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Cancer cell specific cytotoxic gene expression mediated by ARF tumor suppressor promoter constructs

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ABSTRACT

In current cancer treatment protocols, such as radiation and chemotherapy, side effects on normal cells are major obstacles to radical therapy. To avoid these side effects, a cancer cell-specific approach is needed. One way to specifically target cancer cells is to utilize a cancer specific promoter to express a cytotoxic gene (suicide gene therapy) or a viral gene required for viral replication (oncolytic virotherapy). For this purpose, the selected promoter should have minimal activity in normal cells to avoid side effects, and high activity in a wide variety of cancers to obtain optimal therapeutic efficacy.

In contrast to the AFP, CEA and PSA promoters, which have high activity only in a limited spectrum of tumors, the E2F1 promoter exhibits high activity in wide variety of cancers. This is based on the mechanism of carcinogenesis. Defects in the RB pathway and activation of the transcription factor E2F, the main target of the RB pathway, are observed in almost all cancers. Consequently, the E2F1 promoter, which is mainly regulated by E2F, has high activity in wide variety of cancers. However, E2F is also activated by growth stimulation in normal growing cells, suggesting that the E2F1 promoter may also be highly active in normal growing cells. In contrast, we found that the tumor suppressor ARF promoter is activated by deregulated E2F activity, induced by forced inactivation of pRB, but does not respond to physiological E2F activity induced by growth stimulation. We also found that the deregulated E2F activity, which activates the ARF promoter, is detected only in cancer cell lines. These observations suggest that ARF promoter is activated by E2F only in cancer cells and therefore may be more cancer cell-specific than E2F1 promoter to drive gene expression.

We show here that the ARF promoter has lower activity in normal growing fibroblasts and shows higher cancer cell-specificity compared to the E2F1 promoter. We also demonstrate that adenovirus expressing *HSV-TK* under the control of the ARF promoter shows lower cytotoxicity than that of the E2F1 promoter, in normal growing fibroblasts but has equivalent cytotoxicity in cancer cell lines. These results suggest that the ARF promoter, which is specifically activated by deregulated E2F activity, is an excellent candidate to drive therapeutic cytotoxic gene expression, specifically in cancer cells.

1. Introduction

In current cancer treatment regimens, such as radiation and chemotherapy, side effects on normal cells, especially normal proliferative cells, are problematic. One of the important consequences is immune deficiency caused by damage to immune cells, which may lead to death by infectious diseases. In many cases, the dose and efficacy of cancer therapy is limited by such adverse side effects. This is major obstacle to radical treatment of cancer, necessitating the development of more cancer cell specific strategies.

Among such approaches under development, suicide gene therapy and oncolytic virotherapy, which utilize cancer specific gene expression using cancer specific promoters, are focuses of interest. By regulating a cytotoxic gene such as *HSV-TK* or a pro-apoptotic gene under the control of cancer specific promoters, the gene is expressed specifically in cancer cells and causes cell death [1]. Alternatively, by regulating a viral gene required for viral replication under the control of these promoters, the gene is expressed specifically in cancer cells, allowing cell lysis induced

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by viral replication in a cancer cell-specific manner [2]. In these approaches, therapeutic effects and side effects are dependent on the relative promoter activity in cancer and normal cells. The higher the cancer specificity of the promoter, the higher the cancer specificity of the treatment. Therefore, it is important to use a promoter with optimal cancer cell specific activity.

For a promoter to be cancer specific, it should have two important characteristics. First, the promoter should have low activity in normal cells to avoid side effects and second, it should exhibit high activity in a wide variety of cancer cells for maximum therapeutic effectiveness. The alpha-fetoprotein (AFP) promoter [3], the carcinoembryonic antigen (CEA) promoter [4,5] and the prostate-specific antigen (PSA) promoter [6–8] have been used as cancer specific promoters. However, *AFP*, *CEA* and *PSA* genes are overexpressed only in specific types of cancers, suggesting that these promoters show high activity only a subset of cancer cells, limiting the utility of this approach.

