# LβT2 gonadotroph cells secrete follicle stimulating hormone (FSH) in response to activin A

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## Abstract

Secretion of luteinizing hormone in response to gonadotropin releasing hormone (GnRH) has been described in the recently developed LβT2 gonadotroph cell line. We evaluated the expression of follicle stimulating hormone (FSH)β mRNA and secretion of FSH from LβT2 cells in response to GnRH and activin A. LβT2 cells were treated with activin A in doses from 0 to 50 ng/ml, with or without a daily 10 nM GnRH pulse, or with GnRH alone. FSH secretion was stimulated over 6-fold by concomitant GnRH and activin A in a dose-responsive fashion at 72 h of treatment. FSHβ mRNA was detectable by ribonuclease protection assay only in cells treated with activin A with or without GnRH. The demonstration of FSHβ gene expression in LβT2 cells further validates these cells as mature, differentiated gonadotrophs and as an important tool for the study of gonadotroph physiology.

## Introduction

Studies of multiple aspects of the regulatory pathways of the gonadotropin follicle stimulating hormone (FSH) have been severely hampered by the lack of a highly-differentiated gonadotroph cell line that expresses the FSHβ gene and secretes FSH. Similar problems with the luteinizing hormone (LH) gene have been circumvented by expression of this gene in placental cell lines as well as in transgenic animals (Keri, *et al.* 1994, McNeilly, *et al.* 1996). Recently, the LβT2 cell line was derived from a pituitary tumor induced by targeted oncogenesis utilizing promoter sequences from the rat LHß gene linked to the protein-coding sequences of the SV40 T-antigen (Tag) oncogene (Turgeon, *et al.* 1994). These cells were reported to express and secrete LH, but not FSH (Turgeon, *et al.* 1996). We report here inducible expression of FSHβ subunit mRNA and secretion of FSH in LβT2 cells.

## Materials and Methods

### *Cell culture*

LβT2 cells were generously provided by Dr P Mellon and were grown in 6 well plates to approximately 50% confluence in DMEM containing 10% charcoal-treated fetal calf serum, 20 nM dexamethasone, 0.45% glucose, 50 U/ml penicillin G, 50 ug/ml streptomycin, 0.1 mM nonessential amino acids, and 2 mM L-glutamine. Serum was charcoal-treated to avoid potential inhibitory effects of gonadal steroids. For RNase protection assay, cells were grown in 1:1 DMEM/F12 HAM without phenol red, with L-glutamine and 15mM HEPES supplemented with 1 uM apo-transferrin, 5 ug/ml insulin, 100 uM putrescine and 60 nM sodium selenite. Drug treatments included recombinant human activin A (National Hormone Pituitary Program (NHPP), NIDDK, Dr A F Parlow) in doses ranging from 0-50 ng/ml and/or one hour pulses of 10 nM gonadotropin releasing hormone (GnRH, Sigma, St Louis, MO, USA) one to three times daily or activin alone for up to three days.

### *Radioimmunoassay and statistical analysis*

Conditioned media from approximately $10^6$ cultured cells were collected daily for FSH determination by RIA utilizing a rat FSH kit (NHPP, NIDDK and Dr A F Parlow), as previously described (Kumar *et al.* 1992). Sensitivity was 4.9 ng/ml. Data from six experiments were analyzed together by two-way ANOVA. One-way ANOVA was used to evaluate drug dose and time responses. Post-hoc analyses were done by the Tukey method.

### *RNase protection assay*

Total RNA from approximately $5x10^6$ cultured cells and individual male pituitary glands was isolated and mouse FSHß mRNA was detected by RNase protection assay. Total RNA was hybridized in solution with $2x10^5$ cpm of a low specific activity (estimated $5x10^6$ cpm/ug) [$^{32}$P]-rUTP- labeled anti-sense β-actin riboprobe and $9x10^5$ cpm of a high specific activity (est. $2x10^9$ cpm/ug) mFSHβ-specific riboprobe

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(corresponding to nucleotides 2790-3115 in exon 3 of mFSHβ (Kumar *et al.* 1995*a*)). Unprotected fragments were digested with a mixture of RNase A and T1 (Ambion, Austin, TX, USA). Protected bands were separated on a 5% denaturing polyacrylamide gel and imaged using a phosphorimaging system (Molecular Dynamics, Sunnyvale, CA, USA).

