Serum starvation induces G1 arrest through suppression of Skp2-CDK2 and CDK4 in SK-OV-3 cells

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Abstract. Recent studies have suggested that Skp2, an SCF-type ubiquitin ligase, positively regulates cell cycle through degradation of p27, which is an inhibitor of cyclin-dependent kinase 2 (CDK2), which drives cells from the G1 to S phase of cell cycles. In the present study, we examined key regulatory proteins involved in serum starvation-induced cell cycle arrest in human ovarian cancer cells, SK-OV-3. Cell cycle analysis showed that cells were arrested at the G1 phase after serum starvation. Western blot analysis showed that the protein levels of CDK4 and CDK2 were significantly decreased in SK-OV-3 cells. Consistently, Roscovitine, an inhibitor of CDK2, induced cell cycle arrest in normally proliferating cells and a chemical inhibitor of CDK4, 3-ATA [3-Amino-9-thio(10H)-acridone], was found to induce growth arrest. We also found that the protein level of Skp2 was dramatically decreased in response to serum starvation. Moreover, CDK2 protein, which allows cell cycle transit from the G1 to the S phase, was decreased when the Skp2 expression was inhibited by specific siRNA of Skp2, but CDK4 was not decreased. Therefore, these results suggest that serum starvation induces G1 arrest through suppression of Skp2-dependent CDK2 activity and Skp2-independent CDK4 activity in human SK-OV-3 ovarian cancer cells.

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Introduction

Serum starvation commonly leads to cell cycle arrest in the G0/G1 phase (1-3), and also has been used to arrest the cell cycle (4-6). NIH-3T3 cells and MEF are arrested at the G0 phase by serum starvation for 1 and 2 days, respectively (1). However, cancer cells are usually arrested at the G1 phase (7). When cells are starved, they do not enter the S phase and are not arrested at any restriction points. Cells in G1-arrest induced by serum starvation retain the same amount of DNA as those of G1-phase, since these cells do not start DNA synthesis (1). However, the molecular mechanisms in the G1 arrest induced by serum starvation have not been fully elucidated.

It was previously reported that the level of p27 protein is consistent with low CDKs activity in quiescent (G0 phase) or G1-arrested cells (8). p27 is an inhibitor of cyclin-dependent kinase 2 (CDK2), which drives cells from the G1 to the S-phase of the cell cycle (9,10). p27 is a short-lived protein, and its degradation is regulated by the ubiquitin-proteasome system (11). Recent studies have shown that p27 is ubiquitinated by SCF ubiquitin-protein ligase (12). Skp2 is a member of the F-box family of the specific substrate-recognition subunit of SCF ubiquitin-ligase complexes (13). The level of p27 is high in Skp2 knocked out cells (14), but low in Skp2-overexpressing cells (15-17), indicating that Skp2 positively regulates the cell cycle through the degradation of p27. Skp2 levels are inversely related to p27 levels in oral squamous carcinoma (18), gastric carcinoma (17), lymphoma (19) and colon (20). In addition, Skp2 binds to free forms of cyclin E (14) and then ubiquitinates it. The binding of cyclin E with CDK2 prevents the binding of Skp2, resulting in the stabilization of cyclin E (14), indicating that cyclin E turnover during the cell cycle is controlled by Skp2. Thus, Skp2 is highly significant in cell cycle regulation, its role in serum starvation, however, is not clear.

Ovarian cancer is a common gynecological malignancy with a difficult convalescence period. The research approach

for therapy of this cancer is gene transfer by recombinant adenoviruses. Mutation of p53 is observed in ~10-15% of early-stage and 40-50% of advanced-stage ovarian cancers (21). Several research groups have reported that the growth of various tumors can be slowed by transfecting them with wild-type p53 (22-24). Other groups have demonstrated that transfection with p53 also slows the growth of ovarian cancer cells (25,26). However, this technique can result in genomic alterations in cells. Thus, new methods or targets for ovarian cancer therapy are required. In the present study, we examined the molecular mechanisms of cellular response induced by serum starvation. We also examined new target gene related to the G1-phase of the cell division cycle. As mentioned above, serum starvation can induce G1-arrest in various cancer cells. If one gene can overcome G1-arrest induced by serum starvation, the gene could be a new target for ovarian cancer therapy.

