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Research

Activin-A up-regulates type I activin receptor mRNA levels in human immortalized extravillous trophoblast cells.

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Abstract

Activin is known to play an important regulatory role in reproduction, including pregnancy. To further examine the role and signaling mechanism of activin in regulating placental function, the steady-state level of activin type I receptor (ActRI) mRNA in immortalized extravillous trophoblasts (IEVT) cells was measured using competitive PCR (cPCR). An internal standard of ActRI cDNA for cPCR was constructed for the quantification of ActRI mRNA levels in IEVT cells. ActRI mRNA levels were increased in a dose-dependent manner by activin-A with the maximal effect observed at the dose of 10 ng/ml. Time course studies revealed that activin-A had maximal effects on ActRI mRNA levels at 6 hours after treatment. The effects of activin-A on ActRI mRNA levels was blocked by follistatin, an activin binding protein, in a dose-dependent manner. In addition, inhibin-A inhibited basal, as well as activin-A-induced ActRI mRNA levels. These findings provide evidence, for the first time, that activin-A modulates ActRI mRNA levels in human trophoblast cells.

Introduction

Although activins and inhibins were originally isolated from follicular fluids and identified as stimulators and inhibitors, respectively, of pituitary follicle-stimulating hormone (FSH), the identification of inhibins and activins in a wide variety of tissues suggest that these factors play much greater roles than the control of FSH secretion [1–5]. Also, it has become evident that these factors exert their effects mostly in autocrine/paracrine manners.

Similar to other members of the transforming growth factor-β (TGF-β) family, activins exert their actions by interacting with both type I and type II membrane serine/threonine kinase receptors [6,7]. Two type I (ActRI and ActRIB) and two type II (ActRII and ActRIIB) receptors have been shown to interact with activins [5] and their mRNAs have been detected in human placental trophoblast cells [8–10], as well as in choriocarcinoma cells [11]. Activins, particularly activin-A, has been shown to be produced by human placenta [5,11–13]. Many studies have demonstrated that activin-A plays important regulatory roles in human placenta, including stimulation of cytotrophoblast differentiation into invasive extravillous cytotrophoblast [14]; stimulation of progesterone [11,14–16], human chorionic gonadotropin (hCG)[15,17,18], estradiol [11], gonadotropin-releasing hormone (GnRH) [15] and oxytocin [19] secretion. However, regulation of activin signaling at the receptor level has not been examined.

The human placenta provides specialized functions during gestation that is critical for the development of the embryo and fetus. Among these important functions are the production of hormones, cytokines and growth factors that contribute to the gestational coordination of maternal, extraembryonic, and embryonic tissues. Development of the human placenta depends on proliferation and differentiation of certain trophoblast cells as well as invasion to the endometrium and its vasculature by a highly proliferative, migratory and invasive subpopulation of extravillous trophoblast (EVT) cells [20].

To further study activin signaling in human placenta, we have developed a competitive quantitative PCR to measure ActRI mRNA levels in an immortalized EVT cell line, HTR8/SVneo. We report here the first evidence that activin-A regulates its own receptor mRNA levels in a dose- and time-dependent manner and this effect can be blocked by its binding protein, follistatin, or its antagonist, inhibin-A in human placenta.

Materials and Methods

Cell line

The use of placental tissue samples and cell lines was approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects, University of British Columbia. The HTR-8/SVneo trophoblast cell line was generously provided by Dr. PK Lala (University of Western Ontario). This cell line was obtained from human first trimester placenta explant cultures and immortalized using SV40 large T antigen [21]. Cells were cultured in RPMI 1640 medium containing 10% FBS and antibiotics (Invitrogen Canada Inc., Burlinton, ON) as previously described [21].

