Histol Histopathol (2006) 21: 167-178
DOI: 10.14670/HH-21.167

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Identification of gene networks modulated by activin in LßT2 cells using DNA microarray analysis

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Summary. Activins, members of the TGFß family of proteins, are widely expressed in a variety of tissues. First identified based on their ability to regulate biosynthesis and secretion of follicle-stimulating hormone (FSH), activins have also been shown to modulate development, cell growth, apoptosis, and inflammation. Despite their many known functions, the precise mechanisms and downstream signaling pathways by which activins mediate their diverse effects remain unknown. We have used a DNA microarray assay to identify genes that are regulated by activin, alone or in combination with gonadotropin-releasing hormone (GnRH), another major regulator of FSH, in a murine gonadotrope-derived cell line (LßT2). We used mRNA from these cells to screen Affymetrix Mu74av2 mouse Gene Chip oligonucleotide microarrays, representing approximately 12,400 mouse genes. Treatment of LßT2 cells with activin A, a gonadotropin-releasing hormone agonist (GnRHA) or activin A plus GnRHA resulted in alterations in levels of gene expression that ranged in magnitude from 15 to 67-fold. Data analysis identified 268 transcripts that were up- or down-regulated by two-fold or more. Distinct sets of genes were affected by treatment with activin, GnRHA and activin plus GnRHA, suggesting interactions between activin and GnRHA. Changes in expression of seven randomly selected representative genes identified by the microarray technique were confirmed by real-time quantitative PCR and semi-quantitative reverse transcription/PCR (RT/PCR). Modulation of expression of genes by activin suggests that activin may mediate its effects through a variety of signaling pathways.

Key words: Activin, GnRH, FSH, LH, Microarray

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Introduction

Activins and inhibins are structurally related members of the transforming growth factor-ß (TGFß) family of growth factors, and were initially isolated based on their activity in regulating follicle-stimulating hormone (FSH) release from the anterior pituitary. Activins stimulate whereas inhibins inhibit FSH secretion (Woodruff and Mather, 1995; Woodruff, 1998). Activins are secreted by the gonads and within the pituitary gland, where they act as autocrine or paracrine regulators of the reproductive axis (Ying, 1988; Corrigan et al., 1991). In addition, activins are found to be expressed in many other tissues, including testis (Vliegen et al., 1993), ovary (Gurusinghe et al., 1995), endometrium (Otani et al., 1998), prostate (Mellor et al., 2000), breast (Di Loreto et al., 1999), adrenal gland (Spencer et al., 1992), retinal epithelium (Coulombe and Kos, 1997), placenta (Petraglia et al., 1992), bone marrow stroma (Yamashita et al., 1992), oocyte (Uchiyama et al., 1994) and pancreas (Ogawa et al., 1993). Activins are also expressed in several cancers, including ovarian prostate, testicular, breast, pituitary, endometrial, placental and liver cancers (Risbridger et al., 2001). In recent years, activins have been shown to regulate a number of additional biological functions, including erythroid differentiation, neural induction and nerve cell survival, Xenopus laevis embryonic mesoderm induction, bone growth promotion, somatostatin induction, cell proliferation, differentiation, and apoptosis (Chen et al., 2002; Welt et al., 2002).

The mature form of activin is a homodimer held together by a single disulfide bond. Five isoforms of the

Abbreviations: GnRH, Gonadotropin releasing hromone; FSH, Follicle-stimulating hormone; and TGFß, Transforming gorowth factor, Aebp1, AE binding protein 1, WNT5A, WNT-1 related protein 5A, Ptrj, protein tyrosine-like phosphatase receptor j, SDF-1, stormal cell derived factor, Myh8, perinatal skeletal myosin heavy chain polypetide 8, Ephx2, epoxide hydrolase 2, Tyrp1, tyosine-related protein, Erg1, early growth response1 and Egr2, early growth response 2.

activin monomeric subunits have been identified to date in different species. These include: ßA, ßB, ßC, ßD, and ßE (Woodruff, 1998; Welt et al., 2002). ßA and ßB being the most common and best-characterized). Much as activins share structural homology with TGFß, the activin receptor and signaling system similarly resemble, and share components with the TGFß receptor system (Nakao et al., 1997; Whitman, 1998; Yue and Mulder, 2000; Risbridger et al., 2001). Activation of the activin signaling pathway is initiated by direct binding of activin to a transmembrane type II serine-threonine kinase receptor, ActRII or ActRIIB. Upon ligand binding, the type II receptor associates with and phosphorylates a type I receptor, ActRIB (ALK4), which in turn leads to phosphorylation and hence activation of members of the SMAD transcription factor family. The activation of SMADs, SMAD2 and SMAD3, leads to their association with the common mediator SMAD4, and translocation to the nucleus. Once in the nucleus, the SMAD complex participates in binding to specific DNA sequences, the consensus element having been defined as 5'-GTCTAG[N]C-3' (SMAD binding element or SBE), to regulate gene transcription (Welt et al., 2002).

Several mechanisms exist to achieve diversity in responses to activin. Cellular specificity of the activin signal is believed to be achieved in part by interaction of SMADs with cell-specific transcriptional coregulators at the level of DNA binding and transcriptional activation. SMADs have also been reported to interact with additional DNA binding partners such as AP-1, ATF2, and the vitamin D receptor, which in turn are activated by other signaling pathways, providing a mechanism for integrating a variety of signals to regulate cellular response. Extracellularly, binding of activin to its receptor is inhibited by follistatin, an activin binding protein (Schneyer et al., 1994; Chapman et al., 2002), and inhibin antagonizes the actions of activin by binding to betaglycan on the cell surface, sequestering ActRII into nonfunctional complexes (Lewis et al., 2000; Chapman et al., 2002; Gray et al., 2002). In addition, there is accumulating evidence for a role of other intracellular signaling pathways, such as activation of MAP kinase pathways, in mediating at least some of the actions of activin (Murase et al., 2001; Funaba et al., 2002). The MAP kinase pathway, in turn, may modify SMAD activity. The precise mechanisms by which activin mediates its diverse biological effects remain incompletely understood. A greater and more sophisticated knowledge of the molecular pathways and mediators of the actions of activins will lead to a better understanding of the diverse physiologic responses to activin.

