu/nu) nude (athymic) male and heterozygous (nu/+) female mice were used. The parent mice were purchased from the Ezeiza Atomic Center, Ezeiza, 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. On postnatal day 1, each experimental heterozygous and homozygous pup received a single bilateral i.m. (hindlegs) injection of 10^8 plaque-forming units (pfu) with RAd-FTS or RAd-GFP used as a control vector, in 10-μl vehicle (5 μl per side). On postnatal day 71, mice were bled and immediately sacrificed by cervical dislocation. The pituitary glands were immediately dissected, fixed and processed for histology and immunohistochemistry as appropriate.

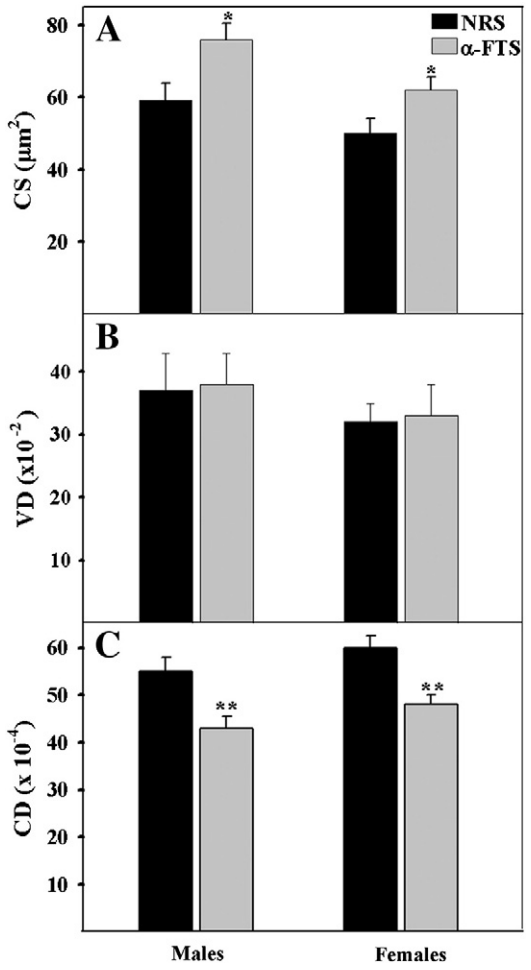

Fig. 2. Impact of thymulin quenching on the somatotrope population morphometry in C57BL/6 mice. The somatotrope population showed a significant decrease in cell density (CD, C) and an increment in cell size (CS, A) in both the male and female α-FTS groups as compared to NRS controls. Five animals in each group were analyzed. Asterisks indicate the level of significance of differences: *: P<0.05; **: P<0.01.

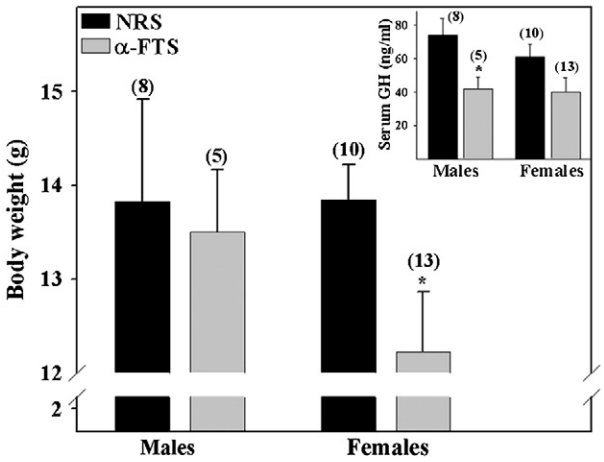

Fig. 3. Effects of thymulin quenching on the body weight and serum GH. Body weight was significantly lower in quenched females but not males compared with their respective controls. Conversely, serum GH was significantly lower in the α-FTS males but not females compared with their corresponding control males and females (inset). The number of mice per group is indicated in parentheses. Asterisks indicate the level of significance of differences: *: P<0.05.

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 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.

Growth hormone determination

Serum GH was determined by radioimmunoassay using the mouse materials provided by Dr. A. F. Parlow, Pituitary Hormones and Antisera Center, UCLA Med. Center, U.S.A. Iodination grade GH was radiolabeled by the Iodo-Gen® method and purified on PD-10 Sephadex® G-25 M columns (Pharmacia, Uppsala, Sweden) equilibrated with 0.01 M phosphosaline, pH 7.6. A 1/10 goat anti-rabbit IgG in 0.9% NaCl was used to separate bound from free hormone. Serum GH was expressed in terms of mGH AFP-10783B.

