Cell-specificity of transforming growth factor-β response is dictated by receptor bioavailability

Magdalena I Suszko1 and Teresa K Woodruff1, 2

1Department of Neurobiology and Physiology, Northwestern University, 2205 Tech Drive, Evanston, Illinois 60208, USA
2Department of Medicine, Northwestern Medical School, Chicago, Illinois 60611, USA

(Requests for offprints should be addressed to T K Woodruff; Email: tkw@northwestern.edu)

Abstract

Members of the transforming growth factor-β (TGFβ) family control diverse cellular responses including differentiation, proliferation, controlled cell death and migration. The response of a cell to an individual ligand is highly restricted yet the signaling pathways for TGFβ, activin and bone morphogenetic proteins share a limited number of receptors and activate similar intracellular cytoplasmic co-regulators, Smads. A central question in the study of this family of ligands is how cells titrate and integrate each TGFβ-like signal in order to respond in a cell- and ligand-specific manner. This study uses the pituitary gonadotrope cell line, LβT2, as a model to delineate the relative contribution of TGFβ and activin ligands to follicle-stimulating hormone (FSH) biosynthesis. It was found that pituitary gonadotrope cells do not express the TGFβ type II (TβRII) receptor and are therefore not responsive to the TGFβ ligand. Transfection of the receptor restores TGFβ signaling capabilities and the TGFβ-mediated stimulation of FSHβ gene transcription in LβT2 cells. Consequently, we evaluated the presence of the TβRII in the adult mouse pituitary. TβRII does not co-localize with FSH-producing cells; however it is detected on the cell surface of prolactin- and growth hormone-positive cells. Taken together, these results suggest that the bioavailability of the TGFβ-specific receptor rather than TGFβ dictates pituitary gonadotrope selectivity to activin, which is necessary to maintain normal reproductive function. It is likely that the ligand-restricted mechanisms employed by the gonadotrope are present in other cells, which could explain the distinct control of many cellular processes by members of the TGFβ superfamily.

Journal of Molecular Endocrinology (2006) 36, 591–600

Introduction

The ability of a single cell to respond to a particular cue is vital for normal function and survival of all organisms. As transforming growth factor-β (TGFβ) ligands control a variety of cellular responses, cells are constantly challenged by signals from multiple members of the superfamily. These ligands are key regulators of cell development, differentiation and proliferation, as well as migration and apoptosis. To date, 42 members of the TGFβ superfamily have been identified in the human genome and, based on a sequence similarity, two subfamilies of TGFβ-related proteins have been delineated. In addition to the TGFβ/activin/nodal subgroup, a bone morphogenic protein/growth and differentiation factors/Mullerian inhibiting substance (BMP/GDF/MIS) subfamily has been characterized. Interestingly, the diverse TGFβ ligands signal through a limited number of receptors and intracellular signaling proteins to elicit different cellular responses. There are five type II and seven type I serine/threonine kinase receptors available for initiation of the TGFβ signaling cascade. Ligand-activated receptors phosphorylate receptor-restricted Smad (R-Smad) proteins, which results in a propagation of the downstream intracellular signal. There are only five R-Smads that have been identified in mammals and, by convention, they fall into two groups based on their association with a particular TGFβ subfamily (reviewed in Shi & Massague 2003). It is intriguing that, given a limited number of available receptors and Smad molecules, the cell can still sense and respond to each ligand in a very precise manner.

A variety of mechanisms have been implicated in the tight control of cellular responses to the members of the TGFβ superfamily. The most obvious way of regulating ligand function is its spatial and temporal bioavailability. During Xenopus development, an increasing dose of activin leads to the sequential transcription of Xenopus brachyury (Xbra) mesoderm gene, which is followed by expression of goosecoid (gsc) and eomesoderm (eomes) genes (reviewed in Gurdon & Bourillot 2001). Another way of limiting ligand activity is through the expression of a bioneutralizing protein. Several high-affinity binding proteins have been identified for the TGFβ superfamily. These factors include follistatin, noggin, chordin/SOG, and proteins of the DAN family (reviewed in Bernard et al. 2001, Shi & Massague 2003, Zwijsen et al. 2003).
While these molecules can antagonize effects of the TGFβ ligands through direct binding, inhibin blocks activin action by sequestering the activin receptors (Lewis et al. 2000, Chapman & Woodruff 2001). This regulatory mechanism controls ligand access to receptors and requires membrane-anchored proteins that act as co-receptors, such as betaglycan, cripto and endoglin. Thus, this constitutes an important way of regulating ligand-specific responses in a cell- and tissue-specific manner.