PCR Primers

Oligonucleotide primers were synthesized based on the published sequences of the human ActRI [22], and β-actin [23]. The upstream primer (ActRI-1), 5'-GATGAGAAGTCATGGTTCAGG-3', and downstream primer (ActRI-2), 5'-TATGTTTGGCCTTTGTTGATC-3' were designed such that the predicted sizes of PCR products are 700 bp for native ActRI cDNA. Primers are chosen to flank introns so that the amplified ActRI cDNA is readily distinguished from a possible contaminating genomic DNA. Another pair of primers, ActRI-3 (5'-GATGAGAAGTCATGGTTCAG G-3') and ActRI-4 (5'-TATGTTTGGCCTTTGTTGATC-3") were synthesized and used as nested primers to validate the internal standard and measuring the regulation of ActRI mRNA levels in HTR8/SVneo cells. The human β-actin primers, used as another internal control to normalize cDNA amount in different samples, are AC1 upstream and AC2 downstream primers of which sequence are 5'-GGACCTGACTGACTACCTCATGAA-3' and 5'-GGTGGAAGGTGGTCAACACCTAG-3', respectively.

Hormonal treatment

Human recombinant activin-A, inhibin-A and follistatin-288 were kindly provided by Dr. Parlow at the National Hormone and Pituitary Program. The HTR8/SVneo cells were plated in 24-well culture plates in FBS supplemented RPMI medium. Two days after plating when cells reach about 70% confluence, the culture medium was replaced with serum-free RPMI 1640. For dose-response studies, cells were treated with various doses (0.1 to 30 ng/ml) of activin-A for 12 hours. For time-course experiments, cells were cultured in the presence or absence of 10 ng/ml activin-A for 1, 3, 6, 12, 24 hours. To determine the interaction between activin and follistatin, cells were treated with activin-A (10 ng/ml) alone, or activin-A with different concentrations of follistatin-288 (10, 20, 50, or 100 ng/ml) for 6 h. Similarly, to examine the effect of inhibin on basal and activin-A-stimulated ActRI mRNA levels, cells were treated with Activin-A, inhibin-A, either alone or in combination for 6 h. There are four wells in each treatment group and each experiment was conducted three times.

Construction of an internal standard for comparative PCR

Cloning of native ActRI PCR product using primers ActRI-1 and ActRI-2 was performed by ligating the PCR product into a pDirect (BD Biosciences Clontech, Palo Alto, CA), followed by sequencing analysis to confirm the identity. To construct an internal standard for comparative PCR, a 156 base pair segment was removed from the cloned native ActRI PCR product by restriction digestion using StyI and Eco47III (Fig. 1). With the same pair of primers is used in PCR, the subcloned mutant ActRI template yielded a PCR product 156 base pairs smaller than that from the native cDNA template.

Total RNA extraction, Reverse transcription and Competitive PCR

Total RNA from HTR-8/SVneo cells was extracted using the RNaid Kit (Bio/Can Scientific Inc., Mississauga, ON) following manufacture's suggested procedures. Two μg of total RNA was used to synthesized cDNA using First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech Inc., Oakville, ON) as previously described [9]. cPCR is similar to PCR except that it includes an internal standard to monitor the variation between different tubes as well as among different experiments. In the cPCR reaction, target sequences of native cDNA or sample cDNA combined with 0.5 pg/μl of the internal standard of ActRI cDNA (internal standard, IS) are co-amplified in a tube with a volume of 50 μl containing 5 μl of 10 × PCR buffer [200 m*M* Tris-HCl (pH 8.4), 500 m*M* KCl], 1.5 μl MgCl2 (50 m*M*), 1 μl dNTPs (10 m*M*), 0.5 μl Taq DNA polymerase (5 units/μl) (Gibco BRL), 38 μl sterile Milli-Q water, 1 μl each (5 μ*M*) of specific primers (ActRI-3 and ActRI-4) and equal volume (1 μl) of IS and cDNA sample or native