## Results

As previously reported by Turgeon *et al.* 1996, under basal conditions, FSH secretion from LβT2 cells was minimal and did not change over 72 h in culture (data not shown). Similarly, cells treated with a single (Fig. 1a) or thrice daily (not shown) one hour pulse of 10 nM GnRH alone also did not demonstrate significant FSH secretion. In contrast, activin A treatment of cells dramatically increased FSH secretion starting at 48 h of treatment (Fig. 1b). The response to activin A was potentiated at earlier time points (24 and 48 h) by simultaneous treatment with a once daily pulse of GnRH, but the maximal response was unchanged (Fig. 1c). The response to activin A was dose-dependent with significant increases in FSH secretion seen at 48 h starting at doses of 25 ng/ml (Fig. 1d, shown is treatment with activin A at various doses with a concurrent once daily pulse of GnRH; dose responsiveness was also seen with activin A treatment alone, data not shown).

Mouse FSHβ mRNA was undetectable in cells under basal conditions nor with GnRH stimulation. However, similar to the FSH secretory response, treatment with various doses of activin A with or without a daily one hour 10 nM GnRH pulse induced mFSHβ mRNA detected by RPA (Fig. 2).

## Discussion

This is the first report of an established gonadotroph cell line that expresses and secretes both FSH and LH. In the previous report of LH secretion from LβT2 cells (Turgeon *et al.* 1996), FSHβ expression was not detected, however this may have been due to the fact that only GnRH and steroid treatments

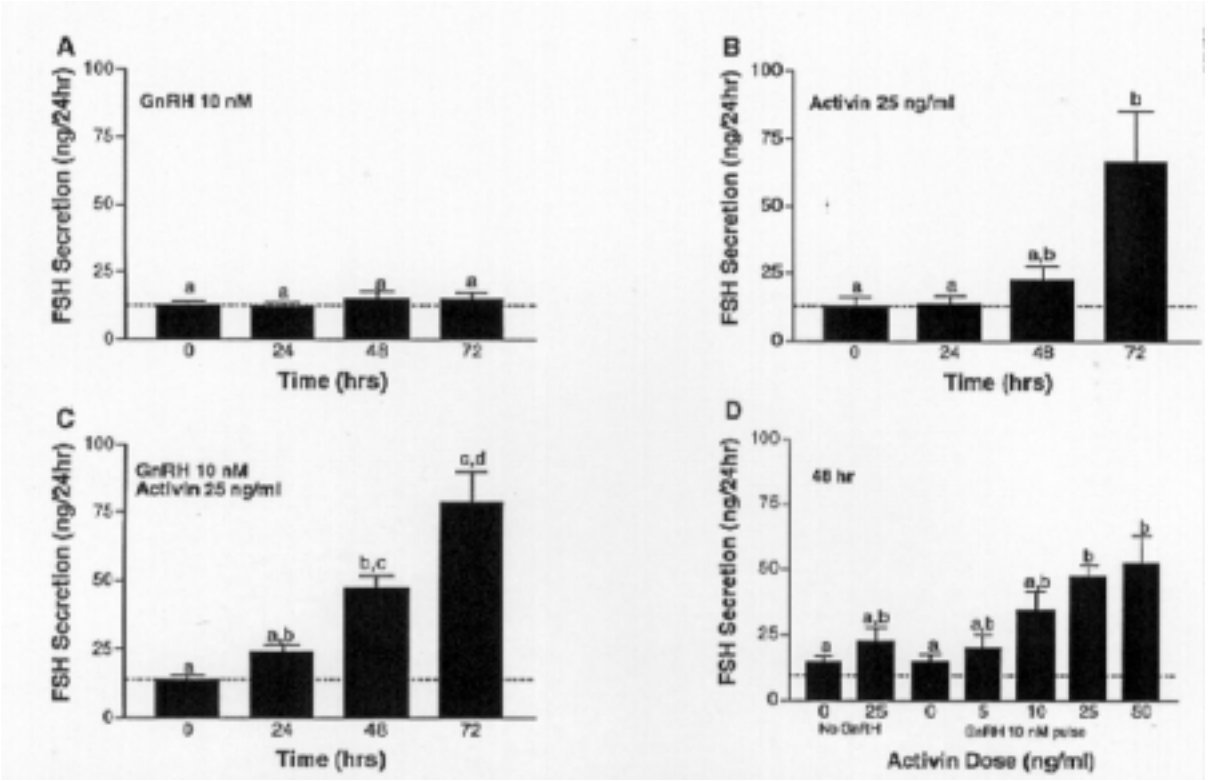


**Figure 1** FSH secretion from LβT2 cells in response to GnRH and activin A. For each panel, treatments that differ significantly by post-hoc analysis have no letters in common. Assay sensitivity is denoted by the dotted line. FSH secretion is minimal from untreated cells (not shown) or a) cells treated with a one hour daily pulse of 10 nM GnRH. b) Treatment with 25 ng/ml activin A stimulates FSH secretion 1.7-fold at 48 h and 5.0-fold at 72 h ($P$=0.02). c) Concurrent treatment with 25 ng/ml activin A and one hour daily pulse of GnRH potentiates FSH secretion, with a 3.6-fold increase by 48 h and 6.0-fold increase by 72 h ($P$<0.01). Maximal FSH response to activin A is not changed by addition of GnRH. d)FSH secretion at 48 h in response to increasing doses of activin A with a daily pulse of GnRH ($P$<0.005).

