行政院國家科學委員會專題研究計畫 成果報告

腫瘤生長因子-beta 對卵巢癌細胞生長抑制作用之訊息傳遞路徑研究

研究成果報告(精簡版)

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（計畫名稱）

中文(名稱)：腫瘤生物因子-β對卵巢癌細胞生物抑制作用之訊息傳遞途徑研究

英文(名稱)：Studies of the signaling pathways of growth-inhibitory effect of TGF-β on ovarian cancer cells

計畫類別：■ 個別型計畫　□ 整合型計畫
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目　錄

中文摘要

中文關鍵詞: 卵巢癌細胞; 腫瘤生長因子-β; Smad 蛋白; 蛋白激酶異形酶; 訊息傳遞

腫瘤生長因子-β (TGF-β) 是細胞增生的抑制劑，目前已知 TGF-β對於生長抑制的作用牽涉到細胞內受動器 Smad 蛋白訊息傳遞路徑。由於 TGF-β訊息傳遞路徑的最終作用是抑制細胞增生，因此 TGF-β傳遞路徑若受到去活性則勢必助長腫瘤的形成。因此本實驗的目的是探討卵巢細胞中 TGF-β的信號傳導情況及 TGF 信號通路各成分在卵巢癌發生中的作用。

TGF-β1 是藉由誘發細胞周期依賴性激酶抑制子 (cyclin-dependent kinase inhibitor) 中的 p15，p21 和 p27 蛋白作用來使得細胞生長週期停止在 G1 期。研究顯示在形成抑制子 p15 和 p21 蛋白的基因調節區塊中有 Smad 蛋白結合序列的存在，這也表示了 Smad 蛋白會調節受 TGF-β誘發的抑制子 p15 和 p21 蛋白的表現。

我們的實驗結果顯示出在卵巢癌細胞株 NIH: OVCAR-3 中，TGF-β1 抑制細胞生長的作用主要是藉由刺激 p21 蛋白而非 p15 蛋白所造成。TGF-β1 同時也會刺激 Smad 蛋白的表現。然而受 TGF-β1 所調控的細胞內受動器 Smad 蛋白的完整的作用機轉仍需要更進一步的探討。

英文摘要

Keywords：Ovarian cancer cells; TGF-β; Smad; Signaling

TGF-β is a potent inhibitor of cell proliferation. The growth-inhibitory effect of TGF-β is involving the Smads pathways. Because TGF-β signaling generally has a negative effect of cell growth, inactivation of any pathways of TGF-β signaling contributes to tumorigenesis. The purpose of this study was to determine the response of ovarian cancer cells to TGF-β1 and to investigate the roles of components of the TGF-β signaling pathway in carcinogenesis of ovarian cancer.

TGF-β1 inhibits growth by causing cell cycle arrest in G1 phase after inducing the expression of cyclin-dependent kinase inhibitor p15, p21 and p27. There is Smad-binding element (SBE) within the regulatory region of the TGF-β-regulated gene p15 and p21, suggesting that Smad may regulate TGF-β-inducible p15 and p21 expression.

Our data imply that TGF-β1 induces growth arrest in NIH: OVCAR-3 cell line is mainly by inducing p21 expression not p15. TGF-β1 also induces Smad expression. However, the distinctly mechanism of regulation of Smad expression by TGF-β1 require further investigation.

Introduction

Transforming growth factor βs (TGF-βs), activins and bone morphogenetic proteins (BMPs) are structurally related proteins, and are collectively termed the TGF-β superfamily. Members of the TGF-β superfamily are extracellular peptides that control cell fate by regulating the expression of genes encoding cell cycle regulators, differentiation factors, cell-adhesion molecules and many other components that are key determinants of cell phenotypes (Alexandrow and Moses, 1995). Two types of single transmembrane serine/theronine kinase receptors, the type I and type II, have been found to mediate the cellular effects of TGF-β family ligands (Bassing et al., 1994). In the absence of ligand, while the type I receptor kinase I is inactive, the type II receptor kinase is constitutively active and the receptor is autophosphorylated (Luo and Lodish, 1997). Binding of TGF-β to type receptor results in the formation of a heteromeric complex containing type I and type II receptor followed by transphosphorylation of the GS domain of type I receptor by type II receptor (Wrana et al, 1994). Then the type I receptor kinase can phosphorylate cytoplasmic substrates to mediate the downstream signaling of TGF-β.

