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The application of multiple miRNA response elements enables oncolytic adenoviruses to possess specificity to glioma cells

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ABSTRACT

Adenovirus-mediated virotherapy is one of the promising therapeutic approaches for glioma treatment. However, its replication efficiency and specificity still failed to meet the requirements for clinical treatment. To improve the anti-tumor activity and specificity of oncolytic adenoviruses (OA), we applied multiple miRNA response elements (MREs) of *miR-124*, *miR-128*, *miR-146b* and *miR-218*, whose expressions were downregulated in glioma cells, to enable OA to be specific to glioma. Adenoviral E1A protein regulated by these 4 MREs (OA-4MREs) was shown to be highly expressed in glioma cells, but not in normal cells. The selective E1A expression led to glioma-specific replication and cytotoxicity of OA-4MREs. Animal experiments also showed that OA-4MREs exhibited improved anti-tumor activities for both subcutaneous and intracranial glioma xenografts, without significant toxicity to normal brain and liver tissues. Collectively, we demonstrated that oncolytic adenovirus, whose replication was regulated by MREs, may be promising biological agents for glioma treatment.

Introduction

As the most common intracranial neoplasm, malignant glioma has high invasiveness, metastasis and recurrence (Reardon et al., 2011). So far, traditional treatments have limited outcome, including surgery, chemotherapy and radiotherapy. Thus, novel therapeutic strategies are urgently required for efficient glioma treatment.

Oncolytic adenovirus (OA, also known as conditionally replicative adenovirus, CRAd) is a prospective biological agent against various types of cancer (Alvarez-Breckenridge et al., 2012; Chen et al., 2011; Fulci et al., 2007; Post et al., 2003; Xu et al., 2012). OA displays a higher anti-tumor activity than replication-incompetent (E1A-null) adenoviruses, mainly due to its remaining replication in cancer cells and continuous infection of surrounding tumor cells. However, natural adenoviruses also have cytotoxicity to normal cells, thereby limiting their clinical application for cancer therapy. To confer OA with specificity to cancer cells, researchers have developed many strategies to enable adenoviruses to discriminate between neoplastic and normal cells for selective replication.

Onyx-015 (deletion of 55 kDa protein in E1B region) is the first attempt to generate a cancer-specific adenovirus. However, its specificity is still not satisfactory, since its replication is greatly impaired after modification (Su et al., 2004). Similarly, 24 bp deletion in CR2 domain of E1A region ensures selective replication of adenoviruses in cancer cells, but weakens their replication efficiency (Zhang et al., 2007). Human telomerase reverse transcriptase (TERT) is found to be overexpressed in a variety of cancers, and thus, is widely employed to construct tumor-specific viral vectors. TERT promoter is also used to regulate E1A activity of adenoviruses in cancer cells. However after this modification, replication of OA is usually weaker than wild type adenovirus, whose E1A expression was driven by its original promoter (He et al., 2011; Luo et al., 2008; Zhang et al., 2007). Low replication efficiency of the current OAs urge scientists to search for novel strategies to improve OA systems.

miRNAs are found to be differentially expressed between cancerous and normal tissues. Downregulated expression profile of specific miRNAs raises the possibility of utilizing these miRNAs to enable adenovirus to specifically replicate in cancer cells, such as malignant glioma (Gomez-Manzano and Fueyo, 2010). So far, there are no reports about constructing glioma-specific oncolytic adenoviruses by utilizing differential expression profile of miRNAs between glioma and normal cells.

In this study, we selected *let7* and 19 other microRNAs (abbreviated as miR), whose expressions have been confirmed to be downregulated in glioma samples and/or cell lines, including *miR-7* (Kefas et al., 2008; Silber et al., 2008; Skalsky and Cullen,

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2011), *miR-16*, *miR-19a*, *miR-20a* (Malzkorn et al., 2010), *miR-26b* (Wu et al., 2011), *miR-124* (Silber et al., 2008; Skalsky and Cullen, 2011), *miR-128* (Godlewski et al., 2008; Skalsky and Cullen, 2011), *miR-129*, *miR-137* (Silber et al., 2008), *miR-139* (Skalsky and Cullen, 2011), *miR-140* (Malzkorn et al., 2010), *miR-146b* (Xia et al., 2009), *miR-181b* (Conti et al., 2009), *miR-184* (Malzkorn et al., 2010), *miR-205* (Yue et al., 2012), *miR-218* (Rao et al., 2010; Silber et al., 2008; Skalsky and Cullen, 2011), *miR-219-5p* (Rao et al., 2010), *miR-328* (Malzkorn et al., 2010) and *miR-451* (Nan et al., 2010), to evaluate the feasibility and effectiveness of their miRNA response elements (MREs) to regulate the specific replication of OA in glioma cells.

Results

Differential miRNA expression profile between glioma and normal brain cells

First of all, *let7* and 19 other microRNAs (miRNAs), whose expressions have been reported to be downregulated in glioma samples and/or cell lines, were listed as candidates to ensure glioma-specificity. To experimentally confirm the differential expression profiles of these miRNAs, we investigated their expressions in 11 glioma tissues from patients. Inconsistent with previous reports, *miR-19a* abundance was not reduced, but increased in malignant glioma tissues, compared with their corresponding noncancerous brain tissues. Also, there were no significant differences in the expressions of *miR-16*, *miR-20a*, *miR-184* and *miR-328* between cancerous and noncancerous samples. However, other miRNAs were all found to be underexpressed in glioma tissues (Fig. 1A).

miRNA response elements (MREs) of miR-124, miR-128, miR-146b and miR-218 were effective regulators for glioma-specific gene expression

To evaluate if these miRNAs could be used for glioma-specific expression of exogenous genes, we transfected U-87 MG glioblastoma cell line and normal embryonic astrocytes with a series of luciferase reporter plasmids, whose luciferase expressions were modulated by MREs of these miRNAs. The results on U-87 MG cells showed that luciferase expressions were suppressed by more than 20% when MREs of *miR-7*, *miR-19a*, *miR-20a*, *miR-26b*, *miR-139*, *miR-184*, *miR-205*, *miR-219-5p*, *miR-328* and *let-7* were inserted, excluding the possibility that these MREs can be used to ensure high expression of E1A in glioma cells during subsequent adenovirus modification. In contrast, MREs of other miRNAs only slightly affected luciferase activity in glioma cells (Fig. 1B).

The data on normal embryonic astrocytes indicated that luciferase expressions were significantly suppressed when these MREs were used, except that of *miR-16*. Furthermore, the application of MREs of *miR-124*, *miR-128*, *miR-139*, *miR-146b*, *miR-181b*, *miR-218* and *miR-219-5p* suppressed luciferase expression by more than 70% (Fig. 1C).

Finally, MREs were selected for glioma-specific expression of exogenous genes according to the following criteria. (1) miRNAs should be downregulated by more than 50% in glioma tissues compared with noncancerous brain tissues. (2) The inhibitory effect of MRE on luciferase expression should be lower than 20% in U-87 MG glioma cells. (3) MRE should suppress the luciferase activity by more than 70% in normal embryonic astrocytes. Based on the above standards, the MREs of *miR-124*, *miR-128*, *miR-146b* and *miR-218* were selected to be used for glioma-specific exogenous expression.

Combination of the MREs of miR-124, miR-128, miR-146b and miR-218 conferred higher glioma-specificity than single MREs

Although the application of MREs of *miR-124*, *miR-128*, *miR-146b* and *miR-218* alone suppressed luciferase expression by 75–85% in normal embryonic astrocytes, there was still a possibility that 15–25% of remaining expressions of exogenous genes were sufficient to affect cell survival and/or function. To further improve the specificity of MRE-based regulation, we simultaneously utilized these 4 MREs to modulate luciferase expression (Fig. 2A). The results demonstrated that combination of MREs of *miR-124*, *miR-128*, *miR-146b* and *miR-218* suppressed luciferase expression by 97.8% and 98.7% in two separate primary normal embryonic astrocytes, respectively, while single application of these MREs lead to only 70.9–81.7% and 73.6–77.6% reduction in luciferase expressions in the same cells, respectively (Fig. 2B). These data indicated that application of multiple MREs had an additive suppressing effect on exogenous gene expression.

To test if the combination of 4 MREs affected gene expression in glioma cells, luciferase reporter assays were also performed on U-87 MG and primary glioma cells. Luciferase activity was found to be higher than 80% when pluc-4miRNAs were transfected into these cells, although its expression level is slightly lower compared with pluc-miR-124, pluc-miR-128, pluc-miR-146b or pluc-miR-218 alone (Fig. 2B).

Collectively, combined application of 4 MREs enabled exogenous gene expression to have higher glioma-specificity, without significantly affecting its expression level in glioma cells.

MRE-regulated exogenous gene expression depended on the level of miR-124, miR-128, miR-146b and miR-218

To confirm the regulation of *miR-124*, *miR-128*, *miR-146b* and *miR-218* on exogenous gene expression under their MREs, luciferase assays were performed in the pluc-4miRNAs-transfected cells after the level of these miRNAs was changed. The expression of endogenous *miR-124*, *miR-128*, *miR-146b* and *miR-218* was suppressed by 37.3–54.7% in normal embryonic astrocytes by the mixture of these 4 miRNA inhibitors (Fig. 3A). Under this condition, luciferase expression was significantly upregulated in pluc-4miRNAs-transfected cells (Fig. 3B). Consistently, luciferase expression was greatly decreased in the pluc-4miRNAs-transfected U-87 MG cells, in which *miR-124*, *miR-128*, *miR-146b* and *miR-218* levels were elevated by the treatment of mixed 4 miRNA mimics (Fig. 3C and D).