To obtain high activity in a wide variety of cell types, the activity of the promoter should be based on changes generally detected in many types of cancers, such as defects in the RB pathway, one of the two major tumor suppressor pathways [9–11]. The pathway is composed of the tumor suppressor pRB, which is the main effector in this pathway, and upstream regulators Cyclins/Cyclin-dependent kinases (CDKs) and CDK inhibitors such as $p16^{INK4a}$. pRB plays an important role in tumor suppression by binding to the transcription factor E2F and decreasing expression of its target, growth-promoting genes. The tumor suppressor $p16^{INK4a}$ inhibits Cyclin D/CDK4, 6, which phosphorylates and inactivates pRB. In almost all cancers, defects in the RB pathway such as deletion or dysfunction of pRB or $p16^{INK4a}$, and overexpression of cyclin D1 or mutation of CDK4, which makes CDK4 insensitive to CDK inhibitors, are detected. Consequently, E2F regulation by pRB is disrupted and E2F activity up-regulated. The increased E2F activity has been utilized to specifically target cancer cells via the E2F1 promoter [12], a physiological target of E2F [13]. Since this approach is based on the mechanism of carcinogenesis, it is expected to be useful for wide variety of cancer cell types. However, pRB is also down-regulated by growth stimulation in normal cells, with consequent activation of the E2F1 promoter [13]. Therefore, to avoid side effects on normal growing cells, a more cancer specific promoter is required.

In contrast to growth-related E2F target promoters, which are activated by both physiological and deregulated E2F activity, the tumor suppressor ARF promoter is specifically activated by deregulated E2F activity. ARF is an upstream activator of the tumor suppressor p53 and the *ARF* gene plays an important role in sensing oncogenic signals in the p53 pathway. We reported that deregulated E2F activity induced by forced inactivation of pRB, which mimics dysfunction of the RB pathway, has distinct functional sequelae compared to physiological E2F activity induced by growth stimulation. Deregulated E2F activity, detected only in cancer cells [14], selectively activates the tumor suppressor *ARF* gene, which is not induced by physiologic E2F activity [14]. These observations suggest that the ARF promoter is activated by E2F only in cancer cells and that the ARF promoter may be more cancer cell-specific than E2F1 promoter. In this study, to examine utility of the ARF promoter in cancer specific therapeutic approaches, we compared the relative activity and specificity of the ARF and E2F1 promoters in a variety of normal and cancer cell lines.

2. Material and methods

2.1. Cell culture

Normal human fibroblasts (HFFs, WI-38) and human cancer cell lines (Saos-2, C-33 A, 293A, HeLa, H1299, A549, U-2 OS) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). Another human cancer cell line (5637) was cultured in RPMI 1640 medium containing 10% FCS.

2.2. Plasmid

pARF-Luc(-736) has been described [14]. pE2F1-Luc(-728) was made by subcloning E2F1 promoter in pGL2-Basic (Promega) [13] into pGL3-Basic (Promega). The expression vectors for E2F1 (pENTR-E2F1) [15] and the 2RG form of adenovirus 12S E1a [16], which binds to and inhibits all members of the pRB family but does not interfere with p300/CBP co-activators, have been described.

2.3. Luciferase assay

Transfection was performed using FuGENE 6 (Promega). To adjust reporter activity by difference of transfection efficiency between the samples, ARF and E2F1 luciferase reporter plasmids were co-transfected with a CMV promoter-driven *Renilla luciferase* construct as an internal control to monitor transfection efficiency. Luciferase and *R. luciferase* activities were determined using Dual-Luciferase Reporter Assay System (Promega), and luciferase activity was normalized to *R. luciferase* activity to adjust by the difference of transfection efficiency. All assays were repeated at least three times and values are shown as means ± SD.

2.4. Recombinant adenovirus

Ad-Con has been described [14]. pARF-TK and pE2F1-TK were generated by subcloning *HSV-TK* from pTK5 (RIKEN BRC) and ARF promoter from pARF-Luc(-736) or E2F1 promoter from pE2F1-Luc(-728) into pENTR (Life Technologies). Ad-ARF-TK and Ad-E2F1-TK were generated from pARF-TK and pE2F1-TK, respectively, using Vira Power Adenoviral expression system (Life Technologies) according to the supplier's protocol. Infection with recombinant adenoviruses proceeded as previously described [16].

2.5. FACS analysis

Cells were fixed with 70% ethanol and stained with propidium iodide (50 μg/ml) containing RNase (50 μg/ml). Cell samples were analyzed with a FACSCalibur (Becton Dickinson). All assays were repeated at least three times and values are shown as means ± SD.

