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Overexpression of inhibin α (1-32) fusion protein promotes apoptosis and cell cycle arrest in a cervical cancer cell model (Hela cells)

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ABSTRACT: Inhibins play important roles in the reproductive system. To evaluate whether inhibin α (1-32) fusion protein plays a role in cervical cancer growth, the plasmid pVAX-inhα was constructed and its effect on proliferation and apoptosis of the human cervical cancer cell line (Hela) was checked by flow cytometry and real-time PCR. The expression and localization of inhibin α protein were detected by RT-PCR and confocal microscopy which showed that inhibin α protein was expressed and localized in the nucleus of Hela cells. Over expression of inhibin α gene significantly induced cell apoptosis and ceased S phase of cell cycle. Furthermore, cell proliferation was significantly suppressed 96 h post-transfection and mRNA level of anti-apoptosis genes (Bcl-2, NFκB) were decreased but pro-apoptosis genes (Bax, wild type p53) and inhibin co receptor (TGFβR3) were increased, indicating that inhibin, through its co-receptor, might activate apoptotic and cell growth cascades which regulate proliferation and apoptosis in Hela cells. These results suggest that inhibin α (1-32) fusion protein, located in the cell nucleus, can regulate Hela cells growth and apoptosis by induction of apoptotic pathways such as NFκB, Bcl-2 and p53 families. These findings may have a significant impact on future research regarding cervical cancer cell lines

Introduction

The second most common malignancy in human reproduction is cervical cancer with proliferation of 500,000 new cancer cases each year worldwide. Although presently handled as pandemic disease, the incidence rate is radical in less developed countries. Moreover, the availability of new tumor markers for cervical adenocarcinoma is scarce (Burges *et al.* 2011; Bergauer *et al.* 2009). Recently, many studies identified the involvement of different members (TGF-β, GDF-9, inhibin) of transforming growth factor-beta super family in transforming cellular growth and proliferation in endometrial and cervical epithelial tumors (Miyazono *et al.* 2011; Wang *et al.* 2010; Drummond and Fuller, 2012). Similarly, p53, p16INK4α, Villin1 and inhibin are likely to exhibit as the potential markers of the endometrial and cervical tumors (Masatsugu *et al.* 2006; Negri *et al.* 2003; Nakamura *et al.* 2009; Tsiqkou *et al.* 2008). The same is observed for inhibin as strong tumor suppressor in different female reproductive anomalies (Stenvers and Findlay, 2010; Meczekalski and Podfiqurna, 2009).

In 1995, Cooke and colleagues found that inhibin-α gene acts as putative tumor suppressor in mice gonads and adrenal glands proceeded by the detection of other inhibin-subunits in endocrine tumors (Mylonas, 2011). This revised the subtitle of inhibin as endocrine regulator in female reproductive system and ultimately, taken into account in different gynecologic cancer developments (Burges *et al.* 2011; Wasowska *et al.* 2009).

The conditional knock down of inhibin-α gene first disclosed its properties as tumor suppressor as this prompt the primary gonadal sex cord-stromal tumors in both male and female mice (Mylonas, 2011). Recently, a remarkably lower expression of inhibin α has been validated in well differentiated adenocarcinomas compared to normal and hy-

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perplastic endometrial tissues (Mylonas *et al.* 2009). Moreover, cultured endometrial stromal or epithelial cells and cervical epithelial cells have admired the presence of inhibin α, activin βA and βB, and receptor type III mRNAs (Bergauer *et al.* 2009). Meanwhile, inhibin subunits are detected in normal and neoplastic human uterine tissues, including cervical cancer and cervical cancer cell lines (Hela and CaSKi) (Mylonas and Dian, 2011; Bergauer *et al.* 2009). Recently, a cohort group of 302 endometrial cancer patients displayed the role of inhibin α as an efficient marker in identification and selection of high-risk and therapeutic patients, respectively (Mylonas *et al.* 2009).