The Smad proteins are essential components of the signaling cascade initiated by members of the TGF-β family. To date, at least nine Smad have been cloned in Xenopus, mouse and human (Massague et al, 1997). All Smad proteins share considerable homology in their primary sequence and most contain two highly conserved Mad homology domains: MH1 in the N-terminal and MH2 in the C-terminal separated by a proline-rich linker with variable amino acid sequence and length. Based on their structures and functions, the Smad proteins can be sorted into three classes. Pathway-restricted or receptor-regulated Smads (R-Smads) are phosphorylated directly on the serine-serine-X-serine (SSXS) motif located at their C-terminals (Macías-Solva et al., 1996), in which at least two serine residues are phosphorylated by the active type I receptors (Abdollah et al., 1997; Souchelnytskyi et al., 1997). Smad1, Smad5 and Smad8 are specifically involved in BMP signaling whereas Smad2 and Smad3 are TGF-β/activin pathway-restricted. Common mediator Smad (Co-Smad) that contain the MH1and MH2 domains, but no SSXS motif therefore can't be phosphorylated by type I receptor, is required by all distinct pathways. Smad4 is the only vertebrate Co-Smad identified thus far. Inhibitory Smads either lack MH1 domain or contain a diverged MH1 domain. Smad6 and Smad7 belong to this class that have been shown to act as inhibitors of these signaling pathways by interfering with activation of the pathway-restricted Smads (Hayashi et al., 1997;). The MH1 domains of some Smads have DNA binding activity (Kim et al., 1997; Zawel et al., 1998), whereas the MH2 domains have transactivating activity. In the basal state, the MH1 and MH2 domains can interact, inhibiting each other's function (Hata et al., 1997). Upon stimulation by TGF-β1, Smad2 and Smad3 interact with the TGF-β receptor complex, the SSXS residues are phosphorylated by the active type I receptors. This phosphorylation overcomes the autoinhibitory state of the receptor-regulated Smads, Smad2 and Smad3 between their N- and C-terminals, inducing their conformational changes and form

heteromeric complexes with Smad4. These Smad complexes translocate to the nucleus where they cooperate with general transcription factors such as Sp1, AP1 and co-activators CBP/p300 binds that directly to the phosphorylated MH2 domain of both Smad2 and Smad3. These co-activators interact directly with RNA polymerase II and modify chromatin structure with their histone acetyltransferase activity, thereby activating transcription of TGF-β responsive genes. E1A, an adenoviral oncoprotein, inhibits transcription by binding to co-activators. Screening of Smad3 and Smad4 binding sites using gel shift-PCR selection of oligonucleotides identified an 8-bp palindromic sequence (5'-GTCTAGAC-3') called the Smad binding element (SBE) (Zawel et al., 1998).

Objectives

Our present proposal is attempting to explore the fundamental TGF-β1 signaling pathway relationship among Smad proteins and specific PKC isozyme(s) in human ovarian cancer cells, which at last will hopefully provide useful information for future molecular targeting of human ovarian cancer therapy.

Literature Review

Epithelial ovarian cancer is often diagnosed at an advanced stage of disease and is the leading cause of death from gynecological neoplasia. The genetic changes that occur during the development of this carcinoma are poorly understood. Using PCR and DNA sequencing, Chen et al showed that 33% of primary ovarian cancer harbor somatic changes in exons 2, 3, 4,and 6 of the TGF-β receptor I (TbetaR-I) gene (Chen et al., 2001). These results indicate TbetaR-I gene is frequently mutateted in ovarian cancer and suggest that resistance to TGF-β-mediated growth inhibition may frequently involve alterations of the TbetaR-I gene. It has been proposed that IGF-IIR, TGF-β1and TbetaR-II act as a functional unit in the TGF-β growth inhibitory pathway. Francis-Thickpenny et al examined these 3 genes in 25 ovarian carcinomas, and demonstrated a total of 3 somatic mis-sense mutations in the TbetaR-II gene (Francis-Thickpenny et al., 2001). The disruption of the TGF-β1 autocrine growth-suppressive circuit is a major and early event mediating the malignant transformation of normal ovarian epithelium. Zeinoun et al. have investigated the effect of restoring the growth-inhibitory autocrine circuit in ovarian cancer cells by transduction of the cancer cells with a recombinant adenovirus containing a TGF-β cDNA and encoding for a constitutively bioactive TGF-β peptide (Zeinoun et al., 2003). They found these cancer cells restore a potent growth-inhibitory effect in vitro, and moreover the transduced cells lost their tumorigenicity in nude mice.

