Thymulin gene therapy prevents the reduction in circulating gonadotropins induced by thymulin deficiency in mice

Rodolfo G. Goya[1,*], **Paula C. Reggiani**[1,*], **Silvan M. Vesenbeckh**[1,2,3], **Jean M. Pléau**[2], **Yolanda E. Sosa**[1], **Gloria M. Cónsole**[1], **Rüdiger Schade**[3], **Peter Henklein**[4], and **Mireille Dardenne**[2]

[1]*Instituto de Investigaciones Bioquimicas de La Plata-Histology B-Comision de Investigaciones Cientificas de la Provincia de Buenos Aires, Facultad de Medicas, Universidad Nacional de La Plata, La Plata, Argentina*

[2]*Centre National de la Recherche Scientifique Unité Mixte de Recherche 8147, Université Paris V, Hôpital Necker, Paris, France*

[3]*Institut für Pharmakologie und Toxikologie, Charité-Universitätsmedizin Berlin, Berlin, Germany*

[4]*Institut für Biochemie, Charité-Universitätsmedizin Berlin, Berlin, Germany*

Abstract

Integrity of the thymus during perinatal life is necessary for a proper maturation of the pituitary-gonadal axis in mice and other mammalian species. Thus congenitally athymic (nude) female mice show significantly reduced levels of circulating gonadotropins, a fact that seems to be causally related to a number of reproductive derangements described in these mutants. Interestingly, a number of in vitro studies suggest that the thymic peptide thymulin may be involved in thymus-pituitary communication. To determine the consequences of low serum thymulin in otherwise normal animals, we induced short (8 days)- and long (33 days)-term thymulin deficiency in C57BL/6 mice by neonatally injecting (intraperitoneally) an anti-thymulin serum and assessed their circulating gonadotropin levels at puberty and thereafter. Control mice received an irrelevant antiserum. Gonadotropins were measured by radioimmunoassay and thymulin by bioassay. Both long- and short-term serum thymulin immunoneutralization resulted in a significant reduction in the serum levels of gonadotropins at 33 and 45 days of age. Subsequently, we injected (intramuscularly) an adenoviral vector harboring a synthetic DNA sequence (5′-ATGCAAGCCAAATCTCAAGGTGGATCCAACTAGTAG-3′) encoding a biologically active analog of thymulin, methionine-FTS, in newborn nude mice (which are thymulin deficient) and measured circulating gonadotropin levels when the animals reached 52 days of age. 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 that typically emerges in these mutants after puberty. Our results indicate that thymulin plays a relevant physiological role in the thymus-pituitary-gonadal axis.

Keywords

reproductive derangements; thymulin immunoneutralization; synthetic gene

DURING PERINATAL LIFE, the integrity of the thymus is necessary for a proper maturation of the pituitary-gonadal axis as revealed by the endocrine alterations caused by neonatal thymectomy or congenital absence of the thymus in mice. In effect, congenitally athymic (nude) female mice show significantly reduced levels of circulating gonadotropins, a fact that seems to be causally related to a number of reproductive derangements described in these mutants (24). Thus, in homozygous (*nu/nu*) females, the times of vaginal opening and first ovulation are delayed (2), fertility is reduced (24), and follicular atresia is increased such that premature ovarian failure results (19). Similar abnormalities result from neonatal thymectomy in normal female mice (20,22). 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 compared with the heterozygous counterparts (14).

Thymulin is a well-characterized thymic factor discovered, purified, and sequenced during the 1970s (1). It consists of a biologically inactive nonapeptide component, FTS (an acronym for serum thymus factor in French), coupled in an equimolecular ratio to the ion zinc (12), which confers biological activity to the molecule (10). The metallopeptide active form bears a specific molecular conformation that has been evidenced by nuclear magnetic resonance (7). 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 (29). 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 (5).

In the present study we report that experimental induction of thymulin deficiency in normal mice mimics the alterations in serum gonadotropins documented in neonatally thymectomized and nude mice. Furthermore, using a recombinant adenoviral (RAd) vector, RAd-metFTS, which harbors a synthetic DNA sequence encoding a biologically active analog of thymulin, methionine-FTS (metFTS), we have been able to demonstrate that neonatal thymulin gene therapy in nude mice completely prevents the reduction in circulating gonadotropin levels that typically emerges in these mutants after puberty.

MATERIALS AND METHODS

Immunoreagents and FTS Synthesis

Anti-FTS was raised by immunizing rabbits with synthetic FTS coupled to keyhole limpet hemocyanin (KLH; Sigma Aldrich, St. Louis, MO) as carrier. Anti-KLH, used as an irrelevant antiserum, was raised by immunizing rabbits with KLH alone. In some experiments, normal rabbit serum (NRS) was used as control serum instead of anti-KLH.

Antisera were initially tested and selected by dot blot with synthetic thymulin or KLH as antigens. A nonapeptide possessing the amino acid sequence of native FTS (PyrGlu-AKSQGGSN) was synthesized, employing an ABI 433A automated peptide synthesizer (Applied Biosystems, Darmstadt, Germany), using the Fmoc/tBu strategy. The crude peptide was purified by reverse-phase HPLC under standard conditions, with spectrophotometric monitoring at $\lambda = 220$ nm, which led to obtaining a single peak of pure peptide.

Animals and Experimental Procedures

Immunoneutralization experiments—Pregnant C57BL/6 female mice were purchased from Janvier (Le Genest, Saint-Isle, France) and kept in the animal facilities of Hôpital Necker, Paris. The offspring of these mice were submitted to the quenching experiments described below. Animals had free access to food and water and were kept at 22°C with a 12:12-h light-dark cycle. Animal experiments with C57BL/6 mice were conducted in conformity with the rules established by the European Union ethics committee for animal research.

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 (ACFNULP), Argentina. All mice were maintained on a γ-irradiate chow diet and sterilized water. Animals had free access to food and water and were kept at 22°C with a 12:12-h light-dark cycle. All experiments on nude animals were done following the Animal Welfare Guidelines of the National Institutes of Health (Instituto de Investigaciones Bioquimicas de La Plata's Animal Welfare Assurance No. A5647-01).

Prepubertal *nu/nu* mutants from ACFNULP have minimal levels of circulating thymulin. During a short time window after puberty, they display a slight increase in serum thymulin levels, which fall to nondetectable levels at around 3 mo of age, remaining so afterward. At postnatal *day 1* or *2*, each experimental pup (both *nu/nu* and *nu*/+) received a single bilateral intramuscular (hindlimb) injection of 10^8 plaque forming units of RAd-metFTS or RAd-GFP/TK (a control vector; see *Adenoviral Vectors Used*) in 10 µl of vehicle (5 µl per side). On postnatal *days 51* and *52*, mice were bled and immediately killed by cervical dislocation. On postnatal *day 13*, three mice from each group were also bled for thymulin determination.

