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Krüppel-like Factor 4 (Gut-enriched Krüppel-like Factor) Inhibits Cell Proliferation by Blocking G₁/S Progression of the Cell Cycle*

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Abstract

Krüppel-like factor 4 (KLF4) is an epithelial cell-enriched, zinc finger-containing transcription factor, the expression of which is associated with growth arrest. Previous studies show that constitutive expression of *KLF4* inhibits DNA synthesis but the manner by which KLF4 exerts this effect is unclear. In the present study, we developed a system in which expression of *KLF4* is controlled by a promoter that is induced upon treatment of cells containing the receptors for the insect hormone, ecdysone, with ponasterone A, an ecdysone analogue. The rate of proliferation of a stably transfected colon cancer cell line, RKO, was significantly decreased following addition of ponasterone A when compared with untreated cells. Flow cytometric analyses indicated that the inducible expression of *KLF4* caused a block in the G₁/S phase of the cell cycle. A similar block was observed when ecdysone receptor-containing RKO cells were infected with a replication-defective recombinant adenovirus containing an inducible *KLF4* and treated with ponasterone A. Results of these studies provide evidence that the inhibitory effect of KLF4 on cell proliferation is mainly exerted at the G₁/S boundary of the cell cycle.

Krüppel-like factor 4 (KLF4;¹ also called gut-enriched Krüppel-like factor) is a recently identified zinc finger-containing transcription factor that is related to the *Drosophila melanogaster* segmentation gene product, Krüppel (1–3). It belongs to a rapidly expanding family of mammalian Krüppel-like factors with erythroid Krüppel-like factor (KLF1) (4) serving as a prototype member (5–7). Within this family, KLF4 is most closely related to KLF1 and KLF2 (lung Krüppel-like factor or LKLF) (8). Through studies involving gene knockout, all three KLFs have been shown to be important in regulating development of tissue-specific functions (9–15). KLF4, for example, was shown to be necessary for the development of the barrier function of the skin (15).

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¹The abbreviations used are: KLF4, Krüppel-like factor 4; AdEGI, adenovirus containing EGFP; AdEGI-KLF4, adenovirus containing EGFP and KLF4; CHO, Chinese hamster ovary; EcR, ecdysone receptor; EGFP, enhanced green fluorescence protein; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; LKLF, lung Krüppel-like factor; PA, ponasterone A; PBS, phosphate-buffered saline; RXR, retinoid X receptor; EcRE, ecdysone receptor element; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

Expression of *KLF4* is particularly enriched in epithelial tissues such as the intestine and skin (1,2,15–17). In the intestinal epithelium, *KLF4* is abundantly expressed in the post-mitotic, differentiated epithelial cells outside the proliferating zone of crypt cells (1,2,16). In addition, in conjunction with several other KLFs, *KLF4* is highly expressed in naive, quiescent B lymphocytes and becomes rapidly down-regulated upon mitogenic activation (18). The *in vitro* pattern of expression of *KLF4* mirrors its *in vivo* expression. For example, *KLF4* is primarily expressed in a growth-arrested state brought on by serum deprivation, contact inhibition, DNA damage, or growth-inhibitory cytokines (1,19,20). Conversely, expression of *KLF4* is decreased in conditions associated with increased proliferation such as in neoplasm of the intestinal tract (21,22). These studies, therefore, demonstrate a correlation between *KLF4* expression and a process of growth arrest.

Consistent with the notion that KLF4 may be important in regulating growth, previous studies have demonstrated that KLF4 is an inhibitor of cellular proliferation. For example, constitutive expression of *KLF4* in transfected fibroblasts or colon cancer cells resulted in the inhibition of DNA synthesis (1,22). This may in part be explained by the ability of KLF4 to activate the p21^{WAF1/Cip1} promoter (19), a cell cycle inhibitor, and to suppress the *cyclin D1* promoter (23), a positive cell cycle regulator. However, the exact mechanism by which KLF4 inhibits cell proliferation is somewhat unclear, with evidence suggesting that it causes either apoptosis (20) or cell cycle arrest (22). To further define the molecular mechanism by which KLF4 regulates the cell cycle, we developed a system using an inducible promoter to control the expression of *KLF4*. We show by two independent means that induced *KLF4* expression results in an accumulation of cells in the G₁ phase of the cell cycle with a concomitant decrease of those in the S phase. The results of our study therefore provide evidence that KLF4 is a cell cycle regulator and does so by creating a block in the G₁/S transition phase of the cell cycle.

EXPERIMENTAL PROCEDURES

Plasmid Constructs

pVgRXR, a plasmid engineered to constitutively express both the VgEcR and RXR receptor subunit, was obtained from Invitrogen (Carlsbad, CA). VgEcR was generated by fusing the DNA-binding domain of the *D. melanogaster* ecdysone receptor (EcR) to a modified transactivation domain of the herpes simplex virus 1 VP16 protein (24,25). RXR (retinoid X receptor) is the mammalian homologue of USP (ultraspiracle), the natural partner to the *Drosophila* ecdysone receptor (26,27). Upon binding to ecdysone, the heterodimeric VgEcR and RXR form a functional ecdysone receptor that is required for the optimal interaction with the ecdysone receptor element (EcRE).