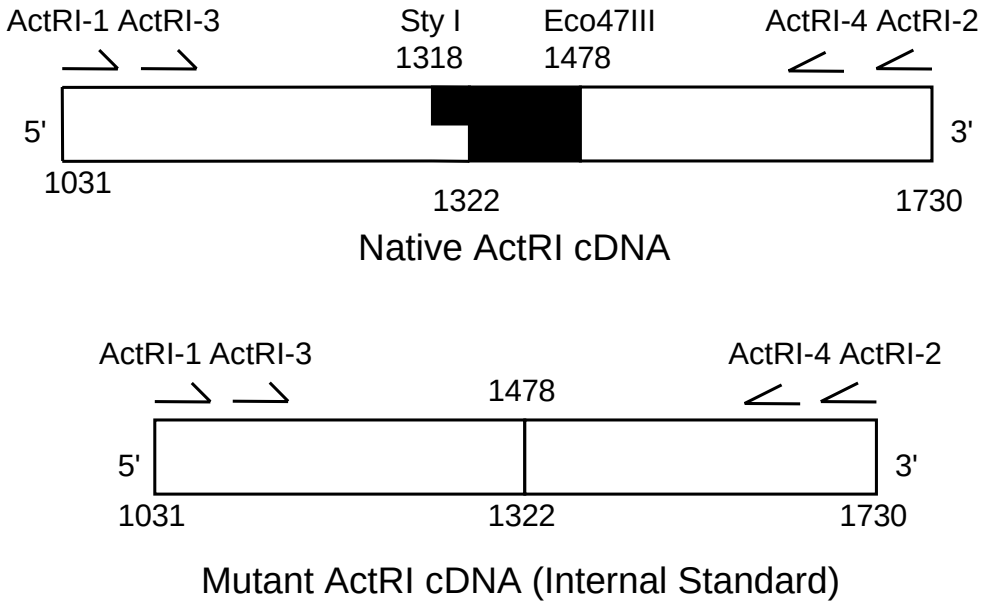

Figure 1
Schematic structure of the ActRI cDNA and primers used in PCR or cPCR. An 156 bp internal deletion has been done between the restriction sites Sty I and Eco47 III. Co-amplification of the ActRI native cDNA and mutant cDNA template using primer ActRI-1 and primer ActRI-2 resulted in 700 bp and 544 bp PCR product, respectively. Co-amplification of the ActRI native cDNA and mutant cDNA template using primer ActRI-3 and primer ActRI-4 resulted in 650 bp and 494 bp PCR product, respectively.

cDNA. PCR amplifications were performed for 35 cycles in a Perkin-Elmer/Citus DNA thermal cycler with denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds and extension at 72°C for 90 seconds. The final cycle was followed by a 15 minutes extension step at 72°C. PCR for β-actin was conducted for 25 cycles using similar conditions as described for ActRI expect that 2 μl of cDNA samples were used and annealing temperature was 55°C.

Assessment and quantification of ActRI mRNA levels

After cPCR, 10 μl from each PCR reaction was subjected to gel electrophoresis and recorded with a negative film. The intensity of PCR products was determined using a densitometer and expressed as the ratio of ActRI and IS. β-actin mRNA levels were used to normalize the samples for variation in cDNA concentrations. Statistical significance of the data was determined by one-way analysis of variance followed by Scheffe's test. $p < 0.05$ was considered significant.

Results

Validation of cPCR

To validate the cPCR for measurement of ActRI mRNA levels, PCR was conducted using a constant amount of IS (0.5 pg) and an increasing amount of native ActRI cDNA (0.01, 0.1, 1, 10 pg). As shown in Fig. 2A, the ratio of band intensity (native ActRI cDNA/IS), measured by densitometry scanning, was nearly linear when plotted as a function of the amount of native ActRI cDNA in the cPCR