In addition, receptor availability and activity in general can influence a particular pathway. In fact, receptor interactions at the level of the plasma membrane have been implicated as an important mechanism for the regulation of TGFβ signaling events. The type I TGFβ receptor has been found associated with cholesterol-rich membrane microdomains, known as caveolae, and this receptor positioning within a membrane suppresses TGFβ-mediated phosphorylation of Smad2. Conversely, ligand-bound TGFβ receptors can undergo endocytosis to early endosomes, where they phosphorylate Smads (Razani et al. 2001, Lu et al. 2002). The activity of receptors and, more importantly, the duration of their active state is also crucial for facilitating a particular cellular response. Receptor activity is required to maintain active Smad complexes in the nucleus and directly reflects the level of the specific signal up to the point of saturation and desensitization of the receptor complex.

In the reproductive axis, activin is essential for the biosynthesis of follicle-stimulating hormone (FSH) β. This regulation is specific to the anterior pituitary gonadotrope cells, where locally produced activin directly stimulates FSH production. Activity of this ligand is reduced by both follistatin and inhibin. Interestingly, both activin and inhibin share a number of their signaling components with a transduction cascade initiated by the TGFβ ligand. For example, both TGFβ and activin signal via the intracellular mediators, Smad2 and Smad3. In addition, both inhibin antagonism of activin action and TGFβ signaling require the co-receptor betaglycan (Lewis et al. 2000, Chapman & Woodruff 2001). Therefore, it became important to determine the relative contribution of these ligands to FSH biosynthesis and to delineate the gonadotrope-restricted TGFβ superfamily signaling pathways.

This report addresses essential questions regarding the ability of the pituitary gonadotrope to titrate and integrate different TGFβ signals. Characterization of processes by which the cell manages a discrete response, such as a precise FSH surge, is necessary for the understanding of normal cell function. These data demonstrate that one of the mechanisms that can regulate cell-specific responses is receptor bioavailability. More importantly, the data reveal that the gonadotrope cells are unable to discriminate between signaling pathways initiated by activin and the TGFβ receptor complex. In the light of these findings, receptor assembly, activation and desensitization become even more critical as a mechanism of regulating diverse cellular responses to TGFβ ligands.

Materials and methods

Recombinant ligands

Recombinant human (rh) activin A (activin) was produced in our laboratory (Pangas & Woodruff 2002). TGFβ1 ligand was purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

DNA constructs, cell culture and transient transfections

Reporter construct containing the rat FSHβ promoter gene fused to the luciferase reporter gene in the pXP2Δ2 vector (described previously by Suszko et al. 2003) and the p3TP-Lux reporter plasmid were provided by Dr J Massague’s laboratory. Constitutively active receptor (CA-ALK4 and CA-ALK5) expression vectors were a generous gift of Dr Y Chen and have been described previously (Chen et al. 1997, Nagarajan & Chen 2000).

The pituitary gonadotrope cell line, LβT2, was carried on plates coated with matrigel (BD Biosciences, Bedford, MA, USA) in F12:DMEM supplemented with 5% fetal bovine serum (FBS), 0.45% glucose and 1% antibiotic and kept in a humidified atmosphere (37 °C) of 5% CO₂. All transfections and experimental treatments have been described before (Suszko et al. 2003). Briefly, cells were plated one day before transfection in 24-well plates and transfected with 250 ng of the reporter DNA, 25 ng of the expression vector and 50 nM of the indicated small interfering (si) RNA oligoduplexes (Dharmacon, Inc. Lafayette, CO, USA) per well using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Empty vectors were used to balance DNA where necessary. Cells were treated with control media or appropriate ligands in phenol-free, serum-free F12:DMEM (Invitrogen). We attempted to utilize internal controls for all transfection experiments by dual luciferase and β-galactosidase assays. Unfortunately, co-transfection of both renilla-luciferase and β-galactosidase expression vectors caused a significant decrease in activin response. LβT2 cells were grown on a matrigel matrix, which interfered with normalization of luciferase activity to protein content. The data shown here reflect the mean fold over control of multiple repeatable transfection experiments.

