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The Thymus–Neuroendocrine Axis:

Physiology, Molecular Biology, and Therapeutic Potential of the Thymic Peptide Thymulin

Paula C. Reggiani^{a,b,*}, Gustavo R. Morel^{a,b,*}, Gloria M. Console^b, Claudio G. Barbeito^c, Silvia S. Rodriguez^{a,b}, Oscar A. Brown^{a,b}, Maria Jose Bellini^{a,b}, Jean-Marie Pléau^d, Mireille Dardenne^d, and Rodolfo G. Goya^{a,b}

^a*Institute for Biochemical Research, Faculty of Medicine, CONICET, National University of La Plata, La Plata, Argentina*

^b*Histology B- Scientific Research Commission of the Province of Buenos Aires, Faculty of Medicine, National University of La Plata, La Plata, Argentina*

^c*Faculty of Veterinary Medicine, National University of La Plata, La Plata, Argentina*

^d*Centre National de la Recherche Scientifique UMR 8147, Université Paris V, Hôpital Necker, Paris, France*

Abstract

Thymulin is a thymic hormone exclusively produced by the thymic epithelial cells. It consists of a nonapeptide component coupled to the ion zinc, which confers biological activity to the molecule. After its discovery in the early 1970s, thymulin was characterized as a thymic hormone involved in several aspects of intrathymic and extrathymic T cell differentiation. Subsequently, it was demonstrated that thymulin production and secretion is strongly influenced by the neuroendocrine system. Conversely, a growing core of information, to be reviewed here, points to thymulin as a hypophysiotropic peptide. In recent years, interest has arisen in the potential use of thymulin as a therapeutic agent. Thymulin was shown to possess anti-inflammatory and analgesic properties in the brain. Furthermore, an adenoviral vector harboring a synthetic gene for thymulin, stereotaxically injected in the rat brain, achieved a much longer expression than the adenovirally mediated expression in the brain of other genes, thus suggesting that an anti-inflammatory activity of thymulin prevents the immune system from destroying virus-transduced brain cells. Other studies suggest that thymulin gene therapy may also be a suitable therapeutic strategy to prevent some of the endocrine and metabolic alterations that typically appear in thymus-deficient animal models. The present article briefly reviews the literature on the physiology, molecular biology, and therapeutic potential of thymulin.

Keywords

thymulin; neuroendocrine control; hypophysiotropic activity; artificial gene; gene therapy; anti-inflammatory; ovarian dysgenesis

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Address for correspondence: Rodolfo G. Goya, INIBIOLP, Faculty of Medicine, UNLP, CC 455, 1900 La Plata, Argentina. Voice: 54-221-425-6735; fax: 54-221-425-0924/425-8988. E-mail: rgoya@netverk.com.ar (please CC to E-mail: preggiani@fcm.uncu.edu.ar).
*These two authors contributed equally to this work.

Conflicts of Interest

The authors declare no conflicts of interest.

Relevance of the Thymus in the Immune–Neuroendocrine Homeostatic Network

The immune system is functionally linked to the nervous and endocrine systems thus constituting an integrated homeostatic network.¹ Within this network, the neuroendocrine system monitors and controls the physical and chemical variables of the internal milieu. On its part, the immune system perceives, through antigenic recognition, an internal image of the macromolecular and cellular components of the body and reacts to alterations of this image, effectively participating in the “biological” homeostasis of the organism.

In mammals, the interaction of the thymus gland with the neuroendocrine system seems to be particularly important during perinatal life when the thymus and the neuroendocrine system influence the maturation of each other. This was initially suggested by early findings showing that in species in which neonatal thymectomy does not produce any evident impairment of immune capacity,² neuroendocrine functions are already highly developed at birth.³ In mice, the importance of the thymus for proper maturation of the neuroendocrine system is revealed by the endocrine alterations caused by neonatal thymectomy or congenital absence of the thymus. In effect, congenitally athymic (nude) female mice show significantly reduced levels of circulating and pituitary gonadotropins, a fact that seems to be causally related to a number of reproductive derangements described in these mutants.⁴ Thus, in homozygous (nu/nu) females the times of vaginal opening and first ovulation are delayed,⁵ fertility is reduced,⁴ and follicular atresia is increased such that premature ovarian failure results.⁶ Similar abnormalities result from neonatal thymectomy of normal female mice.^{7,8} Ovaries of athymic mice respond normally to exogenous gonadotropins, suggesting that the defect is at the level of the hypothalamic–pituitary axis.^{9,10} In homozygous, adult, nude CD-1, male mice, thyrotropin (TSH), prolactin (PRL), growth hormone (GH), and gonadotropin responses to immobilization and cold stress are reduced as also are serum basal levels of the same hormones compared to the heterozygous counterparts.^{11–13} A functional impairment of the hypothalamic–adrenal axis has been reported in nude mice, suggesting that humoral thymic factors may play a role in the maturation of this axis.¹⁴