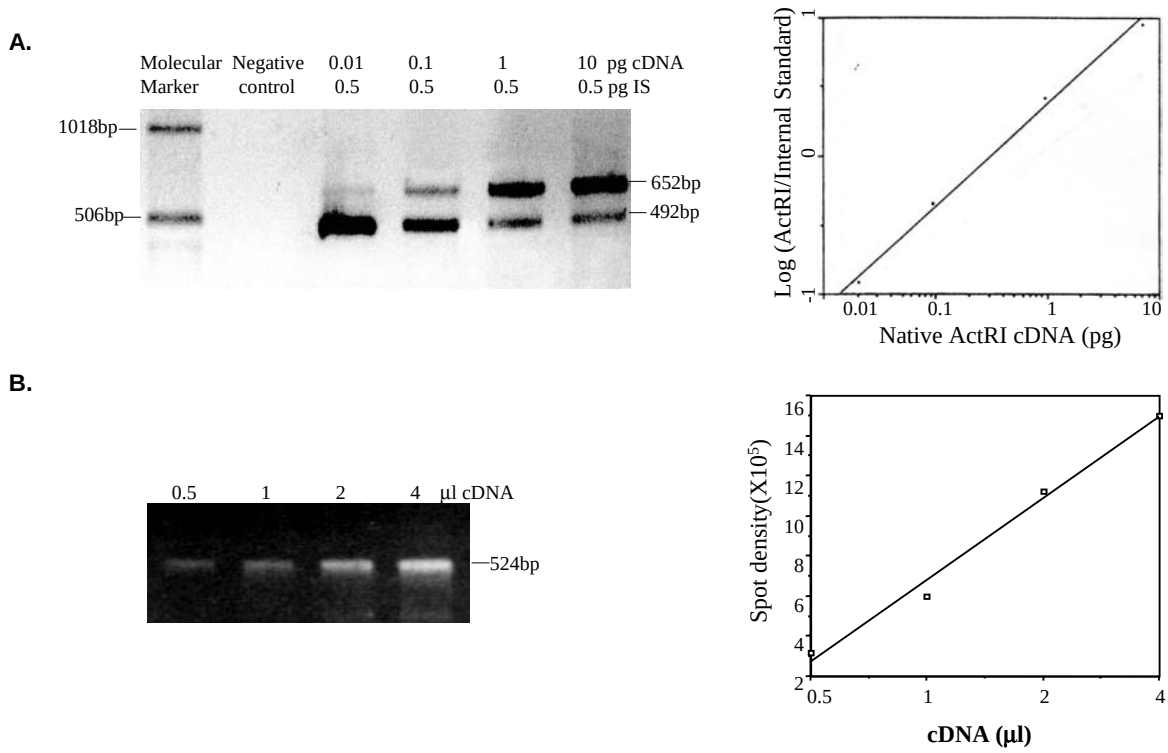

Figure 2
Validation of PCR. A) Competitive PCR for ActRI was performed using a constant amount of internal standard (0.5 pg) and increasing amount of native ActRI cDNA. A linear relationship was found when the ratio of native ActRI cDNA versus internal standard was plotted as a function of the amount of native ActRI cDNA. B) β-actin PCR was conducted using different amount of template cDNA. There is a linear relationship between the amount of cDNA used and the amount of PCR product generated.

reaction. To control for variation in sample cDNA concentrations, β-actin mRNA levels in each sample was also measured and used to normalize the ActRI mRNA levels. To validate a semi-quantitative PCR for β-actin, PCR was first conducted for 20 to 40 cycles. There is a linear relationship between the amount of PCR product generated and the number of PCR cycle when PCR was conducted between 20 to 30 cycles (data not shown) and therefore, subsequent β-actin PCR was conducted for 25 cycles. In addition, when different amount of cDNA (from 0.5 to 4 µl) was used in PCR, there is a linear relationship between the amount of initial cDNA and PCR product (Fig. 2B).

Follistatin blocked the effect of activin-A on ActRI mRNA expression

Activin-A stimulated ActRI mRNA expression

The ActRI mRNA level, measured at 12 hours after treatment, was increased in a dose-dependent manner by activin-A (Fig. 3). A significant stimulation ($P < 0.05$) of ActRI mRNA levels was observed at the dose of 3, 10 and 30 ng/ml. When IEVT cells were treated with activin-A (10 ng/ml) for different period of time, a significant increase in ActRI mRNA level was found at 3 hours ($P < 0.05$) and the maximal response observed at 6 hours ($P < 0.01$) after treatment. By 24 h after treatment, ActRI mRNA levels in activin-A treated cells were not significantly different from those in the control cells (Fig. 4).

The activin-binding protein, follistatin, has been shown to neutralize the effects of activin in several biological