3. Results

3.1. ARF promoter is more cancer cell-specific than E2F1 promoter

To investigate the potential use of the ARF promoter to cancer specific gene therapy, we evaluated the cancer cell-specific activity of the ARF promoter in comparison to the E2F1 promoter. We first examined the relative responsiveness of the ARF and E2F1 promoters to deregulated E2F activity induced by ectopic expression of E2F1 or forced inactivation of pRB by adenovirus E1a, which mimics dysfunction of the RB pathway, a prototype of oncogenic change. Promoter activity was assessed, using luciferase reporter constructs, in normal cells (human foreskin fibroblasts; HFFs). We monitored transfection efficiency by including a CMV promoter-driven *R. luciferase* construct as an internal control and normalized luciferase activity to that of *R. luciferase* to adjust by the difference of transfection efficiency. Each reporter plasmid was transfected into HFFs with or without expression vector for E2F1 or E1a, and fold activation by E2F1 or E1a was determined. In response to ectopic expression of E2F1, ARF and E2F1 promoters showed 75- and 16-fold activation, respectively (Fig. 1A). In

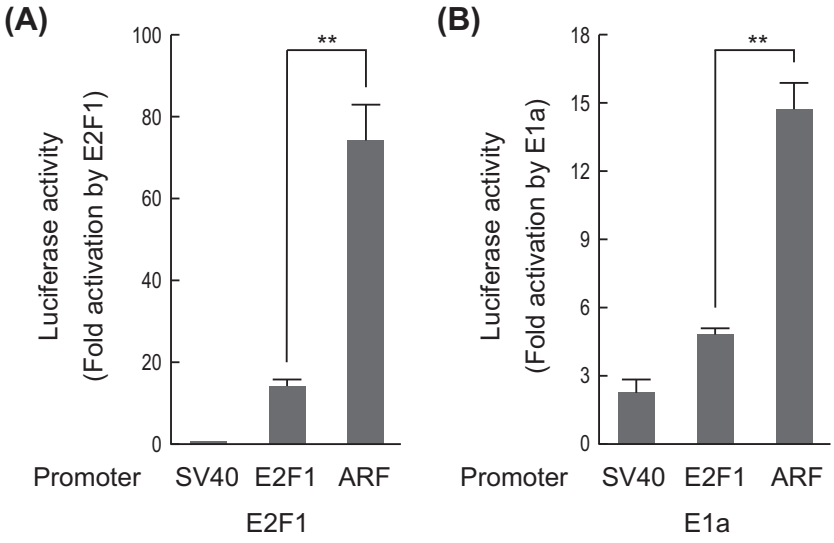

Fig. 1. The ARF promoter shows higher responsiveness to deregulated E2F activity than the E2F1 promoter. (A,B) HFFs were transfected with pE2F1-Luc(-740) or pARF-Luc(-736) reporter plasmid along with expression vector for E2F1 (A) or Δ2–11 form of adenovirus E1a (B). pRL-CMV, in which the *Renilla luciferase* gene is driven by CMV promoter, was included as an internal control to monitor transfection efficiency. pGL3-Prom, which has SV40 core promoter, was used as a negative control. The cells were cultured for 24 h after transfection and were harvested. Luciferase activity was measured, normalized by *Renilla luciferase* activity to adjust by transfection efficiency and presented as fold activation by E2F1 or E1a. $^{**}p < 0.01$.

response to E1a, ARF and E2F1 promoter showed 15- and 5-fold activation, respectively (Fig. 1B). These results indicate that the ARF promoter is more sensitive to deregulated E2F activity induced by ectopic expression of E2F1 and forced inactivation of pRB than the E2F1 promoter. Since deregulated E2F activity is retained in cancer cells by additional defects in the p53 pathway, these results also suggests that the ARF promoter may have high activity in cancer cells and show enhanced cancer cell specificity.

We thus examined whether the ARF promoter is more cancer cell-specific than the E2F1 promoter by comparing activity of both promoters in normal cells and cancer cells. For this purpose, we first examined activity of both promoters in human normal growing cells (HFFs) and the human cervical cancer cell line (C-33 A). To adjust reporter activity by difference of transfection efficiency between different cell lines, we included a CMV promoter-driven *R. luciferase* construct as an internal control and normalized luciferase activity to that of *R. luciferase*. In HFFs, the ARF promoter showed lower activity (about 19%) than the E2F1 promoter, as expected (Fig. 2A). On the other hand, in C-33 A, the ARF promoter showed similar activity (about 71%) to the E2F1 promoter (Fig. 2B). These data suggest that the ARF promoter has comparable activity to the E2F1 promoter in cancer cells, while exhibiting much weaker activity in normal growing cells. As an indicator of cancer cell-specificity of each promoter, we expressed fold promoter activity in a cancer cell line compared to that in normal cells according to the following equation.

Cancer specificity = activity in a cancer cell line/activity in normal cells

When cancer cell-specificity was calculated between C-33 A and HFFs, the ARF promoter showed higher cancer specificity (about 33-fold) than the E2F1 promoter (8-fold) (Fig. 3). These results suggest that the ARF promoter has lower activity in normal cells and may be more cancer cell-specific than the E2F1 promoter.

