infected and uninfected tumor tissues. Similarly, a combination therapy that involves Ad-p53 and bevacizumab, a monoclonal antibody specific for VEGF-A, or FasL (CD95L) transduction may be more effective than monotherapy with Ad-p53 in completely eradicating tumor cells (**Figure 4**).

4. Preclinical studies of replication-competent CRAd-p53 vectors

Although clinical studies have demonstrated that replication-deficient Ad-p53 vector was safe, feasible and well tolerated in patients with various cancers (**Table 1**), it would be impossible to induce profound exogenous p53 expression in every tumor cell via this Ad-p53 vector. The low transduction rate of *p53* gene transfer via Ad-p53 vector is a major problem that must be overcome to improve the clinical outcomes of patients with advanced cancers. Tumor-specific, replication-competent oncolytic adenoviruses are being developed as novel vectors for anticancer gene therapies; in these vectors, the promoters of cancer-related genes are used to regulate virus replication in a tumor-dependent manner. There are several types of p53-expressing conditionally replicating adenovirus CRAd-p53 vectors, such as AdDelta24-p53 [89], SG600-p53 [90] and OBP-702 (**Figure 1B**) [91]. Next, we discuss the therapeutic potential of CRAd-p53 vectors in adenovirus-mediated p53 cancer gene therapy.

4.1 AdDelta24-p53

van Beusechem *et al.* previously constructed a novel p53-expressing CRAd vector, AdDelta24-p53, in which the RB protein-binding CR2 domain (24 base pairs) of the E1A region was deleted and the p53 expression cassette under the regulation of simian virus 40 early promoter was inserted into the E3 region (**Figure 1B**) [89]. AdDelta24-p53 suppressed the viabilities of many types of human cancer cells more efficiently that AdDelta-24. Moreover, AdDelta24-p53 enhanced the sensitivity of radiation in human glioma cells [92]. However, some human cancer cells with overexpression of the p53-negative regulator MDM2 were resistant to AdDelta24-p53 because MDM2 protein efficiently suppresses exogenous p53 expression. Therefore, a novel CRAd-p53 vector expressing an MDM2-resistant p53 variant, AdDelta24-p53 (14/19), has been developed (**Figure 1B**) [93]. AdDelta24-p53 (14/19) induces exogenous expression of a variant form of p53 that is incapable of binding to MDM2 and is resistant to MDM2-dependent degradation. AdDelta24-p53(14/19) was 10 times more effective than AdDelta24-p53 in killing MDM2-overexpressing human cancer cells. These findings suggest that suppression of MDM2-dependent p53 negative regulation is an effective strategy for enhancing the antitumor efficacy of adenovirus-mediated p53 cancer gene therapy.

4.2 SG600-p53

Wang *et al.* recently developed a triple-regulated CRAd carrying a p53 gene expression cassette, SG600-p53, in which the *E1A* gene with a deletion of 24 nucleotides in the CR2 region is controlled by the human telomerase reverse transcriptase promoter (hTERT-p) and the *E1B* gene is regulated by the hypoxia response element and the *p53* gene cassette controlled by the cytomegalovirus promoter is inserted between the E1A and E1B regions (**Figure 1B**) [90]. SG600-p53 was more cytopathic than Ad-p53 vector in the suppression of *in vitro* cell viability and *in vivo* tumor growth in human tumor cells [90], whereas intravenous or intramuscular injection of SG600-p53 had no adverse effects in rodents and nonhuman primates [94]. These findings suggest that CRAd-p53 vector is a safe and effective therapy for inducing antitumor effects.

4.3 OBP-702

We previously developed a telomerase-specific replication-competent oncolytic adenovirus, OBP-301 (Telomelysin), in which the hTERT-p drives the expression of two adenoviral genes, *E1A* and *E1B*, that are linked to an internal ribosome entry site [74]. OBP-301 induces tumor-selective oncolysis in a telomerase-dependent manner [74-76]. In a Phase I clinical study, OBP-301 was well tolerated [95]. Since the combination therapy of Ad-p53 and OBP-301 enhanced p53 expression and resulted in a more profound antitumor effect when compared to monotherapy with either OBP-301 or Ad-p53 [77], we generated an armed OBP-301 variant (OBP-702) (**Figure 1B**) that expresses the wild-type *p53* gene; this variant suppressed the viabilities of both OBP-301-sensitive and OBP-301-resistant tumor cells more efficiently than Ad-p53 or OBP-301 in epithelial and mesenchymal tumor cells [83,91]. Ad-p53 and OBP-301 mainly induce apoptotic and autophagic cell death, respectively, whereas OBP-702 can cause both apoptotic and autophagic cell deaths via exogenous p53 overexpression in tumor cells. These results suggest that CRAd-p53 vector efficiently induces both apoptotic and autophagic cell death via p53 overexpression.