Mechanisms of resistance to TGF-β caused by dysregulation of the TGF-β postreceptor signaling pathway are not fully understood. The study of Xi et al showed overexpression of CDC25A gene and decreased expression of Smad4 in 14 ovarian cancer cell line (Xi et al., 2004). This overexpression of CDC25A correlates with increased tumorigenicity of ovarian cancer cell lines. The loss of sensitivity to TGF-β is not associated with a lack of TbetaR-II. Alterations of TGF-β receptor-interacting proteins in ovarian cancer are suggested to be involved in resistance to TGF-β -mediated growth inhibition. From ovarian cancer tissues, Ding et al. discovered a novel form of km23, missing exon 3(Deltaexon3-km23) encoding 107-amino-acid residues (Delta107km23), instead of the wild-type 96-amino-acid form of km 23 (Ding et al, 2005). The results of Ding et al. point out that km23 alterations found in ovarian cancer tissue result in an inhibition of TGF-β -dependent transcriptional activation of both the p3TP-lux and activin responsive element reporters.

In summary, there are several distinct developmental pathways responsible for human ovarian cancer. Disruption of TGF-β1 autocrine growth inhibition circuit and dysfunction of the TGF-β1 postreceptor signaling transduction pathways have been discovered by many investigators. Early correction of any defect of the above events might in the future lead to cancer preventive and therapeutic strategies.

Materials and Methods

1. Cell culture.

NIH: OVCAR-3 cells were maintained in RPMI-1640 media containing 10% fetal bovine serum and nonessential amino acids. NIH: OVCAR-3 cells were plated in 10-cm culture plates at a density of 10^6 cells per plate. Cells were incubated in the presence or absence of human TGF-β1 (5 ng/ml) for indicated time.

2. Cellular fractionation.

At the end of the experiment, cells were washed three with cold PBS and scraped into 1 ml cold homogenization buffer containing protease and phophatase inhibitors (50 mM Tris-HCL, pH 7.5, 5 mM EDTA/EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium vanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). The cells were homogenized in a Dounce homogenizer with 30 strokes of the pestle, and the homogenate was centrifuged at 100,000 × g for 60 min at 4℃.The supernatant was used as cytosol fraction. The pellet was gently stirred for 30 min at 4℃ in 100 μl homogenization buffer added with 1% Triton X-100. After centrifugation at 100,000 × g for 30 min at 4℃, the obtained supernatant was diluted to 1 ml with homogenization buffer and was used as the Triton-soluble membrane fraction.

3. Immunoprecipitation

The Triton-soluble membrane fraction (containing ~200μg protein) was mixed for 1 h at 4℃ with 5 μg of mouse monoclonal antibody followed by a further 30 min with 10 μl of goat anti-mouse IgG antibody (Sigma). Each sample was mixed with 10 μl 50% Protein A Plus Protein G agarose beads (Oncogene) for 30 min at 4℃. After centrifugation (13000 × g, 5 min) the immunoprecipitate pellet was washed four times with 500 μl of kinase buffer (50 mM HEPES, pH7.2, 100 mM NaCl, 75 mM KCl, 20 mM β-glycerophosphate, 10 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM sodium orthovanadate, 1 mM dithiothreitol) and then resuspended in 20 μl of kinase buffer.

4. Western blot analysis.

Cells were lysed in RIPA (1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate) with protease and phosphatase inhibitors. Cell lysates were denatured heating to 95℃ for 5 min in 2 × SDS sample buffer prior to fractionation on a 10%SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane according to standard procedures by semidry blotting system (Gelman Sciences, BioTrans Model A). Blocking and antibody incubations were performed in 5% dried milk and 0.1% Tween 20 in

phosphate-buffered saline for 1 h at room temperature. Antibodies used were: anti-Smad4 (Santa Cruz Biotechnology, C-20), anti-PKCα, anti-PKCβ (Transduction Laboratories) and horseradish peroxidase-conjugates secondary antibody (Amersham Pharmacia Biotech). Immunoreactive proteins were detected using the Renaissance Western Blot Chemiluminescence Reagent *Plus* (NEN). All Western blots were replicated with at least tree different cell preparations.