To confirm and extend these observations, we examined cancer cell-specificity in other 7 cancer cell lines originated from different tissue types (H1299, A549, HeLa, 293A, U-2 OS, Saos-2 and 5637) (Fig. 3). The ARF promoter showed higher cancer cell-specificity than the E2F1 promoter in 5 cancer cell lines including C-33 A (C-33 A, H1299, A549, HeLa, 293A) (Fig. 3). On the other hand, the E2F1 promoter showed higher cancer specificity than the ARF promoter only in one cancer cell line (5637) (Fig. 3). Therefore, the ARF promoter showed higher cancer cell-specificity than the E2F1 promoter in the majority of cancer cell lines. We also examined promoter activity in another human normal lung cell line (WI-38). In proliferating WI-38 cells, the ARF promoter also showed lower activity (about 19%) than the E2F1 promoter (Fig. 2A). Similar results were obtained, when cancer cell specificity was calculated with WI-38 as the baseline (Fig. 2A) (data not shown). These results suggest that ARF promoter may be more cancer cell-specific than E2F1 promoter with lower activity in normal cells, underscoring utility of the ARF promoter in cancer specific therapeutic approaches.

3.2. Ad-ARF-TK causes cell death more cancer cell-specifically than Ad-E2F1-TK

To examine whether the ARF promoter drives cytotoxic gene expression more specifically in cancer cells than the E2F1 promoter, we generated recombinant adenovirus expressing *HSV-TK* under the control of the ARF promoter (Ad-ARF-TK) or the E2F1 promoter (Ad-E2F1-TK). We examined cytotoxicity of the viruses by infecting normal cells and cancer cells with varying multiplicity of infection (MOI). The infected cells were cultured for 5 days in the presence of ganciclovir (GCV). Cells expressing HSV-TK should be killed in 5 days by metabolizing inactive GCV to an active form. Cytotoxicity was judged by % of population of cells with sub-G1 DNA content, which represents apoptotic cells (Fig. 4). As normal and cancer cells, HFFs and the 7 cancer cell lines (C-33 A, H1299, A549, HeLa, U-2 OS, Saos-2, 5637) were used, respectively. In normal HFFs, cells infected with Ad-ARF-TK showed a significantly lower population of cells with sub-G1 DNA content (about 3, 5, 6% at MOI 10, 25, 50, respectively) than those infected with Ad-E2F1-TK (about 5.5, 10.5, 15% at MOI 10, 25, 50, respectively). On the other hand, in 6 cancer cell lines (C-33 A, H1299, HeLa, U-2 OS, Saos-2, 5637), the cells infected with Ad-ARF-TK and Ad-E2F1-TK showed almost same % of population of cells with sub-G1 DNA content. In one cancer cell line (A549), the cells infected with Ad-ARF-TK showed higher sub-G1 population than

