LOCALIZATION AND REGULATION OF mRNA TRANSCRIPTS ENCODING ACTIVIN RECEPTORS IN HUMAN PLACENTAL TROPHOBLAST CELLS

by

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ABSTRACT

There is increasing evidence to suggest that activin can function as an autocrine/paracrine regulator in various tissues, including the reproductive system. At the cellular level, activin acts via a family of activin receptor (ActR) subtypes which includes two type I (ActRI and ActRIB) and two type II (II and IIB) receptors. The role of activin in the human placenta is not clearly understood. In this study, the detection of inhibin/activin subunit and ActRI mRNA were examined in first trimester cytotrophoblasts, term cytotrophoblasts, extravillous trophoblast (EVT) cells, immortalized extravillous trophoblast (IEVT) cells, JEG-3 cells, decidual tissue, and decidual cells. Regulation of ActRI mRNA levels was also studied in IEVT cells using competitive polymerase chain reaction (cPCR).

With the exception of JEG-3 cells, ActRIIB mRNA was detected in all cell populations, mRNA transcripts encoding inhibinα, inhibinβA, inhibinβB, ActRI, ActRII and ActRIIB were detected in the whole cell populations. However, ActRIB mRNA could not be detected in all cell populations.

With Northern blot hybridization analysis, it was found that the ActRI mRNA level was greatest in first trimester EVT cells followed in decreasing order of first trimester cytotrophoblasts, JEG-3 cells and term cytotrophoblasts.

An internal standard of ActRI cDNA for cPCR was constructed for the precise quantitation of the ActRI mRNA levels in IEVT cells. The ActRI mRNA accumulation was stimulated in a dose-dependent manner

by activin-A with a maximal effect three times that of control culture at a dose of 10 ng/ml activin-A. The time course of the activin-A effects on ActRI mRNA levels showed an early response with the maximal increase at 6 hours. The effects of follistatin, an activin binding protein, with and without the concomitant presence of activin-A, were also examined. Follistatin had no effect on ActRI mRNA levels in the absence of activin-A. Co-treatment of activin-A and follistatin demonstrated the blocking effect of follistatin on activin-A with dose dependent manner.

This study provides evidence for the first time that activin-A and follistatin modulate the ActRI mRNA levels in an autocrine/paracrine manner in human placental trophoblast cells.

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LIST OF ABBREVIATION

ActR	activin receptor
ActRI	activin receptor type I
ActRIB	activin receptor type IB
ActRII	activin receptor type II
ActRIIB	activin receptor type IIB
BSA	bovine serum albumin
CAM	cell adhesion molecule
cAMP	cyclic adenosine 5'-monophosphate
Cdk2	cyclin-dependent protein kinase 2
cDNA	complementary DNA
cPCR	competitive PCR
CSF	colony stimulating factor
DEPC	diethylene perchlorate
DIG	digoxigenin
DNA	deoxyribonucleic acid
E_2	estradiol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EVT	extravillous trophoblast
FBS	fetal bovine serum
FGF	fibroblast growth factor
FRP	FSH releasing protein
FS	follistatin
FSH	follicle-stimulating hormone
g	gravitational constant
GnRH	gonadotropin releasing hormone
GnRHR	gonadotropin releasing hormone receptor
hCG	human chorionic gonadotropin
IEVT	immortalized extravillous trophoblast
IGF	insulin-like growth factor
IL	interleukin
IS	internal standard
kDa	kilodalton
LH	luteinizing hormone

LIF	leukemia inhibitory factor
M	Molar (mole/liter)
MADR	mothers against dpp-related proteins
MMP	metalloproteinase
mRNA	messenger RNA
Q-PCR	quantitative PCR
P_4	progesterone
PAI	plasminogen activator inhibitor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
Q-PCR	quantitative PCR
Rb	the product of the retinoblastoma gene
RNA	ribonucleic acid
RT-PCR	reverse transcription-PCR
SDS	sodium dodecyl sulfate
SEM	standard error of sample mean
SSC	sodium chloride and sodium citrate solution
TBE	Tris + Boric acid + EDTA
TGFβ	transforming growth factor-beta
TIMP	tissue inhibitor of metalloproteinase
TPR-I	Receptors I of TGF-β
TPR-II	Receptors II of TGF-β
VEGF	vascular endothelial cell growth factor

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CHAPTER ONE:
INTRODUCTION AND LITERATURE REVIEW

Introduction

Reproduction is controlled by the hypothalamus through a delicately designed network of gonadotropin-releasing hormone (GnRH) neurons that translate neural signals into endocrine messages to act on the anterior pituitary gland. The synthesis and secretion of GnRH is controlled by higher neural centers in the brain, allowing integration of a variety of influences. The pituitary hormones that regulate gonadal function are luteinizing hormone (LH) and follicle-stimulating hormone (FSH); these are synthesized in the gonadotropes in response to stimulation by GnRH. Gonadal hormones also directly regulate gonadotropin subunit gene expression through the negative or positive feedback mechanism (Nilson et al., 1983; Paul et al., 1990; Girmus and Wise, 1992). The hypothalamo-pituitary-gonadal axis is an anatomical and functional unit, integrating neuronal, metabolic and hormonal factors to produce the patterns of circulating gonadotropins and gonadal steroids in peripheral plasma, which determine reproductive success or failure.

The critical role played by gonadal steroids and GnRH has been investigated thoroughly over many years. However, there are known circumstances involving gonadal damage in laboratory animals (Clarke et al., 1986) and clinical observations in which differential release of FSH and LH takes place that cannot be fully explained by the regulatory mechanisms of GnRH and the gonadal steroids. More recently, interest has focused on the regulatory influences of inhibin and activin, purified from porcine follicular fluid and best known for the selective regulation of pituitary FSH secretion.

Inhibin and related peptides such as activin specifically regulate the secretion of FSH by direct actions at the level of the pituitary gland, without any effect on the secretion of LH (Clarke et al., 1986).

The subsequent identification of inhibin and activin in a wide variety of reproductive and non-reproductive tissues suggested a greatly expanded role for these factors beyond the control of FSH secretion. Also, it has become evident that many of these effects are exerted within the secreting tissues (DePaolo et al., 1991; Bilezikjian and Vale, 1992), acting locally either on adjacent cells (paracrine effects) or on the secreting cell itself (autocrine effects).

Other than hypothalamo-pituitary-gonadal axis, the placenta provides specialized functions during gestation that are 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. Activin, a potent growth factor produced by human placenta (Rabinovici et al. 1992), exerts its action via its membrane receptors. The biological activity of activin in the placenta especially on the implantation has not been studied yet.

A. Placenta

1. Introduction

The human placenta, hemochorial type in classification, has a multiplicity of functional activities that are essential for fetal development to the birth of a normal baby at term. Most importantly, it establishes a connection between the mother and fetus, that is the first priority of the embryo so that subsequent development during pregnancy is ensured.

Human pregnancy is a physiological event that involves complicated hormonal secretion and regulation. The establishment and maintenance of pregnancy after conception is highly dependent upon contributions made by the blastocyst, the trophoblasts, the embryo, and then the fetus. A biomolecular communication system is established between the baby and mother that is operative from before the time of nidation and persists through the time of parturition.

The link between mother and fetus is operative as follows: maternal blood (spurting out of the uteroplacental vessels) directly bathes the syncytiotrophoblast; but fetal blood is contained within fetal capillaries, which traverse within the intravillous spaces of the villi. The link is established through the anatomical and biomolecular juxtaposition of chorionic villi and decidual tissue. Therefore, at all sites of direct cell-to-cell contact, maternal tissues (decidua and blood) are juxtaposed to extraembryonic cells (trophoblasts) and not to embryonic cells of fetal blood.

In order for the embryo to survive, the trophoblast cells within the placenta have to alter the maternal endocrine system that creates the appropriate hormonal medium within the uterus throughout gestation. Trophoblasts need to come in direct contact with maternal blood and develop a circulatory loop between mother and fetus for nutrient and gaseous exchanges. The paternal proteins that interact directly with maternal immune cells without rejection are expressed within trophoblast cells.

The human placenta is an endocrine organ of considerable capacity and diversity, producing many hormones, cytokines and growth factors. Specific receptors for those hormones and factors are present in the cytoplasm or on the membranes of placental cells. The normal course of gestation undoubtedly depends upon the absolute and relative amounts of the hormones and factors produced by the placenta which exert various actions to control the normal progress of gestation.

2. Gross anatomy

During early pregnancy, the structure of the placenta is changing dynamically through the process of blastocyst apposition, attachment, penetration, and invasion of EVT cells.

The central columns of cytotrophoblast cells, covered by syncytiotrophoblast, develop a villous core containing the feto-placental vessels between days 12 and 21 post-fertilization (Kaufmann and Burton, 1994). Central invasion of the villous core by mesenchymal cells derived from the allantoic part of the embryonic disc is followed by the appearance of hemangioblastic cells and the formation of capillaries (Demir et al., 1989). By day 28, the villi start to be perfused by the fetal circulation. These villi are the basic structures from which subsequent differentiation and growth take place.

This basic structure of the fetal placenta is established during the first trimester, with subsequent development being one of growth and differentiation of the individual placentome. The placentome is the placental functional unit, consisting of a fetal villous tree arising from the chorionic plate, perfused centrifugally by a maternal spiral artery. The umbilical arteries ramify across the chorionic plate, penetrating it in about 50 places to start

individual villous trees. The development of the villous tree during early gestation takes place by proliferation of the immature intermediate villus forming new generations of muscularized stem villi (Benirschke and Kaufmann, 1995). Subsequent growth during later gestation is directed towards formation of mature intermediate and terminal villi.

The term placenta, minus membranes and cord, weighs between 450 and 550 grams. It is an oval or round disc that measures about 18 cm in diameter and 2 cm in thickness. Almost all of it is fetal in origin, the decidua andmaternal vessels being the two exceptions. On the fetal surface lies the detachable, amniotic surface membrane. Beneath it is the chorionic membrane that carries fetal blood vessels. These vessels have a nearly invariable arrangement, with the arteries crossing over the veins. At their termination, these vessels dip into the villous tissue, each ramification supplying one cotyledon. The umbilical cord normally inserts near the center of the placenta. There are normally two arteries and one vein in the cord. The two arteries have an anastomosis near the placental surface in most placentae. The umbilical cord contains no nerves.

The membranes attach to the edge of the placenta and contain the decidua capsularis on the outside and the amnion on the inside, followed by chorion with remnants of fetal vessels and villi, and some atrophying trophoblast cells.

3. Histology

Although primate placentae are generally classified as hemochorial, the structures of the implantation sites and early placenta vary among different

primates. To understand the histologic aspects, the components of the human placenta can be divided into five different parts.

a. Decidua

The decidua gradually changes from predecidua to the well-formed tissue present at birth. Initially, there is some hyperplasia of glandular epithelium, with polypoidy of epithelial nuclei and hypersecretion, that is, vacuolization of cytoplasm. This change is often referred to as Arias-Stella phenomenon and has achieved some clinical importance since it is also found in ectopic pregnancies. Later the epithelium of glands atrophies markedly so that, at term, the remains of glands may be mistaken for endothelium-linked channels. There is regularly some necrosis of decidua around the site of implantation, and the regular presence of necrosis in early implantation sites has been confirmed (McCombs and Craig, 1964).

During the early stage of placentation, irregular infiltrates of lymphocytes and granulocytes in the area of implantation is also found. At term, they are rare in the decidua basalis and much more common in the decidua capsularis.

At the site of implantation, fibrin is also deposited in an irregular fashion, the quantity increasing with gestation. The fibrin layers are invariably found in the delivered placenta, and several layers of decidua are found beneath the fibrin.

b. Umbilical cord

Aside from major fetal vessels, the cord is composed largely of connective tissue that contains a substantial amount of mucopolysaccharides and mast cells. Peculiarly, very few macrophages are present within the cord.

Wharton's jelly, which makes up a substantial part of the cord, is readily compressible and varies remarkably in quantity.

c. Amnion

In general, the amnion is composed of a single layer of flat-to-columnar epithelial cells that frequently show some squamous metaplasia. The epithelium is affixed to a basement membrane and some underlying connective tissue. A large population of macrophages are also seen within the connective tissue of the amnion.

d. Chorion

It contains similar macrophage populations as the amnion and, on its undersurface, has a layer of trophoblast cells that are frequently enmeshed in dense fibrin deposits.

e. Villous tissue

Early stages of human placental development includes prelacunar stage, lacunar stage, primary villous stage, secondary villous stage, and tertiary villous stage (Kaufmann and Burton, 1994). Secondary villi are the villous structures formed when the mesenchymal cord, presumably derived from cytotrophoblasts, invade the trophoblastic columns (primary villi). The bulk of the mature placenta is composed of tertiary villi, emerging from the mesenchymal cord by angiogenesis (Benirschke and Kaufmann, 1995). The human placenta arises primarily through proliferation and differentiation of embryonic trophoblast cells which constitute the first epithelial layer lining of the preimplantation blastocyst. Further development results in several distinct trophoblast population within the human placenta. Those lining the large

number of chorionic villi include two subsets: an inner, proliferative cytotrophoblast layer, inclusive of trophoblast stem cells, and an outer, differentiated, non-proliferative syncytiotrophoblast layer. The cells nearer the fibrous core of the villi are mononuclear, and consists of a single layer of Langhans cells in early pregnancy. Syncytiotrophoblast arises by proliferation and fusion of the underlying cytotrophoblasts and provides an extensive absorptive surface, as well as endocrine functions. The syncytium is clearly the most complex of placental cells. It has a remarkable membrane in which the amitotic nuclei float freely, having no real cell border, and in which there is an enormous variety of cytoplasmic structures.

The EVT cells are highly migratory, proliferative and invasive cells that originate from the stem cells within the villus. These cells emerge from the tips of anchoring villi, invade the uterine decidua and remodel the utero-placental vessels (Graham and Lala, 1992; Kurman, 1992).

4. Trophoblasts

Our knowledge of the early phases of human embryogenesis and the establishment of extraembryonic tissue is limited. The early development of trophectoderm is vital for proper implantation. It is possible that proteases produced from the trophoectoderm of the embryonic pole facilitate blastocyst implantation and has been implicated in tissue invasion by metastasizing cancer cells (Strickland et al., 1976). Proliferation and differentiation of trophoblasts *in vivo* usually take place at the area of contact between maternal and embryonic tissues.

Several different populations of placental trophoblast cells can be distinguished on the basis of morphology and anatomical distribution. The use

of monoclonal antibodies directed against a range of trophoblast-specific antigens has revealed an even greater heterogeneity of trophoblast populations (Bulmer and Johnson, 1985).

The human placenta undergoes dramatic structural reorganization during pregnancy, which synchronizes with the development of the embryofetal and maternal compartments. The spatiotemporal patterns of these events are dynamically controlled by processes, which include cell invasiveness, cell proliferation, and cell differentiation.

a. Differentiation

Morphologically, trophoblasts are either cellular or syncytial, and may appear as mononuclear cells. At implantation, some of the innermost trophoblasts, the cytotrophoblasts that are contiguous with and invading the endometrium, coalesce to become an amorphous, multinucleated, continuous membrane that is uninterrupted by intercellular spaces, the syncytium. There are no individual cells in the syncytiotrophoblast layer, only a continuous lining. The mechanism of syncytial growth, however, was a mystery in view of the discrepancy between as increase in the number of nuclei in the syncytiotrophoblast and only equivocal evidence of intrinsic nuclear replication. Mitotic figures are completely absent from the syncytium, being confined to the cytotrophoblasts, the cellular progenitors of the syncytiotrophoblast.

In vitro system, cytotrophoblasts freshly isolated from term placental tissue and cultured in serum-supplemented medium or in serum-free medium on surfaces coated with extracelluar matrix proteins undergo morphological differentiation to form syncytia (Kliman et al., 1986; Kao et al., 1988). The mononucleate cells are seen to move in a random fashion by time-lapse video

microscopy in the initial hours after plating. When the cells make contact, they adhere and may continue to move, but in a more circumscribed area. The motile cells are rounded, whereas cells that have made contact with other cells tend to spread on the culture substrate. With time in culture, cell aggregates increase in number and size. The extent and kinetics of aggregation are dependent on plating density. The aggregated cells ultimately fuse to form multinucleated giant cells that show relatively little motility compared to the freshly isolated cytotrophoblasts. The kinetics of cell fusion and the size of the syncytia formed are also related to plating density (Kliman et al., 1986).

Cyclic adenosine 5'-monophosphate (cAMP) plays a role in trophoblast differentiation. Trophoblasts possess adenylate cyclase, G-proteins, and cAMP-dependent protein kinase (Nulsen et al., 1988). Interestingly, adenylate cyclase is localized to the basal membranes of the syncytiotrophoblast that abut against those of the underlying cytotrophoblasts (Matsubara et al., 1987), suggesting that interactions of these two trophoblast cell types regulate cAMP production.

Morrish et al. (1991b) reported that transforming growth factor β1 (TGFβ1) inhibited the morphological differentiation of cytotrophoblasts in culture, whereas these authors found that epidermal growth factor (EGF) enhanced the transformation of cytotrophoblasts into syncytia. These observations indicate that trophoblast differentiation can be modified by substances acting in a paracrine mode. Whether TGFβ1 or EGF have such roles in situ remains to be established. Cytotrophoblast aggregation is dependent upon protein synthesis and calcium (Babalola et al., 1990). There is evidence that the calcium-dependent cell adhesion molecule, E-cadherin, is involved in the aggregation process because it is localized on the surface of

cytotrophoblasts in chorionic villi (Eidelman et. al., 1989) and detected by immunocytochemistry at areas of contact between trophoblast cells *in vitro*. Desmosomes develop between the cells; and as the cytotrophoblasts fuse, the expression of E-cadherin diminishes (Coutifaris et al., 1991). The mRNA expression of cadherin-11, a new member of the cadherin superfamily, increases when E-cadherin expression decreases in epithelial cells of the human placenta. The fact of cadherin-11 existing in syncytiotrophoblast and EVT cells but not in the villous trophoblasts, suggested its role in modulating trophoblast-endometrium interactions (MacCalman et al., 1996).

As a member of TGFβ superfamily, activin has diverse and widespread effects within living organisms at many stages during growth and development. In cell proliferation and differentiation, the inhibitory effects on cell growth and differentiation that have been observed upon treatment of cells with activin suggest that further understanding of the bioactivity of this molecule and its characterization on a molecular level may aid in a more complete understanding of trophoblast cell growth and differentiation.

b. Invasion

Successful human placentation requires that trophoblast cells which are of embryonic origin rapidly invade genetically dissimilar maternal decidual tissue during the early stages of pregnancy. The timing and extent of invasiveness is precisely regulated spatiotemporally. Trophoblast cells have also the property to differentiate and form multinucleated syncytia which acquire the ability to secrete many growth factors, cytokines and hormones. Growth factors, cytokines, and hormones have been implicated in the control of the invasive process of placental implantation.

Giant multinucleated trophoblast cells are present in the decidua basalis and the myometrium. Endovascular or "intra-arterial" cytotrophoblasts migrate against the blood stream into the intervillous uteroplacental spiral arteries carrying the maternal blood into the intervillous space of the placenta. The deported trophoblasts disseminate through the uteroplacental veins into the maternal circulation, and embolize in the lungs.

