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Competing interests statement
The authors declare no competing financial interests.

DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/gene
ALK6 | *ANGPTL4* | *SOX2* | *SOX4*
OMIM: http://www.ncbi.nlm.nih.gov/omim
BCL6 | *CTGF* | *IL11* | *LIF* | *MYC*
UniProtKB: http://www.uniprot.org
ALK1 | ALK5 | BMP4 | CD24 | CD44 | DEC1 | EVI1 | INK4B | MEL1 | p21 | p300 | PDGFA | PDGFB | RBL1 | RUNX3 | SKI | SKIL | SMAD1 | SMAD2 | SMAD3 | SMAD4 | SMAD5 | SMAD8 | SNAI1 | SNAI2 | Tax | TGFβ1 | TGFβ2 | TGFβ3 | TGFBR2 | TTF1

FURTHER INFORMATION
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Context-dependent regulation of the expression of c-Ski protein by Arkadia in human cancer cells

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Arkadia is a positive regulator of transforming growth factor-β (TGF-β) signalling, which induces ubiquitylation and proteasome-dependent degradation of negative regulators of the TGF-β signalling pathway, *i.e.* Smad7, c-Ski and SnoN. In the present study, we examined the roles of Arkadia in human cancer cells. We first examined the expression of Arkadia in 20 cancer cell lines and 2 non-cancerous cell lines, and found that it was expressed ubiquitously at both the mRNA and protein levels. Interestingly, levels of expression of c-Ski protein, one of the substrates of Arkadia, were not correlated with those of c-Ski mRNA. Arkadia induced down-regulation of c-Ski protein expression in many cell lines examined, but did not in certain cell lines with high levels of expression of c-Ski protein. We also found that knockdown of Arkadia attenuated the induction of TGF-β target genes, whereas ectopically expressed Arkadia enhanced it. Notably, over-expression of Arkadia inhibited the growth of HepG2 cells in the presence as well as the absence of TGF-β stimulation. Arkadia thus regulates the levels of expression of c-Ski protein in cell-type-dependent fashion, and exhibits a tumour suppressor function by inhibiting tumour cell growth.

Keywords: cancer/degradation/Ski/TGF-β/ubiquitin ligase.

Abbreviations: CBP, CREB-binding protein; E3, ubiquitin-protein isopeptide ligase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, haemagglutinin; HDACs, histone deacetylases; MCS, multi-cloning site; MEF: mouse embryonic fibroblast; PCR, polymerase chain reaction; PAI-1, plasminogen activator inhibitor-1; RT, reverse transcription; Sno, Ski-related novel gene; TGF-β, transforming growth factor-β.

Transforming growth factor-β (TGF-β) has a diverse array of activities, including growth inhibition, regulation of motility, extracellular matrix production, differentiation and apoptosis, in various target cells (*1*). TGF-β signalling must be tightly controlled, since its abnormality has been reported to cause progression of various diseases, including cancer and fibrosis (*2*). TGF-β plays dual roles in the progression of cancer (*3*, *4*). In the early stages of carcinogenesis, TGF-β acts as a tumour suppressor by inhibiting cell growth. In contrast, TGF-β exerts tumour-promoting effects by inducing invasion and metastasis in advanced stages of cancer. Levels of expression of TGF-β are positively correlated with clinical stage in certain tumours.

TGF-β signal is transduced through two distinct serine–threonine kinase receptors, termed type I and type II (*5*–*8*). Upon binding of TGF-β to type II receptor, type I receptor is recruited to the ligand–receptor complex and is phosphorylated by the constitutively active type II receptor kinase. Type I receptor is then activated, and phosphorylates receptor-regulated Smads (R-Smads), Smad2 and Smad3. Phosphorylated Smad2 and Smad3 form complexes with Smad4, a common-mediator Smad (co-Smad), and translocate into the nucleus. The activated Smad complexes then bind to promoter regions of target genes either directly or together with other transcription factors, and regulate their transcription in collaboration with transcriptional co-activators and co-repressors (*7*, *9*).

Arkadia was originally identified by gene-trap mutagenesis in mice as a factor required for induction of the mammalian node in extraembryonic lineages (*10*), and was found to induce mesendoderm by enhancing nodal-related signalling (*11*). Arkadia is a nuclear protein with 989 amino acid residues, including a characteristic RING domain at its C-terminus. We previously found that Arkadia is an E3 ubiquitin ligase that enhances TGF-β signalling by targeting negative regulators, *i.e.* c-Ski/SnoN and Smad7 (*12*, *13*).

c-Ski and SnoN are members of the Ski family of oncoproteins (*9*, *14*). Ski was originally identified as the transforming protein (v-Ski) of the avian retrovirus that induces oncogenic transformation of chicken embryo cells (*15*). The Ski family of nuclear oncoproteins represses TGF-β signalling principally through interaction with Smad proteins (*16*, *17*). c-Ski and SnoN interact with Smad2/3 and Smad4 in activated Smad complexes (*18*, *19*). They also bind directly to mSin3A and N-CoR and form a complex containing histone deacetylases (HDACs), thus repressing transcription (*20*). In addition, c-Ski and SnoN have been shown to compete with transcriptional co-activator p300 and/or CREB-binding protein (CBP) for binding to Smad complexes (*18*, *19*, *21*) and to stabilize inactive Smad complex on the promoter regions of target genes

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(22). In contrast, Smad7, an inhibitory Smad (I-Smad), competitively inhibits phosphorylation of Smad2 and Smad3 through binding to activated type I receptor kinase of TGF-β in the cytoplasm (23, 24).

Misexpression of these negative regulators has been implicated in various pathological conditions. Increased expression of Smad7 has been found in inflammatory bowel disease (25) and pancreatic cancer (26). Reduction of Smad7 protein has been reported in human fibroblasts of patients with scleroderma (27) and in tissues with renal fibrosis in mice (28). Increased expression of SnoN or c-Ski has been implicated in the progression of oesophageal squamous cell carcinomas (29, 30), melanomas (31), estrogen-receptor-positive breast carcinomas (32) and colorectal carcinomas (33). Some of these cancers exhibit gene amplification of c-Ski or SnoN (29, 33, 34). Since increased expression of c-Ski or SnoN has been reported to be associated with poor prognosis, overactivity of SnoN and c-Ski may cause cancer. In contrast, systemic deletion of one copy of the *Sno* or *Ski* gene causes increased susceptibility to chemical carcinogens (35, 36). Control of the levels of expression of these negative regulators within appropriate ranges thus appears to be important.

Arkadia appears to play important roles in cancers through regulation of the protein expression of c-Ski/SnoN and TGF-β signalling. However, the roles played by Arkadia in tumours have yet to be fully determined. We describe here the relationship between expression of Arkadia and that of c-Ski/SnoN, as well as the roles played by Arkadia in tumour cells.

Materials and Methods

Cell culture

Cells were cultured in the medium shown in Supplementary Table I, in a 5% CO_2-humidified atmosphere at 37°C.

Lentiviral production and infection

Lentivirus expression vectors (37) for Arkadia and multi-cloning site (MCS) were constructed as previously described (38). Briefly, haemagglutinin (HA)-tagged mouse Arkadia or MCS was inserted into pENTR vectors (Invitrogen), and then transferred to pCSII-EF-RfA vectors using LR clonase (Invitrogen). 293FT cells (6×10^6 cells; Invitrogen) were transfected using Lipofectamine 2000 (Invitrogen) with pCSII-EF-RfA containing Arkadia or MCS, pCAG-HIVgp and pCMV-VSV-G-RSV-Rev. The culture supernatants were collected 72 h after transfection and used for transduction of HepG2 and OCUM-2MLN cells. HepG2 cells were infected with the lentivirus twice.

RNA interference

RNA interference using siRNA oligonucleotides was performed as described below. Sequences of RNA oligonucleotides used to knock down human Arkadia, human c-Ski and human SnoN were as follows: siArkadia RNF111-HSS123238 (forward, 5′-UAACACUUC UCGUUUCUUCCUCUGC-3′; reverse, 5′-GCAGAGGAAGAAA CGAGAAGUGUUA-3′), siArkadia RNF111-HSS123240 (forward, 5′-AACACAAUUCUGCACAUACGAAGGG-3′; reverse, 5′-CCCUUCGUAUGUGCAGAAUUGUGUU-3′), sic-Ski SKI-HSS109772 (forward, 5′-UUGUGCGAGUGCACCACGAACUU GU-3′; reverse, 5′-ACAAGUUCGUGGUGCACUCGCACAA-3′) and siSnoN SKIL-HSS109774 (forward, 5′-AAUAAACCCUGAC AUUUGCCUAGGC-3′; reverse, 5′-GCCUAGGCAAAUGUCAG GGUUUAUU-3′). For knockdown of Arkadia, RNF111-HSS123238 or RNF111-HSS123240 was used. Similar knock-down efficiency was confirmed with these two siRNAs. Pre-annealed oligonucleotides (Stealth RNAi oligonucleotides) were obtained from Invitrogen. Oligonucleotides for negative controls were also purchased from Invitrogen. Transfection of these oligonucleotides was performed using HiPerFect transfection reagent (Qiagen) at the same time as seeding of cells. Oligonucleotides were used at final concentrations of 50 nM or 100 nM for silencing of Arkadia expression, with 50 nM used for SnoN expression and 100 nM for c-Ski expression. Cells were cultured 40 or 60 h (for MKN45 cells) before analysis.

Immunoblotting

MDA-MB-231 cells and mouse embryonic fibroblasts (MEFs) (39) were treated with 1 ng/ml TGF-β (TGF-β1, R & D Systems) before analysis, where indicated. Cells were lysed with a buffer containing 1% Nonidet P-40, 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 1% Trasyrol, 50 μM MG132 (Peptide Institute) and 5 mM EDTA. Cleared cell lysates were separated by SDS–PAGE and transferred to Fluoro Trans W membrane (Pall). Immunoblotting was performed as described previously (13) using the following antibodies: anti-Arkadia 3AP4 (13) for simultaneous detection of both endogenous human Arkadia and exogenous mouse Arkadia, anti-RNF111 (Abnova) for immunoblotting of endogenous Arkadia in human cell lines, anti-c-Ski (Millipore) for detection of endogenous c-Ski in human cell lines and in MEFs, anti-SnoN H-317 (Santa Cruz Biotechnology) for immunoblotting of endogenous SnoN, and anti-tubulin DM 1A (SIGMA). Bands of immunoblotting were quantified using Quantity One 1-D Analysis software (Bio-Rad Laboratories).

Semi-quantitative RT–PCR

Total RNAs from wild-type and *Arkadia*$^{-/-}$ MEFs were extracted using the RNeasy Mini Kit (Qiagen). Reverse transcription and semi-quantitative RT–PCR was performed as described previously (13). The primer sequences used for detection of mouse c-Ski were: forward, 5′- GAGGGTGCCCCGGGTCTCAG-3′; reverse, 5′- AC GGTGGTGCAGGGTGGACT-3′.