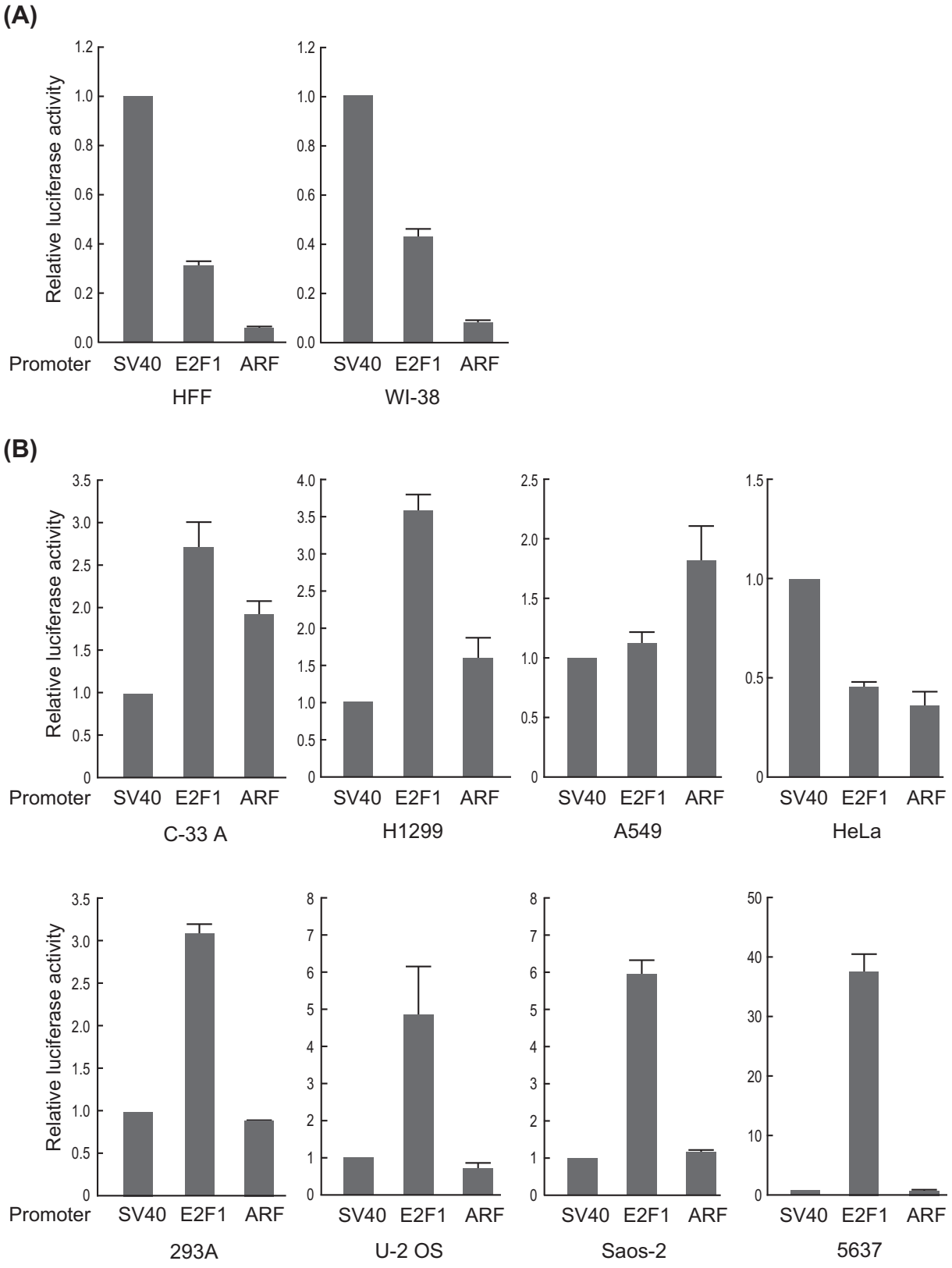

Fig. 2. ARF promoter shows lower activity in normal growing cells and relatively higher activity in cancer cell lines than E2F1 promoter. (A and B) Normal growing cells (HFFs and WI-38) and cancer cell lines (C-33 A, H1299, A549, HeLa, 293A, U-2 OS, Saos-2 and 5637) were transfected with pE2F1-Luc(-740) or pARF-Luc(-736) reporter plasmid. pGL3-Prom was used as a reference and pRL-CMV was included as an internal control to monitor transfection efficiency. The cells were cultured for 1 day and harvested. Luciferase activity was measured, normalized by *Renilla luciferase* activity to adjust by transfection efficiency and presented as relative activity to that of SV40 core promoter.