Injections of Antisera and Thymulin

Antiserum injections—On postnatal *days 1* and *2*, each C57BL/6 pup received an intraperitoneal injection of 20 µl of the corresponding undiluted serum. In the long-term quenching experiments, the initial two intraperitoneal antiserum injections were followed by intraperitoneal antiserum injections every 7 days at a dose of 8 µl serum/g body wt.

Synthetic FTS injections—At the indicated times, C57BL/6 pups received an intraperitoneal injection of 60 pg of thymulin/g body wt. FTS-Zn was prepared 30 min before injection by mixing equimolecular (10 nM) volumes of synthetic FTS and $ZnCl_2$, both prepared in phosphate-buffered saline (PBS).

Adenoviral Vectors Used

RAd-metFTS—A DNA sequence coding for the biologically active thymulin analog metFTS, referred to as a synthetic gene for thymulin, was constructed (Fig. 1, *A* and *B*). A recombinant adenoviral vector harboring the synthetic gene for thymulin was constructed by a variant of the two-plasmid method (16), employing the AdMax plasmid kit (Microbix, Toronto, ON, Canada). This kit uses a shuttle plasmid (pDC515) containing a multiple cloning site (MCS) and an FLP recognition target (FRT) site for the yeast FLP recombinase. This cassette is flanked by sequences of the adenovirus type 5 (Ad5) E1 region. The second plasmid of the kit, the genomic plasmid pBHGfrt(del)E1,3 FLP, consists of the entire genome of Ad5, containing deletions in the regions E1 and E3. Upstream of the E1 deletion, pBHGfrt(del)E1,3 FLP contains an expression cassette for the gene of yeast FLP recombinase, and immediately downstream of the E1 deletion, an FRT site has been inserted. Once the thymulin synthetic gene was inserted into the shuttle, both plasmids were cotransfected into HEK-293 cells. In cotransfected HEK-293 cells, FLP recombinase is readily expressed and efficiently catalyzes the site-directed recombination of the expression cassette of pDC515-metFTS into the left end of pBHGfrt(del)E1,3 FLP, thus generating the genome of the desired recombinant adenoviral vector, RAdmetFTS (Fig. 1*C*). The newly generated RAd was rescued from HEK-293 cell lysates and plaque purified. It was further purified by ultracentrifugation in CsCl gradient and titrated 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 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 fused to herpes simplex virus type 1 thymidine kinase (GFP/TK)$_{fus}$ (a kind gift from Dr. Jacques Galipeau, McGill University, Montreal, Canada). This hybrid gene is driven by the mouse cytomegalovirus promoter. The vector was expanded in HEK-293 cells and was purified and titrated as indicated above.

Thymulin Bioassay

Biologically active thymulin was measured in serum by a rosette bioassay described in detail elsewhere (8). 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 femtograms per milliliter of bioactive thymulin. This bioassay has been previously validated against an ELISA for thymulin (25).

Pituitary Hormone Assays and Immunohistochemistry

Serum levels of 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, University of California, Los Angeles Medical Center. Serum concentrations of LH and FSH were expressed in terms of mouse LH RP-2 and rat FSH RP-2, respectively.

Pituitary sections (4 μm) were incubated for 1 h at room temperature with primary antibodies against LH or FSH (murine; Dako, Carpinteria, CA) diluted 1:100. Thoroughly washed sections were then treated for 30 min with a ready-to-use EnVision reaction system (Dako) using the peroxide-sensitive chromogen diaminobenzidine for color development.

Statistical Analysis

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

RESULTS

In Vitro and In Vivo Immunoneutralization of Thymulin

The anti-FTS serum used was highly effective in quenching the biological activity of thymulin in vitro. The specificity of this immunoneutralizing activity was demonstrated by the fact that neither anti-KLH serum nor NRS displayed any significant immunoneutralizing activity on thymulin (data not shown).

A single intraperitoneal injection of anti-FTS serum (8 μl/g body wt) markedly reduced the serum activity of endogenous thymulin in C57BL/6 infantile mice. This inhibition lasted for at least 10 days (Fig. 2, *inset*).

Long-term thymulin immunoneutralization experiments—Long-term quenching of serum thymulin (antiserum injections done every 7 days) from postnatal *days1* and *2* to postnatal *day 33* induced a significant fall in the serum levels of LH and FSH in both male and female C57BL/6 mice (Fig. 2).

Short-term immunoneutralization experiments—Serum thymulin was immunoneutralized during a short postnatal time window as follows: pups were intraperitoneally injected with an anti-FTS or anti-KLH serum on postnatal *days 1* and *2*. On postnatal *day 8*, three animals from each experimental group were bled to confirm that serum thymulin quenching had been successful; immediately afterward, all animals received an

intraperitoneal injection of synthetic thymulin. On *day 9*, all pups received a second injection of thymulin, and 2.5 h later, three animals from each group were bled to determine serum thymulin levels. As expected, anti-FTS serum, but not control serum, induced a strong reduction in circulating thymulin. The injection of synthetic thymulin saturated the anti-FTS serum and returned serum thymulin to control values until postnatal *day 45*, when mice were killed (Fig. 3).

Thymulin immunoneutralization during an 8-day neonatal window was still effective in causing low serum LH and FSH levels when the mice achieved 45 days of age (Fig. 4). At the end of both short- and long-term quenching experiments, the pituitaries of anti-FTS-treated animals showed a mild decrease in the number of LH and FSH cells as well as a slight hypertrophy of the same cells (Fig. 5).

Restorative Effect of RAd-metFTS Administration on Serum Thymulin in Nude Mice

A single neonatal intramuscular injection of RAd-metFTS, but not RAd-GFP/TK (a control vector), increased the circulating levels of biologically active thymulin in both heterozygous and homozygous nude mice tested at 51-52 days of age (Fig. 6). At 13 days of age, the RAd-metFTS-treated *nu*/*nu* mice achieved serum thymulin levels comparable to those of control *nu*/+ counterparts. At the same time point, RAd-metFTS-treated heterozygous mice showed a clear increase in the serum levels of thymulin compared with control counterparts (Fig. 6).

Neonatal Thymulin Gene Therapy Prevents Serum LH and FSH Deficits in Adult Nudes

A single neonatal intramuscular injection of RAd-metFTS, but not RAd-GFP/TK, prevented the development of hypogonadotropinemia in postpubertal nudes and induced supraphysiological serum levels of LH and FSH in the heterozygous mice (in *nu*/+ mice, this increase only achieved statistical significance for LH; Fig. 7).

