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ORIGINAL PAPER

# Proliferative effects of excretory/secretory products from *Clonorchis sinensis* on the human epithelial cell line HEK293 via regulation of the transcription factor E2F1

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**Abstract** *Clonorchis sinensis* is one of the most prevalent parasitic helminths of humans in East Asia. Although several complications in bile duct epithelial cells are caused by *C. sinensis* infection, the mechanism is not clearly understood. To clarify the effects of *C. sinensis* excretory–secretory products (ES products) on bile duct epithelial cells, we investigated their effects on the human embryonic kidney epithelial cell line HEK293 in vitro. Our results show that ES products alter the proportion of cells in each stage of the cell cycle and induce HEK293 cell proliferation. Among cell cycle-related proteins, the expression of cyclin E increased markedly after treatment with ES products, indicating that the G1/S transition occurred. In addition, the expression of the transcription factor E2F1 was up-regulated by the addition of ES products. Small interfering RNA (siRNA) was used to demonstrate that the transcription factor E2F1 is a key factor in the control of cell proliferation in HEK293 cells. The present results demonstrate that ES products from *C. sinensis* stimulate cell proliferation by inducing E2F1 expression. We suggest that the ES products released from *C. sinensis* during infection may play an important role in the development of cholangiocarcinoma via the overgrowth of the bile duct epithelium.

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## Introduction

*Clonorchis sinensis* is one of the most prevalent parasitic helminths of humans in Asian countries such as Korea, China, Taiwan, and northern Vietnam. Human clonorchiasis occurs as the result of consuming raw or uncooked freshwater fishes, and symptoms comprise severe histopathological changes in the intrahepatic bile duct and the surrounding liver tissue, including desquamation, proliferation, glandular changes, metaplasia of the cholangial epithelium, gallstone formation, and cholangial carcinoma (Hong 2003). In particular, clonorchiasis induces biliary epithelial hyperplasia and metaplasia, which eventually result in the carcinogenesis of the biliary epithelial cells (Cho et al. 2006; Choi et al. 2006; Lim et al. 2006; Rana et al. 2007). Although a large and compelling body of evidence links clonorchiasis and cholangiocarcinoma, the mechanisms involved in this process are still unknown.

*C. sinensis* secrete excretory–secretory products (ES products) during infection in the host. In other parasites, including *Paragonimus westermani*, *Fasciola hepatica*, and *Spirometra mansoni*, it has been reported that these ES products play a role in worm maturation (Chung et al. 1995; Jin et al. 2006), migration in the host tissues (Rhoads and Fetterer 1997), and immune modulation (Carmona et al. 1993; Kong et al. 1994). In rats and hamsters infected by *C. sinensis*, proliferation and differentiation of the bile duct epithelium are induced, but the mechanism is unidentified yet (Hong et al. 1993; Lee et al. 1997). Thuwajit et al. (2004) reported that ES products from *Opisthorchis viverrini*, a closely related liver fluke with *C. sinensis*, induced cellular proliferation in mouse fibroblast NIH-3T3 cells.

All somatic cells regulate proliferation via cell cycle control mechanisms that are mediated by cell cycle proteins, including cyclins, CDKs, CDK inhibitors, Rb family proteins, and E2F transcription factors (King and Cidlowski 1998). Cyclins, which function in the G1 phase of the cell cycle, play an especially important role in cellular growth regulation. Cyclin E is a particularly important regulator of the transition from the G1 to S phase (Geng et al. 1996). E2F transcription factors play important roles in cell cycle progression and the control of proliferation by enhancing the transcription of genes that are required for S phase entry and DNA synthesis (Goto et al. 2006). Several cell cycle proteins, such as cdc2, cyclin E, cyclin A, and the *E2F-1* gene itself, possess E2F binding sites in their promoters (Geng et al. 1996; Goto et al. 2006). Therefore, virtually the entire process that activates DNA replication and S phase entry is under the control of the E2F pathway (Martens et al. 1998). Among the E2F family members, the E2F1 protein fulfills diverse functions by forming complexes with the DP family proteins (DP-1 and DP-2) and the pocket proteins RB. During the early G1 phase, RB binds to the E2F1–DP1complex and inhibits its activity. During the mid-G1 phase, phosphorylated RB released from the E2F1–DP1 complex initiates the transcription of genes necessary for the G1/S transition, including cyclin D, cyclin E, cyclin A, cdc2, and DNA polymerase (Matsumura et al. 2003). E2F1 is also a key factor in the development of cancer; several proto-oncogenes involved in cell proliferation, including B-myb, c-myb, N-myc, and c-myc, are regulated by E2Fs (Johnson and Schneider-Broussard 1998; Mundle and Saberwal 2003).

