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

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Abstract

Members of the transforming growth factor- β (TGF β) 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 TGF β , activin and bone morphogenic 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 TGF β -like signal in order to respond in a cell- and ligand-specific manner. This study uses the pituitary gonadotrope cell line, L β T2, as a model to delineate the relative contribution of TGF β and activin ligands to follicle-stimulating hormone (FSH) biosynthesis. It was found that pituitary gonadotrope cells do not express the TGF β type II (T β RII) receptor and are therefore not responsive to the TGF β ligand. Transfection of the receptor restores TGF β signaling capabilities and the TGF β -mediated stimulation of FSH β gene transcription in L β T2 cells. Consequently, we evaluated the presence of the T β RII in the adult mouse pituitary. T β RII 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 TGF β -specific receptor rather than TGF β 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 TGF β superfamily.

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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 (TGF β) 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 TGF β superfamily have been identified in the human genome and, based on a sequence similarity, two subfamilies of TGF β -related proteins have been delineated. In addition to the TGF β /activin/nodal subgroup, a bone morphogenic protein/growth and differentiation factors/Mullerian inhibiting substance (BMP/GDF/MIS) subfamily has been characterized. Interestingly, the diverse TGF β 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 TGF β signaling cascade. Ligand-activated receptors phosphorylate receptor-restricted Smad (R-Smad) pro-

teins, 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 TGF β 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 TGF β 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 *comesodermin* (*eomes*) genes (reviewed in Gurdon & Bourillot 2001). Another way of limiting ligand activity is through the expression of a bionutralizing protein. Several high-affinity binding proteins have been identified for the TGF β 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 Massagué'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 5×10^4 cells per well. Cells were allowed to attach overnight and then treated with 30 ng/ml activin A or 10 ng/ml TGF β 1 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 L β T2 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 μ g) 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

L β T2 cells were plated in 6-well plates and transfected with 1 μ g empty vector or expression vector containing TGF β type II receptor (T β RII) cDNA, and were then treated for 1 h with control media, activin A (30 ng/ml) or TGF β 1 (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

Table 1 PCR primers

	Primer sequence	Expected size (bp)
Gene		
ActRIIA	5' ATGGGAGCTGCTGCAAAGTTG 3' GGTAGGCCATCTTGTGATGTCTGT	505
ActRIIB	5' ATCTACTACAACGCCAACTGGGA 3' TGGCTCGTACGTGACTTCTGG	297
T β RII	5' TGGCCGCTGCATATCGTC 3' GCATCTTCCAGAGTGAAGCCG	320, 395
BMPRII	5' ATGACTTCCTCGCTGCATCG 3' GCCTCCATCATGTTTCATGCTG	572
ALK1	5' ATGACCTTGGGGAGCTTCAGA 3' GATGGGCATCAACTTCTGGCT	352
ALK2	5' TGGAGTAATGATCCTTCTGTGC 3' TCTTACACGTCATCTTCCCCTG	239
ALK3	5' CATCAGATTACTGGGAGCCTGTCT 3' CCGACAACATTCTATTGTCCTGC	358
ALK4	5' CAGACCAACTACACGTGTGAGACAG 3' ATGATAAGGAAGAGGAGGAAGACG	247
ALK5	5' GTCCGCAGCTCCTCATCGT 3' GACAGTGCGGTTATGGCAGAT	431
ALK6	5' TGTCCGGAAGACTCAGTCAACA 3' CAGACAGTCACAGATAGCAAGG	293
ALK7	5' CACTGAGCCTGGCCCTCC 3' TTGGCGATGCTGTGGGAA	291

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/phospho-Smad1 (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, L β T2 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-hemagglutinin (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 T β RII IgG (10 μ g/ml; R&D Biosystems) and rabbit antisera directed against rat

Figure 1 TGF β does not stimulate FSH β transcription and FSH secretion. L β T2 cells were transiently transfected with -338 rFSH β -Luc (A) or p3TP-Lux (B) promoter constructs and treated with control media, activin A (30 ng/ml) or TGF β 1 (10 ng/ml) for 6 h. Data are plotted as the mean fold increase of the ligand-stimulated promoter activity over untreated control $\pm$ 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 TGF β 1 (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 $\pm$ S.E.M. of $n=4$ samples. ^{**} $P<0.01$, ^{***} $P<0.001$.

FSH β , luteinizing hormone β (LH β), 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 $\times$ 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 $\pm$ 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 TGF β induces FSH β transcription and FSH secretion

We have previously demonstrated that the intracellular signaling molecule Smad3 is involved in activin-mediated stimulation of FSH β gene transcription (Suszko *et al.* 2003, 2005). As both activin and TGF β can induce Smad3 phosphorylation and nuclear

translocation, we investigated whether TGF β can, in a similar manner to activin, induce activity of the rat FSH β promoter. The mouse gonadotrope-derived cell line, L β T2, was transiently transfected with 338 base pairs of the 5'-flanking region of rat FSH β gene fused to the luciferase reporter gene (-338 rFSH β -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 TGF β 1 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 TGF β (Fig. 1A,B).