5. Molecular mechanism of antitumor effect induced by CRAd-p53 vector

CRAd-p53 vector induces higher p53 expression and stronger antitumor effects through induction of cell death than Ad-p53. Although the molecular mechanism by which CRAd-p53 vector is superior to Ad-p53 vector to induce cell death remains to be elucidated, we recently demonstrated that CRAd-p53 vector induces a profound antitumor effect via E1A-dependent enhancement of viral replication and the p53-mediated cell death signaling pathway. We next discuss advances in the understanding of the molecular mechanism of the CRAd-p53-mediated antitumor effect.

5.1 p53-mediated cell death signaling pathway

When tumor cells were infected with a similar dose of Ad-p53 or CRAd-p53 (OBP-702), OBP-702 induced a much higher level of p53 expression than Ad-p53 [83,91]. However, despite

the higher p53 expression, the expression levels of p53-downstream targets p21 and MDM2 were lower in the OBP-702–infected tumor cells than in the Ad-p53-infected tumor cells [91]. This discrepancy between the expression levels of p53 and p53-downstream target genes was due to adenoviral E1A accumulation, which was involved in the suppression of p21 and MDM2 and contributed to the profound antitumor effect. Thus, OBP-702 induces an antitumor effect more efficiently than Ad-p53 via E1A-dependent enhancement of virus replication and p53-mediated cell death signaling pathway.

5.2 E1A-dependent miRNA regulatory network

CRAd-p53 vector possesses the *E1A* gene under the regulation of a tumor-specific promoter for viral replication, although a replication-deficient Ad-p53 vector is E1A-deficient. Recently, we demonstrated that adenoviral E1A-dependent activation of the transcription factor E2F1 upregulates two miRNAs, *miR-93* and *miR-106b*, which efficiently suppress p21 expression in OBP-702-infected tumor cells; this suppression of p21 leads to the enhancement of p53-induced apoptosis and autophagy in these cells (**Figure 5**) [83]. Interestingly, E2F1 has also been suggested to suppress MDM2 expression by inducing upregulation of *miR-25* and *miR-32*, which target MDM2 [96]; therefore, the E1A-dependent miRNA regulatory network may be implicated in the fine-tuning of the p53-mediated cell death signaling pathway. Exploration of the crosstalk between the MDM2–p53-p21 pathway and the E1A–E2F1–miRNA pathway may clarify the molecular mechanism of p53-induced apoptosis and autophagy in OBP-702-infected tumor cells.

6. Conclusion

Adenovirus-mediated p53 cancer gene therapy is a promising antitumor strategy to induce a profound p53-mediated cell death signaling pathway in tumor cells. Clinical studies of replication-deficient Ad-p53 vectors (Advexin, Gendicine and SCH-58500) have shown that administration of Ad-p53 vector by intratumoral, intraperitoneal and intravesical approaches is a safe, feasible and effective antitumor strategy against many types of cancers. However, Ad-p53-mediated p53 activation is often insufficient for efficiently inducing cell death pathways in tumor tissues; therefore, replication-competent oncolytic adenoviruses that express p53, such as AdDelta24-p53 [89], SG600-p53 [90] and OBP-702 [91], have recently been developed to improve the clinical outcome of adenovirus-mediated p53 cancer gene therapy (**Figure 1**). Moreover, given the underlying molecular mechanisms of the p53-mediated tumor suppression network induced by Ad-p53 and CRAd-p53 vectors, we should make an effort to develop safe and effective cancer gene therapies that are based on the potent tumor suppressor *p53* gene.