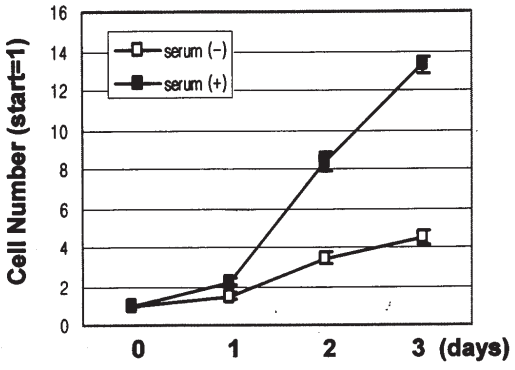


Figure 1. Effect of serum starvation on cell proliferation. Cells ($2x10^5$) were seeded in 60 mm dish. After 24 h, serum was removed. The cell number was determined by counting at each time point analysis the cell growth rate. Cells were maintained by complete media (closed square) and serum starved-cells (open square). Each point represents the average result of three independent experiments.

Materials and methods

Cell culture, materials and DNA transfection. SK-OV-3, human ovarian carcinoma cell line, was maintained in RPMI supplemented with 10% FBS (Life Technologies, Inc., Grand Island, NY, USA) and penicillin-streptomycin (50 U/ml). Roscovitine (A.G. Scientific, Inc., San Diego, CA, USA) as CDK2-specific inhibitor (27), and 3-ATA [3-Amino-9-thio (10H)-acridone] (Alexis Biochemicals, Carlsbad, CA, USA) as CDK4-inhibitor (28).

Serum starvation and cell proliferation analysis. For serum starvation, $2x10^5$ cells were seeded in 60 mm dish. After 24 h, serum was removed at time 0, 24, 48 and 72 h. The cells were counted in each time for analysis of cell growth rate. Cells were treated with either 20 μM of roscovitine or 20 μM of 3-ATA [3-Amino-9-thio(10H)-acridone] for the indicated time, and then live and dead cells were counted for cytotoxicity of serum starvation using the trypan blue exclusion assay.

Cell cycle analysis. Either the normally proliferating cells or the starved-cells at 24, 48 and 72 h were trypsinized, washed three time with cold PBS, and centrifuged. The pellet was resuspended in 1 ml of 70% ethanol and then incubated for 1 h at 4˚C. The cell were centrifuged and washed twice with cold PBS, suspended in 500 μl of PI-staining solution with RNase and incubated at 37˚C for 30 min. The cells were stained with propidium iodide (final concentration, 50 μg/ml) for 15 min and analyzed by flow cytometry.