Although the effect of clonorchiasis on the cell cycle is not fully understood, it is probable that a tight linkage exists between *C. sinensis* infection and cell cycle regulation. We examined the effect of ES products from *C. sinensis* on the human epithelial cell line HEK293 and determined that these factors induce proliferation via the up-regulation of the cell cycle proteins cyclin E and E2F1.

## Materials and methods

### Preparation of ES products

Metacercariae of *C. sinensis* were collected from naturally infected freshwater fish (*Pseudorasbora parva*) at an endemic site in Korea. Pepsin–HCl was used to digest the flesh of the fish to obtain the metacercariae, which were then introduced into 4- to 6-week-old Sprague–Dawley rats. After 3 months, the adult worms of *C. sinensis* were collected from the bile duct and washed several times with phosphate-buffered saline (PBS) containing 100 μg/ml of penicillin and 100 U/ml of streptomycin. Freshly isolated adult worms were incubated in sterile PBS for 24 h under an atmosphere of 5% $CO_2$ at 37°C. After incubation, the medium was centrifuged for 30 min at 15,000 rpm, and then filtered with a syringe-driven 0.2 μm filter unit (Choi et al. 2003). The amount of protein in the extract was measured using the Bradford assay (Bio-Rad, Hercules, CA, USA).

### Cells and reagents

Human embryonic kidney cells (HEK293) were from ATCC (American Type Culture Collection) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine, 100 μg/ml penicillin, and 100 U/ml streptomycin at 37°C under a humidified atmosphere of 5% $CO_2$. Polyclonal or monoclonal antibodies were used to detect several cell cycle molecules. These included anti-cyclin E (sc-247), anti-cyclin B1 (sc-245), anti-E2F1 (sc-193), anti-CDK2 (sc-163), and anti-CDK4 (sc-260). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz) and used as 1:1,000 dilutions. The antibody against calnexin (BD 610523) was purchased from Transduction Laboratories (BD Biosciences) and used as a 1:2,000 dilution. Anti-mouse and anti-rabbit Ig antisera conjugated with horseradish peroxidase (HRP) were purchased from DAKO (Glostrup).

### Cell proliferation assay

We used the XTT formazan method to evaluate the degree of cell proliferation. XTT (1 mg/ml) was dissolved in warm medium (without phenol red), and 1.25 mM phenazine methosulfate (PMS) was prepared in PBS. The cells were grown in tissue-culture grade, 96-well, flat-bottom microtiter plates with 100 μl of culture medium per well. After incubating for the indicated periods, 50 μl of the XTT–PMS mixture (final XTT concentration, 0.3 mg/ml) was added to each well. The microtiter plates were incubated for 4 h at 37°C, and the formazan product was quantified by measuring the absorbance at 492 and 690 nm on a microtiter plate reader (Martens et al. 1998; Kuo et al. 2006). All experiments were performed in triplicate.

### Western blotting

Cells were washed in PBS and solubilized using 1% Nonidet P-40 in a buffer containing 150 mM NaCl, 10 mM NaF, 1 mM PMSF, 200 μM $Na_3VO_4$, and 50 mM HEPES, pH 7.4. After the insoluble pellets were removed,

the lysates were separated by 6, 8, 10, or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon; Millipore). The membranes were incubated with primary antibodies for cell cycle-related proteins. Immune complexes were tagged using goat anti-rabbit or rabbit anti-mouse antibodies conjugated with HRP and then visualized using an enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech).