Despite the differential expression profile of inhibin subunits in different carcinomas (Worbs *et al.* 2007), many previous studies have shown that small inhibin peptides, including α 1-32, 1-26 and 7-28 of inhibin (Zhang *et al.* 1996; Sun *et al.* 1996) play substantial roles in animal and human reproductive system. Moreover, the effect of inhibin subunit α (1-32) has been proven better than that of other fragments (Burges *et al.* 2011). Therefore, we selected inhibin α (1-32) fragment in this study.

Members of TGF-β super family have been shown to configure activation of Smad cascades via serine/threonine kinases receptors. These receptor-Smad complexes translocate to the nucleus where they alter the nuclear transcriptional processes (Drummond and Fuller, 2012). Simultaneously, TGF-β mediates transcriptional activation via NFκB binding sites (Solt and May, 2008; Zhang *et al.* 2008) and Smad-3 and -4 proteins negotiate with the NFκB subunits p65 and p52. In the mirror image, NFκB increases Smad 6 expression which is negatively regulated by the TGF-β signaling pathway through negative feedback loop (Li *et al.* 2007). However, the precise function of the inhibin-α subunits in the regulation of growth and differentiation of endometrioid cancer cells are still quite unclear. Therefore, in the present study, the cellular localization, cell proliferation and apoptosis were studied by over-expressing inhibin α (1-32) fusion protein in Hela cells. Furthermore regulatory mechanisms in apoptosis and growth factors (Bax, p53 and NFκB) were also tested in these cells.

Materials and Methods

Cell lines, plasmids and reagents

Plasmids, pEGISI (pEGFP-ISI), pVAX-asd-gfp (Liang *et al.* 2009), and pVAX-asd were constructed previously in our laboratory. pVAX-asd-ISI-gfp was constructed and named pVAX-Inhα; pVAX-asd-gfp was named pVAX-control respectively. Hela cells were routinely and passagely cultured in our lab. DMEM high glucose medium, Dulbecco's phosphate-buffered saline (PBS), and trypsin with EDTA solutions were obtained from Gibco (Grand Island, NY, USA). Lipofectamine™ 2000 kit was purchased from Invitrogen, China. Apoptosis Detection kit (Annexin V-FITC) was purchased from Pharmingen (Becton Dickinson Company, San Jose, CA, USA), while large scale plasmid extraction kit and RNAprep pure Cell Kit were acquired from Tiangen biochemistry technology limited China. Unless otherwise stated, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Construction of inhibin recombinant plasmid pVAX-inhα

At first, the DNA fragment ISI-EGFP, isolated from the

TABLE 1

Primers for real-time PCR about apoptosis genes

Gene	Primer Name	Primer sequence (5′→3′)	Annealing temperature	Product size
β-actin	R1	GTCCACCGCAAATGCTTCTA		
	F1	TGCTGTCACCTTCACCGTTC	60	190
P53	R2	CCCTCCTCAGCATCTTATCCG	60	264
	F2	CTGGCACAAACACGCACCTC		
Bax	R3	TTGCTTCAGGGTTTCATCCA		
	F3	CAGCCTTGAGCACCAGTTTG	58	298
Bcl-2	R4	CATTGGGAAGTTTCAAATCAGC	58	305
	F4	CTTTGCATTCTTGGACGAGG		
NFKB	R5	GACCAACAACAACCCCTTC		
	F5	ACCTCAATGTCCTCTTTCTGC	58	273