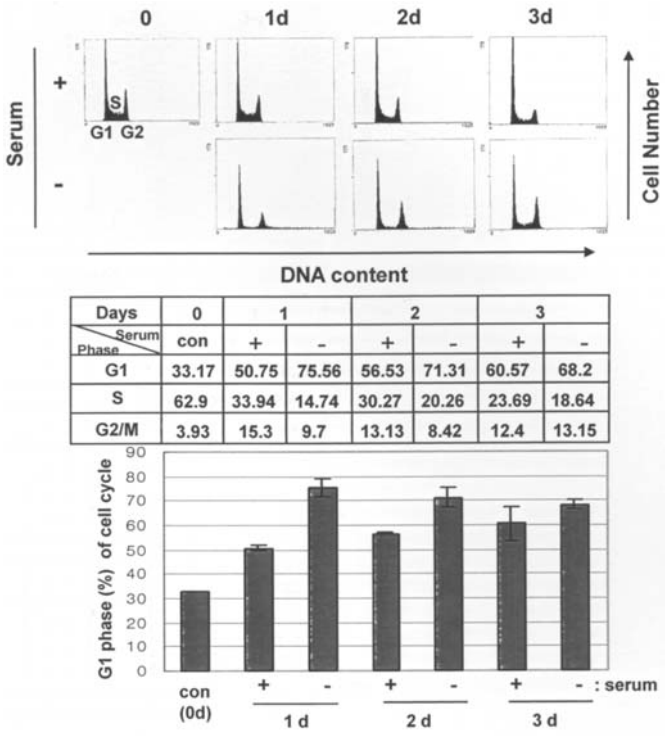
RNA interference. SK-OV-3 ovarian cancer cells were transfected with scramble siRNA or human Skp2 siRNA (200 pmole/60 mm dish, skp2 5'-GAGGAGCCCGACAG TGAGA-3', the C terminus of skp2 5'-GTTGGAGCTGG ATTGCT-3, (29) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in a 60-mm dish. Scrambled RNA (5'-GAAGCAGUCGCAGUGAAGAdTdT-3) as control siRNA was obtained from Proligo LLC (Boulder, CO, USA).

Western blot analysis. Cells lysates were prepared with RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 μM EGTA, 1% Triton X-100, 50 mM NaF, 5 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 mM DTT). Protein extracts were normalized for concentration by the Bradford assay and 20 μg of total cell protein per sample were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a PolyScreen membrane (NEN, Boston, MA). Membranes were blocked with 5% non-fat dry-milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) and probed with one of the following antibodies: anti-Skp2 (sc-7164), anti-CDK2 (sc-6248), anti-CDK4 (sc-260), and anti-γ-tubulin antibody (sc-7396) purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Secondary antibodies: rabbit secondary antibody (NA934V) from GE Healthcare Bio-Sciences Corp and goat secondary antibody (sc-2020) from Santa Cruz Biotechnology.

Results

Serum starvation induces G1-arrest in SK-OV-3 ovarian cancer cells. It was previously reported that serum starvation induces cell arrest at the G0/G1 phase in the cell division cycle (1-3). However, SK-OV-3, human ovarian carcinoma cell line, has not been reported for G1-arrest induced by serum starvation until now and the genes related to this event have not been discovered. So, we first examined the effect of serum starvation on SK-OV-3 ovarian cancer cells. Cell proliferation analysis has shown that the cell growth is inhibited by serum starvation (Fig. 1) without the induction of cell death (data not shown). We performed cell cycle analysis to examine in which phase of cell cycle cell growth was inhibited. It was observed that the cells were arrested at the G1-phase of cell cycle growth based on flow cytometric analysis (Fig. 2), indicating that serum starvation induces G1-arrest in SK-OV-3 ovarian carcinoma cells.

Serum starvation induces G1-arrest through the suppression of CDK4 and CDK2. Activation of cyclin-dependent kinases (CDKs) regulates an orderly progress in the cell cycle (30).



Days	0	1		2		3	
Serum / Phase	con	+	-	+	-	+	-
G1	33.17	50.75	75.56	56.53	71.31	60.57	68.2
S	62.9	33.94	14.74	30.27	20.26	23.69	18.64
G2/M	3.93	15.3	9.7	13.13	8.42	12.4	13.15

Figure 2. Serum starvation-induced G1-arrest. Cell cycle of either the normally proliferating cells or the starved-cells was analyzed by flow cytometry at the indicated times. Data in the table are representative of three different experiments.

It was previously reported that both CDK4 and CDK2 can be controlled at the G1-phase of the cell cycle (31,32), and that the activity of these CDKs was decreased in G1-arrested cells (31-33). Thus, we examined whether the expression of both CDK 4 and CDK2 in fact did decrease after serum starvation. First, it was confirmed that the expression of CDK4 was significantly decreased in the absence of serum (Fig. 3A), but not in normally proliferating cells. Consistently, cell numbers were largely decreased at 2 days after treatment of a kinase inhibitor of CDK4, 3-ATA [3-Amino-9-thio (10H)-acridone] (28), in normally proliferating cells by serum (Fig. 3B), suggesting that CDK4 protein is related to G1-arrest induced by serum starvation in SK-OV-3 cells.