Various TGF-β isoforms within the uterus are expressed in specific cell-types and at distinct times, suggesting that they have specific role during the peri-implantation period. In the mouse uterus, TGF-β1 is primarily expressed in endometrial luminal and glandular epithelium during the preimplantation period and in decidua of the post-implantation period (Tamade et al., 1990). TGF-β2 is synthesized by epithelial, myometrial and decidual cells and its secretion may be regulated by estradiol (E_2), whereas TGF-β3 is primarily synthesized by myometrial cells, independent of ovarian steroids (Das et al., 1992).

In humans, TGF-β1 messenger ribonucleic acid (mRNA) is equally distributed in endometrial glands and stroma, and levels are lowest in the proliferative phase, increasing two-fold in the secretory phase, and about five-fold in the maternal endometrium (decidua) of pregnancy. The ability to detect TGF-β1 mRNA in a cycle-dependent manner suggests that it may contribute to the inhibition of cellular proliferation during periods when endometrial cellular differentiation, induced by TGF-β or other growth factors, is dominant. In addition, the marked abundance of TGF-β mRNA levels in decidua also suggests a possible role for this growth factor in implantation (Lala and Graham, 1990). It has been proposed that TGF-β may inhibit trophoblast invasion, since it promotes differentiation of human

cytotrophoblasts into noninvasive multinucleated giant trophoblast cells (Graham et al., 1992).

The invading trophoblast secretes a variety of proteases (Lala and Graham, 1990), including plasminogen activators and matrix metalloproteinases (MMPs). Since TGF-β induces plasminogen activator inhibitor (PAI) mRNAs and induces the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in a variety of cell types, it has been postulated that TGF-β may act to control trophoblast invasion by regulating proteases and their inhibitors in the embryo and maternal decidua. In addition, the latent form of TGF-β1 is activated by plasmin, and it has been postulated that maternal decidua stores latent TGF-β in the extracellular matrix which is cleaved subsequently by the invading trophoblast by its production of plasmin. TGFβ could then increase PAIs and inhibit trophoblast-derived MMPs. The net result would be limiting trophoblast invasion. In support of the latter hypothesis is the observation that TGF-β produced by first trimester decidual cells in culture inhibits the *in vitro* invasiveness of first trimester human trophoblasts, due to the induction of synthesis and secretion of TIMP-1 (Graham et al., 1993b).

Activin is a member of TGFβ superfamily. It might have the same effects as TGFβ on trophoblast invasion by regulating extracellular matrix (ECM) components or TIMP-1 production.

c. Trophoblast cell culture

Human placental functions can be studied only outside the human body. Placental explants and organ cultures have been investigated extensively. Interpretation of results from these experiments is difficult owing to the presence of many different cell types and the rapid necrosis suffered by

tissue fragments when immersed in culture medium. Trophoblast culture after cell separation is the most useful preparation for studying the different biochemical functions of this cellular layer *in vitro*.

In addition, this model can be used for understanding many of the important phenomena of cell biology such as cellular differentiation, proliferation, invasion, fusion, and fetal oncogen expression.

Trophoblast cells are the parenchyma cells of the placenta. They serve an important role in creating the nutritional, immunological, and hormonal milieu for the developing embryo/fetus.

Multinucleated cell masses form in human trophoblast cultures, but it appears impossible to obtain a pure culture of human syncytiotrophoblast *in vitro*, the layer which is the site of the most essential placental functions *in vivo*.

However, cytotrophoblasts are easier to obtain with the method of trophoblast cell preparation from term (Kliman et al, 1986) or first trimester placenta (Graham et al, 1994).

- **Trophoblast cell preparation**

Trophoblast biologists are often uncertain as to what cell types they are investigating because the mononuclear cell populations prepared from trypsinization of human first-trimester chorionic villi are morphologically very similar. Zuckermann et al. (1986) described that the characteristics of the trophoblasts based on (a) being fetally derived, (b) epithelial cells, and (c) capable of progesterone secretion.

There are several ways of obtaining isolated cells from the placenta for subsequent culture. Anti-trophoblast histocompatibility antigen-A, B, C monoclonal antibodies (18B/A5 and 18A/C4), acting as immunocytochemical

markers, were found to be very efficient in identifying cytotrophoblasts (Butterworth, 1985).

Over 99% of fibroblasts could be absorbed onto an antibody directly against CD9 antigen conjugated to magnetic beads (Morrish et al., 1991a). Using sequential trypsinization, percoll gradient centrifugation, and negative selection with anti-CD9, immunomagnetic separation is another method for the simultaneous preparation of highly enriched human placental trophoblast populations (villous and extravillous) from first-trimester placental villi (5 to 12 weeks) (Aboagyemathiesen et al., 1996). A simple method is described for the isolation of trophoblast cells from both first trimester and term placenta to distinguish these cells from mesenchyme and endothelium (Yeger, 1989). Highly purified human trophoblasts of first trimester can be obtained by flow cytometric sorting (King et al., 1996). The availability of highly purified extravillous cytotrophoblasts by flow cytometry permits precise investigation of trophoblast function (Shorter, 1990). A monoclonal antibody, HSA-10, was shown to react specifically to surface antigens of human trophoblast cells and acrosome-reacted human sperms, but not in any other human tissues, such as brain, heart, kidney, liver, muscle, and blood cells (Yoshiki et al., 1995). The method we used to obtain mononuclear cytotrophoblasts from term is modified from Kliman's method (Kliman et al., 1986) or first trimester from the protocol of Graham et al. (1994).

Highly purified EVT cells can be acquired by collecting cells which migrate from primary explants of mechanically derived chorionic villi fragments on culture dishes with or without coating Matrigel. As a result of cell proliferation and migration, the number of EVT cells increased during the culture period of 4 days in Matrigel. By day 4 of culture, 5 to 10 % of EVT

cells ceased to migrate, firmly attached to the substratum (Genbacev et al., 1993).

In 1993, Graham et al. reported an immortalized EVT (IEVT) population (HTR-8/SVneo) which has shared a number of phenotypic properties with the parental trophoblast cells except the ability to sustain prolonged growth in culture. Those cells are an important tool for the study of placental function and tumor invasion.

5. Implantation

The existence of a maternally-derived receptive phase or "window" for embryo implantation was demonstrated in the mouse (McLaren and Michie, 1956) and in the rat (Dickmann et al., 1960). In each species, the receptive phase is less than 24 hours in length. Human embryo transfer data in assisted reproduction indicates that the window is of approximately 4 days duration in women, from days 20-24 of the cycle (Bergh and Navot, 1992); timing from the LH peak, which precedes ovulation by about 36 hours, gives a window lasting from approximately day LH peak +7 to +11. Just before implantation, the zona pellucida disappears and the blastocyst embraces the endometrial surface; this is the time of apposition, when the blastocyst adheres to the endometrial epithelium. Most commonly, implantation takes place on the upper part of the endometrium, and more often on the posterior wall of the uterus. After gentle erosion between epithelial cells of the surface endometrium, the invading trophoblasts burrow deeper into the endometrium and the blastocyst soon becomes totally encased within the endometrium; specifically, the implanting blastocyst becomes completely buried in and covered over by the endometrium.

Uterine endometrium is programmed to develop in a precise fashion in response to changes in circulating ovarian-derived steroid hormones (Palter et al., 1996), culminating in a state of receptivity to an implanting embryo. In the proliferative phase, there is intense proliferation of endometrial surface and luminal glandular epithelium, stromal cells, and vascular endothelium. In the secretory phase, differentiation of endometrial components occurs reflected primarily in glandular secretion and stromal decidualization. Proliferation and differentiation of the indigenous cellular population and cellular recruitment and activation of the macrophage population are dependent on E_2 and progesterone (P_4), and locally produced growth modulators (growth factors and cytokines), presumed mediators of steroid hormone action. The primary purpose of the endometrium is for the initiation and continuation of pregnancy. In the absence of implantation, in primates, the endometrium is sloughed and cycle begins anew. The opportunity for implantation (the so-called "window" of implantation) is temporally and spacially restricted in endometrium of humans and in other species and is dependent on appropriate endometrial growth and development (Yoshinaga, 1988). This includes appropriate expression of recognition molecules for embryo apposition and adhesion to the epithelium and appropriate controls for the process of trophoblast invasion. Implantation itself is not highly efficient as approximately 65% of conceptions ending in unrecognized loss (Clark et al., 1991). Apposition of the embryo near the maternal endometrial epithelium occurs without adhesion or invasion in about 45% of these losses, implantation failure in about 30%, and development failure after implantation in about 25% (Clark et al., 1991). It is regarded that much of the "programming" of human endometrium for becoming receptive is autonomous, and once the implantation process begins, intense

communication between the blastocyst and the maternal endometrium occurs. Opportunities for this cross-talk occur while the blastocyst is hatching, when it attaches to and passes through the endometrial epithelium, and when it invades into the endometrial stroma. Precisely how growth factors and cytokines are involved in these processes is not well understood, although major advances in this field have developed within the past few years. Much of the information about cytokine action on endometrial cells comes from *in vitro* studies, and their potential roles in implantation derive from molecular biology techniques, including gene knock-out and transgenic animal models.

A number of tissue compartments are involved in the process of implantation, including blastocyst, uterine epithelium, basement membrane, and decidua.

As the blastocyst begins to implant, the trophoblast and in particular the invasive trophoblasts produce laminin and fibronectin. At the same time, receptors for these glycoproteins appear on the trophoblast. The appearance of basement membrane compounds at the apical surface of uterine epithelium may play a part in disorganizing the epithelium at the implantation site in some species (Foidart et al., 1990). Endometrial stromal cells that undergo decidual transformation have components in their basement membranes, such as laminin, fibronectin, entectin, heparan sulphate proteoglycan and collagen type IV. The plasminogen activator and matrix metalloproteinase systems are probably involved in degrading collagen type IV, the main component of basement membrane of uterine epithelium that interacts with the blastocyst during the penetration process (Lala and Graham, 1990).

Recently, knowledge of cell adhesion mechanisms has increased significantly, and this has led to investigations of the subsequent interstitial penetration of the embryo. The simplest hypothesis for the control of

attachment is upregulation under steroidal control of one or more cell adhesion molecules (CAMs) at the luminal epithelial cell surface of the endometrium. These receptors would then interact with cognate ligands on the outer trophectodermal surface of the hatched blastocyst. Considerable advances have been made in describing the composition of these two cell surfaces in human and mouse. A second hypothesis has also emerged: that anti-adhesion molecules, such as Muc1 (an epithelial apical surface mucin), may play a role in regulating implantation (Surveyor et al., 1995).

B. Growth factors

It is now recognized that at least some of the growth regulatory actions of both steroid and peptide hormones on reproductive cells are medicated by local growth factors and cytokines. They exert their actions by autocrine, paracrine, juxtacrine, and/or endocrine mechanisms. Both growth factors and cytokines are peptides or polypeptides that interact with specific cell membrane receptors in an autocrine, paracrine and/or endocrine manner to initiate intracellular signaling pathways which results in cellular division, differentiation, growth arrest, or cellular activation (Carpenter, 1987).

Growth factors interact with specific cell surface receptors that have extracellular ligand binding, transmembrane, and cytoplasmic domains. Most growth factor receptors have been found to possess tyrosine or serine/threonine kinase activity in their cytoplasmic domains, which is activated upon binding of the growth factor to the receptor and activate potent protein kinases that act directly or indirectly on a set of transduction molecules that form several signaling pathways to the cytosol or nucleus and result in regulating gene expression, cellular metabolism, and mitosis.

Growth factors act at distinct phases of the cell cycle. For actively dividing cells, the cycle is comprised of four phases; G_1, S, G_2, and M. DNA synthesis occurs during the S-phase. G_1 is the interval between the end of mitosis and the beginning of the S phase. G_2 is the interval from the end of the S-phase to the beginning of cell division. The M phase is the period of mitosis and cytokinesis. Growth-arrested cells are in a dormant state (G_0 phase). Two critical control points (G_1/S boundary and G_2/M boundary) at which growth factors act are temporally distinct and respond to different mitogens. These are the restriction points where a cell is committed to entering the S phase, occurring about two hours before the onset of deoxyribonucleic acid (DNA) synthesis, and for growth-arrested cells, the point at the beginning of the transition from G_0 to G_1. Among the factors that regulate the cell cycle checkpoints, proteins encoded by the *cdc*2 family of genes and the cyclin proteins appear to play particularly important roles (Murray and Kirschner, 1989). The accumulation and degradation of cyclins regulate the checkpoint at the G_1/S boundary. Mitosis is initiated by activation of the *cdc*2 gene at the G_2/M boundary (Lewin, 1990). The *p*53 tumor suppressor gene also appears to participate in delay of the cell cycle in order for DNA repair to be completed. However, growth factors exert positive or negative effects upon the cell cycle by influencing gene expression related to events that occur at the G_1/S cell cycle boundary.

Growth factors and cytokines are expressed in the proliferative and secretory phase endometrium and in uterine decidua, as well as in blastocyst, but to different degrees (Giudice and Saleh, 1995). Regarding endometrial development, growth factors and cytokines are well suited to participate in these cyclic endometrial events, because of their known mitotic and differentiative properties. The major growth factors participating in growth

and development of endometrial cellular components (Giudice and Saleh, 1995; Giudice, 1994) are epidermal growth factors (EGF), insulin-like growth factors (IGF-I, IGF-II), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and vascular endothelial cell growth factor (VEGF). Regarding apposition and attachment of embryo and endometrium in implantation, EGF family (Giudice and Saleh, 1995), colony stimulating factor-1 (CSF-1) (Giudice, 1994), leukemia inhibitory factor (LIF) (Stewart, 1994), interleukin-1 (IL-1) (Simon et al., 1994) are believed to be involved.

Cytokines, growth factors and hormones have been implicated in the control of invasive process. EGF, TGFα, IGF and TGFβ are among the factors that involve the trophoblast invasion.

C. Activin and TGFβ family

- **Activin and TGFβ superfamily**

Inhibin and activin are intimately relevant peptides whose structures have been clarified recently by protein chemistry and cloning techniques (Vale et al., 1986).

1. Activin and Inhibin

As suggested by their names, inhibin decreases and activin stimulates gonadotrope function. Inhibin is a 32 kDa heterodimer gylcoprotein composed of an α-subunit and one of two related β-subunits, termed inhibin A (α-βA) or inhibin B (α-βB). During the purification of inhibin from porcine follicular fluid, Vale et al. (1986) purified an FSH releasing protein (FRP) that

stimulated the secretion of FSH by cultured anterior pituitary cells. The structure of FRP was found to be a homodimer formed by two inhibin βA subunits linked by disulfide bonds. FRP was named as "activin A" lately. Activins are homodimeric glycoproteins composed of two β subunits with molecular weight of 24 kDa. Three different forms of activins are recognized: activin A (βA/βA), activin AB (βA/βB), and activin B (βB/βB). Recently, two new inhibin β subunits, βC and βD, have been cloned from humans and *Xenopus*, respectively. These two subunits have the same general structure as inhibin βA and βB subunits with nine conserved cysteine residues (Hotten et al., 1995; Oda et al., 1995).

The activins are dimeric growth factors (Thomsen, 1990) which are members of the TGFβ superfamily (Massague, 1987). This family of growth factors is recognized to have function involved in regulating differentiation and development, including TGFβs, activin, inhibin, bone morphogenic proteins, Mullerian inhibiting substance, Dorsalin, decapentaplegic, Vgr60A, Nodal, gene product from *Xenopus* (Vale et al., 1986) and growth differentiation factor (Dong et al., 1996). This class of molecules may serve as important autocrine and paracrine factors (Chen et al., 1993) in the modulation of cellular proliferation and differentiation in different tissues (Yu et al., 1987).

TGFβ is a family of proteins that display multiple effects on a large number of cell types and have been associated with fundamental developmental events. Some of these events include dorsoventral patterning, gut morphogenesis, and proximal-distal outgrowth of imaginal disks in *Drosophila*, mesoderm induction in *Xenopus*, and sexual development, pituitary hormone control, and bone and cartilage formation in mammals. At

the cellular level, TGFβ can inhibit cell proliferation by causing growth arrest in the G_1 phase of the cell cycle.

All known TGFβ-related factors act as dimers, and most have been identified as homodimers, though several examples of heterodimers have been shown. In the case of activin and inhibin, homodimers and heterodimers have opposite effects upon their target cells. TGF-β inhibits the proliferation of epithelial cells by altering the expression or function of various components of the cell cycle machinery (Vivien and Wrana, 1995). Expression of these components, like cyclin A, is inhibited by TGFβ treatment (Feng et al., 1995). TGFβ has been shown to arrest the cell cycle in the middle to late G_1 phase. Cells treated with TGFβ contained normal amounts of cyclin E and cyclin-dependent protein kinase 2 (Cdk2) but failed to stably assemble cyclin E-Cdk2 complexes or accumulate cyclin E-associated kinase activity. TGFβ prevents the phosphorylation of Rb, the product of the retinoblastoma gene, during the G1 phase, retaining Rb in a hypophosphorylated state that may suppress progression into the S phase.

- **Functions of Activin**

Activins, produced in the gonads and extragonadal tissues (including the pituitary, brain, placenta, and kidney), have been implicated in the regulation of many biological processes, including the proliferation of many cell lines (Gonzalez-Manchon and Vale, 1989; Hedger et al., 1989; Kojima and Ogata, 1989; Belezikjian et al., 1992), control of the secretion and expression of the anterior pituitary hormones (FSH, growth hormone, and adrenocorticotropic hormone) but not LH (Vale et al., 1986; Dye et al., 1992), neuronal survival (Hashimoto et al., 1990; Schubert et al., 1990),

hypothalamic oxytocin secretion (Sawchenko et al., 1988), folliculogenesis, oocyte maturation (Hiller, 1991), erythropoiesis (Yu et al., 1987), early embryonic development (Green and Smith, 1990; Thomsen et al., 1990; van den Eijnden et al., 1990) and *Xenopus* mesoderm formation (Yeger, 1989). Activin, inhibin and GnRH have been found to be produced by the same cells in the human placenta (Petraglia et al., 1992). The biological action of hCG in the initiation and maintenance of human pregnancy stresses the importance of GnRH, inhibin and activin in early pregnancy.

Among the myriad of functions now associated with activins are stimulation and repression of gene expression, proliferative and antiproliferative effects, and differentiative activities. The effects of activin on reproductive tissues (Thomsen et al.,1990), hematopoietic cells (Vale et al., 1990), *Xenopus* embryonic development as well as various other organ systems (Dye et al., 1992) have been recently reviewed.

However, there has been very little data reported on activin secretion during menstrual cycles showed that the plasma level of activin does not change significantly (Demura et al., 1993) and the data about serum activin concentration during pregnancy is also limited. The production of activin in the placenta has been reported (Rabinovici et al., 1992; Petraglia et al, 1994a). The expression of mRNAs for inhibin α-, βA, and βB- subunits has been detected in human placental tissues during pregnancy. Northern blot analysis has shown that human placenta produces all inhibin subunits (α, βA, and βB) with a profile related to gestational ages (Petraglia et al., 1991).

D. Follistatin

Follistatin is not a member of TGFβ family, but can bind activin tightly, resulting in the inhibition of activin action. At least three isoforms were found in term human placenta with molecular weights of 31 kDa, 35 kDa, and 38 kDa (de Krester et al., 1994).