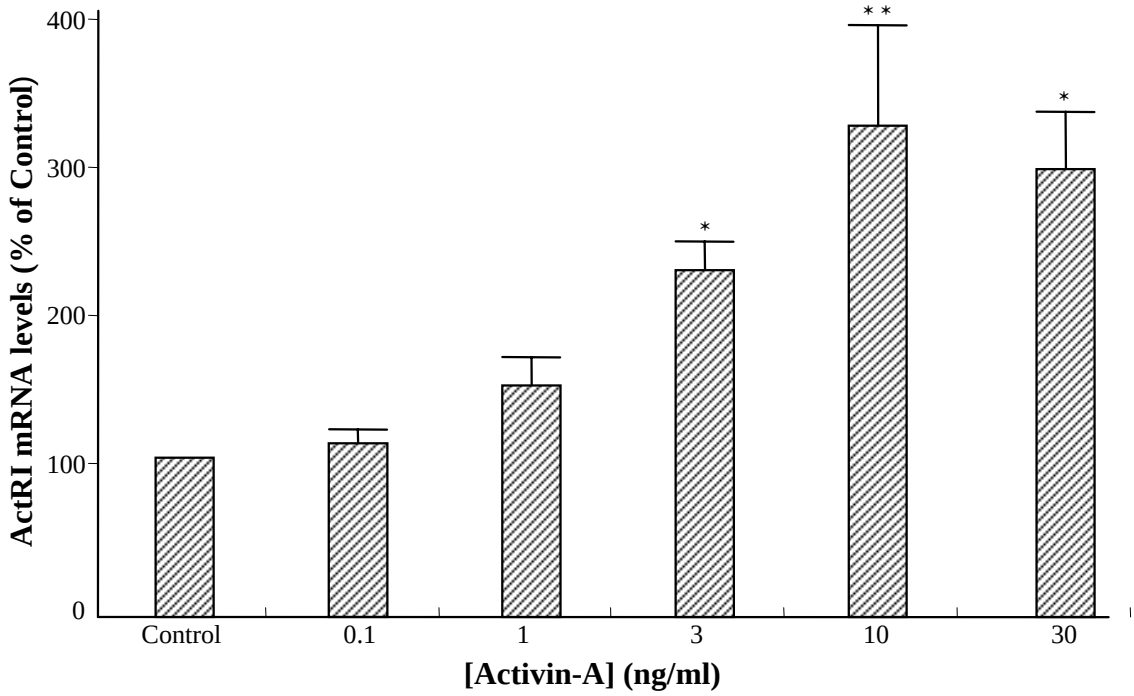

Figure 3
Dose-dependent stimulation of ActRI mRNA levels by activin-A. Cells were incubated for 12 h in the absence or presence of activin-A (0.1–30 ng /ml). cPCR was performed to measure ActRI mRNA levels. The data were pooled from 3 separate experiments and expressed as a percentage of control value (mean ± SEM). Data were normalized for β-actin mRNA levels measured by PCR. *($p < 0.05$) or **($p < 0.01$) versus control.

systems [24–27]. To determine the specificity of activin-A action, we examined the effects of increasing concentrations of recombinant human follistatin-288 on activin-A-induced ActRI mRNA levels. Treatment with activin-A alone resulted in a significant increase in ActRI mRNA levels ($P < 0.05$); however, when activin-A and follistatin were added to the culture together, the activin-A-stimulated ActRI mRNA levels were decreased. At the doses of 50 and 100 ng/ml, follistatin-288 completely blocked the effects of activin-A on the ActRI mRNA levels (Fig. 5).

Inhibin-A antagonized the effect of activin-A on ActRI mRNA expression

Inhibin-A has opposing effects as activin-A in many biological systems. In this study, we also examined the effect of inhibin-A on ActRI mRNA levels. Inhibin-A (10 ng/ml) induced a 60% reduction in ActRI mRNA levels ($p < 0.01$) within six hours of treatment. When inhibin-A was applied together with activin-A (10 ng/ml), significant reductions in activin-A-stimulated ActRI mRNA levels were observed (Fig. 6).

Discussion

For many years the placental cytotrophoblasts obtained by disaggregation of placental tissue have served as the most widely investigated model for the study of placental function *in vitro*. However, the purity of cytotrophoblasts was not consistent. The IEVT cells used in the study exhibit similar cellular function to normal EVT cells [21] and they