These data demonstrated that exogenous gene expression regulated by MREs of *miR-124*, *miR-128*, *miR-146b* and *miR-218* depended on the levels of these 4 miRNAs in the transfected cells.

Expression level of miR-124, miR-128, miR-146b and miR-218 controlled the replication of MRE-regulated adenovirus

A recombinant oncolytic adenovirus (OA-4MREs) was constructed by inserting MREs of *miR-124*, *miR-128*, *miR-146b* and *miR-218* immediately following E1A-encoding open reading frame (ORF), allowing the corresponding miRNAs to suppress E1A expression in normal cells (Fig. 3E). In normal embryonic astrocytes with high expression levels of the 4 miRNAs, E1A protein and mRNA expression was greatly suppressed when the cells were infected with OA-4MREs. However, the treatment of miRNA inhibitors restored E1A expression in OA-4MREs-infected cells (Fig. 3F and H). Consistently, transfection of miRNA mimics significantly reduced the expression of E1A protein and mRNA in U-87 MG cells, in which endogenous levels of *miR-124*, *miR-128*, *miR-146b* and *miR-218* were low (Fig. 3G and I).

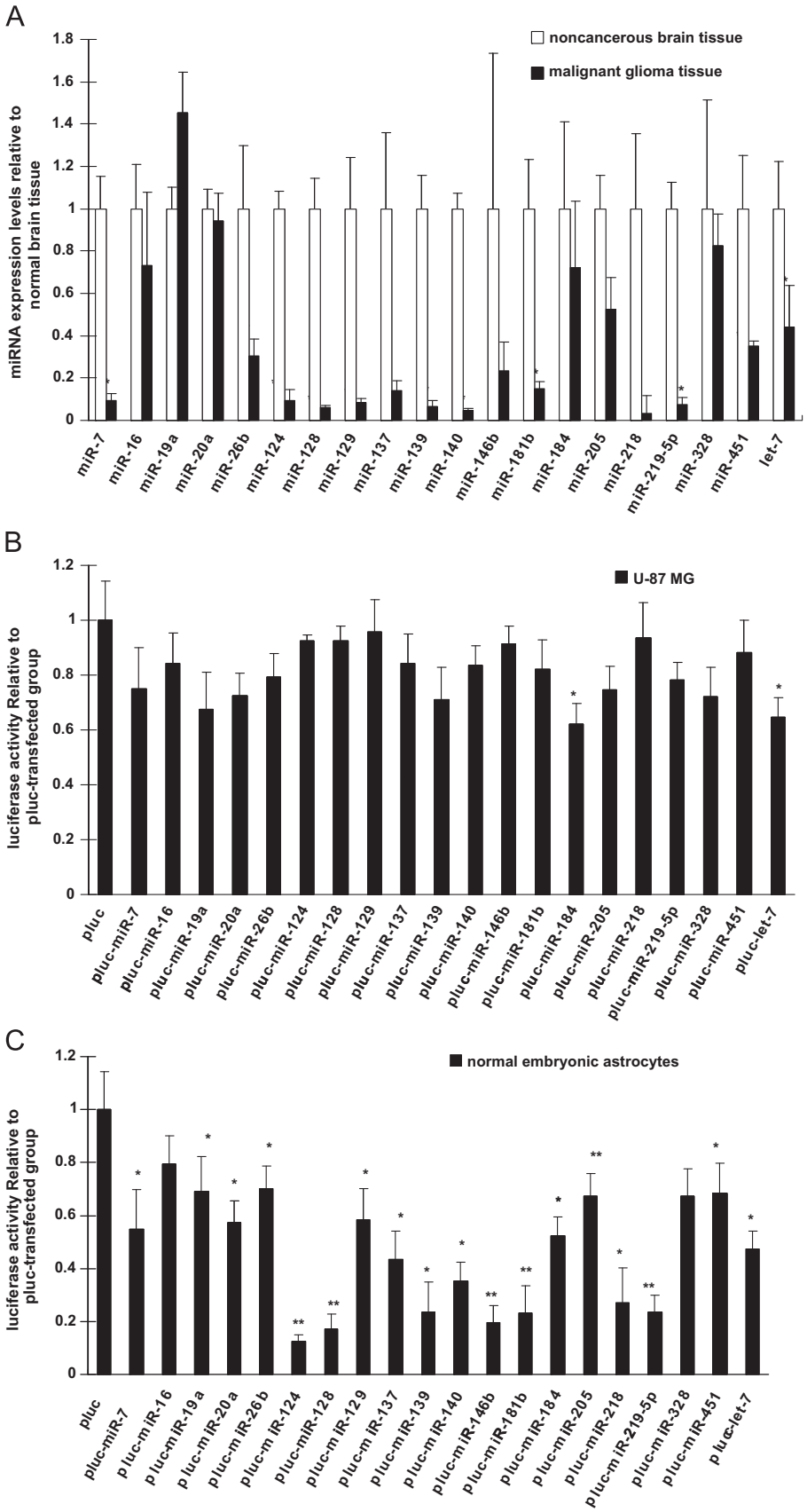


Fig. 1. miRNA response elements (MREs) of *miR-124*, *miR-128*, *miR-146b* and *miR-218* were effective regulators for glioma-specific gene expression. (A) Expression levels of different miRNAs were detected in glioma and corresponding noncancerous brain tissues. miRNA level in the corresponding noncancerous brain tissues was considered as standard and *U6* was selected as the endogenous reference. Means ± SD of three independent experiments are shown. (B and C) Luciferase constructs containing MREs of these miRNAs were transfected into U-87 MG cells and normal astrocytes. Relative luciferase activity in the cells transfected with pluc was considered as standard. Means ± SD of three independent experiments are shown.

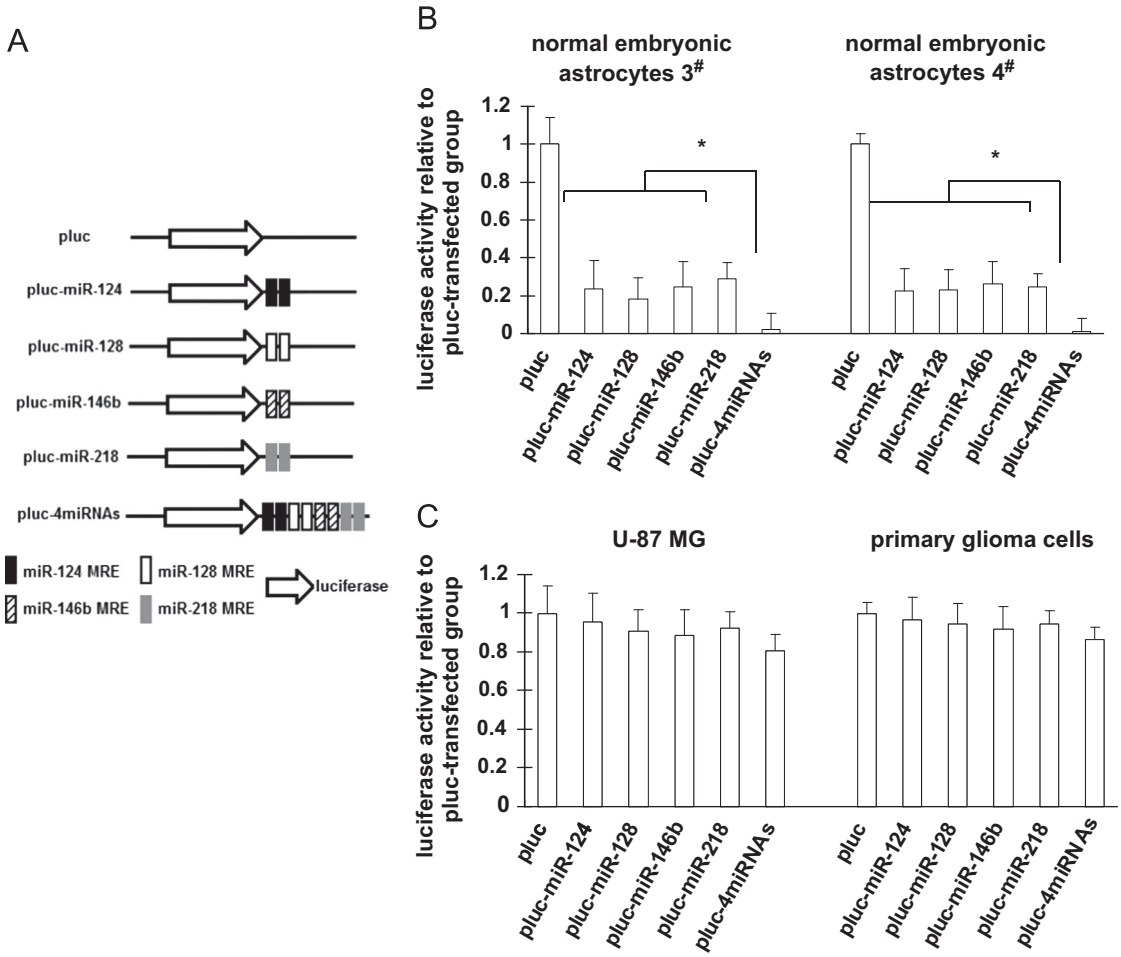


Fig. 2. Combined application of MREs of *miR-124*, *miR-128*, *miR-146b* and *miR-218* resulted in higher glioma-specificity than the usage of single MREs. (A) Schematic illustration of the involved vectors in the luciferase assays. Two copies of MREs of *miR-124*, *miR-128*, *miR-146b* or/and *miR-218* were immediately inserted following the luciferase-encoding regions. (B and C) Luciferase expression was quantified in normal embryonic astrocytes and glioma cells transfected with luciferase reporter plasmids. Relative luciferase activity in the cells transfected with pluc was considered as standard. Means ± SD of three independent experiments are shown.