In this report, we have used a DNA microarray assay to identify genes that are regulated by activin, using a murine gonadotrope-derived cell line (LßT2) as our model. In the case of FSH, another major regulator in addition to activin is gonadotropin-releasing hormone (GnRH). Interestingly, it has been suggested that stimulation of FSHb mRNA by pulsatile GnRH is activin-dependent, and that follistatin attenuates the GnRH stimulation of FSHß gene expression (Weiss et al., 1992; Meriggiola et al., 1994; Pernasetti et al., 2001). We have therefore studied the effects of activin both alone and in combination with GnRH.

Materials and methods

Cell culture, treatment of cells, and purification of mRNA

LßT2 cells were obtained from Dr. Pamela Mellon (University of California, San Diego). Cells were plated in 15 cm culture flasks and maintained at 37°C in 5% CO_2 in high glucose DMEM supplemented with 10% fetal bovine serum (FBS) (Hyclone). For experiments, 4-5x10^6 cells were seeded in each flask. After 24 h, media were replaced with fresh media containing human recombinant activin A (30 ng/ml; R&D System, Minneapolis, MN). LßT2 cells were treated with activin A for 48 h because early studies revealed that the FSHß mRNA expression by activin is delayed (Graham et al., 1999) although more recent studies using PCR revealed a much earlier response (Bernard, 2004). After 48 h of treatment, media were again replaced with fresh media, this time containing 0.1% FBS and activin A (30 ng/ml). One hour later, 100 nM GnRH agonist, ([D-Ala6]GnRH, GnRHA), or vehicle was added for an additional 1 h. Cells were treated with GnRHA for 1 h because prolonged treatment down regulates GnRH receptor and post receptor signaling (Junoy et al., 2002; Liu et al., 2003). The cells were then rinsed with PBS and lysed with Trizol reagent (Invitrogen). Total RNA was prepared from the three samples in each treatment group, using manufacturer's instructions. The quality of the RNA from each sample was examined on a denaturing agarose gel. Equal amounts of total RNA from each sample were used to purify poly $(A)^+$ RNA using an Oligotex mRNA purification kit (Qiagen, Valencia, CA).

Affymetrix genechip experiments

Poly $(A)^+$ RNA was processed according to the Affymetrix Expression Analysis Technical Manual and as described previously (Kakar et al., 2003). Briefly, 2 μg of poly$(A)^+$ RNA was used from each sample in each experiment. cDNA was generated using SuperScript Reverse Transcriptase (Invitrogen) and an oligo-dT primer linked to a T7 RNA polymerase-binding site sequence. The resulting cDNA was used to synthesize biotin-labeled cRNA via an in vitro transcription reaction, using the ENZO Bioarray High-Yield RNA transcript labeling kit (Affymetrix, Inc.). Labeled cRNA was purified using the RNeasy column (Qiagen). Purified, biotinylated cRNAs were quantitated, and 20 mg were subjected to a fragmentation reaction (40 mM Tris, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate) followed by heating at 88°C for 33 min, to randomly generate fragments ranging from 35 to 200 bases. Fifteen mg of labeled and fragmented cRNA

was used to make the hybridization cocktail, containing a control oligonucleotide B2 and four control bacterial and phage cRNAs (BioB, BioC, BioD, Cre).

The hybridization cocktail was used to hybridize to the murine MU74Av2 GeneChip expression array (Affymetrix, Inc.). This array includes approximately 12,400 genes from the mouse genome. Probes consist of 16 pairs of 25-mer oligonucleotides for each gene. One member of each pair contains a single base point mutation, and the signals of pairs are compared to assess specificity of hybridization. Hybridization, staining, scan, and analysis were conducted as per recommended protocols using the Affymetrix GeneChip Fluidics Workstation 400. After binding with phycoerythrin-coupled avidin, microarrays were scanned on a Hewlett-Packard Gene Array Scanner (Hewlett-Packard Co., Palo Alto, CA). An Affymetrix software filter was applied to mask transcripts with incorrect orientation in the public databases. Alterations in RNA transcript levels were analyzed using the Affymetrix Analysis Suite 5.0 software. Differences in levels of fluorescence intensity, which represent levels of hybridization, between the 25-base oligonucleotides and their mismatches, were analyzed by multiple decision matrices to determine the presence or absence of gene expression. The entire experiment was repeated, with the two experiments separated by a one-month interval. The repeat experiment was designed to be a true replicate, taking into account experimental variability in cell culture conditions and sample preparation. Values for the mean and standard deviation of the two replicate average difference scores from the two independent experiments were calculated for each gene. The fold-change in expression between control and treatment groups was calculated from the mean average difference scores.

Quantitative Real-time PCR

For quantitative real-time RT/PCR, the ABI Prism Sequence Detection System 5700 and Primer Express (PE Biosystem) were used. Two μg of total RNA from each sample was used to synthesize first strand cDNA in a 20 μl reaction mixture by using the first strand cDNA synthesis kit from Invitrogen. Twenty ng of cDNA was utilized for the PCR reaction, using SYBR green PCR mixture (Applied Biosystem, Foster City, CA) and 400 nM of each primer, under the following conditions: initial denaturation for 10 min at 95°C, followed by 40 cycles consisting of 30 sec at 94°C and 1 min at 68°C. Amplification was carried out using primer pairs specific for mouse:

Typr1 (5'-TGGGAGCACTGTAACCTTCC-3' and 5'-TGGACCAATCAGGAGAAACC-3'), Aebp1 (5'-GCACCACAGCTACAAGGACA-3' and 5'-GTATTGCATGATGAGCAGGAGCA-3'), Egr1 (5'-CTGACACGAAGCATTGTCAC-3' and 5'-GCTCTGCAATGTTCCTTCTT-3'), Egr2 (5'-CATCTATTCAGGAGTTGGAG-3' and 5'-AAACAACAGGCTACAGTTTC-3'), Gadd45b (5'- GAGAGAAGCCAGAATCAGGT-3' and 5'-GAAGAGATGGGTTTCTAAGG-3'), FSHß (5'-TTGATCCAGCTTTGCATCTT-3' and 5'-CACTGAAAGGAGCAGTAGCTG-3'), SDF1 (5'AGAGCCAACGTCAAGCATCT-3'and 5'-TAATTTCGGGGTCAATGCACA-3'), Ptprj (5'-ACTCAGGCGCTTCAGAATGT-3' 5'-TGTGTCACACCCACAGAGGT-3') and ß-actin (5'-CGAGAAGATGACCCAGATCA-3' and 5'-GATGTCCACGTCACACTTCA-3'). Fold induction and expression ratios were calculated from differences in threshold cycles at which an increase in reporter fluorescence above a baseline signal could first be detected (CT) between two samples and averaged for duplicate experiments.