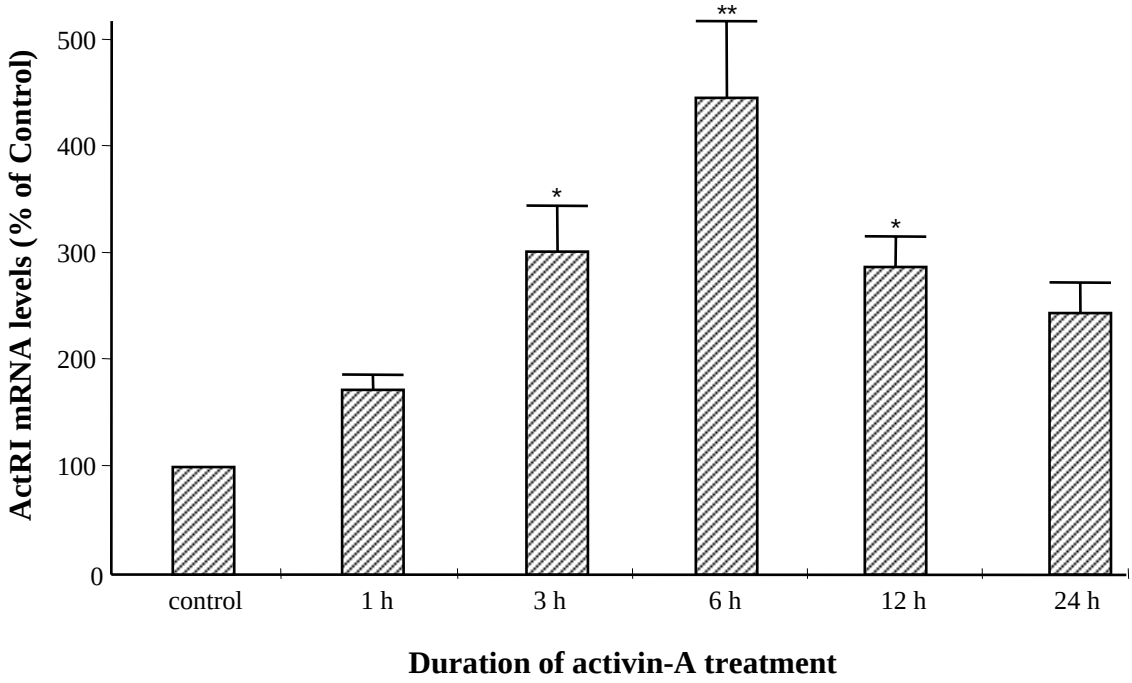

Figure 4
Time course effects of activin-A on ActRI mRNA levels. IEVT cells were incubated with or without activin-A (10 ng/ml) for 1, 3, 6, 12, 24 hours. cPCR was performed to measure ActRI mRNA levels and data were also normalized for β-actin value. *,($p < 0.05$) versus control. **,($p < 0.01$) *vs* control. Data present mean ± SEM of three experiments.

provide a promising model to study the function of EVT cells. Using these cells, we have demonstrated that activin-A stimulates, while inhibin-A inhibits, ActRI mRNA levels.

Our results showed that ActRI mRNA accumulation at 12 hours after treatment was stimulated in a dose-dependent manner by activin-A. The time course of the activin-A effect on ActRI mRNA showed responses at 3 to 12 hours after treatment, with a maximal increase occurring at 6 hours. This response pattern of activin-A on ActRI mRNA suggests that activin-A exerts a positive feedback effect on its own receptor and that the action of activin-A on ActRI mRNA level is transient. The transient effect of activin-A is likely due to the termination of activin signaling. Activin signaling has been shown to be terminated by negative feedback mechanisms involving the activation of Smad7 by activin, and in turn, Smad7 inhibits further activin signaling by blocking the phosphorylation of Smad2/3 by activin type I receptors [28,29]. In addition, activin signaling can also be terminated by the degradation of Smad proteins through the ubiquitination pathway [30].

The changes in ActRI mRNA levels as determined by cPCR in this study could have resulted from changes in the transcriptional rate of ActRI gene and/or the stability of the ActRI mRNA. Activin is known to regulate gene expression at the level of transcription via the Smad signaling pathway [5–7] and expression of Smads in trophoblast cells including the HTR8/SVsno have been demonstrated [31,32], it is therefore possible that activin-A may regulate its receptor expression at the level of gene transcription. Future