Real-time RT–PCR

Total RNA from HEK293, HaCaT, and 20 tumour cell lines was prepared using the RNeasy Mini Kit. cDNA synthesis was performed as described previously (13). Quantitative RT–PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) or FastStart Universal SYBR Green Master [Rox] (Roche) and a 7500 Fast Real-Time PCR System (Applied Biosystems). The primer sequences used were as follows: human SnoN (forward, 5′-CTGTGTTGGAAGGGGAATCT-3′; reverse, 5′-TTTGCTGGAGTGTAAATTCTCG-3′) and human p15^{INK4b} (forward, 5′-GCCGCCCACAACGACTTTAT-3′; reverse, 5′-GCT TGCAGGCTTACAGGCTTTC-3′). Primers for human Arkadia, human Smad7, and human GAPDH were previously described (12). Primers for human c-Ski, human plasminogen activator inhibitor-1 (PAI-1), and human p21^{WAF} were also previously described (40).

Results

Accumulation of c-Ski protein by knockdown of Arkadia

We previously reported that ectopic expression of Arkadia induces ubiquitylation and proteasome-dependent degradation of c-Ski (13). To determine whether endogenous Arkadia affects expression of c-Ski protein, we compared the levels of expression of c-Ski protein in Arkadia-knocked-down and control MDA-MB-231 cells. Since c-Ski has been reported to undergo degradation in response to TGF-β stimulation (41), we also compared the time courses of protein expression of c-Ski after TGF-β stimulation. As shown in Fig. 1A, c-Ski protein accumulated in Arkadia-silenced cells in the absence of TGF-β stimulation, suggesting that Arkadia induces c-Ski degradation under resting conditions. Upon stimulation with TGF-β,

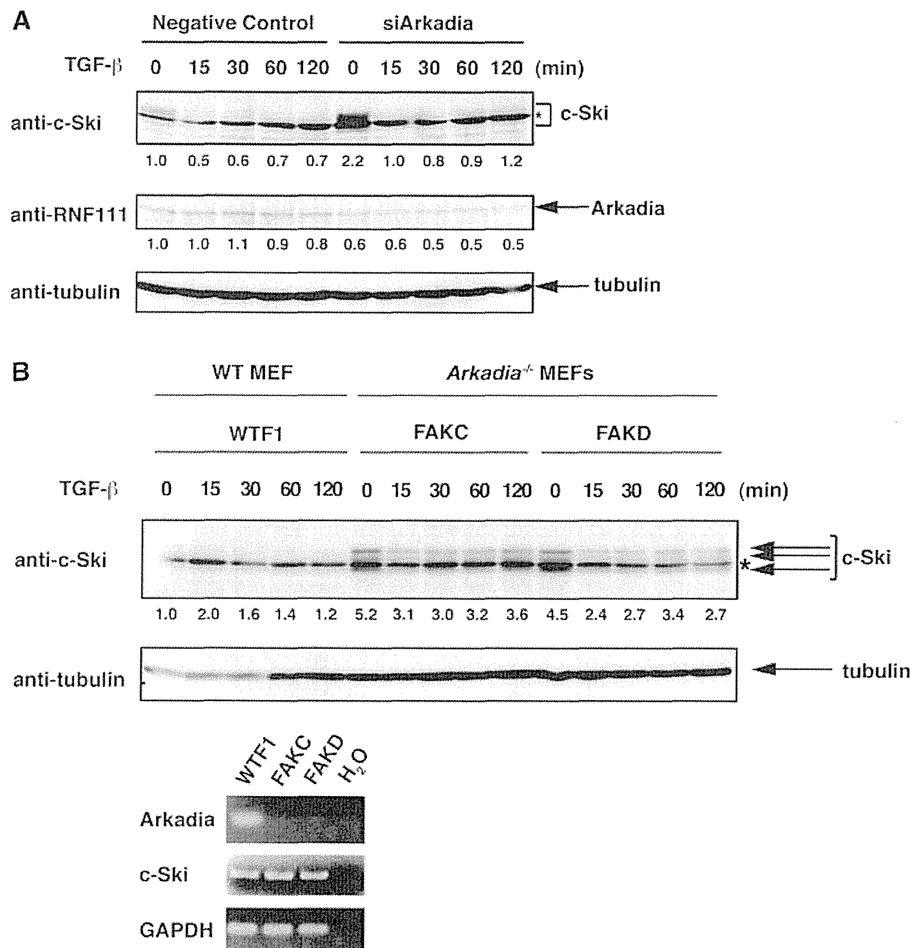

Fig. 1 Arkadia contributes to degradation of c-Ski protein. (**A**) Knockdown of Arkadia was performed by siRNA. MDA-MB-231 cells were transfected with siArkadia oligonucleotide or negative control oligonucleotide at the same time as seeding. Cells were treated with 1 ng/ml TGF-β and harvested at the indicated time points. Cell lysates were subjected to SDS–PAGE, followed by immunoblotting using anti-c-Ski antibody (top panel), anti-RNF111 (Arkadia) antibody (middle panel) and anti-tubulin antibody (bottom panel). Asterisk denotes non-specific bands. The specific bands of c-Ski and Arkadia were quantified and normalized to those of tubulin. Values shown at the bottom of corresponding panels were relative to those of negative control at 0 min. (**B**) Wild-type (WT) MEF cell line (WTF1) and *Arkadia*$^{-/-}$ MEF cell lines (FAKC and FAKD) were treated with 1 ng/ml TGF-β and harvested at the indicated time points. Time courses of expression of c-Ski protein upon TGF-β stimulation were examined (top panel). Asterisk denotes non-specific bands. The specific bands of c-Ski were quantified and normalized to those of tubulin. Values shown at the bottom of top panel were relative to that of WT MEF at 0 min. Expression of Arkadia and c-Ski mRNA are shown at the bottom.

c-Ski protein was degraded within 15 min in the control cells, consistent with previous reports (*41*). TGF-β-induced degradation was not abrogated in Arkadia-knocked-down cells, suggesting the possibility of involvement of E3 ubiquitin ligases other than Arkadia. We also performed a similar experiment using *Arkadia*$^{-/-}$ and wild-type MEFs, and obtained similar results (Fig. 1B). c-Ski protein was hardly detected in wild-type MEFs, but accumulated in *Arkadia*$^{-/-}$ MEFs, whereas expression levels of c-Ski mRNA were similar between WT and *Arkadia*$^{-/-}$ MEFs. c-Ski protein was significantly reduced 15 min after TGF-β stimulation in *Arkadia*$^{-/-}$ MEFs. These findings suggest that Arkadia is involved in the degradation of c-Ski protein in the absence of TGF-β stimulation. Although Arkadia may also play a role in TGF-β-induced degradation of c-Ski, other ubiquitin ligases may also play a role in it.

Broad expression of Arkadia in various cancer cell lines

TGF-β has two opposing effects on the progression of cancer. Since Arkadia enhances TGF-β signalling (*12*), we hypothesized that Arkadia may affect cancer progression via enhancement of TGF-β signalling. We first examined Arkadia expression in 20 cancer cell lines. As shown in Fig. 2A, the levels of expression of Arkadia mRNA in these cell lines were not very different (3-fold at a maximum; top panel). Expression of Arkadia protein was also observed widely in these cell lines, although the levels of expression were different when the intensities of the immunoblot bands were compared (7-fold at a maximum; second panel).

We then examined the levels of expression of c-Ski and SnoN. Increased expression of c-Ski and SnoN has been reported in several human cancers (*30–32*), although in some cases this was accompanied by

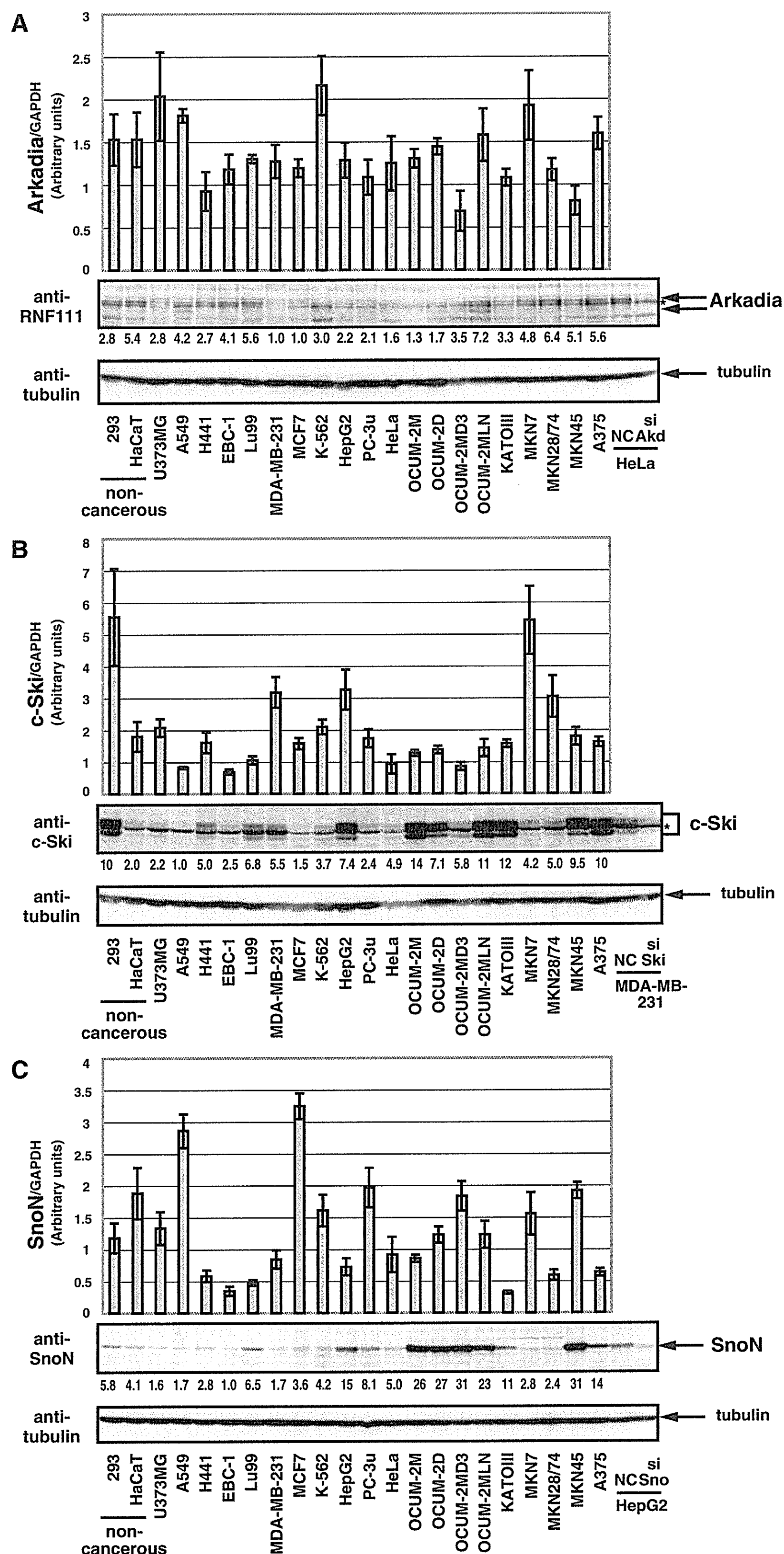

Fig. 2 mRNA and protein expressions of Arkadia, c-Ski and SnoN in various tumour cell lines. (A–C) Levels of mRNA expression of Arkadia (A), c-Ski (B) and SnoN (C) were compared among 20 tumour cell lines and two non-cancerous cell lines (top panels). Vertical axis shows relative expressions of human Arkadia (A), c-Ski (B) or SnoN (C) normalized to human GAPDH determined by real-time RT–PCR. Cell lysates were obtained in parallel with RNA preparation from the 20 tumour cell lines and two non-cancerous cell lines. The lysates were subjected to SDS–PAGE followed by immunoblotting with anti-RNF111 (A), anti-c-Ski (B) or anti-SnoN (C) (middle panels). Bands for each protein were confirmed with siRNA of Arkadia (siAkd) in HeLa cells, that of c-Ski (siSki) in MDA-MB-231 cells, and that of SnoN (siSno) in HepG2 cells (right two lanes in each panel). NC denotes negative control oligonucleotide-transfected cells. Asterisks denote non-specific bands. The specific bands of Arkadia (A), c-Ski (B) and SnoN (C) were quantified and normalized to those of tubulin. Values shown at the bottom of corresponding panels were relative to that of cells with the lowest expression of each protein.

