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RESEARCH ARTICLE

The cyclo-oxygenase 2 promoter is induced in nontarget cells following adenovirus infection, but an AU-rich 3′-untranslated region destabilization element can increase specificity

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Abstract

Background Cyclo-oxygenase 2 (Cox-2) is expressed in many types of tumors, but typically undetectable in normal tissues. However, Cox-2 is known to be induced following infection by many microbial agents, which might threaten the tumor selectivity of the Cox-2 promoter in the context of virotherapy or viral gene delivery. Cox-2 expression is regulated in part post-transcriptionally by stimulation or inhibition of mRNA degradation by 3′-untranslated region (3′-UTR) AU-rich elements. In the present study, we investigated the induction of the Cox-2 promoter both in normal and tumor cells after adenovirus infection and explored the utility of AU-rich elements for regaining promoter selectivity.

Methods Nontumor and tumor cells were transfected *in vitro* and *in vivo* with plasmids containing the Cox-2 or cytomegalovirus immediate early promoter driving luciferase (with or without 3′-UTR elements) followed by adenoviral infection. Selectivity and activity of the promoters and 3′-UTR elements were analysed by luciferase assay and in-vivo imaging.

Results The Cox-2 promoter was induced in both normal and tumor cells following infection with E1 containing replicative adenoviruses but not in the absence of E1. Utilization of AU-rich elements counteracted promoter induction *in vitro* and *in vivo* in nonmalignant cells but not in cancer cells, thus increasing the selectivity of the approach ten-fold without loss of potency.

Conclusions Adenoviral infection induces the Cox-2 promoter in normal and tumor cells, which might compromise specificity of the promoter. Utilization of AU-rich destabilization elements can rescue the tumor selectivity of the promoter.

Keywords adenovirus; cancer gene therapy; cyclo-oxygenase 2; promoter; 3′-UTR; AU rich element

Introduction

Adenoviral gene transfer might be useful for treatment of various diseases. However, trials have demonstrated the inherent toxic potential of unregulated high-dose gene delivery and expression [1].

Therefore, selective expression of genes in target cells would improve the safety of many approaches [2,3]. Furthermore, this might allow utilization of a higher dose for improved efficacy. Viruses can be targeted on a transductional and transcriptional level [4,5]. The former implies restriction of viral entry to target cells, whereas the latter involves selective gene expression in the cells of interest.

The most common approach to realize transcriptional targeting is utilization of tissue or tumor specific promoters [6]. Although a large number of promoters have been used with apparent success, the cyclo-oxygenase (Cox-2) promoter has been among the most popular candidates [7–15]. Cox-2 catalyses the conversion of arachidonate to prostaglandin H (PGE-2), which is the rate limiting step in prostaglandin biosynthesis. Cox-2 expression is stimulated by growth factors, cytokines and extracellular stimuli, and high Cox-2 levels are found in areas of inflammation, infection and injury [16–18]. However, the utility of the Cox-2 promoter for cancer gene therapy stems from high Cox-2 expression in many type of advanced tumors [19]. Moreover, in many tumor types, Cox-2 expression appears to correlate with tumor aggressiveness and inversely with prognosis [20]. Conceivably, sustained Cox-2 expression at high levels is useful for carcinogenesis. Mechanisms include stimulation of angiogenesis and metastatic potential, whereas immune recognition and apoptosis are inhibited [21].

The Ras/mitogen-activated protein kinase (MAPK) group of pathways is among the central growth control pathways and may be aberrant in most if not all human solid tumors. Mutations may occur in Ras, or on other levels of the signal transduction chain, which include Rac-1/MEKK1/JNK, Raf-1/MEK/ERK, PI3-K/Akt and p38/MAPK. A common downstream effect of these changes is high expression of Cox-2, which typically associates with high activity of the Cox-2 promoter [22–24].

For these reasons, the Cox-2 promoter has been a useful approach for transcriptional targeting of cancer gene therapy. However, because Cox-2 is physiologically induced following infection and inflammation, infection of normal cells by adenoviruses might induce Cox-2 and thereby counteract tumor selectivity of the promoter [9]. In the worst case, this could lead to expression of the transgene or replication of a Cox-2 controlled oncolytic virus in also normal cells, which could cause toxicity.

In addition to transcriptional upregulation of Cox-2, studies have shown that Cox-2 is also regulated on a post-transcriptional level by the RAS/p38/MAPK pathway [25]. Regulation of Cox-2 mRNA stability is mediated in part through AU-rich elements within the 3′-untranslated region (3′-UTR). Such elements, often present in 3′-UTRs of many proto-oncogenes and cytokines mRNAs, are often referred to as mRNA instability determinants [26,27]. The physiological function of the element used here is to rapidly downregulate Cox-2 mediated signalling, when the threat (e.g. infection) has passed. However, carcinogenesis has learned to take advantage of this mechanism, and the Cox-2 mRNA is unphysiologically stabilized through Ras pathway defects affecting the 3′-UTR AU rich elements in tumor cells [23,26,28].

In the present study, we sought to determine whether adenovirus infection *per se* results in induction of the Cox-2 promoter in tumor and normal cells. Furthermore, we hypothesized that specificity might be regained by utilization of 3′ AU-rich destabilization elements [29].