Activin response assay

To verify the presence of functional activin receptors and activin responsiveness in the LßT2 cell line used for our studies, we used a reporter construct containing the mouse GnRH receptor gene promoter region fused to a luciferase reporter gene (GnRHR-Luc), used previously to demonstrate the regulation of GnRH receptor gene transcription by activin (Norwitz et al., 2002). LßT2 cells were plated in 6-well plates. After 24 h in culture, cells were co-transfected with GnRHR-Luc and renilla-luc (Promega) using Fugene6 reagent, as described previously (Kakar et al., 2003). After 24 h of transfection, media were removed and replaced with fresh media containing activin (30 ng/ml). After an additional 24 h, the cells were washed with PBS and harvested. Luciferase and renilla-luciferase activities were assayed using the dual reporter kit from Promega (Madison, WI), as described previously (Kakar et al., 2003). Each assay was performed in triplicate and in three independent experiments.

GnRH Response assay

To confirm the expression of biologically active GnRH receptors in LßT2 cells, we plated the cells in 6 well plates. Forty-four hours later, the cells were washed with DMEM containing 0.1% bovine serum albumin and loaded with 2 mCi of [myo-^{3}H] inositol (Amersham Biosciences, Piscataway, NJ) for 18 h at 37°C. Cells were treated for 2 h with increasing concentrations of GnRHA and levels of inositol triphosphate (IP3) were measured, as described previously (Kakar et al., 2003).

Results

LßT2 cells express biologically active activin and GnRH receptors

LßT2 cells are a mouse pituitary gonadotrope-derived cell line that is known to secrete FSH and LH in response to GnRH, and express activin receptors. To verify that these cells express biologically active receptors for GnRH and are responsive to GnRH under our culture conditions, we treated the cells with GnRHA

and measured the resulting levels of inositol triphosphate. As shown in Fig. 1A, treatment of cells with GnRHA resulted in an increase in IP3 levels. Dose-response studies demonstrated that the increase in levels of IP3 were dose dependent, with an EC50 value of ~ 0.1 nM.

To verify the expression of biologically active activin receptors and demonstrate responsiveness to activin, we transfected the LßT2 cells with GnRHR-Luc (mouse GnRH receptor gene promoter sequence fused to a luciferase reporter gene), and treated them with activin A. As shown in Fig. 1B, exposure of transfected cells to activin (30 ng/ml) for 24 h resulted in a 3-fold increase in luciferase activity, compared to vehicle-treated cells. Together, these results suggest that LßT2 cells express high affinity receptors relevant for signal transduction by both GnRH and activin. These results are consistent with those of Turgeon et al. (1996) and Yuen et al. (2002) for the expression of GnRH receptors and with Norwitz et al. (2002) and Pernasett et al. (2001) for the expression of activin receptors.

Gene expression profiles induced by activin, GnRHA, and both activin plus GnRHA

Despite the rapid progress in understanding the signaling mechanisms activated by activin receptors, the mechanisms by which activin increases FSH biosynthesis and release remain incompletely understood (Magdalena et al., 2001; Dupont et al., 2003). Furthermore, the mechanisms by which activins exert their diverse effects on many other biological functions and genes remain poorly characterized. In the gonadotrope, interactions between GnRH and activin have been suggested but the mechanisms have not been elucidated. In addition, a systematic search for target genes of activin in gonadotropes is lacking. In this study, we have examined the gene expression patterns induced by activin and GnRHA, alone and in combination, in LßT2 cells using DNA microarray analysis. For this analysis, we have used the Affymetrix mouse MU74Av2 GeneChip, which represents approximately 12,400 genes. Quantitation of target hybridization allowed us to determine the relative expression levels of each gene, and the increase or decrease in expression of each gene compared to controls. The hybridization intensity of the arrays was uniform for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase, cylophilin, and a large number of ribosomal proteins, indicating that the expression profiles were consistent with other standards for studying gene expression.

To identify genes in LßT2 cells that are regulated by activin or GnRHA, alone and in combination, replicate data points representing gene expression levels in each treatment group were analyzed by pair wise comparison with expression levels in vehicle-treated LßT2 cells. Genes that increased or decreased by more than 2.0-fold (mean value) were identified (HYPERLINK "http://www.louisville.edu/~sskaka01/" www.louisville.edu/~sskaka01/) (Fig. 2). Treatment of LßT2 cells with activin for 49 h resulted in changes in expression levels of 70 genes. Of these 70 genes, 39 were up-regulated, and 31 were down-regulated. Treatment of LßT2 cells with GnRHA for one h resulted in changes in expression levels of 213 genes, of which 135 were up-regulated and 78 were down-regulated. Treatment of LßT2 cells with both activin and GnRHA in combination resulted in changes in expression levels of 169 genes, of which 132 were up-regulated and 37 were down-regulated. Importantly, most of the genes that we previously identified (Kakar et al., 2003) to be regulated by GnRHA were also detected in this study,