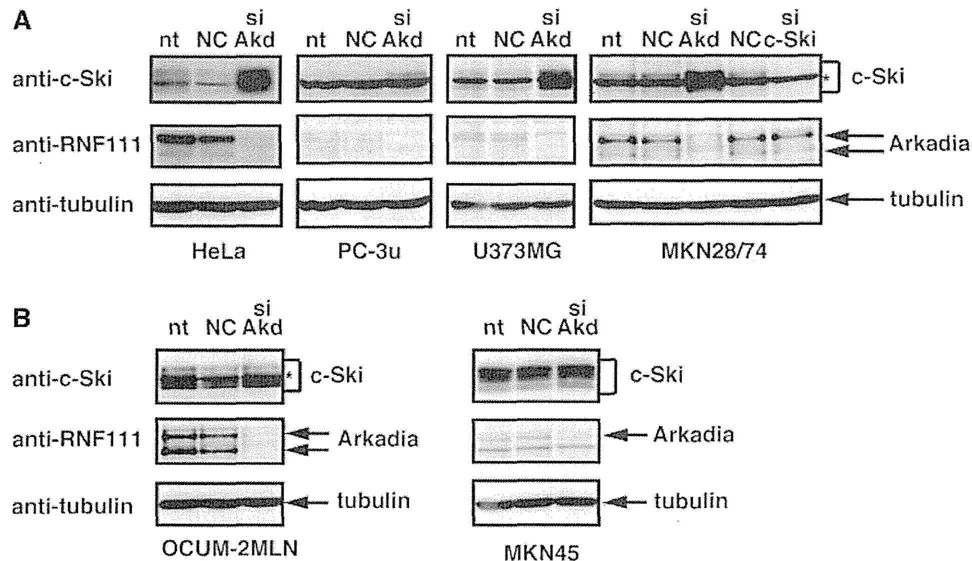

Fig. 3 Effect of knockdown of Arkadia on c-Ski protein expression. (**A** and **B**) Expression of endogenous Arkadia was knocked down by transfection of siArkadia oligonucleotide into the indicated tumour cell lines. Lysates from non-transfected cells (nt), control oligonucleotide-transfected cells (NC) and Arkadia-knocked down cells (siAkd) were subjected to SDS–PAGE followed by immunoblotting with anti-c-Ski (top panels). Bands of c-Ski were confirmed by siRNA of c-Ski in MKN28/74 cells (right two lanes in the right top panel of A). Knockdown of Arkadia was confirmed by immunoblotting with anti-RNF111 (middle panels). Asterisks denote non-specific bands.

gene amplification of c-Ski or SnoN (*29*, *33*, *34*). As shown in Fig. 2B and C, mRNA levels of c-Ski and SnoN varied markedly among the cell lines tested. Levels of c-Ski mRNA differed nearly 8-fold between EBC-1 and MKN7 (Fig. 2B, top panel), and levels of SnoN mRNA differed nearly 10-fold between KATOIII and MCF7 (Fig. 2C, top panel). Levels of expression of c-Ski/SnoN proteins were also varied markedly among these cancer cell lines. When bands of immunoblotting were quantified, the levels of expression of c-Ski protein differed 14-fold between A549 and OCUM-2M (Fig. 2B, second panel), and those of SnoN protein differed >30-fold between EBC-1 and OCUM-2MD3 (Fig. 2C, second panel).

In some cell lines, the levels of expression of c-Ski were high at the mRNA but low at the protein level (MKN7 and MKN28/74), whereas in other cell lines they were low at the mRNA level but high at the protein level (OCUM-2M, OCUM-2D, OCUM-2MLN, KATOIII, MKN45 and A375). Similar results were obtained for SnoN (Fig. 2C; A549 and MCF7).

Arkadia was thus ubiquitously expressed in various cancer cell lines at both the mRNA and protein levels, whereas in some cell lines levels of expression of c-Ski and SnoN varied at both mRNA and protein levels and levels of expression of mRNA and protein were not correlated.

Dysfunction of Arkadia in degradation of c-Ski in some cancer cell lines

Since Arkadia was expressed in all the 22 cell lines tested, we examined whether Arkadia functions as an E3 ubiquitin ligase in these cell lines. We knocked down Arkadia and determined protein expression of c-Ski. c-Ski protein accumulated in MDA-MB-231 (Fig. 1A), HeLa, PC-3u, U373MG and MKN28/74 cells upon knockdown of Arkadia (Fig. 3A), indicating that Arkadia down-regulates c-Ski in these types of cells. However, as shown in Fig. 3B, accumulation of c-Ski protein was not observed in OCUM-2MLN and MKN45 cell lines upon silencing of Arkadia. These findings suggest that Arkadia does not degrade c-Ski in some cancer cell lines. The high levels of expression of c-Ski protein in these cell lines can be attributed to dysfunction of Arkadia.

Enhancement of TGF-β target gene expression by endogenous Arkadia

We next examined mRNA expression of TGF-β target genes upon knockdown of Arkadia. HeLa cells were transfected with siRNA oligonucleotide and treated with TGF-β for the indicated periods of time. SnoN is one of the target genes of TGF-β (*42*), and, as shown in Fig. 4A, induction of SnoN mRNA was reduced (left panel) when expression of Arkadia was silenced (right panel), suggesting that endogenous Arkadia contributes to enhancement of TGF-β signalling. We also used OCUM-2MLN cells in which c-Ski protein did not accumulate upon knockdown of Arkadia. As shown in Fig. 4B, induction of target genes including *SnoN* (left top panel), *PAI-1* (left bottom panel) and *Smad7* (right bottom panel) was attenuated when Arkadia was silenced (right top panel). These findings suggest that Arkadia functions as an enhancer of TGF-β signalling in OCUM-2MLN cells, although it does not function as an E3 ubiquitin ligase for c-Ski.

Reduction of c-Ski protein expression by exogenous Arkadia

We further performed gain-of-function experiments, and examined the effects of exogenous Arkadia on c-Ski protein expression. HepG2 cells were used since the levels of expression of c-Ski and SnoN proteins

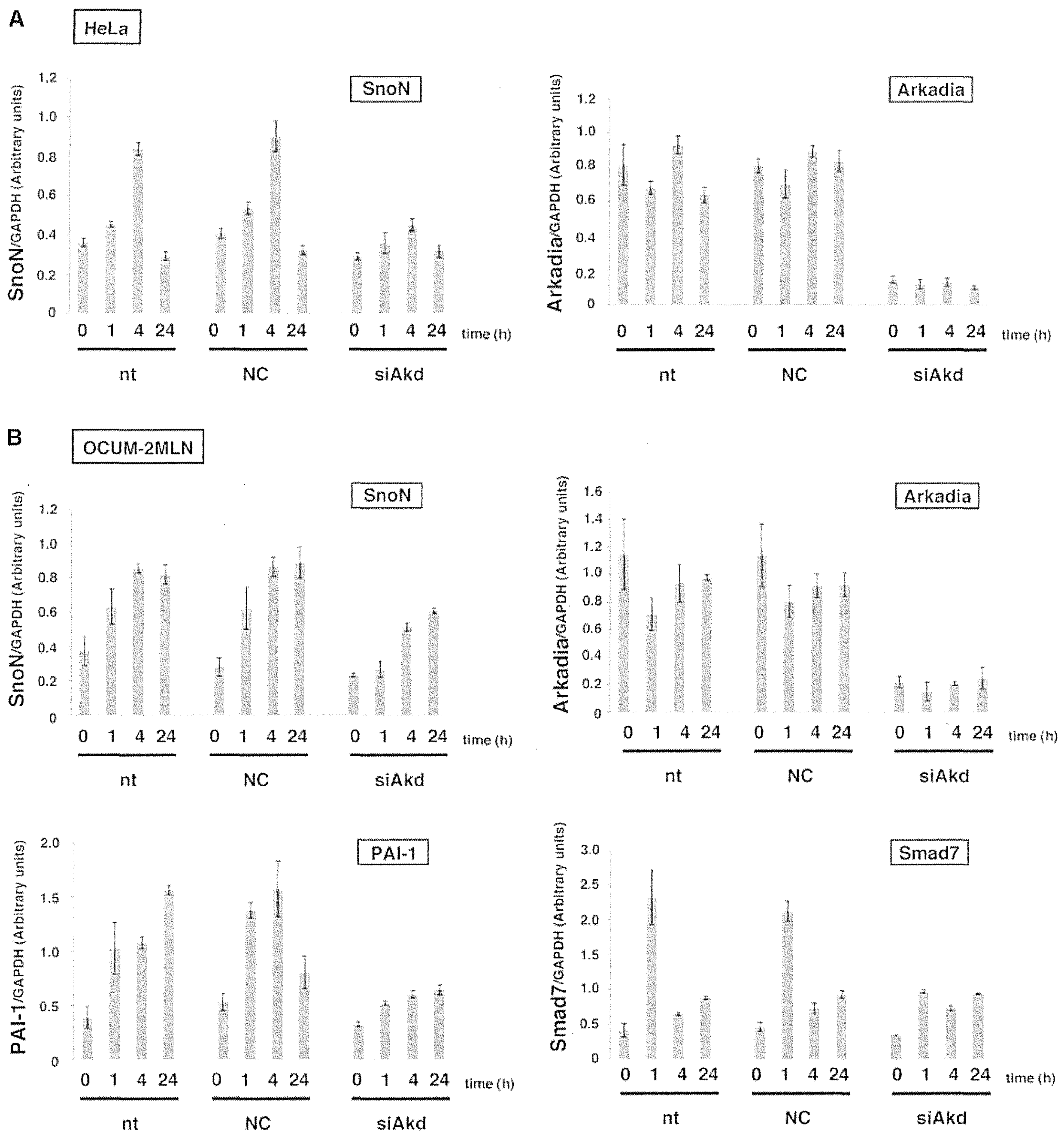

Fig. 4 Knockdown of Arkadia attenuates induction of TGF-β target genes. (**A** and **B**) HeLa cells (A) and OCUM-2MLN cells (B) were transfected with siArkadia oligonucleotide (siAkd) for silencing of endogenous Arkadia expression, or control oligonucleotide (NC), or remained untreated (nt). Cells were treated with 1 ng/ml TGF-β and harvested at the indicated time points. mRNA expression of SnoN, Arkadia, PAI-1 and Smad7 was determined by real-time RT–PCR. Vertical axis shows relative expressions of these genes normalized to human GAPDH.