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

Constitutively active TGF β -specific type I receptor ALK5 stimulates FSH β transcription

Ligands of the TGF β 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 TGF β -specific type I receptor, ALK5, can trigger molecular events leading to stimulation of the rat FSH β promoter. L β T2 cells were transiently co-transfected with the -338 rFSH β -Luc or p3TP-Lux

Figure 2 TGF β pathway is inducible in the L β T2 cell line. L β T2 cells were transiently transfected with p3TP-Lux or -338 rFSH β -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 $\pm$ 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.

reporter construct and with constitutively active activin or TGF β type I receptor expression vectors (CA-ALK4 or CA-ALK5), and luciferase activity was measured (Fig. 2). As expected, p3TP promoter was highly responsive to overexpression of both ALK4 and ALK5 receptors. Interestingly, FSH β was also significantly activated not only by ALK4 but also by the TGF β -specific ALK5 receptor. In fact, stimulation of this promoter by ALK4 reached 2.1-fold over empty vector control, whereas ALK5 induced transcription by up to 1.7-fold.

Gonadotrope-derived cell line L β T2 and adult mouse pituitary gonadotrope cells lack T β RII

As constitutively active TGF β -specific type I receptor can induce FSH β promoter activity in L β T2 cells, we concluded that downstream components of the TGF β ligand signaling are intact in these cells. Therefore, we examined the presence of receptors necessary for initiation of signaling cascades by TGF β ligands in the L β T2 cell line (Fig. 3A,B). The presence of mRNA transcripts for both activin type II receptors, ActRIIA and ActRIIB, as well as for the BMP-specific type II receptor, BMPRII, was detected in these cells. Importantly, T β RII mRNA was not detected in L β T2 cells, although both transcript variants of this gene were found in the mouse pituitary. All mRNA transcripts of type I receptors of the TGF β superfamily, including TGF β -specific ALK5, were detected in L β T2 cells.

As T β RII is absent in the gonadotrope-derived L β T2 cell line, we examined whether this receptor is expressed in anterior pituitary, specifically in gonadotrope cells. Results of the immunofluorescence experiment show that T β RII 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 FSH β and LH β subunits (panels a and b). These data suggest that the absence of the TGF β receptor from gonadotropes restricts TGF β 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 T β RII and is TGF β responsive (Yamashita *et al.* 1995). As expected, the rat FSH β promoter was not responsive to either activin or TGF β , whereas generic p3TP promoter was significantly induced by both ligands in these cells (data not shown).

Rescue of TGF β -mediated stimulation of the rat FSH β promoter by T β RII overexpression

As constitutively active ALK5 was able to induce activity of the FSH β promoter, we examined whether TGF β stimulation of FSH β -subunit gene transcription requires a specific type II receptor. L β T2 cells were co-transfected with the -338 rFSH β -Luc or the p3TP-Lux reporter construct and T β RII expression vector, and then treated for 6 h with activin or TGF β 1 (Fig. 4A). Co-transfection of the TGF β -specific receptor led to a 2.7-fold increase in the TGF β -stimulated p3TP promoter activity over untreated controls. The rat FSH β promoter was also significantly stimulated (up to 2.1-fold) when treated with TGF β 1 in the presence of T β RII.

We also examined the effects of T β RII overexpression on activation of downstream signaling molecules, Smads. An immunoblot of lysates from L β T2 cells overexpressing T β RII shows increased phosphorylation of Smad proteins upon TGF β 1 treatment (Fig. 4B). Interestingly, in addition to activin and TGF β -specific Smad molecules (Smad2 and Smad3), BMP-specific Smad (Smad1) was activated with TGF β 1 treatments in the presence of T β RII receptor.

Specific type I receptors mediate activin- and TGF β -stimulated transcription of the FSH β gene

It is known that a high degree of crosstalk between receptors and signaling molecules exists within the TGF β superfamily. To establish specificity of the activin and TGF β pathways in the L β T2 cell line, we used siRNA oligoduplexes to suppress expression of ALK4 and ALK5 proteins independently. First, to confirm the specificity of the siRNA duplexes, L β T2 cells were

co-transfected with HA-tagged ALK4 and ALK5 expression vectors and the indicated siRNA oligoduplexes. 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 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 complementary 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 negative 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.