5. Nuclear extracts.

Nuclear lysates were prepared from control and TGF-β1-treated cells. Confluent sells from 10-cm culture plates were washed twice with PBS buffer. After washing, 1 ml of ice-cold hypotonic lysis buffer (10 mM Tris-HCl, pH7.9, 140 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1% NP-40 with protease and phosphatase inhibitors) was added. The cells were allowed to swell on ice for 5 min. After centrifugation 500 × g for 5 min at 4℃, the pellet was washed once wash buffer (20 mM Tris-HCl, pH7.9, 140 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1% NP-40 with protease and phosphatase inhibitors). The nuclei fraction was recovered by centrifugation and then incubated on ice with 50 μl hypertonic buffer (0.4 M NaCl, 5 mM EDTA, 10 mM HEPES pH 7.5, 20% glycerol with protease and phosphatase inhibitors) for 30 min. After centrifugation, the supernatant was recovered as nuclear extract.

6. Electrophoresis Mobility Shift Assays (EMSA)

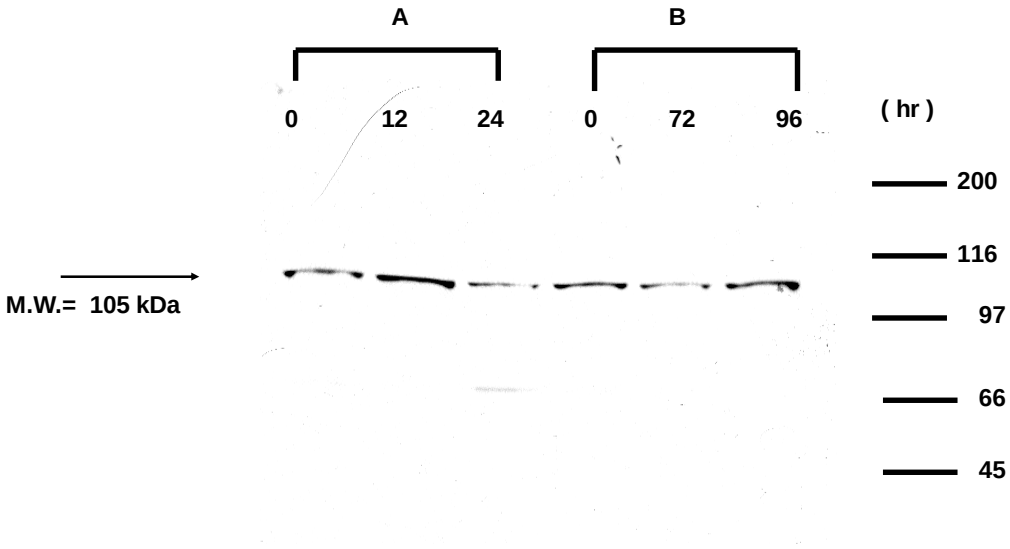
The nuclear extracts were incubated with ^{32}P-labeled probes for 15 min at room temperature in binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH7.5, 0.5 mg/ml poly(dI-dC).poly(dI-dC)). Single-stranded oligonucleotides were end labeled using T_4 polynucleotide kinase, annealed and the unincorporated nucleotides were removed from DNA probe by ethanol precipitation. Double-stranded oligonucleotide probes used in the EMSA assay are AP-1 probe (containing the synthetic consensus AP-1 sequence 5’ -CGCTTGATGAGTCAGCCGGAA-3’ purchase from Promega), SP-1 probe (containing the synthetic consensus SP-1 sequence 5’ -ATTCGATCGGGGCGGGGCGAGC-3’ purchase from Promega) and SBE (containing SBE site found in the p21waf1 promoter region 5’ -AGACAGACAAT**GTCTAGTC**TATTTGAAATGCCTGA-3’). ^{32}P-labeled protein-DNA complexes were resolved on a 5% nondenaruring polyacrylamide gel in 0.5 × TBE.