The plasmid pAdLoxEGI-KLF4 was obtained by subcloning the full-length coding region of the *KLF4* cDNA into pAdLoxEGI (28). The latter was constructed from the pAdLox plasmid (29) by substituting the ecdysone inducible promoter from pIND (Invitrogen) for the cytomegalovirus promoter and by inserting an expression cassette containing the enhanced green fluorescence protein (EGFP) (30), which was followed by an internal ribosome entry site (31). The *KLF4* cDNA was inserted between the *Xho*I and *Eco*RI sites in the multiple cloning sites following the internal ribosome entry site.

Transfection and Immunocytochemistry

Transfection was accomplished using the LipofectAMINE reagent from Life Technologies (Gaithersburg, MD). To test the co-expression of *EGFP* and *KLF4*, EcR-CHO cells, a Chinese hamster ovary (CHO) cell line containing the stably integrated pVgRXR vector, were cultured on glass coverslips in 6-well dishes. Cells were transfected with pAdLoxEGI-KLF4 and treated for 24 h with 5 μ M ponasterone A (Invitrogen). The coverslip was washed twice with phosphate-

buffered saline (PBS) and fixed in 3.5% paraformaldehyde in PBS for 8 min followed by washing twice with PBS. Cells were then permeabilized in 0.1% Nonidet P-40 in PBS for 15 min, washed twice with PBS, and incubated in 10% fetal bovine serum (FBS) in PBS for 20 min. Fifty μl of a 1:100 diluted anti-KLF4 serum in PBS containing 10% FBS was added to the coverslip and incubated at 4 °C overnight. The coverslip was washed 3 times with 10% FBS in PBS followed by the addition of 100 μl of a 1:200 dilution of Cy3-labeled secondary antibody and incubated for 1 h at room temperature. The coverslip was then washed with 0.01% Tween 20 in PBS for 30 min and placed face down in 100 μl of mounting media containing 50% glycerol in PBS. Stained cells were visualized under a confocal microscope using filters designed to detect GFP and Cy3. The primary antibody was eliminated in control experiments.

Establishment of Stable Cell Lines

The EcR-RKO/pAdLoxEGI-KLF4 cell line was generated by co-transfecting pVgRXR and pAdLox-EGI-KLF4 at a molar ratio of 1:20 into the RKO human colon cancer cells. Two days following transfection, 150 $\mu\text{g}/\text{ml}$ Zeocin was added to the medium to select for resistant clones. To identify clones that contained the inducible *KLF4*, cells were treated with 5 μM ponasterone A for 24 h. Individual clones that exhibited green fluorescence were expanded and further sorted by Star Plus (Becton Dickinson) following induction to enrich for cells stably expressing EGFP. The level of *KLF4* induction was tested by Northern and Western blot analyses upon hormonal treatment.

EcR-RKO cells were similarly obtained as above except that transfection was performed with pVgRXR alone. Zeocin-resistant clones were selected and the degree of RXR expression measured by Western blot analysis.

Generation of Recombinant Adenovirus and Conditions of Infection

The recombinant adenovirus containing EGFP and KLF4 (AdEGI-KLF4) or GFP alone (AdEGI) was generated by Crelox recombination of purified ψ5 viral DNA (29) and pAdLoxEGI-KLF4 or pAdLoxEGI, respectively, as previously described (28,29). The recombinant products were plaque-purified and expanded by infecting HEK293 cells for 8 h. Forty-eight hours after infection, cells were freeze-thawed 3 cycles to release the viruses, which were purified by CsCl gradient centrifugation to achieve a titer of $\sim 10^{10}$ plaque forming units/ml.

EcR-RKO cells were grown to 90% confluence in 10-cm dishes and replenished with fresh media containing 2% FBS followed by the addition of 10^8 plaque forming units of recombinant virus per dish. Infected cells were incubated at 37 °C for 6–8 h, at which time the media were changed and incubation continued overnight. Cells were then treated with 5 μM ponasterone A for 24 h and then collected for further analyses.

Northern Blot and Western Blot Analyses

RNA was extracted from cells using the TriZol reagent (Life Technologies) following the manufacturers recommendations. Twenty μg of total RNA was resolved by electrophoresis in 1.2% agarose gels containing 2.4 M formaldehyde and transblotted onto nylon membranes (Hybond-N; Amersham Pharmacia Biotech). Hybridization and washing were performed under high stringency conditions using radioactively labeled *KLF4* and glyceraldehyde-3-phosphate dehydrogenase cDNA probes.

Western blot analyses were performed using standard procedures. Protein samples were dissolved in a loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, and 0.01% bromphenol blue), heated at 100 °C for 3 min, and loaded onto a SDS-polyacrylamide gel in running buffer containing 25 mM Tris-HCl, pH 8.3, 250 mM glycine, and 0.1% SDS. At

the completion of electrophoresis, proteins were electrophoretically transferred to a membrane, which was immunoblotted with a rabbit anti-KLF4 serum (1:1,000 dilution; Ref. 1) or anti-RXR serum (1:1,000 dilution; Santa Cruz). Following incubation with the secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit IgG; Santa Cruz), KLF4 or RXR was visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech).

Cell Proliferation Assays

EcR-RKO/pAdLoxEGI-KLF4 cells were plated on 60-mm dishes at a density of 2.8×10^5 cells/dish. After 24 h, ponasterone A was added to a final concentration of $5 \mu\text{M}$. Media containing ponasterone A were changed every other day. Control cells received vehicle (ethanol) only. Cell numbers were measured with a hemocytometer daily up to 8 days following seeding.