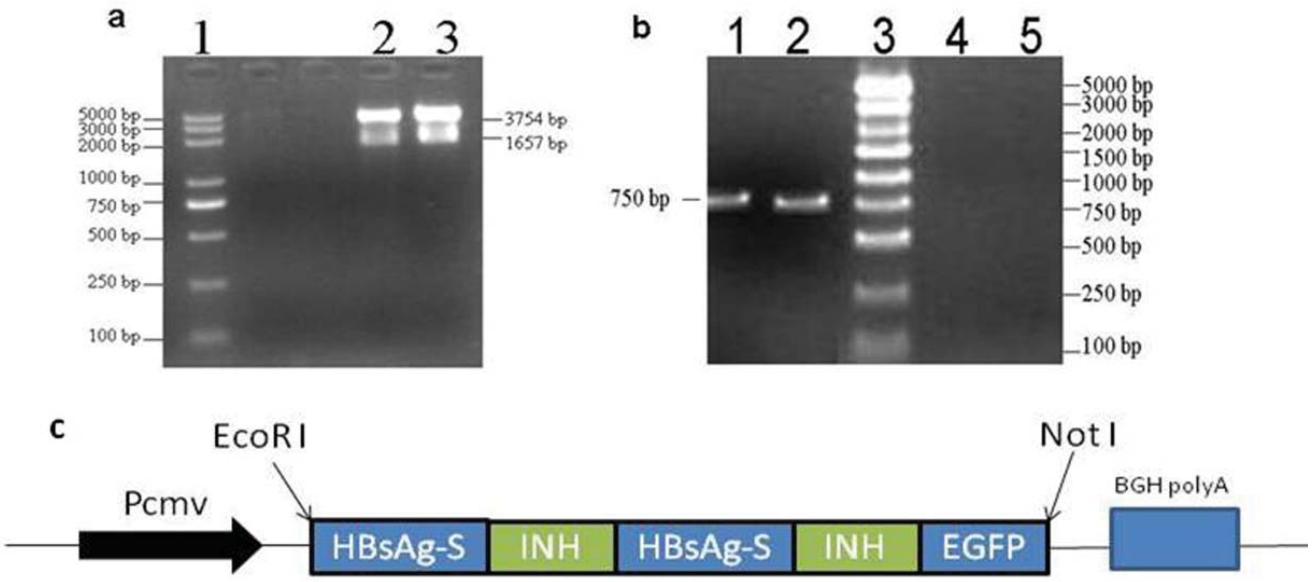

FIGURE 1. Identification of pVAX-inhα plasmid: **a:** Identification of pVAX-inhα by restriction endonucleases. Lane 1: 5000bp DNA maker; Lane 2-3: pVAX- inhα was digested with EcoR I and Not I. **b:** Detection of inhibin α (1-32) gene in transfected Hela cells at mRNA level. Total RNA was extracted from Hela cells, reverse transcribed into cDNA, submitted for RT-PCR and analyzed different products after electrophoresis. Lane 1 and 2: inhibin α (1-32) fusion gene; lane 3: DNA marker; lane 4: the polymerization products of the extracted RNA without reverse transcriptase are shown as negative controls; Lane 5: second negative control using ddH2O as template. **c:** Schematic diagram of Inhα insertion in pVAX vector containing GFP and specific restriction sites.

plasmid pEGISI harboring resistance gene for kanamycin, was inserted into the plasmid pVAX-asd, digested with the same of EcoR I/Not I, resulting in a new plasmid named pVAX-inhα encoding two copies of the inhibin α (1-32) fragments, hepatitis B surface antigen gene *S* (HBsAg-S), enhanced green fluorescent protein gene (EGFP) and characterized by the nutritional selector marker-*asd* gene instead of the kanamycin resistant gene (Fig 1C). The integrity of these coding sequences was further validated by restriction endonuclease and sequence analysis.

Transfection of Hela cells

Hela cells were divided into three groups: (a) control (without transfection); (b) pVAX-control group (transfection with pVAX-control and liposome); and (c) pVAX-inhα group (transfection with pVAX-inhα and liposome). The transfection of these vectors was executed by Lipofectamine™ 2000 Kit (Invitrogen) as per manufacturer's protocol. After 5h, the medium was removed, washed with PBS and replenished with DMEM containing 10% FBS. To examine transfection efficiency, cells transfected with pVAX-inhα were observed after 48h under a fluorescent microscope to detect the expression of GFP.