The influence of the neuroendocrine system on thymus (and immune) function seems to continue during adult life either through a direct action of pituitary hormones or via peripheral hormones, both of which act on the thymic epithelial cells (TEC) and/or on immature thymocytes within the gland (for a review, see Ref. 15).

Thymulin

Thymulin is a thymic metallopeptide involved in several aspects of intrathymic and extrathymic T cell differentiation.¹⁶ Thymulin, which is exclusively produced by the thymic epithelial cells,¹⁷ 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,¹⁸ which confers biological activity to the molecule.¹⁹ The metallopeptide active form bears a specific molecular conformation that has been evidenced by nuclear magnetic resonance.²⁰

Neuroendocrine Control of Thymulin Production

The control of thymulin secretion seems to be dependent on a complex network of events. Initial studies showed that the hormone itself exerts a controlling feedback effect on its own secretion both *in vivo* and *in vitro*.^{21,22} Additionally, thymulin production and secretion is influenced directly or indirectly by the neuroendocrine system. For instance, GH can influence thymulin synthesis and secretion. *In vitro*, human GH can stimulate thymulin release from TEC lines,²³ which are known to possess specific receptors for GH.²⁴ Animal studies have shown

that treatment of aged dogs with bovine GH partially restored their low thymulin serum levels.²⁵ In old mice, treatment with ovine GH increased their low circulating thymulin levels and enhanced the concanavalin A-dependent proliferative response of their thymocytes as well as interleukin-6 production.²⁶ In old rats, combined treatment with GH and thyroxine (T₄) was also able to partially restore their reduced thymulin levels.²⁷ In clinical studies, it was reported that in congenitally GH-deficient children who consistently exhibited low plasma thymulin levels GH therapy succeeded in increasing thymic hormone levels to near normal values.²⁸ Acromegalic middle-aged patients have elevated thymulin serum levels compared to age-matched normal subjects.^{23,28} It is likely that these effects of GH are mediated, at least in part, by insulin-like growth factor 1 (IGF-1) as suggested by the fact that the GH-induced enhancement of thymulin production could be prevented by previous treatment with antibodies against IGF-1 or IGF-1 receptor.²³

There is also evidence for a PRL–thymulin axis. Thus, it is known that TEC possess PRL receptors²⁹ and that PRL can stimulate thymulin synthesis and secretion both *in vitro* and *in vivo*.³⁰ Furthermore, administration of PRL to old mice elevated their reduced circulating levels of thymulin.³⁰

The thyroid axis also influences thymulin secretion. Thus, T₄ has been shown to stimulate thymulin synthesis and secretion in mice.³¹ *In vivo* treatment of mice with triiodothyronine enhanced thymulin secretion, whereas treatment of the animals with propylthiouracil, an inhibitor of thyroid hormone synthesis, decreased their circulating thymulin levels.³² In humans, hyperthyroidism brings about an increase in circulating thymulin levels, whereas hypothyroid patients show depressed levels of this thymic hormone.³³ In *in vitro* studies, it was shown that thyroid hormones stimulate thymulin secretion by a direct action on TEC.^{34,35} Interestingly, it has been shown that treatment of aged animals with T₄ can reverse their decreased thymulin levels.^{31,35}

Although there are no studies documenting a direct effect of gonadotropins or adrenocorticotropic hormone (ACTH) on thymulin secretion gonadectomy or adrenalectomy in mice is known to induce a transient decrease in serum thymulin levels. This effect is potentiated by the simultaneous removal of the adrenals and gonads.³⁴ In TEC cultures it was shown that exposure to physiological levels of glucocorticoids or gonadal steroids enhanced thymulin concentration in the cell supernatants.^{36,37}