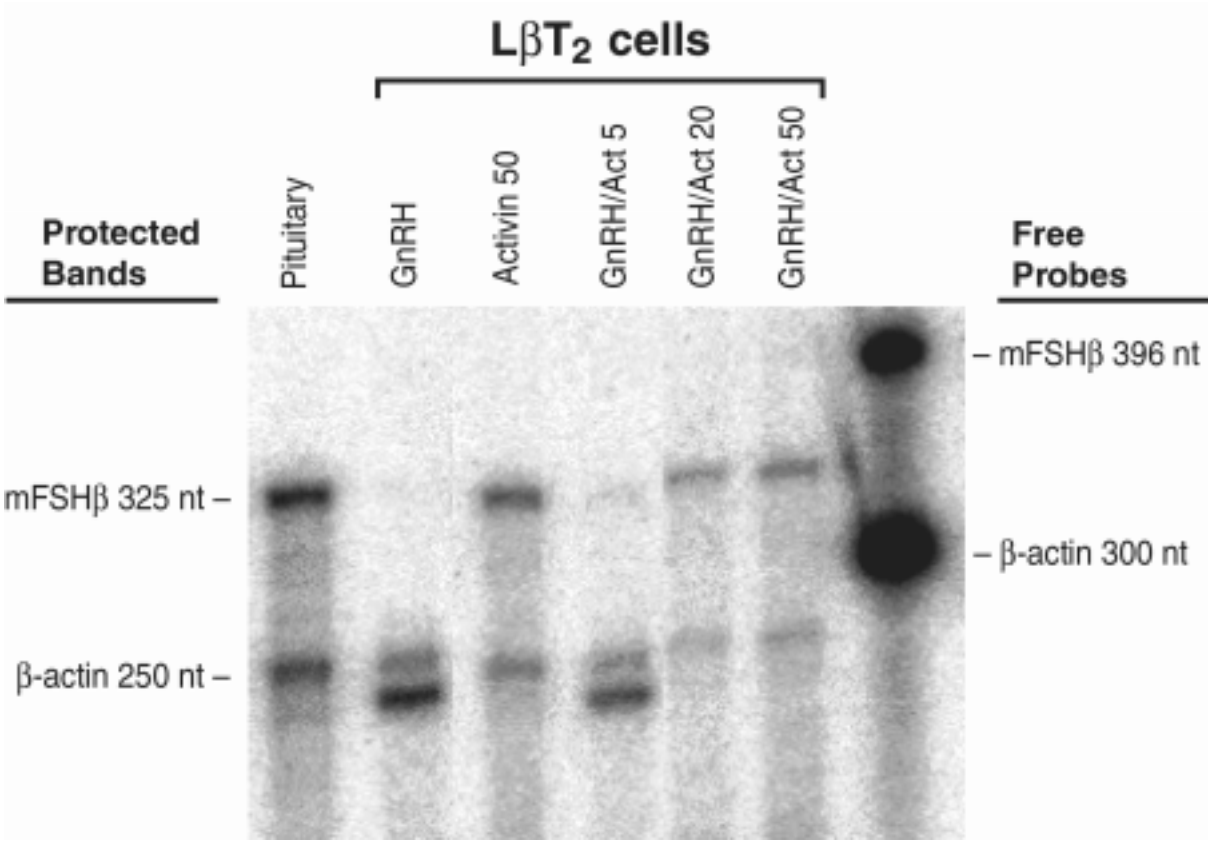


**Figure 2** RNase protection assay demonstrating expression of mouse FSHβ mRNA in LβT2 cells treated with GnRH alone, activin alone, increasing doses of activin A (5-50 ng/ml) and a daily one hour pulse of GnRH, all for 48 h. The bands shown are full length mFSHβ free probe (396 nt), full length β-actin free probe (300 nt) in the last two lanes, and protected mFSHβ (325 nt) and β-actin fragments (250 nt) for each of the LβT2 and control pituitary samples.

were given. The current study is consistent with those previous findings in that activin was required for stimulation of FSHβ expression and FSH secretion. Basal FSH secretion was low and the response to a single daily pulse of GnRH was also minimal. However, in the presence of activin A, FSH secretion increased dramatically on successive days, with synergism observed between the stimulatory effects of GnRH and activin A. The potentiation of FSH secretion on successive days of treatment was also similar to the previous report demonstrating greater maximal LH on successive days.

