Acta Med. Okayama, 2013
Vol. 67, No. 6, pp. 333–342

Acta Medica Okayama

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Review

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

Hiroshi Tazawa[a,b]*§, Shunsuke Kagawa[b], and Toshiyoshi Fujiwara[b]

[a]*Center for Innovative Clinical Medicine, Okayama University Hospital,* [b]*Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700–8558, Japan*

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

Received August 30, 2013; accepted September 26, 2013.
*Corresponding author. Phone:+81-86-235-7491; Fax:+81-86-235-7492
E-mail:htazawa@md.okayama-u.ac.jp (H. Tazawa)
§The winner of the 2012 Incentive Award of the Okayama Medical Association in Medical Science.

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]

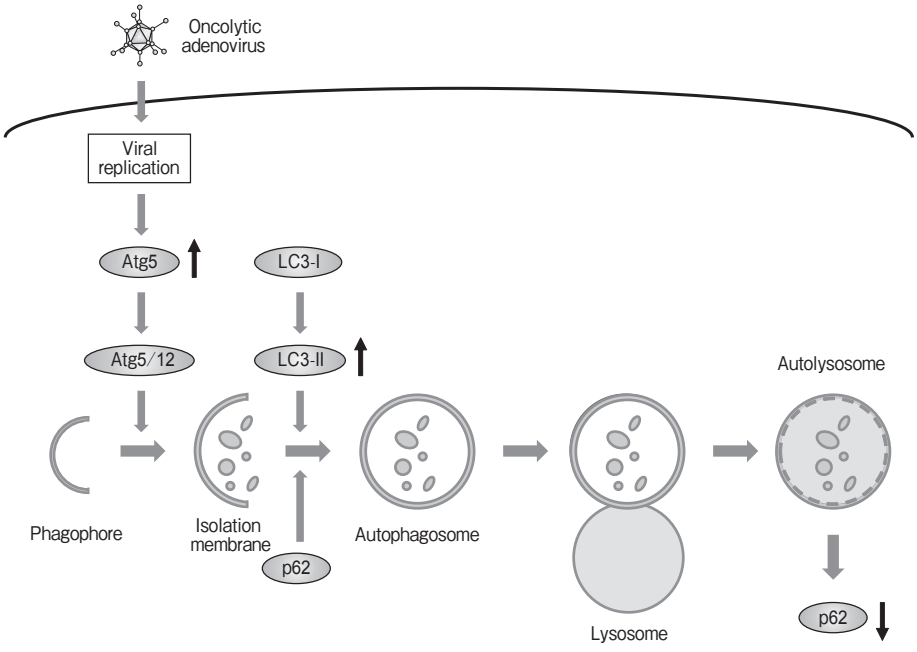

Fig. 1 Schematic diagram of oncolytic adenovirus-mediated autophagy induction. In tumor cells infected with oncolytic adenovirus, Atg5 expression is upregulated following viral replication. The Atg5-Atg12 complex binds to the isolation membrane. After conversion from LC3-I to LC3-II, LC3-II, p62, and 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

through interaction with Atg5 and FADD [31]. In contrast, the adenoviral E4 protein inhibits autophagy. E4 protein activates the mammalian target of rapamycin (mTOR) signaling pathway [32], which suppresses autophagy through suppression of unc-51-like autophagy activating kinase 1 (ULK1) activity by phosphorylation [33]. In fact, E4 protein inhibits the oncolytic potential of the E1B-55K-deleted oncolytic adenovirus ONYX-015 through activation of the mTOR signaling pathway [34]. Suppression of the mTOR signaling pathway by rapamycin and everolimus enhances the autophagic cell death induced by oncolytic adenoviruses (Table 1). Thus, oncolytic adenoviruses may strictly induce autophagy through regulation of the E1-dependent activation signaling pathway and the E4-dependent inhibitory signaling pathway.

Small Noncoding RNAs Regulated by Oncolytic Adenovirus

Infection with wild-type Ad5 regulates 2 different types of small noncoding RNAs in the infected cells, viral miRNAs (mivaRNAs) and cellular miRNAs, which are respectively derived from virus-associated RNA (VA RNA) and cellular primary miRNA transcripts (Fig. 3) [35]. Recently, these 2 types of small noncoding RNAs have been suggested to be involved in antiviral defense and viral replication. VA RNAs consist of 2 types of 160-nucleotide-noncoding RNAs, VA RNAI and VA RNAII, which are transcribed by cellular RNA polymerase III from the adenoviral genome. VA RNA induces the expression of type I interferon (IFN), which is associated with innate