Rat primary pituitary cell culture and radioimmunoassay

All animals were treated in full accordance with the NIH Guide for the Care and Use of Laboratory Animals.
Immature female Sprague–Dawley rats (Charles River Laboratories, Inc., Wilmington, MA, USA) were asphyxiated in a CO₂ chamber and decapitated. Pituitaries were collected and cells were dispersed with 0·4% porcine pancreatic trypsin dissociation medium as previously described, with modifications (Wilfinger et al. 1984, Krummen & Baldwin 1988). Cells were plated in 24-well plates at the confluency of 5 × 10⁴ cells per well. Cells were allowed to attach overnight and then treated with 30 ng/ml activin A or 10 ng/ml TGFβ1 in α-MEM medium supplemented with 10% charcoal-stripped FBS and 1% antibiotic (Invitrogen). Media were collected after 24 h treatment and submitted for FSH radioimmunoassay (Ligand Assay and Analysis Core Laboratory, Center for Research in Reproduction, University of Virginia, Charlottesville, VA, USA). FSH was determined by RIA with NIH-FSH RP-2 (NIDDK) as standard and anti-rFSH (S-11) as primary antibody. The limit of detection in the assay was 0·7 ng/ml with less than 0·5% cross-reactivity with other pituitary hormones. Intact intra-assay variation was 3·5% for the low control and 1·3% for the QCRS2 control. The average intra-assay and interassay coefficients of variation are from 8·7 to 14·4% respectively.

RT-PCR analysis

Total RNA from LβT2 cells and female mouse pituitaries was isolated using the TRIzol reagent (Invitrogen) and samples were treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and phenol–chloroform extracted. RNA samples (5 µg) were then primed with random hexamers and reverse transcribed with M-MLV reverse transcriptase (Promega) according to the manufacturer’s instructions. The cDNA from the original RT reaction was subjected to PCR amplification for 35 cycles under the following conditions: 94°C for 60 s, 54°C for 60 s, 72°C for 60 s. Negative controls were run using water and RNA that had not been reverse transcribed with M-MLV. Primers used to amplify ligand-specific type I and type II receptors are presented in Table 1.

Immunoblot analysis

LβT2 cells were plated in 6-well plates and transfected with 1 µg empty vector or expression vector containing TGFβ type II receptor (TβRII) cDNA, and were then treated for 1 h with control media, activin A (30 ng/ml) or TGFβ1 (10 ng/ml). Cells were lysed in buffer containing 50 mM Tris, pH 7·5, 10% glycerol, 5 mM EDTA, 150 mM NaCl and 0·5% NP-40 and supplemented with Complete Mini Protease Inhibitor Cocktail tablets (Roche, Indianapolis, IN, USA) and Phosphatase Inhibitor Cocktail II (Sigma, St Louis, MO, USA). Samples were run under reduced conditions on NuPage 4–12% Bis-Tris denaturing gels (Invitrogen) and transferred onto nitrocellulose. An immunoblot was performed using rabbit polyclonal antibodies. The phospho-Smad2 (3101) and phospho-Smad3/Smad4 (9514) antibodies were acquired from Cell Signaling Technologies (Beverly, MA, USA) and actin antibody was purchased from Sigma. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was obtained from Zymed Laboratories (San Francisco, CA, USA). For siRNA analysis, LβT2 cells were plated in 24-well plates and co-transfected with 100 ng of the indicated ALK or Smad expression vector and siRNA oligoduplexes for 24 h. Cells were lysed and cell lysates were run on a NuPage 4–12% gel as described above. An immunoblot analysis was performed using rabbit anti-hemagluttin (Zymed), mouse anti-Flag or mouse anti-Myc (Sigma) antibodies. Immunoblot results were visualized using the ECL detection reagent (Amersham Biosciences, Piscataway, NJ, USA).

Immunofluorescence

Pituitary paraffin sections mounted on slides were washed in PBS and incubated with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in PBS/0·3% Triton X-100 (PBST) buffer for 1 h at room temperature. Sections were then incubated overnight at 4°C with cocktails of affinity purified goat antihuman TβRII IgG (10 µg/ml; R&D Biosystems) and rabbit antiserum directed against rat