Inhibins and activins bind to follistatin, α2-macroglobulin, and activin receptors *in vitro* (Tano et al., 1995). Like inhibins and activins, follistatin was originally isolated and purified from ovarian follicular fluid (Ueno et al., 1987) and has the ability to bind activin via their common inhibin-β chains to control activin action in various tissues in an autocrine/paracrine manner to regulate cell growth, differentiation (Shimonaka et al., 1991) and pituitary FSH secretion (Ying et al., 1995). In contrast to inhibin and activin, follistatin is a single-chain monomeric protein (Cataldo et al., 1994) that binds to activin with high affinity comparable to that of activin with its receptors, and to inhibin with lower affinity (Schneyer et al., 1993). Using primary cultures of human placental cells, Petraglia et al. (1994b) found that follistatin mRNA is expressed in human placenta, fetal membranes, and decidua. The functional role of follistatin in the local control system regulating placental hormone production has also demonstrated.

E. Activin receptors

Many hormones and other ligands that regulate cell function circulate at subnanomolar concentrations and exert their actions by binding to specific receptors located on the cell surface or within the cell. In general, steroid hormones diffuse into the cell and are bound by receptor proteins in the cytoplasm and/or nucleus. Once occupied by the hormone, steroid receptors undergo activation and translocation to the nucleus where they influence gene transcription and the expression of specific proteins. On the other hand,

peptide hormones are bound by receptors residing on the plasma membrane and activate processes that release molecular signals (second messengers) into the cytoplasm of the cell. Growth factors are peptides that bind to membrane receptors and exert their function for cell differentiation, proliferation, immigration, apoptosis and etc..

In contrast to the signaling mode of G protein-coupled receptors, with the formation of small second messenger molecules that activate potent serine/threonine protein kinases located throughout the cell, receptors for growth factors and cytokines operate through a network of enzymatic proteins that act directly or indirectly on sets of transduction molecules that form several signaling pathways to the nucleus. These pathways are initiated at the cell surface by ligand-induced dimerization or oligomerization of several types of receptors, leading to activation of their intrinsic tyrosine or serine/threonine kinase activity or to interaction with other proteins that possess tyrosine kinase activity or activate such enzymes in the cytoplasm. The ligand-induced dimerization of tyrosine kinase receptors, or ligand binding to the preformed oligomeric receptors, leads to tyrosine phosphorylation of the receptor molecules or recruitment of cytoplasmic tyrosine kinases, followed by the activation of several major cellular signaling pathways. This tyrosine or serine/threonine kinase activity is considered to be the primary effector system in transmembrane signaling and results in phosphorylation of intracellular proteins which regulate pathways altering gene expression, cellular metabolism, and mitosis.

TGF-β and activins transduce their signals through the formation of heteromeric complexes of two different types of serine/threonine kinase receptors, approximately 50-55 kDa and approximately 70-80 kDa. On the basis of cross-linking experiments with TGF-β-1, receptors were designated

type I, II, and III in increasing order of molecular mass corresponding to 54-70, 80-100, and 300 kDa. The type III receptor is a betaglycan, which is a membrane-anchored 853 amino acid protein core with an extracellular domain consisting of covalently attached chondroitin sulfate and heparan sulphate chains.

Type I and type II receptors belong to a family of transmembrane serine/threonine kinases. Type II receptors for activin require association with the type I receptor for ligand binding. The type II kinase phosphorylates the type I receptor at a conserved, type I-specific GS domain, resulting in its activation. Neither type I nor type II receptors appear to be able to signal alone. These proteins are therefore considered to form heteromeric signaling receptor complexes that contain type I and type II receptors.

Type II receptors are able to interact with different type I receptor isoforms. The specificity of the biological response to a ligand in a given cell type appear to be defined by the particular type I receptor engaged in the complex. Type II receptor is phosphorylated by cellular kinases at multiple sites and by itself at additional sites and this autophosphorylating activity is not regulated by ligand binding, i.e., TGF-β-II is constitutively active. Unlike this, the type I receptor is not an autophosphorylating kinase and is directly phosphorylated by the type II receptor kinase.

In the rat, mouse and human, activin acts via a family of activin receptors (ActRs) that includes at least two type I (ActRI and ActRIB) and two type II (ActRII and ActRIIB) subunits (Dalkin et al., 1996; Tsuchida et al., 1995). The activin receptors have been characterized to be serine/threonine kinase receptors that contain an intracellular protein kinase domain phosphorylating serine and threonine residues (Lin et al., 1992). The basic structure of the receptors includes relatively small extracellular ligand

binding domain, a single spanning transmembrane domain and an intracellular enzyme-linked kinase domain (Fig. 1). Interestingly, the two types of receptors in the presence of activin, can form a stable receptor complex by dimerization and becomes functional to initiate the intracellular signal transduction (Mathews, 1994).

Using the ActRIB and ActRIIB receptor isoforms, Attisano et al. (1996) found activin binds directly to ActRIIB, and this ligand-receptor complex associates with ActRIB, which becomes phosphorylated. Investigating the mechanism of activin receptor activation showed that ActRIIB acts as a primary activin receptor and ActRIB acts as an intracellular downstream transducer of activin signals. The interaction between activin type I and type II receptors is necessary for activin signal transduction, as has been shown for two different type I receptors (Attisano et al., 1993; Carcamo et al., 1994).

Mothers against dpp related proteins (MADR) are essential intracellular components of TGFβ signaling pathways of serine/threonine kinase receptors and are regulated by phosphorylation. Interaction of MADR2 with receptors and phosphorylation requires activation of receptor I by receptor II and is mediated by the receptor I kinase. Mutation of the phosphorylation sites generates a dominant negative MADR2 that blocks TGFβ-dependent transcriptional responses, stably associates with receptors, and fails to accumulate in the nucleus in response to TGFβ signaling (Macias-Silva et al., 1996).

The cDNA encoding type I and type II activin receptors have been cloned from mouse (Mathews and Vale, 1991), rat (Shinozaki et al., 1992), and human testes (Donaldson et al., 1992). Four activin receptor subtypes (ActRI, ActRIB, ActRII, and ActRIIB) have been identified in various tissues

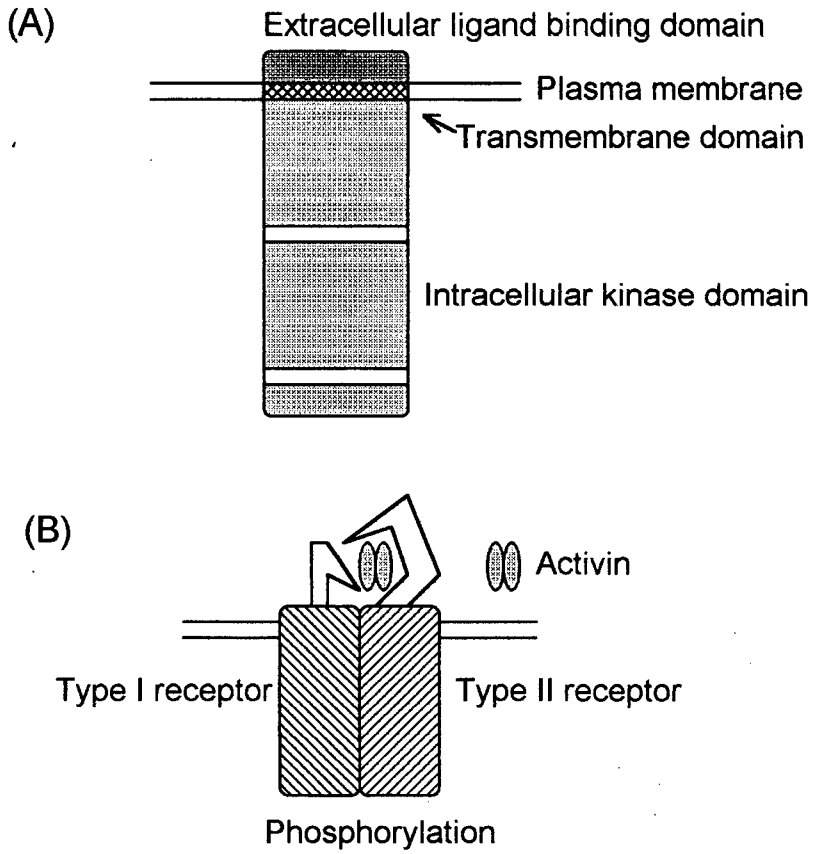


Figure 1. Common structural features of activin receptors Type I and II (panel A). Small white striped region of intracellular part indicates the inserts characteristics of this serine/threonine kinase family. A model for activin signaling receptor system is shown in panel (B). The signal transmission requires the presence of activin receptor types I and II, receptor dimerization, and involve a receptor coupled serine/threonine kinase activity.

(Matzuk et al., 1992; ten Dijke et al., 1994; Peng et al., 1996; Peng et al., 1993; Feng et al., 1993). The literature reports of activin subunits and ActRs gene expression and regulation in different tissue and different species are summarized in Table 1. However, there has not been a report regarding gene regulation of activin receptors in the human placenta.

Table 1. Some studies on the detection of activin subunits and ActRs mRNA

	1991 Petraglia	1992 Matzuk	1993 Peng	1993 Feng	1994 Jaatinen	1996 Tunri	1996 Peng
Inhibin-α	Hg				Ho	Hg	
Inhibin-βA	Hp	Hg			Ho	Hg	
Inhibin-βB	Hp	Hg			Ho		
ActRI							Hg
ActRIB							Hg
ActRII		Rgt	Hp*t*	Rot			Hg
ActRIIB		Rt		Rot			Hg

H: Human
R: Rat
g: granulosa cells
t: testes
o: ovary
t: trophoblasts
p: placenta

F. Gene expression

Introduction

The genome of a cell contains in its DNA sequence the information to make many thousands of different RNA and protein molecules. Gene expression involves multistep process of converting a gene sequence into a

functional protein. The main steps in this process are transcription of a DNA sequence into RNA and translation of RNA into protein.

A cell typically expresses only a fraction of its genes, and the different types of cells in multicellular organisms arise because different sets of genes are expressed. Moreover, cells can change the pattern of genes they express in response to changes in their environment, such as signals from other cells or the extracellular matrix. Although all of the steps involved in expressing a gene can in principle be regulated, for most genes the initiation of RNA transcription is one of the most important points of control.

The regulation of gene expression may play an important role in many aspects of cellular function. Many steps in the pathway from RNA to protein are regulated by cells to control gene expression. Most genes are thought to be regulated at multiple levels, although control of the initiation of transcription (transcriptional control) usually predominates. Some genes, however, are transcribed at a constant level and turned on and off solely by posttranscriptional regulatory processes. These regulatory processes include (1) attenuation of the RNA transcript by its premature termination, (2) alternative RNA splice-site selection, (3) control of 3'-end formation by cleavage and poly-A addition, (4) control of transport from the nucleus to the cytosol, (5) localization of mRNAs to particular parts of the cell, such as endoplasmic reticulum, (6) RNA editing, (7) control of translational initiation, (8) regulating mRNA degradation, and (9) translational recoding. Most of these control processes require the recognition of specific sequences or structures in the RNA molecule being regulated. This recognition can be accomplished by either a regulatory protein or a regulatory RNA molecule.

A complete understanding of the expression of genes encoding peptide hormones and growth factors, as well as hormone receptors and binding proteins, includes the analysis of the mRNA transcripts which encode them.

- **Measuring gene expression**

To estimate the expression of a given gene, we can measure rate of mRNA production or steady-state level of mRNA as well as the protein product. Northern blot and solution hybridization assay are two methods to measure steady-state mRNA levels in cells before and after treatment. In most cases, total RNA preparations are adequate for these analyses. The exception is when either the mRNA transcript is of low abundance or when ribosomal RNA cross-hybridizes with the mRNA transcript to be detected. In these cases, analysis of the poly(A^+) RNA transcript will be necessary.

Both polymerase chain reaction (PCR) and Northern blot are important methods for quantifying RNA levels. The Northern blot typically requires 5-30 μg of RNA, restriction enzyme digestion, transfer, and hybridization to a labeled probe, all of which may take a week or longer. With the advent of PCR, molecular biology drastically changed. Now minute quantities (nanogram amounts) of DNA could be amplified at least one million-fold in a matter of hours, resolved on agarose gels, stained with ethidium bromide, and photographed on the same day without the use of labeled probes. This process is routinely used clinically in the diagnosis of any genetic disorders and has been used to amplify DNA and RNA from even a single cell.

G. PCR and Competitive PCR

The technique and application of PCR

The target gene makes up a minority of sequences in total chromosomal DNA. PCR allows for the exponential amplification of the gene of interest among total DNA so that its structure can be studied. One important prerequisite for PCR is that the sequence of the gene or at least the borders of the region of DNA to be amplified is known. Each PCR cycle has three basic steps. In the first step, DNA is heated to 94°C to render it single stranded. In the second step, the temperature is lowered to 37-55°C (the exact temperature must be determined empirically), which results in DNA renaturing. Primers, which have been added to the tube, are short pieces (oligonuleotides) of DNA (usually about 20-30 base pairs in length), which are complementary to the 3' ends of each piece of the double stranded DNA to be amplified. When the temperature is lowered, these primers will bind to their complementary regions of the DNA. The third step involves raising the temperature to about 72°C, and in the presence of an enzyme (a heat stable DNA polymerase such as Taq polymerase) and the deoxy-forms of the nucleotides, the synthesis of the second complementary strand of DNA will be completed. In actuality the primers, buffer, deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), and enzyme are all added together along with the DNA in a 50 μl reaction volume in a tube and placed in an incubator (the thermal cycler) that changes temperature rapidly. The exact cycling duration and temperatures are empirically determined. After 30 to 35 cycles, the gene of interest may be amplified over one million fold, such that it may be visualized as a band on an agarose gel without the need for DNA probes.

Gene expression may be studied through the use of PCR. Extracted RNA has been successfully amplified by copying the RNA to cDNA with the enzyme reverse transcriptase (RT), and subjecting the cDNA to PCR. RT-PCR is particularly important for the study of gene regulation when the copy

number of transcripts is too low to be determined by Northern blot analysis. The promoter and exact start site of transcription of the GnRH receptor gene has been characterized using RT-PCR in our laboratory (Fan et al., 1994). The abundance of RNA available for study was too low to use any other techniques. This procedure is now commonplace in expression studies, and can even be used for the very low level mRNAs transcripts present in peripheral blood.

- **Competitive polymerase chain reaction (cPCR)**

Understanding regulation of gene expression depends largely on our ability to accurately measure mRNA concentrations in a defined cell population. Conventional methods of mRNA analysis, such as Northern blotting, RNA in situ hybridization and even ribonuclease protection assay are not sensitive enough to detect the extremely low concentration of mRNA transcripts. All these methods involve using radioactive substance. Classical Northern blot analysis for measuring mRNA requires more cells for analysis.

PCR, first described by Saiki et al. (1985), is a highly sensitive and specific methodology for detection of nucleic acids and an useful tool for quantitation of the amount of specific nucleic acids present in a sample. The great utility of the method lies in its exquisite sensitivity. Quantitation of nucleic acids by PCR has been used in an array of situations in both basic and clinical research (Bej et al., 1991; Clementi et al., 1993). Quantitative PCR (Q-PCR) is capable of relatively quantitating mRNA levels with great precision. The most precise quantitation of DNA and RNA can be obtained by cPCR (Gilliland et al., 1990) or competitive RT-PCR (Zimmermann and Manhalter, 1996).

cPCR, one method of Q-PCR, has emerged as a valuable method for determining the relative levels of mRNA transcripts; for example, a change in mRNA expression levels or to estimate the actual number of mRNA transcript. Perhaps the greatest advantage of cPCR is that useful data can be obtained during either the exponential phase or plateau phase of PCR amplification (Nedelman et al., 1992). In other methods, such as co-amplification of the target gene with an endogenous housekeeping gene, the PCR products must be obtained before the plateau phase of the reaction occurs. This is necessary for both the housekeeping gene and the target gene, which often exhibit different amplification kinetics.

In cPCR, an exogenous DNA fragment, the internal standard containing the same primer sequences as the target fragment, is added to compete in the same tube with the target for primer binding and amplification. Experimentally, cPCR reaction tubes containing the target samples are reacted with a dilution series of the competitor fragment. Since the amount of internal standard is known, the amount of target thus can be determined. The competitor fragment can be either a DNA sequence or a synthetic RNA containing the same primer binding sequences as the target gene. In order to differentiate between the PCR products generated from the target and the competitor, the competitor can be engineered to be slightly larger or smaller than the target (Gilliland et al., 1990). The resulting amplified products with different sizes are easily distinguished on polyacrylamide or agarose gel. A unique restriction site can be added or removed from the internal standard which has the same size molecule as the target (Becker-Andre and Hahlbrock, 1989) and the PCR products digested with a restriction enzyme before gel electrophoresis.

Although the cPCR internal standard shares the same primer binding sites as the target template, the intervening DNA sequence differs, making it possible for the internal standard and target to be amplified at different efficiencies. It is therefore necessary to show that their amplification efficiencies are similar, which is achieved by validating the kinetic value of these two competitive cDNAs. The data is plotted as a function of the log (target/internal standard, or internal standard/target) against the amount of internal standard, or target cDNA.

The easiest way to distinguish between wild-type template and the internal standard is by differences in the size of the two products. This can be achieved by constructing standards which have the same sequence as the specific target but contain a deletion or an insertion. The simplest construction procedure is to use a composite primer containing two specific target sequences at a predetermined distance from each other and a second primer specific for the opposite strand (Riedy et al., 1995). The amplification of wild-type templates with such primers results in a PCR product that is shorter than the wild-type template and can be easily identified. Alternatively, elongated internal standards can be constructed using a "looped oligo" method by amplifying cDNA with a primer containing a non-templated nucleotide insertion between template sequences (Sarkar and Bolander, 1994).

CHAPTER TWO: OBJECTIVES

A. Background & Rationale

1. Background:

Normal human reproductive function is maintained through the elaborate interplay of hypothalamic, pituitary, gonadal, and placental factors. Factors produced by hypothalamo-pituitary-gonadal axis or the placenta are important for achieving and maintaining the pregnancy well.

In the 1980s, inhibin and activin were successfully purified from follicular fluid of the ovary. Since then, our understanding of the important roles of these two new hormones in the reproductive endocrine system has advanced rapidly (Vale et al., 1990). The physiological significance and relationship between inhibin and activin or its related peptides represents one of the most challenging and interesting research focuses in developmental and reproductive endocrinology.

Human hemochorial placentation involves proliferation of maternal endometrial epithelial cells, followed by fetal trophoblastic cells. The consequent morphogenesis of heterologous tissues is probably regulated by autocrine or paracrine growth factors. Among the potential growth factors are the family of TGFβs that are important embryonic morphogens controlling tissue growth and remodeling (Massague, 1990).

Activin and inhibin, members of TGFβ superfamily, have various paracrine and autocrine local actions. Recent progress in their purification and determination of molecular structure indicates important roles of inhibin and activin in regulating FSH secretion from the pituitary gland. They also manifest many other biological activities. In women, these peptides play roles

in the regulation of hormonal production, folliculogenesis and oocyte maturation in the ovary and embryofetal development (Mather et al., 1992).