MTS assays were performed based using the manufacturers recommendations (Promega, Madison, WI). EcR-RKO and EcR-RKO/pAdLox-EGI-KLF4 cells were plated in 96-well microtiter plates at a density of 1.0×10^3 cells/well in Dulbecco's modified Eagle's medium with 10% FBS and $150 \mu\text{g/ml}$ Zeocin. After 24 h, the medium was changed to Dulbecco's modified Eagle's medium containing 10% FBS, $150 \mu\text{g/ml}$ zeocin, and either $5 \mu\text{M}$ ponasterone A or vehicle alone. The number of cells at this time ($t = \text{day } 0$) and at 1, 3, and 5 days was determined using the colorimetric MTS assay. Results are depicted as absorbance at 490 nm as a function of time.

Cell Cycle Analysis

DNA content was measured following staining of cells with propidium iodide. EcR-RKO/pAdLoxEGI-KLF4 cells were treated with $5 \mu\text{M}$ ponasterone A or vehicle alone for 24 h. Cells were subsequently trypsinized, washed once in cold PBS, and fixed in 70% ethanol at -20°C overnight. Fixed cells were pelleted and stained in the propidium iodide solution ($50 \mu\text{g/ml}$ propidium iodide, $50 \mu\text{g/ml}$ RNase A, 0.1% Triton X-100, and 0.1 mM EDTA) in the dark at 4°C for 1 h prior to flow cytometric quantification of DNA by a FACScan (Becton Dickinson). Similarly, FACS analyses were conducted in EcR-RKO cells infected with recombinant AdLoxEGI-KLF4 or AdLoxEGI viruses followed by treatment with $5 \mu\text{M}$ ponasterone A or vehicle alone for 24 h.

RESULTS

To investigate the mechanism by which KLF4 regulates the cell cycle, we first established an inducible system in which *KLF4* expression could be controlled. We generated a DNA construct called pAdLoxEGI-KLF4 that would produce a bicistronic message of enhanced green fluorescence protein (EGFP) and KLF4 separated by an internal ribosomal entry site (31). Expression of *EGFP* and *KLF4* was controlled by a promoter that contained a response element to a hormone-activated EcR (24). A CHO cell line, called EcR-CHO, that had a stably integrated dimeric ecdysone receptor and RXR (26,27) derived from the plasmid pVgRXR was transiently transfected with pAdLoxEGI-KLF4 and treated with an ecdysone analogue, ponasterone A. As seen in Fig. 1, GFP and KLF4 were produced in the same transfected cells (*panels A and B*, respectively), as evidenced by direct fluorescence visualization of the former and by immunocytochemical staining of the latter. As a control, transfected cells (Fig. 1C) from which the primary KLF4 antibody was eliminated during staining lacked any fluorescence (Fig. 1D). These results indicate that the pAdLoxEGI-KLF4 construct was able to produce both proteins in transfected cells upon induction.

We then established a cell line that contained stably integrated pVgRXR and pAdLoxEGI-KLF4 by co-transfecting a human colon cancer cell line, RKO. Clonal derivatives were selected

by the antibiotic Zeocin. We chose RKO cells because of their exceedingly low level of *KLF4* mRNA, which is probably due to mutations in upstream genes that regulate *KLF4* expression (32). A stably transfected clone, called EcR-RKO/pAd-LoxEGI-KLF4, was treated with ponasterone A or vehicle alone for 24 h and visualized with a fluorescence microscope. As shown in Fig. 2A, the addition of ponasterone A resulted in the appearance of numerous green cells in the culture (+PA), whereas, the uninduced control cells exhibited no fluorescence (-PA). The addition of ponasterone A also resulted in a significant increase in the level of the *KLF4* transcripts as demonstrated by Northern blot analysis (Fig. 2B).

To begin investigating the effect of induced *KLF4* expression on cell proliferation, we seeded EcR-RKO/pAdLoxEGI-KLF4 cells in low densities and treated them with ponasterone A or vehicle alone for up to 8 days. Cell numbers were assessed by direct daily counting (Fig. 3A) or by MTS assay every other day (Fig. 3B). As seen, the ponasterone A-treated cells did not proliferate during the first 4 days of treatment when compared with the control cells. After 4 days of treatment, the number of induced cells did begin to increase but at a slower rate compared with the uninduced cells. As demonstrated by the Western blot analysis in Fig. 3C, the level of *KLF4* protein was low in uninduced cells (day 0) but was increased 1 day following induction and maintained at a similar level up to 6 days. These results suggest that the decreased proliferation in induced cells is a consequence of *KLF4* production.

To address the mechanism by which *KLF4* inhibits cell proliferation, we performed FACS analyses of EcR-RKO/pAdLox-EGI-KLF4 cells treated or not with ponasterone A for 24 or 48 h. As shown in Fig. 4A, a great majority of cells exhibited green fluorescent after 24 h of ponasterone A treatment. This was accompanied by a change in the cell cycle profile as illustrated in Fig. 4B. Specifically, the induction of *KLF4* caused a statistically significant increase in the percentage of cells in the G₁ phase of the cell cycle and a statistically significant decrease in the percentage of cells in the S phase (Fig. 4C). The difference in the percentage of cells in the G₂ phase between treated and control groups was not significant. In addition, FACS analyses showed no evidence of apoptosis in the induced cells, which would have been manifested by a DNA content, that is less than G₁ as in Fig. 4B. The results obtained after 48 h of induction were similar to those at 24 h (results not shown). Importantly, accompanying the G₁/S block associated with hormonal induction was a concomitant increase in the amount of p21^{WAF1/Cip1} mRNA (Fig. 4C, *inset*). These results suggest that the ability of *KLF4* in blocking the cell cycle is mediated by p21^{WAF1/Cip1}.