7. Expert opinion

Adenovirus-mediated p53 cancer gene therapy is a promising antitumor therapy to restore the wild-type p53 function, because many human cancers lose p53 function due to genetic alterations in the *p53* gene. Over the past decade, clinical studies have shown that replication-deficient Ad-p53 vector administered with various injection approaches is safe, feasible and well tolerated in patients with malignant tumors. However, the antitumor efficacy of Ad-p53 vector in clinical studies has been limited in some cancer patients, unlike the antitumor effect of Ad-p53 vector in preclinical experiments. To improve the therapeutic potential of Ad-p53 vector, we must develop an effective strategy for Ad-p53-based cancer gene therapy. Since mesenchymal types of malignant tumors, including osteosarcomas, are sensitive to p53 restoration in preclinical experiments [97-100], sarcoma patients may also be good candidates for treatment with Ad-p53-based cancer gene therapy. Based on preclinical experiments for the improvement of Ad-p53-mediated antitumor efficacy, several combination therapies with E1A-expressing replication-competent adenovirus, MDM2 inhibitors and p21-targeted siRNA/miRNA would enhance the therapeutic potential of Ad-p53 vector via an increased p53-mediated cell death signaling pathway. Moreover, antiangiogenic therapy with bevacizumab and proapoptotic therapy via the Fas receptor/ligand system would also promote the bystander effect of Ad-p53 therapy. In contrast, replication-competent p53-expressing CRAd-p53 vector may be superior to Ad-p53 vector in inducing the p53-mediated cell death signaling pathway via not only viral replication but also E1A-dependent suppression of p21/MDM2 expression. Exploration of the interaction between p53- and E1A-mediated signaling pathways is needed to understand the molecular mechanism of the CRAd-p53-mediated antitumor effect. In the near future, clinical studies of CRAd-p53 vectors should be conducted to evaluate the safety and antitumor efficacy of CRAd-p53 in cancer patients. Moreover, to improve the clinical outcome of adenovirus-mediated p53 cancer gene therapy in patients with advanced cancers, we must develop a delivery system for intravenous administration of Ad-p53 and CRAd-p53 vectors because metastatic tumors are often directly inaccessible. In particular, tumor-specific delivery system of adenoviral vectors using carrier cells or nanotechnologies would be a promising antitumor strategy to overcome preexisting or induced immunity to adenoviral vectors. Thus, the development of potent p53-expressing adenovirus vectors and delivery systems would provide great opportunities to treat p53-inactivated primary and metastatic tumors.

Declaration of interest

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Review

Oncolytic Adenovirus-Induced Autophagy: Tumor-Suppressive Effect and Molecular Basis

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Autophagy is a catabolic process that produces energy through lysosomal degradation of intracellular organelles. Autophagy functions as a cytoprotective factor under physiological conditions such as nutrient deprivation, hypoxia, and interruption of growth factors. On the other hand, infection with pathogenic viruses and bacteria also induces autophagy in infected cells. Oncolytic virotherapy with replication-competent viruses is thus a promising strategy to induce tumor-specific cell death. Oncolytic adenoviruses induce autophagy and subsequently contribute to cell death rather than cell survival in tumor cells. We previously developed a telomerase-specific replication-competent oncolytic adenovirus, OBP-301, which induces cell lysis in tumor cells with telomerase activities. OBP-301-mediated cytopathic activity is significantly associated with induction of autophagy biomarkers. In this review, we focus on the tumor-suppressive role and molecular basis of autophagic machinery induced by oncolytic adenoviruses. Addition of tumor-specific promoters and modification of the fiber knob of adenoviruses supports the oncolytic adenovirus-mediated autophagic cell death. Autophagy is cooperatively regulated by the E1-dependent activation pathway, E4-dependent inhibitory pathway, and microRNA-dependent fine-tuning. Thus, future exploration of the functional role and molecular mechanisms underlying oncolytic adenovirus-induced autophagy would provide novel insights and improve the therapeutic potential of oncolytic adenoviruses.

Key words: oncolytic adenovirus, autophagy, E2F1, microRNA

Autophagy is a catabolic process that produces energy through the lysosomal degradation of cytoplasmic organelles in autophagosomes [1]. Physiological conditions such as nutrient deprivation [2], hypoxia [3], and abrogation of growth signaling [4] induce autophagy as a cytoprotective function. On the other hand, infection with pathogenic viruses and bacteria can also activate the autophagic machinery in infected cells [5, 6]. Virus-mediated autophagy functions as an antiviral defense to eliminate viral components in the innate immune system and as virus replication machinery to produce virions in the viral life cycle [5]. Oncolytic virotherapy with replication-competent oncolytic viruses is a promising antitumor strategy to induce tumor-specific cell death [7]. Among the oncolytic viruses, oncolytic adenoviruses frequently induce autophagy and consequently contribute to cell death in tumor cells [8-10]. We previously generated a telomerase-specific, replication-competent oncolytic adenovirus, OBP-301, which drives the adenoviral *E1A* and *E1B* genes under the control of the