Materials and methods

Cell lines

Human NSCLC cell line A549 (ATCC CCL-185) and human transformed embryonic kidney cell line 293 (ATCC CRL-1573) were obtained from ATCC (Manassas VA, USA). Human fibroblasts Hs 173We (ATCC CRL-7834) were kindly provided by Gerd Bauerschmitz, University of Helsinki, Finland). Cell lines were cultured as recommended. 293 and A549 cells were used *in vivo*, because they form tumors and allow effective (293) or adequate (A549) levels of plasmid transduction.

Plasmid constructs

Luciferase containing plasmid constructs were created as follows: for construction of pShuttleCox-2Lluc and pShuttleCox-2Lluc + 3′-UTR, the Cox-2 l promoter (−1492/+59) was amplified by polymerase chain reaction (PCR) from full-length Cox-2 contained pGL3/Cox-2 (from Ari Ristimäki, University of Helsinki, Finland) using forward primer 5′-AAAAGATCTGAGGTACCTGGT-3′ to place a *Bgl*II site at 5′-end of the Cox-2 l and reverse primer 5′-AAAAAGCTTCGCTGCTGAGGA-3′ to place a *Hind*III site at 3′-end. This fragment was called Cox-2 l to allow comparison to previous reports [8,13,30]. A 603-bp long 3′-UTR fragment from Cox-2 was amplified from pZeo/Luc3 + 3′-UTR (kind gift from Ari Ristimäki, University of Helsinki, Finland), which contains the full-length UTR using forward primer 5′-AAAATCTAGAAAGTCTAATGATC-3′ and reverse 5′-AAAATCTAGAAACTTTAAG-3′ to place *Xba*I at both ends. The Cox-2 l promoter and 3′-UTR were cloned into luciferase containing plasmid pGL3basic (Promega, Madison, WI, USA). The final constructs: pShuttleCox-2Lluc and pShuttleCox-2Lluc + 3′-UTR were created by digesting pGL3Cox-2Lluc and pGL3Cox-2Lluc + 3′-UTR with *Kpn*I and *Sal*I and subcloning into pShuttle (Stratagene, La Jolla, CA, USA). pShuttleCMVluc and pShuttleCMVluc + 3′-UTR were also created: the luciferase gene and 3′-UTR were digested with *Kpn*I and *Sal*I from pGL3basic and inserted into pShuttleCMV (Stratagene). All plasmids were purified by agarose gel electrophoresis.

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Semiquantitative PCR

Transfected Hs173We cells were incubated with Proteinase K (Qiagen, Valencia, CA, USA) at 37 °C for 30 min. Proteinase K was then inactivated by 20 min of incubation at 95 °C. Amplification (30, 40 and 60 cycles, annealing at 50 °C) of a 77-bp DNA fragment was carried out by using primers that were positioned at the left end of adenovirus genome, between the left inverted terminal repeat and the packaging signal. Upstream: 5′-GCGACGGATGTGGCAAAAGT-3′ and downstream: 5′-CCTAAAACCGCGCGAAAA-3′.

Recombinant adenoviruses

One replication deficient and two conditionally replicative oncolytic adenoviruses were used in addition to wild-type adenovirus (Ad300wt, ATCC). Ad5-LacZ contains a β-galactosidase gene in a place of deleted E1 region. Ad5-Δ24E3 has a 24-bp deletion in the constant region 2 region of the E1A, resulting in selective replication in cells where the Rb/p16 pathway is defective, whereas Ad5/3-Δ24 is similar but features a fiber chimeric for the serotype 3 knob for enhanced infectivity of tumor cells [31,32]. Replication deficient viruses were propagated on 293 and oncolytic viruses on A549 cells. All viruses were purified with standard cesium chloride gradients. The viral particle (vp) concentration was measured at 260 nm and standard plaque assay was performed to determine functional units. For cell types that cannot be effectively infected with Ad300wt, Ad5/3-Δ24 was sometimes used instead.

In vitro transfection

Transfections of 293, Hs173we, and A549 cells were accomplished by plating 1.5×10^5 cells into 12-well plates 17–20 h prior to transfection; 1 μg of each plasmid were transfected with Fugene 6 reagent (Roche, Basel, Switzerland) in a ratio of 3 : 2 (Fugene 6/plasmid) following the manufacturer's instructions.

In vitro luciferase assay

24 h (293 cells and fibroblasts) or 48 h (A549 cells) after transfection, cells were infected at 10 and 1000 vp/cell in 2% GM in 24-well plates for 30 min at room temperature. Afterwards, cells were washed once with 10% medium and complete medium was added and incubation continued at 37 °C for 24 h before cells were lysed and luciferase assay was performed by the Luciferase Assay System (Promega).

In vivo studies

Female NMRI nude mice were purchased from Taconic (Ejby, Denmark); 1×10^6 293 and 5×10^5 A549 cells, respectively, were injected subcutaneously into both flanks of 6-week-old mice. Tumors were allowed to develop for 30 days (293) or 14 days (A549) to reach 5 mm in diameter and thereafter transfected with 10 μg of plasmid. Transfection was done by using *in vivo*-JetPei cationic polymer transfection reagent (Poly transfection, Illkirch, France) following the manufacturers protocol. Twenty-four hours after transfection, 5×10^9 vp/tumor of Ad300wt in 100 μl was injected intratumorally. Tumors were collected for luciferase analysis as described previously [33]. All animal experiments were approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland.