Results

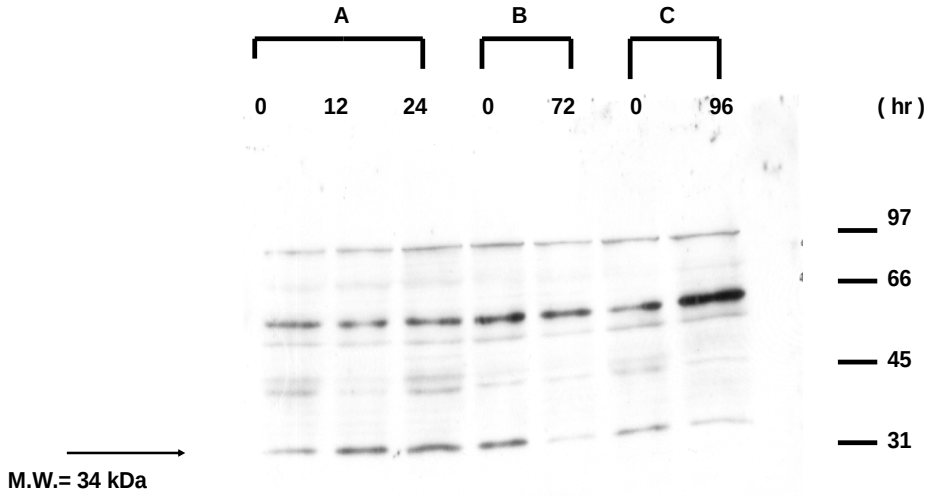
TGF-β1 inhibits growth by causing cell cycle arrest in G1 phase after inducing the expression of cyclin-dependent kinase inhibitor p15, p21 and p27; the binding of these inhibitors to specific cyclin-dependent kinase complexes block their activity. There is Smad-binding element (SBE) within the regulatory region of the TGF-β-regulated gene p15 and p21, suggesting that Smad may regulate TGF-β-inducible p15 and p21 expression. Many TGF-β responsive genes contain AP-1 consensus sequences in their regulatory region, such as plasminogen activator inhibitor type-1.

The following results are also observed:

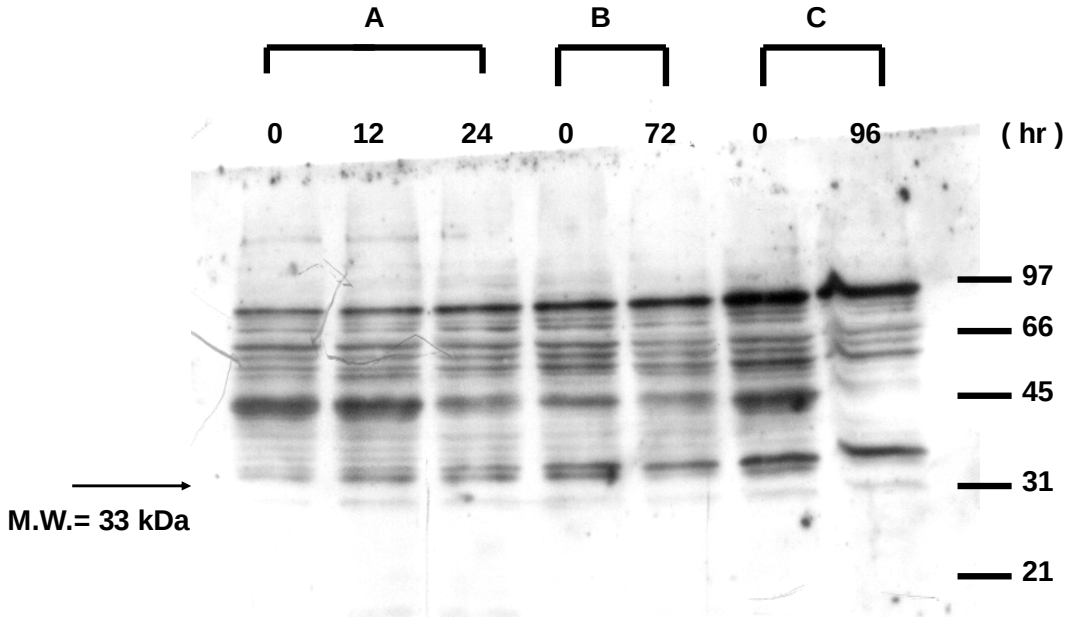
1. TGF-β1 increased RB expression in 12 hrs on OVCAR-3 cells.