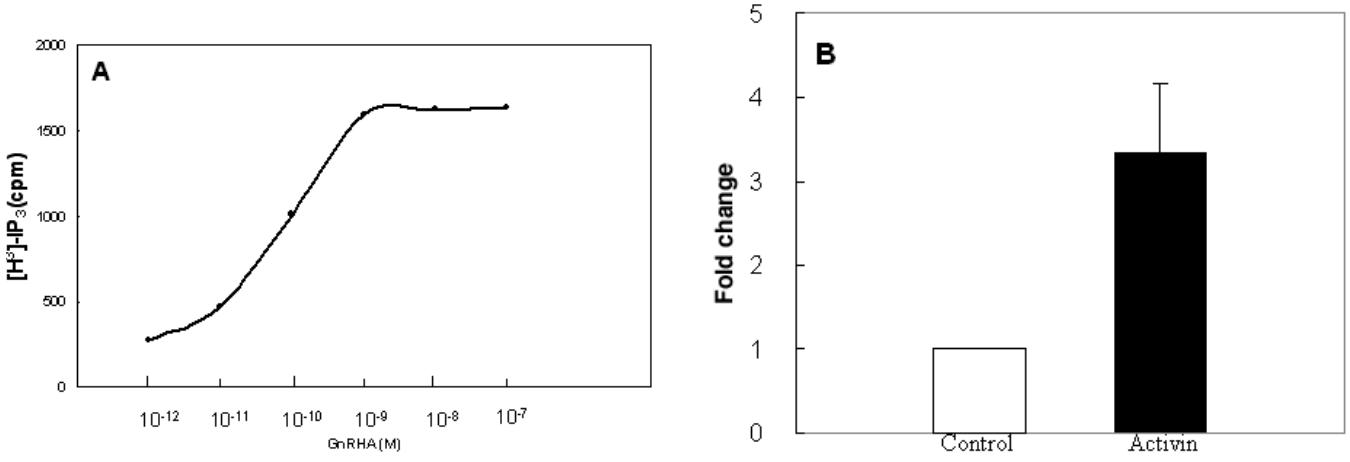

Fig. 1. LßT2 cells express biologically functional receptors for GnRH and activin. **A.** LbT2 cells were treated with various concentrations of GnRHA for 2 h. IP_3 levels were measured as described in the Materials and Methods. The values represent the mean of two independent experiments. **B.** LbT2 cells were transiently transfected with GnRHR-Luc. After 24 h of transfection, cells were treated with activin A (30 ng/ml) for 24 h. Cells were lysed and assayed for luciferase activity. The values represent mean ± SEM of three independent experiments.

validating both the cell system and the microarray analysis. Overall, 268 genes out of 12,400 genes (2.1%) showed changes in expression levels by at least one of the three treatments studied. Interestingly, many of the genes which were regulated by activin or GnRHA alone were not affected when activin and GnRHA were used together. On the other hand, a new set of genes was identified by the combined treatment that was not regulated by either treatment individually. These could be due to the increase in GnRH receptor expression (3.1-fold) on activin treatment, thus making LßT2 cells more responsive to GnRH action.

Using RT/PCR, RNase protection assays, and transfection assays, an increase in FSHß mRNA and GnRH receptor levels by activin, GnRH and activin plus GnRH has been reported in rat primary pituitary cells and LßT2 cells (Weiss et al., 1992; Meriggiola et al., 1994; Turgeon et al., 1996; Pernasetti et al., 2001; Norwitz et al., 2002; Kakar et al., 2003). Our microarray analysis of the LßT2 cells showed 3-fold increase in GnRH receptor mRNA levels by activin and activin plus GnRH and 1.5-fold by GnRH alone. However, no effect of activin on FSHß gene expression was observed by microarray analysis. To validate our system, we used real-time PCR analysis to determine the expression of FSHß mRNA in LßT2 cells and the effects of treatment with GnRHA, activin and activin plus GnRHA. As shown in Fig. 3, a significant increase in FSHß mRNA was observed following treatment of cells with activin or activin plus GnRHA (3- to 4-fold respectively). No change or only a small increase in FSHß mRNA levels was observed following treatment of cells with GnRHA alone. These results are consistent with previous reports (Weiss et al., 1992; Meriggiola et al., 1994; Turgeon et al., 1996; Magdalena et al., 2001; Norwitz et al., 2002; Pernasetti et al., 2001; Kakar et al., 2003), and suggest a lower sensitivity of cDNA microarray analysis compared to real-time PCR, absence of the *FSHβ* gene on the chip or presence of different variant on the chip.

When analysis was restricted to those genes showing four-fold or more alterations in levels of expression by activin, GnRHA or activin plus GnRHA, the size of the regulated group of genes was decreased to 60. Of these, 25 encoded proteins with as yet unknown functions (Table 1). Division of genes into functional classes revealed that the two largest groups consist of genes that encode products involved in (1) transcriptional regulation, and (2) cell growth/maintenance. All genes in these two groups except two (AE binding protein 1 and perinatal skeletal myosin heavy chain polypeptide 8) were up-regulated. Many genes that were identified after treatment of LßT2 cells with both GnRHA and activin, did not show any changes when cells were treated with either activin or GnRHA alone. The effects of activin plus GnRHA were not additive, indicating differential

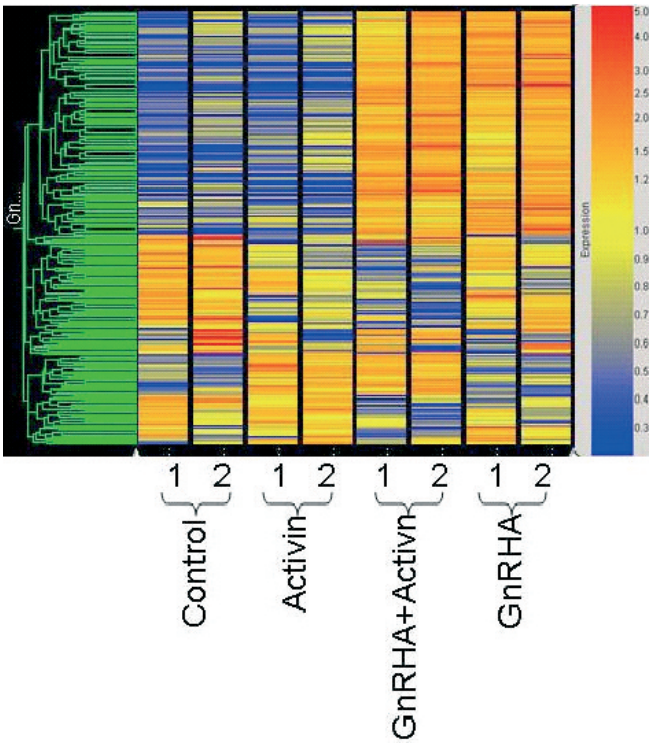

Fig. 2. Cluster analysis of microarray data. Data obtained from microarray analysis were used to generate a cluster analysis. Each horizontal line represents a gene. Genes that were changed by 2-fold or higher in LßT2 cells compared with background are shown. Colors indicate high expression (red) to low expression (blue) and correspond to relative expression changes between 0 and 4.0 of normalized values. 1 and 2 represent two independent experiments.