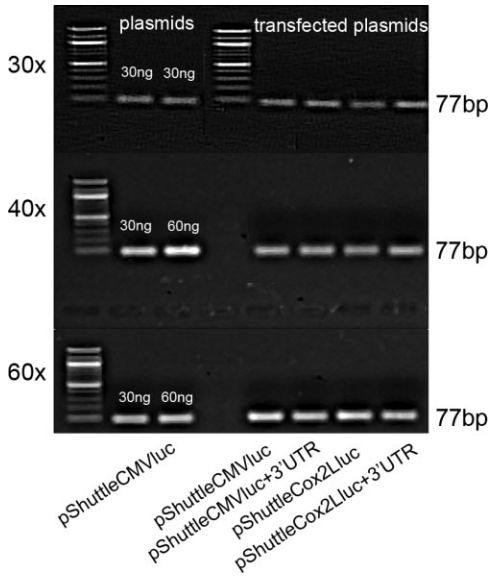

Figure 1. Semiquantitative PCR was performed to check that each plasmid construct is transfected at the same level, as expected due to identical plasmid backbones. PCR reaction was performed for 30, 40 and 60 cycles with identical results between plasmids. To confirm that the sensitivity of the assay was sufficient for detection of small changes in plasmid DNA, 60 ng of DNA was compared to 30 ng in the ×40 and ×60 experiments, and a clearer signal could be seen in the 60 ng lane

In vivo imaging

Tumors were imaged for luciferase expression on the day of transfection (day 0; data not shown), on the day of Ad300wt injection (day 1) and 24 h later (day 2). Before imaging, 4.5 mg of D-luciferin (Promega) was injected intraperitoneally in a total volume of 100 μl of 0% RPMI growth medium. After 15 min, images were taken by IVIS Imaging System Series 100 (Xenogen, Alameda, CA, USA).

Statistical analysis

Data are reported as the means ± SE. Data were transformed to the log scale for normality if indicated. Pairwise comparisons were performed with the two tailed

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Student's *t*-test. In the animal experiment, Multtest, version 9.1 (SAS Institute Inc., Cary, NC, USA) was used to examine change in means. $p < 0.05$ was considered statistically significant.

Results

The Cox-2 promoter is induced in nonmalignant and malignant cells following adenoviral infection

Transfection efficiency between different plasmids was checked using a semiquantitative PCR method (Figure 1) Following transfection of 293 cells with luciferase containing plasmid pShuttleCox-2Lluc, infection with a wild-type adenovirus (Ad5wt) or a conditionally replicating virus (Ad5-Δ24E3) resulted in significant Cox-2 induction ($p < 0.05$ and $p < 0.01$, respectively; Figures 2A and 2B). In addition, infection with Ad5LacZ appeared to induce Cox-2 in these E1 containing cells, which allows replication of E1-deleted viruses ($p < 0.05$; Figure 2C).

To investigate whether Cox-2 induction by adenovirus was a cell type specific effect, human fibroblasts were transfected and then infected. Induction of the Cox-2 promoter was again seen with both replication competent viruses ($p < 0.01$; Figures 3A and 3B). By contrast to 293 cells, there was no induction of the promoter following infection with Ad5LacZ in these cells, which do not allow replication of E1 deleted viruses (Figure 3C).

A549 human lung cancer cells, which express a relatively high level of Cox-2 [34], were transfected and then infected to investigate whether Cox-2 expression