As an alternative and independent means to measure the effect of *KLF4* on the cell cycle, we constructed a recombinant adenovirus, called AdEGI-KLF4, using the pAdLoxEGI-KLF4 plasmid as a shuttle vector (see "Experimental Procedures"). We also established a RKO cell line, called EcR-RKO, which had a stably integrated pVgRXR construct (Fig. 5A, *lane 3*). Following infection with the AdEGI-KLF4 recombinant virus, EcR-RKO cells were treated with ponasterone A or vehicle alone for 24 h and the degree of *KLF4* expression assessed by Northern and Western blot analyses. As shown in Fig. 5B, both the levels of *KLF4* mRNA and protein were significantly increased in infected and induced cells when compared with infected but uninduced cells (Fig. 5B, *upper and lower panel*, respectively). The induced *KLF4* expression was accompanied by the appearance of green fluorescence in cells treated with ponasterone A (Fig. 5C). To control for a possible effect of EGFP on the cell cycle, we also generated a recombinant adenovirus, called AdEGI that contained only EGFP. As seen in Fig. 5D, EcR-RKO cells infected with the AdEGI virus and induced with ponasterone A also exhibited intense green fluorescence at 24 h. Importantly, FACS analysis of infected cells showed only those infected by AdEGI-KLF4 and induced with ponasterone A had a statistically significant increase in the G₁ population and a statistically significant decrease in the S population when compared with the infected but uninduced cells (Fig. 5E). There was no difference in the cell cycle profiles of AdEGI virus-infected cells between induced and

uninduced conditions (Fig. 5F). These results suggest that the alteration in cell cycle profile upon ponasterone A addition is the result of induction in *KLF4* and not in *EGFP* expression.

DISCUSSION

KLF4 was first identified some 5 years ago as a gene whose expression is temporally associated with growth arrest (1). This correlation has been documented in several independent *in vitro* conditions (1,19,20,23). *In vivo*, the predominant expression of *KLF4* in differentiated cells of several epithelial tissues also suggests that it is a growth arrest-specific gene (1,2,15,16,33). It is particularly telling that *KLF4*, along with several other Krüppel-like factor-encoding genes, is highly expressed in naive, quiescent B lymphocytes and is rapidly down-regulated upon activation due to mitogenic stimulation (18). These observations suggest that *KLF4* may have an inhibitory function in regulating cell proliferation. Consistent with this notion, *KLF4* was shown to inhibit DNA synthesis when constitutively produced in transfected cells (1).

The present study established the mechanism by which *KLF4* exerts its negative effect on cell growth. This was accomplished by the development of an inducible system for *KLF4* expression. By either stable transfection or adenovirus-mediated infection of RKO cells, we demonstrated that induced expression of *KLF4* caused a significantly decreased rate of proliferation, which was likely due to a block in the cell cycle at the G₁/S boundary. The result of our study is consistent with that from a previous one, which examined the effect of constitutive *KLF4* expression in a stably transfected colon cancer cell line, HT-29 (22). It is also consistent with the finding that, among the genes regulated by *KLF4* are p21^{WAF1/Cip1} and *cyclin D1*, two genes with key, albeit opposite, effects in controlling G₁/S transition in the cell cycle (35–37). As *KLF4* is an activator of transcription of p21^{WAF1/Cip1} (19) and a suppressor of transcription of *cyclin D1* (23), a logical consequence of *KLF4*'s activation is the impediment in the transition from G₁ to S phase as observed in the present study. The physiological implication of our finding extends our previous observation that cell cycle arrest due to DNA damage is accompanied by p53-dependent activation of *KLF4* expression (19). Moreover, an activated *KLF4* transcriptionally induces p21^{WAF1/Cip1} expression. This is further supported by the present study showing that the level of p21^{WAF1/Cip1} transcript is significantly increased due to the induction of *KLF4* (Fig. 4C). Combining the results of these studies, it appears likely that *KLF4* is an essential member of the p53 tumor suppressor network of cell cycle regulators.

A recent study showed that expression of *KLF4* is up-regulated by treatment of the human colon cancer cell line, HT-29, with the cytokine interferon- γ (20). In this system, interferon- γ induced growth arrest in HT-29 cells by causing apoptosis. Moreover, transient transfection of several colon cancer cell lines with a *KLF4*-expressing plasmid was shown to promote apoptosis as evidenced by DNA fragmentation (20). It was proposed that *KLF4* may be a downstream target gene of interferon- γ and may mediate the apoptotic effect of interferon- γ . In our system, however, we did not observe any evidence of apoptosis by FACS or DNA staining (results not shown) during the first 48 h of induction in either stably transfected or infected cells. As the same group has reported in a different study that stable expression of *KLF4* in HT-29 cells caused cell cycle arrest but not apoptosis (22), it is unclear at this time whether *KLF4* is in fact a pro-apoptotic factor.