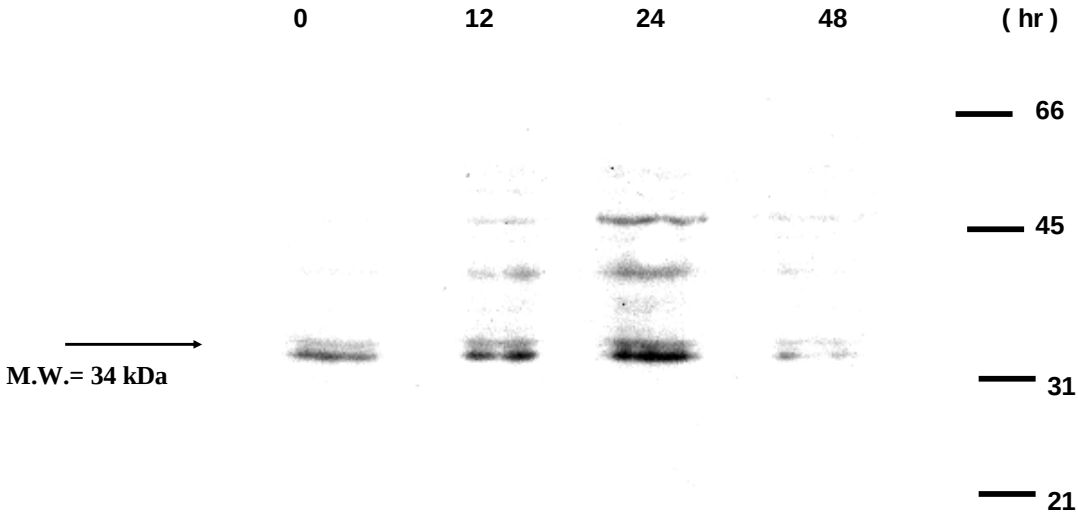
2. TGF-β1 induced expression of CDK4 in 24 hrs on OVCAR-3 cells.



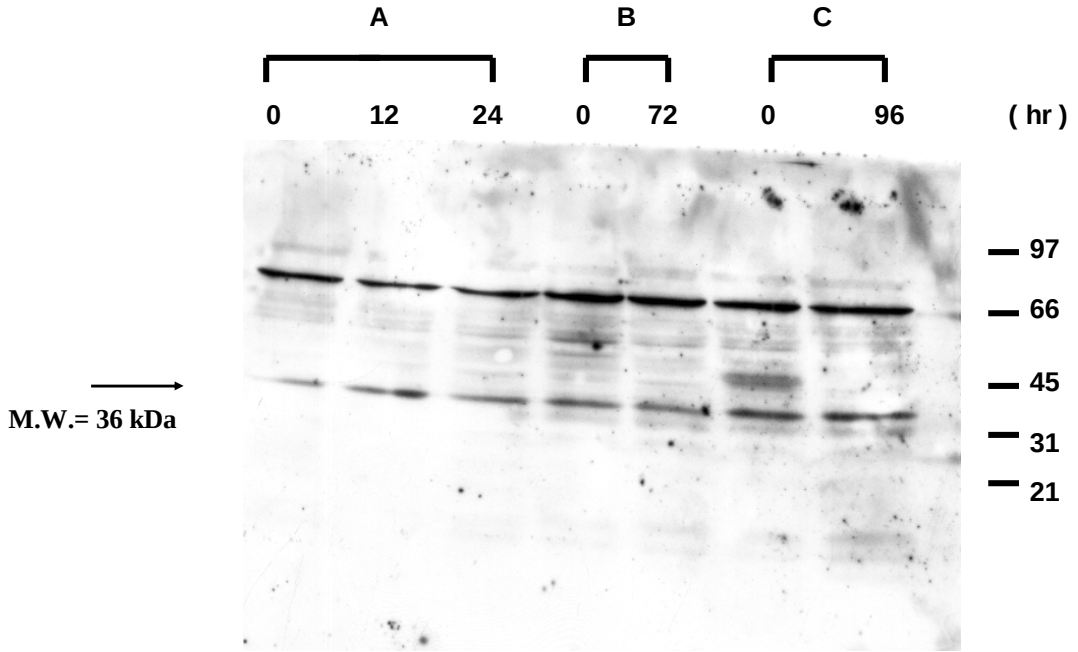
3. TGF-β1 did not affect the expression of CDK2 in OVCAR-3 cells