Histology-immunohistochemistry

Stated in brief, pituitary glands from five animals per group were fixed in Bouin's fluid and embedded in paraffin. Serial sections of 4 μm were obtained at two levels of the blocks following a ventral-to-dorsal sequence. The pituitary sections were immunostained, and then incubated for 1 h at room temperature with a primary anti-GH antibody (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 PBS.

Anterior pituitary morphometry

Morphometry was performed as reported in detail previously (Cónsole et al., 2002). Measurements of immunostained somatotrope cells were made by means of an image-analysis system (Imaging Technology, Optimas 5.2, USA). The cells per 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 were subsequently calculated: cell size (CS, expressed in μm^2), volume density (VD = Σ cell area/RA) and cell density (CD = number of cells/RA). 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 density according to generally accepted criteria. The 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. 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

Effect of thymulin quenching on the somatotrope population in C57BL/6 mice

Immunostained GH-cells of the *pars distalis* exhibited an ochre definite granular cytoplasmic pattern. The decrease of immunolabeled

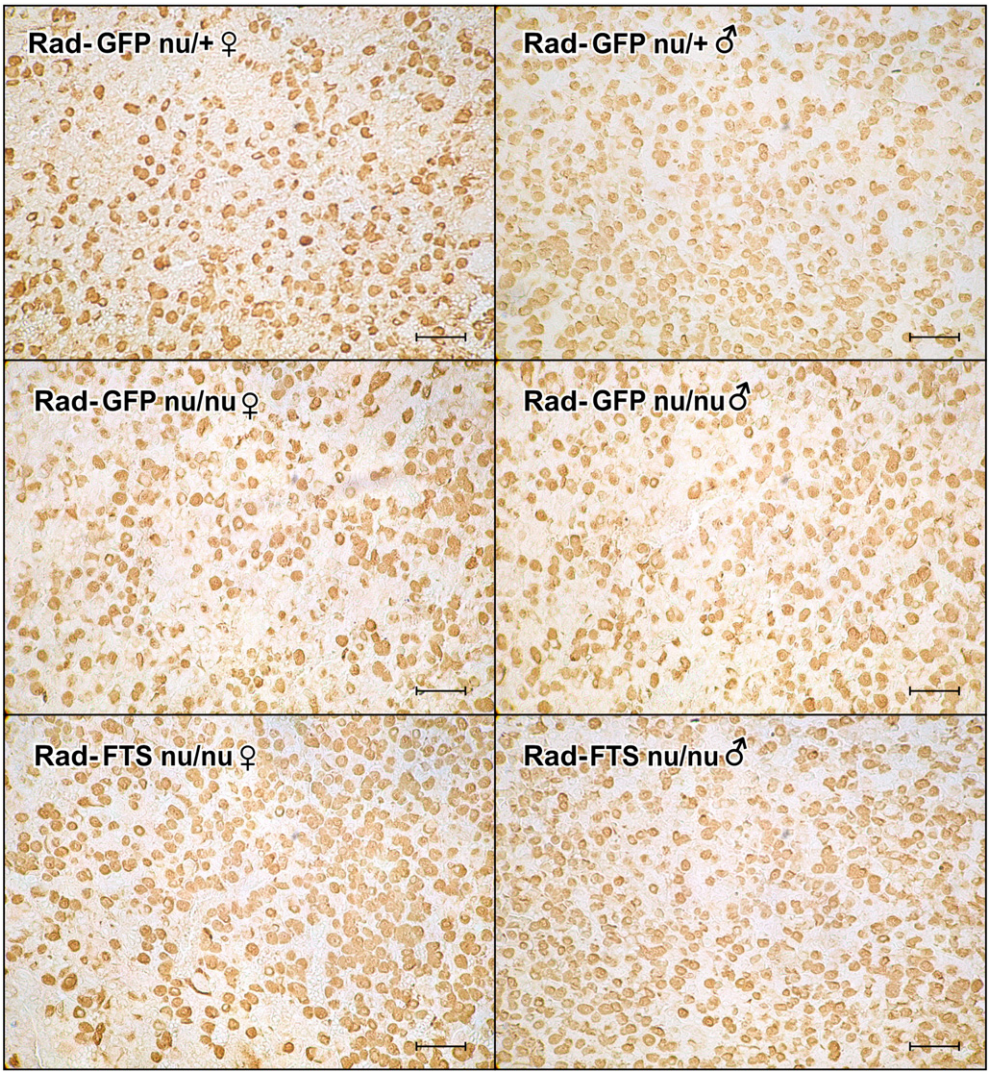

Fig. 4. Impact of thymulin gene therapy on the somatotrope cell population in nude mice. Representative fields of specifically immunostained GH-cells in the three experimental groups: the nu/nu RAd-FTS groups showed an increase in CD with respect to the nu/nu RAd-GFP groups of both sexes. EnVision system peroxidase. Bar: 45 μm.

somatotropes of the α-FTS groups as compared to control groups NRS corresponding to representative fields of the histometry performed is shown in Fig. 1.