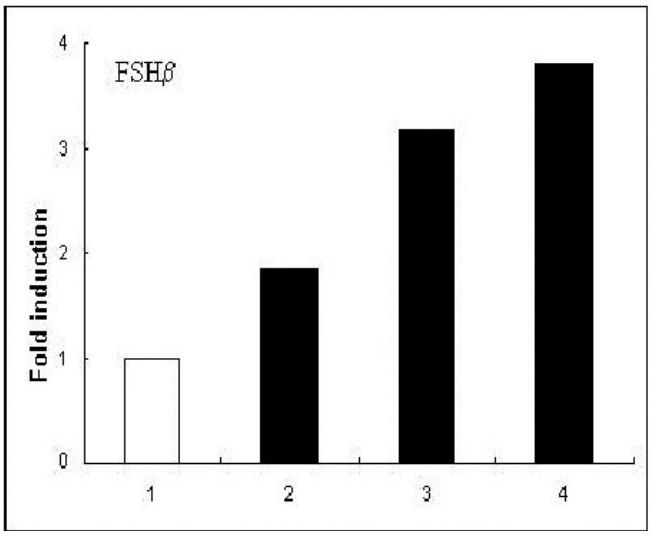

Fig. 3. Effect of GnRHA and Activin A on expression of the *FSHβ* gene. LbT2 cells were treated with vehicle (1) GnRHA (2), Activin (3) or GnRHA + Activin A (4) as described in the Materials and Methods. Real-time PCR expression of FSHß was normalized to ß-actin expression levels. The value represent the mean of two independent experiments.

regulation by activin, GnRHA and activin plus GnRHA.

Validation of regulation of gene expression by real-time quantitative PCR

To verify the data obtained in the microarray assays, we performed quantitative real-time PCR. We used specific oligonucleotide primers derived from the coding sequences of seven selected genes, Tyrp1, Aebp1, Ptprj, SDF1, Gadd45b, Egr1, and Egr2 . As shown in Fig. 4, the changes in expression levels induced by activin, GnRH, or both activin plus GnRHA in combination for these seven genes were highly correlated by the two protocols. Although the fold changes were not identical, the increases or decreases in mRNA levels as measured by quantitative real-time PCR for each treatment group mirrored those changes measured by the microarray assay. Similar results were obtained using semiquantitative RT-PCR (data not shown).

Discussion

Activins are a family of proteins which consist of disulfide-linked homodimers and heterodimers of the ß subunits of inhibin, termed ßA and ßB. These three proteins, called activin A (ßA-ßA), activin B (ßB-ßB) and activin AB (ßA-ßB), are members of the TGFß superfamily of proteins. The known physiological roles of the activin family of proteins are based largely on the established properties of activin A. Although the activins were originally isolated for their ability to stimulate FSH secretion, they have been shown to influence many additional diverse biological functions, including

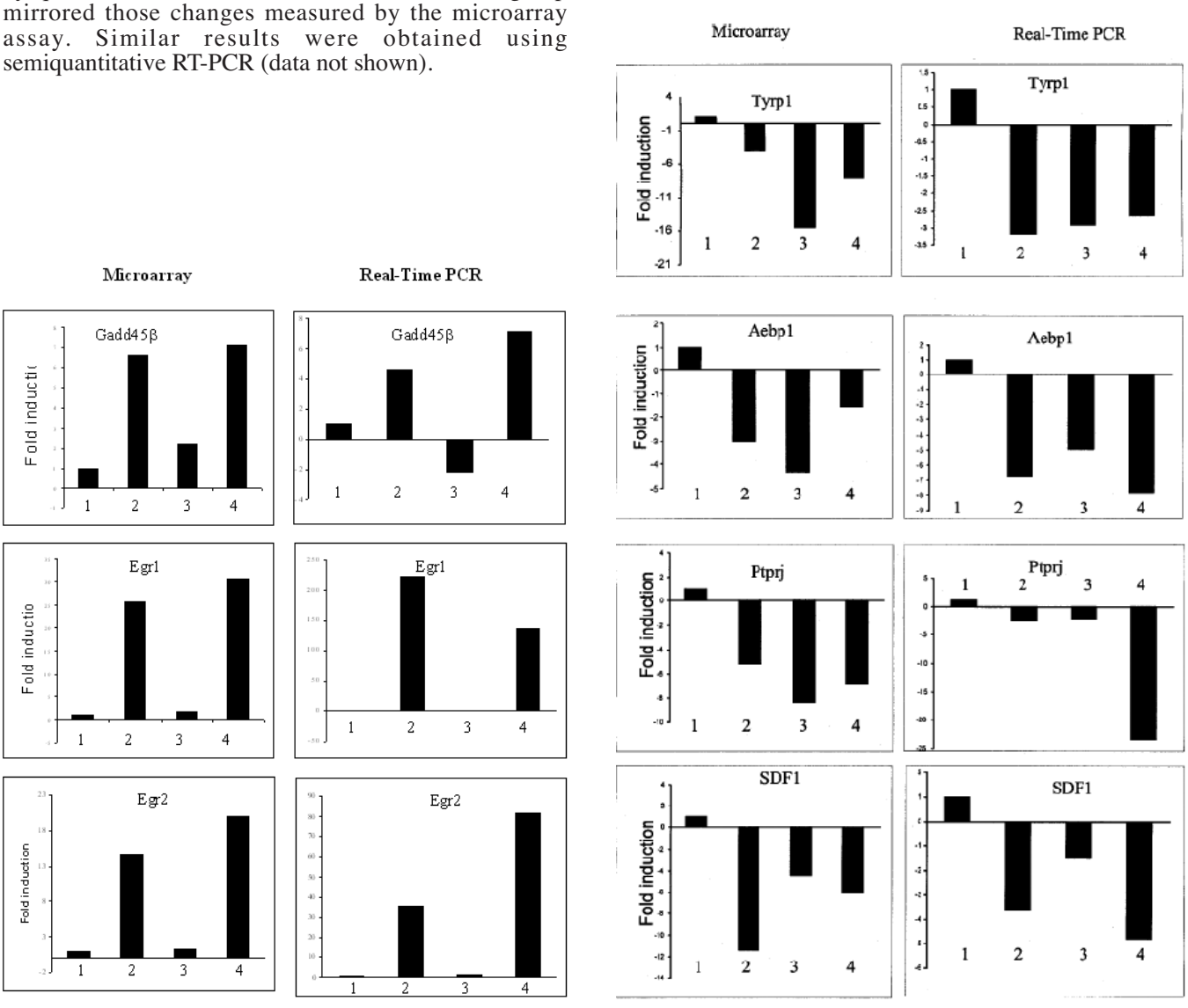

Fig. 4. Comparative analysis of expression profiles between microarray data and real-time PCR analysis for seven genes, as indicated. Real-time PCR expression was normalized to ß-actin expression levels. The values represent the mean of two independent experiments. In each panel, 1: no treatment, 2: activin, 3: GnRHA, 4: GnRHA + activin.

proliferation, differentiation, apoptosis, haemopoiesis, embryogenesis, cell division, prostate biology and angiogenesis (Risbridger et al., 2001; Chen et al., 2002; Welt et al., 2002). Even though considerable progress has been made in determining the signaling cascades downstream of the activin receptor, relatively little is known about the genes that are regulated by activin and its signaling pathways.