Table 1 PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Expected size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ActRIIA</td>
<td>5’ ATGGGAGCTGCTGCAAAATTG 3’</td>
<td>505</td>
</tr>
<tr>
<td></td>
<td>3’ GGTAGGCCATCTGTGACTTGT 5’</td>
<td></td>
</tr>
<tr>
<td>ActRIIB</td>
<td>5’ ATCTACATAACGCCAAGTGGGA 3’</td>
<td>297</td>
</tr>
<tr>
<td></td>
<td>3’ TGCTGCTAGTGACTTCTTGG 5’</td>
<td></td>
</tr>
<tr>
<td>TβRII</td>
<td>5’ TGGCGCCTGCTACATGC 3’</td>
<td>320, 395</td>
</tr>
<tr>
<td></td>
<td>3’ GCATCTTCAGAGTGAAAGCG 5’</td>
<td></td>
</tr>
<tr>
<td>BMPRII</td>
<td>5’ ATGACCTCCTGGTGACATCG 3’</td>
<td>572</td>
</tr>
<tr>
<td></td>
<td>3’ GGCCTCATGTTCTAGCTG 5’</td>
<td></td>
</tr>
<tr>
<td>ALK1</td>
<td>5’ ATGACCTGGGACCTTCCAAGA 3’</td>
<td>352</td>
</tr>
<tr>
<td></td>
<td>3’ GATGGGCGATGACTTCTGCTG 5’</td>
<td></td>
</tr>
<tr>
<td>ALK2</td>
<td>5’ TGGAGTAAATGATCCTTCTCTGTGC 3’</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>3’ TCTTACAGCTACCTCCTTCCCCGT 5’</td>
<td></td>
</tr>
<tr>
<td>ALK3</td>
<td>5’ CATCAGATTACTGGGAGGCTCTGCTT 3’</td>
<td>358</td>
</tr>
<tr>
<td></td>
<td>3’ CCGAACAACATTCTATTGCTCGCTC 5’</td>
<td></td>
</tr>
<tr>
<td>ALK4</td>
<td>5’ CAGACCACTACACCTGAGGAACACAG 3’</td>
<td>247</td>
</tr>
<tr>
<td></td>
<td>3’ ATGATAAGGAAAGGGAGAAAGGCAG 5’</td>
<td></td>
</tr>
<tr>
<td>ALK5</td>
<td>5’ GTCGCCAGCTCCTCCTGCTGT 3’</td>
<td>431</td>
</tr>
<tr>
<td></td>
<td>3’ CAGAAGGCGTTATGGGCAATAC 5’</td>
<td></td>
</tr>
<tr>
<td>ALK6</td>
<td>5’ TGCACGGAAGACTAGTTACAAAAA 3’</td>
<td>293</td>
</tr>
<tr>
<td></td>
<td>3’ CAGACAGTACAGAGAATAAGACGAG 5’</td>
<td></td>
</tr>
<tr>
<td>ALK7</td>
<td>5’ CACTGAGCTGGGCCCTCC 3’</td>
<td>291</td>
</tr>
<tr>
<td></td>
<td>3’ TTGGCGATGCTGTGGAAGA 5’</td>
<td></td>
</tr>
</tbody>
</table>
FSHβ, luteinizing hormone β (LHβ), prolactin or growth hormone (diluted 1:50 in PBST; NIDDK’s National Hormone and Pituitary Program). Following three washes in PBS, tissues were incubated for 1 h at room temperature with Cy3-conjugated donkey anti-goat IgG (1:400 in PBST) and FITC-conjugated donkey anti-rabbit IgG (both from Jackson ImmunoResearch Laboratories, Inc.). Slides were washed three times in PBS, briefly dried, mounted with Vectastain (Vector Laboratories, Inc., Burlingame, CA, USA) and sealed. Slides were viewed using fluorescence microscopy and digital images were collected at 40× magnification using a SpotRT monochrome digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) and analyzed using the Metamorph image analysis system (v.4.5; Universal Imaging Corp., West Chester, PA, USA).

**Statistical analysis**

The values are expressed as the mean fold over control ± s.e.m. Analysis of variance (ANOVA) followed by Tukey’s post hoc test was used to evaluate differences between treatment groups. P<0.01 and P<0.001 were considered statistically significant.

**Results**

**Activin but not TGFβ induces FSHβ transcription and FSH secretion**

We have previously demonstrated that the intracellular signaling molecule Smad3 is involved in activin-mediated stimulation of FSHβ gene transcription (Suszko et al. 2003, 2005). As both activin and TGFβ can induce Smad3 phosphorylation and nuclear translocation, we investigated whether TGFβ can, in a similar manner to activin, induce activity of the rat FSHβ promoter. The mouse gonadotrope-derived cell line, LBT2, was transiently transfected with 338 base pairs of the 5’-flanking region of rat FSHβ gene fused to the luciferase reporter gene (–338 rFSHβ-Luc) or to the plasminogen activator inhibitor-1 promoter ligated upstream of the luciferase reporter gene (p3TP-Lux) (Dennler et al. 1998). Cells were treated with control media, activin or TGFβ1 for 6 h and luciferase assays were performed. Transcriptional activity of both promoters was significantly stimulated by activin (P<0.001). However, neither promoter directed luciferase production in response to TGFβ (Fig. 1A,B).