### Flow cytometric analysis

HEK293 cells were plated in six-well cell culture plates at $2\times10^5$ cells/well in 2 ml DMEM containing 10% FBS. After 24 h, the medium was replaced with DMEM containing only 2% FBS (serum-free medium). The cells were incubated with the ES products from *C. sinensis* for an additional 24 h, followed by propidium iodide (PI) staining for cell cycle analysis. PI-stained cells were analyzed using a FACSCalibur multicolor flow cytometer (Becton-Dickinson), and the data were analyzed using CellQuest software (Becton-Dickinson).

### Transient transfection and luciferase assay

To analyze E2F1 transcriptional activity, HEK293 cells ($1\times10^6$ cells per 60 mm plate) were transfected with either 5 μg of the pXP2-luciferase reporter gene (control) or the dihydrofolate reductase (*dhfr*) promoter-driven luciferase reporter construct. Transfection was performed using Lipofectamine™ 2000 (Invitrogen). After 24 h, the transfected cells were treated with ES products from *C. sinensis* at the indicated concentrations for an additional 24 h. Cell extracts were prepared using 1× passive lysis buffer (Promega, Madison), and luciferase activity was measured for a 15-s time course using the luciferase assay system and a luminometer (Turner Designs TD-20/20; Promega). Only one-tenth of the total volume of cell extract was used in the analysis.

### siRNA transfection

To suppress E2F1 expression, we used the human E2F1 small interfering RNA (siRNA) SureSilencing™ kit (Invitrogen), which contains nonspecific control siRNA (Stealth™ negative control; Invitrogen) and two specific siRNAs for E2F1: E2F1 no. 1 (ID HSS103391; 5′-AGA CCA GCC ACA UUC ACU UUG UCC C-GGG ACA AAG UGA AUG UGG CUG GUC U-3′) and E2F1 no. 2 (ID HSS103392; 5′-AUA ACU CAA UAG CUU GGU UGA AUC C-GGA UUC AAC CAA GCU AUU GAG UUA U-3′). Transfection was performed using Lipofectamine™ 2000.

## Results

### ES products induced cell proliferation in HEK 293 cells

Infection by *C. sinensis* stimulates the proliferation and differentiation of small ductular or periductal cells in rats and hamsters (Hong et al. 1993; Lee et al. 1993; Lee et al. 1994; Lee et al. 1997). The XTT assay was used to investigate the role of these ES products in cell proliferation in vitro. To minimize the effect of growth factors present in FBS, the cells were incubated in 2% FBS for 1 day before treatment with ES products. We also conducted experiments using 0.5 or 1% FBS; however, the cells required a minimum of 2% FBS to survive (data not shown). As shown in Fig. 1, cell proliferation in HEK293 cells treated with ES products increased approximately 10−25% in comparison to the control. Our data show that ES products from *C. sinensis* stimulate cell proliferation in HEK293 epithelial cells.

### Effect of ES products on cell cycle distribution in HEK293 cells

We hypothesized that increased cell proliferation is the result of cell cycle progression driven by ES products, and we, thus, monitored cell cycle progression using PI staining for DNA content (Fig. 2). When HEK293 products were treated with ES products for 24 h, the cell population in the G0/G1 phase decreased. In contrast, the cell popula-

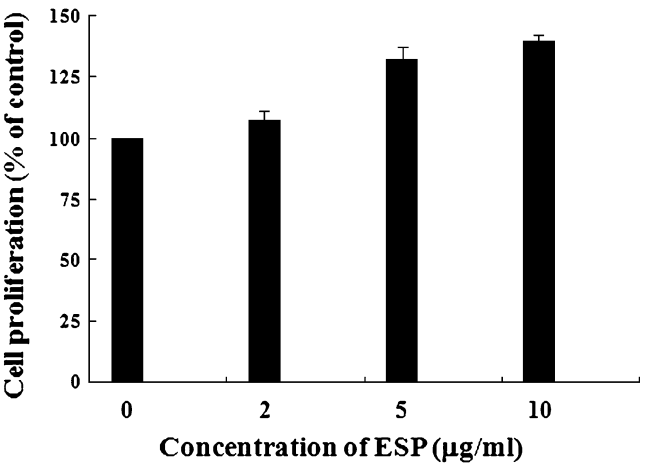


**Fig. 1** Effect of ES products on cell proliferation in HEK293 epithelial cells. Cells were plated in 96-well plates ($1.5\times10^4$ cells/well). After a 24-h incubation, the medium was replaced with serum-free medium (2% FBS-RPMI1640 without phenol red). The cells were incubated in PBS (vehicle) or various concentrations of ES products in PBS for 24 h. Cell proliferation in each group was determined using the XTT assay. The histograms represent cell proliferation as a percentage of the control±SD ($n$=4)