Figure 4 TGF β type II receptor is necessary to confer TGF β responsiveness in the L β T2 cell line. (A) L β T2 cells were transiently co-transfected with -338 rFSH β -Luc or p3TP-Lux promoter constructs and vector encoding TGF β type II receptor cDNA. Cells were treated with control media, activin A (30 ng/ml) or TGF β 1 (10 ng/ml) for 6 h. Data are plotted as the mean fold increase of the ligand-stimulated promoter activity over untreated control $\pm$ S.E.M. of four independent experiments, each performed in quadruplicate. *** P <0.001, statistically significant difference between treatment groups and untreated control. (B) L β T2 cells were transfected with empty vector or T β RII expression vector. Cells were treated with control media, activin A or TGF β 1 for 1 h. Phosphorylation of the Smad proteins was evaluated by the immunoblot using polyclonal anti-phosphoSmad antibodies.

the -338 rFSH β -Luc reporter construct and T β RII expression vector, as well as control, Smad1- or Smad3-specific siRNA duplexes (Fig. 6C). The cells were then treated with control media, activin or TGF β 1 for

Figure 5 ALK5 mediates the TGF β pathway in the L β T2 cell line. L β T2 cells were transfected with HA-tagged type I (ALK4 or ALK5) receptor expression vectors and the indicated siRNA oligoduplexes. Expression of ALK proteins was evaluated by the immunoblot using polyclonal anti-HA antibody. cont., control. (B) L β T2 cells were transiently transfected with -338 rFSH β -Luc promoter construct, T β RII expression vector and the indicated control or type I receptor (ALK4 or ALK5) siRNA oligoduplexes. Cells were treated with control media, activin A or TGF β 1 for 6 h and luciferase assay was performed. Data are plotted as the mean fold increase of the ligand-stimulated promoter activity over untreated control $\pm$ S.E.M. of four independent experiments, each performed in quadruplicate. *** P <0.001, statistically significant difference between treatment groups and untreated control.

6 h. As expected, downregulation of Smad3 reduced both activin- and TGF β -mediated induction of the FSH β 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 TGF β -dependent FSH β gene transcription in L β T2 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

Figure 6 Smad3 is involved in TGFβ-mediated activation of the FSHβ promoter. LβT2 cells were transfected with Flag-tagged Smad1 (A) or Myc-tagged Smad3 (B) expression vectors and increasing amounts of Smad-specific siRNA oligoduplexes. Expression of Smad proteins was evaluated by immunoblot using monoclonal anti-Flag or anti-Myc antibody respectively. (C) LβT2 cells were transiently transfected with -338 rFSHβ-Luc promoter construct, TβRII expression vector and the indicated control, Smad1 or Smad3 siRNA oligoduplexes. Cells were treated with control media, activin A or TGFβ1 for 6 h. Data are plotted as the mean fold increase of the ligand-stimulated promoter activity over untreated control $\pm$ S.E.M. of three independent experiments, each performed in quadruplicate. *** $P < 0.001$, statistically significant difference between treatment groups and untreated control.

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 (α GSU), 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 homeo-domain 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 cells 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

TGF β -mediated FSH β gene regulation. In fact, the siRNA results suggest that Smad3 is important for mediating the TGF β signal that leads to FSH β transcription. These data suggest that the gonadotrope cell cannot discriminate between Smad3 phosphorylated via the activin and the TGF β pathways and it responds to this signaling molecule in a similar manner. Intracellular signals initiated by TGF β were able to stimulate FSH β 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 TGF β levels are low during lactation to restrict hyperproliferation of the tissue (Jeruss *et al.* 2003). Also, temporal expression of activin and TGF β 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 T β RII is restricted to the granulosa cells of antral follicles (Bristol & Woodruff 2004). Similarly, in our gonadotrope cell model, the lack of T β RII 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 TGF β superfamily confer wide biological responses in different biological contexts. As the pituitary gonadotrope cells are sensitive to activin and not to TGF β ligand, the question arises regarding the physiological relevance of such restricted FSH β 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 TGF β 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 TGF β 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 TGF β 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 TGF β 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.