To address whether the rat FSHβ promoter may lack elements necessary for TGFβ response, we investigated TGFβ-mediated secretion of FSH in a primary pituitary cell system. Dispersed rat pituitary cells were treated with activin or TGFβ1 and FSH levels in the media were measured (Fig. 1C). Activin, but not TGFβ, stimulated FSH secretion from the primary pituitary cells confirming the results of the transfection experiment.

**Constitutively active TGFβ-specific type I receptor ALK5 stimulates FSHβ transcription**

Ligands of the TGFβ superfamily initiate the signaling cascade through binding to a specific type II serine/threonine kinase receptor, which complexes with and phosphorylates a type I receptor. We examined whether a TGFβ-specific type I receptor, ALK5, can trigger molecular events leading to stimulation of the rat FSHβ promoter. LBT2 cells were transiently co-transfected with the –338 rFSHβ-Luc or p3TP-Lux promoter constructs and treated with activin A (30 ng/ml) or TGFβ1 (10 ng/ml) for 6 h. Data are plotted as the mean fold increase of the ligand-stimulated promoter activity over untreated control ± s.e.m. of three independent experiments, each performed in triplicate. (C) Dispersed rat pituitary cells were treated with control media, activin A (30 ng/ml) or TGFβ1 (10 ng/ml) for 24 h and FSH secretion was measured by radioimmunoassay. Hormone secretion is reported in ng per ml media and plotted as the mean ± s.e.m. of n=4 samples. **P<0.01, ***P<0.001.

**Figure 1.** TGFβ does not stimulate FSHβ transcription and FSH secretion. LBT2 cells were transiently transfected with –338 rFSHβ-Luc (A) or p3TP-Lux (B) promoter constructs and treated with control media, activin A (30 ng/ml) or TGFβ1 (10 ng/ml) for 6 h. Data are plotted as the mean fold increase of the ligand-stimulated promoter activity over untreated control ± S.E.M. of three independent experiments, each performed in quadruplicate. (C) Dispersed rat pituitary cells were treated with control media, activin A (30 ng/ml) or TGFβ1 (10 ng/ml) for 24 h and FSH secretion was measured by radioimmunoassay. Hormone secretion is reported in ng per ml media and plotted as the mean ± S.E.M. of n=4 samples. **P<0.01, ***P<0.001.
As TβRII is absent in the gonadotrope-derived LβT2 cell line, we examined whether this receptor is expressed in anterior pituitary, specifically in gonadotrope cells. Results of the immunofluorescence experiment show that TβRII localizes to somatotrope and lactotrope cells of adult mouse pituitary (Fig. 3C). Yellow overlay that is produced by co-localization of the receptor and either prolactin or growth hormone (panels c and d) is not present in cells immunoreactive for FSHβ and LHβ subunits (panels a and b). These data suggest that the absence of the TGFβ receptor from gonadotropes restricts TGFβ activity in this cell type and provides a mechanism of cell-specific ligand action. Consistent with our immunolocalization studies, the somato-lactotrope cell line, GH3, expresses TβRII and is TGFβ responsive (Yamashita *et al.* 1995). As expected, the rat FSHβ promoter was not responsive to either activin or TGFβ, whereas generic p3TP promoter was significantly induced by both ligands in these cells (data not shown).

Rescue of TGFβ-mediated stimulation of the rat FSHβ promoter by TβRII overexpression

As constitutively active ALK5 was able to induce activity of the FSHβ promoter, we examined whether TGFβ stimulation of FSHβ-subunit gene transcription requires a specific type II receptor. LβT2 cells were co-transfected with the –338 rFSHβ-Luc or the p3TP-Lux reporter construct and TβRII expression vector, and then treated for 6 h with activin or TGFβ1 (Fig. 4A). Co-transfection of the TGFβ-specific receptor led to a 2.7-fold increase in the TGFβ-stimulated p3TP promoter activity over untreated controls. The rat FSHβ promoter was also significantly stimulated (up to 2.1-fold) when treated with TGFβ1 in the presence of TβRII.