Activin has been shown to stimulate progesterone, GnRH, and human chorionic gonadotropin (hCG) production by cultured human placental cells (Petraglia et al., 1989). Using first trimester cells in a perfusion system, our laboratory has shown that activin (but not inhibin) rapidly stimulates hCG secretion (Steele et al., 1993). Inhibin and activin subunits and their mRNAs have been identified in decidual cells (Petraglia et al., 1990). The α subunits and its mRNA are found in the cytotrophoblast region of the villi, whereas the βB is observed in the syncytial layer. The βA protein is expressed in both inner and outer layers. The expression of all three subunits in the trophoblast cells increases with the progression of pregnancy (Petraglia and Vale, 1988). The presence of these subunits expression suggest that activin and inhibin play a key role in the autocrine and paracrine function of the placenta.

The discovery of follistatin adds a further dimension to the regulation of these peptides. More recently, the cloning of inhibin and activin, and the observation of their close homology to the TGFβ family of peptides, has led to an interest in their possible role as growth and differentiation factors. Activin, inhibin and follistatin are expressed in embryonic and fetal tissues, as well as in the placenta. Although activin is a potent regulator of growth and differentiation in a number of cell types, their role in embryonic/fetal development and implantation has yet to be determined.

2. Rationale:

The primary purpose of this research was to study the existence and regulation of activin receptors mRNA levels in human placental trophoblast

cells. In order to achieve this goal, we have investigated the mechanism of activin action by characterizing the expression and function of the multiple activin receptor forms and then determining the signal transduction events initiated by activin binding to each receptor. ActRI is involving the signal transduction and the cDNA of its whole sequence had been cloned (ten Dijike et al, 1993).

We established a cPCR method to accurately assess the comparative amount of mRNA in different cell populations. We have cloned a mutant ActRI PCR product within *p*Direct for subsequent using as an internal standard for cPCR procedure. This study of the expression and regulation of ActRs gene in trophoblasts is necessary as a first step for understanding the role of activin in the human placenta.

B. Hypothesis and objectives

1. Hypothesis:

The hypothesis to be tested was that activin functions in an autocrine or paracrine fashion to regulate the steady-state level of ActRI mRNA in human placental trophoblast cells.

2.Objectives:

The goal of this project was to understand the function of the activin via its receptors in different *in vitro* cell populations of human placental cells, including first trimester decidual cells, first trimester cytotrophoblasts, first trimester EVT cells, term cytotrophoblasts, IEVT cells, and JEG-3 cells (cell line of placental choriocarcinoma). These cells were used to demonstrate the

action of activin on the ActRI mRNA levels, as measured by cPCR. Using this system, the following specific objectives were addressed:

1. To verify the internal standard for cPCR
2. To validate the internal standard for optimizing cPCR
3. To quantify the relative amount of cDNA in each sample using β-actin PCR as an adjusting parameter.
4. To investigate the existence of ActRs and activin subunit mRNA in different cell populations using PCR.
5. To investigate the ActRI mRNA levels in different cell populations using Northern blot analysis.
6. To investigate the effects of activin-A on the ActRI mRNA levels in cultured IEVT cells using cPCR.
7. To investigate the effects of follistatin on the ActRI mRNA levels in cultured IEVT cells using cPCR.
8. To investigate the interaction between follistatin and activin-A on the ActRI mRNA levels in cultured IEVT cells using cPCR.
9. To investigate the effects of GnRH, inhibin, inhibin plus activin-A , and TGFβ1 on the ActRI mRNA levels in cultured IEVT cells using cPCR.

CHAPTER THREE: MATERIALS AND METHODS

A. Materials

- **Sources of tissue and cells**

The use of placental tissue samples was approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects, University of British Columbia. First trimester placentae were obtained from patients undergoing elective pregnancy termination and term placentae from patients undergoing elective Cesarean section before onset of labor. EVT cells were prepared from first trimester placenta. IEVT cells were generously provided by Dr. PK Lala (University of Western Ontario). JEG-3 cells were obtained from ATCC (American Type Culture Collection). Decidual cells from uterine content of first trimester abortion material were prepared by Dr. George Chen in Dr. MacCalman's laboratory.

B. Methods

1. Cell preparation

- Term and first trimester cytotrophoblast preparation

Cytotrophoblasts were collected from term placenta by dissecting chorionic villi from the placenta, enzymatic dissociation, and Percoll gradient centrifugation. The protocol used for term placenta is modified from that of Kliman et al. (1986) as follows:

Normal human term placentae were obtained after elective Cesarean section. The placenta was transported on ice to the laboratory, and aseptically

processed 30 to 60 minutes after delivery. Cotyledons were rinsed several times in phosphate-buffered saline (PBS) at room temperature. The villous tissues were separated from connective tissues and vessels. Approximately 50 grams of villous material was coarsely minced into 1-2 mm pieces, collected in 150 ml of Hank's solution, and incubated with trypsin (0.125%) and deoxyribonuclease I (DNAse I, 0.2 mg/ml) at 37°C for 30 minutes with gentle agitating in water bath. The supernatant of the first enzymatic digestion was discarded, and the supernatant of second, third and/or fourth enzymatic digestion were pooled. The mixture was filtered through a 40 μm sterile mesh and then layered (about 13 ml) onto 1.5 ml fetal bovine serum (FBS). After centrifuging at 1000x g (gravitational constant) for 10 minutes at room temperature, the cell pellets were then collected, resuspended, and pooled together in 24 ml M199. The cell suspension was centrifuged at 1000x g for a further 10 minutes. The cell pellet was resuspended and layered over 5% to 70% discontinuous Percoll gradient. Following centrifugation at 500x g for 30 min at room temperature, the cells in the middle layer (corresponding to a specific gravity of 1.048-1.062) were collected, diluted with M199 containing 10% FBS to the total volume of 25 ml and centrifuged at 1000x g for 5 minutes. Cell viability was checked using trypsin blue exclusion. The cells were then resuspended at the density of 5-10 x 10^5 cells/35-mm well for hormonal treatment group or 20 x 10^6 cells/100-mm dish for observing trophoblast cell differentiation in culture. Cells were incubated in a humidified incubator (5 per cent CO_2 in air at 37°C).

First trimester cytotrophoblasts were collected by picking out the villi from decidua. The cell preparation was achieved by following the steps modified from the procedures described by Graham et al. (1994).

2. Immunohistochemical staining of human trophoblast cells

In order to know the purity of the trophoblasts collected, we performed indirect immunofluorescence and peroxidase immunohistochemistry.

With immunofluorescence method, the monoclonal body HSA-10, (generously provided by Dr. CY Gregory Lee, University of British Columbia) reacts specifically against surface antigen of acrosome-reacted sperms. HSA-10 also reacts specifically with human trophoblast cells but not other human cells (Lee et al., 1993). Immunofluorescence was performed as follows: Cells grown on cover slides were air-dried, fixed in 95% methanol for 15 minutes, and air-dried again. After a 5 minute incubation with PBS-BSA (PBS 1x + BSA 0.5% + 0.1% NaN_3) in a humid chamber, PBS-BSA was changed to 1:100 (or 500) dilution of fluorescein isothiocianate (FITC)-HSA-10 and incubated for 30-60 minutes. A negative control and a positive control (BeWo cells) were required. Following three washes with PBS-BSA, cells were observed under microscope for immunofluorescence.

The biotin-avidin immunoperoxidase method was used for trophoblasts staining as follows: Cells grown on cover slides fixed as described above before being incubated with freshly prepared methanol/2% hydrogen peroxide solution for 20 minutes at room temperature. After being washed in 1% BSA for 5 minutes twice, cells were incubated with blocking reagent (horse serum) for 20 minutes at 37°C, followed by incubation with anti-human cytokeratin antibody for 45 minutes at 37°C. The next three steps were preceded by a wash in 1% BSA for 5 minutes twice. Cells were incubated with biotinylated horse anti-mouse immunoglobulin G (IgG) diluted 1 in 200 for 30 minutes at 37°C. Incubation with S-A-HRP (streptavidin-horseradish peroxidase conjugate, diluted 1 in 100) was proceeded for 30 minutes at 37°C. Cells

were immersed in fresh DAB (diaminobenzidine)/H_2O_2 solution for 5 minutes to show a brown reaction product, washed in running water, counterstained with hematoxylin, and then covered in a synthetic mounted media.

3. Cell culture

a. Culture for term and first trimester cytotrophoblasts

Term or first trimester cytotrophoblasts were plated as monolayers at a density of 2 to 5 x 10^5 cells/well on 35-mm dishes and cultured in 2.5 ml of medium (M199 for term cytotrophoblasts and DMEM for first trimester cytotrophoblasts) supplemented with 10% FBS, and antibiotics (10 mg/ml Streptomycin, 10 IU/ml Penicillin) at 37°C in 5% CO_2 humidified incubator. Cell culture medium was changed every second day. Cells for RNA extraction were collected soon after cell dissociation or after culture them for at least two days. In order to determine the cell biology of the cytotrophoblast *in vitro*, some cells were cultured for up to 14 days for observation.

b. Culture for EVT cells

EVT cells were grown in DMEM with 10% FBS, and 1% Gentamicin at 37°C in 5% CO_2 humidified incubator. They were harvested several days after splitting.

c. Culture for IEVT cells

IEVT cells were cultured in RPMI supplemented with 10% fetal bovine serum, and antibiotics (10 mg/ml Streptomycin, 10 IU/ml Penicillin) at

37°C in 5% CO_2 humidified incubator. Before treatment, the cells were washed twice with PBS.

d. Culture for first trimester decidual cells

Decidual cell were also cultured in DMEM medium as for the EVT cells above.

4. Hormonal treatment

Two days after passing, IEVT cells were transfer to serum-free RPMI medium. In order to determine whether activin-A effected on ActRI mRNA levels in a dose-dependent manner, cells were treated with 0.1-30ng/ml (0.1ng, 1ng, 3ng, 10ng, 30ng/ml) activin-A for 12 hours. For time-course experiments, cells were cultured in the presence or absence of 10ng/ml recombinant activin-A for 1, 3, 6, 12, 24 hours. To test the effects of recombinant follistatin (follistatin-288) on basal and activin-A stimulated ActRI mRNA levels, 10 to 100 ng/ml of follistatin were first incubated with or without activin-A at 37°C for 1 hour in 50 μl PBS-0.1% BSA before being given to the cells in 1 ml serum-free RPMI for 6 hours. The final components of the medium contain follistatin (10, 20, 50, or 100 ng/ml) with or without activin-A (10ng/ml). IEVT cells were also treated with vehicle, follistatin (3-100 ng/ml), inhibin (10 ng/ml) and/or activin-A (10 ng/ml), GnRH (10^{-6} *M*, 10^{-7} *M*), TGFβ (0.1ng, 1ng, 10ng/ml) and collected six hours after being in serum-free medium.

5. RNA extraction

Total RNA from placental tissue was isolated using the guanidine/cesium chloride method (Chomczynski and Sacchi, 1987). Placenta tissue was homogenized in guanidine thiocyanate buffer (4 *M* guanidine thiocyanate, 5 m*M* sodium citrate with pH 7, 0.1 *M* β-mercaptoethanol, 0.5% sarcosyl). The supernatant was subsequently layered onto 3 ml of CsCl-EDTA solution (pH 8.0, 5.7 *M* CsCl with 0.1*M* ethylenediaminetetraacetic acid). After ultracentrifugation at 28,000 rpm at 18°C for 18 hours, the cell pellet was resuspended and rinsed with Tris/EDTA/SDS solution (10 μ*M* Tris with pH 7.4, 5m*M* EDTA, 0.1% sodium dodecyl sulfate). Total RNA was then extracted with chloroform:butanol (4:1) and precipitated with ethanol in the presence of 0.3 *M* sodium acetate.

Total cellular RNA was extracted using the RNaid Kit (Bio/Can) following manufacture's suggested procedures. Briefly, the cultured cells were lysed and collected with guanidine thiocyanate buffer (4 *M* guanidine thiocyanate, 5 m*M* sodium citrate with pH 7, 0.1 *M* β-mercaptoethanol, 0.5% sarcosyl) followed by RNA extraction with a modified acidic phenol and chloroform:isoamyl alcohol (24:1) solution. RNA was then purified by absorption to the RNAMATRIX and eluted with diethylene perchlorate (DEPC)-treated water and stored at -70°C. RNA concentration was determined with a spectrophotometer by measuring absorbency at a wavelength of 260 nm (nanometer).

6. Northern blots analysis

Total RNA samples (20-30μg) were denatured by heating at 60°C for 15 minutes in a buffer containing 50% deionized fomamide, 10 m*M* 4-morpholinepropanesulphonic acid (MOPS) and 17% formaldehyde. RNAs

were separated by electrophoresis (1.2% agarose), transferred to nylon membrane (Hybond-N, Amersham) by pressure blotting in 10x SSC (1.5 *M* NaCl and 0.15 *M* sodium citrate, at pH 7.0), fixed under UV light, prehybridized and hybridized with antisense RNA probe for ActRI mRNA, and labeled with digoxigenin (DIG). The membranes were then washed in 2x SSC with 0.5% SDS for 15 minutes at room temperature and in 1x SSC with 0.5% SDS for 15 minutes at 60°C. Washed membranes were exposed to X-ray film with intensifying screen for several minutes. The intensity of signals on the X-ray film was quantified using video densitometer (Bio-Rad, model 620).

7. Reverse transcription

Double-stranded RNA:cDNA hetero-complex was synthesized using First Strand cDNA Synthesis Kit (Pharmacia). Reactions were carried out using 2.0 μg of total RNA obtained from placental tissue or trophoblast cells. The 15 μl of reaction mixture contains 2 μg of total RNA in 8 μl DEPC water (preheated at 70°C for 10 minutes), 1 μl dithiothreitol (DTT), 1 μl oligo-dT (0.5 μg/ml) and 5μl of bulk reaction mixture (reverse transcriptase buffer, dNTP, RNAse inhibitor and Avian Myeloblasts Virus reverse transcriptase). After incubation at 37°C for at least 1 hour, the reaction mixture was heated at 100°C for 10 minutes, spinned briefly, and stored at -20°C until use in PCR.

8. PCR and Primers

Oligonucleotide primers were synthesized based on the published sequences of the human gene of ActRs (Matsuzaki et al., 1993; ten Dijike et al.,1993; Donaldson et al., 1992; Hilden et al., 1994), inhibin subunits (Voutilainen et al., 1991), and β-actin (Ng et al., 1985). To prevent self-annealing, primers were chosen not to be complementary to each other. The dissociation temperature of primers in a PCR pair is approximately the same. Pairs of primers are selected to give a single PCR product. Table 2 shows the positions of primers on the genes, sequence of each primer and the annealing temperature for each pair of primers.

PCR was performed in a final volume of 50 μl. Since multiple samples were often amplified in the same experiment, a PCR master mix was prepared. The PCR reaction mixture contains 49 μl of PCR master mix and 1 μl of the DNA template in water. The recipe for PCR master mix (for 1 reaction tube) is as follows:

Ingredients	Amount (μl)
10x PCR buffer [KCl (500 m*M*), Tris-HCl (200 m*M*, pH 8.4)]	5.0
MgCl2 (50 m*M*),	1.5
dNTP (10 μ*M*)	1.0
Upstream primer (5 μ*M*)	1.0
Downstream primer (5 μ*M*)	1.0
Sterile Milli-Q water	39.0
Taq DNA polymerase (5 units/μl)	0.5
Total (without cDNA)	49.0

To each reaction, 1 μl of the DNA template is added to 49 μl of PCR master mix in sterile milli-Q water; and the reaction mixture was

Table 2. Primers (Human ActRs, activin subunit, and β-actin cDNA)

Primers	Annealing temperature, Size of PCR product Sequence	Position
ActR I	53°C, 652 bps	
ActR-IA 1 (f)	5' GAT GAG AAG TCA TGG TTC AGG 3'	1060-1080
ActR-IA 2 (r)	5' TAT GTT TGG CCT TTG TTG ATC 3'	1691-1711
ActR IB	59°C, 674 bps	
ActR-IB 1 (f)	5' GAT ATA CCA GAC GGT CAT GCT 3'	747-767
ActR-IB 2 (r)	5' GTT GGC ATA CCA ACA CTC TCG 3'	1440-1420
ActR II	53°C, 456 bps	
ActR-II 1 (f)	5' ACC AGT GTT GAT GTG GAT CTT 3'	942-962
ActR-II 2 (r)	5' TAC AGG TCC ATC TGC AGC AGT 3'	1377-1397
ActR IIB	59°C, 699 bps	
ActR-IIB 1 (f)	5' TTC TGC TGC TGT GAA GGC AAC 3'	287-307
ActR-IIB 2 (r)	5' GAG GTC GCT CTT CAG CAA TAC A 3'	964-985
Inhibin α	59°C, 503 bps	
	5' CAG CCA CAG ATG CCA GCT GT 3'	217-236
	5' CTC CGC AGG CCT CTG CAG CAG GCC CAG 3'	693-719
Inhibin βA	57°C, 787 bps	
	5' GTG TGC CGA GTC AGG AAC AG 3'	611-630
	5' GAG GTT GGC AAA GGG GCT ATG GCC CCG CAT 3'	1368-1397
Inhibin βB	59°C, 618 bps	
	5' TTC GCC GAG ACA GAT GGC CT 3'	276-295
	5' GTG GAA GGA GGA GGC AGA GCC GGG GAC CCC 3'	864-893
β-actin	55°C, 524 bps	
AC3 (f)	5' GGA CCT GAC TGA CTA CCT CAT GAA 3'	590-614
AC2 (r)	5' TGA TCC ACA TCT GCT GGA AGG TGG 3'	1090-1113

(f)= forward (r) = reverse

covered with approximately 50 μl of mineral oil.

PCR amplifications were performed in a Perkin-Elmer/Citus DNA thermal cycler with a typical temperature profile as follows:

	Temperature (°C)	Duration	Cycles
Initial denaturation	94	5 min	1
Annealing	53, 59, or 55*	30 sec	34
Extension	72	1.5 min	34
Denaturation	94	30 sec	34
Final annealing	53, 59, or 55*	30 sec	1
Final extension	72	15 min	1

*, 53°C for ActRI and ActRII 59°C for ActRIB and ActRIIB, 55°C for β-actin.

The final cycle was followed by a 15 minute extension step at 72°C to ensure that the amplified DNA was double stranded.

After thermocycling was completed, an aliquot of the PCR mixture (10 μl) was run within an 1% agarose gel in 1X TBE (Tris + Boric acid + EDTA). The PCR product was stained by ethidium bromide and visualized with UV transillumination.

9. Cloning

Cloning of each ActR was performed by ligating the PCR product into a plasmid, followed by the transformation into E. Coli with heat induction. After minipreparing plasmid, enzymatic digestion, and confirming the

molecular weight of the digested products, large scale plasmid preparation was performed, followed by DNA sequence analysis to confirm the identity.

ActRI PCR product was ligated with pDirect (Clontech). ActRII cDNA in pBlueScript(KS+) was obtained from Dr. WW Vale (Salk Institute). The ActRIIB PCR product was cloned into pCR™II (Invitrogen) and the procedure was performed as that for cloning ActRI PCR product. Only ActRI PCR product was mutated to be an internal standard for cPCR.

10. Mutagenesis

An internal standard was constructed from a cloned native ActRI PCR product. Removing a segment of 160 base pairs from PCR product within plasmid was done by StyI and Eco47III (Fig. 2). With the same pair of primers for PCR, the subcloned mutant plasmids template yielded a PCR product 160 base pairs smaller than that from native cDNA template. Validation of mutagenic plasmid to determine its optimal amount as an internal standard for cPCR quantification had to be determined.