The morphometry of the somatotrope population showed a highly significant ($P<0.01$) decrease in CD, and an increment ($P<0.05$) in CS, in both the male and female α-FTS groups as compared to NRS (Fig. 2, A–C). VD did not show significant changes.

Impact of thymulin immunoneutralization on serum thymulin and GH and body weights

A single i.p. injection of anti-thymulin serum (8 μl/g BW) markedly reduced the serum activity of endogenous thymulin in C57BL/6 infantile mice. This inhibition lasted for at least 10 days (data not shown). Quenching of serum thymulin (antiserum injections done every 7 days) from postnatal day 1 to 35 induced a significant ($P<0.01$) fall in the serum levels of thymulin which fell from 90 ± 16 fg/ml in controls to 16 ± 2.3 fg/ml in the quenched animals at age 35 days. The body weight gain was lower in the quenched than in the control mice (Fig. 3) and serum GH was significantly lower in quenched than in control males but not females (Fig. 3 inset).

Thymulin gene therapy on the somatotrope population in nude mice

Immunostained GH-cells stood out in sharp relief, exhibiting an ochre definite granular cytoplasmic pattern. The increment of immunolabeled somatotrope cells of the RAd-FTS groups as compared to RAd-GFP controls corresponding to representative fields of the histometry performed in the three experimental groups is shown in Fig. 4.

Neonatal thymulin gene therapy significantly ($P<0.01$) increased the CD and VD of the somatotrope population in nu/nu mice of both sexes submitted to RAd-FTS *vs.* RAd-GFP (Fig. 5, A–C), thus preventing the alteration observed in the somatotrope population of control athymic mice.

Effects of thymulin gene therapy on serum thymulin and body weight in nude mice

A single neonatal i.m. injection of RAd-FTS, but not RAd-GFP increased ($P<0.01$) the circulating levels of biologically active thymulin in both heterozygous and homozygous nude mice tested at 71 days of age. Thus, serum thymulin in control nudes was 8 ± 1.2 fg/ml and rose to 102 ± 19 fg/ml in nudes submitted to thymulin gene therapy. Thymulin gene therapy induced a significant ($P<0.05$) body weight gain in the female homozygous nudes but not in males (Fig. 6).

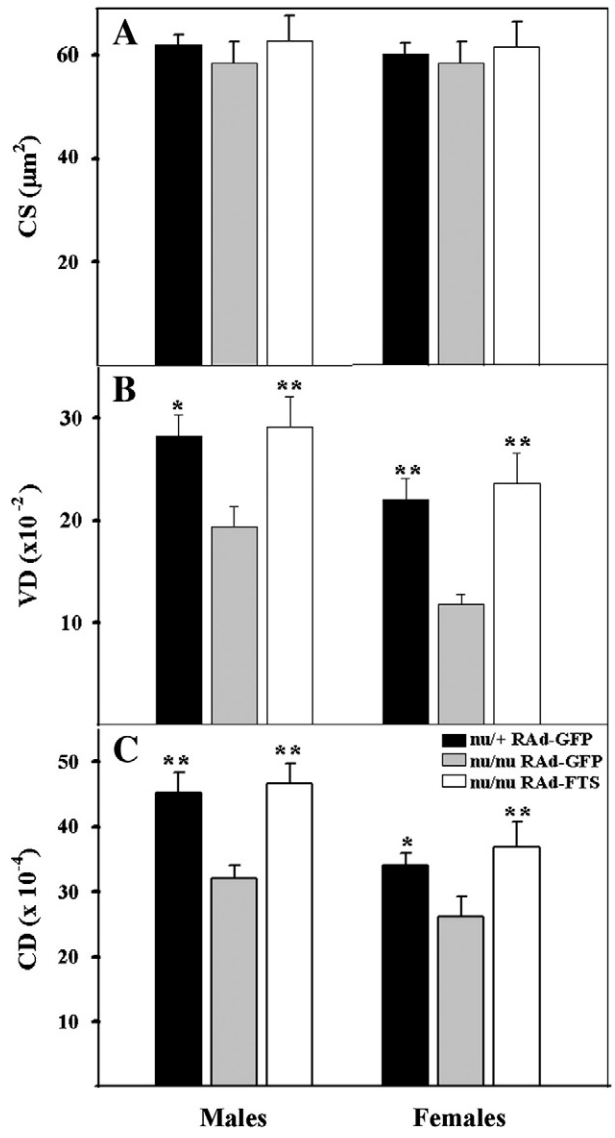

Fig. 5. Effect of neonatal thymulin gene therapy on the somatotrope population in nude mice. Somatotrope VD (B) and CD (C) in the nu/nu RAd-FTS groups showed a significant increase as compared with the nu/nu RAd-GFP groups of both sexes. Five animals in each group were analyzed. Asterisks indicate the level of significance of differences: *: $P<0.05$; **: $P<0.01$.