We also examined the effects of TβRII overexpression on activation of downstream signaling molecules, Smads. An immunoblot of lysates from LβT2 cells overexpressing TβRII shows increased phosphorylation of Smad proteins upon TGFβ1 treatment (Fig. 4B). Interestingly, in addition to activin and TGFβ-specific Smad molecules (Smad2 and Smad3), BMP-specific Smad (Smad1) was activated with TGFβ1 treatments in the presence of TβRII receptor.

Specific type I receptors mediate activin- and TGFβ-stimulated transcription of the FSHβ gene

It is known that a high degree of crosstalk between receptors and signaling molecules exists within the TGFβ superfamily. To establish specificity of the activin and TGFβ pathways in the LβT2 cell line, we used siRNA oligoduplexes to suppress expression of ALK4 and ALK5 proteins independently. First, to confirm the specificity of the siRNA duplexes, LβT2 cells were

---

**Figure 2** TGFβ pathway is inducible in the LβT2 cell line. LβT2 cells were transiently transfected with p3TP-Lux or –338 rFSHβ-Luc promoter constructs and empty vector or the indicated constitutively active type I (CA-ALK4 or CA-ALK5) receptor expression vectors. Data are plotted as the mean fold increase of the ALK-stimulated promoter activity over empty vector control ± S.E.M. of at least n = 5 independent experiments, each performed in quadruplicate. ***P < 0.001, statistically significant stimulation compared with empty vector control.
co-transfected with HA-tagged ALK4 and ALK5 expression vectors and the indicated siRNA oligo-
duplexes. As expected, there was a selective downregulation of ALK protein expression with the introduction of ALK4- or ALK5-directed siRNA (Fig. 5A). As Smad3 is important for FSHβ transcription we confirmed that ALK-specific siRNA had no e-

effect on expression of this protein. Next, we examined whether downregulation of endogenous ALK proteins has an effect on activin and TGFβ-mediated stimulation of FSHβ promoter. LβT2 cells were co-transfected with the –338 rFSHβ-Luc reporter construct and TβRII expression vector, as well as control, ALK4- or ALK5-specific siRNA duplexes (Fig. 5B). The cells were then treated with control media, activin or TGFβ1 for 6 h. The results of the luciferase assay show that downregulation of ALK4 reduced activin- and not TGFβ-mediated induction of the FSHβ promoter. Likewise, ablation of ALK5 protein expression caused a specific and significant decrease in stimulation of the promoter by TGFβ ligand. Similar results were observed with the p3TP-Lux promoter (data not shown). These data suggest that activin and TGFβ engage different receptor complexes in order to propagate specific signal transduction cascades.

Smad3 mediates induction of the FSHβ promoter by TGFβ ligand

We have previously demonstrated that Smad3 is necessary and sufficient for transcriptional regulation of the FSHβ-subunit in LβT2 cells (Suszko et al. 2003). Therefore, we wanted to investigate whether this cytoplasmic co-regulator is also involved in mediating the TGFβ response in our system. In addition, we have observed that in the presence of over-expressed TβRII this ligand can stimulate phosphorylation of Smad1. Hence, we also examined whether this BMP-specific factor has any biological significance in the regulation of FSHβ gene transcription. To address these questions, we employed siRNA oligoduplexes to suppress expression of Smad1 and Smad3 proteins independently. The specificity of the siRNA was confirmed by co-transfecting Smad expression vectors with the complimentary siRNA oligoduplexes and examining protein abundance by immunoblot analysis of cell lysates. As expected, there was a selective downregulation of Smad protein expression with the introduction of specific siRNAs (Fig. 6A,B). Next, we examined whether downregulation of endogenous Smad proteins has an effect on activin and TGFβ-mediated stimulation of FSHβ promoter. LβT2 cells were co-transfected with