11. Validation of cPCR

Before validating the internal standard (IS) of ActRI, verification of the IS was performed. Either or both of the internal standard and native ActRI cDNA were placed in a reaction tube. A cycle program of 35 cycles was performed.

In order to determine the ideal concentration of internal standard for the co-amplification, two sets of experiments were performed. In the first set of experiment, a fixed amount (0.05 pg) of the internal standard was co-amplified in the presence of increasing amount (0.01 to 10.0 pg) of the native

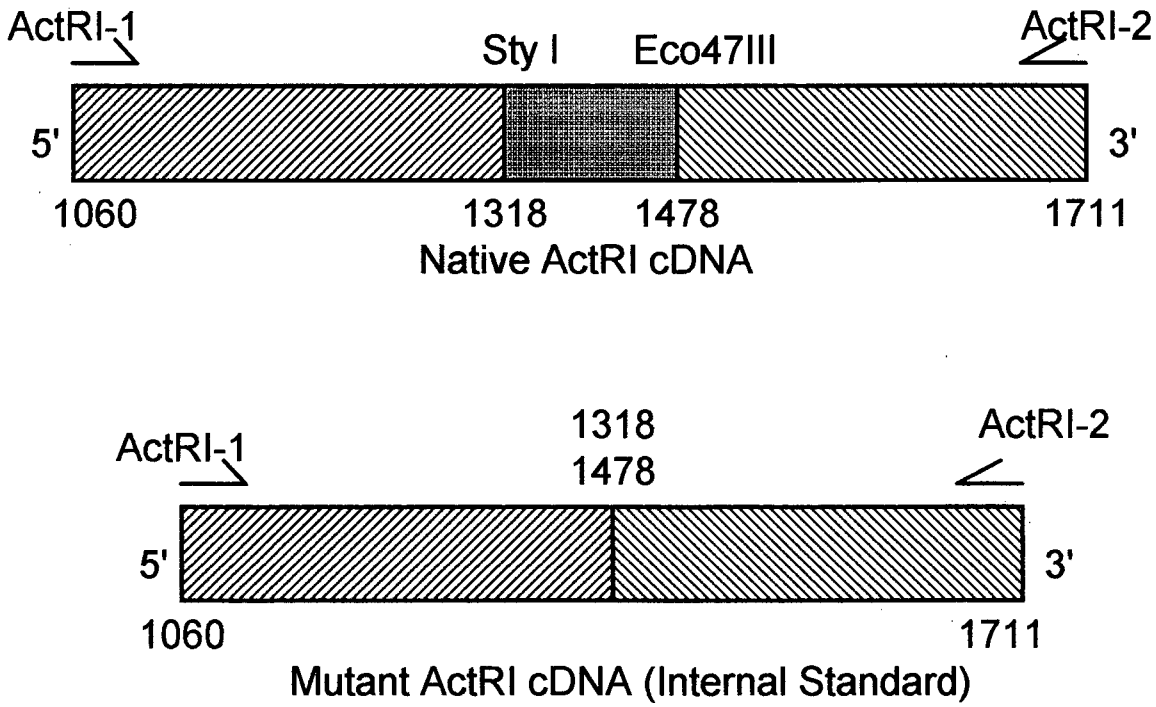


Figure. 2. Schematic structure of the ActRI cDNA and primers used in PCR or cPCR. An 160 bp internal deletion has been done at the restriction sites with Sty I and Eco47 III. Co-amplification of the ActRI native cDNA and mutant cDNA template using primer ActR-1 and primer ActRI-2 resulted in 652 bp and 492 bp PCR product, respectively.

ActRI.

In the second set of experiment, a fixed amount of cDNA, prepared from 2μg of IEVT cells total RNA, was co-amplified with increasing amount of the internal standard (0.01 to 0.5 pg). Quantification by densitometry scanning after gel electrophoresis was performed to compare the relatives amount of PCR products. From these studies, we determined the ideal concentration of internal standard for co-amplifying with cDNA that was made from 2 μg total RNA of IEVT cells.

12. Competitive PCR

Competitive polymerase chain reaction (cPCR)

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 a cPCR reaction, target sequences of native cDNA combined with the internal standard of ActRI is co-amplified by Taq DNA polymerase in the same tube in which the same ActRI primers were used. cPCR reactions were performed in a volume of 50 μl containing 5 μl 10X PCR buffer [200 m*M* Tris-HCl (pH 8.4), 500 m*M* KCl], 1.5 μl $MgCl_2$ (50 m*M*), 1 μl dNTPs (10 μ*M*), 0.5 μl Taq DNA polymerase (5 units/μl) (Gibco), 38 μl sterile Milli-Q water, and 1 μl each of the 5'ActR-1 and 3'ActRI-2 sequence specific primers (5 μ*M*). Equal volume (1 μl) of sample cDNA and mutant cDNA were added into each reaction mixture. 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 to ensure that the amplified DNA was double-stranded.

To measure the relative amounts of ActRI mRNA in each different sample after adjusting the cDNA concentrates, β-actin PCR products were also obtained from each sample. The amount of β-actin PCR product was measured with video densitometer (Bio-Rad, model 620).

13. Analysis

Assessment and quantification of PCR products

After cPCR, 10 μl of each PCR product was subjected to gel electrophoresis using 1% agarose-TBE (Tris, Boric acid, EDTA). Under ultraviolet light illumination, distinct PCR product bands of native cDNA and/or internal standard were viewed after staining with ethinium bromide and recorded with negative film. Similarly the β-actin PCR product of sample cDNA were subjected to electrophoresis. Since the amount of internal standard is known, the amount of native ActR mRNA was calculated using video densitometer. Utilizing β-actin as another standard, the relative amount of IEVT ActRI mRNA in different samples can be figured out accurately.

14. Statistical Analysis

Statistical significance of the data were determined by one-way analysis of variance followed by Scheffe's test ($p<0.05$).

CHAPTER FOUR: RESULTS

Term cytotrophoblasts, first trimester cytotrophoblasts, EVT cells, and IEVT cells were immunostained for cytokeratin 8 and 18. Purity of the cell preparations was determined to be at least 95% using this immunoperoxidase method and was confirmed using immunoflurorecence with an antibody, HSA-10, directed against the surface antigen of acrosome-reacted sperm and placental trophoblast cells. These observation suggested that these cells are epithelial cells and are trophoblasts suitable for experiments.

Term placental cytotrophoblasts, released by trypsin digestion of placental villi and purified on Percoll gradient and grown in serum-containing medium, attached to the culture dishes within 6 hours of plating. These cells differentiate within 24 to 48 hours in culture from mononucleated cytotrophoblasts at the beginning of culture to aggregated cells or multinucleated giant (syncytiotrophoblast-like) cells. After culturing for 4 to 7 days, the syncytia showed increasing amounts of vacuolation in the cytoplasm. Most cells died 14 days after platting.

A. Verification of the internal standard for optimizing cPCR

Before validating the internal standard (IS) of ActRI, verification of the internal standard was performed. Either or both of IS and native ActRI cDNA was put in the reaction tube. The PCR program was performed for 35 times. As shown in Figure 3, when either IS or native ActRI cDNA was put in the reaction tube, there is only one PCR product band in each lane, corresponding to the size of the added template. The lane refer to both templates in the ingredient showed two bands with different size, and lighter density due to

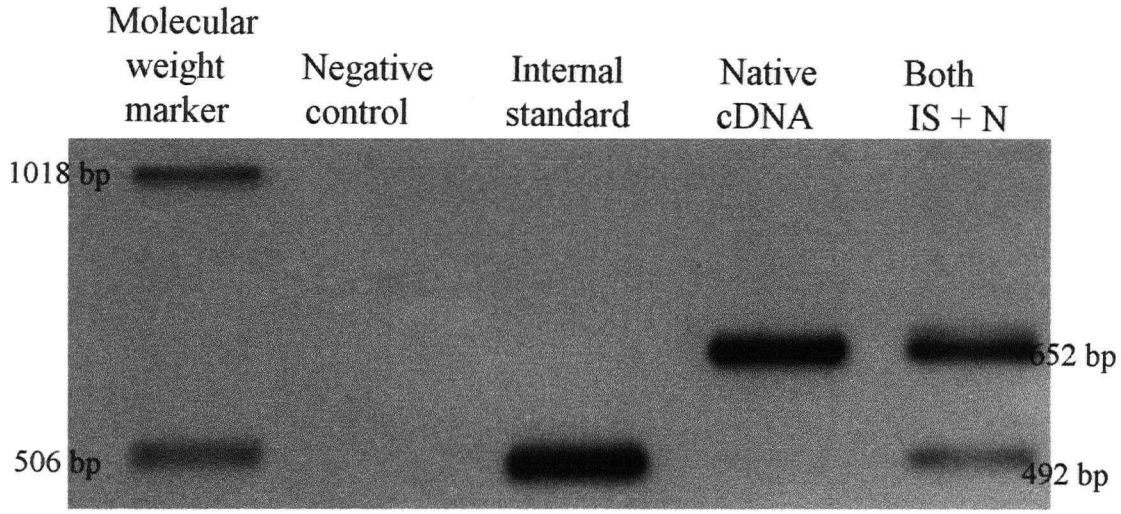


Figure 3. Verification of PCR products from the cloned native ActRI cDNA and internal standard. Either native ActRI cDNA or internal standard was amplified. Both templates were also coamplified in one tube. Two expected products with size of 652bp and 492bp were detected after running gel electrophoresis.

competition. There is no band in the negative control lane. This result indicated that the mutant ActRI cDNA is eligible for use as an IS of ActRI cDNA for cPCR.

B. Validation of cPCR

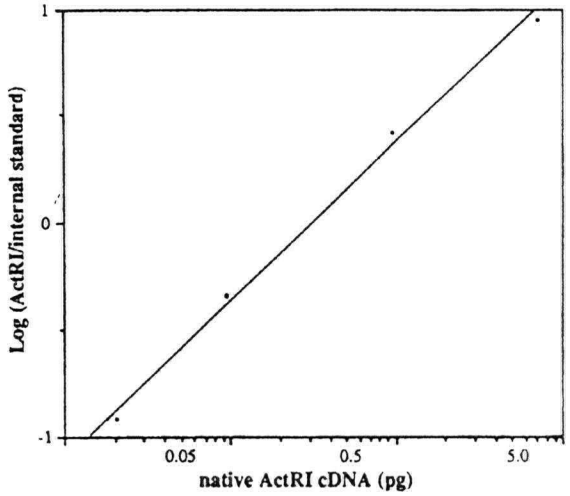
The PCR product obtained from different amount of native ActRI cDNA (0.01, 0.1, 1, 10 pg) with same amount of IS (0.05 pg) in each sample is shown in Figure 4. The ratio of band intensity (native ActRI cDNA/IS), measured by densitometry scanning, was nearly linear when plotted as a function of the amount of native ActRI cDNA in the cPCR reaction. The linear result was as same as that of Telenti et al. (1992).

A fixed amount of cDNA, prepared from 2 μg total RNA of IEVT cells, was co-amplified (Fig. 5) with an increasing amount of the IS (10, 30, 100, 300, 500 ng). Quantification by densitometry scanning indicated that there was a linear relationship between the relative intensity of the IS and the IEVT cells cDNA bands when plotted against the increasing amount of IS cDNA template in the PCR.

C. The detection of activin subunit and ActRs mRNA in different cell populations using PCR

With the exception of JEG-3 cells that did not express ActRIIB mRNA, all cell populations express mRNA of inhibinα, inhibinβA, inhibinβB, ActRI, ActRII and ActRIIB (Table 3). However, ActRIB mRNA could not be detected in all cell populations. Even though we did not perform quantitative

(A)



(B)

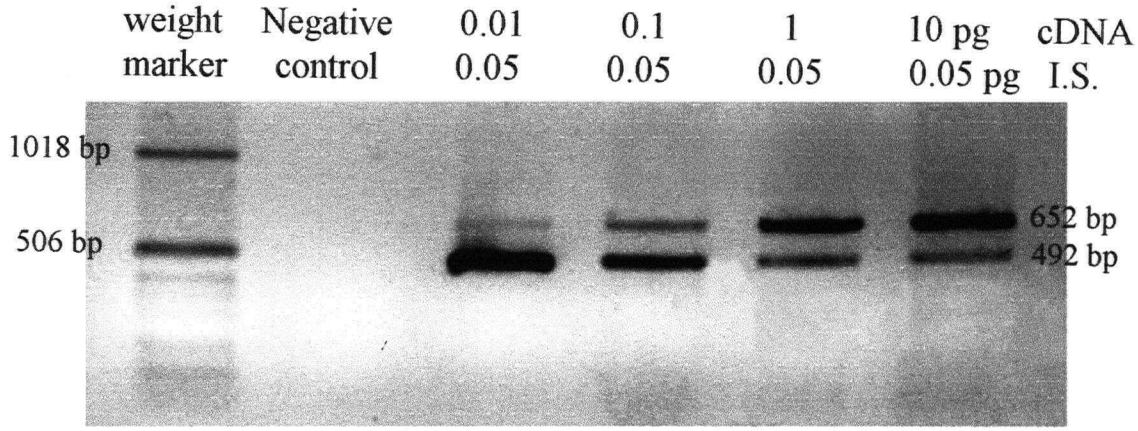
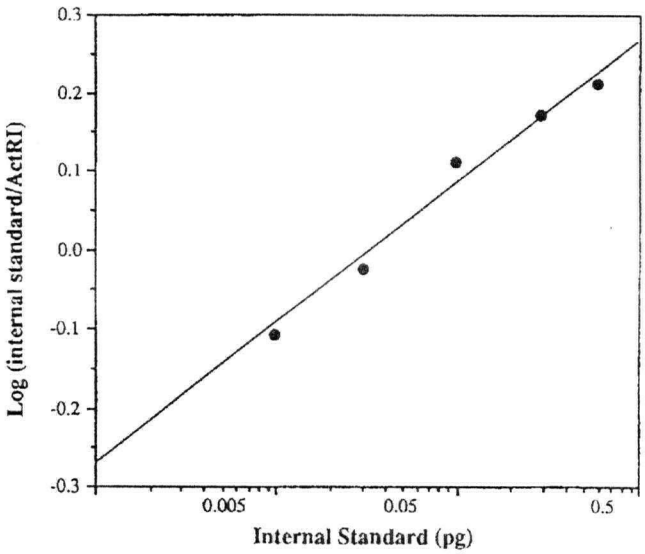


Figure 4. Graphic analysis of negative image shown in panel (B). The film was scanned with video densitometer. A linear relationship was found when the ratio of native ActRI cDNA versus internal standard was plotted as a function of the amount of added native ActRI cDNA. Panel (A) discloses a serial decrease of internal standard PCR product when increasing amount of native ActRI cDNA was added.

(A)



(B)

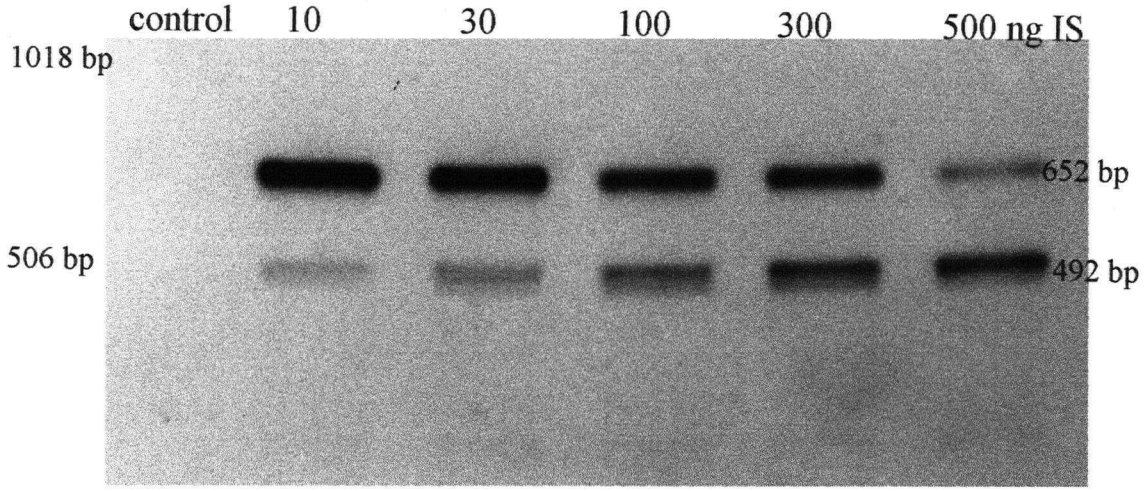


Figure 5. Graphic analysis of negative image shown in panel (B). The film was scanned with video densitometer. A linear relationship was found when the ratio of internal standard versus IEVT ActRI cDNA was plotted as a function of the amount of added internal standard. (A) discloses a serial decrease of IEVT cDNA ActRI PCR product when increasing amount of internal standard was added.

PCR, conventional PCR showed that ActRI, ActRII, and ActRIIB mRNAs levels was highest in IEVT cells.

D. Comparing ActRI mRNA levels in different cell populations using Northern blot analysis

Northern blot analysis (n=1) using DIG method was performed to see the levels of ActRI mRNA transcripts in different cell populations (Fig. 6). The ratio of the level of 3.6-kb ActRI mRNA in these cells was approximately 3.5 in EVT cells, 2 in first trimester cytotrophoblasts, 1.5 in JEG-3 cells, and 1 in term cytotrophoblasts. No statistic analysis was performed for this experiment.

E. The effects of activin-A on the ActRI mRNA levels in cultured IEVT cells cPCR.

The ActRI mRNA level, measured at 12 hours after treatment (Fig. 7), was stimulated in a dose-dependent manner by activin-A, becoming significant at a concentration of 3 ng/ml ($P<0.05$), and with a maximal response three time greater in control cultures at a dose of 10 ng/ml ($P<0.01$). The time course of the activin-A effects on ActRI mRNA levels showed an early response at 3 hours and 6 hours ($p<0.05$) with maximal level occurring at 6 hours after treatment ($P<0.01$). ActRI mRNA levels declined at 24 hours of treatment (Fig. 8).