The negative effect of *KLF4* on the cell cycle progression suggests that *KLF4* may play an important role in regulating the transition of cells from a proliferative to a non-proliferative state such as those observed *in vitro* and *in vivo* (1,2,15,16,18–20,23,33). It may also be reasonable to speculate that one of *KLF4*'s functions in terminally differentiated epithelial cells in organs such as the gut or skin is to prevent the cells from re-entering the cell cycle. Conversely, in situations where there is increased proliferation such as that encountered in

neoplasm, one may expect to see a decrease in *KLF4* expression, which may in turn contribute to the tumorigenic phenotype. Indeed, expression of *KLF4* is diminished in benign intestinal adenomas when compared with the surrounding normal intestinal mucosa in both mouse and human models of a hereditary polyposis syndrome (21). Similarly, levels of *KLF4* mRNA have been shown to be lower in sporadic cases of colonic adenomas and carcinomas (22). However, it should be cautioned that the expression pattern of *KLF4* in cancer tissues might not always be consistent with its putative role as a tumor suppressor. For example, increased expression of *KLF4* has been observed in dysplastic squamous epithelium and squamous cell carcinoma of the oral cavity (38), and in breast cancer (39). Moreover, evidence suggests that *KLF4* may be deregulated or misexpressed in certain tumorous tissues (22,38). A potential explanation for the apparent discrepancies stated above is that certain tumors may contain mutated forms of *KLF4*, which may exert a dominant negative effect on cell function. Previous studies indicating that *KLF4* interacts with multiple regulatory proteins including Sp1 (40), p53 (19), Zf9 (41), and p300/CBP (34) distinctly suggest that this latter hypothesis may be a possibility.

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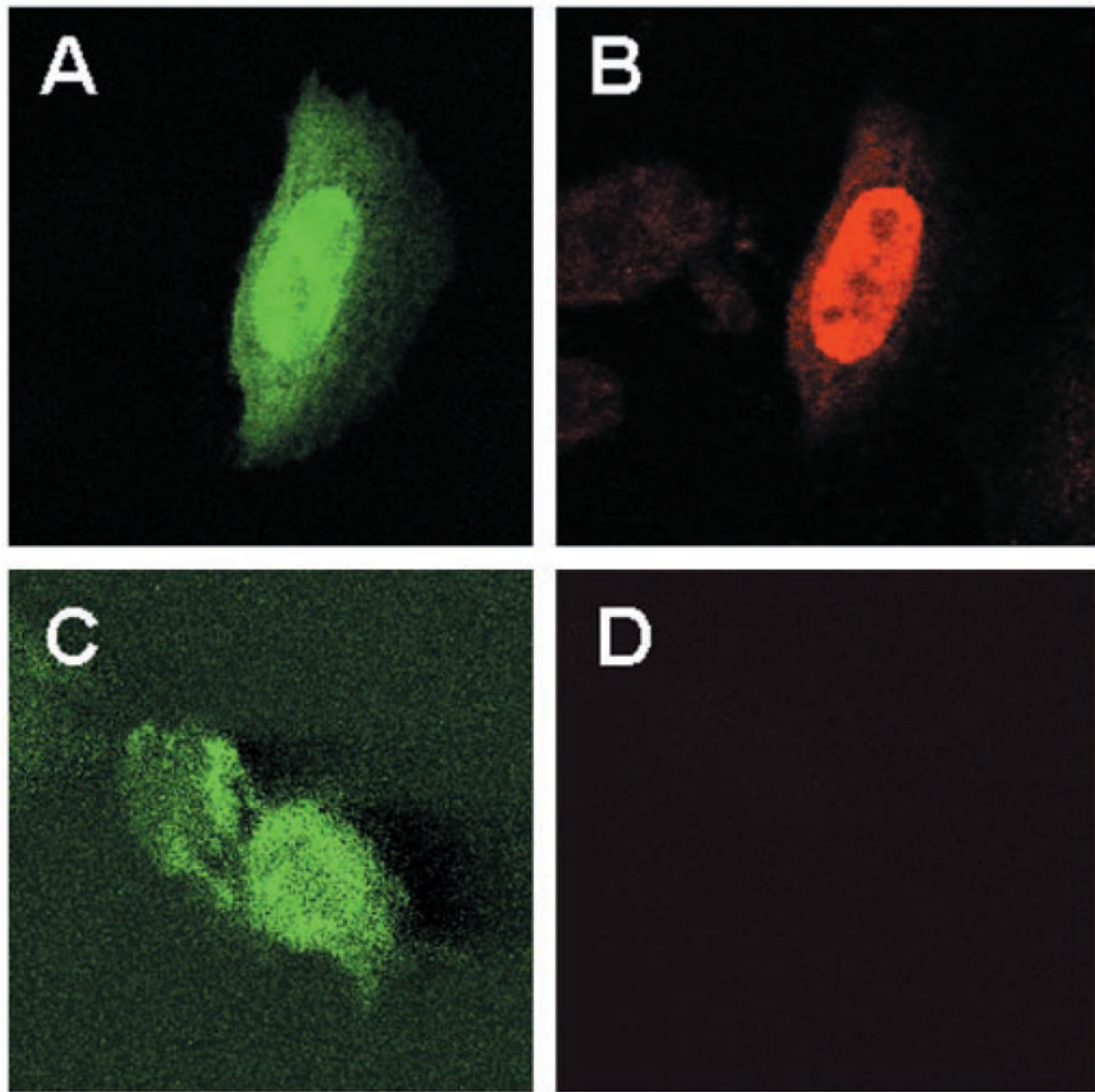