Subsequently, replication of MRE-regulated adenovirus was evaluated both in U-87 MG cells and normal embryonic astrocytes. OA-4MREs produced much lower titer of progenies in normal cells than wild type adenovirus (Ad-WT), and treatment of miRNA inhibitors increased progeny production of OA-4MREs (Fig. 3J). In U-87 MG cells, OA-4MREs had a similar yield of virus progeny to Ad-WT. However, increasing the abundance of the 4 miRNAs lead to a great reduction in the replication of the MRE-regulated adenovirus (Fig. 3K).

These data showed that E1A expression and replication of MRE-regulated adenovirus were both dependent on the levels of *miR-124*, *miR-128*, *miR-146b* and *miR-218*.

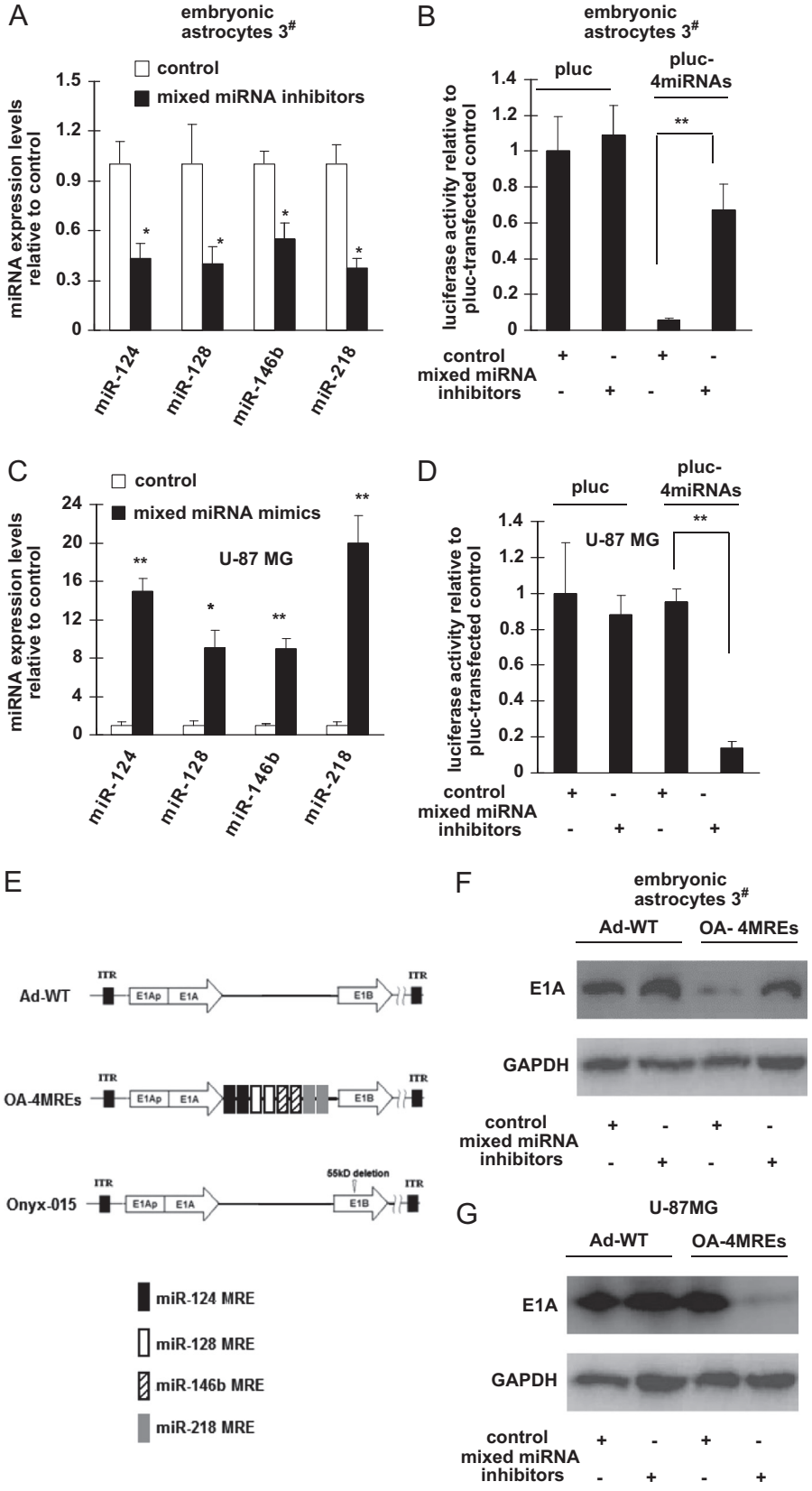
The replication and cytotoxicity of MRE-regulated adenovirus was suppressed in the cells derived from normal tissue

E1A expression was subsequently estimated in normal embryonic astrocytes infected by OA-4MREs, compared with Ad-WT and Onyx-015. OA-4MREs had no detectable E1A expression in the astrocytes, while there was high level of E1A proteins both in Ad-WT- and Onyx-015-infected cells (Fig. 4A). qPCR assay also confirmed the differential expression of E1A mRNA in the cells infected with indicated adenoviruses (Fig. 4B). Accordingly, OA-4MREs also had lower yield of progenies in the two primary astrocytes, compared with Onyx-015

Fig. 3. Adenoviral E1A expression regulated by the 4 MREs was strictly dependent on the levels of their corresponding miRNAs. (A) Synthetic inhibitors of *miR-124*, *miR-128*, *miR-146b* or *miR-218* were mixed and transfected into normal embryonic astrocytes (40 nM). Expression levels of these miRNAs were assessed by qPCR with *U6* as endogenous reference and were shown as values relative to control groups. Means ± SD of three independent experiments are shown. (B) Co-transfection of embryonic astrocytes with indicated constructs and mixed miRNA inhibitors or controls (40 nM). 48 h later, luciferase expression was evaluated. Relative luciferase activity in the cells transfected with pluc and control inhibitors was considered as standard. Means ± SD of three independent experiments are shown. (C) Synthetic mimics of *miR-124*, *miR-128*, *miR-146b* or *miR-218* (40 nM) were mixed and transfected into U-87 MG cells. Expression levels of these miRNAs were assessed by qPCR with *U6* as the endogenous reference and were shown as values relative to control groups. Means ± SD of three independent experiments are shown. (D) Co-transfection of U-87 MG cells with indicated constructs and mixed miRNA mimics or controls (40 nM). 48 h later, luciferase expression was evaluated. Relative luciferase activity in the cells transfected with pluc and control inhibitors was considered as standard. Means ± SD of three independent experiments are shown. (E) Schematic illustration of the involved adenoviruses in the manuscript. Wild type serotype 5 of adenovirus (Ad-WT) was used as control. OA-4MREs has MREs of *miR-124*, *miR-128*, *miR-146b* and *miR-218* inserted immediately following E1A gene. E1B 55 kDa protein in Onyx-015 was completely deleted. (F) Normal astrocytes were transfected with mixed miRNA inhibitors or control (40 nM), followed by indicated adenovirus infection (5 MOI). 48 h later, E1A protein level was determined by immunoblotting assay and GAPDH was selected as the endogenous reference. (G) U-87 MG cells were transfected with mixed miRNA mimics or control (40 nM), followed by indicated adenovirus infection (5 MOI). 48 h later, E1A protein level was also determined with GAPDH as the endogenous reference. (H and I) E1A mRNA expression was detected using qPCR methods in the miRNA inhibitor-treated normal astrocytes and miRNA mimics-treated U-87 MG cells after infection of Ad-WT and OA-4MREs (5 MOI). *GAPDH* was selected as the endogenous reference. Means ± SD of three independent experiments are shown. (J and K) Progeny production of the normal astrocytes and U-87 MG cells with indicated treatments were evaluated by replication assays. Progeny yields are presented as pfu/ml. Means ± SD of three independent experiments are shown.

(Fig. 4C). Viability analysis indicated that OA-4MREs only exerted a slight cytotoxicity to normal astrocytes, at the dose of up to 100 MOI (Fig. 4D). However, the survival of these astrocytes was affected by the infection of Onyx-015 of higher than 20 MOI (Fig. 4D).