Figure 3 TGFβ type II receptor is not expressed in the LβT2 cell line and it does not localize to gonadotrope cells of the anterior pituitary. LβT2 and mouse pituitary mRNA were reversely transcribed to cDNA. (A) Specific primer pairs were used to amplify cDNA of activin (ActRIIA and ActRIIB), TGFβ (TβRII) and BMP (BMPRII) type II receptors. (B) cDNA of type I receptors (ALK) for the TGFβ ligands were amplified. PCR products were resolved on a 2% agarose gel. (+) indicates positive control with no reverse transcriptase enzyme and (+) shows the RT-PCR product of appropriate size. (C) Mouse pituitary sections were co-incubated with antibodies specific to FSHβ (a), LHβ (b), prolactin (PRL; c) or growth hormone (GH; d) and TGFβ type II receptor protein. Immunoreactive signal was detected by immunofluorescence using FITC-conjugated anti-rabbit and Cy3-conjugated anti-goat secondary antibodies. Pictures were taken at 40× magnification. Overlay of FITC and Cy3 signals is colored yellow and indicated by arrowheads.
Progression of the female reproductive cycle depends on synchronized and repetitive synthesis and secretion of a variety of hormones. The gonadotropin hormones, FSH and LH, are integral components of the female reproductive system. FSH is primarily responsible for the maturation of ovarian follicles, while LH triggers ovulation and luteinization of the corpus luteum.

The -338 rFSHβ-Luc reporter construct and TβRII expression vector, as well as control, Smad1- or Smad3-specific siRNA duplexes (Fig. 6C). The cells were then treated with control media, activin or TGFβ1 for 6 h. As expected, downregulation of Smad3 reduced both activin- and TGFβ-mediated induction of the FSHβ promoter. In contrast, decreased Smad1 protein expression had no significant effects on the promoter activity of either ligand. Similar results were observed with the p3TP-Lux promoter (data not shown). Taken together, these results suggest that Smad3, and not Smad1, mediates TGFβ-dependent FSHβ gene transcription in LβT2 cells.

**Discussion**

Progression of the female reproductive cycle depends on synchronized and repetitive synthesis and secretion of a variety of hormones. The gonadotropin hormones, FSH and LH, are integral components of the female reproductive system. FSH is primarily responsible for the maturation of ovarian follicles, while LH triggers ovulation and luteinization of the corpus luteum.
reproductive axis and require specific cues from ovarian steroid and peptide hormones, as well as pituitary and hypothalamic regulatory factors. Both hormones are secreted by gonadotropes of the anterior pituitary and play important roles in follicular development. Structurally, the gonadotropins share an α-subunit (αGSU), which is common to all members of the pituitary glycoprotein family, while the β-subunit is distinctive to each member. Activin is a major regulator of FSH, which targets FSH through transcriptional regulation of the unique FSH β-subunit (Carroll et al. 1989, Weiss et al. 1995). Previous work demonstrated that activin stimulates FSHβ gene transcription directly and this regulation involves a proximal promoter region that binds both Smad3 and Smad4 proteins (Suszko et al. 2003, Gregory et al. 2005).

The limited number of receptors and intracellular Smad molecules that are available for transducing TGFβ signals suggests that there are mechanisms by which a cell, such as a gonadotrope, manages different hormonal inputs and confers cell type-specific responses. In fact, activin affects the expression of many genes but activin-regulated FSH biosynthesis is exclusive to pituitary gonadotropes. One way by which cells achieve specificity of ligand action is through transcriptional co-regulators. A number of tissue- and cell-specific transcription factors were identified, including the pituitary-specific members of the bicoid-related homeodomain proteins, Pitx1 and Pitx2, which permit the cell type-restricted FSH response (Zakaria et al. 2002, Suszko et al. 2003). However, given that members of the TGFβ superfamily can activate the same transduction pathways and that any cell can potentially respond to these signals, it is still a puzzle as to how the pituitary gonadotropes respond specifically to activin, and not to TGFβ, by producing FSH.

In our model gonadotrope-derived cell line, TGFβ does not induce FSHβ promoter transcription. We have established that TGFβ type II is not expressed in the LβT2 lineage, which explains the lack of TGFβ response in these cells. Indeed, overexpression of TβRII confers some degree of TGFβ responsiveness to the rat FSHβ promoter. Thus, one mechanism by which the gonadotrope controls its responses to TGFβ ligands is through the complement of expressed receptors. This is, in fact, the basis for specificity of many endocrinology and reproductive systems. For example, only ovarian granulosa cells can respond to FSH because only these cell express FSH receptor.