DISCUSSION

Thymulin is a thymic metallopeptide involved in several aspects of intra- and extrathymic T-cell differentiation (1). Circulating levels of this molecule fall sharply in humans affected by pathologies such as acquired immunodeficiency syndrome (17) and DiGeorge syndrome, a syndrome characterized by the congenital absence of the thymus and parathyroid glands (18), as well as during normal aging (6). Thymulin, which is exclusively produced by the thymic epithelial cells (9), exerts a controlling feedback effect on its own secretion (28). In addition, thymulin production and secretion are stimulated directly by growth hormone and prolactin and indirectly by thyrotropin through thyroid hormones. Corticotropin and gonadotropins inhibit thymulin secretion via a direct action of adrenal and gonadal steroids, respectively, on thymic epithelial cells (13). Considering the multihormone control effected by the pituitary gland on thymulin secretion, it seems logical to hypothesize that thymulin may in turn exert feedback actions on the hypophysis and the hypothalamus.

To our knowledge, this is the first report documenting that immunoneutralization of a circulating molecule specifically produced by the thymus can reproduce the gonadotropin deficiencies caused by neonatal thymectomy or congenital absence of the thymus in mice. Consequently, the present findings strongly point to thymulin as the physiological mediator, or at least one of the mediators, of the perinatal influence of the thymus on the maturation of the pituitary-gonadal axis. This is in line with studies showing that injection of synthetic thymulin in the hypothalamus of prepubertal female mice has a facilitatory action on equine chorionic gonadotropin-induced ovulation (11). The present results also indicate that thymulin deficiency during the first 8 days of life is enough to induce low serum levels of gonadotropins as well as morphological changes at pituitary level in the adult animals.

We can only speculate on the physiological mechanism by which thymulin exerts its influence on gonadotropic hormone secretion. Thymulin is known to possess hypophysiotropic activity in vitro (3-5,15,29) and therefore may act directly on the adenohypophysis in vivo, modulating the response of the gland to hypothalamic or other secretagogues or inhibitors. There also is evidence that thymulin stimulates cytokine release from certain types of lymphocytes (23). Therefore, thymulin could also influence gonadotropin production by stimulating the release of cytokines or other neuroactive molecules from thymulin-responsive immune cells.

Since the gene for native FTS has not been cloned, we constructed an artificial DNA sequence, optimized for expression in rat systems, which codes for a biologically active analog of FTS, metFTS (25). This sequence was used to construct RAd-metFTS, an adenoviral vector able to achieve long-term restoration of circulating thymulin levels when intramuscularly injected in thymectomized rats and mice (25). Adenovirally delivered metFTS in the brain of immunocompetent rats showed a much longer expression than adenovirally delivered GFP and β-galactosidase (21). The ability of RAd-metFTS to achieve long-term expression in vivo was also observed in the present study, where a single intramuscular injection of the vector resulted in sustained expression of the transgene 51 days after the treatment despite the severalfold increase in the size (and volemia) of the animals from the beginning to the end of the study. Since thymulin and certain thymulin analogs have been reported to possess anti-inflammatory activity in the brain (26,27), we hypothesize that an anti-inflammatory activity of thymulin may have protected the adenovirally transduced cells from being destroyed by the immune system of the host animals.

The finding that neonatal thymulin gene therapy prevents the well-documented deficit of circulating gonadotropins in adult nudes complements our thymulin immunoneutralization results in normal mice and lends further support to the view that thymulin is an important player in the thymus-gonadotropic axis. In addition, our data suggest that thymulin gene therapy may be an effective strategy to approach reproductive deficits associated with thymus dysfunction.
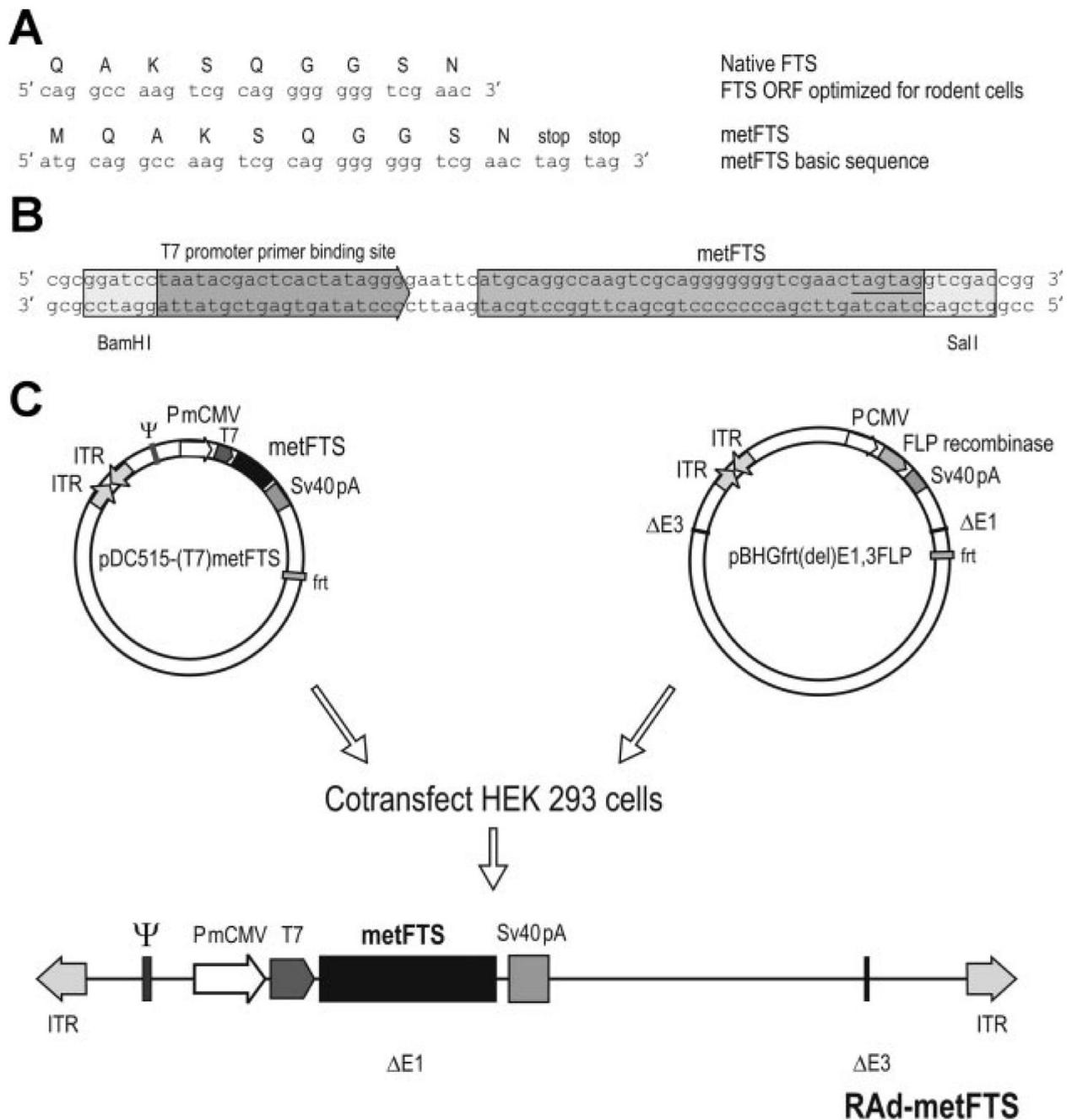
GRANTS