Besides astrocytes, normal cells originating from other tissues were also investigated for the specificity of OA-4MREs replication and cytotoxicity. Expression levels of *miR-124* and *miR-128* were both significantly upregulated in HCN-2 neural cells, HUV-EC-C



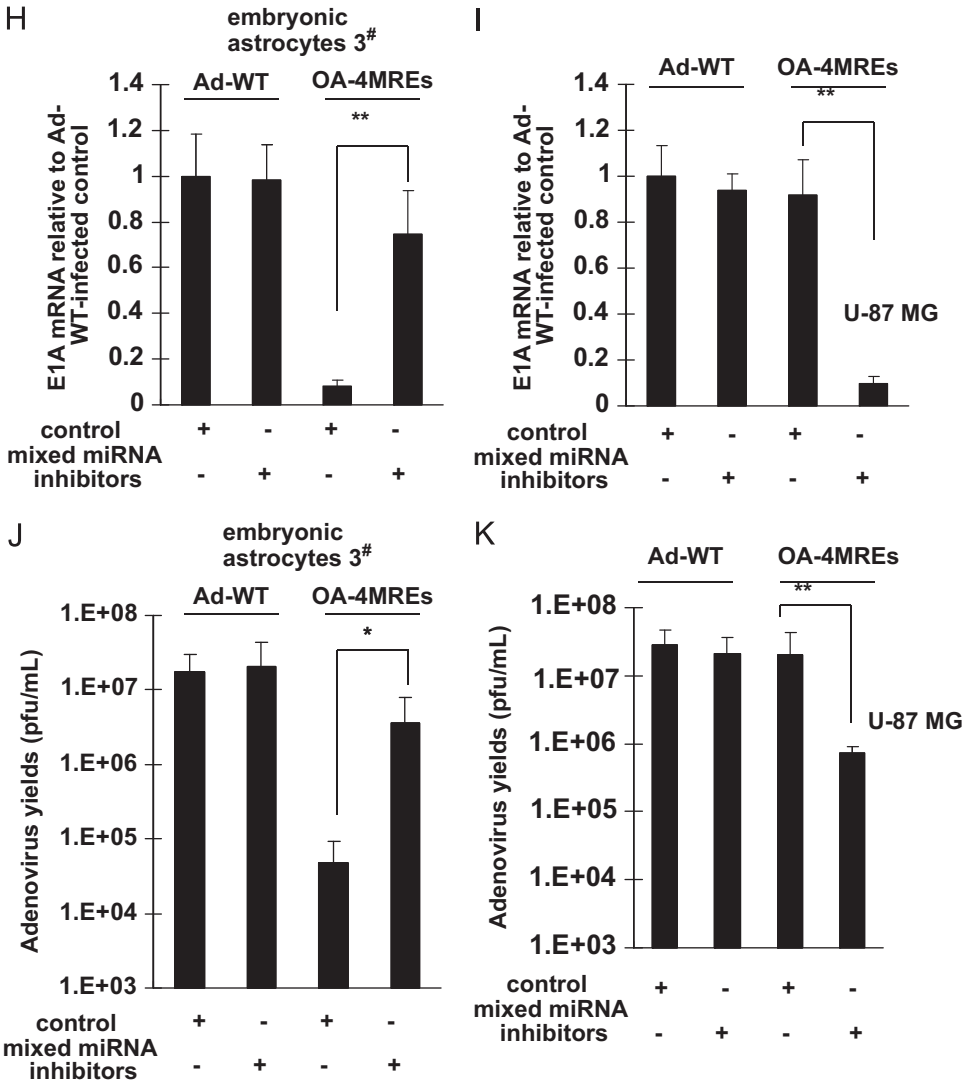


Fig. 3. (*continued*)

endothelial cells, and L-02 liver cells, in comparison with glioma samples from patients (Fig. 4E). HCN-2 and HUV-EC-C cells also had significantly higher copies of *miR-146b* and *miR-218* than glioma tissues (Fig. 4E). Compared with Ad-WT, the replication of OA-4MREs was highly suppressed in these normal cells (Fig. 4F). Similar to the data on normal astrocytes, MRE-regulated adenovirus also produced lower titers of progenies than Onyx-015 in HCN-2, HUV-EC-C and L-02 cells (Fig. 4F). OA-4MREs did not reduce the viability of these normal cells, whereas Onyx-015 showed cytotoxicity at high MOIs (Fig. 4G).

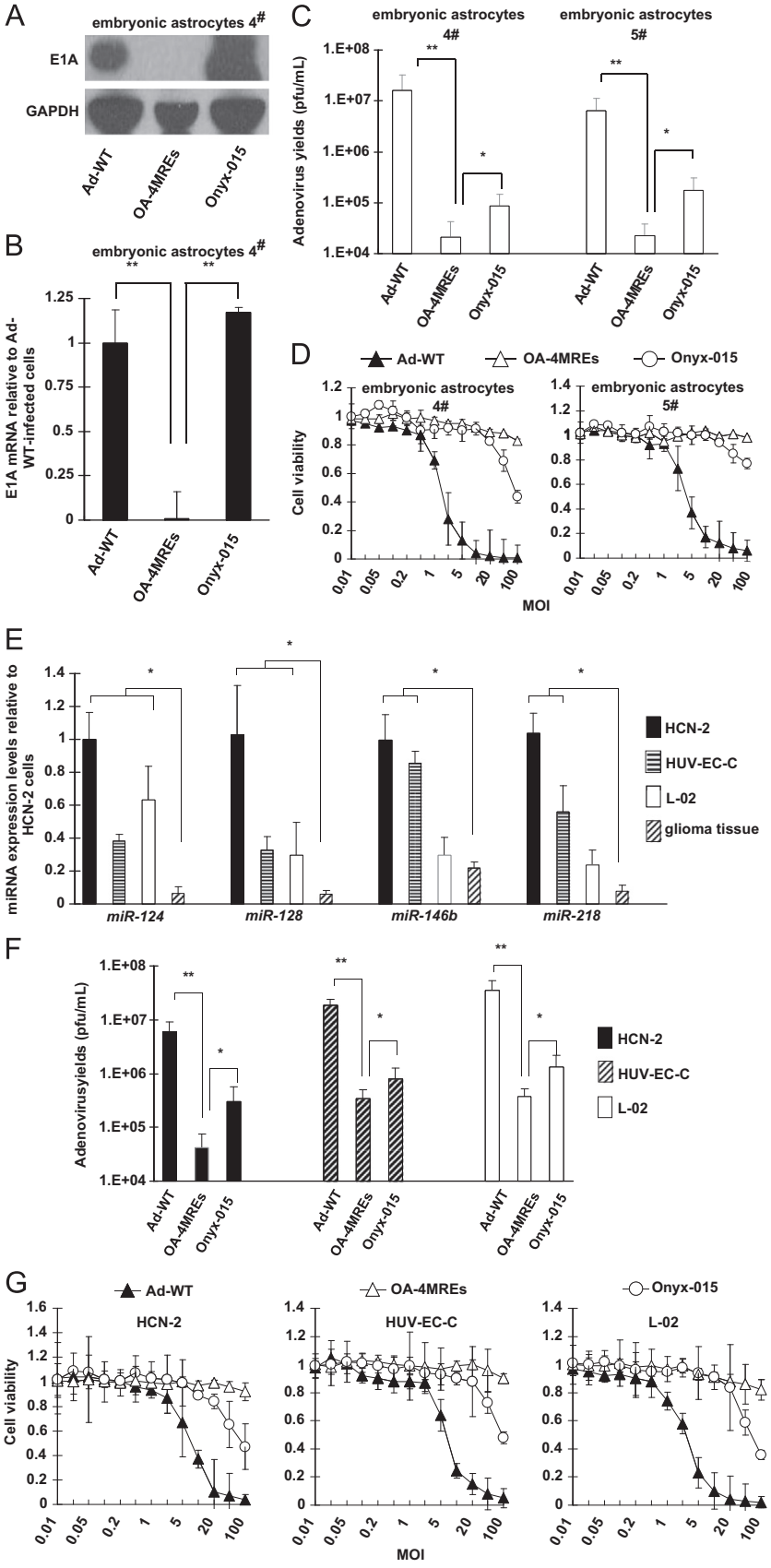
The above results suggested that replication of OA-4MREs was significantly suppressed in normal cells, compared with Ad-WT and Onyx-015. OA-4MREs had no obvious cytotoxicity to normal cells.

OA-4MREs efficiently replicated in glioma cells and exerted a survival-reducing activity

Anti-tumor capacity of OA-4MREs was investigated using both glioma cell lines and primary glioma cells. E1A expressions were shown to be similar between OA-4MREs- and Ad-WT-infected U-87 MG cells both on protein and mRNA levels (Fig. 5A and B). Replication assay showed that OA-4MREs produced similar amount of progenies to Ad-WT in U-87 MG, U-251 MG, U-371 MG and

Fig. 4. OA-4MREs had no cytotoxicity to normal cells originating from brain and other tissues. (A) Normal embryonic astrocytes were infected with Ad-WT, OA-4MREs and Onyx-015 (5 MOI). 48 h later, E1A protein level was determined by immunoblotting assay and GAPDH was selected as the endogenous reference. (B) E1A mRNA expression was detected using qPCR methods in adenovirus-treated normal astrocytes (5 MOI). *GAPDH* was selected as the endogenous reference. Means ± SD of three independent experiments are shown. (C) Progeny production of the normal astrocytes treated with indicated adenoviruses (5 MOI) were evaluated by replication assays. Progeny yields are presented as pfu/ml. Means ± SD of three independent experiments are shown. (D) Cell viability was evaluated on embryonic astrocytes infected with different concentrations of adenoviruses by MTT assays on day 7 after infection. Means ± SD of three independent experiments are shown. (E) Expression levels of *miR-124*, *miR-128*, *miR-146b* and *miR-218* were detected in HCN-2, HUV-EC-C, and L-02 cells and *U6* was selected as the endogenous reference. Means ± SD of three independent experiments are shown. (F) Progeny production of HCN-2, HUV-EC-C and L-02 cells treated with indicated adenoviruses (5 MOI) were evaluated by replication assays. Progeny yields are presented as pfu/ml. Means ± SD of three independent experiments are shown. (G) Cell viability of HCN-2, HUV-EC-C and L-02 cells was tested on day 7 after infection of different concentrations of adenoviruses evaluated by MTT assays. Means ± SD of three independent experiments are shown.