We next examined protein level of CDK2 in both normal and serum-free condition. The expression of CDK2 protein was dramatically decreased after serum starvation, but not in the presence of serum (Fig. 3A). After treatment with roscovitine, a kinase inhibitor of CDK2 (27), in serum-existed cells, the cell number was decreased at day 2 (Fig. 3C). The same result occurred with CDK4. Consistently, flow cytometic analysis showed that cells were arrested at the G1-phase in response to both the CDK4 inhibitor and the CDK2 inhibitor in the presence of serum (Fig. 3D), implying that G1-arrest induced by serum starvation in SK-OV-3 ovarian cancer cells is induced through the suppression of both CDK4 and CDK2. Therefore, we next studied regulators that control both CDK4 and CDK2.

The suppression of CDK4, -2 by serum starvation is a result of the suppression of oncogenic protein, Skp2. It has been reported that Skp2 binds to cyclin E. This binding appears to

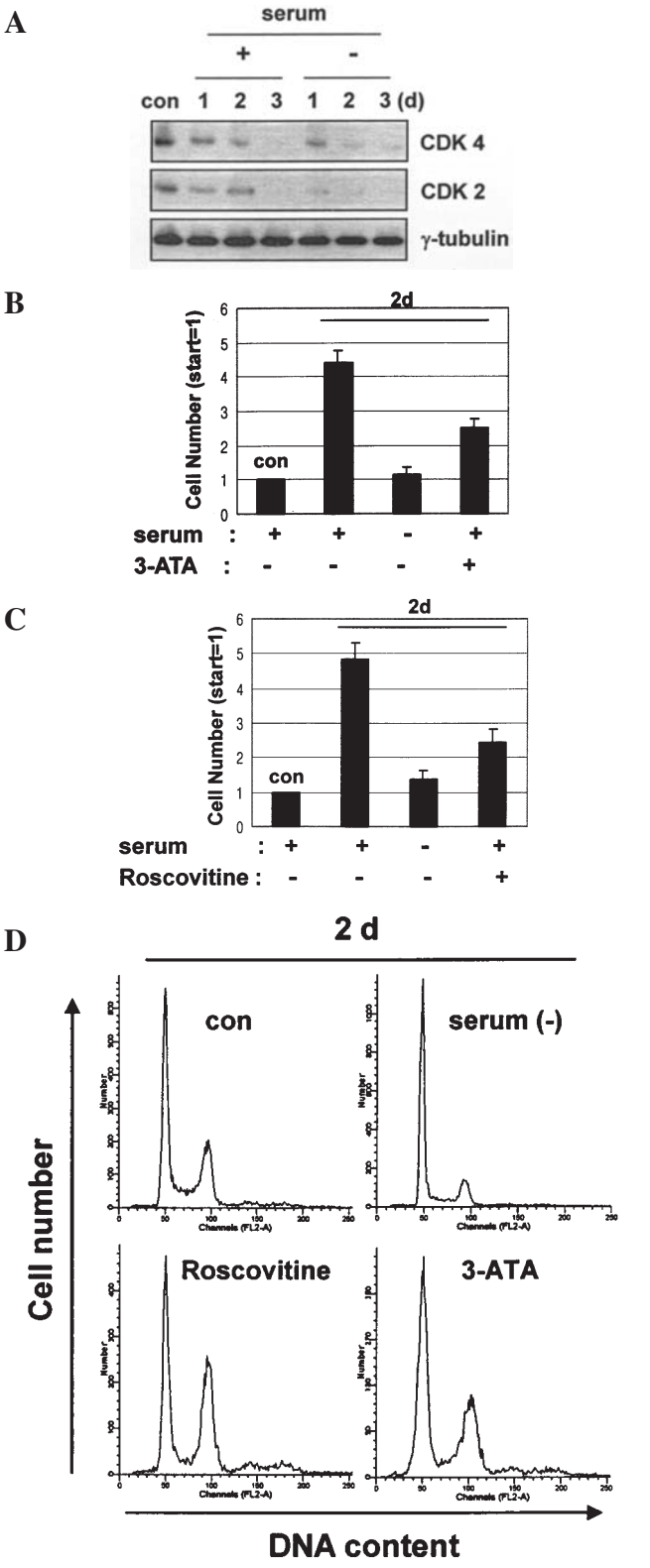


Figure 3. Serum starvation induces the suppression of CDK4 and CDK2 protein. (A) Cells ($5x10^5$) were seed in a 100-mm dish. After 24 h, serum was removed and then cultured for the indicated times. Protein levels of CDK4 and CDK2 were detected by Western blotting, loading control as γ-tubulin. Cell lysates were prepared from 30 μg of total lysates and applied. (B) The cells were