Although there is no rigorous evidence proving the existence of hypothalamic factors able to influence thymulin production by a direct action on TEC, there are two studies that suggest that this may be the case. Treatment of old mice with hypothalamic extracts from young mice resulted in the reappearance of detectable levels of circulating thymulin.³⁸ Hypothalamic and pituitary extracts from young mice stimulated thymulin release from TEC cultures, but this stimulation declined when the pituitary and hypothalamic extracts were obtained from old mice.³⁹

Hypophysiotropic Activity of Thymulin

The multilateral influence that the neuroendocrine system exerts on thymulin secretion suggests that this metalloprotein could, in turn, be part of a feedback loop acting on neuroendocrine structures. This possibility is now supported by a significant body of evidence indicating that thymulin possesses hypophysiotropic activity. Thus, thymulin has been shown to stimulate luteinizing hormone (LH) release from perfused rat pituitaries⁴⁰ and ACTH from incubated rat pituitary fragments, the latter being an effect mediated by intracellular cAMP and cGMP accumulation.⁴¹ In an *in vitro* study using pituitary cells obtained from female rats in different days of the estrous cycle, it was observed that thymulin modulates the stimulatory activity of gonadotropin-releasing hormone on LH and follicle-stimulating hormone (FSH)

release.⁴² Thymulin has been found to stimulate GH, PRL, TSH, and gonadotropin release in dispersed rat pituitary cells at doses from 10^{-8} to 10^{-3} mol/L,^{43–45} whereas others have reported that thymulin doses of 10^{-11} mol/L stimulate LH, inhibit PRL release, and have no effect on GH secretion in incubated rat pituitary fragments.⁴⁰ The stimulatory effect of thymulin on hormone release in rat pituitary cells declines with the age of the cell donor, which suggests that aging brings about a desensitization of the pituitary gland to thymic signals.^{43–45}

There is *in vitro* and *in vivo* evidence suggesting that thymulin plays a role in the regulation of female spontaneous puberty, possibly through effects on pituitary gonadotropin release and ovarian steroidogenesis.^{40,42,44–47} Thymulin also modulates gonadotropin-induced testicular steroidogenesis.⁴⁸

Recent immunoneutralization studies have strengthened the hypothesis that thymulin is a physiological 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 most anterior pituitary endocrine cell populations when the animals reached puberty.⁴⁹ Thymulin immunoneutralization from birth to puberty in normal mice also induced serum gonadotropin⁵⁰ and serum TSH, PRL, and GH reduction (Goya *et al.*, unpublished results) when the animals reached puberty.

Construction of Synthetic Genes for Thymulin

The prospect of implementing thymic hormone gene therapy appears as an interesting avenue of research aimed at restoring endocrine thymic activity when thymus function is compromised. However, none of the genes coding for the known thymic peptides have been cloned, a situation that hinders the implementation of gene or other molecular therapies for thymic hormones. It was suggested that a possible way to overcome this problem could be to construct “artificial genes” coding for those thymic peptides whose amino acid sequences were short and required no posttranslational processing.⁵¹ This has been recently achieved for thymulin and the corresponding DNA sequence cloned in a recombinant adenoviral (RAD) vector that was subsequently used in a number of experimental gene therapy studies (see below).

In an early study aimed at upscaling thymulin production, a synthetic DNA sequence coding for FTS was inserted into a bacterial expression vector and successfully used to obtain large quantities of purified thymulin retaining full biological activity.⁵² More recently, a DNA sequence coding for the biologically active FTS analogue called *metFTS* was constructed and cloned in an adenoviral vector.⁵³ The design of the DNA sequence for *metFTS* 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 (Fig. 1A). A variant of this sequence was used to construct RAD-*metFTS*, an adenoviral vector that harbors the synthetic gene for *metFTS* driven by the mouse cytomegalovirus promoter (PmCMV) (Fig. 1B and C). When intramuscularly (i.m.) administered to thymectomized (Tx) mice and rats (whose circulating levels of thymulin are nondetectable), RAD-*metFTS* induced sustained supraphysiological serum levels of biologically active thymulin that remained high for at least 112 days in mice⁵³ and for over 320 days in rats. Interestingly, adenovirally mediated expression of the synthetic gene for *metFTS* in the substantia nigra and hypothalamus of adult Tx rats had a significantly longer duration than adenovirally mediated expression of the gene for green fluorescent protein or *Escherichia coli* β -galactosidase in the same brain regions.⁵⁴ This phenomenon could be a result of the anti-inflammatory activity in the brain reported for thymulin and some thymulin analogues.^{55,56} Additionally, results from experiments using intracerebroventricular injection of thymulin in rats with experimentally induced brain inflammation suggested that this peptide has a neuroprotective role in the central nervous system and indicate a possible