M059J cells (Fig. 5C). Interestingly, this MRE-regulated oncolytic adenovirus had higher production of progenies than Onyx-015 in glioma cells (Fig. 5C). OA-4MREs had an anti-tumor activity that is similar to Ad-WT on the tested glioma cell lines (Fig. 5D). Also, the survival-reducing effect of OA-4MREs on glioma cells was stronger than Onyx-015 (Fig. 5D).



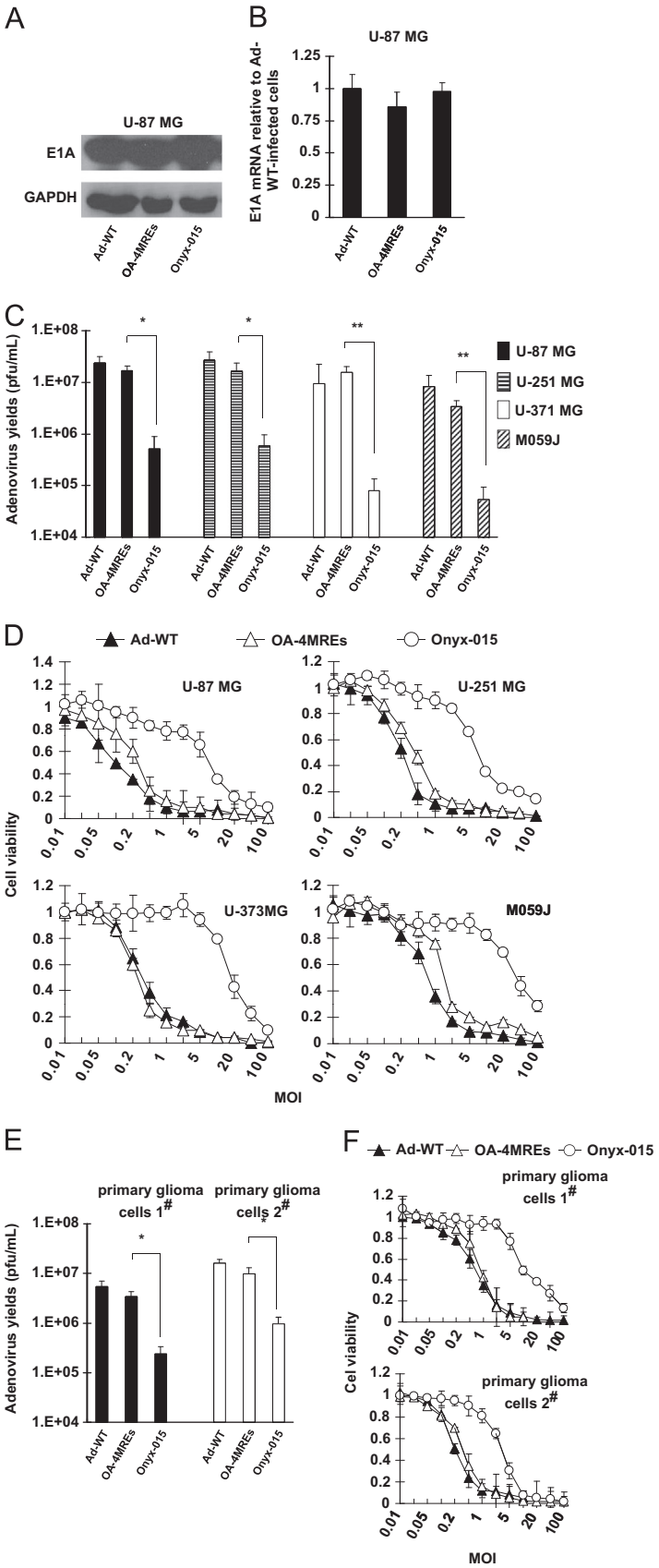


Fig. 5. OA-4MREs potently reduced the survival of glioma cells. (A) U-87 MG cells were infected with Ad-WT, OA-4MREs and Onyx-015 (5 MOI). 48 h later, E1A protein level was determined and GAPDH was selected as the endogenous reference. (B) E1A mRNA expression was also detected using qPCR methods in adenovirus-treated U-87 MG cells. *GAPDH* was selected as the endogenous reference. Means ± SD of three independent experiments are shown. (C) Progeny production of U-87 MG, U-251 MG, U-371 MG and M059J cells treated with indicated adenoviruses (5 MOI) were evaluated by replication assays. Progeny yields are presented as pfu/ml. Means ± SD of three independent experiments are shown. (D) Cell viability was evaluated on U-87 MG, U-251 MG, U-371 MG and M059J cells infected with different concentrations of adenoviruses on day 7 by MTT assays. Means ± SD of three independent experiments are shown. (E) Progeny production of two primary glioma cells treated with indicated adenoviruses were evaluated by replication assays. Progeny yields are presented as pfu/ml. Means ± SD of three independent experiments are shown. (F) Cell viability of two primary glioma cells, which were infected with different concentrations of adenoviruses, was evaluated by MTT assays. Means ± SD of three independent experiments are shown.

Consistent with the data on glioma cell lines, OA-4MREs also replicated more efficiently than Onyx-015 in primary glioma cells from the patients (Fig. 5E). Its cytotoxicity to these primary cells was also higher than Onyx-015 (Fig. 5F).

Therefore, OA-4MREs retained a high replication ability in glioma cells and greatly suppressed their viability.

OA-4MREs suppressed the growth of glioma xenograft without toxicity to normal tissues

We further tested the anti-glioma activity of OA-4MREs on glioma xenograft in different mouse models. In a subcutaneous xenograft model, intratumoral injection of OA-4MREs inhibited the growth of both U-87 MG- and primary glioma-derived tumors. The therapeutic outcome of OA-4MREs is similar to that of Ad-WT and there was no significant difference in the anti-tumor capacity between OA-4MREs and Ad-WT (Fig. 6A and B). In contrast, Onyx-015 was shown to reduce the growth of tumors by no greater than 40% (Fig. 6A and B).

In an orthotopic U-87 MG xenograft model, OA-4MREs treatment markedly prolonged the survival of tumor-bearing mice (mean $\pm$ SD, 54.67 $\pm$ 6.25 days) (Fig. 6C). Onyx-015 had only a limited survival-prolonging effect on mice (mean $\pm$ SD, 20.00 $\pm$ 5.18 days), compared with the PBS (mean $\pm$ SD, 14.00 $\pm$ 5.51 days) (Fig. 6C). Ad-WT also prolonged the survival of tumor-bearing mice (47.00 $\pm$ 11.40 days), although its effect is moderately weaker than OA-4MREs (P=0.188) (Fig. 6C). Strong E1A staining indicated high proliferation in tumor sections both from Ad-WT- and OA-4MREs-treated mice. Weak E1A staining showed that Onyx-015 had relatively limited proliferation ability (Fig. 6D). Importantly, there was no obvious E1A staining in the brain section from OA-4MREs-injected mice. In contrast, extensive E1A expression was easily observed in normal brain derived from the animals treated with Ad-WT and Onyx-015 (Fig. 6D).

To evaluate the hepatotoxicity induced by adenovirus treatment, we collected the blood from another 4 groups of tumor-free mice systemically injected with Ad-WT, PBS, Onyx-015 and OA-4MREs. The results showed that both Ad-WT and Onyx-015 treatment significantly increased the level of liver damage indicator, alanine aminotransferase (ALT), in the serum. In contrast, there was no obvious difference in serum level of ALT between PBS- and OA-4MREs-treated mice (Fig. 6E). Immunohistological analysis indicated that there was no obvious E1A staining in the liver section from OA-4MREs-injected mice. In contrast, E1A was widely expressed in normal liver tissues from the mice injected with Ad-WT or Onyx-015 (Fig. 6F).

The above data demonstrated that OA-4MREs were able to suppress the growth of glioma in vivo, but not damaged normal tissues of the treated animal.

Discussion

We compared miRNA expression profiles of glioma and its corresponding noncancerous brain tissue. The data showed that most of the tested miRNAs had significantly reduced expression level in glioma. However, *miR-19a* level was increased, but not reduced, in these glioma samples, inconsistent with previous reports (Malzkorn et al., 2010). The explanation may be that we used primary glioma for miRNA detection while Malzkorn et al. studied this miRNA using secondary glioblastoma samples, and there may be differential expression profile of *miR-19a* between the two cases.

As one of the most widely studied tumor-suppressing miRNA, *let-7* expression showed only a moderate reduction in glioma (approximately 55%) compared with some other miRNAs (*miR-124*, *miR-128*, *miR-139*, *miR-140* and *miR-218*), which weakened its feasibility and effectiveness to prevent adenovirus replication from normal brain cells.

Luciferase reporter assay revealed that 7 MREs suppressed luciferase expression by more than 70%. The strong inhibitory activity of these MREs may be due to their enrichments in normal brain tissues (Cheng et al., 2009; Ciafre et al., 2005; Sempere et al., 2004). MREs of *miR-7*, *miR-26b*, *miR-181b*, *miR-184*, *miR-205*, *miR-328* and *let-7* were also found to affect luciferase expression in glioma cells, which may impair adenovirus replication if applied. Although these miRNAs were downregulated in glioma cells, the remaining RNA appeared to suppress exogenous gene expression to some extent.