Fig. 1. Inducible expression of EGFP and KLF4 in transiently transfected cells

EcR-CHO cells were transfected with pAdLoxEGI-KLF4 and treated with $5 \mu\text{M}$ ponasterone A for 24 h. Cells were fixed and stained with an anti-KLF4 antibody (*panel B*) or without any primary antibody (*panel D*), followed by a Cy3-labeled secondary antibody. Shown in *panels A and B* is a representative cell that contained EGFP (*green*) and KLF4 (*red*), respectively. In contrast, neither of the two green cells in *panel C* stained red in the absence of the primary antibody (*panel D*) (magnification, $\times 100$).

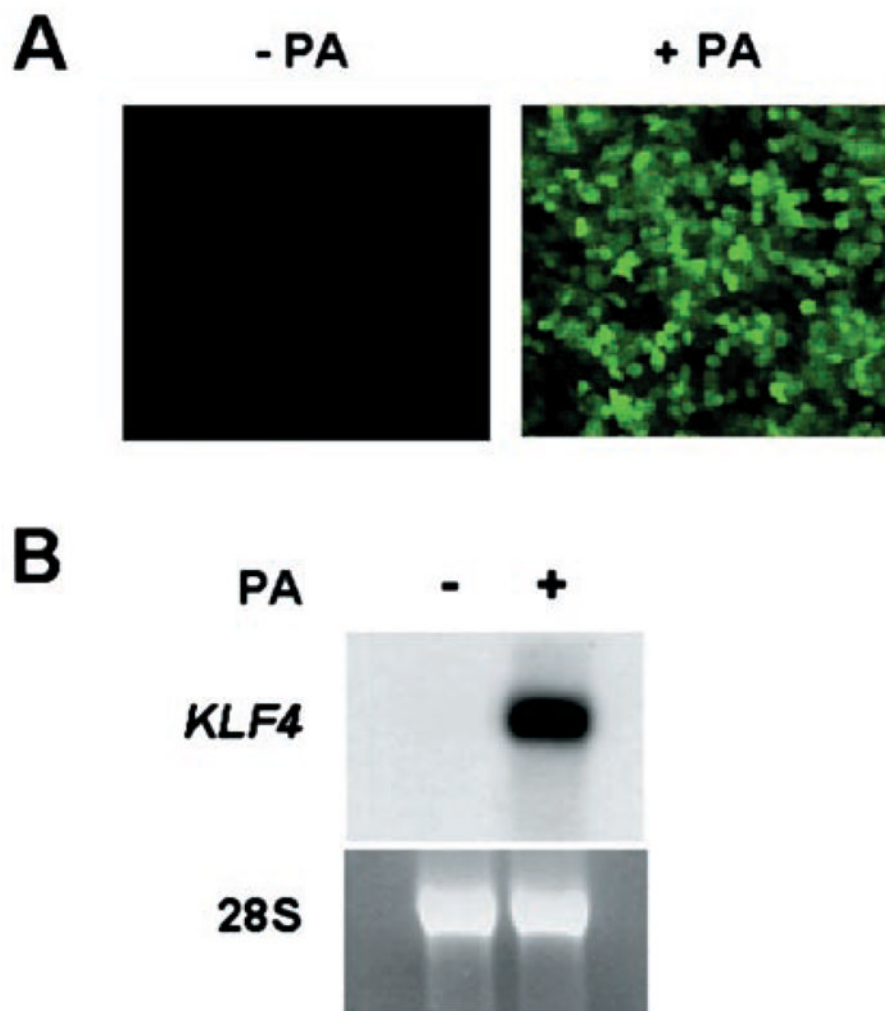


Fig. 2. Inducible expression of KLF4 in stably transfected RKO cells

A stable RKO cell line (EcR-RKO/pAdLoxEGI-KLF4) was established by co-transfection with pVgRXR and pAdLoxEGI-KLF4. After treatment with or without $5 \mu\text{M}$ ponasterone A (PA) for 24 h, cells were inspected under an inverted fluorescence microscope for the presence of GFP (*panel A*). *Panel B* shows the result of a Northern blot analysis of RNA isolated from uninduced (-) and induced (+) cells, and probed with a labeled *KLF4* cDNA fragment (*upper panel*). A photograph of the 28 S ribosomal RNA (*lower panel*) from both sets of cells is included to indicate equal loading.