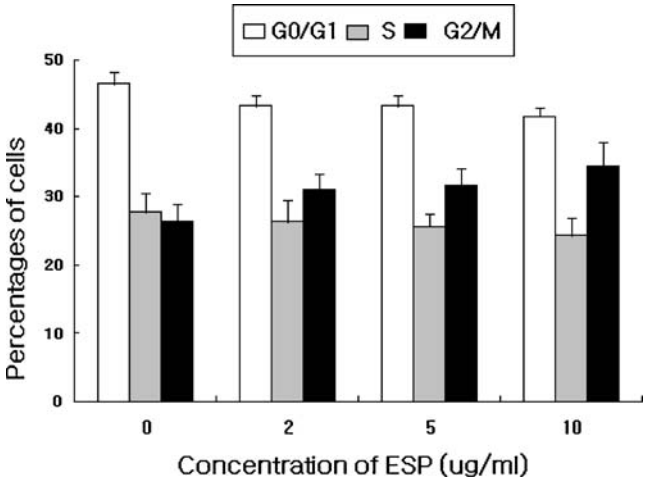


**Fig. 2** Effect of ES products on cell cycle distribution in HEK293 cells. After incubation in serum-free medium, HEK293 cells were treated with different concentrations of ES products for 24 h. PI staining was performed to determine the percentage of cells in each phase. The histograms represent cell proliferation as a percentage of the control±SD ($n$=4)

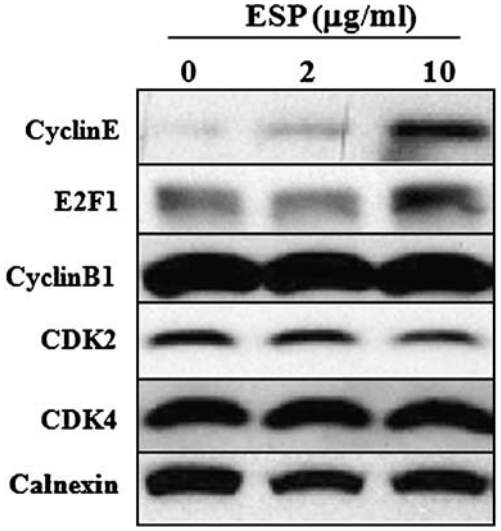


**Fig. 3** Expression of cell cycle-related proteins after treatment with ES products. HEK293 cells were incubated with either PBS (vehicle) or various concentrations of ES products for 24 h, and the cells were collected for protein extraction. Approximately 30−50 μg of protein extract from each sample was subjected to immunoblot analysis and probed with the indicated antibodies

tion in the G2/M phase, which contained cells undergoing rapid proliferation, increased approximately 10−15% compared to the control in a dose-dependent manner (Fig. 2). Interestingly, the percentage of cells in the S phase did not change.

## ES products enhanced the expression of cell cycle-related proteins

We examined whether ES products affect the expression of cell cycle-related proteins. We performed immunoblotting against several such proteins and calnexin as a loading control. Because ES products appear to drive progression from the G1 to G2/M phase, we first focused on cell cycle proteins related to the G1/S transition (Fig. 2). After incubation with ES products, the expression of cyclin E and the transcription factor E2F1 significantly increased in comparison to the control (Fig. 3). However, the expression of cyclin B1, CDK2, and CDK4 did not change in response to treatment with ES products.