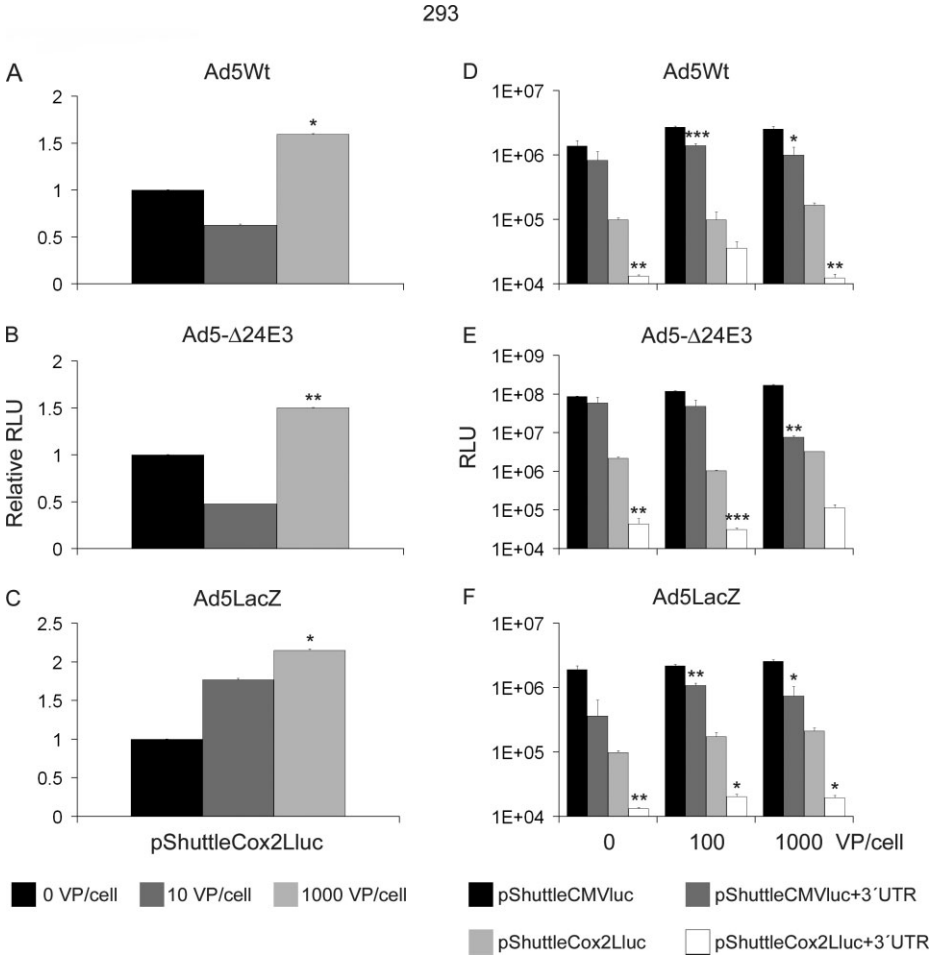

Figure 2. The Cox-2 promoter is induced in nonmalignant 293 cells following adenoviral infection, but induction can be downregulated by 3′-UTR AU-rich elements. Cells were transfected with a plasmid (pShuttleCox2Lluc) featuring the Cox-2 promoter controlling luciferase (A–C). The plasmid transfected cells were then infected with a wild-type adenovirus Ad5wt (A, D), a selectively oncolytic adenovirus Ad5-Δ24E3 (B, E), or an E1 deleted adenovirus (C, F). An increase in luciferase (relative light units, measured at 24 h) indicates induction of the Cox-2 promoter following adenoviral infection. To study the effects of the UTR, cells were transfected with Cox-2 plasmids with or without the UTR (D-F). To determine whether the effects of the 3′-UTR were promoter specific, also plasmids featuring the CMV promoter were transfected (pShuttleCMVluc, pShuttleCMVluc + 3′-UTR). All viruses induced the Cox-2 promoter, and the 3′-UTR element was effective in downregulating promoter induction. Note that 293 cells stably express E1. Error bars indicate the SEM, $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus no virus (A–C) or no UTR (D–F). VP, virus particles; RLU, relative light units (D–F). Relative RLU = light emitted by plasmid transfected cells without virus (A–C)

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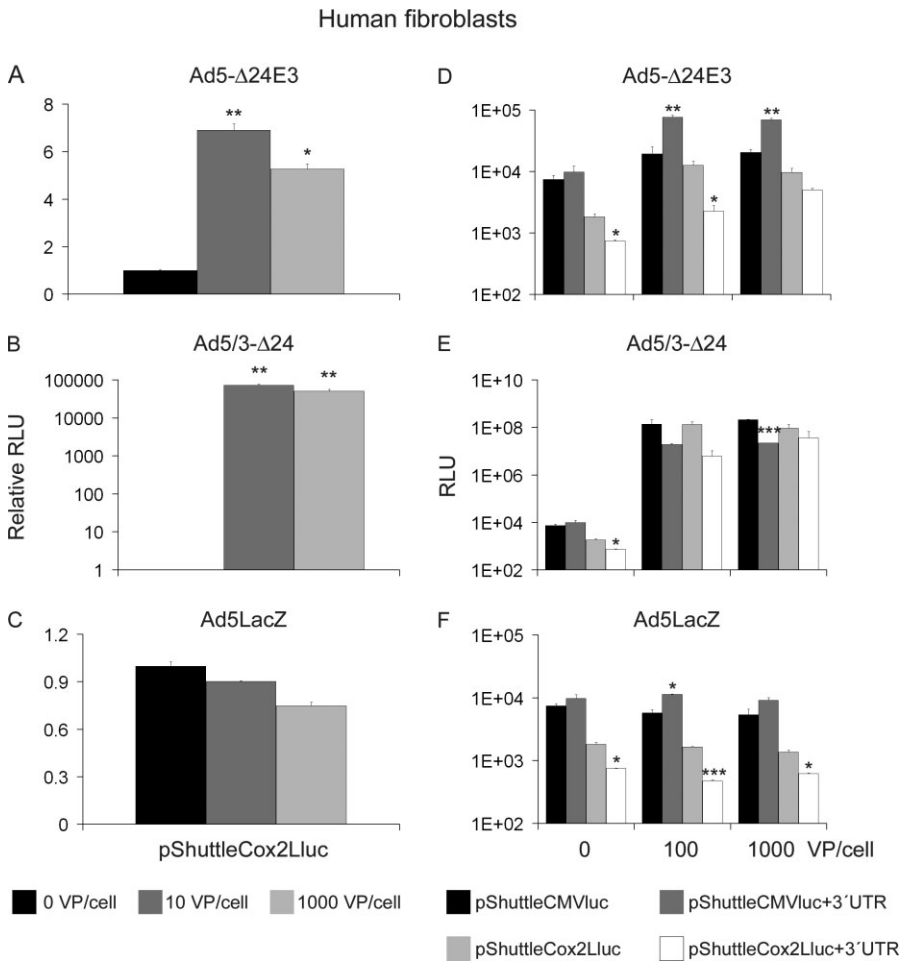