therapeutic use as analgesic and anti-inflammatory drug.⁵⁷ The anti-inflammatory activity could prevent the immune system of the vector-injected animals from mounting a destructive response against the transduced cells. The same rationale could explain the long-term persistence of high concentrations of transgenic metFTS in the circulation of RAd-metFTS-injected Tx rodents.⁵³ Because thymulin has no known toxic effects even at high doses, i.m. injection of RAd-metFTS could generate sustained pharmacologically effective levels of serum and brain thymulin for the amelioration of pathologies involving chronic brain inflammation. This would represent a distinct advantage over alternative anti-inflammatory approaches that use direct brain injection of viral vectors that block the production or actions of pro-inflammatory cytokines.⁵⁸

Gene Therapy for Thymulin

Neonatal Thymulin Gene Therapy Prevents the Disruptive Impact of Athymia on the Reproductive System of Female Mice

A single i.m. injection of RAd-metFTS in newborn nude mice (nude mice have undetectable circulating levels of thymulin) elicited long-term restoration of serum thymulin in these mutants. This treatment was able to prevent the deficits in serum LH and FSH that typically appear in adult female nude mice.⁵⁰ Furthermore, neonatal thymulin gene therapy in nude female mice has been found to significantly prevent the ovarian dysgenesis that usually develops in 70-day-old, female, nude mice.⁵⁹

Effect of Neonatal Thymulin Gene Therapy on the Metabolic Dyshomeostasis in Nude Mice

There is evidence that the endocrine thymus may participate in glucose homeostasis. Thus, it has been reported that after 1 month of age, nude BALB/c mice develop spontaneous hyperglycemia and impaired glucose tolerance.^{60,61} Furthermore, these animals show peripheral insulin insensitivity with normal pancreatic β -cell reserves and normal lean body mass.⁶² Assessment of pancreatic islet cell populations in hyperglycemic nude mice revealed an increase in the D cell population (somatostatin producing). Also, somatostatin content in pancreatic tissue was higher in the athymic nude mice compared to heterozygous sex- and age-matched counterparts.⁶³ Adult thymectomy in Wistar rats was reported to increase circulating insulin levels without significant changes in blood glucose.⁶⁴ Other studies point to a modulatory activity of thymic factors on lipid metabolism. Thus, a thymic protein factor was reported to reduce serum cholesterol levels in rodents, increase low-density lipoprotein catabolism, and inhibit the activity of hepatic 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, the rate-limiting enzyme in cholesterol synthesis.^{65–67} In adult nude mice it has been reported that the relative percentage of 16:0, 18:1 n9, and 18:1 n7 fatty acids is lower whereas that of 18:0, 20:4 n6, and 22:6 n3 fatty acids is higher in hepatic phospholipids of nu/nu animals compared to nu/+ counterparts. Some of these alterations were completely or partially prevented by neonatal thymulin gene therapy.⁶⁸ Neonatal thymulin gene therapy completely prevented the adult-onset hyperglycemia of 70-day-old nude mice (Garcia-Bravo *et al.*, unpublished results).

Concluding Remarks

Thymulin is probably the best characterized of all putative thymic hormones and seems to play a physiological role in thymus–pituitary communication, particularly during perinatal life. Interest in the therapeutic use of thymulin flourished during the 1970s and 1980s when efforts were almost exclusively focused on using thymulin (and other thymic peptides) for the treatment of autoimmune and other immunopathologies as well as cancer.^{69,70} Subsequent studies, most of them carried out during the last 15 years, established that thymulin is active on the hypophysis and the brain. This awareness and the recent availability of a synthetic gene

for metFTS have opened new avenues for the exploration and eventual exploitation of the therapeutic potential of this metallopeptide.