Many researchers have modified adenovirus for its tumor-specific replication by inserting MRE of single miRNA to modulate E1A expression (Cawood et al., 2011; Jin et al., 2011; Leja et al., 2010). However, adenovirus replication was not effectively suppressed if the selected miRNA was not highly expressed in some specific normal cells, thereby causing a nonselective cytotoxicity. In our study, we simultaneously utilized 4 MREs of different miRNAs to control E1A expression and viral replication. Our data verified a superiority of our strategy over using single MRE to limit exogenous gene expression in normal tissue-derived cells, showing that application of multiple MREs is a promising strategy to construct oncolytic adenoviruses with higher specificity. In fact, the application of multiple MREs has already been tested for gene therapy and virotherapy for cancers (Liu et al., 2013; Ylosmaki et al., 2008).

The expression profiles of *miR-124*, *miR-128*, *miR-146b* and *miR-218* in normal cells were also investigated in our studies. Compared with glioma tissue, *miR-124* was found to have an elevated level in neuron, endothelial cells and hepatic cells. Interestingly, overexpression of *miR-124* was able to facilitate reprogramming of fibroblast to functional neuron (Yoo et al., 2011). It was also reported that *miR-124* had a high expression level in normal liver tissues (Zheng et al., 2012). *miR-128* level was also highly expressed in these normal cells. In fact, *miR-128* was among the most enriched miRNAs in neuron (Smirnova et al., 2005). Although *miR-146b* expression was downregulated in mature neuron, in comparison with neuronal progenitor cells (Liu et al., 2012), we demonstrated that its abundance was significantly higher in neuron cells than glioma tissues. The tested normal cells also had higher expression level of *miR-218,* which has been shown to be important for vascularization of retina and enriched in neuron (Small et al., 2010).

Consistently, OA-4MREs have lower E1A expression and replication in normal cells than other oncolytic adenoviruses. Viral replication assays showed that OA-4MREs had only a limited amount of adenoviral progenies in normal cells, HCN-2, HUV-EC-C and L-02. Consistently, OA-4MREs had no significant cytotoxicity to the tested normal cells. In animal experiments, OA-4MREs prolonged animal survival most efficiently among the tested adenoviruses. Liver function evaluation and immunohistological analysis both showed that MRE-regulated adenoviruses had no toxicity to normal liver and brain tissues. Therefore, we concluded that OA-4MREs possessed a high safety and can be further tested for clinical applications.

qPCR and immunoblotting assay revealed that E1A expression was almost not affected in OA-4MREs-infected glioma cells. Onyx-015 has the lowest progeny production in glioma cells among the tested adenoviruses, because the deletion of E1B 55 kDa protein greatly decreased the efficiency of adenovirus replication. This reduction in adenovirus replication has also been reported by other groups (Su et al., 2004).

In future, MREs of other miRNAs can also be used to further improve the safety of OA. For example, *miR-122* is the most abundant

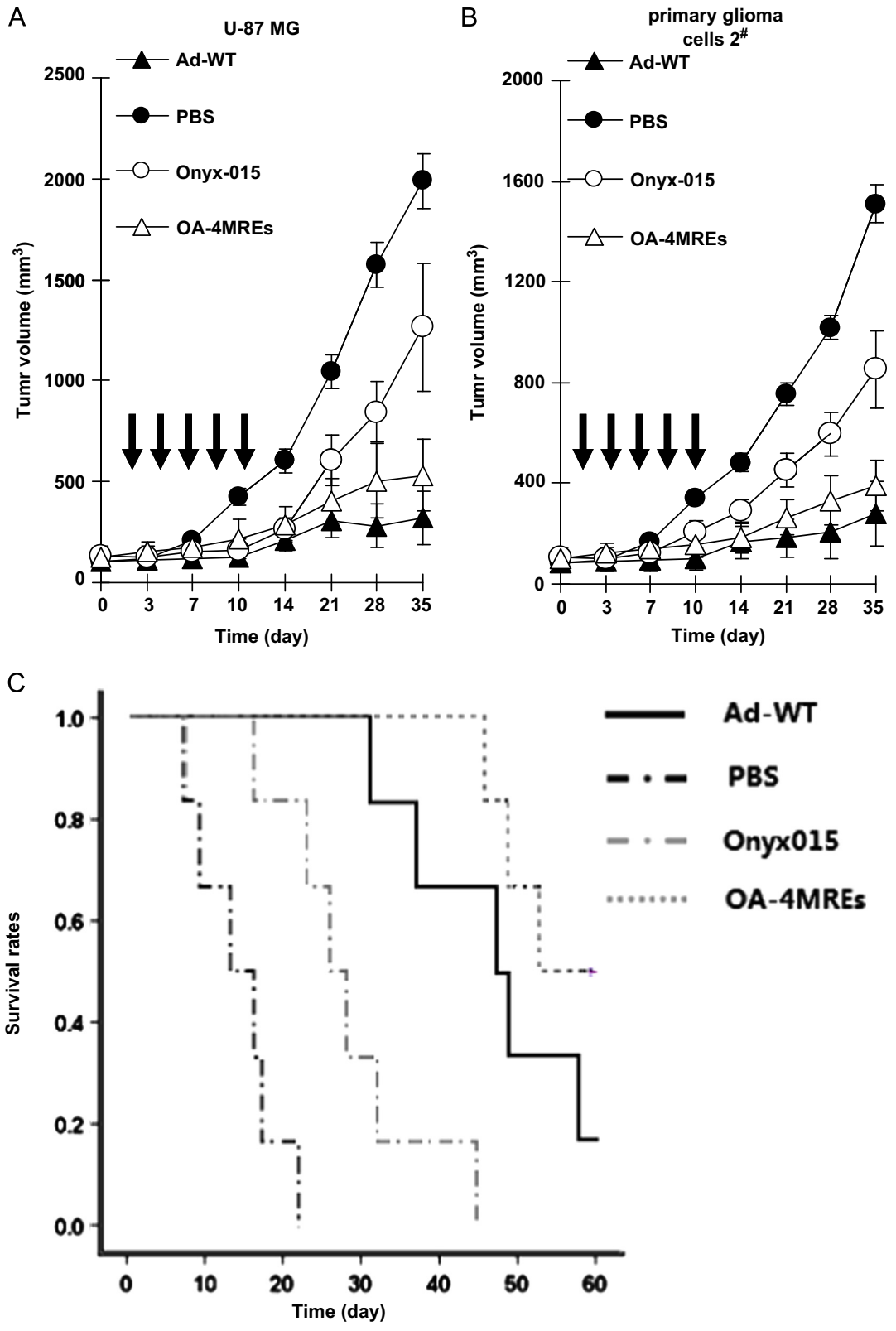


Fig. 6. OA-4MREs suppressed the growth of glioma xenograft in vivo without damage to normal tissues. (A) Subcutaneous U-87 MG glioma xenograft was established by inoculating 2×10^6 cells into left flanks of male BALB/c nude mice ($n=6$). 1×10^9 pfu of Ad-WT, Onyx-015 and OA-4MREs were treated and the tumor volumes were periodically measured. Means $\pm$ SD of tumor sizes are shown. The arrows indicate time-points of adenovirus injection. (B) Subcutaneous primary glioma xenograft was established by subcutaneously injecting 1×10^6 cells into lower right flanks of male BALB/c nude mice ($n=5$). 1×10^9 pfu of different adenoviruses were treated and the tumor volumes were periodically measured. Means $\pm$ SD of tumor sizes are shown. The arrows indicate time-points of adenovirus injection. (C) Intracranial U-87 MG glioma xenografts were established by injecting 1×10^6 cells into the forebrain of male BALB/c nude mice ($n=6$). 1×10^9 pfu of different adenoviruses were directly injected into the established tumors. The deaths of mice were recorded every day. The Kaplan–Meier method and the Cox–Mantel log-rank test (two-sided) were used to assess the statistical significances of differences in survival time. (D) Histological staining was performed to detect the expression of E1A, which indicated the proliferation of adenovirus, in the tumor and brain sections from adenovirus-injected mice that bear intracranial glioma xenografts (200 $\times$). (E) 20 male BALB/c mice ($n=5$, no tumor xenograft) were intravenously injected with 1×10^9 pfu of different adenoviruses every other day for five times. On day 11, their blood was harvested for the measurement of ALT levels. Means $\pm$ SD of ALT serum levels are shown. (F) Histological staining was also performed to detect E1A expression in liver tissue from tumor-free mice systemically injected with adenovirus (200 $\times$).