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human telomerase reverse transcriptase (*hTERT*) promoter for tumor-specific virus replication and induces oncolytic cell death in tumor cells with telomerase activities [11]. OBP-301 induces autophagy-related cell death primarily in tumor cells [12, 13]. To enhance the antitumor effect of OBP-301, we generated an armed OBP-301 variant (OBP-702) that expresses the tumor suppressor *p53* gene. OBP-702 exhibits a more profound antitumor effect in association with autophagic and apoptotic cell death than OBP-301 [14]. Interestingly, we found that the E1A-mediated microRNA (miRNA) signaling pathways were involved in the OBP-301- and OBP-702-mediated autophagic death of tumor cells [13, 14]. In the present review, we focus on the tumor-suppressive role of autophagy induced by oncolytic adenoviruses and the molecular mechanisms underlying the oncolytic adenovirus-induced autophagic cell death of tumor cells.

Tumor-Suppressive Role of Oncolytic Adenovirus-Induced Autophagy

Recent evidence in oncolytic virotherapy has shown that autophagy induction is associated with both cell death and cell survival in tumor cells infected with oncolytic adenoviruses (Table 1). Most oncolytic adenoviruses induce autophagy and subsequently contribute to cell death rather than cell survival in tumor cells. For example, a conditionally replicating oncolytic adenovirus, hTERT-Ad, which contains a 255-bp hTERT promoter fragment in the E1A promoter region for tumor-specific virus replication, induces autophagic cell death in malignant brain tumor cells [8]. Our hTERT promoter-driven oncolytic adenovirus, OBP-301, which contains a 455-bp hTERT promoter, also induces autophagic cell death in tumor cells with telomerase activities [12, 13]. An RGD fiber–modified OBP-301 variant (OBP-405) and a p53-expressing OBP-301 variant (OBP-702) also induce more profound autophagic cell death than OBP-301 in malignant brain tumor cells [15] and mesenchymal tumor cells [14], respectively. Tumor-specific survivin promoter-driven oncolytic adenoviruses, CRAd-S-pk7 and CRAd-S-RGD, which contain modified fiber knobs with PK7 and RGD motifs, respectively, also induce autophagic cell death in malignant brain tumor cells [10, 16]. In contrast, an oncolytic adenovirus, Delta-24-RGD, which lacks 24 bps (919–943) in the E1A region that binds to tumor suppressor retinoblastoma (Rb) protein and contains RGD-modified fiber knobs, induces autophagic cell death in malignant brain tumor cells [9, 17–19]. Human chorionic gonadotropin (hCG)-expressing oncolytic adenovirus Ad5/3Δ24hCG, which lacks a 24-bp segment (919–

Table 1 Role of autophagy induced by oncolytic adenoviruses

Oncolytic adenovirus	E1 Promoter	E1A region	E1B region	Fiber knob	Transgene	Function of autophagy
hTERT-Ad	hTERT	+	+	wild-type	–	Cell death
OBP-301	hTERT	+	+	wild-type	–	Cell death
OBP-301	hTERT	+	+	wild-type	–	Cell death
OBP-405	hTERT	+	+	RGD	–	Cell death
OBP-702	hTERT	+	+	wild-type	p53	Cell death
CRAd-S-pk7	Survivin	+	+	PK7	–	Cell death
CRAd-S-RGD	Survivin	+	+	RGD	–	Cell death
Delta-24-RGD	wild-type	del (919–943)	+	RGD	–	Cell death
Delta-24-RGD	wild-type	del (919–943)	+	RGD	–	Cell death
Delta-24-RGD	wild-type	del (919–943)	+	RGD	–	Cell death
Delta-24-RGD	wild-type	del (919–943)	+	RGD	–	Cell death
Ad5/3Δ24hCG	wild-type	del (919–943)	+	Ad3	hCG	Cell death
dl922-947	wild-type	del (922–947)	+	wild-type	–	Cell survival
dl922-947	wild-type	del (922–947)	+	wild-type	–	Cell survival

hTERT, human telomerase reverse transcriptase; RGD, arginine-glycine-aspartate motif; PK7, polylysine motif; hCG, human chorionic gonadotropin; LC3, microtubule-associated protein 1 light chain 3; AVO, acidic vesicular organelle; Atg5, autophagy-related 5; mTOR, mechanistic target of rapamycin; EGFR, epidermal growth factor receptor; FADD, Fas-associated via death domain; DRAM, DNA-damage regulated autophagy modulator.