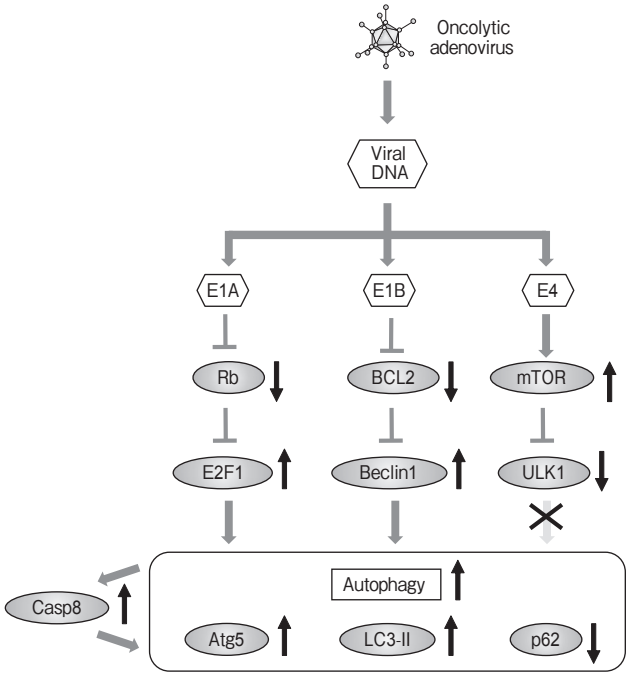

Fig. 2 Schematic diagram of the molecular mechanism of oncolytic adenovirus-mediated autophagy induction. Oncolytic adenovirus induces the accumulation of adenoviral DNA-derived E1A, E1B, and E4 proteins. E1A protein upregulates E2F1 expression by suppressing Rb activity. E1B protein upregulates Beclin1 expression through the dissociation of the Beclin1-BCL2 complex. In contrast, E4 protein downregulates ULK1 expression by activating the mTOR signaling pathway. E1A and E1B proteins activate, but E4 protein inhibits, autophagy in association with Atg5 upregulation, LC3-II upregulation, and p62 downregulation. Moreover, oncolytic adenovirus-mediated autophagy can be enhanced by caspase-8 (Casp8) activation.

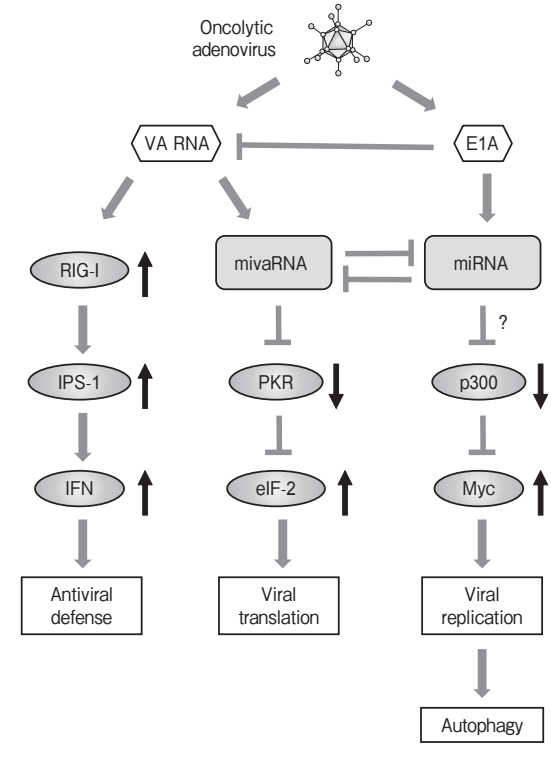

Fig. 3 Schematic diagram of antiviral and proviral processes regulated by small noncoding RNAs. When infected with oncolytic adenovirus, two types of small noncoding RNAs, viral microRNA (mivaRNA) and cellular microRNA (miRNA), are induced by virus-associated RNA (VA-RNA) and E1A, respectively. VA RNA regulates antiviral defense and viral translation through the RIG-I-IPS-1-IFN signaling pathway and mivaRNA-PKR-eIF-2 signaling pathway, respectively. E1A regulates viral replication and autophagy through the miRNA-p300-Myc signaling pathway.