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References

- Alexander JM, Bikkal HA, Zervas NT, Laws ER Jr & Klibanski A 1996 Tumor-specific expression and alternate splicing of messenger ribonucleic acid encoding activin/transforming growth factor-beta receptors in human pituitary adenomas. *Journal of Clinical Endocrinology and Metabolism* **81** 783–790.
- Bernard DJ, Chapman SC & Woodruff TK 2001 An emerging role for co-receptors in inhibin signal transduction. *Molecular and Cellular Endocrinology* **180** 55–62.
- Bristol SK & Woodruff TK 2004 Follicle-restricted compartmentalization of transforming growth factor beta superfamily ligands in the feline ovary. *Biology of Reproduction* **70** 846–859.
- Carroll RS, Corrigan AZ, Gharib SD, Vale W & Chin WW 1989 Inhibin, activin, and follistatin: regulation of follicle-stimulating

- hormone messenger ribonucleic acid levels. *Molecular Endocrinology* **3** 1969–1976.
- Chapman SC & Woodruff TK 2001 Modulation of activin signal transduction by inhibin B and inhibin-binding protein (INhBP). *Molecular Endocrinology* **15** 668–679.
- Chen Y, Bhushan A & Vale W 1997 Smad8 mediates the signaling of the ALK-2 [corrected] receptor serine kinase [published erratum appears in *PNAS* 1998 **95** 1968]. *PNAS* **94** 12938–12943.
- Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S & Gauthier JM 1998 Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO Journal* **17** 3091–3100.
- Gregory SJ, Lacza CT, Detz AA, Xu S, Petrillo LA & Kaiser UB 2005 Synergy between activin A and GnRH in transcriptional activation of the rat follicle-stimulating hormone beta (FSH β) gene. *Molecular Endocrinology* **19** 237–254.
- Gurdon JB & Bourillot PY 2001 Morphogen gradient interpretation. *Nature* **413** 797–803.
- Jeruss JS, Santiago JY & Woodruff TK 2003 Localization of activin and inhibin subunits, receptors and SMADs in the mouse mammary gland. *Molecular and Cellular Endocrinology* **203** 185–196.
- Krummen LA & Baldwin DM 1988 Regulation of luteinizing hormone subunit biosynthesis in cultured male anterior pituitary cells: effects of gonadotropin-releasing hormone and testosterone. *Endocrinology* **123** 1868–1878.
- Lewis KA, Gray PC, Blount AL, MacConell LA, Wiater E, Bilezikjian LM & Vale W 2000 Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. *Nature* **404** 411–414.
- Lu Z, Murray JT, Luo W, Li H, Wu X, Xu H, Backer JM & Chen YG 2002 Transforming growth factor beta activates Smad2 in the absence of receptor endocytosis. *Journal of Biological Chemistry* **277** 29363–29368.
- Nagarajan RP & Chen Y 2000 Structural basis for the functional difference between Smad2 and Smad3 in FAST-2 (forkhead activin signal transducer-2)-mediated transcription. *Biochemical Journal* **350** 253–259.
- Pangas SA & Woodruff TK 2002 Production and purification of recombinant human inhibin and activin. *Journal of Endocrinology* **172** 199–210.
- Razani B, Zhang XL, Bitzer M, von Gersdorff G, Bottinger EP & Lisanti MP 2001 Caveolin-1 regulates transforming growth factor (TGF)-beta/SMAD signaling through an interaction with the TGF-beta type I receptor. *Journal of Biological Chemistry* **276** 6727–6738.
- Shi Y & Massague J 2003 Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113** 685–700.
- Suszkowski MI, Lo DJ, Suh H, Camper SA & Woodruff TK 2003 Regulation of the rat follicle-stimulating hormone beta-subunit promoter by activin. *Molecular Endocrinology* **17** 318–332.
- Suszkowski MI, Balkin DM, Chen Y & Woodruff TK 2005 Smad3 mediates activin-induced transcription of follicle-stimulating hormone β -subunit gene. *Molecular Endocrinology* **19** 1849–1858.
- Weiss J, Guendner MJ, Halvorson LM & Jameson JL 1995 Transcriptional activation of the follicle-stimulating hormone beta-subunit gene by activin. *Endocrinology* **136** 1885–1891.
- Wiater E & Vale W 2003 Inhibin is an antagonist of bone morphogenetic protein signaling. *Journal of Biological Chemistry* **278** 7934–7941.
- Wilfinger WW, Larsen WJ, Downs TR & Wilbur DL 1984 An *in vitro* model for studies of intercellular communication in cultured rat anterior pituitary cells. *Tissue Cell* **16** 483–497.
- Yamashita H, Okadome T, Franzen P, ten Dijke P, Heldin CH & Miyazono K 1995 A rat pituitary tumor cell line (GH3) expresses type I and type II receptors and other cell surface binding protein(s) for transforming growth factor-beta. *Journal of Biological Chemistry* **270** 770–774.
- Zakaria MM, Jeong KH, Lacza C & Kaiser UB 2002 Pituitary homeobox 1 activates the rat FSH beta (rFSHbeta) gene through both direct and indirect interactions with the rFSHbeta gene promoter. *Molecular Endocrinology* **16** 1840–1852.
- Zwijnen A, Verschueren K & Huylebroeck D 2003 New intracellular components of bone morphogenetic protein/Smad signaling cascades. *FEBS Letters* **546** 133–139.

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