F. The effects of follistatin on the ActRI mRNA levels in cultured IEVT cells using cPCR.

The activin-binding protein, follistatin has been shown to neutralize the effects of activin in several biological systems (Cataldo et al., 1995; Michel et

	α-subunit	βA-subunit	βB-subunit
Negative control	N	N	N
Term CTBs	Y	Y	Y
EVT cells	Y	Y	Y
IEVT cells	Y	Y	Y
JEG-3 cells	Y	Y	Y
Decidual tissue	Y	Y	Y
Decidual cells	Y	Y	Y

	ActRI	ActRIB	ActRII	ActRIIB
Negative control	N	?	N	N
Term CTBs	Y	?	Y	Y
EVT cells	Y	?	Y	Y
IEVT cells	Y	?	Y	Y
JEG-3 cells	Y	?	Y	N or ?
Decidual tissue	Y	?	Y	Y
Decidual cells	Y	?	Y	Y

Table 3. Detection of mRNA transcripts encoding activin/inhibin subunits and activin receptors in term cytotrophoblasts (CTBs), EVT cells, IEVT cells, JEG-3 cells, decidual tissue, and decidual cells using PCR.
N: negative band, undetectable
Y: positive band with right size
?: uncertain result

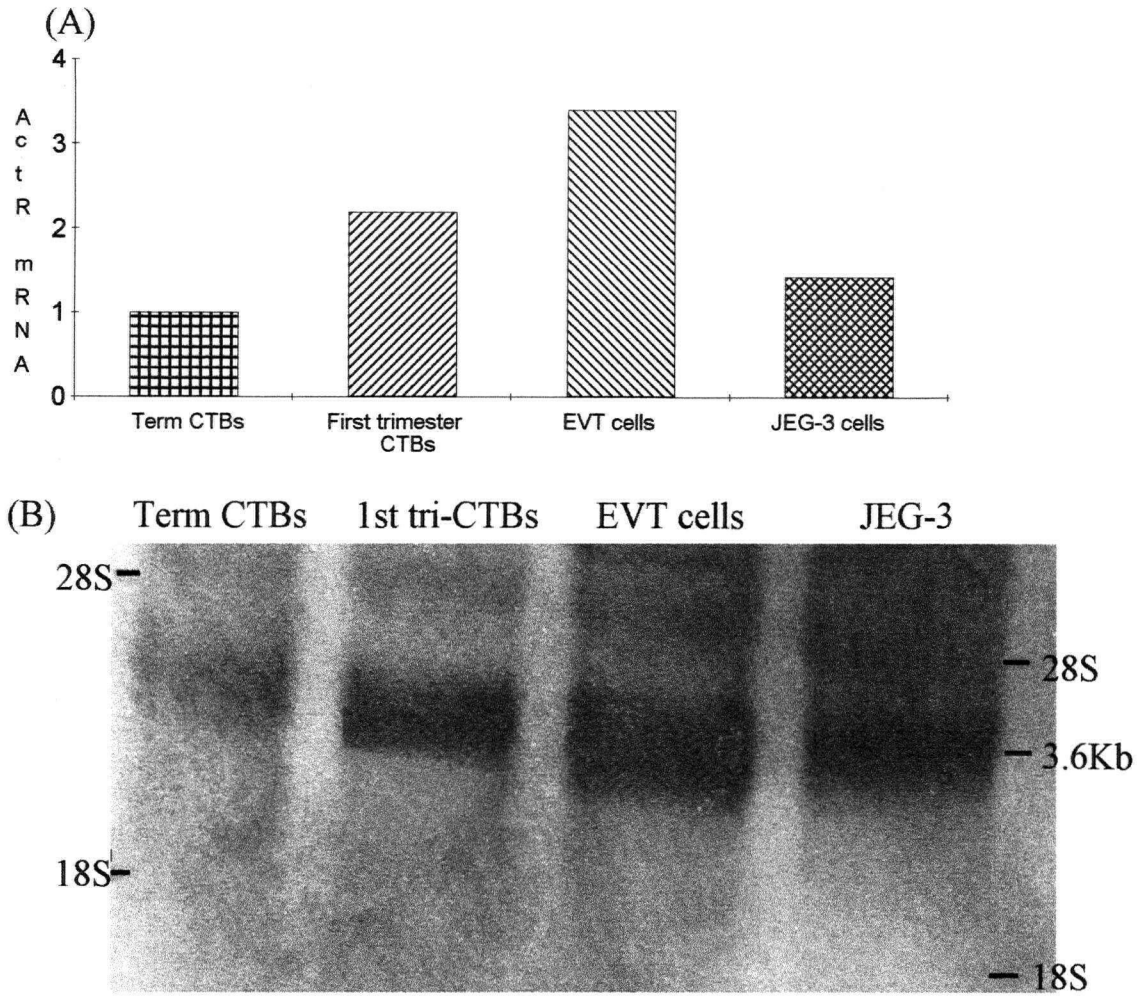


Figure 6. Comparing ActRI mRNA levels in different cell populations (n=1) using Northern blot analysis with DIG. Total RNA was extracted from term cytotrophoblasts (CTBs), first trimester CTBs, EVT cells, and JEG-3 cells. A chemoilluminant picture was developed and quantified by approximately same amount of RNA with adjustig the RNA amount with prior diagnostic RNA gel.

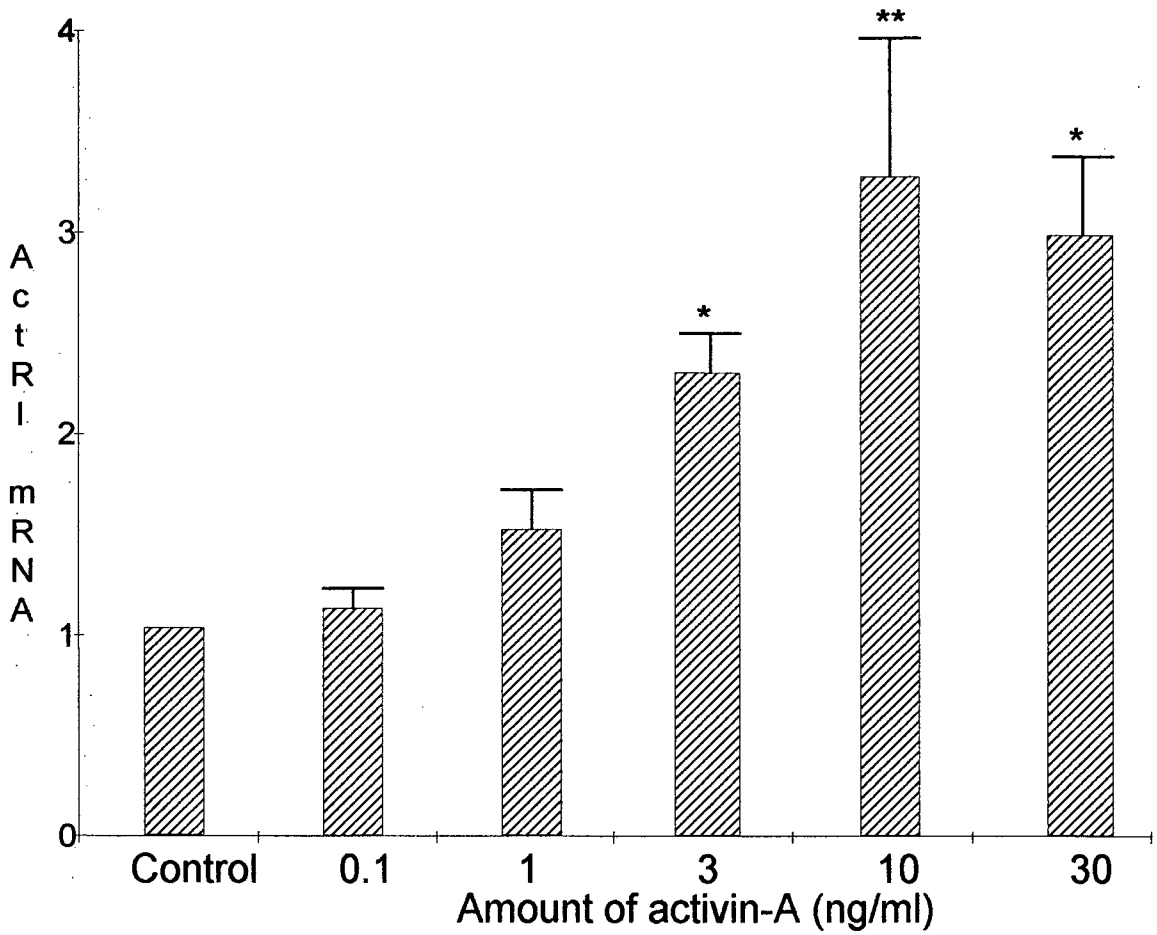


Figure 7. Dose dependent stimulation of ActRI mRNA levels after the addition of activin-A. Cells were incubated for 12 h in the absence or presence of activin-A (0-30 ng /ml). cPCR was performed to obtain negative film for scanning measurement. The data were pooled from 3 separate experiments and expressed as a percentage of control value (±SEM). Data were normalized for β-actin mRNA levels measured by PCR and expressed relative to the control value. *($p<0.05$) or **($p<0.01$) versus control.

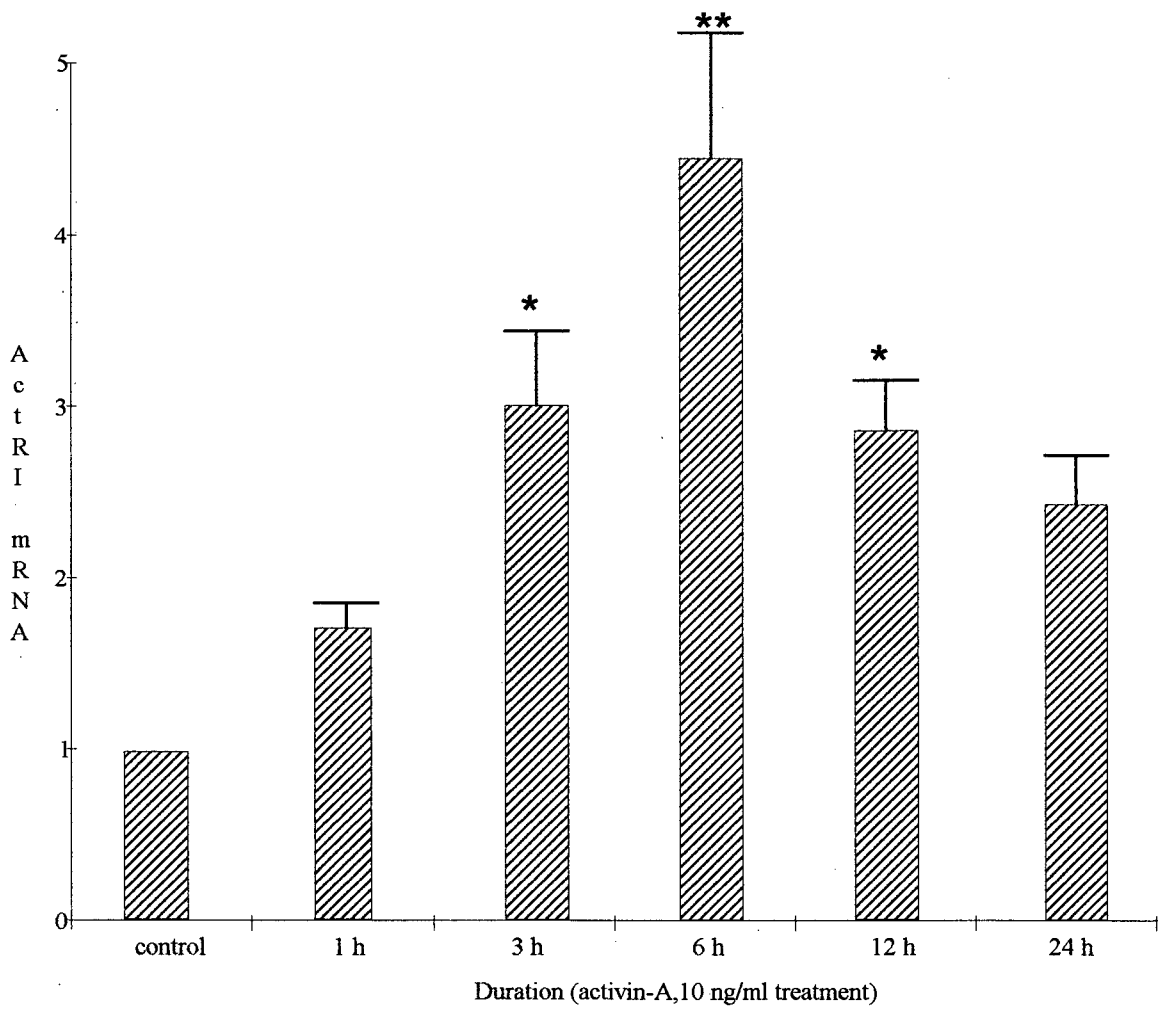


Figure 8. ActRI mRNA levels of IEVT cells (n=3) after 0, 1, 3, 6, 12, 24 hours of culture in the presence or absence of activin-A (10 ng/ml). cPCR was performed to obtain negative film for scanning measurement. Data were normalized for β-actin mRNA levels obtained by PCR and expressed relative to the control value. *,($p<0.05$) versus control. **,($p<0.01$) versus control.

al., 1993). In view of these observation, we examined the effects of increasing concentrations of recombinant human follistatin on the basal ActRI mRNA levels. Different concentration of follistatin had no effect on ActRI mRNA levels. A slight decrease in the intensity of ActRI mRNA levels was observed. The time course of the follistatin effects on ActRI mRNA showed no significant difference ($p>0.05$) within 24 hours of treatment (Fig. 10).

G. Interaction between follistatin and activin-A on the ActRI mRNA levels in cultured IEVT cells using cPCR.

An increase in ActRI mRNA levels was observed when these cell cultures were treated with activin-A. Co-treatment of activin-A and follistatin demonstrated that follistatin blocked the effects of activin-A on the ActRI mRNA levels in a concentration-dependent manner (Fig. 11). The inhibitory effect becomes more prominent as the concentration of follistatin increases with the maximal effect at 50 or 100 ng/ml on the stimulatory effect of activin-A 10 ng/ml.

The time course effects of activin-A (10 ng/ml) and follistatin (50 ng/ml) combination on the ActRI mRNA levels in IEVT cells is shown in Figure 12. The combination of activin-A and follistatin did not have a stimulatory effect on ActRI mRNA levels.

The concentration of follistatin at 50 ng/ml inhibited the effects of activin-A on the ActRI mRNA levels in IEVT cells.

H. The effects of GnRH, inhibin, inhibin plus activin-A , TGFβ1, on the ActRI mRNA levels in cultured IEVT cells using cPCR (Fig. 13).

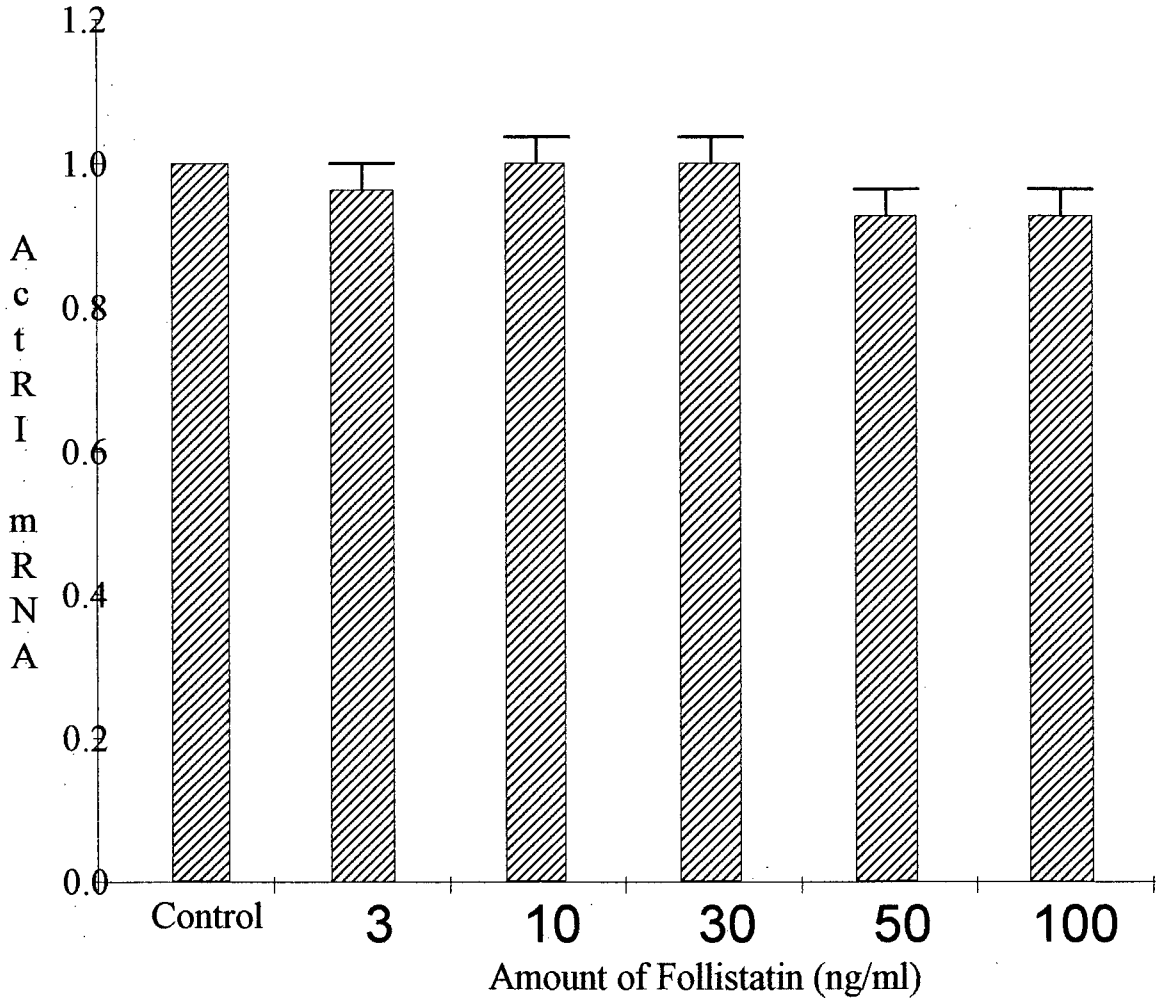


Figure 9. The effects of varying dose of follistatin on ActRI mRNA levels in IEVT cells. cDNA was synthesized from total RNA of IEVT cells incubated for 6 hours without follistatin (control) or with increasing dose of follistatin (3-100 ng/ml). cPCR was performed to obtain negative film for scanning measurement. Data were normalized for β- actin mRNA levels measured by PCR and expressed relative to the control value. Columns denote means ± SEM of 2 experiments. No significant finding was detected at any different concentration of follistatin.