immune responses [36–38]. In terms of the molecular mechanism of VA RNA-mediated IFN induction, the involvement of IFN-β promoter stimulator-1 (IPS-1) and retinoic acid-inducible gene I (RIG-I) has been suggested [37–39], whereas the role of VA RNA-derived mivaRNAs remains to be elucidated. VA RNA further activates the eukaryotic translation initiation factor 2α (eIF-2) through suppression of protein kinase R (PKR), contributing to the translation of viral mRNA [40–42]. However, a recent report has suggested that VA RNA-derived mivaRNAs are not involved in adenovirus-mediated cell lysis [43]. In contrast to this report, evidence also supports the utility of VA RNA-deleted oncolytic adenovirus to induce more profound antitumor effects in tumor cells and lower cytotoxicity in normal cells than wild-type adenovirus [44–46]. In contrast, adenoviral E1A protein modulates the expression of cellular miRNAs in tumor cells (Fig. 3) [47]. We also demonstrated the modulation of cellular miRNAs in oncolytic adenovirus-infected tumor cells [13, 14]. These miRNAs are mainly transcribed by RNA polymerase II from the cellular DNA genome. E1A protein is necessary for viral replication because E1A-deleted adenovirus lacks the ability to replicate. E1A protein induces DNA synthesis and viral replication followed by S phase entry through upregulation of c-Myc [48–50], which is caused by suppression of the p300 transcriptional co-activator through an unknown mechanism [51, 52]. Since miR-132 regulates the antiviral defense and viral replication through suppression of p300 after infection with other types of viruses [53, 54], viral replication of oncolytic adenovirus may also be regulated by an E1A-dependent miRNA network through suppression of p300 (Fig. 3). Recent reports have suggested that both p300 knockdown [55] and c-Myc overexpression [56] induce autophagy; thus, viral replication induced by p300 downregulation and c-Myc upregulation may contribute to autophagy induction. These findings suggest that VA RNA-derived mivaRNAs mainly induce antiviral defense in the innate immune response, whereas E1A protein supports viral replication in the adaptive immune response. E1A-mediated viral replication may further promote the autophagic machinery. Moreover, VA RNA-derived mivaRNAs and cellular miRNAs competitively inhibit the RNAi machinery [57–59], whereas E1A protein suppresses the accumulation of VA RNAs through suppression of RNA polymerase III [60]. Therefore, the crosstalk between VA RNA and E1A protein may finally decide the cellular fate with respect to cell survival or cell death in oncolytic adenovirus-infected cells.

E2F1-Regulated miRNAs in Oncolytic Adenovirus-Induced Cell Death

Adenovirus infection activates E2F1 expression through the E1A-dependent suppression of Rb protein [27]. Recent evidence in tumor biology has shown the presence of the E2F1-regulated small noncoding miRNAs as tumor-suppressive factors [61] or oncogenic factors [62]. The crosstalk between E2F1 and the E2F1-regulated miRNA network has been suggested to be critical for determining the prosurvival and antiproliferative effects of E2F1 in cancer [63]. We recently demonstrated the involvement of E2F1-regulated miRNAs in oncolytic adenovirus-induced cell death (Fig. 4) [13, 14]. When human cancer cells were infected with the hTERT promoter-driven oncolytic adenovirus OBP-301, miR-7 expression was upregulated in a time- and dose-dependent manner [13]. Overexpression of exogenous E2F1 induced miR-7 expression, whereas pretreatment with E2F1 siRNA attenuated the OBP-301-induced miR-7 upregulation in human cancer cells, suggesting the E2F1-dependent regulation of miR-7 expression. Interestingly, ectopic expression of exogenous miR-7 induced autophagy-related cell death through suppression of oncogenic epidermal growth factor receptor (EGFR) [13]. Another report also supports that suppression of EGFR expression by EGFR siRNA induces autophagic cell death in human cancer cells [64]. The EGFR-downstream phosphatidylinositol 3'-kinase -Akt-mTOR signaling pathway suppresses the autophagy machinery [65]. We reported that OBP-702 in p53-expressing OBP-702-infected tumor cells suppressed the expression of p21, which functions as both an anti-apoptotic [66] and anti-autophagic factor [67], through upregulation of miR-93 and miR-106 [14, 68]. miR-93/106-mediated p21 downregulation enhanced the apoptotic and autophagic cell deaths induced by p53-mediated pro-apoptotic BCL2-associated X protein (BAX) and pro-autophagic DNA-damage regulated autophagy modulator (DRAM) proteins [14]. miR-93 and miR-106 are upregulated by E2F1