were high and that of Arkadia was low in them (Fig. 2). OCUM-2MLN cells were also used since endogenous Arkadia did not contribute to degradation of endogenous c-Ski protein in them (Fig. 3B). Wild-type Arkadia (WT) or a RING finger domain-deleted mutant of Arkadia (ΔC) was expressed in these cells using a lentivirus vector expression system. Expression of Arkadia was confirmed using anti-Arkadia antibody. As shown in Fig. 5 (top panels), expression of exogenous Arkadia was higher than that of endogenous Arkadia in control cells. In both cell lines examined, c-Ski protein was down-regulated in cells expressing Arkadia-WT but up-regulated in those expressing Arkadia-ΔC. These findings showed that exogenous Arkadia induced degradation of endogenous c-Ski in these cells through its ubiquitin ligase activity.

Inhibition of growth of HepG2 cells by Arkadia in the presence and absence of TGF-β stimulation

To examine the effects of Arkadia in cancer cells, growth assay was performed using cells that express

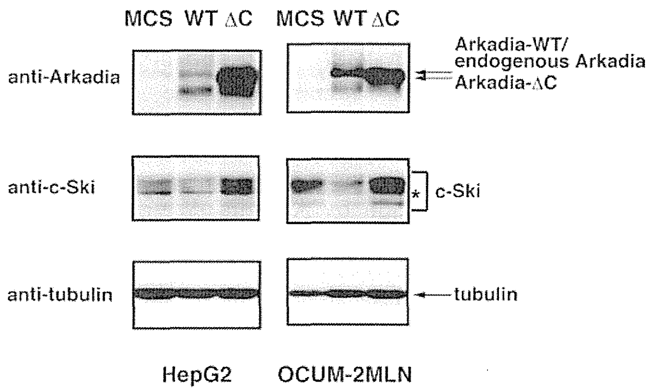

Fig. 5 Exogenous Arkadia reduces protein expression of c-Ski. HepG2 and OCUM-2MLN cells were infected with lentivirus harbouring multi-cloning site control (MCS), Arkadia-WT (WT) or Arkadia-ΔC (ΔC). Lysates from these cells were subjected to SDS–PAGE followed by immunoblotting with anti-Arkadia antibody (top panels), anti-c-Ski antibody (middle panels) and anti-tubulin antibody (bottom panels). Asterisk denotes non-specific bands.

Arkadia-WT or Arkadia-ΔC. We used HepG2 cells, since OCUM-2MLN cells do not respond to TGF-β for growth inhibition (*38*). As previously reported (*43*), growth of HepG2 cells was inhibited by treatment with TGF-β (Fig. 6A MCS). Cell growth was inhibited by expression of Arkadia-WT but not by Arkadia-ΔC in the absence of ligand. In addition, Arkadia-WT, but not Arkadia-ΔC, enhanced TGF-β-induced growth inhibition. These findings suggest that Arkadia represses HepG2 cell growth in the presence as well as the absence of TGF-β.

We then examined mRNA expression of TGF-β target genes in Arkadia-expressing HepG2 cells. Control cells (MCS), wild-type Arkadia-expressing cells (WT) and Arkadia-ΔC-expressing cells (ΔC) were treated with TGF-β for the indicated periods of time. Arkadia-WT, but not ΔC, enhanced induction of target genes of TGF-β, including *SnoN* and *Smad7* (Fig. 6B, top panels), suggesting that ectopic Arkadia enhanced TGF-β signalling through its C-terminal RING domain. Since TGF-β has been reported to

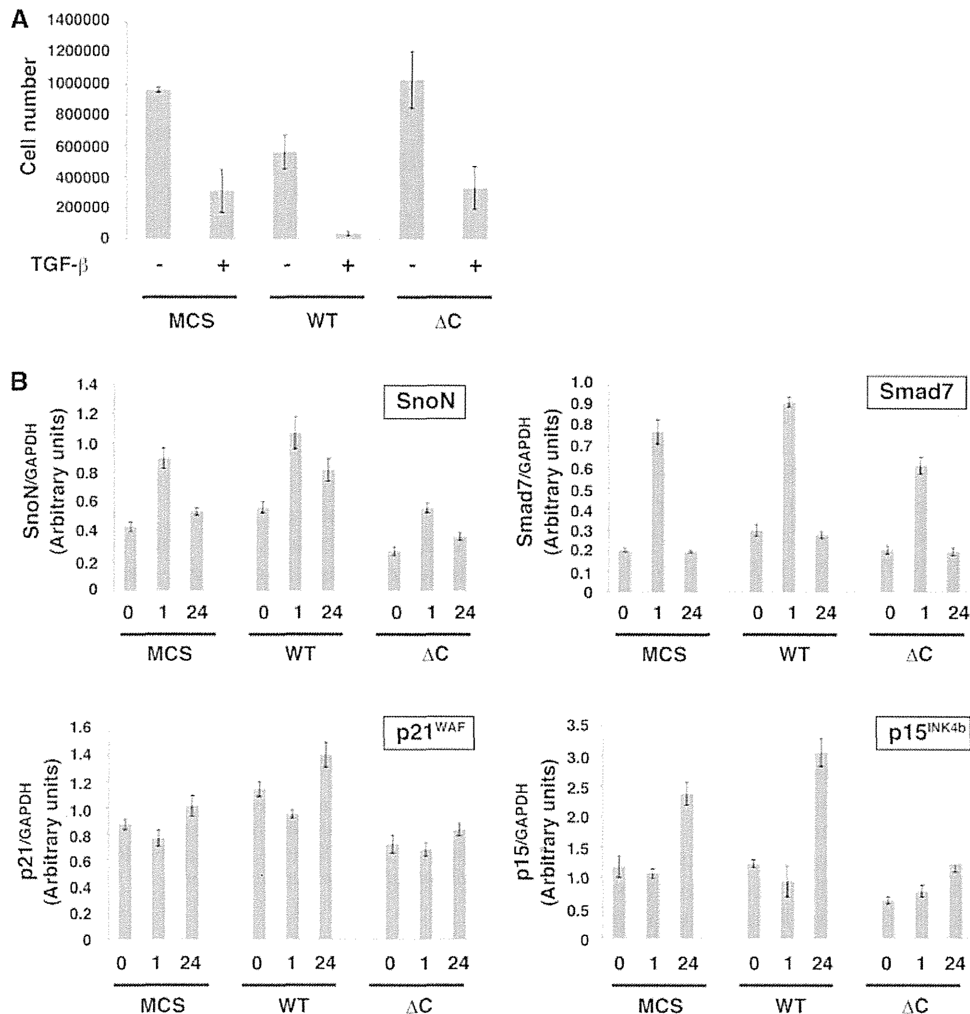

Fig. 6 Arkadia inhibits HepG2 cell growth. **(A)** Cell growth assay was performed as previously described (*43*). Numbers of HepG2 cells expressing Arkadia-WT (WT) or Arkadia-ΔC (ΔC) and control cells (MCS) were counted at day 4 with or without treatment with 0.5 ng/ml TGF-β. **(B)** HepG2 cells expressing Arkadia-WT (WT) or Arkadia-ΔC (ΔC) or control cells (MCS) were treated with 0.5 ng/ml TGF-β for the indicated periods of time. mRNA expressions of p21, p15, SnoN and Smad7 were determined by real-time RT–PCR. Vertical axis shows relative expressions of these genes normalized to human GAPDH.

inhibit cell growth by regulating expression of cell cycle regulators (*44*), we examined the expression of $p21^{WAF}$ and $p15^{INK4b}$. As shown in Fig. 6B, expression of $p21^{WAF}$ was up-regulated in WT cells but not in ΔC cells, in the presence as well as the absence of TGF-β stimulation (left bottom panel). Expression of $p15^{INK4b}$ was also up-regulated in WT cells but not in ΔC cells in the presence of TGF-β stimulation (right bottom panel). These findings suggest that Arkadia inhibits HepG2 cell growth, at least in part through induction of $p21^{WAF}$ and $p15^{INK4b}$.

Discussion

c-Ski has been reported to undergo degradation in response to TGF-β stimulation (*41*). The E3 ubiquitin ligases involved in this process have yet to be fully determined. Recently, Le Scolan *et al.* (*45*) reported that knockdown of Arkadia abrogated TGF-β-induced degradation of c-Ski, suggesting that Arkadia is responsible for the degradation of c-Ski. In our study, however, knockdown of Arkadia in MDA-MB-231 cells failed to attenuate the decrease in c-Ski protein upon TGF-β stimulation (Fig. 1A). We also found that TGF-β-stimulation resulted in down-regulation of c-Ski in *Arkadia*$^{-/-}$ MEFs (Fig. 1B), although the down-regulation was incomplete. These findings suggest that ubiquitin ligase(s) other than Arkadia are involved in TGF-β-induced degradation of c-Ski protein, at least under some experimental conditions. The differential effects of E3 ubiquitin ligases may depend on cell type or cellular context.

Arkadia was expressed broadly in various cancer cell lines. In contrast, the levels of expression of c-Ski/SnoN protein varied markedly among these cancer cells (Fig. 2). Interestingly, in some cancer cell lines examined, levels of expression of c-Ski/SnoN at the mRNA and protein levels were not correlated. These findings suggest that c-Ski and SnoN are regulated at the post-transcriptional level in these cancer cells. The lack of correlation between expressions of c-Ski/SnoN at the mRNA and protein levels may be due in part to dysfunction of Arkadia, since Arkadia did not degrade c-Ski protein in some of these cancer cell lines. Regulation by ubiquitin ligases other than Arkadia or regulation at translational level may also account for this lack of correlation.

In OCUM-2MLN cells, Arkadia degrades neither c-Ski protein (Fig. 3B) nor SnoN protein (our unpublished data), but does enhance TGF-β signalling (Fig. 4B). These findings indicate that endogenous Arkadia enhances TGF-β signalling through ubiquitylation of substrates other than c-Ski or SnoN. In HepG2 cells, c-Ski, SnoN and Smad7 are important substrates of Arkadia in maximal enhancement of TGF-β signalling (*13*). It remains to be determined whether Arkadia degrades Smad7 in OCUM-2MLN cells, since Smad7 protein was not detected by immunoblotting in the present study (data not shown). Thus, the possibility cannot be excluded that substrate(s) of Arkadia other than c-Ski, SnoN, or Smad7 are involved in the negative regulation of TGF-β signalling in OCUM-2MLN cells.