4. TGF-β1 induced the expression of CDC2 in OVCAR-3 cells in 24 hrs.



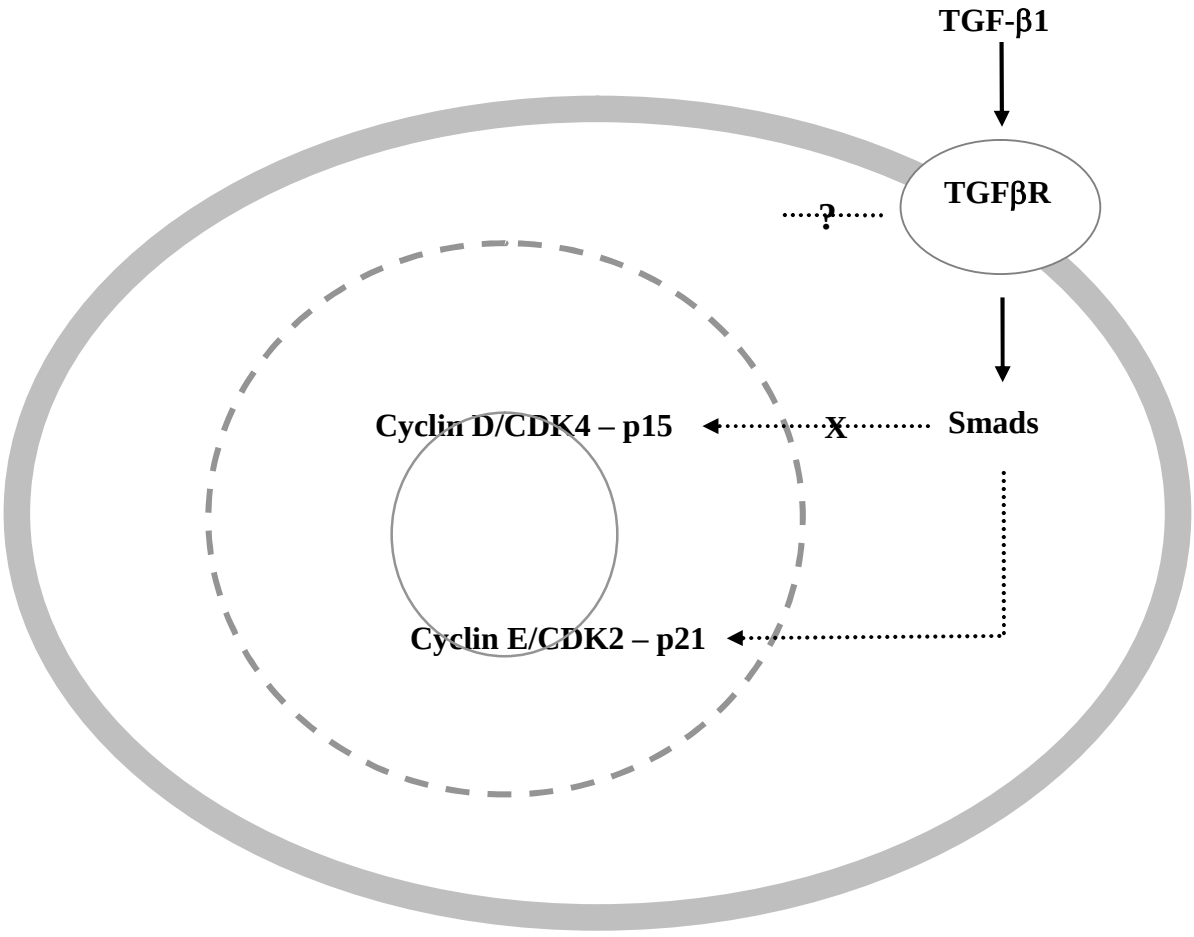
5. TGF-β1 did not increase the expression of cyclin D in OVCAR-3 cells.



Discussion

According to the results of present studies, the growth of OVCAR-3 cell line was inhibited by the treatment of 5 ng/ml TGF-β1 and the cell cycle also arrested at G1 phase. Our data imply that TGF-β1 induces growth arrest in NIH: OVCAR-3 cell line is mainly by inducing p21 expression not p15. TGF-β1 also induces Smad expression. However, the distinctly mechanism of regulation of Smad expression by TGF-β1 require further investigation.

A number of cellular systems, including vascular smooth muscle cells (Sasaguri et al. 1993), vascular endothelial cells (Zhou et al. 1993; Kosaka et al. 1996), melanoma cells (Coppock et al. 1992), IMR-90 fibroblast (Hamada et al. 1996), intestinal epithelial cells (Frey et al.1997), and hematopoietic cells (Tiefenbrun et al. 1991), demonstrating that PKC could mediate cell growth arrest at the G1/S boundary and/or in G2/M phase. Further investigations to address that PKCs molecules are involved in the TGF-β signaling toward cell growth arrest are expected.



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