Figure 3. The Cox-2 promoter is induced in human fibroblasts following infection with E1 containing adenoviruses but not following infection with E1 deleted virus. Cox-2 induction can be downregulated by 3′-UTR AU-rich elements. Cells were transfected with plasmids featuring the Cox-2 promoter controlling luciferase (pShuttleCox2Lluc) (A–C). The plasmid transfected cells were then infected with selectively oncolytic adenoviruses Ad5-Δ24E3 (A, D) and Ad5/3-Δ24 (B, E), or an E1 deleted adenovirus (C, F). An increase in luciferase (relative light units, measured at 24 h) indicates induction of the Cox-2 promoter following adenoviral infection. To study the effects of the UTR, cells were transfected with Cox-2 plasmids with or without the UTR (D–F). To determine whether the effects of the 3′-UTR were promoter specific, also plasmids featuring the CMV promoter were transfected (pShuttleCMVluc, pShuttleCMVluc + 3′-UTR). Only E1 containing viruses induced the Cox-2 promoter, and the 3′-UTR element was effective in downregulating promoter induction. Error bars indicate the SEM, $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus no virus (A–C) or no UTR (D–F). VP, virus particles; RLU, relative light units (D–F). Relative RLU = light emitted by plasmid transfected cells without virus (A–C)

would be increased further by adenoviral infection. Replicating virus Ad5/3-Δ24E3 induced Cox-2 ($p < 0.001$), whereas Ad5-Δ24E3 and the nonreplicating virus did not (Figures 4A to 4C).

A 3′-UTR mRNA destabilization element can abrogate adenoviral induced Cox-2 expression in nonmalignant cells but has little effect in malignant cells

When the mRNA destabilization element was placed 3′ of the luciferase gene and 293 cells were infected, viral induction of the Cox-2 promoter could be abrogated. In other words, no significant induction was seen after adenovirus infection, in contrast to the same construct without the UTR element (Figures 2D to 2F). However, induction appeared to be somewhat dependant on conditions because some viral doses seemed to result in a low level of induction, perhaps due to the inherent E1 sequences present in these cells. Nevertheless, luciferase levels following adenoviral infection were always much lower when the UTR was used and the UTR element reduced the level of induction of the Cox-2 promoter (Figures 2D to 2F).

In fibroblasts, the UTR element was not able to completely prevent induction of the Cox-2 promoter. However, a relative reduction in Cox-2 promoter induction was seen, as measured by luciferase expression from the plasmids (Figures 3D to 3F).

The RAS/MAPK pathway is usually aberrant in cancer cells, including A549 cells [35], and therefore mRNA destabilization mediated by AU-rich elements in the 3′-UTR is not expected to occur. This should result in lack of

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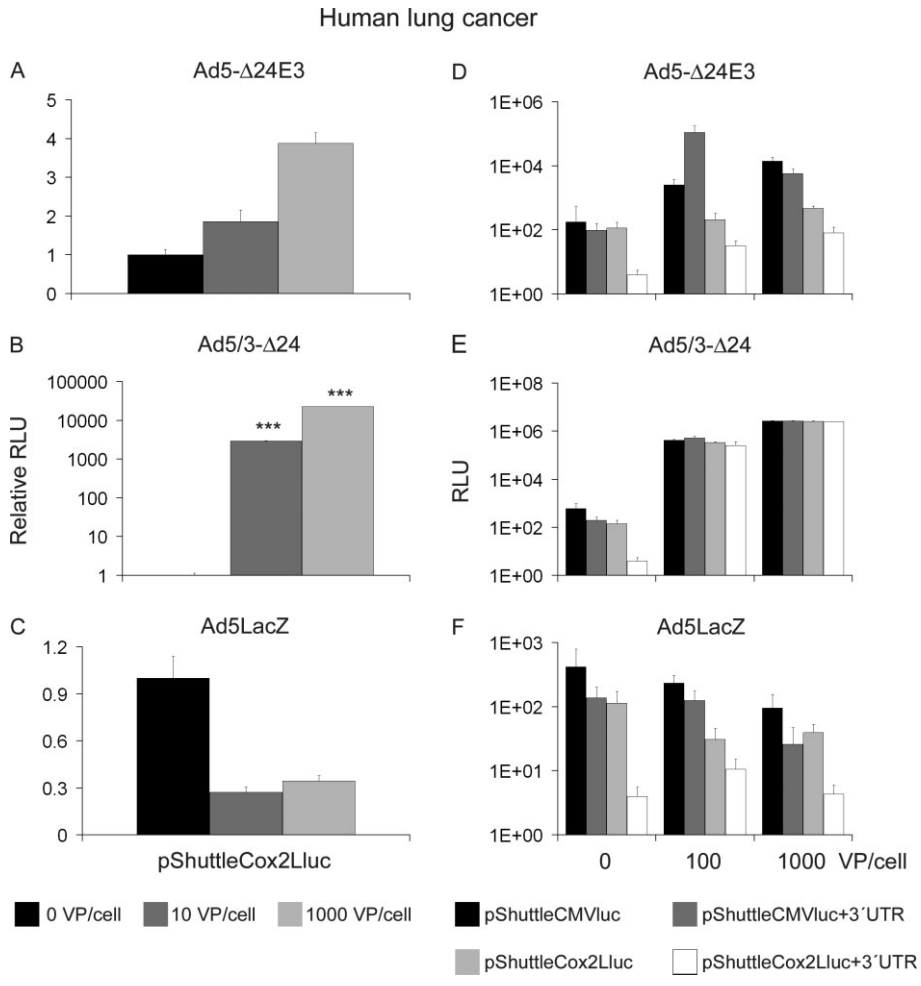

Figure 4. The Cox-2 promoter is induced in A549 cells following infection with replication competent adenoviruses but not following infection with E1 deleted virus (A–C). Due to activation of Ras/MAPK in these cells, and in contrast to nontumor cells, 3′-UTR AU-rich elements had little or no effect on promoter activity thereby increasing the therapeutic window of the approach (D–F). Cells were transfected with plasmids and then infected with selectively oncolytic adenoviruses Ad5-Δ24E3 (A, D) and Ad5/3-Δ24 (B, E), or an E1 deleted adenovirus (C, F). An increase in luciferase (relative light units, measured at 24 h) indicates induction of the promoter following adenoviral infection. Error bars indicate SEM, $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus no virus (A–C) or no UTR (D–F). VP, virus particles; RLU, relative light units

downregulation of Cox-2 induction following adenovirus infection. As expected, the UTR element had little or no effect on Cox-2 induction by replication competent adenoviruses (Figures 4D to 4F).