To determine the global profile of genes that are regulated by activin, we used the Affymetrix GeneChip technology to identify the downstream genes which are regulated by activin to mediate its biological effects. For this purpose, we selected the LßT2 cell line as our model. The LßT2 cell line is a mouse gonadotrope-derived cell line, which was developed utilizing the murine LHb gene promoter for targeted expression of the SV40 T-antigen in transgenic mice (Turgeon et al., 1996). These cells express receptors for both GnRH and activin (Turgeon et al., 1996; Norwitz et al., 2001; Pernasetti et al., 2001; Yuen et al., 2002; Kakar et al., 2003) (Fig. 1), and secrete FSH in response to activin, GnRHA and activin plus GnRHA (Turgeon et al., 1996; Graham et al., 1999; Magdalena et al., 2001; Bedecarrats et al., 2003). These cells thus represent a good model for the identification of genes regulated by activin, alone or in combination with GnRH, as potential mediators of regulatory effects on FSH biosynthesis as well as of other cellular effects of activins.

Treatment of LßT2 cells with activin (30 ng/ml) for 49 h resulted in changes in expression of 70 genes, of which 39 were up-regulated and 31 were down-regulated by two-fold or more (www.louisville.edu/~w0mazh01/). Fourfold or higher alterations in expression were exhibited by 13 genes, 6 of which were found to be ESTs (Expressed sequence tags) or with unknown function (Table 1). The remainder of the genes encode proteins with known functions and include AE binding protein 1(AEBP1), WNT-1 related protein 5A (WNT5A), protein tyrosine-like phosphatase receptor J (PTPRJ), cytokine SDF-1a (also known as chemokine; C-X-C motif ligand 12; CXCL12), perinatal skeletal myosin heavy chain polypeptide 8 (MYH 8), epoxide hydrolase 2 (EPHX2), and tyrosine-related protein 1 (TYRP1).

Treatment of LßT2 cells with 100 nM of GnRHA resulted in changes of 213 genes by 2-fold or higher within 1 h of treatment. Fold-changes in levels of expression of most of these genes are consistent with our previous studies (Kakar et al., 2003) and Wurmbach et al. (2001). Studies from other laboratories have shown increased levels of GnRH receptor mRNA in LßT2 cells when treated with activin or GnRH (Turgeon et al., 1996; Norwitz et al., 2002). Consistent with these data, our microarray analysis revealed 3.1-fold and 1.5-fold increase in GnRH receptor mRNA by activin and GnRHA respectively, validating the cell system and microarray analysis.

GnRH is known to modulate the expression of LHß and FSHß genes under certain conditions; however, we did not observe an increase in the expression of LHß or FSHß by GnRH. These results are consistent with our previous findings (Kakar et al., 2003) and those of Wurmbach et al. (2001), who also failed to observe an increase in the expression of LHß and FSHß genes in LßT2 cells when treated continuously with GnRH for 1, 3 or 6 h. This lack of expression could be due to the requirement for stimulation by pulsatile GnRH for the activation of the LHß and FSHß gene expression (Turgeon et al., 1996).

Activin A resulted in down regulation of adipocyte enhancer-binding protein 1 (AEBP1) by 4-fold. GnRHA also reduced expression of this gene, by 3-fold. Interestingly, when activin and GnRHA were used together, the effect on AEBP1 expression was reduced. AEBP1 is a transcriptional repressor with carboxypeptidase activity that is down-regulated during adipogenesis (Muise and Ro, 1999). It protects the modulation of MAPK activation , resulting in increased MAPK activity, a biologically relevant process in adipogenesis (Kim et al., 2001). It has been demonstrated that, in fibroblasts and 3T3-L1 cells, sustained activation of MAPK is associated with cell proliferation (Benito et al., 1991; Cowley et al., 1994; Mansour et al., 1994). Activin and its receptors have shown to be expressed in variety of cell types (Ogawa et al., 1993; Woodruff and Mather, 1995; Woodruff, 1998). Our studies suggest that the modulation of AEBP1 expression by activin A in adipocytes could constitute a critical part of the molecular mechanism of adipogenesis. Modulation of MAPK activity may represent a mechanism by which activins influence cellular proliferation in general.

WNT signaling pathways play key roles in carcinogenesis and embryogenesis, and WNT signaling molecules are potent targets for diagnosis, prevention and treatment of cancer as well as for regenerative medicine or tissue engineering. WNT5A is a member of the wingless (WNT) family and is highly expressed in several tumors including gastric, brain, pituitary, colorectal, uterine, and breast tumors (Howng et al., 2002; Saitoh and Katoh, 2002). A role for WNT5A in cell differentiation has also been reported (Yokoi et al., 2003). Concurrently, several types of tumors have been reported to express activin and its receptors (Vliegen et al., 1993; Mellor et al., 2000; Risbridger et al., 2001; Chen et al., 2002; Welt et al., 2002). Antiproliferative effects of activin in breast and prostate cancer and leukemia have been reported (Chen et al., 1992). As shown in Table 1, our results using microarray analysis of the gene profile regulated by activin revealed up-regulation of the WNT5A gene by more than four-fold. GnRHA treatment resulted in only a 2.2-fold increase. The combination of activin and GnRHA together was additive. These results suggest that WNT5A may serve as one of the signaling pathways regulated by activin A to regulate tumor cell growth and proliferation.

PTPRJ is another interesting gene which was identified to be regulated by activin. PTPRJ encodes a membrane-type protein tyrosine phosphatase. It is a

Table 1. Gene expression profile determined by microarray analysis and classified according to biological function (Four-fold change and higher).