The question why Arkadia does not degrade c-Ski in OCUM-2MNL cells remains to be addressed. Expression of c-Ski protein was reduced when Arkadia was ectopically expressed in OCUM-2MLN cells (Fig. 5). Endogenous c-Ski in these cells is thus sensitive to degradation by Arkadia. We detected no mutations in the RING finger domain of endogenous Arkadia in OCUM-2MLN cells (data not shown), consistent with the finding that Arkadia still enhances TGF-β signalling in these cells. It is possible that in OCUM-2MLN cells Arkadia harbors mutation(s) in its c-Ski/SnoN-interacting region. Alternatively, endogenous Arkadia in these cells may be post-translationally modified and thereby lose its effects on c-Ski/SnoN. Investigation of the mutations and intracellular modifications of Arkadia is of importance for further understanding of the regulation of TGF-β signalling in cancer cells.

Overexpression of Arkadia inhibited basal growth of HepG2 cells. We found that expression of $p21^{WAF}$ was higher in HepG2 cells overexpressing Arkadia-WT than in control cells or cells overexpressing Arkadia-ΔC in the absence of TGF-β stimulation (Fig. 6B). It remains to be determined how Arkadia enhances the expression of $p21^{WAF}$ in the absence of TGF-β stimulation. Arkadia may have substrates other than c-Ski, SnoN or Smad7 when it inhibits the basal growth of HepG2 cells, although overexpression of Arkadia may exhibit non-physiological effects.

In the present study, we have shown that endogenous as well as exogenous Arkadia positively regulates the expression of TGF-β target genes in HeLa, OCUM-2MLN and HepG2 cells. In addition, we found that Arkadia inhibits the growth of HepG2 cells in the presence of TGF-β stimulation. Arkadia may function as a tumor suppressor by inhibiting the growth of tumour cells that are sensitive to TGF-β-induced cytostasis. Examination of the roles of Arkadia in late-stage cancer will also be needed in the near future. Further analysis will reveal how Arkadia regulates the dual effects of TGF-β on tumourigenesis and cancer development.

Supplementary Data

Supplementary data are available at JB online.

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Conflict of interest
None declared.

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Review Article

Oncolytic virus therapy for prostate cancer

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Abstract: The use of replication-competent viruses that can selectively replicate in and destroy neoplastic cells is an attractive strategy for treating cancer. Various oncolytic viruses have been taken to clinical trials since a recombinant virus was first applied to cancer patients a decade ago. The concept of the therapy is simple: infectious virus kills the host cancer cells in the course of viral replication. It is important, however, that the virus does not harm the surrounding normal tissue. Oncolytic viruses can be classified largely into two groups: DNA viruses genetically engineered to achieve cancer specificity (e.g. adenovirus, herpes simplex virus and vaccinia) and RNA viruses of which human is not the natural host (e.g. Newcastle disease virus and reovirus). Prostate cancer has always been one of the major targets of oncolytic virus therapy development. The result of six clinical trials for prostate cancer has been published and several trials are now going on. Forty-eight of 83 (58%) patients evaluated in the phase I studies demonstrated a >25% decrease in serum prostate-specific antigen level without evidence of severe toxicities. The result shows the oncolytic virus therapy is promising toward clinical application. Here, we review the recent advances in the field and summarize the results from clinical trials.

Key words: gene therapy, oncolytic, prostate cancer, replication competent, virotherapy, virus therapy.

Introduction

Prostate cancer is the second leading cause of cancer death in the United States,[1] and its incidence is increasing in many countries including Japan. Despite 70–80% of the patients that receive androgen ablation therapy showing an initial response, it leads to only small improvements in the 5-year survival rate, and the majority of patients eventually experience disease progression within 12 to 18 months.[2] New chemotherapeutic reagents such as docetaxel have contributed to improving the quality of life of patients with androgen-independent prostate cancer.[3] However, second-line treatment options are limited, and most patients who present with metastases die within 5 years of diagnosis.[4] Development of therapies with non-conventional approaches is highly needed.

Oncolytic virus therapy is an attractive means of treating prostate cancer. Replication-competent viruses are used that can replicate *in situ* and spread, and, at the same time, exhibit oncolytic activity by a direct cytocidal effect (Fig. 1).[5] Oncolytic viruses are either genetically engineered (e.g. adenovirus, herpes simplex virus type 1 [HSV-1], vaccinia virus), naturally attenuated (e.g. Newcastle disease virus), or non-pathogenic in humans (e.g. reovirus), so that they can replicate selectively in cancer cells but do not harm normal tissues. Whereas the effect of so-called gene therapy is limited by the efficiency of vectors to deliver therapeutic foreign genes (transgenes),[5] genetically engineered replication-competent viruses can also function as vectors and provide amplified delivery of transgenes in cancer. Prostate cancer is especially suited as a target disease for oncolytic virus therapy. The prostate gland is a nonessential organ, and as such its complete removal or ablation is not life threatening.[6] It is also easily accessible for inoculating viruses or obtaining tissue samples via a perineal or trans-rectal route. In addition, serum prostate-specific antigen (PSA) levels may be used to monitor the responses to therapy.

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Adenovirus

The two major strategies to restrict adenovirus replication in tumor cells are (i) to inactivate viral genes, the functions of which can be compensated in cancer cells;[7] and (ii) to place an essential viral gene under the control of a tissue- or tumor-specific promoter.[8] A representative example of an oncolytic adenovirus created by the former strategy is ONYX-015, in which the deletion in the E1B-55K coding region theoretically allows the virus to replicate only in cells with p53 mutation. ONYX-015 was the first oncolytic adenovirus to be tested in humans and 18 phase I and/or II clinical trials have been carried out so far mainly for p53-mutated cancers. Oncolytic adenoviruses created using the latter strategy have been tested more often in prostate cancer patients.

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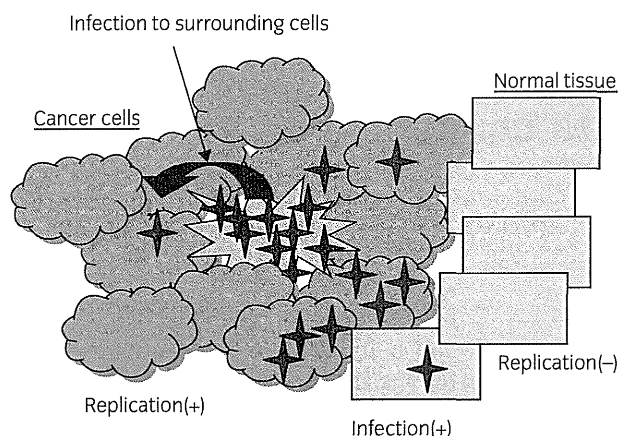

Fig. 1 Oncolytic viruses replicate in and kill tumor cells selectively. The progeny viruses produced within the host cancer cells are then released, spread and further infect surrounding cancer cells. This cycle results in continuous killing of cancer cells. Because the oncolytic viruses cannot replicate in normal cells, the normal tissues are left unharmed.

Oncolytic adenoviruses using tumor/tissue-specific promoters

The discovery of various molecular and genetic changes associated with oncogenesis led to the development of various oncolytic adenoviruses using tumor-specific transcriptional targeting of virus replication.[9] CN706 (also called CG7060 or CV706) was created by inserting the prostate-specific enhancer (PSE), a minimal enhancer/promoter construct derived from the 5′ flank of the human PSA gene, into the adenovirus type 5 (Ad5) genome so as to drive the E1A gene.[10] When infected with CN706, human PSA-producing LNCaP prostate cancer cells expressed high levels of E1A but not non-PSA-producing DU145 prostate cancer cells. A single intratumoral injection with CN706 cured LNCaP xenografts and abolished PSA production in athymic mice. The initial dose-escalation phase I study was carried out in 20 patients with prostate cancer that recurred after radiotherapy.[11]

CV764 is an oncolytic adenovirus with the E1A gene driven by PSE and the E1B gene driven by the human kallikrein 2 (hK2) enhancer/promoter.[12] CV764 was observed to be significantly attenuated in primary human microvascular endothelial cells.[12] In virus yield assays, it has a cell specificity of 10 000:1 for LNCaP cells compared with ovarian cancer OVCAR-3, SK-OV-3 or PA-1 cells.[12]

Another oncolytic adenovirus CV787 (also called CG7870) contains the rat probasin (PB) promoter-driven E1A gene and the PSE-driven E1B gene, together with a wild-type E3 region that suppresses the host immune system.[13] CG7870 destroys PSA-producing cells 10 000 times more efficiently than non-PSA-producing cells. A single tail vein injection with CG7870 eliminates distant LNCaP xenografts in athymic mice. This virus was given intravenously as a single infusion in 23 patients with hormone-refractory prostate cancer in a phase I trial.[14] The combination of oncolytic virus therapy using CG7870 and radiation therapy was significantly more efficacious than either therapy alone.[15] The antitumor efficacy of CG7870 (1×10^7 particles/mm^3 of tumor), local radiation (10 Gy) or the combination was evaluated in established subcutaneous LNCaP xenografts in athymic mice. Serum PSA levels in mice treated with the combination therapy decreased to less than 11% of the baseline by day 46, a significantly greater decrease than in mice treated with CG7870 alone (26%) or radiation alone (38%). Histological analyses of tumors collected from the combination therapy group revealed increases in the area of necrosis and percentage of apoptotic cells.[15]

The human telomerase reverse transcriptase (hTERT) promoter is used to selectively drive transgenes in many human cancer cells expressing hTERT, the catalytic component of the telomerase ribonucleoprotein complex. Irving *et al.* created a conditionally replicating adenovirus where the viral E1A gene is regulated by the hTERT promoter (AdhTERTp-E1A).[16] *In vitro* studies using a variety of cell lines demonstrated that the replication of AdhTERTp-E1A was primarily restricted to telomerase-positive tumor cells. Lytic activity was not observed in normal primary fibroblast and epithelial cells. Intratumoral administration of the virus in athymic mice bearing human liver or prostate cancer xenografts led to a significant inhibition of tumor growth and, in some cases, resulted in a complete tumor regression. AdhTERTp-E1A exhibited no apparent toxicity in the liver in mice when given systemically. The hTERT promoter-driven oncolytic virus was also significantly less toxic to freshly cultured human hepatocytes.[16] An attenuated adenovirus OBP-301 (Telomelysin) has been constructed, in which the hTERT promoter drives expression of E1A and E1B genes linked with an internal ribosome entry site. The intratumoral OBP-301 injections (10^7 PFU per tumor $\times$ 3 days) led to a significant inhibition of the detectable LNCaP prostate tumor in the subcutaneous LNCaP tumor model in nude mice.[17] Ryan *et al.* constructed an oncolytic adenovirus that contains the E2F-1 promoter-driven E1A gene and the hTERT promoter-driven E4 gene (OAS403).[18] The E2F-1 gene is a transcription factor that primarily upregulates genes associated with cell growth. OAS403 showed tumor-selective cell killing in a panel of human cells. OAS403 was less toxic than a control virus that lacked the selective E4 control in human hepatocyte cultures as well as in animals. Systemic administration in mice with established LNCaP tumors resulted in a complete tumor regression at a tolerable dose in more than 80% of animals. Furthermore, the efficacy was significantly improved when the OAS403 therapy was combined with doxorubicin.[18]