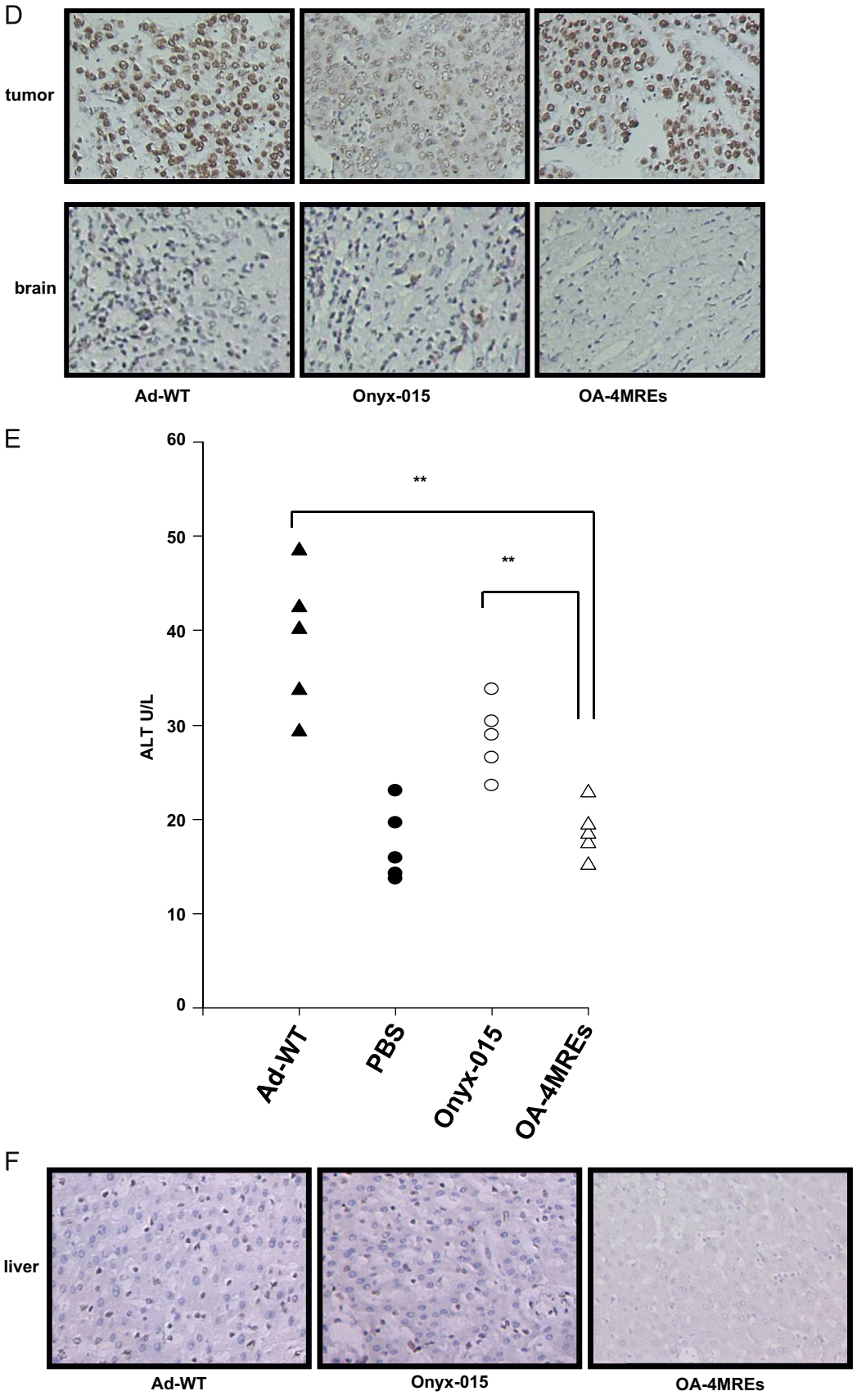


Fig. 6. (*continued*)

miRNA in normal liver cells and acts as a tumor-suppressing miRNA (Ma et al., 2010). Therefore, MRE of *miR-122* can also be applied to prevent liver cells from the cytotoxicity induced by OA.

Collectively, we constructed a glioma-targeting oncolytic adenovirus whose E1A expression was regulated by multiple MREs and demonstrated that this modification retained adenovirus replication and effectively suppressed glioma growth, and meanwhile, showed a limited cytotoxicity to normal cells. This oncolytic adenovirus is a promising anti-glioma biological agent and has the potential to be applied for clinical treatment.

Materials and methods

Cell line cultures

Human glioblastoma cell lines, U-87 MG, U-251 MG, U-373 MG and M059J, human neuronal cells HCN-2 and human endothelial cells HUV-EC-C were purchased from American Type Culture Collection (Manassas, VA). Human normal liver cells L-02, and human embryonic kidney cells HEK-293 cells were obtained from Shanghai Cell Collection (Shanghai, China). The cells were all cultured using DMEM supplemented with 10% of fetal bovine serum (FBS), 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 and humidified atmosphere at 37 °C.

Primary culture/ethics statement

We employed primary cultures derived from malignant glioma and normal astrocytes in this study. For primary glioma culture, fresh cancerous tissue was obtained from patients according to protocols approved by the Ethical Review Board in the Affiliated Hospital of Medical College of Qingdao University (Qingdao, China). The patients have provided their written informed consent to participate in this study. All patients underwent surgical resection of primary glioma at the Department of Neurological Surgery, The Affiliated Hospital of Medical College of Qingdao University (Qingdao, China). Glioma tissues were cut into small pieces. The single cell suspension was obtained by mechanical manipulation. The primary cultures were established initially in DMEM supplemented with 15% FBS and maintained in DMEM supplemented with 10% FBS.

For primary astrocyte culture, the samples were obtained from aborted fetus with informed consent from the pregnant women, according to the previously published procedures approved by Ethical Review Board in the Affiliated Hospital of Medical College of Qingdao University (Qingdao, China). The procedures were briefly described as follows. Brain tissue from anterior fontanel was cut into pieces with the removal of meninges, followed by trypsin digestion. The digested cells were filtered with a steel net and cultured in DMEM supplemented with 15% FBS. GFAP expression was confirmed by immunofluorescent staining.

Quantitative PCR (qPCR)

For miRNA detection in glioma tissues, fresh cancerous and noncancerous brain tissues were obtained with written informed consent from all patients according to protocols approved by the Ethical Review Board in the Affiliated Hospital of Medical College of Qingdao University (Qingdao, China). All patients underwent surgical resection of primary glioma at the Department of Neurological Surgery, The Affiliated Hospital of Medical College of Qingdao University (Qingdao, China).

Total RNA was extracted from glioma and noncancerous brain tissue with Trizol solution (Sigma-Aldrich, MO). Reverse transcription reaction was performed with a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. qPCR was performed using TaqMan® 2 × Universal PCR Master Mix (Applied Biosystems) on CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, CA) supplied with analytical software.

To determine E1A mRNA level in adenovirus-infected cells, 5 MOI of indicated adenoviruses were added to cell cultures. After 48 h, cells were harvested for RNA extraction, followed by being transcribed into cDNAs using Rever Tra Ace qPCR RT Kit (Toyobo, Japan) according to the manufacturer's instructions. qPCR was performed using SYBR premix Ex Taq (Takara) on CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, CA) supplied with analytical software. The primers used for qPCR are previously described (He et al., 2011).

Adenovirus construction

Wild type serotype 5 (Ad-WT) and oncolytic adenovirus Onyx-015 were kindly gifted by Dr. Zhao (General Hospital of Chengdu Military Area Command of Chinese PLA, Chengdu, China). We constructed OA-4MREs mainly according to the procedures described by Jin et al. (2011). Briefly, *BstBI* and *SalI* restriction sites were introduced immediately following adenoviral E1A gene of pXC1 (Microbix Biosystems Inc., Toronto, Canada), generating pXC-MREs. A DNA fragment containing 2 copies of *miR-124* MREs, 2 copies of *miR-128* MREs, 2 copies of *miR-146b* MREs and 2 copies of *miR-218* MREs (TTCGAAACAAACACCCGGCCTGATTCACAACACCAGCTACAAACACCCGGCCTGATTCACAACACCAGCTACAAACACCGAACAGGTAGTCTGAACACTGGGACAAACACCGAACAGGTAGTCTGAACACTGGGACAAACACCCAAACACCATTGTCACACTCCAACAAACACCCAAACACCATTGTCACACTCCAGTCGAC) was introduced into pXC-MREs at the sites of *BstBI*/*SalI* to generate pXC-4MREs. The structure of the recombinant adenovirus was confirmed by DNA sequencing. pXC-4MREs was co-transfected with pAdEasy into HEK293 cells. After plague purification for three times and PCR-based identification, the adenoviruses were harvested and then purified with the CsCl gradient centrifugation. The constructed adenovirus was designated as OA-4MREs. The titers of the involved adenoviruses were quantified with the $TCID_{50}$ method on HEK-293 cells and shown as plaque-forming units per milliliter (pfu/ml). The structures of these adenoviruses are illustrated in Fig. 3E.

Luciferase reporter construction and assay

The sense and antisense sequences containing 2 copies of specific MREs were synthesized, annealed and inserted into the *XhoI* and *NotI* sites of psiCheck2 vectors (Promega, WI) immediately following the Renilla luciferase gene to construct MRE-regulated luciferase reporter, which were designated as pluc-miR-**.

After transfection of U-87 MG cells and normal embryonic astrocytes and subsequent 48 h incubation, cells were harvested and treated with lysis buffer. Firefly and Renilla luciferase activities were determined with the Dual-Luciferase reporter system (Promega, WI) following the manufacturer's procedures.

miRNA mimics and inhibitors

mirVana™ mimics and inhibitors of *miR-124*, *miR-128*, *miR-146b* and *miR-218* (Life Technologies Invitrogen, Karlsruhe, Germany) were mixed thoroughly and added to the indicated cells at the concentration of 40 nM (10 nM for each mimic or inhibitor) using Lipofectamine™ 2000 (Life Technologies Invitrogen, Karlsruhe, Germany) according to the instructions. 48 h later, cells were used for subsequent experiments.

Immunoblotting assay

Cells were infected with indicated adenoviruses at a MOI of 5. 48 h later, proteins were harvested with M-PER® Mammalian Protein Extraction Reagent (Thermo Scientific, IL), separated using polyacrylamide gel electrophoresis and transferred onto 0.45 μm nitrocellulose membranes. After being blocked with 5% fat-free dry milk for 2 h, the membrane was incubated with mouse monoclonal IgG against adenovirus E1A protein (1:200, Santa Cruz Biotechnology, CA) for 2 h. Overnight, the membrane was incubated with horseradish peroxidase-conjugated goat polyclonal antibody against mouse IgG (1:4000, Immunology Consultants Laboratory, OR) and visualized with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, IL).