Induction of the CMV promoter by adenoviral infection

To test whether induction of the Cox-2 promoter by adenovirus was promoter-dependent, we included control plasmids with the CMV (cytomegalovirus immediate early) promoter, with or without the UTR moiety (Figures 2 to 4, panels D to F). Somewhat surprisingly, the CMV promoter was induced to similar degree as the Cox-2 promoter in all three cell lines. Also in parallel with Cox-2, CMV induction was only seen when viruses contained E1 or it was supplied in *trans*. Furthermore, the UTR element had similar activity on the CMV promoter as it did on the Cox-2 promoter.

Non-invasive imaging of the induction of the Cox-2 promoter *in vivo*

Subcutaneous 293 or A549 tumors were allowed to develop 30 and 14 days, respectively, to reach a diameter of 5 mm. Tumors were then transfected with plasmids and infected with Ad5wt on the next day. To study how rapidly Cox-2 is induced *in vivo*, luciferase imaging was performed 15 min after virus injection, and again 24 h later (Figures 5 and 6). Interestingly, a trend for Cox-2 induction could be seen already immediately after infection (day 1). Mean photon emission in tumors only transfected with pShuttleCox2Lluc was 55 722 whereas, when tumors were infected after transfection, mean photon emission was 241 341 (4.3-fold difference, $p = 0.68$; Figure 6A). When imaging was repeated 24 h later (day 2), Cox-2 induction persisted and a similar difference between mean values was seen: 80 463 versus 307 707 (3.8-fold, $p = 0.62$).

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Immediately after infection, the UTR had not had time to reduce Cox-2 induction and mean photon emissions were 4339 (no virus) and 55 039 (with virus), respectively (13-fold, $p = 0.29$). However, when imaging was repeated 24 h later, luciferase expressed by the Cox-2–UTR complex in infected cells decreased from 55 039–51 716 whereas, in uninfected cells, the luciferase continued to increase as expected (from 4339–14 519) and therefore the destabilization signal appeared to be working as predicted. However, the UTR could not completely block induction of the Cox-2 promoter by the virus (photon increase from 14 519–51 716, 3.6-fold difference, $p = 0.67$, Figure 6A). Due to variation typical of *in vivo* luciferase imaging, and the complexities associated with *in vivo* plasmid transfection followed by intratumoral infection, no statistically significant differences between groups could be seen.

Induction of the Cox-2 promoter *in vivo* in nonmalignant and malignant cells

After the second round of imaging (24 h after infection), tumors were excised and the amount of luciferase protein was analysed. The results were well in line with the imaging data. Adenovirus infection induced expression of the Cox-2 promoter in both nonmalignant and malignant cells (Figures 6B and 6C). When the 3′ destabilization signal was added, induction of Cox-2 was not seen in 293 cells. In A549 cells, the UTR appeared to have little effect, as predicted, and suggesting normal tissue specific activity.

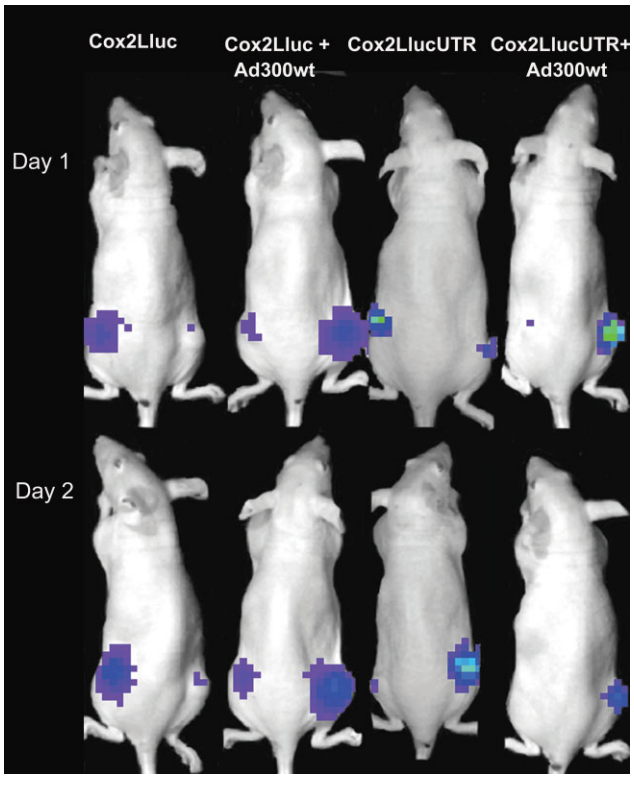

Figure 5. The Cox-2 promoter is induced by adenovirus infection of nonmalignant cells *in vivo*. 293 cells were injected subcutaneously into both flanks of nude mice and when tumor diameter was approximately 5 mm, transfection was performed with the indicated plasmids. Twenty-four hours later, tumors in animals 2 and 4 were infected with a wild-type adenovirus. Luciferase expression was quantifyd non-invasively with the Xenogen IVIS system at 15 min (day 1) and 24 h after virus injection (day 2). RLU, relative light units (D–F). Relative RLU = light emitted by plasmid transfected cells without virus (A–C)