Qualitative and quantitative polymerase chain reaction

After transfection, the cells were washed with PBS, scraped off from petri dish and lyzed for the extraction of RNA using RNAprep pure Cell Kit. Total RNA (2μg) was reverse transcribed into cDNA in a 20μl reaction mixture using First strand cDNA synthesis kit (Toyobo, Osaka, Japan). The synthesized cDNAs were amplified using specified primers (Table 1) in a PCR based detection method. For control samples, reverse transcriptase was omitted at RT steps. Finally, the PCR products were analyzed by electrophoresis on 1.0% agarose gel stained with ethidium bromide. For quantification, real time PCR for all target genes was carried out using TaqMan genotyping Master Mix protocol in an Applied Biosystems Prism (ABI) 7500 sequence detection system. All qPCR results were analyzed by the ΔΔ cycle threshold level using the ABI 7500 System Software (version 1.2.3) and values were normalized to the endogenous reference (actin). After estimation of optimal annealing temperature, melting curve analysis was performed for further insurance. The results were drawn using relative expression of each gene against its control values. All experiments were carried out thrice independently with three replications of each sample.

DAPI staining cells for confocal microscopy

DAPI (40, 6-diamidino-2-phenylindole, Sigma) was used as a DNA-specific probe, which passed through the cell membrane of viable cells with prior fixation for 5min by methanol. Hela cells were cultured on coverslips in 6-well plates, transfected with respective plasmids, washed with PBS for 5 min each and covered with 1μg/ml of DAPI for karyotin. After staining in a dark chamber for 10 min, cells were rinsed with PBS to remove DAPI solution. The coverslips

were then fixed on slides with a drop of glycerol: PBS liquid (75% vs. 25%). Then localization of inhibin α protein were detected by confocal microscopy.

Detection of apoptosis

After transfection, Hela were cultured in 12-well plates for 48h, washed with pre-warmed PBS, digested with trypsin without EDTA and stained with Annexin V-FITC/propidium iodide kit, according to the manufacturer's instructions. Cell apoptosis was detected with flow cytometric analyzer, (Becton-Dickinson FACScan, Mount View, CA, USA).

Cell cycle

After transfection, Hela were cultured in 12-well plates for 48h, washed with PBS and then harvested by digestion with the trypsin without EDTA. Cells were fixed with precooled 75% ethanol for 12 h, and then probed with propidium iodide and 5uL RNase A (10 mg/mL) at 37.5 °C for 30 min. The cell cycle analysis was carried out by flow cytometry in triplicate.

Cell proliferation assay

At the end of the culture period, the effect of transfection of pVAX-inhα on cell viability and proliferation were assessed using MTT. The experiments were performed in 96-well plates at cell densities of 1.0-2.0 × 10^3 cells per well. Briefly, 20ul of MTT solution (5mg/ml, in PBS) and 180ul DMEM supplemented with 10% FBS were added to each well and incubated for 4 h in a humidified incubator at 37.5 °C in atmospheric air with 5% CO^2. Following the removal of supernatant, 200ul DMSO was added to dissolve purple crystallization, and then the assay plates were read at 490 nm with an ELISA microplate reader adjusted for background absorbance at 630 nm (Multiskan MK3, Thermo, USA).

Statistical Analysis

All data were analyzed using the General Linear Models Procedure of Statistical Analysis Systems (SPSS Inc., Cary, NC, USA). A value of $P < 0.05$ was considered to be significant. All data is represented as mean ± SEM of repeated experiments (n = 3).

Results

Identification of pVAX-inhα plasmid

The recombinant plasmid pVAX-inhα was constructed and further identified by double digestion with EcoR I /Not I. Agrose gel electrogram depicted the presence of two bands of 3754bp and 1657bp fragments with EcoR I and Not I digestion, representing the vector plasmid and the fragment include inhibin, respectively (Fig. 1a, c). These fragments