## Treatment with ES products increases the activity of transcription factor E2F1

To confirm that ES products modulate E2F1 expression at the transcriptional level, we performed transient transfection experiments with a *dhfr* promoter-driven luciferase reporter construct. The *dhfr* gene encodes an enzyme that catalyzes the synthesis of purines and thymidylate, which are necessary for DNA replication during the S phase (Slansky and Farham 1996). Furthermore, the E2F family proteins regulate this gene: *dhfr* contains E2F binding elements, and its expression is correlated with E2F1 mRNA expression (Blake and Azizkhan 1989; Fry et al. 1999; Sowers et al. 2003). A schematic of the *dhfr*–luciferase reporter construct containing the Sp1 and E2F elements (DHFR–luc) is shown in Fig. 4a. HEK293 cells were transfected with the DHFR–luc plasmid or the pXP2 plasmid

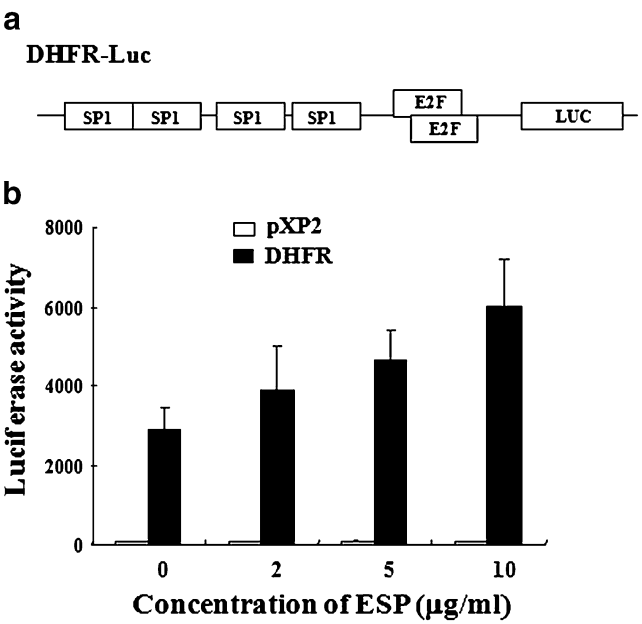


**Fig. 4** ES products up-regulate DHFR promoter-driven luciferase activity in HEK293 cells. **a** The construct contains SP1 binding sites and E2F binding regions. **b** Effect of ES products (ESP) on DHFR promoter activity. HEK293 cells were transfected with the luciferase reporter gene *dhfr*–luc or *pXP2*–luc (control). Luciferase activity is reported as the mean±SD ($n$=3)

(Nordeen 1988) as a negative control. After 24 h, the transfected cells were plated in cell culture plates and treated with ES products at the indicated concentrations for an additional 24 h. Luciferase activity increased 2- to 2.5-fold in DHFR–luc transfected HEK239 cells, suggesting that ES products induce E2F1 expression at the transcriptional level, which resulted in enhanced promoter activity in the *dhfr* gene (Fig. 4b).

E2F1 transcription factor is a key regulator of cell proliferation induced by ES products in HEK293 cells

To investigate the functional role of E2F1 and to determine if E2F1 is sufficient to cause proliferation in HEK293 cells, E2F1 expression was suppressed using siRNA. HEK293 cells were transfected with one of two E2F1 siRNAs and optimized for effective E2F1 knockdown. After 48 h, Western blotting showed a substantial decrease in the expression of E2F1; calnexin was also analyzed as a loading control (Fig. 5a). When the cells were pretreated with siRNA against E2F1 and then incubated with ES products, the proliferative effect of the ES products was inhibited, whereas control siRNA-treated cells still responded to the addition of ES products (Fig. 5b). In contrast to the cells transfected with control siRNA, HEK293 cells transfected with siE2F1 no. 1 or no. 2 did not undergo substantial changes in cell cycle distribution after treatment with ES products (Fig. 5c).