A second difference between the design of the previous study and the current one that may have influenced the ability to stimulate FSH secretion was the frequency of GnRH pulses. It is well established that lower pulse frequencies stimulate FSH secretion whereas higher pulse frequencies favor LH secretion and even decrease FSH expression and secretion in primary cultured rat pituitary cells (Ishizaka *et al.* 1992, Kaiser *et al.* 1995). When four pulses of 15 min duration were given every 90 min, LH secretion was stimulated (Turgeon *et al.* 1996); in the current experiments, a one hour daily pulse stimulated FSH secretion in preference to LH (LH data not shown). The interpulse intervals tested in these two studies differ from those previously optimized in primary rat pituitary cultures. However, it is already known that there are differences between LβT2 cells and rat pituitary cells with regard to GnRH desensitization (Turgeon *et al.* 1996, Weiss *et al.* 1995), although it is not possible to exclude the possibility that these differences were related to the use of static cultures vs. perifusion or paracrine actions from other pituitary cells in the primary culture. In addition, human pituitary adenomas demonstrate aberrant responses to GnRH agonists (Klibanski *et al.* 1989). The optimal pulse frequency will need to be empirically determined in this tumor cell line for both LH and FSH.

The requirement of exogenous activin A administration for FSH stimulation could be explained by a number of factors. There may be a loss of local activin production or over-production of follistatin as part of the oncogenic process or due to prolonged cell culture in the absence of GnRH. Although GnRH has been shown to increase activin production (Liu *et*

*al*. 1996), it was ineffective alone in these cells to stimulate FSH secretion. The dose of activin required for FSH secretion is higher than that observed in previous studies with perifused rat primary pituitary cells (Weiss *et al*. 1993), but similar to that in other studies using a static culture model (Attardi & Miklos 1990, Carroll *et al.* 1989) or in other tumor cells (Fernandez-Vazquez *et al.* 1996). Although activin A has been shown to stimulate the synthesis of GnRH receptors (Braden & Conn 1992), this is unlikely to be a significant factor given the robust LH secretory response to GnRH in the previous study (Turgeon *et al*. 1996) indicating the presence of functional GnRH receptors.

The time course of stimulation of FSH secretion is also similar to that seen in previous studies of activin action. We demonstrated increases in FSH secretion and FSHβ mRNA starting as early as 24 h after treatment. Stimulation of GnRH receptor synthesis has been reported to occur over 24 to 36 h (Fernandez-Vazquez *et al.* 1996) and although FSHβ and activin receptor (ActRI and ActRIIA) expression are observed as soon as 2 h after activin stimulation (Dalkin *et al.* 1996, Carroll *et al*. 1991), FSH secretion in many systems is not observed until after 14 h or more of treatment (Carroll *et al.* 1989, Schwall *et al.* 1988). We have not yet evaluated earlier time points for changes in steady-state FSHβ mRNA, however, secretion clearly increases with continued activin treatment Alternatively, there is evidence that activin has differentiating effects on pituitary cells (Childs & Unabia 1997) and we cannot exclude the possibility that 24 to 48 h of activin treatment induced differentiating changes in these immortalized tumor cells that then allowed FSHβ mRNA expression.

Until now, study of the gonadotropin β-subunit genes has been done almost exclusively in transgenic mice or in primary rat pituitary cultures due to the lack of a well-differentiated gonadotroph cell line. Transgenic expression has the advantage of being 'truer' to expression of the endogenous gene, due to the stringency related to chromatin structure and issues such as imprinting, and is particularly advantageous in evaluating the intact hypothalamic-pituitary-gonadal axis allowing study of short and long feedback loops. Primary rat pituitary cultures have been a significant tool, but are not sustainable in culture. Further, although primary rat pituitary cultures in general demonstrate similar regulation to that observed in physiological studies in humans, we have observed some differences among the human FSHβ and mouse FSHβ genes in transgenic animals and the rat FSHβ gene, specifically with regard to androgen regulation (Kumar & Low 1995*b*). We postulate that the mouse gene may be more comparable to the human gene, making the LβT2 cell line, derived from a mouse pituitary adenoma, a more appropriate model. In addition, we are currently evaluating this cell line for its potential in expressing transfected human FSHβ constructs.

The demonstration of expression of FSHβ mRNA and FSH secretion further validate the LβT2 cell line as a model of mature, fully differentiated gonadotrophs. A more detailed analysis of the response of LβT2 cells to gonadal steroids and peptides and GnRH is underway and will further clarify their usefulness for study of gonadotroph physiology.

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