REFERENCES

1. Bach JF. Thymulin (FTS-Zn). Clin Immunol Allergy 1983;3:133–156.

2. Besedovsky HO, Sorkin E. Thymus involvement in female sexual maturation. Nature 1974;249:356–358. [PubMed: 4858269]

3. Brown OA, Sosa YE, Bolognani F, Goya RG. Thymulin stimulates prolactin and thyrotropin release in an age-related manner. Mech Ageing Dev 1998;104:249–262. [PubMed: 9818729]

4. Brown OA, Sosa YE, Dardenne M, Pléau JM, Goya RG. Growth hormone-releasing activity of thymulin: effects of age. Neuroendocrinology 1999;69:20–27. [PubMed: 9892847]

5. Brown OA, Sosa YE, Dardenne M, Pléau JM, Goya RG. Studies on the gonadotropin-releasing activity of thymulin: changes with age. J Gerontol A Biol Sci Med Sci 2000;55:B170–B176. [PubMed: 10811143]

6. Consolini R, Legitimo A, Calleri A, Milani M. Distribution of age-related thymulin titres in normal subjects through the course of life. Clin Exp Immunol 2000;121:444–447. [PubMed: 10971509]

7. Cung MT, Marraud M, Lefrancier P, Dardenne M, Bach JF, Laussac JP. NMR study of a lymphocyte differentiating thymic factor. J Biol Chem 1988;263:5574–5580. [PubMed: 3356698]

8. Dardenne, M.; Bach, JF. The sheep cell rosette assay for the evaluation of thymic hormones. In: Van Bekkum, DW.; Kruisbeek, AM., editors. Biological Activity of Thymic Hormones. International Workshop and Symposium. Kooyker Scientific Publications, Wiley; Rotterdam, The Netherlands: 1975. p. 235-243.

9. Dardenne M, Papiernik M, Bach JF, Stutman O. Studies on thymus products. III. Epithelial origin of the serum thymic factor. Immunology 1974;27:299–304. [PubMed: 4421188]

10. Dardenne M, Pléau JM, Nabarra B, Lefrancier P, Derrien M, Choay J, Bach JF. Contribution of zinc and other metals to the biological activity of serum thymic factor (FTS). Proc Natl Acad Sci USA 1982;79:5370–5373. [PubMed: 6957870]

11. García L, Hinojosa L, Domínguez R, Chavira R, Rosas P. Effects of injecting thymulin into the anterior or medial hypothalamus or the pituitary on induced ovulation in prepubertal mice. Neuroimmunomodulation 2005;12:314–320. [PubMed: 16166812]

12. Gastinel LN, Dardenne M, Pléau JM, Bach JF. Studies on the zinc-binding site to the serum thymic factor. Biochim Biophys Acta 1984;797:147–155. [PubMed: 6538097]

13. Goya RG, Brown OA, Pléau JM, Dardenne M. Thymulin and the neuroendocrine system. Peptides 2004;25:139–142. [PubMed: 15003367]

14. Goya RG, Cónsole GM, Sosa YE, Gómez Dumm CLA, Dardenne M. Altered functional responses with preserved morphology of gonadotrophic cells in congenitally athymic mice. Brain Behav Immun 2001;15:85–92. [PubMed: 11259083]

15. Hadley AJ, Rantle CM, Buckingham JC. Thymulin stimulates corticotrophin release and cyclic nucleotide formation in the rat anterior pituitary gland. Neuroimmunomodulation 1997;4:62–69. [PubMed: 9483196]

16. Hitt, M.; Bett, A.; Prevec, L.; Graham, FL. Construction and propagation of human adenovirus vectors. In: Celis, J., editor. Cell Biology: A Laboratory Handbook. Academic; New York: 1998. p. 1500-1512.

17. Incefy GS, Pahwa S, Pahwa R, Sarngadharan MG, Menez R, Fikrig S. Low circulating thymulin-like activity in children with AIDS and AIDS-related complex. AIDS Res 1986;2:109–116. [PubMed: 3487329]

18. Kornfeld SJ, Zeffren B, Christodoulou CS, Day NK, Cawkwell G, Good RA. DiGeorge anomaly: a comparative study of the clinical and immunologic characteristics of patients positive and negative by fluorescence in situ hybridization. J Allergy Clin Immunol 2000;105:983–987. [PubMed: 10808180]

19. Lintern-Moore S, Pantelouris EM. Ovarian development in athymic nude mice. The size and composition of the follicle population. Mech Ageing Dev 1975;4:385–390. [PubMed: 1228336]

20. Michael SD, Taguchi O, Nishizuka Y. Effects of neonatal thymectomy on ovarian development and plasma LH, FSH, GH and PRL in the mouse. Biol Reprod 1980;22:343–350. [PubMed: 6769514]

21. Morel GR, Brown OA, Reggiani PC, Hereñú CB, Portiansky EL, Zuccolilli Pleau JMGO, Dardenne M, Goya RG. Peripheral and mesencephalic transfer of a synthetic gene for the thymic peptide thymulin. Brain Res Bull 2006;69:647–651. [PubMed: 16716832]

22. Nishizuka Y, Sakakura T. Ovarian dysgenesis induced by neonatal thymectomy in the mouse. Endocrinology 1971;89:889–893.

23. Palacios R, Fernandez C, Sideras P. Development and continuous growth in culture of interleukin-2 producer lymphocytes from athymic *nu/nu* mice. Eur J Immunol 1982;12:777–782. [PubMed: 6982822]

24. Rebar RW, Morandini IC, Erickson GF, Petze JE. The hormonal basis of reproductive defects in athymic mice. Endocrinology 1981;108:120–126. [PubMed: 6780309]

25. Reggiani PC, Hereñú CB, Rimoldi OJ, Brown OA, Pléau JM, Dardenne M, Goya RG. Gene therapy for long-term restoration of circulating thymulin in thymectomized mice and rats. Gene Ther 2006;13:1214–1221. [PubMed: 16617301]

26. Safieh-Garabedian B, Dardenne M, Pleau JM, Saade NE. Potent analgesic and anti-inflammatory actions of a novel thymulin-related peptide in the rat. Br J Pharmacol 2002;136:947–955. [PubMed: 12110619]

27. Safieh-Garabedian B, Ochoa-Chaar CI, Poole S, Massaad CA, Atweh SF, Jabbur SJ, Saade NE. Thymulin reverses inflammatory hyperalgesia and modulates the increased concentration of

proinflammatory cytokines induced by i.c.v. endotoxin injection. Neuroscience 2003;121:865–873. [PubMed: 14580936]

28. Savino W, Dardenne M, Bach JF. Thymic hormones containing cells. III. Evidence for a feed-back regulation of the secretion of the serum thymic factor (FTS) by thymic epithelial cells. Clin Exp Immunol 1983;52:7–12. [PubMed: 6683135]