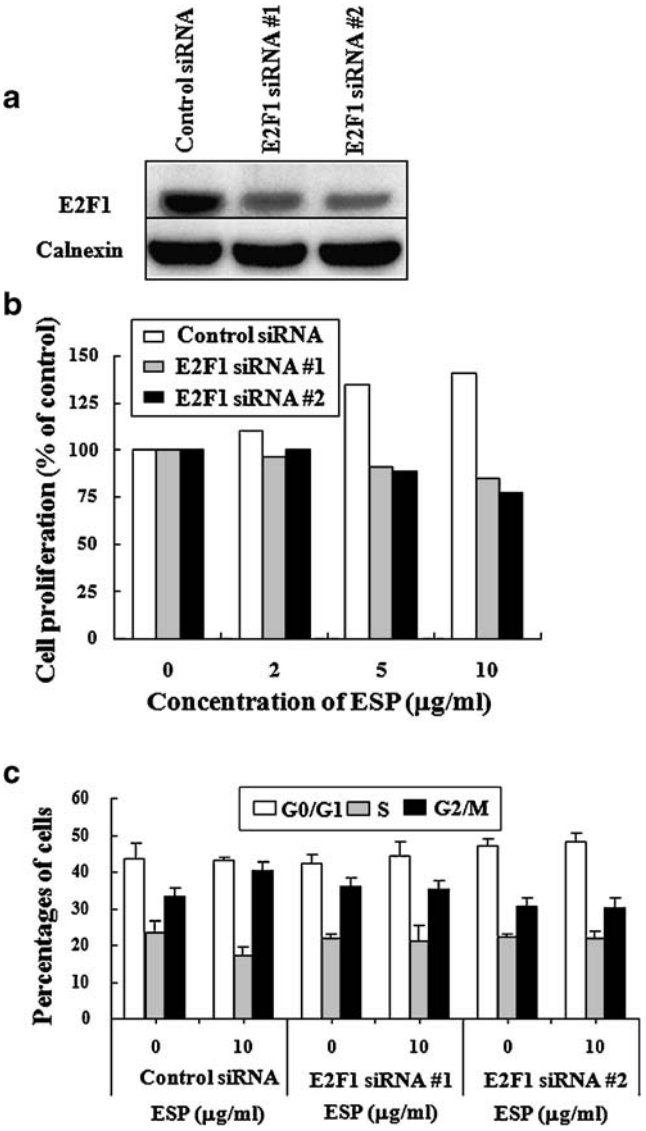


**Fig. 5** E2F1 down-regulation inhibits cell proliferation and cell cycle progression induced by ES products (ESP). **a** HEK293 cells were transfected with E2F1 no. 1, E2F2 no. 2, or control siRNA. Protein extracts from these cells were subjected to immunoblot analysis for E2F1 and calnexin as a loading control. After siRNA treatment, the cells were re-plated in 96-well plates for the XTT assay (**b**) and in six-well plates for cell cycle analysis (**c**). The cells were grown in serum-free medium for 24 h and then ES products were added for an additional 24 h at the indicated concentrations. The histograms represent cell proliferation as a percentage of the control±SD (**b** $n=2$; **c** $n=3$)

## Discussion

Clonorchiasis induces severe histopathological changes in the intrahepatic bile duct and surrounding liver tissue, including desquamation, proliferation, glandular changes, metaplasia of the cholangial epithelium, gallstone formation, and cholangiocarcinoma (Hong 2003). Although *C. sinensis* infection is an important factor in the development of cholangiocarcinoma (Lee et al. 1993, 1997; Choi et al. 2006; Lim et al. 2006; Rana et al. 2007), the mechanisms that promote cell proliferation and carcinogenesis are not clearly understood. In humans, a combination of mechanical irritation caused by physical contact with the parasite and chemical irritation caused by the ES products promotes pathogenesis in infection of *Opisthorchis viverrini* (Thuwajit et al. 2004).

ES products from *O. viverrini* induce proliferation in the mouse fibroblast cell line NIH3T3 (Thuwajit et al. 2004), but infection with *Leishmania*, an obligate intracellular parasite, results in lower proliferation of the host macrophages (Kuzmenok et al. 2005). The present study used the human epithelial cell line HEK293 to examine the in vitro effect of ES products of *C. sinensis* on the growth of bile duct epithelial cells. The cells proliferated in the presence of ES products in a dose-dependent manner (Fig. 1), but cell viability suffered when more than 10 μg/ml of ES products were applied, probably because of changes in osmolality. We incubated HEK293 cells in media containing 2% FBS for 2 days to minimize the effect of growth factors present in FBS. Previously, Lee et al. (1997) had reported that *C. sinensis* infection induced in vivo cell proliferation which preceded the appearance of cholangio-

carcinoma. This is the first study to demonstrate that these ES products of *C. sinensis* also induce in vitro proliferation of human epithelial cells.