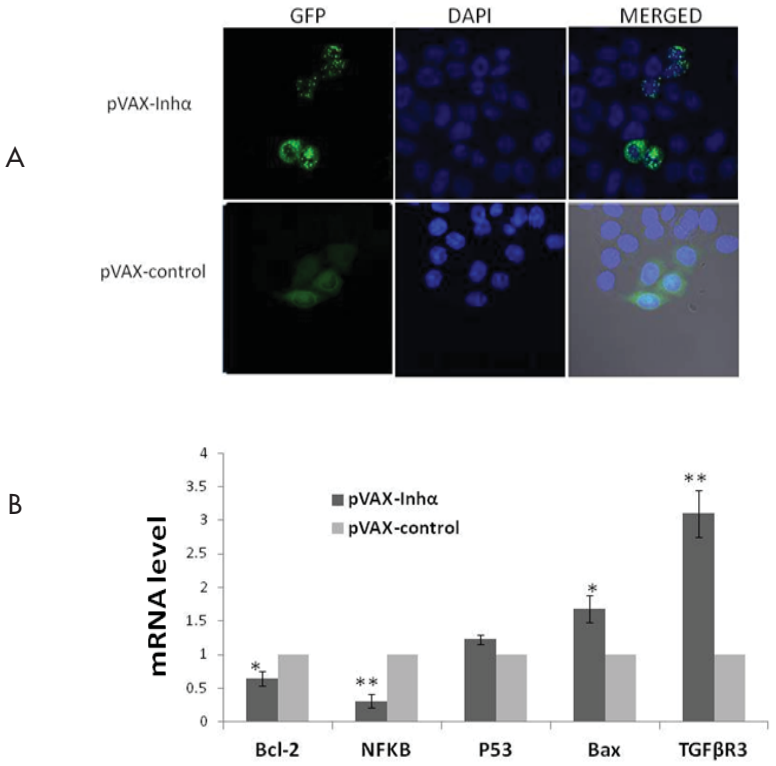

FIGURE 2. Subcellular localization and relative expression (mRNA levels) of different genes in transfected Hela cellsc: Schematic diagram of Inhα insertion in pVAX vector containing GFP and specific restriction sites. **A)** Subcellular localization of inhibin fusion protein in Hela cells. Hela cells were transfected with pVAX-inhα or pVAX-control plasmids respectively, and stained with DAPI after transfection for 48h. Images were taken using a confocal microscope. Cells transfected with pVAX-inhα showing fluorescence (EGFP) only in the nucleus, while fluorescence in the control plasmid (pVAX-control) are (EGFP) diffused over the entire cell. **B)** Relative expression of different genes in Hela cells after transfection with different vectors. Anti-apoptosis genes, Bcl-2 and NFκB were decreased, while pro-apoptosis gene Bax, wild type p53 and TGFβR3 gene were increased compared with other control groups. Data are presented as mean ± SEM of three independent replicates and analyzed by t-test. $P < 0.05$ as significant.

TABLE 2

Effect of pVAX-Inhα transfection for 48 h on apoptosis in Hela cells (mean±SEM, n = 3)

	Viable count	Early Apoptotic cells	later Apoptotic cells
pVAX-Inhα	16.71±1.42	30.75±1.76[b]	47.78±4.71
pVAX-control	18.09±6.55	24.86±1.11[a]	44.76±2.26

Results were evaluated by One-way ANOVA. "a" and "b" indicates level of significance in columns ($P < 0.05$).test).

were later confirmed by sequence analysis from a sequencing company (Sangon).

Expression of inhibin mRNA

To detect the expression of inhibin fusion gene in Hela cell line, RT-PCR analysis was performed after transfection for 48h. Presumably, the transcriptional level of inhibin fusion gene was detected using specific primers against cDNA of Hela cells, resulted in fragment of 750bp (Fig. 1b). Accordingly, no visible bands were observed in two negative controls in which, we omitted the reverse transcriptase for RT steps. These results provides an evidence that inhibin fusion gene is expressed in Hela cells at mRNA level.

Cellular localization of inhibin fusion protein

To examine the cellular localization of inhibin fusion protein in Hela cell lines, the transfected cells were stained with DAPI and observed by laser scanning confocal microscope. Cells transfected with pVAX-inhα showed fluorescence, only in nucleus, however, in control vector the fluorescence was diffused over the entire cell surface (Fig. 2a).