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Ad-hOC-E1 is a conditionally replicating adenovirus whose replication is regulated by the human osteocalcin (hOC) promoter in order to target both epithelial and stromal cells of prostate cancer.[19] Ad-Flk1-Fc is another that expresses a soluble vascular endothelial growth factor (VEGF) receptor capable of inhibiting angiogenesis and tumor growth.[19] Ad-Flk1-Fc markedly inhibited the tubular formation of human umbilical vein endothelial cells (HUVEC) *in vitro*. In a subcutaneous C4-2 xenograft model, an 8-week treatment with either Ad-hOC-E1 or Ad-Flk1-Fc led to a 40–60% decrease in tumor volume compared with controls and, furthermore, the combination therapy resulted in a 90% decrease, with three of 10 animals showing complete tumor regression.[19]

Ad.Δ55.HRE has the deleted E1B55 gene, in which the expression of E1A is regulated under the control of the hypoxia-response element (HRE)-expression system.[20] Ad.Δ55.HRE expressed E1A under normoxia and more E1A under hypoxia, causing oncolytic activities in tumor cells, but showed relatively attenuated cytotoxic effects in normal fibroblasts. Ad.Δ55.HRE exhibited a significant antitumor activity in athymic mice bearing PC-3 prostate cancer that expressed hypoxia-inducible factor (HIF)-1α.[20]

Suicide gene therapy

The two most widely used gene-directed enzyme prodrug therapy systems for prostate cancer are (i) HSV thymidine kinase (HSV-tk) and ganciclovir (GCV) or its analog acyclovir; and (ii) cytosine deaminase (CD) and 5-fluorocytosine (5-FC).[21] Freytag *et al.* developed an oncolytic adenovirus (Ad5-CD/TKrep) that contained the CD/HSV-tk fusion gene.[22] The replicative capability of Ad5-CD/TKrep resulted in a much greater transgene expression per cell (up to 2000-fold) than a replication-defective adenovirus and in a potentially higher gene transduction efficiency (percentage of tumor cells infected) via viral spread. Ad5-CD/TKrep effectively destroyed tumor cells *in vitro* while displaying minimal cytotoxicity toward normal cells. Moreover, the combination of CD/5-FC and HSV-tk/GCV suicide gene systems augmented the tumor cell-specific cytopathic effect of Ad5-CD/TKrep, and the treatment sensitized tumor cells to radiation.[22] Recently, Zhang *et al.* constructed a double suicide gene system, a dual CD and uracil phosphoribosyltransferase (UPRT) expression plasmid system by targeted regulation of prostate-specific membrane antigen (PSMA) promoter and enhancer. Cytotoxicity of the CD and UPRT double suicide gene system treated with 5-FC was greater on prostate cancer cells. Although this has not been applied to oncolytic therapy yet, this construct can specifically target prostate cancer cells and might have a role in oncolytic therapy against prostate cancer.[23] Barton *et al.* further developed a second-generation oncolytic adenovirus, Ad5-yCD/mutTK$_{SR39}$rep-ADP, containing an improved yeast CD (yCD)/mutant$_{SR39}$ HSV-tk fusion (yCD/mutTK$_{SR39}$) gene and the adenovirus death protein (ADP) gene.[24] Relative to Ad5-CD/TKrep, Ad5-yCD/mutTK$_{SR39}$rep-ADP demonstrated greater tumor cell killing *in vitro* and a significantly greater tumor growth suppression in a human prostate cancer model. Quantification of transgene volume following direct injection of the adenovirus into human tumor xenografts and the naive canine prostate demonstrated that ADP enhanced adenoviral spread *in vivo*. Intraprostatic injection of Ad5-yCD/mutTK$_{SR39}$rep-ADP did not result in significantly increased toxicity relative to the parental Ad5-CD/TKrep adenovirus.[24]

Other approaches

Sova *et al.* constructed a capsid-modified adenovirus (Ad5/35.IR-E1A/TRAIL) that replicated selectively in tumor cells and expressed tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to mediate apoptosis and enhance the release of progeny virus from infected cells.[25] The capsid contained short-shafted fibers derived from adenovirus serotype 35, which allowed for efficient infection of malignant tumor cells. This oncolytic adenovirus induced apoptosis in cell lines derived from human colorectal, lung, prostate, and liver cancer. This virus spread efficiently in cell cultures as well as in tumors of animal models.[25] Suzuki *et al.* demonstrated that the fiber knob modification of conditionally replicating adenoviruses allowed coxsackievirus and adenovirus receptor (CAR)-independent infection leading to enhanced viral propagation and oncolytic activities.[26] Such an infectivity-enhanced adenovirus showed improvement of viral replication, infection and cell killing in cultured LNCaP cells, and augmented antitumor efficacy *in vivo*.[26]

The oncolytic adenovirus, dl922/947, was created so that its replication was restricted to cancer cells with a loss of the G1-S cell cycle checkpoint.[27] Co-infection of the replication-deficient Ad-Flk1-Fc with dl922/947 allowed replication and repackaging of Ad-Flk1-Fc and enhanced the expression of soluble VEGF receptor. Co-administration of these viral vectors led to significantly enhanced antitumor effects in colon HCT 116 and prostate PC-3 xenografts in mice. The therapeutic benefit correlated with an increase in copy numbers of the Ad-Flk1-Fc viral genome, an increase in levels of the Flk1-Fc protein, and a decrease in microvessel densities, consistent with enhanced anti-angiogenic activity.[27] AxdAdB-3 has a mutation in the Rb-binding site of E1A and deletion of E1B 55 KD, and is unable to bind pRb and p53, leading not to replicate in normal cells. AxdAdB-3 showed the antitumor effect in the orthotopic prostate cancer model established with DU145 in severe combined immunodeficiency (SCID) mice and significantly improved survival.[28]

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Mantwill *et al.* reported that an oncolytic adenovirus (Xvir03) expressing the viral proteins E1B55k and E4orf6 led to nuclear translocation of the Y-box protein YB-1.[29] YB-1 is known to be involved in the transcriptional activation of the genes, such as MDR1 and MRP1, which play a major role for tumor cells to acquire a multidrug-resistant phenotype. They showed that Xvir03 downregulated the expression of MDR1 and MRP1, and exhibited a strong cytotoxic effect in PC-3 and DU145 cells. Thus, the nuclear translocation of YB-1 by Xvir03 might lead to a resensitization of tumor cells to cytotoxic drugs and to viral replication and cell lysis.[29]

HSV-1

G207, one of the first oncolytic HSV-1 strains taken into clinical trials, was derived from HSV-1 strain F and has deletions in both copies of the *γ34.5* gene and a *lacZ* insertion inactivating the *ICP6* gene. The double mutations permitted viral replication within cancer cells that can complement these mutations but not in normal cells including neurons.[30] G207 has been shown to be effective against human prostate cancer *in vitro*, and also *in vivo* following direct intraneoplastic inoculation[31] as well as intravenous administration.[32] In athymic mice with human prostate cancer xenografts, intratumoral injection with G207 caused a reduction in tumor size and a complete eradication of more than 22% of tumors.[32] Walker *et al.* reported that LNCaP tumors that recurred after radiation therapy remained sensitive to G207 therapy.[32] In addition to G207, another onoclytic HSV-1, NV1020, was shown to cause cytolytic effects *in vitro* in all prostate cancer cell lines tested.[33] Both viruses demonstrated a significant decrease in serum PSA and an inhibition of tumor growth when inoculated into PC-3 or C4-2 subcutaneous xenografts.[33] Systemic administration of NV1023 virus, an HSV-1/HSV-2 recombinant, to spontaneously arising tumors in the transgenic TRAMP (transgenic adenocarcinoma of mouse prostate) mouse inhibited primary tumor growth and metastases to lymph nodes.[34] Jorgensen *et al.* combined the G207 therapy with external beam radiation in subcutaneous prostate cancer models, athymic mice bearing human LNCaP tumors, or athymic or C57BL/6 mice bearing mouse TRAMP-C2 tumors. The virus was delivered intravenously for LNCaP, and intratumorally for TRAMP-C2. Although either G207 or radiation was effective in delaying tumor growth in these models, the combination did not cause an enhanced antitumor effect.[35]

From G207, Todo *et al.* constructed a triple-mutated, oncolytic HSV-1, G47Δ, by creating a further deletion within the nonessential *α47* gene.[36] We recently demonstrated that: (i) G47Δ showed a greater antitumor activity in prostate cancer cells than G207 *in vitro* and *in vivo*; (ii) the combination therapy with G47Δ and androgen ablation exhibited additive effects, resulting in a greater inhibition of tumor growth than either therapy alone; and (iii) G47Δ was also effective against those prostate cancers that once responded to androgen ablation but eventually became refractory and recurred.[37] The combination of castration with the G47Δ treatment resulted in tumor growth suppression that was significantly greater than either castration or G47Δ alone in the TRAMP-C2 subcutaneous model. Similarly, the treatment with flutamide plus G47Δ resulted in a significantly better tumor growth inhibition than either flutamide or G47Δ alone in the human HONDA prostate cancer subcutaneous model. Thus, in both syngeneic and xenogeneic prostate cancer models, the combination of androgen ablation with G47Δ results in superior tumor growth suppression and prolonged animal survival compared with either therapy alone (Fig. 2). While our studies were done using intraneoplastic inoculations of the oncolytic HSV-1, and while such delivery may benefit locally advanced diseases, studies in other settings have demonstrated that the intravenous delivery of oncolytic HSV-1 is feasible and can cause regression of distant prostate cancer deposits.[32] This would be clinically relevant and an area for future investigation for treatment of widely metastatic prostate cancer with G47Δ in combination with androgen ablation.