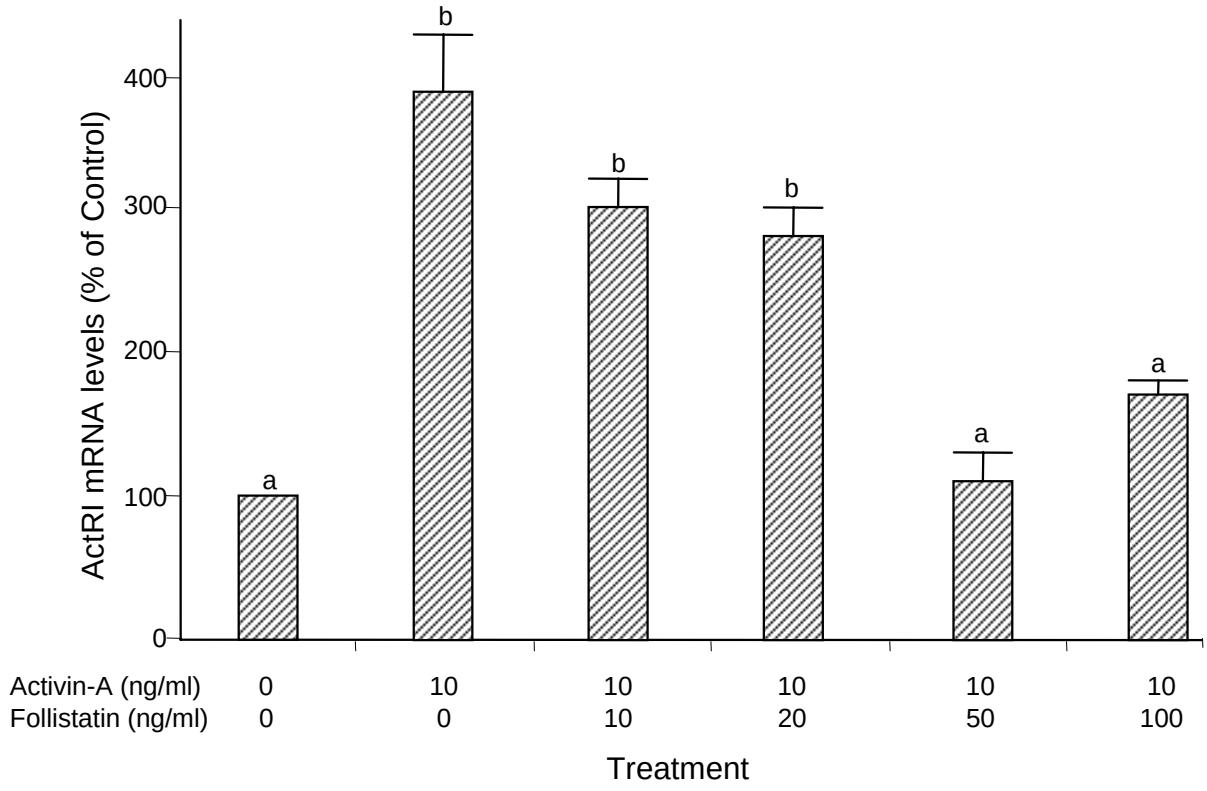

Figure 5
Interaction between activin-A and follistatin-288 on ActRI mRNA expression. Cells were incubated for 6 hours in the presence or absence of activin-A (10 ng/ml) alone, or activin-A(10 ng/ml) incombination with increasing concentrations of follistatin (10–100 ng/ml). cPCR was performed to obtain ActRI mRNA levels which were then normalized for β-actin mRNA levels measured by PCR. Data present mean ± SEM of three experiments. Different letters indicate statistically significant differences ($p < 0.05$).

studies are also needed to confirm that there is a correlated change at the protein level.

Although this is the first study demonstrating that activin-A regulates its own receptor expression in human placenta, several studies in other tissues have also observed modulation of activin signaling by activin itself. In the rat pituitary, activin-A stimulated ActRI and ActRIIB mRNA levels without altering ActRII mRNA expression [33]. On the other hand, activin-A downregulated ActRIB, ActRII and ActRIIB mRNA levels in a cell line derived from a p53(-/-)inhibin-α (-/-) mouse testicular tumor [34].