Effect of inhibin on cell apoptosis

A summary regarding apoptosis in Hela cells are presented in Table 2. The inhibin-induced alterations on the exposure of phosphatidylserine on cell surface were determined with Annexin V-FITC and PI double staining. Based on flow cy-

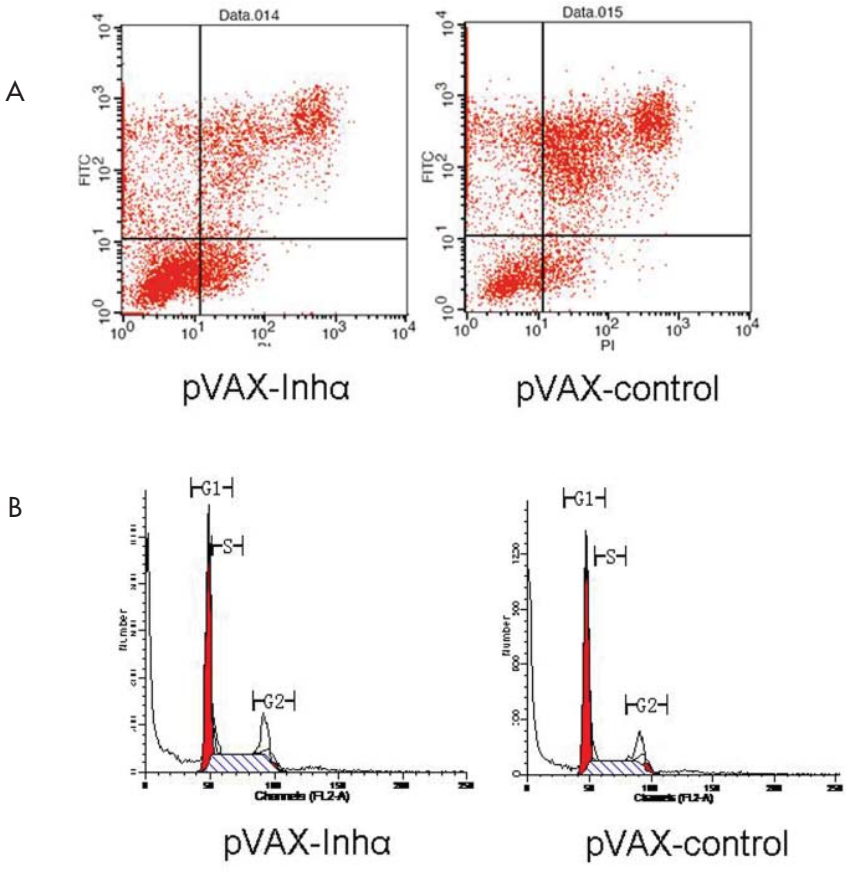

FIGURE 3. Apoptotic and cell cycle analysis in Hela cells after transfection with pVAX-inhα or pVAX-control. **A)** Apoptotic analysis in Hela cells after transfection with pVAX-inhα. The Hela cells were grouped as un-transfected, transfected with pVAX-inhα or pVAX-control for 48 h. Following staining with annexin V-FITC and PI, cells were subjected to flow cytometric analysis. Annexin V-FITC fluorescence is reported on the Y-axis, and PI fluorescence on the X-axis. The lower left areas of the graphs are representative of viable cells (PI and annexin V-FITC negative cells), the upper left part of the graph is representative of annexin V-FITC positive apoptotic cells, whereas, the upper right area of the graph (PI and annexin V-FITC positive cells) represents necrotic cells, the low right part of the graph is representative of PI positive cell debris. The experiment was repeated three times with 3 independent replications. **B)** Detection of cell cycle in transfected Hela cells. Hela cells were transfected with pVAX-inhα or pVAX-control plasmids, respectively. After staining with PI, cells were analyzed through flow cytometric analysis. The experiment was repeated three times with 3 independent replications.

TABLE 3

Effect of pVAX-Inhα transfection for 48 h on cell cycle distribution in Hela cells (mean±SEM, n = 3)

	G0/G1	S	G2/M
pVAX-Inhα	55.75±2.70	39.41±0.49[b]	4.84±0.04[b]
pVAX-control	56.11±2.69	36.20±1.48[a]	7.67±1.05[a]

Results were evaluated by One-way ANOVA. "a" and "b" indicates level of significance in columns ($P < 0.05$).

tometry, the percentage of apoptotic cells in the pVAX-inhα group was significantly higher (30.75±1.76) than in control groups (24.86±1.11 $P < 0.05$) (Fig. 3a, Table 2).