29. Zaidi SAA, Kendall MD, Gillham B, Jones MT. The release of LH from pituitaries perifused with thymic extracts. Thymus 1988;12:253–264. [PubMed: 3256093]

A

Q	A	K	S	Q	G	G	S	N			Native FTS

5′ cag gcc aag tcg cag ggg ggg tcg aac 3′   FTS ORF optimized for rodent cells

| M | Q | A | K | S | Q | G | G | S | N | stop | stop | metFTS |

5′ atg cag gcc aag tcg cag ggg ggg tcg aac tag tag 3′   metFTS basic sequence

B

T7 promoter primer binding site   metFTS

5′ cgcggatcctaatacgactcactataggggaattcatgcaggccaagtcgcaggggggggtcgaactagtaggtcgaccgg 3′
3′ gcgcctaggattatgctgagtgatatccccttaagtacgtccggttcagcgtccccccagcttgatcatccagctggcc 5′

BamHI   SalI

C

Cotransfect HEK 293 cells

RAd-metFTS

Fig. 1.
DNA constructs encoding the thymulin analog methionine-FTS (metFTS) and a recombinant adenoviral (RAd) vector for metFTS (RAd-metFTS). A DNA sequence coding for native serum thymus factor (FTS) was designed for optimal expression in rat cells. By adding an ATG starting codon upstream and 2 stop codons downstream of this sequence, we converted it into an open reading frame (ORF) for the analog metFTS (*A*). This metFTS ORF was used to generate a construct to be cloned in the shuttle vector pDC515. The construct included the phage T7 promoter primer binding site, which was used for sequencing purposes (*B*). The shuttle pDC515-metFTS was generated by inserting the T7-metFTS sequence into the *Bam*HI-*Sal*I sites of the multiple cloning site of the shuttle pDC515. This construct was used to generate