We also investigated the mechanism of this effect and the molecules involved. Cell proliferation is controlled by both the frequency and timing of the cell cycle. Consequently, we analyzed progression through the cell cycle to determine whether any changes occur in the cell cycle phase distribution. As shown in Fig. 2, treatment with ES products promoted cell cycle progression. The percentage of cells in the G1 phase decreased, whereas the percentage of G2/M phase cells increased, indicating that the G1 to S and S to G2/M transitions had occurred (Fig. 2). However, it is unclear why G2/M cells were unable to initiate another cell cycle. We speculate that the G1 to S to G2/M transitions were too rapid in treated cells, and thus, cells accumulated in the G2/M phase. Alternatively, the ES products may not regulate exit from the G2/M phase in HEK293 cells.

Cell cycle progression is tightly controlled by regulatory mechanisms whose components consist of a regulatory subunit referred to as a cyclin and a protein kinase called a cyclin-dependent kinase (CDK). Specific cyclin/CDK complexes are expressed throughout the phases of the cell cycle in which each cyclin has a specific expression profile and kinase activity (Shackelford et al. 1999; Rogoff and Kowalik 2004). Focusing on regulators related to the G1/S transition, we investigated the expression of cyclin E because it is an essential regulator in the transition from the G1 to S phase (Ohtsubo et al. 1995). E2F transcription factors play a pivotal role in cell cycle progression and the control of proliferation. Among these, E2F1 is involved in the G1/S transition along with other regulators, such as pRb and DPs (King and Cidlowski 1998; Matsumura et al. 2003).

After treatment with ES products, the expression of cyclin E and E2F1 was markedly up-regulated, whereas the expression of cyclin B and CDK2/4 remained unchanged (Fig. 3). In fact, cyclin B was functional during the G2/M phase, and as a result, progression from the G2/M phase was not noticeable (Fig. 2); cyclin D expression was only faintly observed in HEK293 cells.

As described above, ES products from *C. sinensis* induced the expression of cyclin E and E2F1. Because the expression of cyclin E is controlled by E2F1, we hypothesized that E2F1 regulation may be a key factor in our system. As determined by the DHFR-promoter assay and siRNA experiments (Figs. 4 and 5), ES products induced E2F1 expression at the transcriptional level, and E2F1 expression alone was sufficient to drive cell proliferation and cell cycle progression. When siRNA for E2F1 was applied, the expression of cyclin E did not change in response to ES products.

The ES products contain several molecules such as proteins, lipids, and carbohydrates. The functions of ES products from *C. sinensis* appeared to be heat-labile, and as a result, we assume that the heat-sensitive proteins in ES products have a role in cell proliferation and cell cycle progression. Furthermore, endotoxin testing did not detect the presence of an endotoxin in the ES products, thus, ruling out the potential effect of endotoxins on cell proliferation. In *O. viverrini*, the morphology of NIH3T3 mouse fibroblasts was altered after incubation with ES products (Thuwajit et al. 2004); in contrast, we observed no morphological changes after the addition of ES products. These results imply that although both *O. viverrini* and *C. sinensis* are liver flukes implicated in cholangiocarcinoma, these organisms activate different mechanisms; alternatively, the response to ES products may vary according to the host cell type. In addition, *Schistosoma haematobium* has been implicated in bladder cancer (Herrera et al. 2005), and this parasite also promotes cell proliferation (Rosin et al. 1994). It is possible that these three parasites share a common carcinogenic protein(s) and signaling cascades.

In conclusion, we demonstrated that ES products from *C. sinensis* stimulate distinctive proliferation of human embryonic kidney cells (HEK293) by inducing E2F1 expression. Because enhanced cell proliferation is an important factor in the formation of cancer, the effects of ES products on cell proliferation and cell cycle progression may explain the pathogenesis of cholangiocarcinoma during *C. sinensis* infection.

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