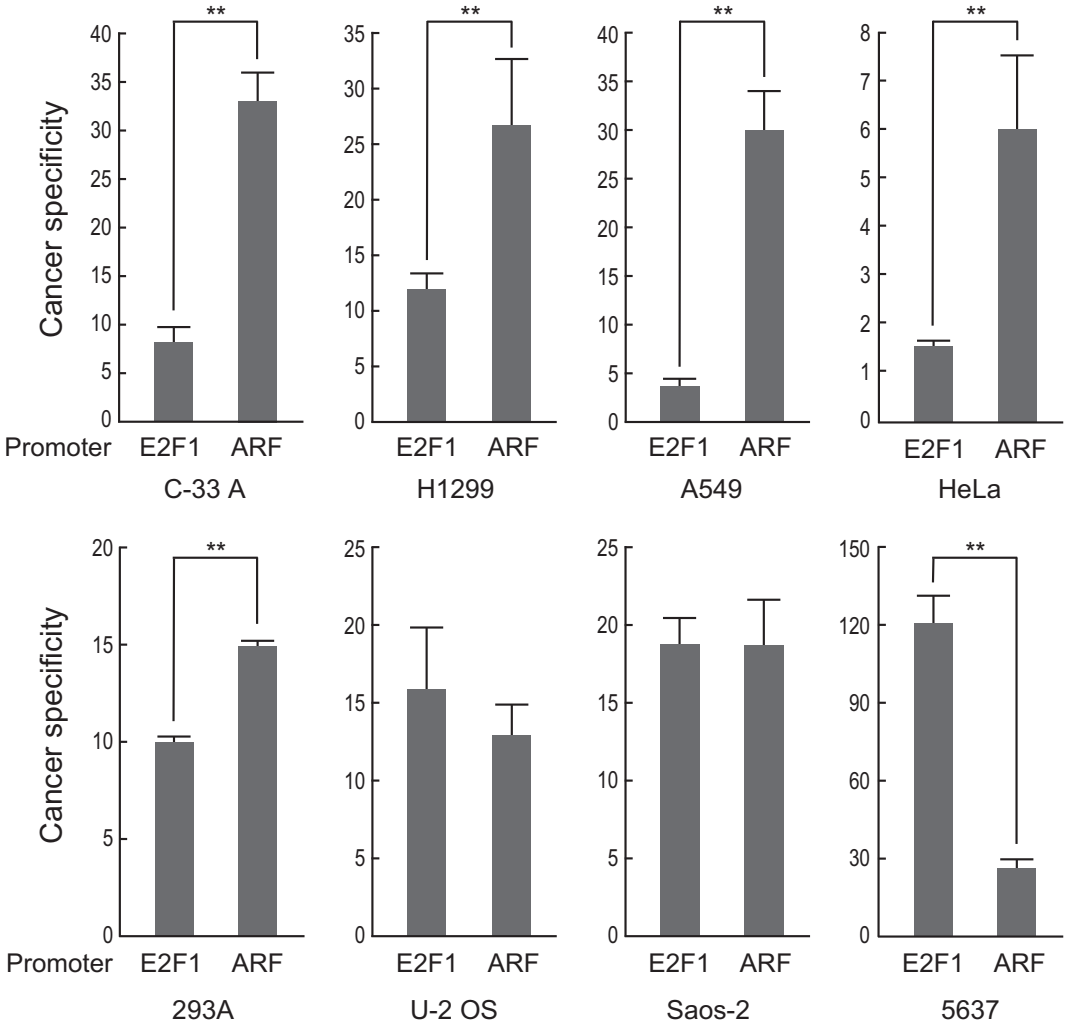

Fig. 3. The ARF promoter shows higher cancer cell-specificity than the E2F1 promoter. Cancer specificity is determined as promoter activity in a cancer cell line divided by that in a normal cell line. Cancer specificity is calculated between 8 cancer cell lines (Saos-2, C-33 A, 293A, HeLa, H1299, U-2 OS, 5637 and A549) and HFFs as normal cells. $^{**}p < 0.01$.

that with Ad-E2F1-TK. These results suggest that Ad-ARF-TK shows comparable or higher cytotoxicity than Ad-E2F1-TK, in cancer cell lines and lower cytotoxicity in normal growing cells. We conclude that the ARF promoter construct is a useful vehicle to mediate cancer specific cytotoxic gene expression and facilitate therapeutic targeting of a variety of malignancies.

4. Discussion

For suicide gene therapy and oncolytic virotherapy, an ideal promoter should have low activity in normal cells to avoid side effects, and high activity in a wide variety of cancer cell types for therapeutic efficacy. In this study, we demonstrated that the ARF promoter exhibits both of these characteristics. In reporter assays using normal growing fibroblasts (HFFs, WI-38), the ARF promoter showed significantly lower activity (19%) than the E2F1 promoter, which has previously been used to drive gene expression specifically in various types of cancers (Fig. 2A). In addition, the ARF promoter showed relatively higher activity in many cancer cell lines compared to normal growing fibroblasts, resulting in a higher cancer cell-specificity than the E2F1 promoter (Fig. 3). Consistent with the results of reporter assays, recombinant adenovirus expressing HSV-TK under the control of ARF promoter (Ad-ARF-TK) showed lesser cytotoxicity than the adenovirus expressing HSV-TK under the control of the E2F1 promoter (Ad-E2F1-TK) in normal growing cells. Most importantly, Ad-ARF-TK killed cancer cells as efficiently as Ad-E2F1-TK (Fig. 4), even in cancer cell lines, in which ARF promoter had lower activity than the E2F1 promoter (Fig. 2B). These observations support the suitability of the ARF promoter for cancer cell-specific therapeutic approaches.

In contrast to the CEA, AFP and PSA promoters, which show strong promoter activity in a limited subset of cancers, the E2F1 promoter displays strong promoter activity in wide variety of cancers, consistent with the underlying mechanism of carcinogenesis. Defects in the RB pathway are observed in almost all cancers and, as a consequence, activity of E2F, the main target of the RB pathway, is enhanced. Expression of the *E2F1* gene is regulated by E2F itself [13], resulting in high E2F1 promoter activity to drive gene expression in wide variety of cancers. However, E2F is also activated in normal growing cells by growth stimulation, suggesting that this approach may also affect normal cells. Indeed, Ad-E2F1-TK killed normal growing fibroblasts at high MOI (Fig. 4). To circumvent this problem, we focused on the ARF promoter, which is specifically activated by deregulated E2F activity induced by dysfunction of pRB. We found that the ARF promoter has weaker activity than the E2F1 promoter in normal growing