Induction of the CMV promoter *in vivo*

As seen *in vitro*, the CMV promoter was induced following adenoviral infection of 293 cells. Confirming the functionality of the UTR element, no Cox-2 induction was seen when the plasmids with the destabilization signals were used (Figure 6B). Furthermore, abrogation of mRNA destabilization (i.e. no reduction in induction) was seen with malignant A549 cells (Figure 6C).

Discussion

Cox-2 is a promising promoter for transcriptional targeting of cancer gene therapy. However, it is well established that physiological induction of the Cox-2 protein occurs during infection, inflammation and tissue damage [19,34,36]. Many viruses are known to induce Cox-2. For example, human cytomegalovirus immediate–early proteins, hepatitis B virus × protein, human leukemia virus type 1 (HTLV-1) Tax protein, Epstein–Barr virus latent membrane protein and human herpes virus 6 immediate–early protein 2 have been implicated in Cox-2 induction [37,38].

The mechanisms behind these phenomena are poorly understood and it is unclear whether Cox-2 induction occurs more as a protective antiviral measure initiated by human cells, or if viruses can also actively promote Cox-2 induction for their own purposes. There is some evidence that, at least in some cases, the latter could be true because certain viruses have been reported to actively participate in Cox-2 induction, presumably because they benefit from the consequent catalytic activity. For example, Cox-2 catalyses production of PGE-2, which induces the CMV transcriptional regulatory protein (IE2), resulting in more effective replication of the virus [39]. It has been shown that PGE-2 has an effect on the replication of also other viruses such as HTLV and herpes [37,38].

It is currently unknown whether adenovirus induces Cox-2 or whether it benefits from Cox-2 expression. However, a preliminary report suggests that the Cox-2 protein is expressed by adenovirus infected cells [9], indicating that adenovirus may resemble the aforementioned viruses in this respect. To our knowledge, whether Cox-2 protein induction by viruses is mainly mediated by increased promoter activity or other mechanisms, such as post-transcriptional and/or translational modulation, has not been studied previously.

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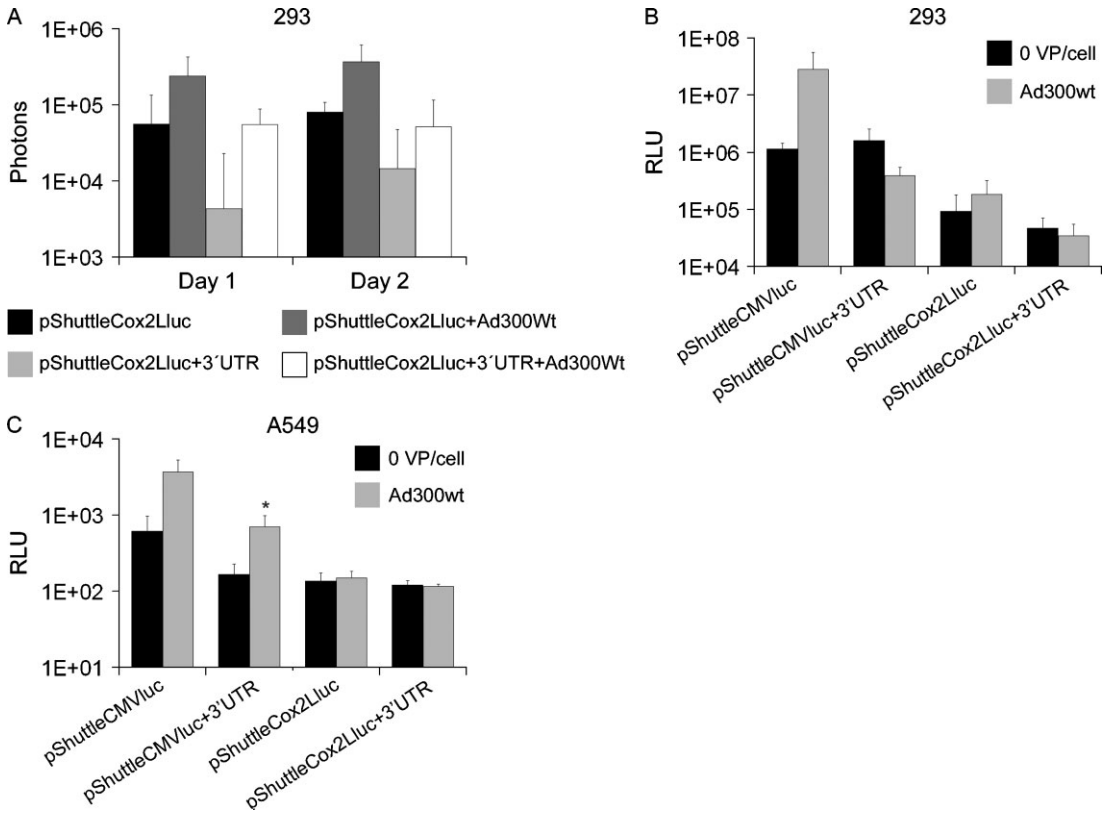