pre-incubated with 20 μM of 3-ATA [3-Amino-9-thio(10H)-acridone] for 1 h following serum starvation. Cell numbers were monitored at the times indicated. Each point represents the average result of three independent experiments. (C) The cells were treated with 20 μM of roscovitine 1 h before serum removal and then analyzed for the cell number at each time period. The data are representative of three different experiments. (D) Cells treated with either a CDK4 inhibitor or a CDK2 inhibitor were analyzed by flow cytometry at the indicated times.

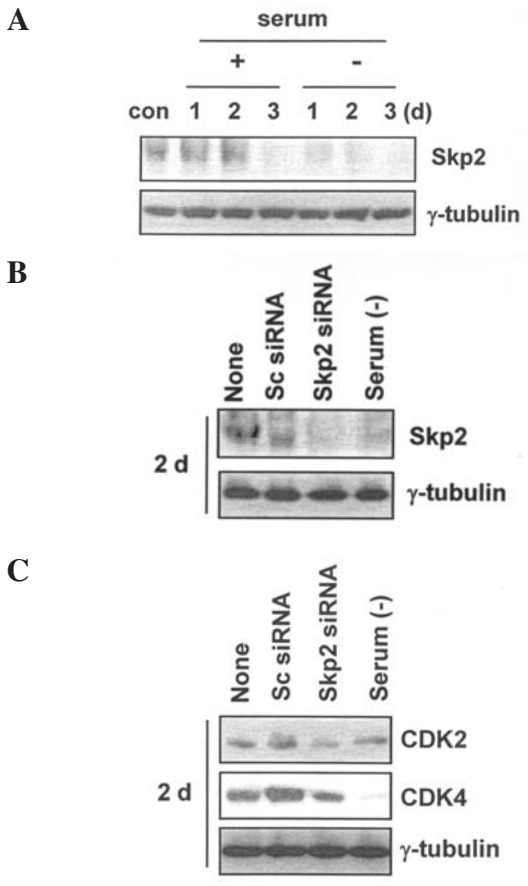


Figure 4. Effect of Skp2-specific siRNA on cell proliferation. (A) Cells were seed in a 100-mm dish. After 24 h, the serum was removed and then cultured for the indicated times. Protein level of Skp2 was detected by Western blotting, loading control as γ-tubulin. Cell lysates were prepared from 30 μg of total lysates and applied. (B) After transfection with scrambled RNA and Skp2 siRNA, the cells were maintained for 2 days and then the cell lysates were prepared for analysis the expression of Skp2, loading control as γ-tubulin. (C) Effect of Skp2-siRNA on expression of either CDK4 or CDK2 protein in the presence of serum. After transfection with Skp2-siRNA as shown in Materials and methods, expression of CDK4, the proteins were analyzed by method mentioned as in (A), respectively.