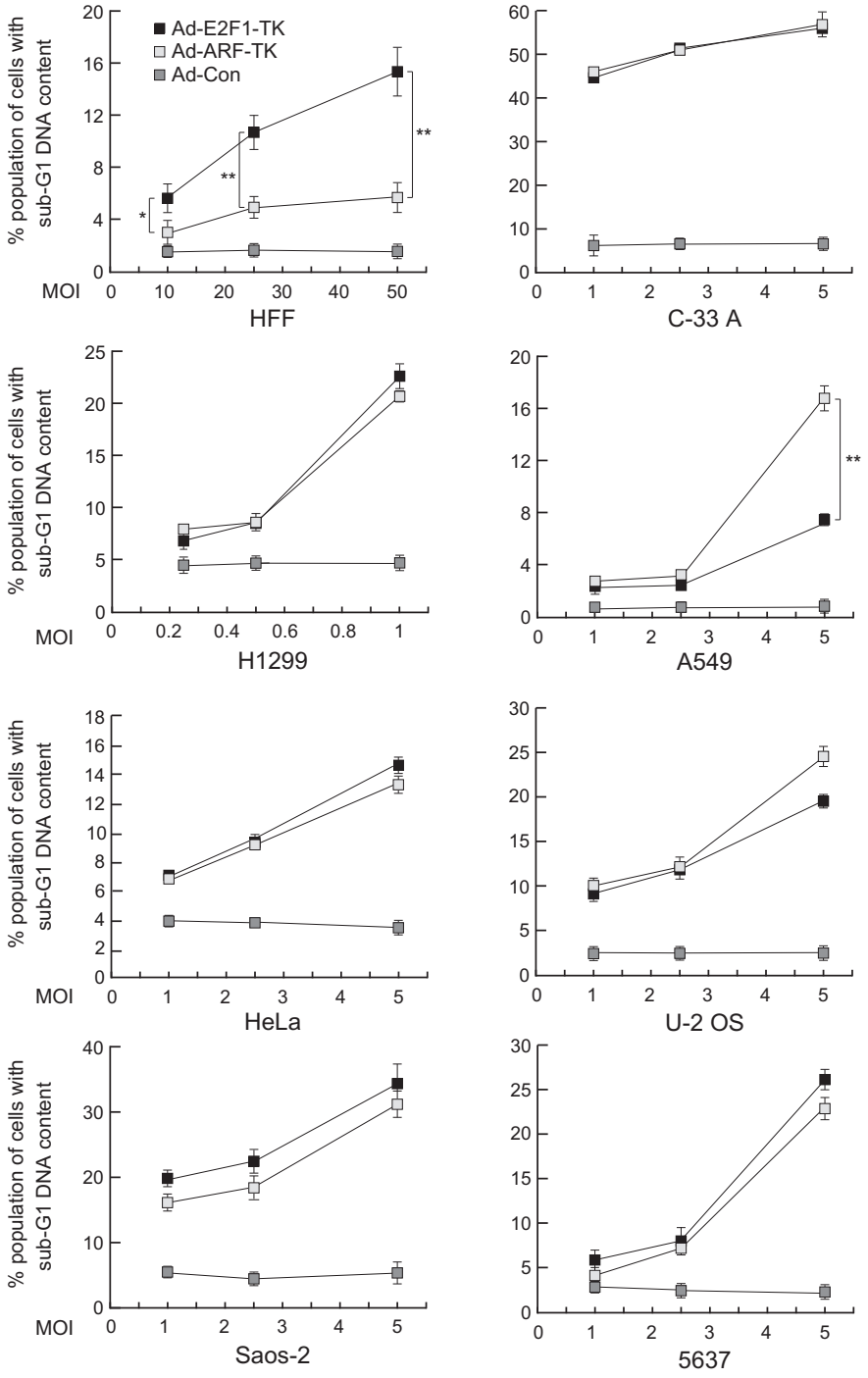

Fig. 4. Ad-ARF-TK shows lower cytotoxicity than Ad-E2F1-TK in normal growing cells and equivalent cytotoxicity to Ad-E2F1-TK in cancer cell lines. HFFs were infected with recombinant adenovirus expressing *HSV-TK* under the control of the ARF promoter (Ad-ARF-TK) or the E2F1 promoter (Ad-E2F1-TK) at MOI 10, 25 and 50. Recombinant adenovirus harboring the CMV promoter without an insert (Ad-Con) was used as a control virus. The cells were cultured for 5 days in the presence of ganciclovir (GCV) (50 μM) and harvested. The cells were fixed with 70% ethanol and stained with propidium iodide. Percentage of cells with sub-G1 DNA content was measured by FACS analysis. Cancer cell lines (C-33 A, A549, HeLa, U-2 OS, Saos-2 and 5637) were infected with Ad-ARF-TK, Ad-E2F1-TK or Ad-Con at MOI 1, 2.5 and 5. A cancer cell line (H1299) was similarly infected at MOI 0.25, 0.5 and 1. The cells were cultured for 5 days in the presence of GCV (50 μM) and harvested. Percentage of cells with sub-G1 DNA content was similarly determined. $^{*}p < 0.05$, $^{**}p < 0.01$.