Figure 6. The Cox-2 promoter is induced by adenovirus infection of both nonmalignant 293 (A, B) and malignant A549 cells (C) *in vivo*. By day 2, the AU rich 3′-UTR destabilization signal reduces unwanted Cox-2 induction in nontumor cells (B) but not in tumor cells (C), thus increasing the transcriptional window. Cells were injected subcutaneously into nude mice and when tumor diameter was approximately 5 mm, plasmid transfection was performed. Twenty-four hours later, tumors were infected with a wild-type adenovirus and imaged for photon emission 15 min later (day 1 in A). A further 24 h later, imaging was repeated (day 2 in A), tumors were excised, homogenized, and luciferase expression (relative light units, RLU) was measured (B–C). Data indicate that Cox-2 induction occurs very rapidly following infection, and UTR mediated destabilization is effective by day 2 but only in normal cells. Error bars indicate the SEM, $n = 4–6$ tumors/group. $^*p < 0.05$

In our experiments, we saw Cox-2 promoter induction following infection with adenovirus. Nevertheless, induction was only seen in with viruses containing E1. Highlighting the role of E1 proteins, E1 containing 293 cells also sustained Cox-2 promoter induction following infection with an E1 deleted virus (Figure 2). It is not completely clear whether induction of Cox-2 relates more to E1 expression *per se*, or whether virus replication is required. In support of the former theory, Cox-2 promoter activity in 293 cells could be effectively reduced by the UTR even in the absence of virus (Figure 2). This effect was much less dramatic in the other cells. Furthermore, the selectively oncolytic viruses used in the present study are not expected to replicate much in fibroblasts, yet they induced the Cox-2 promoter to high degree (Figure 3). Nevertheless, Ad5/3-Δ24, which replicates more effectively than Ad5-Δ24E3 in most cell types [7,32], also induced higher Cox-2 levels (Figures 3 and 4). Although this appears to support the need for replication with respect to Cox-2 induction, faster replicating viruses would probably express higher E1 levels, which again indicates the importance of E1 expression *per se*. Taken together, our data suggest that both E1 expression and virus replication contribute to Cox-2 expression.

A previous study suggested that an E1 deleted virus induced NFκB as a mechanism for Cox-2 protein production [9]. Since many of the constructs used here have the E1 region present, and E1A is known to downregulate NFκB [40], it appears that other mechanisms can also be involved. One possible explanation is that adenovirus E1 A activates the MAPK pathway, which in turn is important in Cox-2 regulation [41,42].

The Cox-2 3′-UTR AU-rich element used in the present study abrogated induction of the promoter in noncancer cells both *in vitro* and *in vivo*. This is in line with previous findings suggesting the importance of Ras/MAPK pathway mediated post-transcriptional mechanisms in Cox-2 protein regulation [22,23]. Because this pathway is aberrant in most if not all tumor cells, including A549 cells [23,26,43], UTR mediated downregulation of the Cox-2 mRNA is not expected in tumor cells. Therefore, the Ras/MAPK pathway influences Cox-2 protein production on at least two levels: upregulation of the Cox-2 promoter and downregulation of mRNA degradation. Here, we utilized both mechanisms for increasing tumor selectivity, by combining the Cox-2 promoter with the Cox-2 3′-UTR destabilization signal, thus increasing the tumor selectivity of the approach.

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In addition to induction of the Cox-2 promoter, the CMV immediate early promoter was induced. If we hypothesize that adenovirus induces Cox-2 to benefit its own replication, similarities between the early promoters of these viruses could explain the phenomenon. For example, it is known that both adenovirus and CMV early promoters have ATF/CREB binding sites that enhance replication. Further, adenovirus E1A upregulates junB, which in turn induces elements that enhance the activity of the CMV immediate early promoter [44–46].

Our studies performed *in vivo* suggest that viral activation of the Cox-2 promoter occurs very rapidly after intratumoral infection because induction could be seen already at 15 min. Given its role as an acute phase protein, it is not completely surprising that induction of the Cox-2 protein at 5 h post infection has been reported [38]. This supports the notion that the Cox-2 promoter is induced even earlier. However, because viral entry has not yet been completed at 15 min, it is unlikely that viral genes would have contributed to Cox-2 activation at 15min. Instead, early Cox-2 activation probably results from the cell reacting to the virus. This suggests that Cox-2 induction following virus infection may initially (from an evolutionary perspective) have been a protective measure by human cells, but later viruses have deviously learned to turn this to their advantage. The effect of the UTR could not be seen at this early time, but a clear trend for UTR activity was seen 24 h later (not statistically significant). This is explained by the role of the UTR as a destabilization signal, where an effect would only be expected some time after induction of Cox-2 expression.

The present data show that adenoviruses induce the Cox-2 and CMV promoters and that induction can be abrogated by the 3′-UTR element of Cox-2. With regard to future directions, it might be interesting to study whether these modifications bring more selectivity to therapeutic approaches such as oncolytic adenoviruses. In addition, other promoters may be controllable by the 3′-UTR element and therefore the approach might have widespread utility. Furthermore, these findings may help shed light on the intriguing relationships between viruses and Cox-2. Finally, given the important role of Cox-2 in tumorigenesis, an increased understanding of mechanisms relating to its regulation may promote our understanding of carcinogenesis.

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