943) of the E1A region and contains Ad5/3-modified fiber, also induces autophagic cell death in human cancer cells [20]. However, one type of oncolytic adenovirus, dl922-947, induces autophagy as a cytoprotective function [21, 22]. A 24-bp segment (922-947) of the E1A region is deleted in dl922-947; this deleted area is similar to the 24-bp deletion (919-943) in the E1A region of Delta-24-RGD. However, infection with dl922-947 induces autophagy as a cell-survival mechanism in ovarian cancer cells [21] and brain tumor cells [22]. The relationship between oncolytic adenoviruses and the function of autophagy is summarized in Table 1. Oncolytic adenoviruses that induce autophagic cell death have tumor-specific promoters for promoting viral replication and/or modified fiber knobs for enhancing virus infection, whereas only dl922-947, which induces cytoprotective autophagy, possesses both the wild-type E1 promoter and wild-type fiber knobs. These findings suggest that oncolytic adenoviruses with tumor-specific promoters and fiber modifications induce a greater amount of autophagy through the enhancement of viral replication and infection efficiency than wild-type adenovirus, probably resulting in cell death rather than cell survival in tumor cells.

Biomarkers for Oncolytic Adenovirus-Mediated Autophagy

When tumor cells are infected with oncolytic adenoviruses, the modulation of autophagy-related marker proteins, such as autophagy-related 5 (Atg5) [23], microtubule-associated protein 1 light chain 3 (LC3) [24], and p62 [25], is observed in the infected tumor cells (Table 1 and Fig. 1). After infection with oncolytic adenoviruses, Atg5 expression is upregulated following viral replication in the infected tumor cells [9]. Atg5 is conjugated with Atg12 to form the Atg5-Atg12 complex, which accumulates in the isolation membrane derived from the phagophore. The long form of LC3-I is then converted to the short form of LC3-II. LC3-II, p62, and intracellular organelles cooperatively bind to the isolation membrane containing the Atg5-Atg12 complex. Autophagosomes fuse with lysosomes to become autolysosomes, which are acidic vesicular organelles (AVOs) in which p62 and intracellular organelles are degraded. Thus, oncolytic adenovirus-induced autophagy can be confirmed by detecting changes in autophagy-related biomarkers, including Atg5 upregulation [9, 13, 14, 17, 18], LC3-II upregulation [8, 9, 13-19, 22], p62 downregulation [13, 14, 18, 19, 22], and formation of cytoplasmic AVO [8-10, 12, 15-22]. Many oncolytic

Table 1 Continued from the opposite page

Autophagy-related markers	Autophagy-inducing factors	References
LC3-II ↑, AVO ↑	Suppression of mTOR-p70S6K pathway	Ito et al. [8]
AVO ↑		Endo et al. [12]
LC3-II ↑, Atg5 ↑, p62 ↓	E2F1-miR-7-EGFR pathway	Tazawa et al. [13]
LC3-II ↑, AVO ↑	Rapamycin (mTOR inhibitor)	Yokoyama et al. [15]
LC3-II ↑, Atg5 ↑, p62 ↓	E2F1-miR-93/106b-p21 & p53-DRAM pathways	Hasei et al. [14]
LC3-II ↑, AVO ↑		Ulasov et al. [16]
AVO ↑	Beclin-1	Ulasov et al. [10]
LC3-II ↑, Atg5 ↑, AVO ↑		Jiang et al. [9]
LC3-II ↑, Atg5 ↑, AVO ↑	Everolimus (mTOR inhibitor)	Alonso et al. [17]
LC3-II ↑, Atg5 ↑, p62 ↓, AVO ↑	FADD/Caspase-8 pathway	Jiang et al. [18]
LC3-II ↑, p62 ↓, AVO ↑	E1B19K-Beclin-1 complex	Piya et al. [19]
AVO ↑	Suppression of Mre11	Rajecki et al. [20]
AVO ↑		Baird et al. [21]
LC3-II ↑, p62 ↓, AVO ↑		Botta et al. [22]