GENE NAME AND CLASSIFICATION	GENE SYMBOL	ACCESS ID	FOLD CHANGE		
			Activin	GnRHA	GnRHA+Activin
Transcription Factors:					
Kruppel-like factor 4 (gut)	Klf4	U20344	1.0±1.0	6.6±1.3	5.6±1.1
Early growth response 1	Egr1	M28845	1.6±1.0	25.8±1.2	30.7±1.5
Kruppel-like factor 9	Klf9	Y14296	1.1±1.4	6.1±1.5	5.6±1.0
Zinc finger protein 36	Zfp36	X14678	1.6±1.7	11.4±1.8	14.1±1.2
Activating transcription factor 3	Atf3	U19118	-0.1±1.1	39±1.2	38±1.0
Early growth response 2	Egr2	M24377	1.3±1.6	14.6±1.3	20.0±1.1
Nuclear receptor subfamily 4, group A, member 1	Nr4a1	X16995	-1.2±1.3	67.6±1.2	52.0±1.6
Jun-B oncogene	Junb	U20735	1.3±1.3	15.0±1.1	22.8±1.3
TG interacting factor	Tgif	X89749	1.3±1.3	5.1±1.1	4.9±1.7
cAMP responsive element modulator	Crem	M60285	-1.3±1.4	4.8±1.3	3.6±1.2
AE binding protein 1	AEbp1	NM009636	-4.2±7.3	-3.0±2.1	-1.6±2.0
Cell communication/ Signaling:					
Wingless-related MMTV integration site 5A	Wnt5a	M89798	4.5±1.3	2.2±2.4	5.9±1.1
Molecule possessing ankyrin-repeats induced by lipopolysaccharide	Mail-pending	AA614971	-1.3±1.3	4.7±1.1	3.1±1.0
Immediate early response, erythropoietin 1	Ierepo1-pending	AW049031	-1.4±1.1	8.5±1.1	10.8±1.3
Regulator of G-protein signaling 2	Rgs2	U67187	1.3±2.6	9.2±1.4	4.3±1.1
Chemokine (C-X-C motif) ligand 10	Cxcl10	M33266	1.1±8.1	11.1±1.5	8.4±3.1
Protein tyrosine phosphatase, receptor type J	ptprj	AY038877	-8.4±1.1	-4.1±3.1	-8.1±1.6
Chemokine (C-X-C motif) ligand 12	Cxcl12	L12029	-4.5±1.3	-11.4±1.6	-6.0±1.3
Cell growth/Maintenance:					
Cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	AW048937	-1.4±1.5	3.2±1.7	4.0±1.5
Ski/sno related	Skir	U10531	2.0±1.3	4.9±0.0	8.2±1.1
Cysteine rich protein 61	Cyr61	M32490	1.2±1.2	4.2±1.4	2.6±2.1
Myosin, heavy polypeptide 8, skeletal muscle, perinatal	Myh8	M12289	-7.4±3.5	-4.1±3.0	-9.1±1.1
FBJ osteosarcoma oncogene	Fos	V00727	1.3±1.4	24.1±1.3	24.1±1.4
Myeloid differentiation primary response gene 116	Myd116	X51829	-1.3±1.4	4.9±1.9	4.3±1.3
FBJ osteosarcoma oncogene B	Fosb	X14897	1.0±0.0	19.2±1.2	20.5±1.2
Growth arrest and DNA-damage-inducible 45 beta	Gadd45b	X54149	2.2±2.4	6.6±1.8	7.1±2.5
Growth arrest and DNA-damage-inducible 45 gamma	Gadd45g	AF055638	-2.3±3.5	5.8±3.4	5.7±3.0
Actin, alpha, cardiac	Actc1	M15501	1.5±4.4	53.4±15.3	9.3±6.5
Actin, alpha 1, skeletal muscle	Acta1	XM134551	-1.5±3.1	7.8±1.8	3.5±3.2
V-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	Maff	AB009694	-2.3±2.3	3.6±2.8	4.7±2.7
Apoptosis:					
Bcl2-associated athanogene 3	Bag3	AI643420	1.1±2.0	4.1±1.1	3.0±1.1
Transport:					
Solute carrier family 2 (facilitated glucose transporter), member 1	Slc2a1	M22998	1.3±1.1	7.4±1.0	6.5±1.0
Metabolism:					
Epoxide hydrolase 2, cytoplasmic	Ephx2	Z37107	4.2±1.1	1.1±1.2	4.0±1.1
Tyrosine-related protein 1	Tyrp1	NM031202	-15.9±1.4	-4.1±3.1	-8.1±1.9
Kallikrein 6	Klk6	M13500	-1.5±1.3	-1.0±1.3	-4.7±2.5
Unknown:					
Fos-like antigen 1	Fosl1	AF017128	-1.2±1.3	26.4±5.3	24.9±4.6
Immediate early response 2	Ier2	M59821	-1.1±1.1	11.8±1.1	14.0±1.1
RAS, dexamethasone-induced 1	Rasd1	AF009246	-1.0±1.4	4.1±1.7	4.4±1.6
Calbindin-28K	Calb1	D26352	-1.9±1.1	-1.1±1.2	-6.5±2.3
RIKEN cDNA 2010005I16 gene	2010005I16Rik	2H3	-2.0±1.8	-5.1±3.1	-1.6±1.7
Pleiotrophin	Ptn	D90225	-1.4±1.8	-1.1±1.4	-5.2±1.4
Ras-related associated with diabetes	Rrad	AF084466	1.1±1.1	25.8±1.5	26.7±1.0
ESTs	ESTs		5.9±4.2	1.1±1.2	6.4±2.1
Immediate early response 3	Ier3	X67644	1.1±1.1	4.9±1.0	6.1±1.2
Ribosomal protein S20	Rsp20	NM026147	-4.1±1.6	-4.3±2.6	-2.0±2.3
Calponin 2	Cnn2	Z19543	1.3±3.7	7.0±10.2	3.7±5.6
Testis expressed gene 21	Tex21-pending	AB000682	-1.7±1.2	-3.2±1.8	-8.5±1.6
Cysteine-rich protein 2	Csrp2	D88792	3.3±1.0	1.2±1.2	4.0±1.0
Telomerase associated protein 1	Tep1	NM9351	4.1±2.0	1.5±1.6	2.3±1.5
Williams-Beuren Syndrome chromosome region 5 homolog (Human)	Wbscr5	AK092904	-4.9±1.9	-4.3±1.7	-2.9±2.1
Heat-responsive protein 12	Hrsp12	AV321289	4.5±1.4	2.5±2.7	4.2±1.5
Cryptochrome 1(photolyase-like)	Cry1	NM007771	-3.5±14.0	-10.0±7.0	1.2±1.8
ESTs	ESTs		-5.5±21.4	-4.4±3.1	3.5±13.7
RIKEN cDNA 1110032C13 gene	1110032C13Rik	AI847051	-1.0±1.2	3.6±1.5	4.6±1.5
RIKEN cDNA E130103E02 gene	E130103E02Rik	AA739263	-1.1±1.1	-1.6±1.3	-6.1±1.3
Nuclear factor, interleukin 3, regulated	Nfil3	U83148	1.2±1.0	4.5±1.3	4.4±1.2
Neoplastic progression 3	Npn3	Z31362	-1.0±1.1	8.5±1.5	5.1±1.0
Ligand of numb-protein X-1	Lnx1	AF034745	-3.4±3.6	-7.7±1.7	-8.4±1.6
B-cell translocation gene 2, anti-proliferative	Btg2	M64292	-1.1±1.1	8.3±1.1	10.4±1.1
Ras homolog gene family, member AB	Arhb	X99963	1.0±1.3	3.5±1.0	5.1±1.1