Effect of inhibin on different cell cycle phases in Hela cells

A summary regarding cell cycle analysis following transfection is presented in Table 3. To better understand the cellular growth induced by inhibin α, we assessed the cell cycle in Hela cells transfected with pVAX-inhα for 48h. Based on flow cytometry, the percentage of S phase in the pVAX-inhα group was significantly higher than in control groups (39.41±0.49% vs. 36.20±1.48%, $P < 0.05$), thus reducing the cells in G2/M phase (4.84±0.04% vs. 7.67±1.05%, $P < 0.01$) (Fig. 3b, Table 3).

Effect of inhibin on cell proliferation in Hela cells

The effect of inhibin on Hela cells proliferation was tested at 24h, 48h, 72h, and 96h after transfection. The inhibition ratio of the pVAX-inhα group was shown in the Table 4. The inhibition ratio of pVAX-inhα group was significantly higher 96 h post-transfection in pVAX-inhα group (0.41±0.08) compared to pVAX-control (0.63±0.09). These data indicate that cell proliferation of the Hela cells transfected with pVAX-inhα was inhibited but not time-dependently compared with other control groups (Table 4).

Expression of genes involved in apoptosis and TGFβR3

We further detected the transcriptional expression of some genes which regulated by inhibin fusion protein. Relative expression of the pro-apoptotic and anti-apoptotic genes was assessed using real-time PCR. In addition, TGFβR3 can modulate testosterone, inhibin/activin, and dysregulate different growth-factor pathways, so we also detected the relative expression of TGFβR3 in the present study. In the pVAX-inhα group, anti-apoptosis genes including, Bcl-2 and NFκB were significantly down-regulated, whereas, relative expression of TGFβR3 and the pro-apoptotic gene Bax and wild type p53 were increased compared to control group at 48h post-transfection (Fig. 2b, Table 1).

Discussion

Although the discovery of inhibin has been primarily evident in human gonads, they are also reported to be synthesized in different endocrine tissues, including normal and pathological human placental (Mylonas *et al.* 2009) and endometrial tissues (Kimmich *et al.* 2010). Many physiopathological studies about expression, localization and functions of inhibin- a, βA and βB subunits in human placental (Blankenstein *et al.* 2011) and endometrial tissues have cor-

TABLE 4

The growth inhibitory effect of pVAX-Inhα transfection on Hela cells (mean±SEM, n = 3)

	24h	48h	72h	96h
pVAX-Inhα	0.56±0.04	0.49±0.034	0.62±0.01	0.41±0.08[b]
pVAX-control	0.58±0.02	0.49±0.05	0.57±0.04	0.63±0.09[a]

Hela cells (4 ×103) were transfected with respective time intervals as indicated.Then, cells were collected and inhibitory effect was checked by MTT assay. Results were evaluated by one-way ANOVA. "a" and "b" indicates level of significance in columns ($P < 0.05$).

roborated inhibin-α as a tumor suppressor gene with crucial functions in endometrial carcinoma development (Mylonas *et al.* 2009). Although, the exact molecular mechanism of an inhibin-α subunit in human cervical cancer cell line is still not clear.