fibroblasts (Fig. 2A). This suggests that using the ARF promoter reduces the likelihood of cytotoxicity in normal cells. Indeed, Ad-ARF-TK showed weaker cytotoxicity than Ad-E2F1-TK in normal growing fibroblasts (HFFs) (Fig. 4). In order to obtain the same % of population of cells with sub-G1 DNA content in HFFs, a three fold higher MOI is required for Ad-ARF-TK compared to Ad-E2F1-TK. Since the cytotoxicity of Ad-ARF-TK in the normal cells is lower than that of Ad-E2F1-TK, dosage of Ad-ARF-TK may be increased to obtain therapeutic effects; a potential advantage of using the ARF promoter in cancer-specific oncolytic or gene therapeutic strategies.

The relatively high ARF promoter activity observed in many cancer cell lines and consequent enhanced cancer cell specificity compared to the E2F1 promoter may be due to the increased

sensitivity of the ARF promoter to deregulated E2F activity. Our results showed that both the ARF promoter and the E2F1 promoter were activated by deregulated E2F activity induced by ectopically expressed E2F1 and forced inactivation of pRB by adenovirus E1a in normal human fibroblasts, HFFs (Fig. 1A and B). Remarkably, the ARF promoter showed higher responsiveness to deregulated E2F activity than the E2F1 promoter (Fig. 1A and B). On the other hand, the E2F1 promoter is activated by physiological E2F activity induced by growth stimulation [13], whereas the ARF promoter is not [14]. This may contribute to the lower activity of ARF promoter in normal growing cells and higher responsiveness to deregulated E2F activity in cancer cell lines. Since global ARF promoter activity is very low in the mouse [17], the ARF promoter may also have similar low activity in humans. The relatively low activity of the ARF promoter in normal cells may, in part, underlie its improved cancer cell specificity and reduced potential side effects.

In 5637 cancer cell line, the E2F1 promoter showed exceptionally high cancer cell specificity, exceeding that of the ARF promoter (Fig. 3). It is reported that, in 5637 cancer cell line, the *E2F3* gene is amplified and E2F3 is over-expressed [18]. Among activator E2Fs, E2F3 is a strong activator of growth-promoting genes including the *E2F1* gene, which may explain the unusually high cancer cell specificity of the E2F1 promoter in 5637 cell line. However, amplification of the *E2F3* gene is not commonly detected in many types of cancers and the cancer specificity of the ARF promoter in 5637 cell line is also much higher than that seen in other cancer cell lines (Fig. 3). Therefore, the observed higher cancer specificity of the E2F1 promoter in 5637 cell line does not undermine the proposed utility of the ARF promoter in cancer specific therapeutic approaches.

Consistent with activities of the ARF and E2F1 promoters in HFFs, Ad-ARF-TK showed significantly lower cytotoxicity than Ad-E2F1-TK in HFFs (Fig. 4). Moreover, Ad-ARF-TK killed cancer cell lines as efficiently as Ad-E2F1-TK (Fig. 4). Surprisingly, this was also observed even in cancer cell lines, in which the ARF promoter showed lower activity than E2F1 promoter in reporter assays, such as C-33 A, H1299, U-2 OS, Saos-2 and 5637 (Fig. 2B and 4). This apparent discrepancy may be a reflection of the use of circular plasmid DNA versus the linear adenoviral genome or a certain level of HSV-TK expression being sufficient for induction of cell death in these cells. However, the evident lower cytotoxicity of Ad-ARF-TK in normal HFFs and equivalent induction of apoptosis in cancer cells clearly support our hypothesis that the ARF promoter construct is a more promising, cancer cell-specific targeting vector.

We previously reported that the ARF promoter is specifically activated by deregulated E2F activity [14]. In this report, our results suggest that the ARF promoter is more cancer cell-specific than the E2F1 promoter and that Ad-ARF-TK shows similar cytotoxicity in cancer cell lines compared to Ad-E2F1-TK, but markedly lower side effects on normal proliferating cells. Based upon these observations, we propose that ARF promoter constructs provide a means to specifically drive gene expression in cancer cells to mediate suicide gene therapy or viral oncolysis.

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