Although TGFβ and activin signals are initiated by different receptor complexes, these ligands share a set of intracellular mediators, Smad2 and Smad3. Smad3 has been shown to be important for activin-mediated transactivation of the FSHβ promoter (Suszko et al. 2003, Gregory et al. 2005). As expected, TβRII overexpression in LβT2 cells permitted TGFβ-mediated phosphorylation of Smad proteins. Interestingly, in addition to Smad2 and Smad3, Smad1 was also phosphorylated in the presence of the TβRII. However, downregulation of Smad1 protein expression by specific siRNA oligoduplexes had no effect on TGFβ-mediated stimulation of FSHβ gene. Whereas it is possible that Smad1 can mediate other responses to TGFβ ligand in LβT2 cells, this factor is not necessary for
TGFβ-mediated FSHβ gene regulation. In fact, the siRNA results suggest that Smad3 is important for mediating the TGFβ signal that leads to FSHβ transcription. These data suggest that the gonadotrope cell cannot discriminate between Smad3 phosphorylated via the activin and the TGFβ pathways and it responds to this signaling molecule in a similar manner. Intracellular signals initiated by TGFβ were able to stimulate FSHβ promoter activity suggesting that availability, assembly and activation of a specific receptor complex, rather than specificity of intracellular mediators, is necessary for a proper gonadotrope response to the extracellular ligand.

Regulation of the specific pathway through bioavailability of ligands and receptors is not entirely a new concept. In mammary tissue, for example, activin signaling components are found during mid to late lactation and then decrease during involution, while TGFβ levels are low during lactation to restrict hyperproliferation of the tissue (Jeruss et al. 2003). Also, temporal expression of activin and TGFβ signaling components is observed throughout follicular development. Expression of activin receptors is significantly more abundant in the early stages of follicles, while the presence of TβRII is restricted to the granulosa cells of antral follicles (Bristol & Woodruff 2004). Similarly, in our gonadotrope cell model, the lack of TβRII expression provides a mechanism of cell-restricted responses. This established system of available receptors may be necessary for normal function of the cell and any disturbance of this network may lead to abnormal gene regulation. Indeed, upregulation of activin type II receptor (ActRIIB) expression is prevalent in pituitary adenomas as compared with normal pituitary tissue (Alexander et al. 1996).

It is obvious that ligands of the TGFβ superfamily confer wide biological responses in different biological contexts. As the pituitary gonadotrope cells are sensitive to activin and not to TGFβ ligand, the question arises regarding the physiological relevance of such restricted FSHβ stimulation. One explanation lies in the negative control of activin action by the potent antagonist, inhibin. Inhibin is an endocrine hormone that is produced in a cycle-dependent manner by the ovary to provide a negative feedback directly at the level of the pituitary. Structurally, inhibin shares a β-subunit with activin and blocks activin signaling by forming an inactive complex with its receptors (Lewis et al. 2000, Chapman & Woodruff 2001). Although, inhibin has been shown to modestly antagonize TGFβ signaling (Wiater & Vale 2003), its primary role is to block activin. Thus the necessary positive and negative inputs exist for a dynamic regulation of the pituitary hormone synthesis and release. If TGFβ was permitted to regulate FSH even at low levels, reproductive health could be significantly compromised. Therefore, it is necessary for the pituitary gonadotrope to be able to respond only to a particular ligand in a very precise manner in order to control both specific hormone stimulation and down-regulation.

In conclusion, the results of these studies reveal a level of regulated cellular responses in the pituitary gonadotrope that may be important for normal reproductive function. These data suggest that receptor bioavailability can dictate specificity of TGFβ action in pituitary gonadotrope cells. More importantly, these results reveal that the gonadotrope cells are unable to discriminate between intracellular signaling pathways initiated by different TGFβ ligands. We predict receptor assembly as well as their activation and desensitization are important mechanisms in this and other systems that require filtering, integrating and discriminating various hormonal signals.

**Acknowledgements**

The authors are grateful to Dr U Kaiser and Dr J Massagué who kindly provided the full-length rat FSHβ and p3TP-Lux promoter constructs respectively. Constitutively active ALK plasmids were generous gifts of Dr Y Chen. Additional thanks go to Dr A F Parlow and the NIDDK National Hormone and Peptide Program for the FSHβ, LHβ, prolactin and growth hormone antisera. We would also like to credit the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (NCHD (SCCPPR) Grant U54-HD28934) for performing the FSH radioimmunoassay. Finally, our sincerest thanks to Dr M Antenos for invaluable help in preparing this manuscript.

This work was supported by NIH grant HD044464 (to T K W). During part of this study M I S was a Fellow of the Training Program in Reproductive Biology (HD00768). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

**References**


Received in final form 24 January 2006
Accepted 15 February 2006