be independent of the phosphorylation status of cyclin E. However, the association of Skp2 and cyclin E was prevented by CDK2-cyclin E complex (14). Another group showed that Skp2 protein positively controls G1-phase of the cell cycle by ubiquitination of p27 of CKIs, CDK inhibitors (12). These results suggest that the oncoprotein, Skp2 regulates the cell division cycle through the control of either p27 or CDK2-cyclin E complex, respectively. Moreover, it has been shown that Skp2 associates with c-myc, which is a positive regulator of CDK4 (34,35). Thus, Skp2 indirectly regulates either CDK4 or CDK2 through the association with p27 or c-myc, respectively. Thus, we first examined whether the expression of Skp2 protein decreases after serum starvation. It was confirmed that the protein level of Skp2 was significantly decreased in the absence of serum, but not in normally proliferating cells (Fig. 4A). Second, we used Skp2-specific siRNA, endogenous Skp2 knock-down (Fig. 4B), to analyze whether the expression of Skp2 affects expression of either CDK4 or CDK2. Expression of CDK2 protein in cells transfected with Skp2-siRNA was dramatically decreased. This is consistent with decreased Skp2, but not in the cells transfected with scrambled siRNA used as control. However, the expression of CDK4 protein was not affected by Skp2-siRNA (Fig. 4C), implying that CDK2, but not CDK4 protein, was Skp2-dependently regulated after serum starvation. Therefore, these results suggest that G1-arrest following serum starvation is induced through the suppression of both Skp2-dependent CDK2 and Skp2-independent CDK4 activity.

Discussion

In this study, we first demonstrated that a cell's response to serum starvation was arrested at the G1-phase by decreasing protein levels of CDK2 and CDK4. Treatment with either an inhibitor of CDK2, roscovitine, or CDK4, 3-ATA [3-Amino-9-thio(10H)-acridone], induced G1-arrest in the presence of serum and this is consistent with effects in absence of serum. We also found that expression of Skp2 protein was significantly decreased after serum removal. Moreover, expression of CDK2 protein was co-related to that of Skp2 protein. However, the protein level of CDK4 was Skp2-independently decreased after serum starvation.

Several research groups have shown that Skp2 interacts with the oncoprotein, c-myc, to induce transcription of c-myc target genes, which requires c-myc expression and activity (34). They also suggested that Skp2 is a coactivator of c-myc-induced transcription. It was previously reported that c-myc regulates the cyclin D-CDK4 complex (36). These reports, therefore, support the hypothesis that the expression of CDK4 protein is Skp2-dependently regulated. However, our data showed that CDK4 protein was Skp2-independently regulated, and has a decreased response to serum starvation. Consistently, SK-OV-3 cells treated with 3-ATA [3-Amino-9-thio(10H)-acridone], kinase inhibitor of CDK4, induced arrest at the G1-phase in the presence of serum.

It was reported that the oncogenic protein, Skp2 binds to p27, CKI and its ubiquitination (12). p27 down-regulates cyclin-dependent kinase 2 (CDK2), which drives cells from the G1 to the S-phase of the cell cycle (9). Thus, Skp2 indirectly positive-regulates CDK2 activity through the suppression of p27 and subsequent transit from the G1 to the S-phase. We showed that the expression of Skp2 protein was significantly decreased after serum removal. Interestingly, knock-down of Skp2 by Skp2-specific siRNA affected expression of CDK2 protein, suggesting that the expression of CDK2 protein is indirectly regulated by Skp2. According to recent reports, Skp2 associates with CKI p27 or cyclin E. However, the manner in which decreased Skp2 in turn decreases CDK2 protein was not confirmed in this study. In addition, cell cycle analysis showed that cells treated with roscovitine, the kinase inhibitor of CDK2, induced G1-arrest as well as slightly G2-arrest. This was probably due to the side-effect of roscovitine as not only a CDK2 inhibitor but also as an inhibitor of CDK1 (5).

Here, we suggest the molecular mechanisms of G1-arrest induced by serum starvation and the role of Skp2 in this event. Our results suggest that Skp2 can be a new target for ovarian cancer therapy. Further studies are required to determine the role of p27 after serum starvation.

Acknowledgements

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