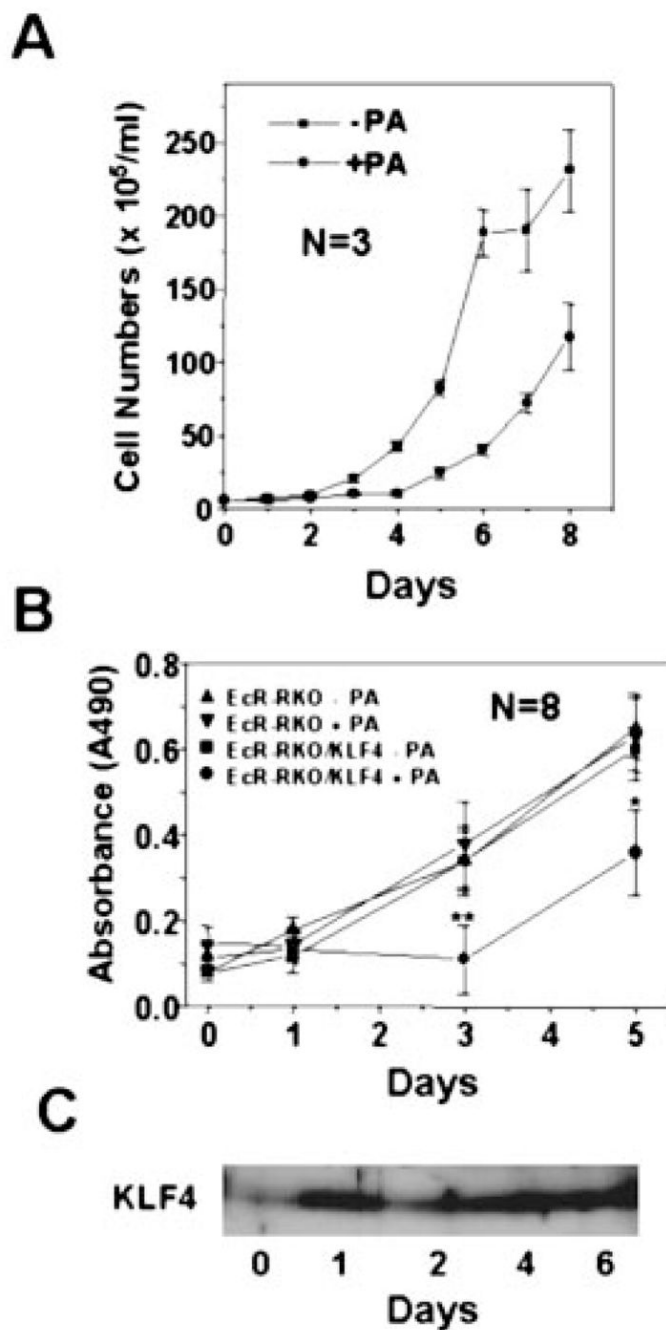


Fig. 3. The effect of induced KLF4 expression on proliferation of RKO cells

In *panel A*, 2.8×10^5 EcR-RKO/pAdLoxEGI-KLF4 cells were seeded and continuously cultured in 60-mm dishes with or without $5 \mu\text{M}$ ponasterone A for up to 8 days. The media were changed every other day. Cells were counted with a hemocytometer daily. Cell numbers are expressed as mean \pm S.D. (*bars*). In *panel B*, EcR-RKO and EcR-RKO/pAdLoxEGI-KLF4 (labeled as EcR-RKO/KLF4 in the figure) cells were plated in 96-well microtiter plates at a density of 1×10^3 cells per well. After 24 h, fresh media containing either $5 \mu\text{M}$ ponasterone A or vehicle only were added. The number of cells was determined using the colorimetric MTS assay on days 0, 1, 3, and 5. Results are depicted as mean \pm S.D of absorbance at 490 nm as a

function of time. *, $p < 0.01$; **, $p < 0.0001$ by paired Student t test. *Panel C* is a Western blot analysis of proteins extracted from induced cells for the presence of KLF on the days indicated.