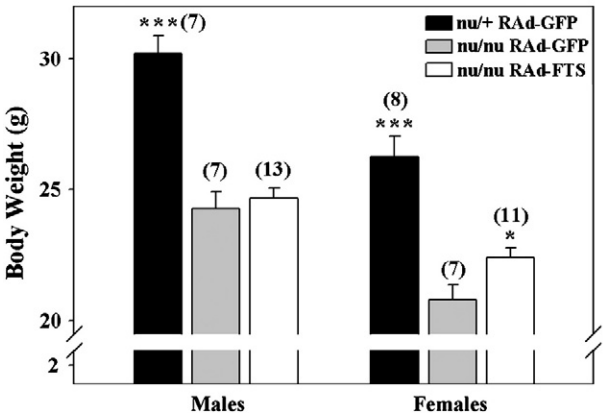

Fig. 6. Effect of thymulin gene therapy on body weight. Body weight was significantly increased in the nu/nu RAd-FTS groups as compared to the nu/nu RAd-GFP groups of both sexes. The number of mice per group is indicated in parentheses. Asterisks indicate the level of significance of differences respect to nu/nu RAd-FTS group: *: $P<0.05$; ***: $P<0.001$.

Discussion

The present results are in line with the idea that thymulin is a relevant physiologic mediator of the thymus–somatotropic axis. Specifically, our data suggest that a deficiency in serum thymulin during early life may compromise body growth. The morphometric data further show that hypothymulinemia has an impact on the somatotrope population, inducing a reduction in the somatotrope number accompanied by an increase in the size of these cells. Since the gross morphology of the pituitary was not different between treated and controls, it is possible that the reduction in somatotropes may be due at least in part, to failure of the various pituitary cell types to expand (proliferate). Therefore, there may be less hormone/cell that can be detected by IHC. Alternatively, this may represent a compensatory response that nevertheless fails to prevent the fall in serum GH levels which was significant in males but only a trend in females. In an early study we reported that 3-month-old male nude mice possess low basal GH levels without gross qualitative changes in the somatotrope population (Goya et al., 1995). Nevertheless, it should be pointed out that since adenovirus-mediated thymulin overexpression has been shown to increase the number of corticotropes and thyrotropes (Martines et al., 2011a,b), the increase in body weight observed here may be due, at least in part, to an overall improved pituitary function. We have also shown that passive immunoneutralization of thymulin leads to a decrease in LH/FSH and TSH and that neonatal gene therapy prevents the typical gonadotropic alterations observed in female nudes. Therefore the reported changes in the somatotrophs might be partly due to the influence of thymulin on the gonadal and thyroid axes.

A single i.m. injection of RAd-FTS in newborn nude mice (nude mice have undetectable circulating levels of thymulin) elicited long-term restoration of serum thymulin in these mutants. In previous studies we could demonstrate that in nude mice neonatal thymulin gene therapy prevented the reduction in the number of gonadotropes (Reggiani et al., 2009), thyrotropes and corticotropes (Martines et al., 2011a,b).

Circulating thymulin levels are known to fall sharply both in animals and humans affected by immunodepressing pathologies like AIDS, Di George Syndrome (a syndrome characterized by the congenital absence of the thymus and parathyroid glands) as well as by chronic or acute stress. Interestingly, AIDS is associated with GH deficiency which may be consequential at least in part, to thymulin deficiency (Falutz, 2011). A number of T cell alterations consequential to congenital athymia or neonatal thymectomy in mice, were found to be reversed by *in vitro* or *in vivo* treatment with thymulin (Bach, 1983; Dardenne et al., 1984). The present report provides evidence for a preventive action of neonatal thymulin gene therapy regarding the deficits in body weight and somatotrope morphology induced by congenital athymia. Given that recombinant thymulin has an impact on other pituitary cell types it is possible that thymulin may play a role in maturation of these cell types and/or modify central input to the pituitary. In particular, our results showing a favorable influence of thymulin on body weight gain and somatotrope maturation during early life are in line with previous reports that the thymus influences the maturation of somatotropic function. Thus, in light microscopy studies it was observed that in mice neonatal thymectomy or congenital absence of the thymus induces significant modifications in the acidophilic cells of the hypophysis (Lintern-Moore and Pantelouris, 1976; Pierpaoli and Besedovsky, 1975).

Conclusion

We conclude that during early postnatal life thymulin plays a relevant role on the maturation of somatotropic cell structure and function in mice.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgments

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