Fu *et al.* inserted the gene encoding a truncated form of the gibbon ape leukemia virus envelope fusogenic membrane glycoprotein (GALV.fus) into the genome of an oncolytic HSV-1, using an enforced ligation procedure.[38] The expression of GALV.fus in the context of an oncolytic HSV-1 significantly enhanced the antitumor effect of the virus in DU145 prostate cancer cells. Furthermore, by controlling the GALV.fus expression by a strict late viral promoter whose activity depends on the initiation of viral DNA replication, the glycoprotein was expressed in tumor cells but not in normal nondividing cells.[38] SCID mice bearing human prostate cancer PC-3M-Pro4 were used to evaluate three different types of oncolytic HSV-1; non-fusogenic Baco-1, singly fusogenic Synco-2, and doubly fusogenic Synco-2D. In a lung metastases model, intravenous administration of Synco-2D significantly reduced the number of tumor nodules by day 40 compared with Synco-2, Baco-1 or phosphate-buffered saline (PBS).[39]

Lee *et al.* demonstrated that a replication-deficient HSV-1 could be complemented by an amplicon vector to restore its oncolytic activity in a tissue-specific and low toxic fashion. A prostate-specific amplicon (ARR(2)PB-ICP4) containing a probasin-derived promoter (ARR(2)PB) upstream of the infected-cell protein 4 (ICP4) gene was constructed, together with a control amplicon CMV-ICP4.[40] When a replication-deficient ICP4(–) HSV-1 was used as a helper virus, the amplicons could complement the ICP4(–) helper virus, and cause it to replicate in and kill prostate cancer cells. Intratumoral injection of LNCaP tumors with either of the amplicons together with the helper virus resulted in a >75% reduction in tumor volume and serum PSA. Histo-

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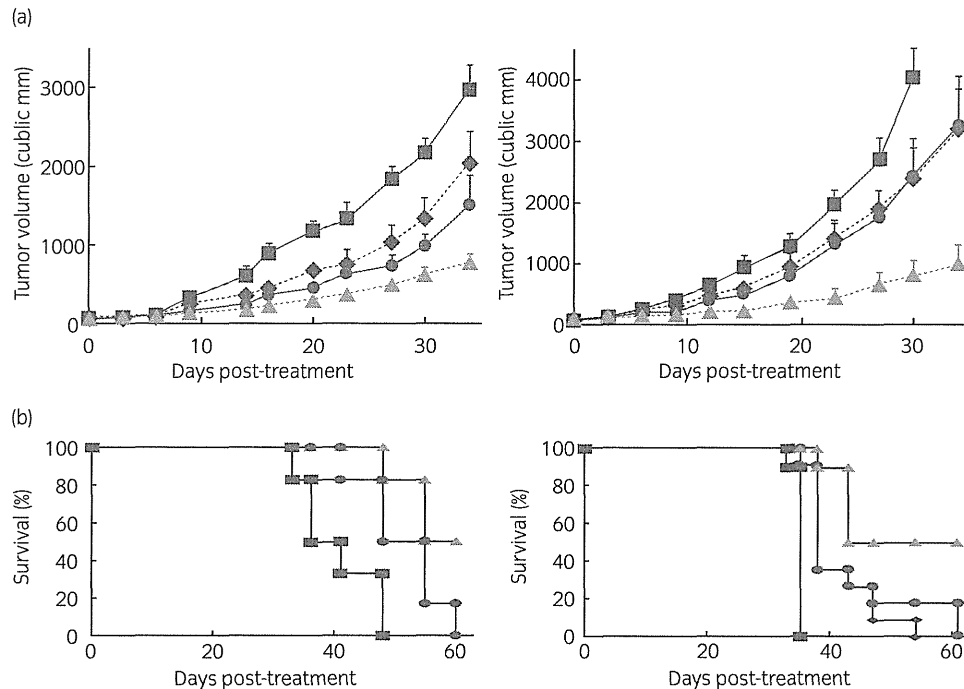

Fig. 2 (a) therapeutic effects of G47Δ in combination with androgen ablation. (Left) C57BL/6 male mice bearing established s.c. TRAMP-C2 tumors were treated with intraneoplastic G47Δ inoculations (5×10^6 pfu, days 0 and 3) in combination with surgical castration or sham operation (day 0). The combination treatment (triangles) was significantly more efficacious than castration alone (lozenges) or G47Δ alone (circles), resulting in smaller tumor volumes ($P < 0.05$ for castration alone on day 9–34 and for G47Δ alone on day 20–23, 30, unpaired *t*-test;). (Right) Male athymic mice bearing established s.c. HONDA tumors were treated intraneoplastically with 2×10^6 pfu of G47Δ on days 0 and 3 in combination with 60-day-long flutamide pellets on day 0. The combination treatment (triangles) was significantly more efficacious than flutamide alone (lozenges) or G47Δ alone (circles) ($P < 0.05$ for G47Δ alone on day 15 and for castration alone on day 23, unpaired *t*-test). The bars represent standard error of the mean (SEM). (b) Kaplan–Meier analyses of survival in experiments described above. The combination therapy significantly prolonged survival of tumor-bearing animals in both models compared with the monotherapies; androgen ablation or G47Δ treatment ($P < 0.05$). Fukuhara H *et al. Clin Cancer Res* 2005; **11**: 7886–90. Left panels: -■-, Mock/sham operation; ··◆··, Castration; -●-, G47Δ; ··▲··, G47Δ/Castration. Right panels: -■-, Mock/placebo; ··◆··, Flutamide; -●-, G47Δ; ··▲··, G47Δ/Flutamide.

logical and quantitative polymerase chain reaction (PCR) analyses indicated that the toxicity in nontumor tissues was much lower with ARR(2)PB-ICP4 than with CMV-ICP4.[40]

Immunomodulation therapy

Accumulating evidence indicates that, aside from the extent of replication capability within the tumor, the efficacy of an oncolytic HSV-1 depends on the extent of induction of host antitumor immune responses. Ways to modify the host immune responses include expression of immunostimulatory molecules using oncolytic HSV-1 as a vector and co-administration of reagents that modulate immune reactions.[41] The oncolytic HSV-1 expressing murine interleukin-12 (IL-12) (NV1042), but not the one expressing murine granulocyte-macrophage colony stimulating factor (GM-CSF) (NV1034), showed greater efficacy than the control HSV-1 (NV1023) in two murine prostate cancer models, one with a high major histocompatibility complex (MHC) class I level (Pr14-2) and the other with a low level (TRAMP-C2).[42] Systemic administration of NV1042 showed better suppression of tumor growth and caused longer survival of animals than NV1023 in mice with established metastatic lung tumors of TRAMP-C2.[43] NV1042-treated mice exhibited a transient increase in serum IL-12 levels at 1 day post-treatment, whereas IL-12 levels in tumor-bearing lungs persisted for at least 2 more days. Splenocytes from NV1042-treated mice, but not those from NV1023-treated ones, responded to TRAMP-C2 cells and displayed natural killer activity.[43] We have recently created four oncolytic HSV-1s by using the triple-mutated G47Δ as the backbone, which are HSV-1 expressing (i) murine IL-18, (ii) murine soluble B7-1, (iii) both of IL-18 and B7-1, or (iv) none (Fig. 3).[44] The *in vivo* efficacy of the armed oncolytic HSV-1 was tested in two immunocompetent mouse tumor models, TRAMP-C2 prostate tumors in syngeneic C57BL/6 mice and Neuro2a malignant brain tumors in syngeneic A/J mice. Intratumoral inoculation of G47Δ double-armed with

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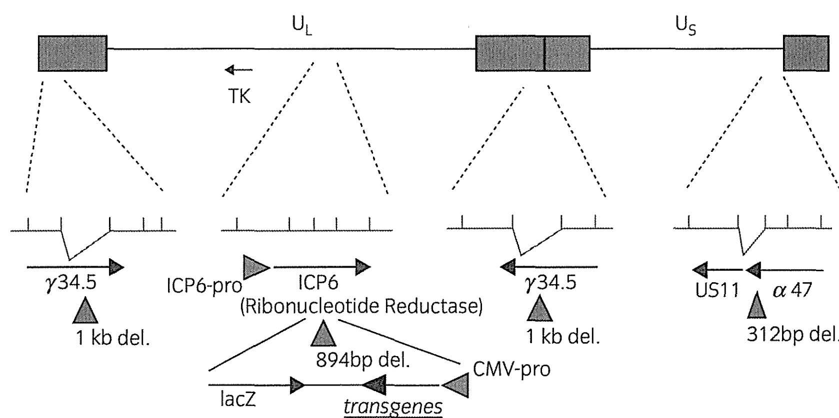

Fig. 3 The structure of 'armed' oncolytic herpes simplex virus-1 (HSV-1) (G47Δ-transgene) constructed using the bacterial artificial chromosomes (BAC) system. The boxes (top line) represent inverted repeat sequences flanking the long (U_L) and short (U_S) unique sequences of HSV-1 DNA. The G47Δ-transgene contains 1.0-kb deletions in both copies of the *γ34.5* gene, a 312-bp deletion in the *α47* gene, and an 894-bp deletion in the *ICP6* gene. The *lacZ* gene and the cytomegalovirus (CMV) promoter-driven transgenes, placed in opposite directions, are inserted in the deleted *ICP6* locus. Thick arrows indicate transcribed regions.

IL-18 and B7-1 caused a significant reduction of tumor growth compared with G47Δ expressing IL-18 alone or G47Δ expressing soluble B7-1 alone.[44] We used bacterial artificial chromosomes (BAC) and two recombinase systems (Cre/loxP and Flp/FRT) to develop a method that allowed a rapid, reliable, and simultaneous construction of multiple 'armed' oncolytic HSV-1 vectors using G47Δ as the backbone.[44] Because the *α47* gene product (ICP47) of HSV-1 inhibits the transporter associated with antigen presentation (TAP) that mediates antigen presentation in the context of MHC class I, *α47*-deficient HSV-1 including G47Δ are especially suited to express immunostimulatory transgenes in humans. The BAC system we constructed might be useful for expedited development of 'armed' oncolytic HSV-1.

Reovirus

It is known that cells with an activated Ras signaling pathway support reoviral replication.[45] Mutations involving Ras occur in approximately 30% of all human cancers and mutations in other elements in the Ras pathway can also lead to cancer development. It was found that normal, untransformed cells were not infectable by reovirus.[46] Coffey *et al.* reported that a single intratumoral injection of the virus resulted in regression of tumors in 65–80% of mice.[47] Oncolytic wild-type reovirus (Reolysin) is in clinical development, and Phase I/II clinical trials for advanced solid tumors including prostate cancer have been conducted in Canada and the UK.[48]

Newcastle disease virus

The first definitive report of efficacy with Newcastle disease virus (NDV) was published in 1964.[49,50] In this report, a patient with myelogenous leukemia responded to intravenous NDV treatment.[50] The virus is not pathogenic to humans. Tumor selectivity is believed to arise from viral induction of tumor necrosis factor (TNF)-α secretion by peripheral blood mononuclear cells and viral enhancement of sensitivity of neoplastic cells to the cytotoxic effects of TNF-α. The most well-characterized oncolytic strain of NDV is 73-T.[51] A significant tumor growth inhibition (77–96%) was seen for prostate (PC-3), epidermoid (KB8-5-11), colon (SW620 and HT29), large cell lung (NCIH460), breast (SKBR3) and low passage colon (MM17387) carcinoma xenografts treated intratumorally with the strain 73-T.[52] In all cases, tumors treated intratumorally with PBS or UV-inactivated NDV displayed rapid growth.[52] No patients with prostate cancer participated in the phase I clinical trials but phase II studies are ongoing for patients with cancer resistant to conventional modalities, including prostate cancer. A naturally attenuated NDV strain (PV701) was isolated and used in a phase I clinical trial in which 79 patients with solid cancers, excluding prostate cancer, that failed conventional therapy were treated with intravenous administration. Three types of side effects were seen but not cumulative toxicity: flu-like symptoms, tumor-site-specific adverse events, and infusion reactions.[22] The doses, schedules, and infusion rates for PV701 were evaluated in other clinical trials, and tumor responses were first noted when higher doses were achieved using desensitization.[53]

Vaccinia virus

Vaccinia virus has a distinctive history as a vaccine used for the eradication of smallpox, and the unique biology of this virus has been extensively investigated.[54,55] Pox viruses are unique in that their entire life cycle occurs in the cytoplasm,

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including transcription and DNA replication, thus avoiding the potentially inefficient process of nuclear translocation.[54,55] Compared with HSV-1 and adenoviruses, vaccinia virus is still at its early stage of development as an oncolytic virus.[55] Oncolytic poxvirus JX-963 was engineered to target cancer cells with activation of the transcription factor E2F and the epidermal growth factor receptor (EGFR) pathway by deletion of the thymidine kinase and vaccinia growth factor genes.[56] JX-963 expresses human GM-CSF[56] and is currently in the pre-clinical phase for lung, renal and prostate cancer.