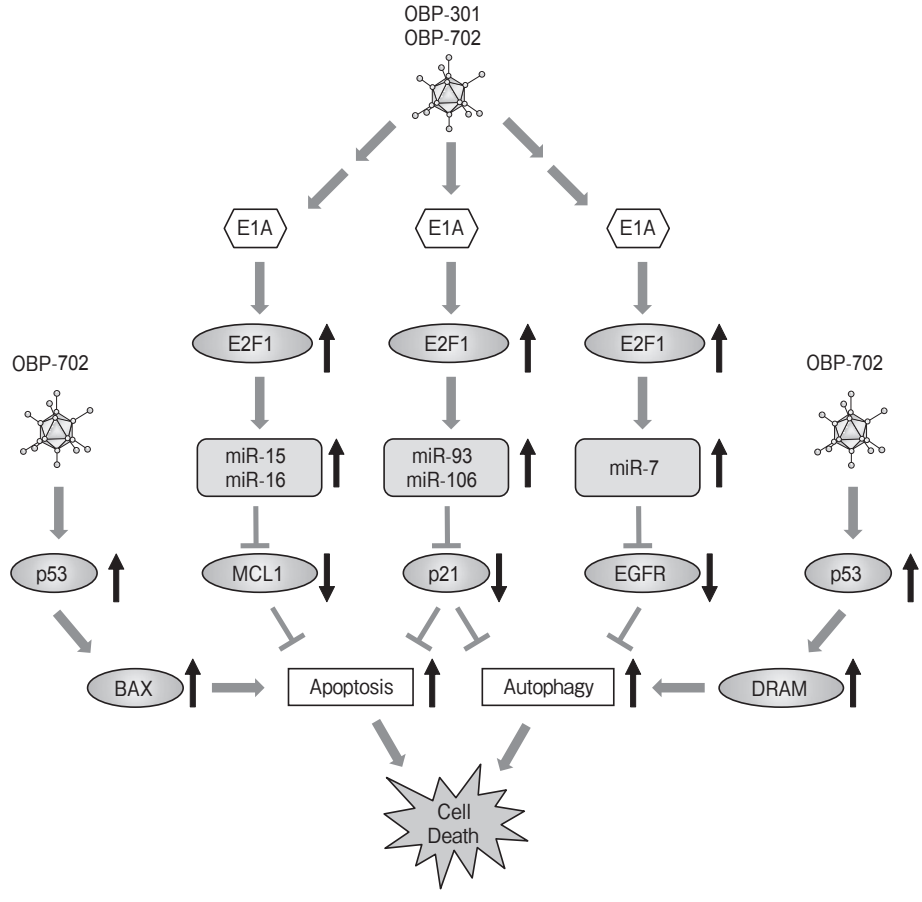

Fig. 4 Schematic diagram of E1A-dependent cell death pathways through the E2F1-regulated miRNA network. Oncolytic adenoviruses, OBP-301 and OBP-702, induce E1A-dependent cell death pathways through regulation of the E2F1-inducible miRNA network. E2F1-inducible miRNAs (miR-7, miR-15, miR-16, miR-93, miR-106) induce apoptotic and autophagic cell death through suppression of anti-apoptotic and anti-autophagic factors (MCL1, p21, EGFR). Overexpression of tumor suppressor p53 also induces apoptotic and autophagic cell death via upregulation of p53-downstream targets, BAX and DRAM, respectively, in OBP-702-infected tumor cells.

and suppress the expression of p21 [69]. We recently observed that, in human tumor cells co-treated with OBP-301 and chemotherapy, OBP-301 downregulated the expression of the anti-apoptotic BCL2 family myeloid cell leukemia sequence 1 (MCL1) through upregulation of miR-15 and miR-16, resulting in the enhancement of chemotherapy-induced apoptotic cell death (our unpublished data). Other types of oncolytic adenoviruses downregulate the expression of MCL1 in infected tumor cells [70, 71]. Moreover, miR-15 and miR-16 can be upregulated by E2F1 [72] and suppress the expression of MCL1 [73]. A recent report also supports the modulation of cellular miRNAs in adenoviral E1A-overexpressed human cancer cells [47]. Downregulation of miR-520h by E1A activation was associated with E1A-dependent antitumor effects [47]. In this report, more interestingly, some E2F1-inducible miRNAs (miR-92, miR-18, miR-15, miR-93, miR-25) were upregulated in the E1A-expressing human cancer cells [47], which was consistent with our recent experimental observations (Fig. 4). These findings suggest that the E2F1-regulated miRNA network is a critical signaling pathway for fine-tuning the oncolytic adenovirus-induced autophagic and apoptotic cell deaths through the suppression of anti-autophagic and anti-apoptotic factors.

Conclusion

Oncolytic virotherapy is a promising antitumor

strategy for eliminating tumor cells without adversely affecting normal cells. Various types of oncolytic adenoviruses have been developed, and these viruses mainly induce autophagy-related cell death in tumor cells. However, the molecular mechanisms underlying the oncolytic adenovirus-induced autophagic cell death remains unclear. Some adenoviral proteins, including E1A, E1B, and E4, have been suggested to be implicated in the regulation of autophagic machinery. Moreover, following E1A accumulation, the E2F1-regulated microRNA network may be involved in fine-tuning of the oncolytic cell death associated with autophagy. An exploration of the functional role and molecular basis of oncolytic adenovirus-mediated autophagy induction would help to improve the therapeutic potential of oncolytic virotherapy.

Acknowledgments. This study was supported by grants from the Ministry of Health, Labour, and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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