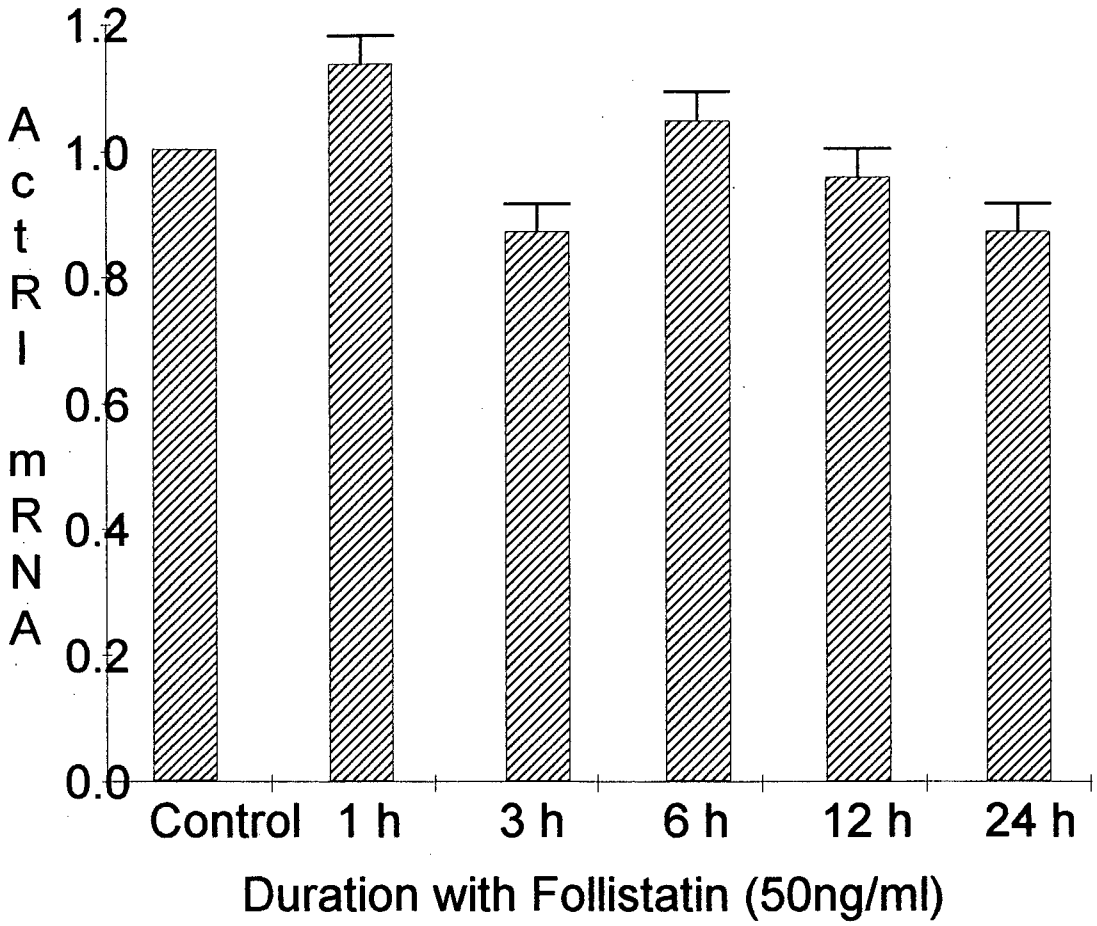


Figure 10. The effects of time on ActRI mRNA levels in IEVT cells cultured in the absence (control) or presence of follistatin (50 ng/ml). cPCR was performed to obtain negative film for scanning measurement. Data were normalized for β-actin mRNA levels measured by PCR and expressed relative to the control value. Columns denote means by PCR and expressed ± SEM of 2 experiments. No significant finding was detected at any different time after exposure to follistatin (50 ng/ml).

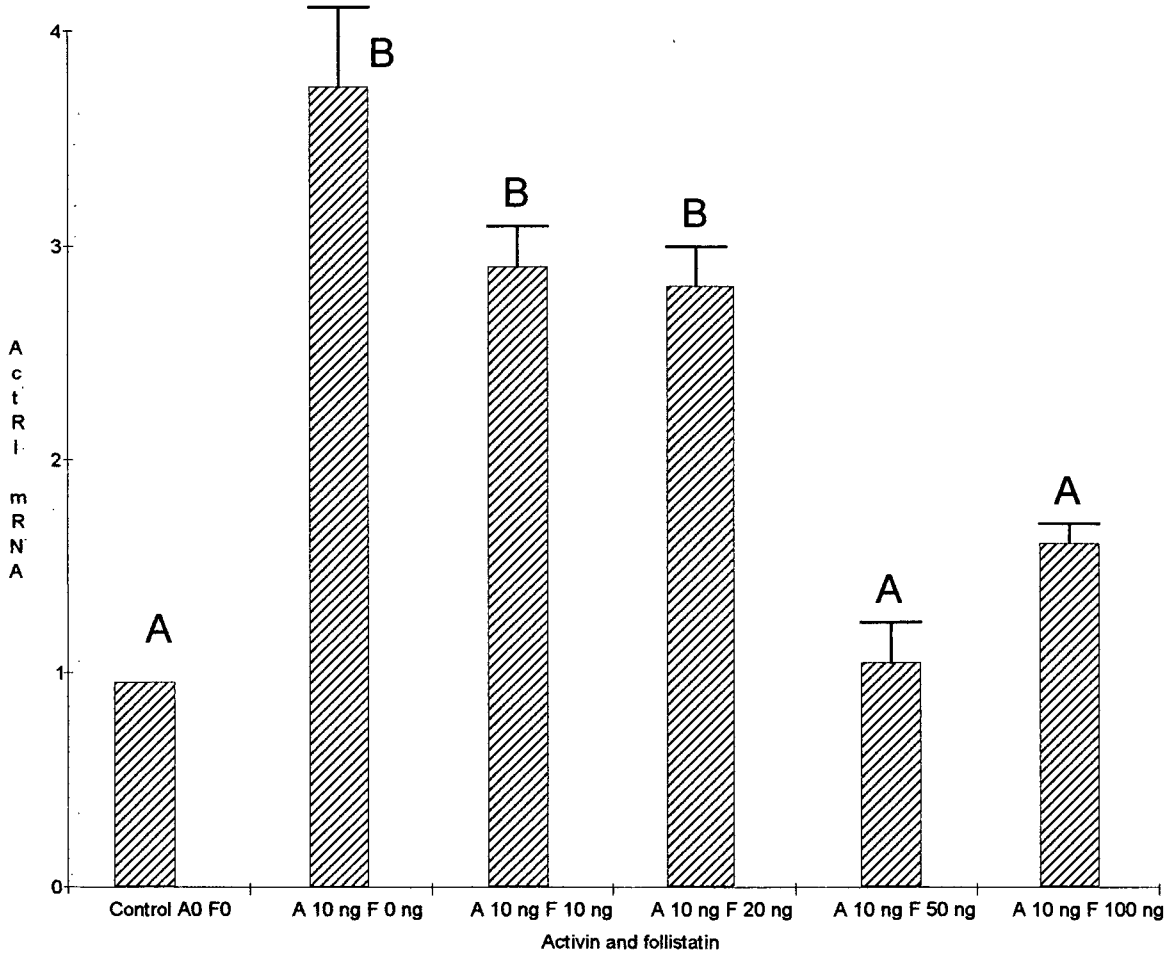


Figure 11. The effects of follistatin on the ActRI mRNA levels induced by activin-A in IEVT cells. Cells were incubated for 6 hours in the presence or absence of activin-A (10 ng/ml) with increasing concentrations of follistatin (0-100 ng/ml). cPCR was performed to obtain negative film for scanning measurement. Data were normalized for β-actin mRNA levels measured by PCR and expressed relative to the control value. Columns denote means ± SEM of 2 experiments. *Different letters* indicate statistically significant differences ($p<0.05$, by Scheffe's test).

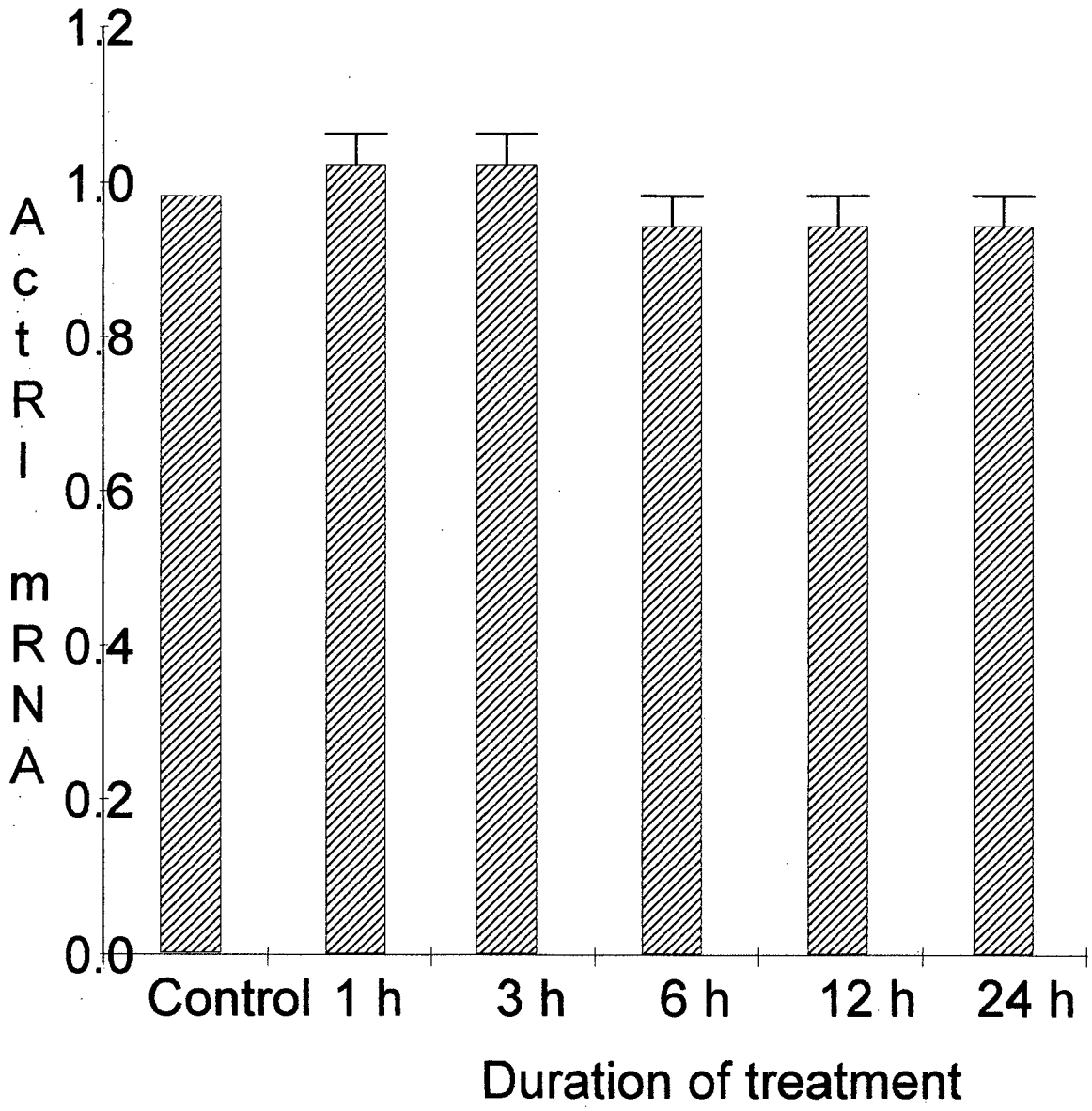


Figure 12. The effects of time on ActRI mRNA levels in the absence or presence of activin-A (10 ng/ml) and follistatin (50 ng/ml) in cultured IEVT cells. cPCR was performed to obtain negative film for scanning measurement. Data were normalized for β-actin mRNA levels measured by PCR and expressed relative to the control value. Columns denote means ± SEM of 2 experiments. No significant finding was detected at any different time after exposure to activin-A (10 ng/ml) plus follistatin (50 ng/ml).

GnRH at a concentration of 10^{-7} *M* and 10^{-6} *M* decreased the ActRI mRNA levels ($p<0.05$). Concentration at 10^{-6} *M* of GnRH had greater inhibitory effect ($p<0.05$).

Inhibin (10 ng/ml) induced a 60% reduction in ActRI mRNA levels ($p<0.01$) with six hours of treatment. Co-treatment of activin-A (10 ng/ml) and Inhibin (10 ng/ml) decreased the stimulatory effect of activin-A on ActRI mRNA level in IEVT cells.

TGFβ1 at a concentration of 0.1 ng/ml and 1 ng/ml increased ActRI mRNA levels ($p<0.01$). However, ActRI mRNA levels were not increased in IEVT cells cultured in the presence of TGFβ1 (10 ng/ml).

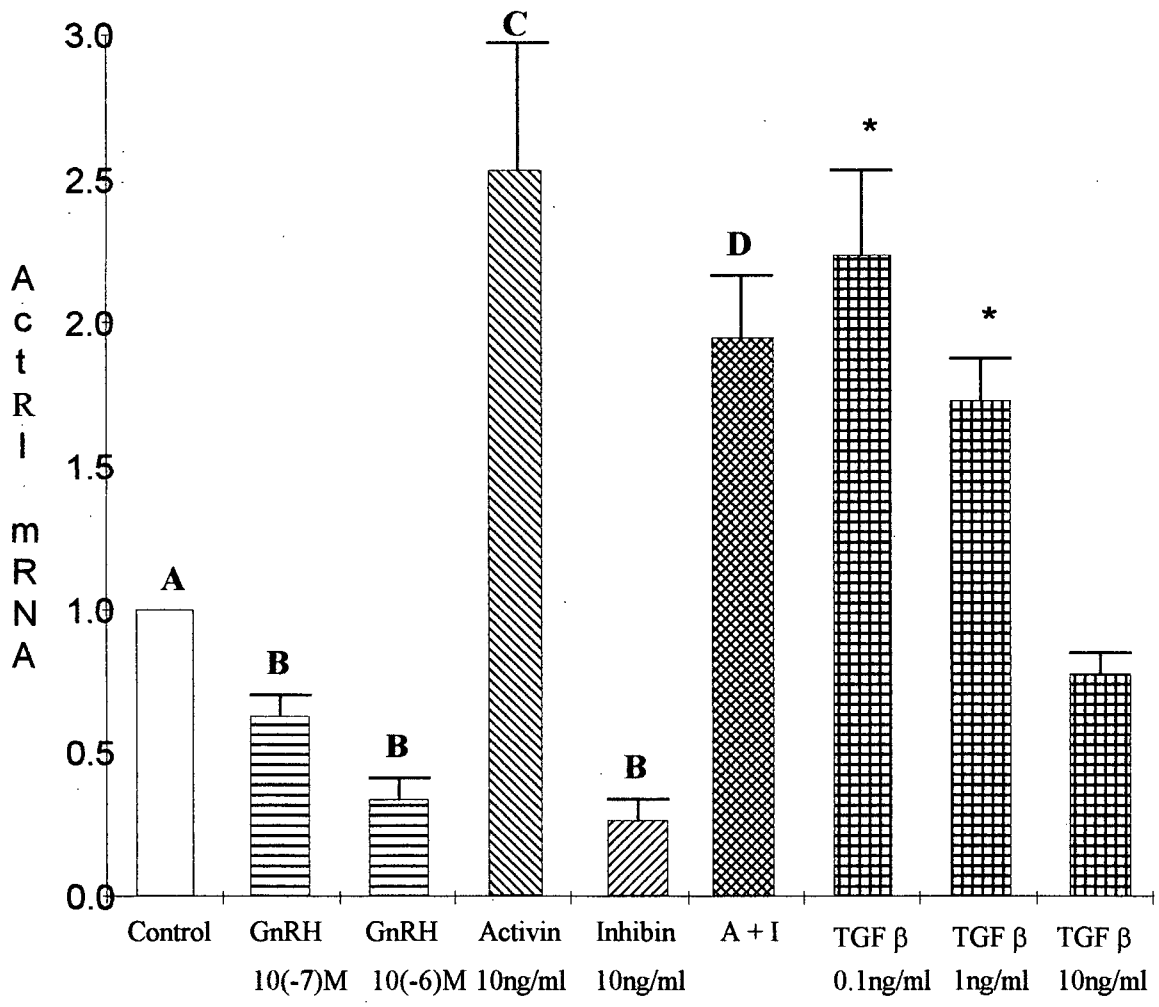


Figure 13. ActRI mRNA levels induced by GnRH, activin-A, inhibin and TGFβ in IEVT cells was evaluated with cPCR. Cells were incubated for 6 hours in the presence or absence of GnRH, activin, inhibin, or TGFβ1 with different concentration. Data were normalized for β-actin mRNA levels measured by PCR and expressed relative to the control value. Columns denote means ± SEM of 1 experiment with duplicate.
*, $P<0.05$ versus control(in the TGFβ group).
Different letters indicate statistically significant differences.

CHAPTER FIVE: DISCUSSION

Studies on human placental functions can only be performed extracorporeally. Placental explants and organ cultures have been studied extensively. However, the interpretation of results from these experiments is difficult owing to the presence of many different cell types and in these cell preparation, and the rapid necrosis suffered by tissue fragments when immersed in culture medium. Multinucleated cell masses form in human trophoblast cultures, but it appears impossible at present to obtain a pure culture of human syncytiotrophoblast, the layer which is the site of the most essential placental functions *in vivo*. 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 those described (Graham et al., 1993a); and they provide a promising model in which to study the function of EVT cells.

Trophoblast cells are formed very early in gestation and circulate in the maternal blood during early pregnancy as a consequence of their invasive and proliferative nature. After enrichment, these cells can be used for noninvasive prenatal diagnosis. Maternal leukocytes could be

separated from red blood cells and trophoblasts. It might be able to get highly purified trophoblast cells from maternal blood for *in vitro* study (van Wijk et al., 1996).

The regulatory effects of hormones or other factors on ActRs mRNA levels have not been reported. The present experiments support the hypothesis that activin regulates ActRI mRNA levels in autocrine/ paracrine fashion in immortalized EVT cells.

A. Verification and validation of the internal standard for optimizing cPCR

In this study, we utilized a cPCR technique that precisely quantitated relative ActRI mRNA levels in IEVT cells. RNA was isolated from cultured IEVT cells, and cDNA was prepared from these RNA samples. The relative abundance of ActRI mRNA in the samples was assessed by a cPCR method, adapted from other reported quantitative PCR methods (Kim et al., 1993) which depends on co-amplification of a control template with the sample cDNA using the same set of primers. cPCR requires construction of a control cDNA template, similar to the target sequence, but yielding a distinguishable product.

Recently several methods for quantitating specific mRNA species using different internal standards have been reported (Celi et al., 1993;

Forster, 1994). The method we used requires plasmid cloning to achieve the internal standard. The internal standard within the plasmid after cloning is more stable than the internal standard from extended PCR product. Any methods creating the internal standard with plasmid cloning are worthy to practice to make the internal standard for cPCR.

In our studies, the target cDNA was co-amplified with a dilution of control template cDNA. Products were compared with an internal standard curve, and estimates of the relative levels of cDNA were determined. The advantage of this technique is that the range in which PCR product is a linear function of starting template copy number is rather narrow, and the internal standard series allows recognition of saturation. In addition, with co-amplification of control and unknown amount of target cDNA in the same reaction, tube-to-tube variability can be identified, and an unsuccessful PCR can be distinguished from a negative result. We also assessed the abundance of β-actin as an additional control (Sun et al., 1996) for variation in RNA purification, cDNA synthesis and the possibly inaccurate measurement of the RNA concentrate using spectrophotometer.

We previously validated the internal standard of ActRI for the term cytotrophoblasts cDNA (unpublished data). The internal standard of ActRI can be used to study this cell population with regard to the

regulation of ActRI mRNA levels *in vitro* without exposure to radioactive substances.

B. Detecting the existence of activin subunits and ActRs mRNA in different cell populations using PCR

a. Inhibin subunits mRNA transcripts

In humans, inhibin subunit mRNA transcripts have been detected in ovarian granulosa cells (Jones et al., 1988), corpus luteum (Davis et al., 1990), adrenals (Voutilainen et al., 1991), placenta (Mayo et al., 1986), and decidua (Petraglia et al., 1990). Recent studies have indicated that activin and inhibin may act as local regulators of cell growth and steroidogenesis in human gonads (Mather et al., 1992). Some placental cells at term can produce and release inhibin, activin, and/or GnRH (Petraglia et al., 1992). By using semi-quantitative PCR technique, we have previously shown that mechanically dispersed trophoblast-enriched cells from human term placenta expressed all inhibin subunits and cAMP increased all three subunits mRNA levels (Li et al., 1993). The basal level of βA and α-subunits mRNA levels was higher than that of βB-subunit as determined by PCR combined with Southern blotting or Northern hybridization. The stimulatory effects of cAMP were being observed at

dose-dependent fashion with maximal effects at the concentration of 150 μ*M*. However, GnRH at the concentration of 100 n*M* increased α- and βB-subunit mRNA levels but not the βA-subunit. Previous finding which indicate that GnRH stimulates inhibin subunits and our present findings that indicating that GnRH regulates ActRI mRNA level support the interaction of activin/inhibin and GnRH in placental cellular function.