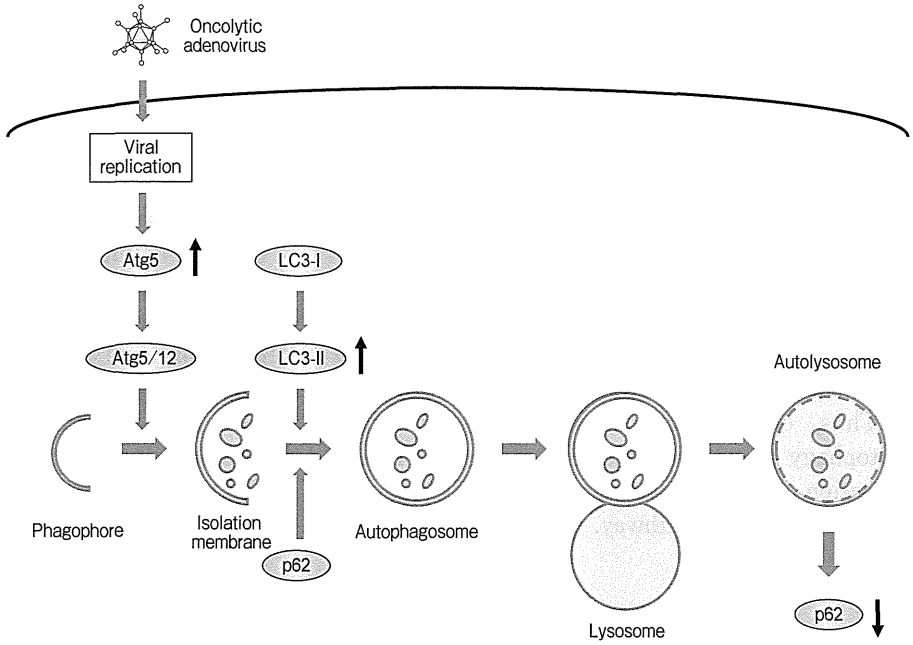

Fig. 1 Schematic diagram of oncolytic adenovirus-mediated autophagy induction. In tumor cells infected with oncolytic adenovirus, Atg5 expression is upregulated following viral replication. The Atg5-Atg12 complex binds to the isolation membrane. After conversion from LC3-I to LC3-II, LC3-II, p62, and intracellular organelles cooperatively accumulate in the isolation membrane, resulting in the formation of autophagosomes, which fuse with lysosomes to form autolysosomes, in which the p62-binding cytoplasmic organelle is degraded under the acidic condition and p62 expression is decreased.

adenoviruses induce these autophagy-related markers in tumor cells (Table 1).

Mechanism of Oncolytic Adenovirus-Mediated Autophagy Induction

With respect to the molecular mechanism of the oncolytic adenovirus-mediated autophagy induction, adenoviral DNA-derived proteins, including E1A, E1B, and E4, function as pro-autophagic and anti-autophagic factors. The E1A and E1B proteins mainly act as autophagy-inducing factors (Fig. 2). In fact, when 3 types of adenovirus vectors with different E1A and E1B regions, *i.e.*, the wild-type adenovirus serotype 5 (Ad5), E1B-deleted Adhz60, and E1A- and E1B-deleted AdlacZ, were compared with respect to their induction of autophagy in human tumor cells, Ad5 induced a higher level of autophagy than E1B-deleted Adhz60, and E1A- and E1B-deleted AdlacZ hardly induced autophagy [26], suggesting the critical role of E1A and E1B in adenovirus-mediated autophagy induction. Adenoviral E1A protein binds to tumor suppressor Rb protein, which results in the activation of transcription factor E2F1 [27]. E2F1 activation induces autophagy through the upregulation of autophagy-related markers, such as Atg5 and LC3, in a transactivation-dependent and a transactivation-independent manner [28, 29]. E1A-mediated E2F1 upregulation may be mainly involved in the upregulation of Atg5 and LC3-II after adenovirus infection. In contrast, adenoviral E1B protein interacts with pro-autophagic Beclin1 through dissociation of the Beclin1-B cell/CLL lymphoma 2 (BCL2) complex, contributing to the induction of Beclin1-dependent autophagy [19]. E1B protein has also been suggested to induce autophagy through the inhibition of Mre11 activity and dissociation of the Mre11-Rad50-NBS1 complex, contributing to the enhancement of radiosensitivity in human cancer cells [20, 30]. E1B may act mainly to support the E1A-mediated autophagy induction. Moreover, oncolytic adenovirus-induced autophagy may be enhanced by activation of the Fas-associated via death domain (FADD)/caspase-8 signaling pathway [18] and result in autophagic cell death