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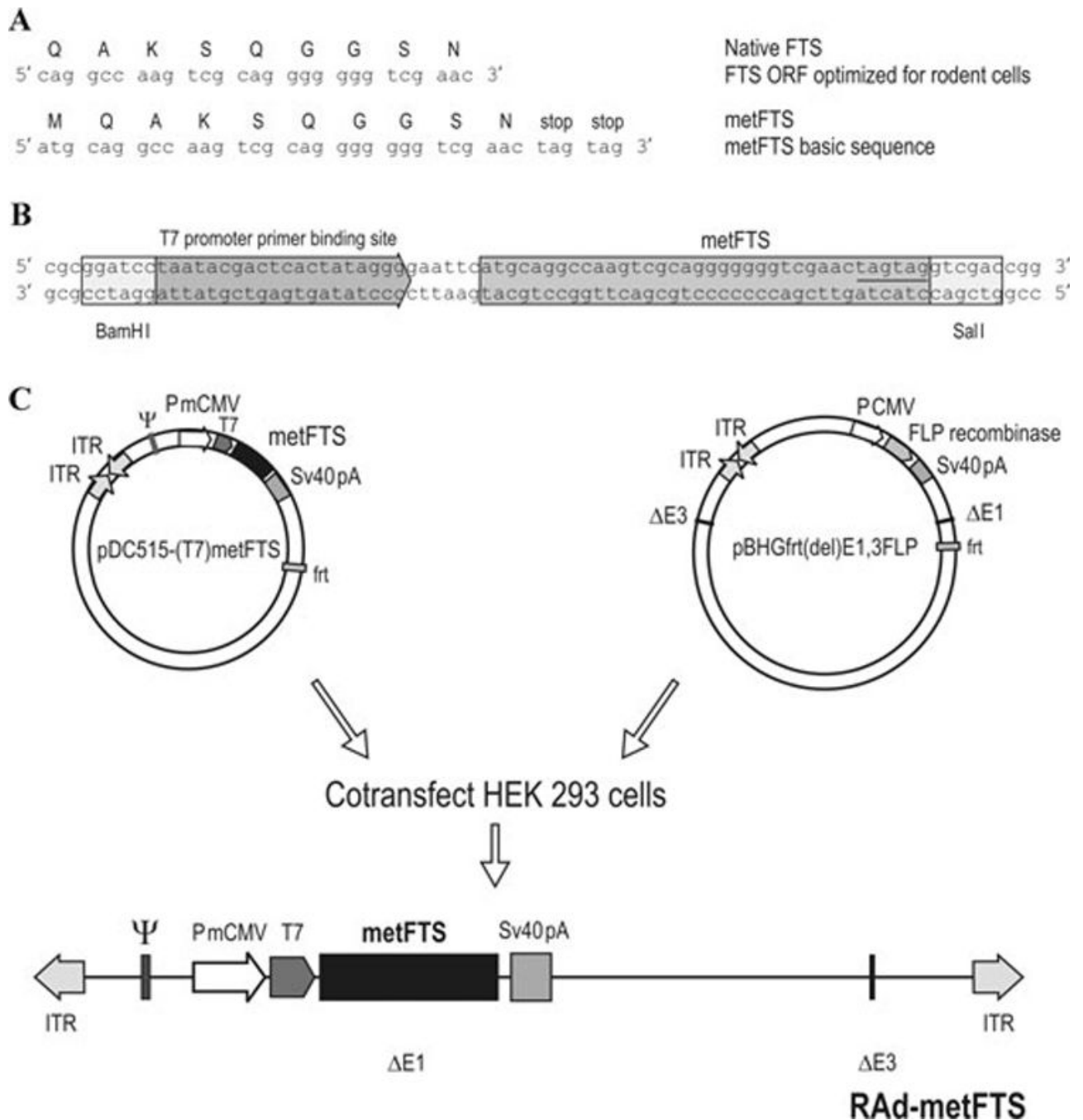


Figure 1. DNA constructs encoding the biologically active serum thymus factor (FTS) analogue (metFTS) and assembly of an adenoviral vector (RAD-metFTS) that harbors the synthetic gene for metFTS. A DNA sequence coding for native FTS was designed for optimal expression in rat cells. By adding an ATG starting codon upstream and two stop codons downstream of this sequence, it was converted into an open reading frame (ORF) for the analogue 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 BamHI and Sall sites of the multiple cloning site of the shuttle

pDC515. This construct was used to generate RAd-metFTS (C). PmCMV, mouse cytomegalovirus promoter; frt, recognition element for the yeast FLP recombinase; ITR, inverted terminal repeats; $\Delta E1$ and $\Delta E3$, deletions in the Ad5 genome; SV40, simian virus 40 polyadenylation signal; ψ , packaging signal. From Ref. 50, used with permission.