Petraglia et al. (1990) reported that human decidua contains and synthesizes inhibin α, βA, and βB subunits using Northern blot analysis. Inhibin α, and βB subunit mRNAs were both detected in first trimester and term pregnancy. The βA subunit mRNA transcripts were detected at very low levels at term. The subunit could not be detected in early pregnancy. Petraglia et al. (1990) did not determined if decidual cells expressed inhibin subunits mRNA transcripts. However, our PCR data demonstrated that all inhibin subunits mRNA are present in first trimester decidual tissue and isolated first trimester decidual cells. The presence of all subunit mRNAs indicates that both inhibins and activins may be produced in the human placenta. Because of their regulatory role in placental hormone secretions (hCG, progesterone, and GnRH) (Petraglia et al.,1989), inhibin and activin have been suggested to play a role in human pregnancy. Inhibin-βA and βB produced by decidual cells may contribute to the elevated concentrations of activin in amniotic fluid during late gestation

(Jenkin et al., 1995). The sequence homology between inhibin, activin, and TGFβ and the large number of growth factors (Vale et al., 1988) that act on fetal placental tissues suggests that a possible paracrine or autocrine action of activin and inhibin as growth factors in modulating cellular differentiation and proliferation of trophoblasts and decidual cells.

b. ActRs mRNA

Four activin receptor subtypes (ActRI, ActRIB, ActRII, and ActRIIB) have been identified in various tissues (Qu and Thomas, 1995). In the placenta, the existence of ActRII mRNA was proved in both syncytiotrophoblast- and cytotrophoblasts-enriched fractions in the first trimester and at term (Peng et al., 1993). ActRIIB mRNA was found in syncytiotrophoblast and amnion cells using in situ hybridization histochemistry (Petraglia et al., 1994a).

We have examined the mRNA of all four ActR subtypes using PCR. With the exception of JEG-3 cells, ActRIIB mRNA was detected in all the cell populations. The mRNA transcripts of ActRI, ActRII and ActRIIB were detected in the whole cell population. However, the ActRIB mRNA transcripts could not be detected in all the cell populations. The above data except ActRIIB mRNA in JEG-3 cells is consistent with previous reports. The absence of ActRIB mRNA may be owing to

extremely low expression of this gene in placental cells or the pair of primers were inappropriate. Conventional PCR showed that IEVT cells had higher levels of ActRI, ActRII, and ActRIIB mRNAs than the other trophoblast cells. The reason why JEG-3 cells did not express ActRIIB mRNA is unknown, but it is worthy to investigate this topic.

C. Comparing ActRI mRNA levels in different cell populations using Northern blot analysis

DIG-labeled antisense RNA probe, coupled with chemiluminescence detection, provides a convenient, nonradioactive procedure for Northern blotting. There is a growing need for methodologies, accessible and nonradioactive, to enable Northern blotting to be undertaken as a routine procedure in a wide range of laboratories.

Northern blot analysis using the DIG-labeled method was performed to evaluate the level of ActRI mRNA expression in different cell populations. A 3.6-kb mRNA transcripts was detected in all four cell populations. The levels of the ActRI mRNA transcripts is greatest by first trimester EVT cells followed in decreasing order of first trimester cytotrophoblasts, JEG-3 cells and term cytotrophoblasts. The results of this experiment indicate that the ActRI gene is expressed in placental trophoblasts and are consistent with the results of Matsuzaki et al. (1993)

who reported that a serine/threonine type I receptor was detected in the human placenta. However, unlike ActRII mRNA transcripts (Peng et al., 1993), the levels of ActRI mRNA transcripts is higher in first trimester cytotrophoblasts, especially EVT cells than term cytotrophoblasts. This suggests that activin and ActRI may be important in cell differentiation or invasion during first trimester pregnancy .

D. The effects of activin-A on ActRI mRNA levels in cultured IEVT cells using cPCR.

A cell population of human trophoblast cells growing out of primary explants of mechanically derived chorionic villus fragments was studied by Irvine et al. (1995). Immunolabelling and immunoprecipitation revealed that this phenotypic profile was retained with complete fidelity in the long-term culture. Therefore, trophoblasts migrating out of first trimester chorionic villus explants and their propagated progeny belong to the invasive extravillous trophoblasts of the placenta. The IEVT cells (HTR-8/SVneo) developed by Graham et al. (1993a) exhibit several major phenotypic characteristics with the parental trophoblast cells except the ability to sustain prolonged growth in culture. The IEVT cells provides a good culture system in which to study cytotrophoblast function and tumor cell biology *in vitro*.

ActRI, ActRII and ActRIIB mRNA transcripts were easily detected in female rat pituitary RNA, with the relative abundance of ActRI > II >IIB (18:9:1) (Dalkin et al., 1996). Activin increased ActRI (4-fold, at 2 hours), ActRIIB (2-fold, at 24 hours), but did not alter ActRII mRNA levels. Activin has been shown to have a differentially positive feedback effect on its own receptors in female rat pituitary (Dalkin et al., 1996). On the other hand, treatment of granulosa cell cultures with FSH (100 ng/ml) resulted in tentative suppression of its own FSH receptor mRNA level 2-6 hours after treatment, with subsequent recovery at 24 hours (Minegishi et al., 1995).

Activin is expressed in embryonic and fetal tissues, as well as in the placenta. Although activin is a potent regulator of growth and differentiation in a number of cell types, its role in embryo-fetal and placental development have yet to be established. Our results show that ActRI mRNA accumulation at 12 hours after treatment was stimulated in a dose-dependent manner by activin-A with a maximal response three times greater than the control cultures using the concentration of 10 ng/ml. Activin-A had no effect on ActRI mRNA levels in IEVT cells at concentrations of 1 ng/ml or lower. The time course of the activin effect on ActRI mRNA showed an early response, with a maximal increase occurring at 6 hours. The stimulation was evident at 3 hours, but not at 1

hour. This response pattern suggests that activin-A exerts a positive feedback effect on its own receptor and that the action of activin on ActRI mRNA level is transient.

Xenopus embryos which contain high levels of ActRIIB mRNA transcripts required lower concentration of activin for maximum stimulation of activin mRNA levels (Mathews et al., 1992). In the present study, the increase of ActRI mRNA levels by activin-A also suggests that lower concentration of activin-A will be required to achieve full physiological action.

E. The effects of follistatin on ActRI mRNA levels in cultured IEVT cells using cPCR.

Follistatins are widely distributed (DePaolo et al.,1991; Michel et al., 1993) and are produced by many activin-responsive tissues or those that express the inhibin/activin β subunits (Meunier et al., 1988) and may, therefore, serve to anatomically and temporally limit the local activities of activins.

Follistatin mRNA transcripts have been detected in placental and decidual cells collected during first trimester or at term, and immunoreactive follistatin is localized in syncytial cells of placental villi at term as well as in decidual cells, amnion epithelium, and in chorionic cells

(Petraglia et al., 1994b). Follistatin, the activin-binding protein, has been shown to neutralize the effect of activin in several biological systems (Cataldo et al., 1995; Michel et al., 1995). In view of these observation, we examined the effect of increasing concentrations of recombinant human follistatin on the basal ActRI mRNA levels. Follistatin had no effect on ActRI mRNA levels at any concentration used in this study. Although not statistically significant, a slight decrease in the intensity of ActRI mRNA levels was observed. The possible explanation for this is that follistatin binds endogenous activin or ActRI and results in a small decrease in ActRI mRNA levels.

F. Interaction between follistatin and activin-A on ActRI mRNA levels in cultured IEVT cells using cPCR.

This experiment examined the hypothesis that follistatin reverses the actions of activin-A on ActRI mRNA levels in cultured IEVT cells.

It is known that activin, inhibin, and follistatin were widely expressed and biologically diverse in their effects on cell growth and differentiation in many organisms (Erickson et al., 1995). Follistatin and activin may be viewed as components of an autocrine/paracrine system that regulate cellular differentiation and growth.

Comparing the prior experiment that showed a dramatic stimulation of ActRI mRNA levels when IEVT cell culture was treated with activin-A alone, co-treatment of activin-A and follistatin in this experiment demonstrated that follistatin blocked the stimulatory effect of activin-A on the ActRI mRNA levels in a dose dependent manner. The inhibitory effect became more prominent as the concentration of follistatin increased, with maximal effects be observed at the 50 or 100 ng/ml. Another experiment demonstrated that the combination of activin-A (10 ng/ml) and follistatin (50 ng/ml) had no stimulatory effect on ActRI mRNA levels over time. Follistatin (50 ng/ml) completely inhibited the effect of activin-A (10 ng/ml) on the ActRI mRNA levels in IEVT cells.

The present study which demonstrates that follistatin acts to modulate the action of activin-A in ActRI mRNA levels in cultured IEVT cells, emphasizes the possible functional role of activin and follistatin in the complex autocrine/paracrine regulation of placental function. This antagonistic effect is dose dependent and consistent with the requirement for two molecules of follistatin to neutralize one molecule of activin. Follistatin can inhibit FSH secretion in cultured pituitary cells with a potency lower than that of inhibin but has subsequently been shown to bind activins with high affinity and neutralize their biological activity (Nakamura et al., 1990). By double ligand blotting, Shimonaka et al.

(1991) have shown that two molecules of follistatin can bind to one molecule of activin-A, whereas only one activin-A molecule can bind to each follistatin molecule. Those observations suggest that a three-in-one complex may form, consisting of one activin-A molecule interacting with two follistatin molecules by each of its two β-subunits.

Our results are consistent with those reported for the interaction of follistatin and activin in cultured rat and bovine granulosa cells (Shukovski et al., 1991; Xiao et al., 1992) that suggested the antagonistic effect of activin on progesterone secretion by follistatin was not dependent on the species or the differentiation state of the granulosa cells.

Our data support the view that follistatin acts as a binding protein which prevents access of the ligand, activin, to its receptor. On the other hand, follistatin had no effect in the absence of activin-A. The widespread tissue localization of follistatin is very similar to that of activin, that suggests follistatin plays a local modulatory role in the various autocrine/paracrine actions of activin.

Follistatin was originally isolated from porcine (Ueno et al, 1987) and bovine ovarian follicular fluid (Ling et al., 1986; Vale et al., 1986; Robertson 1987). Follistatin has the ability to bind activin, and is thought to control activin action in various tissues (Nakamura et al., 1990; Shimonaka et al., 1991). In the ovary, follistain has been found to be

produced by granulosa cells (Nakatani et al., 1991). In addition, the presence of follistatin (Shimasaki et al., 1989; Shimonaka et al., 1991) and inhibin-β mRNA (Meaner et al., 1988) in the ovary suggests a regulatory role for follistatin and activin in follicular maturation.

In human luteinized granulosa cells, follistatin can antagonize the effects of activin-A on steroidogenesis (Cataldo et al., 1994). Activin-A decreased basal progesterone secretion by granulosa cells in a dose-dependent manner. When follistatin was added along with activin-A to granulosa cell cultures, the inhibition of progesterone production by activin-A was reversed by follistatin in a manner dependent on the molar ratio of follistatin to activin-A. When graded doses of follistain were added together with a constant activin-A dose, a dose-response relationship was seen, with reversal of the activin-A effect only occurring when the follistain to activin-A molar ratio exceeded 2:1. The results of Cataldo et al. (1994) confirmed that follistatin functions as an activin-binding protein to regulate progesterone secretion in luteinizing human granulosa cells.

G. The effects of GnRH, inhibin, activin-A with inhibin, and TGFβ1 on ActRI mRNA levels in cultured IEVT cells using cPCR.

Human placenta produces a large variety of bioactive substances with endocrine and neural competence: pituitary and gonadal hormones, hypothalamic-like releasing or inhibiting hormones, growth factors, cytokines and neuropeptides. Immunoreactive GnRH is present in placenta, specifically in cytotrophoblasts, but not in the syncytiotrophoblast (Siler-Khodr and Khodr, 1978). Siler-Khodr (1983) described this substance as hCG-releasing hormone. The human placenta contains low-affinity GnRH binding sites that interact with GnRH agonist and antagonist analogs (Currie et al., 1981). Also, GnRH stimulates the synthesis and release of bioactive hCG in placental cells (Belisle et al., 1984) as well as increases intracellular calcium ion concentrations in first trimester human trophoblast cells (Currie et al., 1993). A specific GnRH-hCG regulation of placental steroidogenesis has been proposed as an internal regulatory system acting on steroids production from human placenta (Petraglia et al., 1989).

We have previously shown that GnRH increased cytosolic calcium ion concentration in a preliminary trial using syncytiotrophoblasts derived directly from a single first trimester placenta and cultured for 3 days (Currie et al., 1993). Intracellular message for GnRH activity may be mediated by transient increase in the free cytosolic calcium concentration. The releasing of GnRH-like immunoreactivity (GnRH-LI) from cultured

human placental cells was shown to be induced by the opening of ionic channels and activation of the adenylate cyclase/cAMP system (Petraglia et al., 1987). Cytotrophoblasts of first trimester placenta synthesize and secrete multiple forms of GnRH that stimulate hCG synthesis and secretion from syncytiotrophoblasts in a dose-dependent manner. Indirect evidence suggests that increased free cytosolic calcium concentration induces hCG secretion (Belisle et al., 1989).

The addition of activin increased progesterone production and potentiated the GnRH-induced release of hCG. Inhibin by itself did not modify placental immunoreactive GnRH, hCG, and progesterone secretion but reversed the activin-induced changes. In addition, activin and inhibin have been proposed as further regulatory substances of the synthesis and secretion of steroids; the addition of activin-A to placental culture augments the secretion of GnRH, hCG and progesterone, and this effect can be significantly reduced by the addition of inhibins.

Our results show that GnRH at the concentrations of 10^{-7} *M* and 10^{-6} *M* decreased ActRI mRNA levels but a concentration at 10^{-6} *M* had strong inhibitory effect. Although it is obvious that not only activin-A, but also GnRH, can regulate ActRI mRNA levels, the mechanism involving the secretion of hCG and progesterone from trophoblast cells by stimulation with activin or GnRH remains to be defined.

Inhibin is a heterodimeric glycoprotein hormone that is produced by human ovarian granulosa cells, testis, and placenta. In conjunction with the large amounts of sex steroid hormones produced in human pregnancy inhibin may serve to inhibit FSH secretion and thereby prevent ovulation during pregnancy. In the placenta, inhibin is produced in cytotrophoblasts and may act, in a paracrine fashion, to stimulate hCG production by the syncytiotrophoblast. In isolated trophoblasts, hCG and cyclic AMP analogues stimulate inhibin production; the finding that antibodies to the inhibin α subunit cause an increase in hCG secretion is suggestive that inhibin may act to regulate hCG release/secretion in placenta (Petraglia et al., 1987).

Inhibin and activin possess opposing activities in several biological systems including pituitary FSH secretion, erythroid differentiation, and gonadal sex-steroid production. Steele et al. (1993) examined human first trimester placental trophoblasts on microcarrier beads in a perfusion system. Both activin-A and GnRH induced a rapid and transient hCG secretory response. Increased hCG secretion by activin-A was not blocked by concomitant treatment with a GnRH antagonist that canceled the action of GnRH. Inhibin did not affect hCG secretion. Co-treatment with inhibin and activin-A resulted in a transient increase in hCG, followed by a decrease in hCG secretion to below pretreatment levels. Steele et al.

(1993) suggested that activin facilitates hCG secretion without stimulating endogenous GnRH release. These results suggest that in addition to GnRH, activin may play a role in the regulation of hCG secretion in first trimester placenta.

Our data showed that inhibin (10 ng/ml) cause a 60 % reduction in ActRI mRNA levels within six hours following treatment. Co-treatment of activin-A (10 ng/ml) and inhibin (10 ng/ml) resulted in a smaller stimulatory effect compared to the effect of activin-A alone. This finding is in agreement with the observation that inhibin counteracts the effects of activin on cultured first trimester trophoblast cells (Steele et al., 1993).

The first trimester immortalized EVT cells were transfected by Simian virus 40 large T antigen, while retaining the normal trophoblast markers, have complete resistance to the anti-invasive but not the anti-proliferative effects of TGFβ (Graham et al, 1993a). Treatment with TGF-β also resulted in decreased secretion of plasminogen activators (PAs) and reduced PA activity. The level of secretion of this enzyme was not significantly affected by TGF-β. The anti-invasive effect of TGF-β is believed to be due, at least in part, to the induction of tissue inhibitor of metalloproteinases (TIMP)-1. The invasiveness of the IEVT cells was not reduced by TGF-β. The transfected HTR-8/SVneo (IEVT) cells share a number of phenotypic properties with the parental trophoblast cells. For

this reason, these transfected trophoblasts may prove to be an important tool for the study of placental function and/or tumor progression.

Only few reports revealed that the dose response effects of TGFβ on cellular function was biphasic. The mitogenic stimulation of connective tissue cells by TGFβ was an example, with lower concentrations increasing and high concentrations decreasing or having no effect (Seifert et al., 1994). Another example is that TGFβ stimulated the EGF receptor mRNA transcripts in normal rat kidney fibroblast cells resulting biphasic dose response pattern (Thompson et al., 1988).

In our experiment, TGFβ1 at the concentration of 0.1 ng/ml and 1 ng/ml promoted ActRI mRNA expression, but suppressed ActRI mRNA expression at the concentration of 10 ng/ml, also showing a biphasic response pattern. A possible explanation for this biphasic pattern is that several subtypes of TGFβ receptors exist that can react with the ligands. TGFβ inhibits cytotrophoblasts syncytial formation in trophoblast cells isolated from term placenta, and inhibit trophoblast invasion. The mechanism of TGFβ action on trophoblast cells may involve the alteration of the expression of activin or activin receptors in these cells.

SUMMARY

Activin has been suggested to play an autocrine/paracrine role in the regulation of placental function. It exerts its cellular function via binding to membrane receptors of TGFβ superfamily. In our laboratory, we have developed a cPCR method for the precise quantification of ActRI mRNA levels in IEVT cells.

We have examined the effect of activin-A on ActRI mRNA levels in cultured human IEVT cells using cPCR. Northern blot analysis indicated the presence of an ActRI mRNA transcript of trophoblasts, approximately 3.6 kb in total RNA prepared from different cell populations. Culture of IEVT for 12h in the presence of increasing concentration of activin-A resulted in a dose-dependent increase in ActRI mRNA levels with a maximal increase of approximately 3-4 folds over control levels in the presence of 10 ng/ml activin-A. Treatment of IEVT cultures with 10 ng/ml of activin-A resulted in slight increase of ActRI mRNA level with maximal effect being observed at 6 hours of treatment. Follistatin blocked activin-A effects on ActRI mRNA levels in dose-dependent manner in IEVT cells. Results of our experiment suggest that activin and follistain modulate ActRI gene expression in an autocrine/paracrine manner in human placental trophoblasts.

In conclusion, we have demonstrated that it is possible to use cPCR methodology to study the expression and regulation of different ActR genes

in different populations of placental trophoblast cells. Clearly, cPCR promises to be an excellent tool in the evaluation of gene regulation.

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