RAd-metFTS (*C*). For further details, see materials and methods and Ref. 25. PmCMV, mouse cytomegalovirus promoter; frt, recognition element for the yeast FLP recombinase; ITR, inverted terminal repeats; ΔE1 and ΔE3, deletions in the Ad5 genome; SV40, simian virus 40 polyadenylation signal; Ψ, packaging signal.
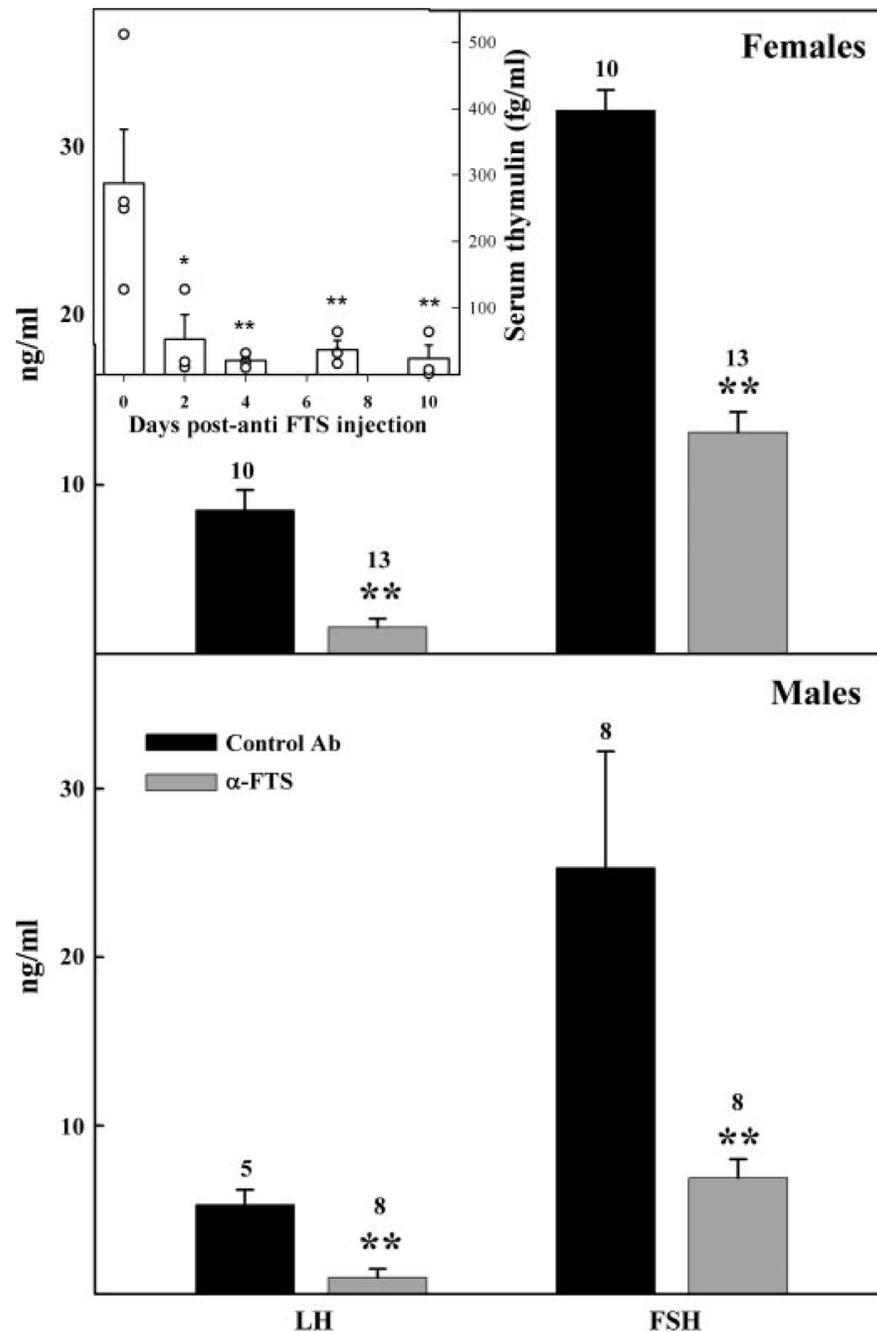
Fig. 2.
Effect of long-term thymulin quenching on gonadotropin serum levels in mice. Experimental animals (shaded bars) received weekly intraperitoneal injections of rabbit anti-FTS serum beginning at birth. Control mice (solid bars) received normal rabbit serum. LH, luteinizing hormone; FSH, follicle-stimulating hormone. All mice were killed at 33 days postinjection. Numbers over bars represent the number of mice assessed for the corresponding data point. *Inset*: in vivo immunoneutralization of serum thymulin in mice. Effects of a single intraperitoneal injection of undiluted rabbit anti-FTS serum (8 μl/g body wt) on thymulin serum level in 15-day-old mice were determined. Error bars represent SE. *$P < 0.05$; **$P < 0.01$, significance of differences between control and experimental mice.
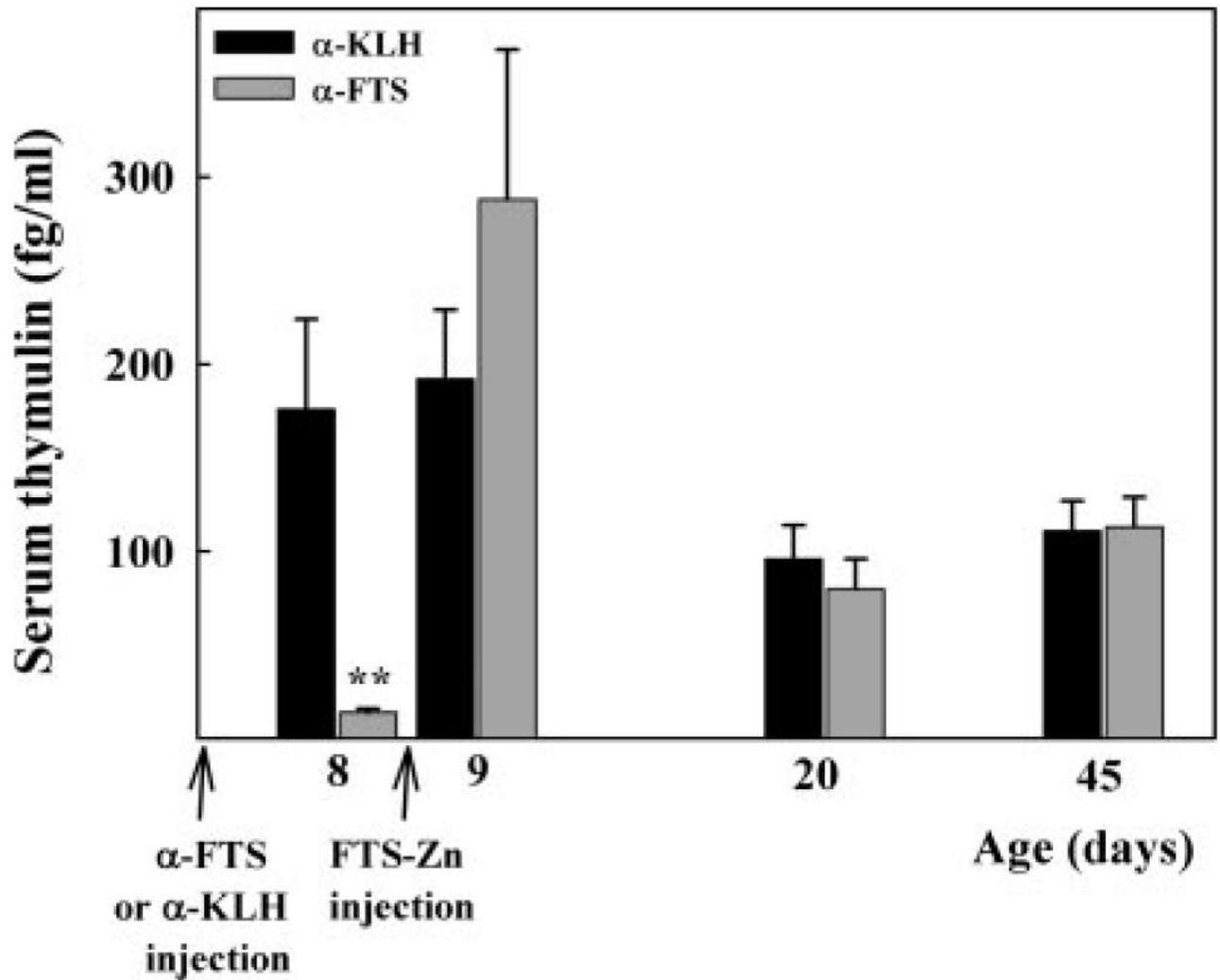
Fig. 3.