tumor suppressor gene and is occasionally deleted or contains missense mutations in a variety of cancers. Its expression levels are related to cellular differentiation. PTPRJ overexpression in undifferentiated cells induces differentiation and inhibits cell growth (Ruivenkamp et al., 2002). Furthermore, in transformed rat thyroid cells that have lost the expression of PTPRJ, Overexpression of PTPRJ in these cells resulted in suppression of the cancer phenotype. This is possibly achieved as a result of increased steady-state levels of $p27^{kip1}$ (Trapasso et al., 2000). LßT2 cells, when treated with activin, showed an 8.5-fold decrease in expression of PTPRJ. GnRHA treatment caused a 4-fold decrease, whereas the combination resulted in an 8-fold decrease in PTPRJ expression levels, indicating that the GnRHA effect is not additive with the activin effect. Together, these results suggest that activin as well as GnRHA may regulate cell growth and differentiation by modulating the expression of the PTPRJ gene.

Stromal cell-derived factor 1 (SDF1) is a highly conserved gene localized to mouse chromosome 6 (Normura et al., 1996). This gene encodes two isoforms, SDF1-α and SDF1-ß, that arise from alternate splicing. These two isoforms differ only in that SDF-1ß contains four additional 3' amino acids (Shirozu et al., 1995). SDF-1-α is the predominant. SDF1 binds to a seven transmembrane G-protein coupled receptor, CXCR4, to modulate several biological functions through signal transduction pathways, including cell proliferation, cell migration, and transcriptional activation. High levels of expression of SDF1 in various cancers (Sehgal et al., 1998; Rempel et al., 2000) and HIV-1 infected patients (Bleul et al., 1996) have been reported, indicating that SDF1 is an important molecule for the regulation of various cellular functions. Our results, as shown in Table 1, revealed a 4.5-fold decrease in expression of SDF1 expression by activin, an 11.4-fold decrease by GnRHA, and a 6.0-fold decrease by activin plus GnRHA suggesting a novel role of activin and GnRHA in regulating SDF1 expression.

Epoxide hydrolases have an important defence function in various organisms due to their conversion of potentially harmful epoxide-containing compounds into diols, which are less reactive and easier to excrete (Meijer and DePierre, 1988). Activin increased the expression of epoxide hydrolase 2 (Ephx2) by 4.2-fold (Table 1). Expression of Ephx2 was not changed by GnRHA, however the effect of activin was retained when activin and GnRHA were used together. The biological significance of activation of Ephx2 transcription by activin remains unclear.

Activin has been demonstrated to inhibit cell growth in many cell types, including prostate cancer, breast cancer, B cell leukemia, vascular endothelial, vascular smooth muscle, peripheral blood granulocyte-macrophage colony-forming unit progenitors, BALB/c 3T3 mouse fibroblasts, and rodent hepatocytes (Chen et al., 2002). However, the mechanisms by which activin exerts its growth-inhibitory effects are poorly understood. In a B cell hybridoma, activin has been reported to stimulate the expression of cyclin-dependent kinase inhibitor p21 CIPI/WAF1 and suppress cyclin D2 expression (Danila et al., 2000), which leads to inhibition of cyclin-dependent kinase Cdk4 activity and consequently to the accumulation of phosphorylated Rb protein. Our results, as shown in Table 1, revealed a marginal change in expression of the p21 gene by activin in LßT2 cells. Activin also has been shown to induce programmed cell death in several types of cells. As shown in Table 1, activin regulates the expression of Gadd45ß and Gadd45γ. These genes are known to play an important role in cell cycle regulation and apoptosis. Together, these results suggest that activin may regulate cell growth and proliferation by modulating the expression of Gadd45ß and Gadd45γ, leading to cell cycle arrest and apoptosis.

The pituitary gonadotropins, FSH and LH are known to play a pivotal role in normal reproductive function. The gonadotrope cells in the anterior pituitary produce both of these hormones. The synthesis and release of each gonadotropin are often discordant in various physiological situations such as the infantile period in the female rat (Wilson and Handa, 1997), estrous cycle in the ewe (Pant et al., 1997), and photoperiod in the hamster (Bernard et al., 2000; Anand et al., 2002). However, precise mechanisms of LH and FSH differential regulation are still unclear. Gonadoatropin biosynthesis and secretion are regulated mainly by GnRH, and by circulating gonadal steroids and polypeptides, including activin, inhibin, and follistatin (Ying, 1988). In our present studies using pituitary gonadotrope LßT2 cells, we showed regulation of a large number of downstream signaling genes by GnRH, activin and in combination. Many investigators using pituitary and pituitary derived cell lines (Lß and αT3-1) have shown regulation of a number of downstream signaling pathways by GnRH (see Kakar et al., 2004 for review) as well as by activin (Dupont et al., 2003). Some of these downstream target genes and signaling pathways appear to be common to both GnRH and activin. How coordination of these downstream target genes and signaling pathways by GnRH and activin is achieved to regulate the expression and secretion of gonadotorpins and to maintain reproductive process remain to be decided.

The downstream genes regulated in LßT2 cells on treatment with activin for 49 h may be different from those at earlier times. Some genes may be transiently regulated by activin (e.g. early response genes) and would be missed at this time point. Also genes changed in a gonadotrope cell line on activin treatment may differ in extra-pituitary tissues.

In summary, we have employed a global gene expression strategy to identify genes that are regulated by activin A, GnRHA, and activin plus GnRHA in combination. Our studies have identified genes that are regulated by each treatment. These genes include oncogenes, and those that encode transcription factors,

ion channel proteins, and cytoskeletal proteins, as well as other proteins that are involved in cell cycle and signal transduction. Since not all genes that were regulated by activin or GnRHA alone were regulated when the ligands were used together, our results suggest interactions and differential regulation by activin and GnRHA. The interrelationship between these genes in regulation of cellular function including reproduction by activin and GnRHA remains to be further studied in greater detail.

Acknowledgements. Supported by NCI grants CA60871 and HD19546 and Kentucky Research Challenge fund.

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Accepted September 30, 2005