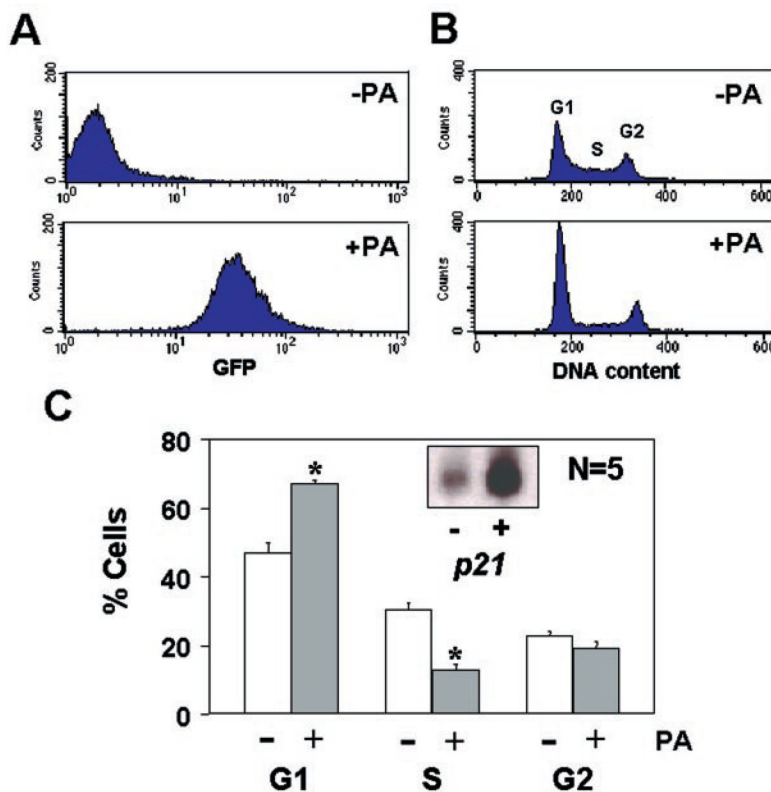


Fig. 4. The effect of induced KLF4 expression on the cell cycle

EcR-RKO/pAdLoxEGI-KLF4 cells were cultured to ~50% confluency and treated with or without 5 μ M ponasterone A for 24 h. Cells were fixed and stained with propidium iodide, and then analyzed by FACS. *Panel A* shows the result of FACS based on the intensity of green fluorescence in uninduced (-PA) and induced (+PA) cells. *Panel B* shows the DNA content as revealed by propidium iodide staining in both conditions. DNA contents corresponding to the 3 phases (G₁, S, and G₂) of the cell cycle are labeled as such. In *panel C*, the mean percentages of cells with DNA content in each of the 3 phases of the cell cycle under the uninduced or induced condition over five independent determinations were shown as mean \pm S.D. (bars). *, $p < 0.001$. Inset shows the result of a Northern blot analysis for p21^{WAF1/Cip1} in the absence (-) or presence (+) of ponasterone A.

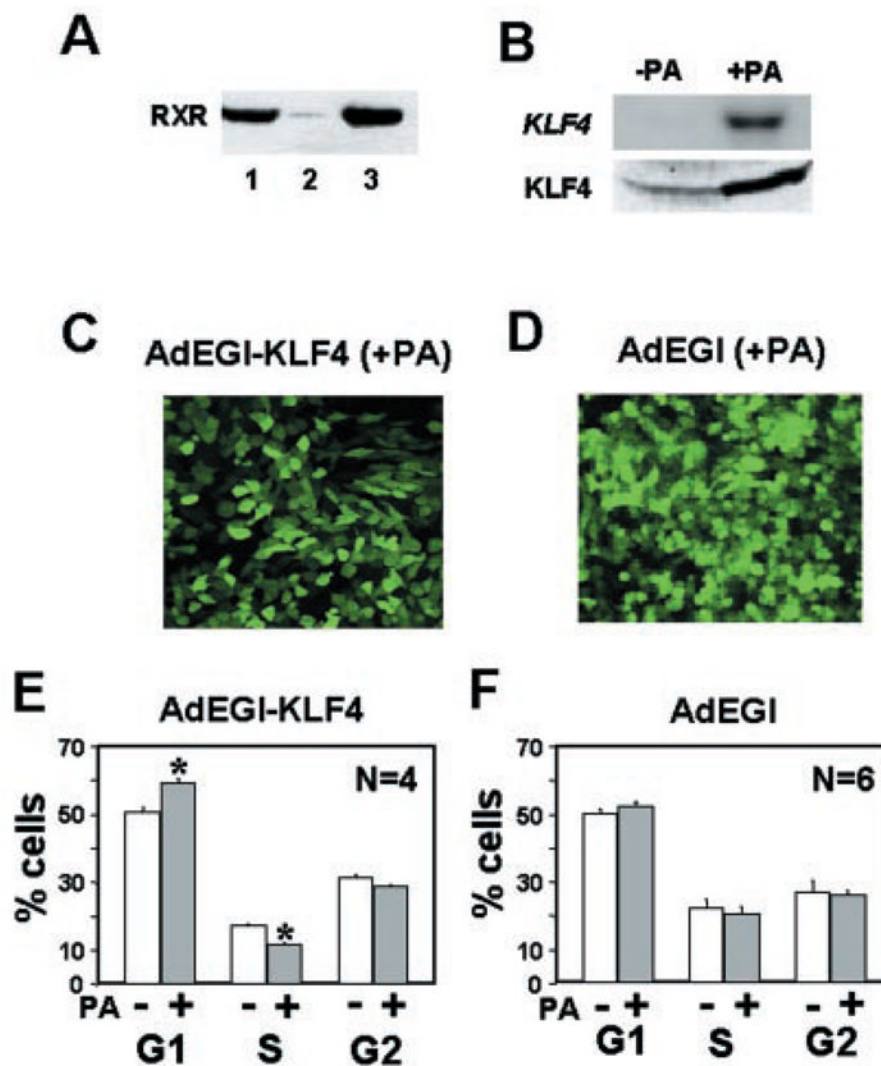


Fig. 5. The effect of inducible expression of KLF4 in cells infected with recombinant adenoviruses containing EGFP and KLF4 or EGFP alone

Panel A is a Western blot analysis for RXR production in CHO cells with stably transfected pVgRXR (*lane 1*), untransfected RKO cells (*lane 2*), and the stably transfected RKO cells, EcR-RKO (*lane 3*). In *panel B*, EcR-RKO cells were infected with the recombinant AdEGI-KLF4 virus and treated (+PA) or not (-PA) with ponasterone A for 24 h and then analyzed for the RNA (*upper panel*) and protein (*lower panel*) content for KLF4 by Northern and Western blotting, respectively. In *panels C* and *D*, EcR-RKO cells were infected with AdEGI-KLF4 and AdEGI, respectively, and treated with 5 μ M ponasterone A for 24 h. Shown are cells observed under a fluorescence microscope. In *panels E* and *F*, EcR-RKO cells were infected with AdEGI-KLF4 and AdEGI, respectively, and treated or not with 5 μ M ponasterone A for 24 h. Cells were stained with propidium iodide and the DNA content analyzed by FACS. Shown are the mean and S.D. of percentages of cells in the 3 phases of the cell cycle. *, $p < 0.001$.