Short-term immunoneutralization of serum thymulin in mice. Experimental animals (shaded bars) were intraperitoneally injected at postnatal *days 1* and *2* with rabbit anti-FTS serum, which was followed by 2 intraperitoneal injections of synthetic thymulin (FTS-Zn; 60 pg/g body wt) on postnatal *days 8* and *9*. Control mice (solid bars) were submitted to the same treatment except that they received a rabbit anti-keyhole limpet hemocyanin (KLH) serum instead of anti-FTS. Asterisks represent significance of differences between control an experimental mice at each time point.
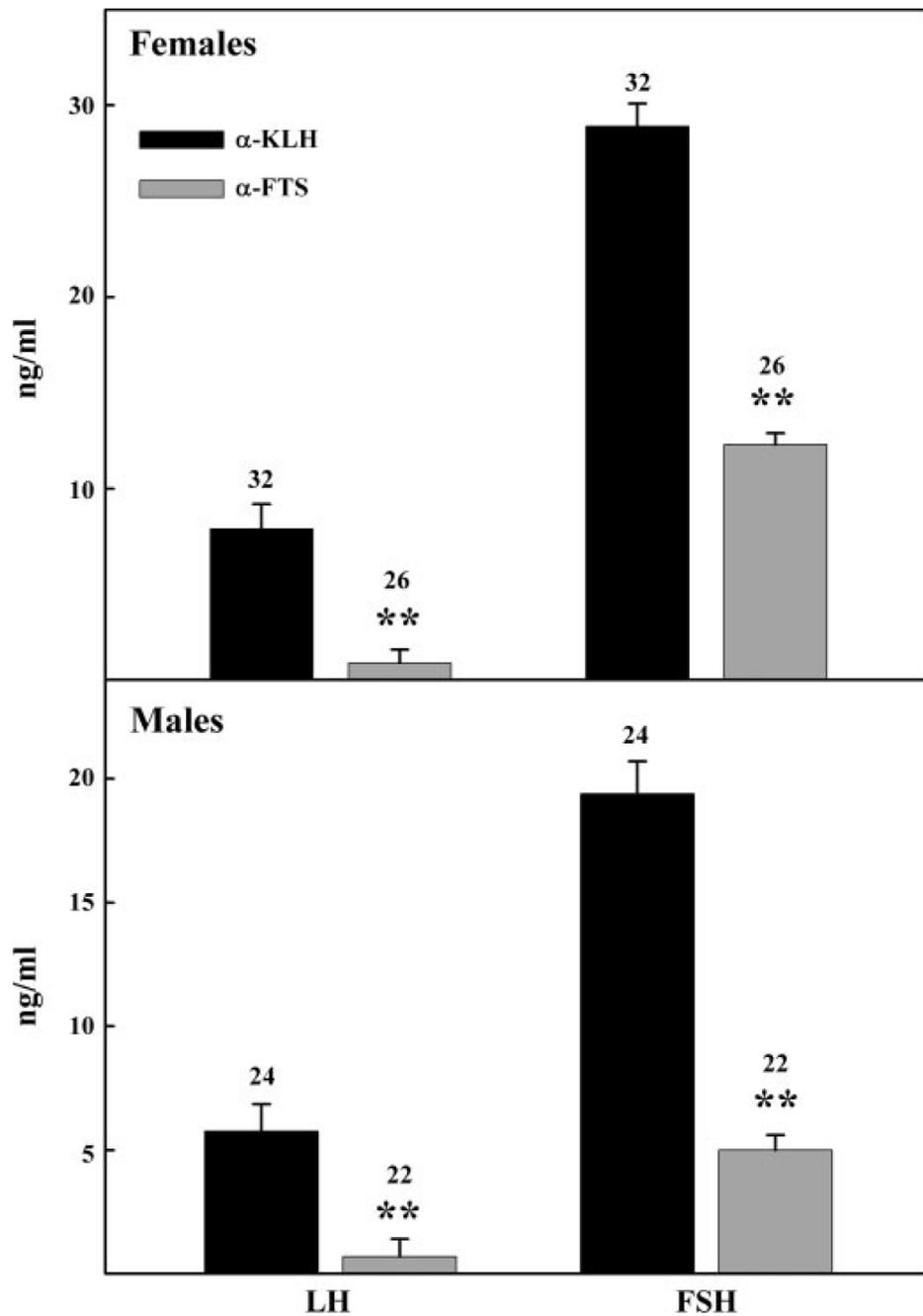
Fig. 4.
Effect of short-term thymulin quenching on gonadotropin serum levels in mice. Short-term thymulin quenching was performed as described in Fig. 3. Control mice received anti-KLH rabbit serum, whereas experimental mice received rabbit anti-FTS serum. All mice were killed at 45 days of age. Other details are as described in Fig. 2. **$P < 0.01$, significance of differences between control and experimental mice.
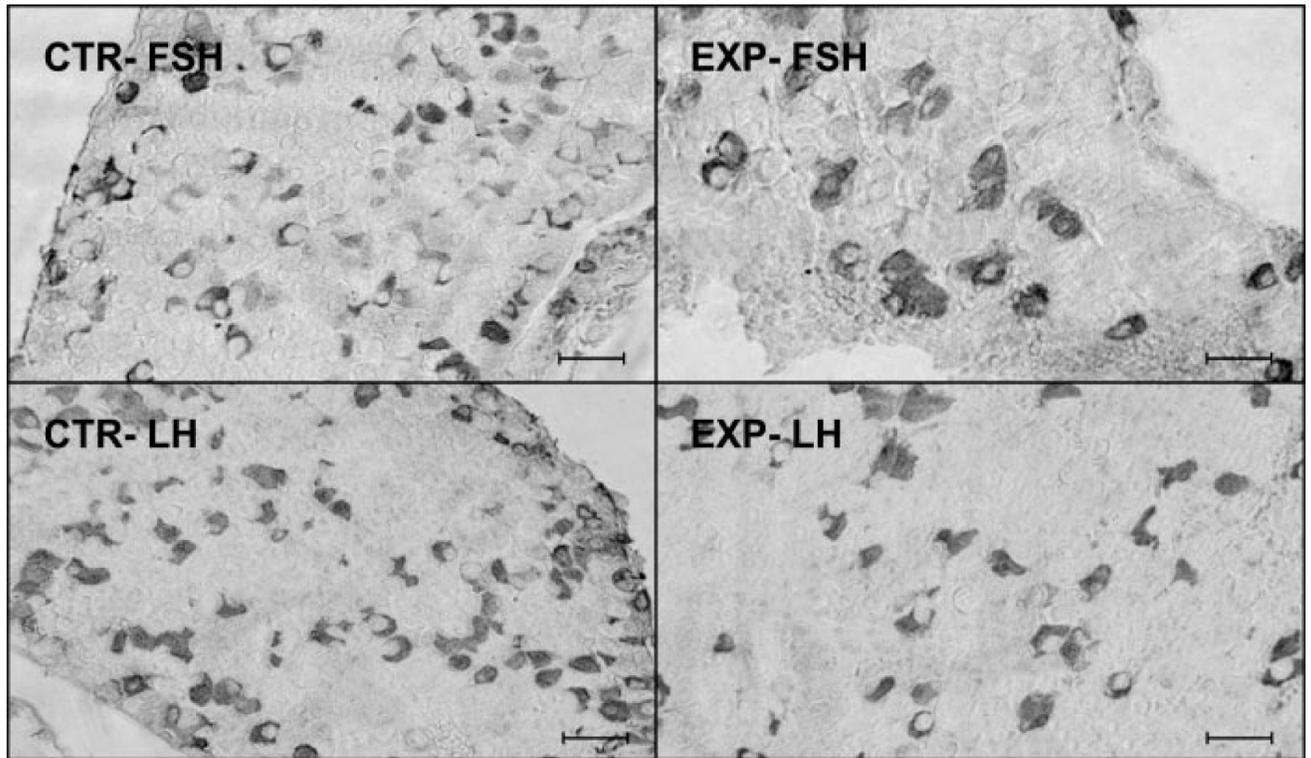
Fig. 5.
Impact of short-term serum thymulin quenching on gonadotropic cell morphology in C57BL/ 6 mice. Mice were submitted to short-term thymulin immunoneutralization as described in Fig. 3 and MATERIALS AND METHODS. Mice were killed at 45 days of age. Pituitary sections were submitted to immunohistochemistry for LH and FSH (see MATERIALS AND METHODS). A trend toward hypertrophy and a decrease in numbers in gonadotrophs can be noticed in the representative experimental (EXP) sections shown compared with the control (CTR) counterparts. Scale bar, 25 μm.