Other viruses

Recently, several other viruses have been reported as the oncolytic virus in 2008–2009. Intratumoral and intraperitoneal injections of the human respiratory syncytial virus (RSV) led to a significant regression of PC-3 prostate cancer *in vivo*. The RSV virus in PC-3 cells led to selective destruction of PC-3 cancer cells *in vitro* and in xenograft tumors *in vivo* due to apoptosis triggered by the downregulation of nuclear factor-kappaB (NF-kappaB) activity in RSV-infected PC-3 cells.[57] Kawaguchi *et al.* examined the direct tumor-killing activity of inactivated Sendai virus particle (hemagglutinating virus of Japan envelope [HVJ-E]) in the hormone-resistant human prostate cancer cell lines PC-3 and DU145. HVJ-E infection produced interferon-α (INF-α) and INF-β and induced apoptosis in cancer cells. Direct injection of HVJ-E into PC3 tumor cells in SCID mice led to the reduction of the tumor volume and 85% of the mice became tumor-free.[58] Liu C *et al.* have constructed a live attenuated strain of measles virus (MV) that infects cells exclusively through the PSMA receptor. The PSMA virus induced tumor regression of LNCaP and PC3-PSMA tumor xenografts and apoptosis was seen in virus-treated tumors.[59] Vesicular stomatitis virus (VSV) is also a candidate oncolytic virus for prostate cancer. However, the efficacy of the virus depends on the type of infected cells. LNCaP cells were sensitive to infection with VSV, while PC-3 cells were relatively resistant to VSV.[60] Berry *et al.* evaluated the low pathogenic enteroviruses: Coxsackievirus A21 (CVA21), as well as a bio-selected variant of Coxsackievirus A21 (CVA21-DAFv) and Echovirus 1 (EV1). Systemic delivery of each of the three viruses induced reduction of xenograft tumor *in vivo*.[61]

Safety

To address the concerns regarding virus dissemination beyond the target tissue, the biodistribution, persistence, toxicity, and potential of germ-line transmission of an oncolytic adenovirus (Ad5-CD/TKrep) following intraprostatic administration were examined in mice.[62] Although the virus persisted in the urogenital tract and the liver for up to 28 days post-injection, the toxic effects observed were expected, minimal, and self-limiting. Ad5-CD/TKrep also persisted in the adult male gonads and may have replicated *in vivo*, but there was no evidence of germ-line transmission in 149 offspring examined.[62]

In preclinical safety evaluations, the second-generation oncolytic HSV-1, G207, displayed no evidence of clinical disease, no shedding of infectious virus, and no spread of the virus into other organs when injected into the prostates of HSV-1-susceptible mice and nonhuman primates.[63]

Clinical trials

Adenovirus

Three clinical trials have been carried out with two oncolytic adenoviruses transcriptionally targeted for prostate cancer (Table 1). CG706 and CG7870 were tested in respective phase I trials, in which patients with local recurrence received an intraprostatic injection of the viruses following primary radiotherapy. Liver function toxicities of greater than grade 1 associated with CV706 administration were not observed.[11] The responses of 20 treated patients were dose-dependent: all five patients that achieved at least a 50% reduction in PSA received the two highest doses of CV706. Intravenous administration with CG7870 was evaluated in a phase I trial with 23 patients with hormone-refractory metastatic prostate cancer.[14] At the highest dose level, transient grade 2–3 transaminitis and grade 1 thrombocytopenia were observed. Mild liver inflammation, associated, in some instances, with elevated D-dimer levels, a marker for disseminated intravascular coagulation, and elevated IL-6 levels as a marker of antiviral response were experienced at a relatively high dose. Five patients showed a decrease in serum PSA of 25% to 49% following a single treatment, including three of eight patients at the highest dose level.[14] The development of this virus will advance to two phase II trials: one in combination with radiotherapy and another in combination with taxane-based chemotherapy.

Ad5-CD/TKrep was injected intraprostatically under transrectal ultrasound guidance into 16 patients with local recurrence of prostate cancer after definitive radiation therapy.[64] Two days later, patients were given 5-fluorocytosine and ganciclovir (5-FC/GC) prodrug therapies for 1 or 2 weeks. There was no dose-limiting toxicity, and the maximum tolerated dose was not defined. Ninety-four percent of the adverse events observed were mild to moderate (grade 1/2) in nature. Seven of 16 (44%) patients demonstrated at least a 25% decrease in serum PSA, and three of 16 (19%) patients demonstrated at least a 50% decrease.[64] The PSA doubling time (PSADT) after the gene therapy significantly increased from a mean of 17 to 31 months at 5-year follow-up.[65] In the second trial, 15 patients received an intraprostatic injection of the virus, followed by 5-FC and valganciclovir prodrug therapy for 1,

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Table 1 Summary of results from closed oncolytic virus clinical trials for prostate cancer

Virus	Phase/delivery	Prodrug or combination therapy	Cancer type (*n*)	Doses	Antitumoral activity	Reference (year)
CG7060 (E1A, E3(–), PSA)	I intratumoral		Local recurrence of prostate cancer after radiation therapy (20)	1×10^{11}–1×10^{13} vp	>50% PSA reduction in 5/20 >25% PSA reduction in 13/20	DeWeese *et al.* (2001)[11]
CG7870 (E1A, E1B, probasin promoter, PSA enhancer)	I intravenous		Hormone-refractory metastatic prostate cancer (23)	1×10^{11}–6×10^{12} vp	>25% PSA reduction in 5/23	Small *et al.* (2006)[14]
Ad5-CD/TKrep	I intratumoral	5-FC/GC	Local recurrence of prostate cancer after radiation therapy (16)	1×10^{10}–1×10^{12} vp	>50% PSA reduction in 3/16, >25% PSA reduction in 7/16	Freytag *et al.* (2002, 2007)[64,65]
Ad5-CD/TKrep	I intratumoral	5-FC/VGC + 3D-CRT	Newly diagnosed prostate cancer (15)	1×10^{12} vp	PSA <0.5 ng/mL for 9 months in 5/10 >25% PSA reduction in 15/15	Freytag *et al.* (2003)[66]
Ad5-CD/TKrep	I intratumoral	5-FC/GC + IMRT	Newly diagnosed prostate cancer (12)	1×10^{11}–6×10^{12} vp	0/12 being positive for cancer at biopsy >25% PSA reduction in 8/9	Freytag *et al.* (2007)[67]
Reolysin	I intravenous		Prostate cancer (five in 33 solid cancers)	up to 3×10^{10} $TICD_{50}$	PSA reduction (from 100 to 50 ng/mL) in one patient	Vidal *et al.* (2008)[48]

3D-CRT, 3 dimensional confocal radiotherapy; 5-FC, 5-fluorocytosine; GC, ganciclovir; IMRT, intensity-modulated radiotherapy vp, viral particle; pfu, plaque-forming units; PSA, prostate-specific antigen; VGC, vanganciclovir.

2 or 3 weeks along with three-dimensional confocal radiotherapy (3D-CRT; 70–74 Gy).[66] There were no dose-limiting toxicities and no significant treatment-related adverse events. Ninety-four percent of the adverse events observed were mild to moderate and self-limiting. Follow-up biopsy specimens demonstrated persistence of transgene expression for up to 3 weeks, implicating that the prodrug therapy should be continued for a longer period to maximize therapeutic benefits. The mean PSA half-life of patients receiving 2–3 weeks of the prodrugs was significantly shorter than that of patients receiving the prodrugs for only 1 week (0.6 months *vs* 2.0 months) and markedly shorter than that reported previously for patients treated with conventional dose 3D-CRT alone (2.4 months).[66] With a median follow-up of only 9 months, five of 10 (50%) patients not treated with androgen-ablation therapy achieved a serum PSA level of less than or equal to 0.5 ng/mL. In the third trial, intensity-modulated radiotherapy (IMRT) was given during administration of the 5-FC/GC prodrugs.[67] This therapy was associated with low toxicity, and there were no dose-limiting toxicities and treatment-related serious adverse events. When the results of these two similar trials were combined, 22% of evaluable patients were positive for adenocarcinoma at their last biopsies, which is significantly better than expected (>40%) for this cohort of patients. When the results were categorized by prognostic risk, most patients that showed treatment benefit belonged to the intermediate-risk group, with none of 12 patients being positive for cancer at their last biopsies.[67]

Reovirus

The UK phase I trial with Reolysin (reovirus Type 3 Dearing) was carried out in patients with solid tumors including prostate cancer (Table 1). Escalating intravenous doses of Reolysin of up to 3×10^{10} $TCID_{50}$ were found to be safe with minimal toxicity. Evidence of viral replication was found from biopsy samples, but neutralizing antibodies were also produced. A reduction in the PSA level (from 100 to 50 ng/mL) in a patient with prostate cancer suggested a potential for efficacy.[48] A phase II trial was carried out in patients with stage T2 (organ-confined) prostate cancer. A single intraprostatic injection of reovirus was given 3 weeks prior to prostatectomy. Evidence of tumor cell apoptosis was seen in four of six tumors and the PSA level fell in one patient. No toxicity was observed and immune cell infiltration was limited to the tumor.[68]

Conclusion

Oncolytic virus therapy is an attractive new means for treating prostate cancer, in which the viruses exhibit a direct antitumor activity through viral replication and spread. Prostate cancer is a good target for oncolytic virus therapy, because the prostate gland is a nonessential organ and easily accessible. In addition, serum PSA levels may be monitored for efficacy evaluation. So far, results from multiple clinical trials of oncolytic virus therapy are encouraging. We believe that the development of oncolytic virus therapy will lead to a promising therapeutic option for prostate cancer in the near future.

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