Follistatin is widely distributed and produced by many activin-responsive tissues [1,25] and may, therefore, serve to anatomically and temporally limit the local activities of activin. Follistatin mRNA transcripts and immunoreactivities have been detected in placental trophoblast cells [16] Similar to many other systems [24,25], follistatin also neutralizes activin actions in human placenta [16]. In the present study, we also found that the stimulatory effect of activin-A on ActRI mRNA levels could be completely blocked by follistatin. Since it is known that follistatin inhibits activin actions by binding to activin and thus preventing the interaction between activin and its type II receptor [35] the neutralization of activin-A action on ActRI mRNA levels by follistatin suggests that the action of activin is a receptor-mediated event.

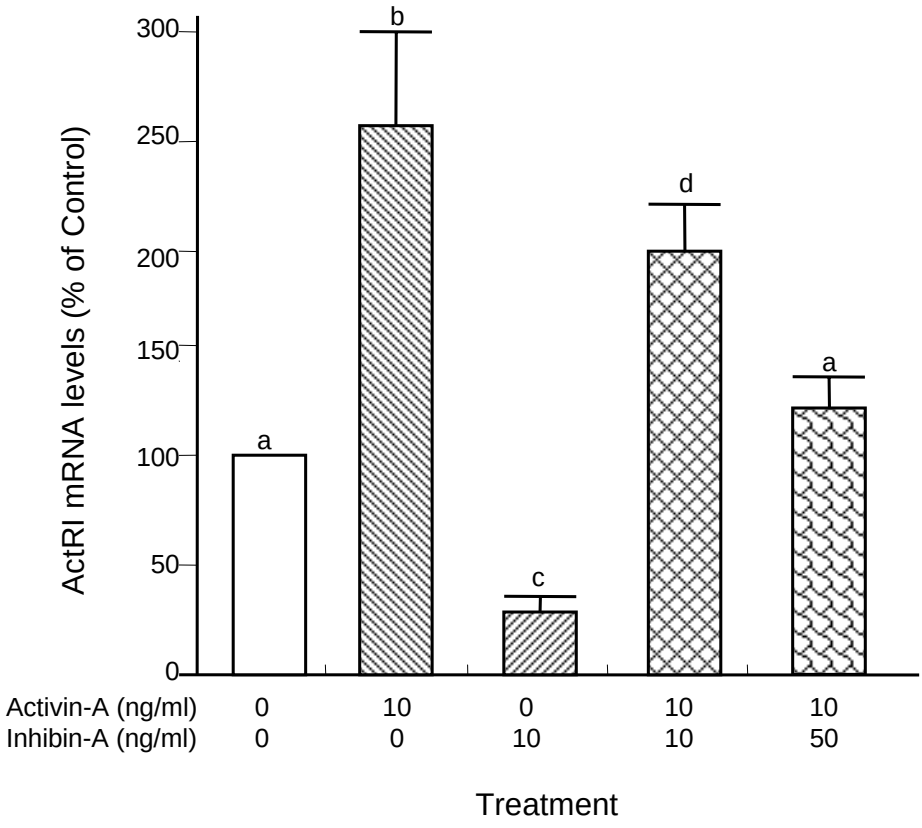

Figure 6
Effects of inhibin-A on basal and activin-A-induced ActRI mRNA levels in IEVT cells. Cells were treated with inhibin-A (10 ng/ml), activin-A (10 ng/ml) or the combination of both for 6 h. ActRI mRNA levels were measured by cPCR and then normalized for β-actin mRNA levels. Data present mean ± SEM of three experiments. Different letters indicate statistically significant differences ($p < 0.05$).

Inhibin and activin possess opposing activities in several biological systems including pituitary FSH secretion, erythroid differentiation, and gonadal sex-steroid production [4]. In the present study, we found that inhibin-A caused a 60 % reduction in basal ActRI mRNA levels and completely inhibited activin-A-induced ActRI mRNA expression. This finding is in agreement with the observation that inhibin counteracts the effects of activin on cultured first trimester trophoblast cells [17].

In summary, we have demonstrated that in activin-A up-regulates mRNA levels of it own receptor ActRI in human extravillous trophoblast cells. Since activin-A has been shown to stimulate the differentiation of extravillous trophoblast cells [14], the up-regulation of ActRI by activin-A suggests a positive feedback regulatory mechanism by which activin-A modulates its own signaling in these cells.

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