The present study was intended to reveal the expression and localization of inhibin α protein in Hela cell line (derived from human cervix) , furthermore the functional incorporation of inhibin α protein in different regulatory mechanisms of Hela cells were estimated using expression plasmid of inhibin α (1-32) in this cell line. The results depicted that both vectors were successfully transfected in Hela cells with efficiency of ~60%. Transfection studies showed that inhibin α fusion protein was localized in the nucleus of these cells as detected by GFP expression following transfection while, after transfection, over-expressed inhibin α (1-32) fusion protein significantly induced apoptosis, suppressed proliferation and cessation of different cell cycle phases in Hela cells which was further corroborated by the up regulation of the mRNA level of Bax and wild type p53 (apoptotic inducer) and down regulation of Bcl-2, and NFκB (apoptotic suppressor). Moreover, expression of inhibin co receptor, TGFβR3 was also significantly up regulated. Members of the TGF-β super family are known to ignite serine/threonine kinases receptors, which subsequently upstream cascades of Smad proteins. These receptor-Smad complexes admire to inhabit in the nucleus, where they alter number of nuclear transcriptional processes (Drummond and Fuller, 2012). In the same context, green fluorescence proteins (GFP) are secreted by variety of cells and most often used in localization of specific proteins and cellular labeling. While in our study, the plasmid was engineered with GFP reporter gene thus, emit fluoresce under ultraviolet (UV) light. After staining with DAPI, transfected cells emitted green fluoresce in the nucleus, confirming the localization of inhibin α gene in Hela cells. As GFP expression localization can be unspecific in intact cells and could disperse through nuclear pores (Liang *et al.* 2009; Seibel *et al.* 2007), we observed fluoresce both around cytoplasm and nucleus in the control samples as detected by laser scanning confocal microscopy.

In this study, we detected the up regulation of expression of TGFβR3 following transfection. This receptor has the dual capacity to cross talk with inhibin as well as TGFβ. Some studies have described TGFβR3 as tumor suppressor in early phases and tumor promoter in metastatic disease models in humans, similarly, gene knock out model for TGFβR3 stated overwhelming pathogenesis in some metastatic cancers by associating inhibin-binding region of betaglycan (Ajiboye *et al.* 2010). From this it can be clued that inhibin may regulate these transcriptional pathways through TGFβ - serine/threonine kinases - Smad pathway in cervical cancer cell line.

Inhibin subunits are characterized by their presence in a wide range of human tissues including placenta, breast tissue, human endometrium (Meczekalski and Podfiqurna, 2009; Mylonas *et al.* 2009; Worbs *et al.* 2007) revealing an inductive role in endometrial proliferation and growth. Interestingly, conditional inactivated model for ovarian cancer depicted that inhibin α might be a candidate as tumor suppressor gene (Matzuk *et al.* 1992). Recently, presence and regulation of inhibin subunits in cervical cancer, neoplasm and in normal and malignant human uterine endometrial tissues is presumed to deregulate TGF-β, leading to loss of cell cycle control (Mom *et al.* 2007). In our study, we observed S-phase cessation of these cells after transfection, indicating its strong influence on cellular growth. This was later substantiated by cell proliferation assay (MTT) which depicted termination of growth phase. There is always some degree of cross talk between cellular arrest and apoptosis. In our study, apoptosis was significantly induced following transfection, which further crosschecked by estimating the apoptotic regulatory factors. Previously, many studies have proved inhibin as modulator of apoptosis and growth stopper by making an imbalance in Bax/Bcl2 family in different cell models (Cai *et al.* 2011). Similarly, wild type p53 is a powerful genomic stabilizer through cellular arrest, DNA repair and apoptosis (Collavin *et al.* 2010) while in an ovarian model, NFκB is critical is cell proliferation through its constitutive action (Solt and May, 2008). Although the role of these crucial factors has been extensively studied in different models, their trafficking with inhibin in cervical tumor cell model is completely unraveled. The results of our study depicted that inhibin can cause apoptosis and cellular cessation by unbalancing Bax/Bcl2 genes and p53, while preliminary results with its association with NFκB showed that inhibin can suppress the cancerous activity by modulating NFκB gene expression. Further studies are required for its incorporation in this cell model.

In conclusion, the present studies disclosed that inhibin α-subunit may regulate cervical tumor cells growth through modulation of its co receptor and through regulation of many correlated pathway such as NFκB and Bcl-2 families. Although the expression of inhibin subunits have been widely studied in cervical tissues, the functions of inhibin subunits in cervical pathogenesis and carcinogenesis are scarce, these results revealed the possible important functions and related regulatory mechanism of inhibin in cervical cancer, which should be further investigated.

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