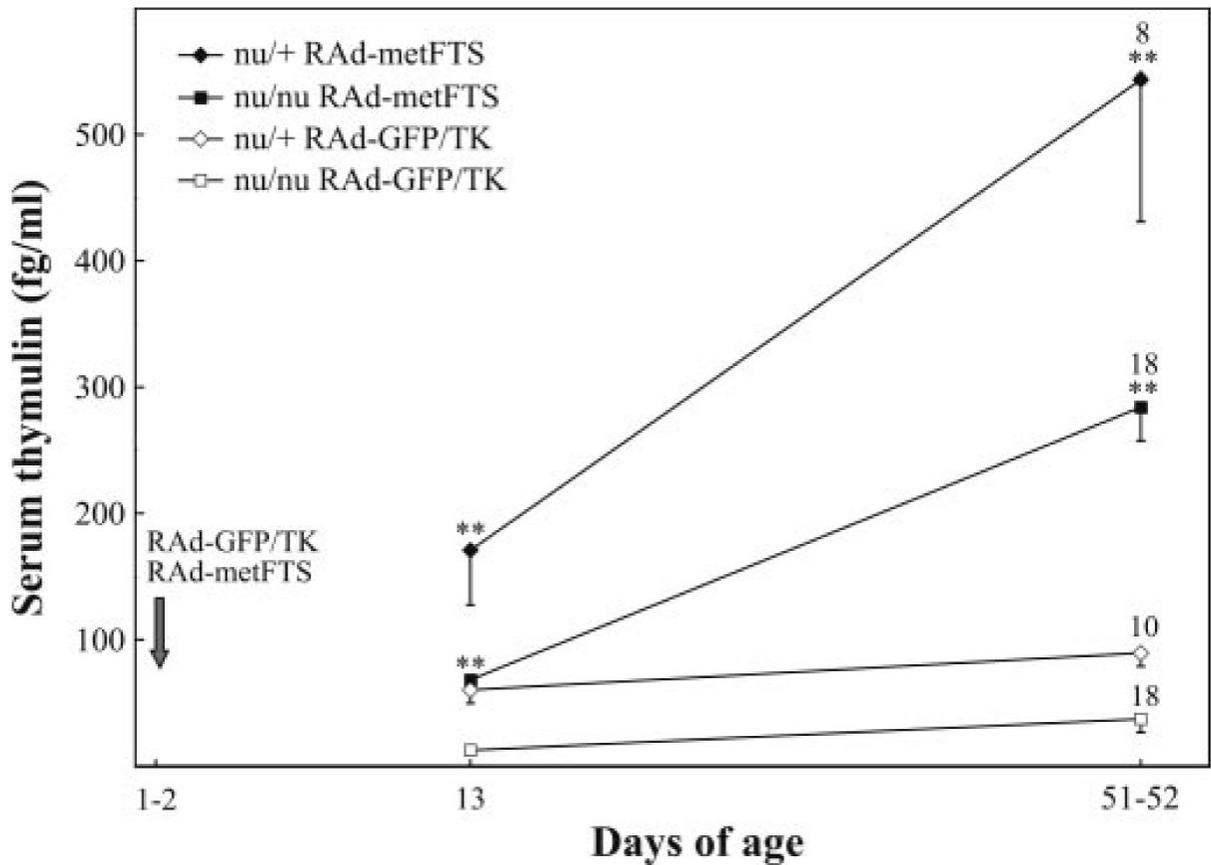
Fig. 6.
Effect of neonatal thymulin gene therapy on serum levels of thymulin in heterozygous (*nu*/+) and homozygous (*nu*/*nu*) nude mice. Vectors were intramuscularly injected on the day of birth or the day after (arrow). At postnatal *day 13*, 3 mice per group were bled and serum thymulin was assayed. On postnatal *days 51* and *52*, the remainder of the animals were bled and thymulin was assayed. Since the volume of serum that can be obtained from newborn mice is exceedingly small, thymulin could not be reliably determined on postnatal *days 1* and *2*. Serum thymulin values (fg/ml) are expressed as means ± SE; *n* values for data points at *days 51* and *52* are shown above symbols. **P < 0.01, significance of differences between RAd-GFP/TK-injected animals and RAd-metFTS-injected counterparts.
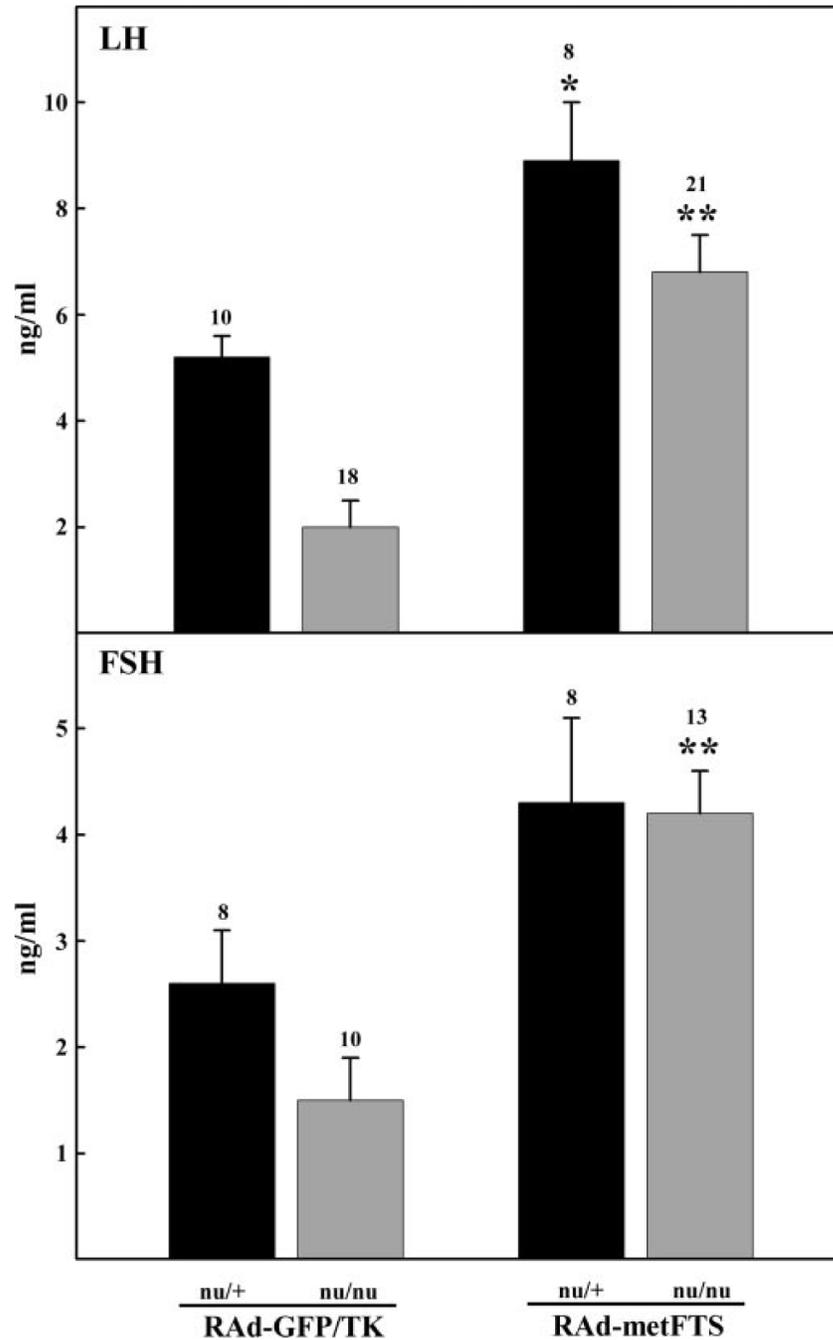
Fig. 7.
Effect of neonatal thymulin gene therapy on serum LH and FSH in nude mice. The indicated vectors were intramuscularly injected at birth, and the animals were bled 51-52 days afterward for serum LH and FSH determination. Other details are as described in Fig. 2. *$P < 0.05$; **$P < 0.01$, significance of differences between RAd-GFP/TK-injected animals and RAdmetFTS-injected counterparts.