Virus replication assay

Cells were infected with indicated adenoviruses at a MOI of 5. 48 h later, cells were harvested and then lysed via freeze and thaw three times. The titers of viral progenies were quantified on HEK-293 cells with the $TCID_{50}$ method.

Cytotoxicity assay

Adenoviral vectors of indicated MOIs were added to cell cultures. 7 Days later, 50 μl of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (1 mg/ml) was added. 4 h later, MTT was removed and 150 μl of dimethyl sulfoxide (DMSO) was added. The spectrophotometric absorbance was measured on a model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA) at 570 nm with a reference wavelength of 655 nm. Cell viability was calculated according to the following formula: cell viability = absorbance value of infected cells/absorbance value of uninfected control cells.

Subcutaneous glioma model

Procedures for animal experiments were all approved by the Committee on the Use and Care on Animals in the Affiliated Hospital of Medical College of Qingdao University (Qingdao, China).

U-87 MG subcutaneous tumor xenograft was established by injecting 2×10^6 cells at the left flanks of 5-week-old male BALB/c nude mice (Institute of Animal Center, Chinese Academy of Sciences, Shanghai, China). As soon as tumors grew to 6–8 mm in diameter, 24 mice were randomly and equally divided into 4 groups ($n=6$). The mice were intratumorally injected with 100 μL PBS with or without 2×10^8 pfu of Ad-WT, Onyx-015 and OA-4MREs. The injections were repeated every other day with a total dosage of 1×10^9 pfu of adenoviruses for five times.

Primary glioma xenograft was established by incubating 1×10^6 cells at the lower right flanks of 5-week-old male BALB/c nude mice. 20 mice were randomly divided into 6 groups with equal number ($n=5$). The doses of used adenoviruses were equal to that for U-87 MG tumor xenograft.

Tumor diameter was measured by periodic measurements with calipers and volume was calculated using the following formula: tumor volume (mm^3) = maximal length (mm) × perpendicular width $(mm)^2/2$.

Intracranial glioma model

To establish an intracranial glioma xenograft mouse model, a previously described procedure was followed (McCutcheon et al., 2000). Briefly, 1×10^6 U-87 MG cells were injected into the forebrain of 5-week-old male BALB/c nude mice with the direction of a guide-screw. 7 Days later, 24 mice were equally divided into 4 groups ($n=6$). 1×10^9 pfu of indicated adenoviruses were intratumorally delivered in a single injection. Since then, the mice were monitored every day for their conditions. The mice were euthanized by carbon dioxide when they showed signs of impending death. At the same time, the death was recorded to determine their survival rates.

The Kaplan–Meier method and the Cox–Mantel log-rank test (two-sided) were used to assess the statistical significances of differences in survival time.

Liver damage evaluation

To evaluate the hepatotoxicity induced by adenovirus treatment, twenty 5-week-old male BALB/c mice were divided into 4 groups ($n=5$) and were intravenously injected with 1×10^9 pfu of indicated adenoviruses every other day for five times. On day 11, their blood (600 ml/mice) was harvested by cardiac puncture, followed by being incubated with 12 U of heparin. Alanine aminotransferase (ALT) levels in blood were detected at the Clinical Laboratory, The Affiliated Hospital of Medical College of Qingdao University (Qingdao, China)

Histological staining

On day 7 after adenovirus injection, one mouse from each group in intracranial tumor models was sacrificed and its tumor and brain were harvested. Liver were obtained from the tumor-free mice intravenously injected with adenoviruses. Histological staining was then performed on formalin-fixed, paraffin-embedded samples using the streptavidin–biotin-peroxidase complex method. Mouse monoclonal IgG against adenovirus E1A protein (1:100; Santa Cruz Biotechnology, CA) was used to specifically recognize and bind the antigen. Incubation with secondary antibody (Immunology Consultants Laboratory Inc, OR; 1:100) was followed and E1A expression was visualized using 3, 3′-diaminobenzidine (Sigma, USA). The sections were finally counterstained with hematoxylin.

Statistical analysis

The statistical tests in this manuscript were two-tailed Student's *t*-test. Differences were considered as statistically significant (*) when $P<0.05$ and statistically very significant (**) when $P<0.01$, except for the survival analysis on the intracranial glioma xenograft mouse model.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.04.007.

References

Alvarez-Breckenridge, C.A., Yu, J., Price, R., Wojton, J., Pradarelli, J., Mao, H., Wei, M., Wang, Y., He, S., Hardcastle, J., Fernandez, S.A., Kaur, B., Lawler, S.E., Vivier, E., Mandelboim, O., Moretta, A., Caligiuri, M.A., Chiocca, E.A., 2012. NK cells impede glioblastoma virotherapy through NKp30 and NKp46 natural cytotoxicity receptors. Nat. Med. 18, 1827–1834.

Cawood, R., Wong, S.L., Di, Y., Baban, D.F., Seymour, L.W., 2011. MicroRNA controlled adenovirus mediates anti-cancer efficacy without affecting endogenous microRNA activity. PLoS One 6, e16152.

Chen, W., Wu, Y., Liu, W., Wang, G., Wang, X., Yang, Y., Chen, W., Tai, Y., Lu, M., Qian, Q., Zhang, Q., Chen, G., 2011. Enhanced antitumor efficacy of a novel fiber chimeric oncolytic adenovirus expressing p53 on hepatocellular carcinoma. Cancer Lett. 307, 93–103.

Cheng, L.C., Pastrana, E., Tavazoie, M., Doetsch, F., 2009. miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. Nat. Neurosci. 12, 399–408.
Ciafre, S.A., Galardi, S., Mangiola, A., Ferracin, M., Liu, C.G., Sabatino, G., Negrini, M., Maira, G., Croce, C.M., Farace, M.G., 2005. Extensive modulation of a set of microRNAs in primary glioblastoma. Biochem. Biophys. Res. Commun. 334, 1351–1358.
Conti, A., Aguennouz, M., La Torre, D., Tomasello, C., Cardali, S., Angileri, F.F., Maio, F., Cama, A., Germano, A., Vita, G., Tomasello, F., 2009. miR-21 and 221 upregulation and miR-181b downregulation in human grade II–IV astrocytic tumors. J. Neurooncol. 93, 325–332.
Fulci, G., Dmitrieva, N., Gianni, D., Fontana, E.J., Pan, X., Lu, Y., Kaufman, C.S., Kaur, B., Lawler, S.E., Lee, R.J., Marsh, C.B., Brat, D.J., van Rooijen, N., Stemmer-Rachamimov, A.O., Hochberg, F.H., Weissleder, R., Martuza, R.L., Chiocca, E.A., 2007. Depletion of peripheral macrophages and brain microglia increases brain tumor titers of oncolytic viruses. Cancer Res. 67, 9398–9406.
Godlewski, J., Nowicki, M.O., Bronisz, A., Williams, S., Otsuki, A., Nuovo, G., Raychaudhury, A., Newton, H.B., Chiocca, E.A., Lawler, S., 2008. Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. Cancer Res. 68, 9125–9130.
Gomez-Manzano, C., Fueyo, J., 2010. Oncolytic adenoviruses for the treatment of brain tumors. Curr. Opin. Mol. Ther. 12, 530–537.
He, X., Liu, J., Yang, C., Su, C., Zhou, C., Zhang, Q., Li, L., Wu, H., Liu, X., Wu, M., Qian, Q., 2011. 5/35 Fiber-modified conditionally replicative adenovirus armed with p53 shows increased tumor-suppressing capacity to breast cancer cells. Hum. Gene Ther. 22, 283–292.
Jin, H., Lv, S., Yang, J., Wang, X., Hu, H., Su, C., Zhou, C., Li, J., Huang, Y., Li, L., Liu, X., Wu, M., Qian, Q., 2011. Use of microRNA Let-7 to control the replication specificity of oncolytic adenovirus in hepatocellular carcinoma cells. PLoS One 6, e21307.
Kefas, B., Godlewski, J., Comeau, L., Li, Y., Abounader, R., Hawkinson, M., Lee, J., Fine, H., Chiocca, E.A., Lawler, S., Purow, B., 2008. microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. Cancer Res. 68, 3566–3572.
Leja, J., Nilsson, B., Yu, D., Gustafson, E., Akerstrom, G., Oberg, K., Giandomenico, V., Essand, M., 2010. Double-detargeted oncolytic adenovirus shows replication arrest in liver cells and retains neuroendocrine cell killing ability. PLoS One 5, e8916.
Liu, D.Z., Ander, B.P., Tian, Y., Stamova, B., Jickling, G.C., Davis, R.R., Sharp, F.R., 2012. Integrated analysis of mRNA and microRNA expression in mature neurons, neural progenitor cells and neuroblastoma cells. Gene 495, 120–127.
Liu, J., Ma, L., Li, C., Zhang, Z., Yang, G., Zhang, W., 2013. Tumor-targeting TRAIL expression mediated by miRNA response elements suppressed growth of uveal melanoma cells. Mol. Oncol. 7, 1043–1055.
Luo, J., Xia, Q., Zhang, R., Lv, C., Zhang, W., Wang, Y., Cui, Q., Liu, L., Cai, R., Qian, C., 2008. Treatment of cancer with a novel dual-targeted conditionally replicative adenovirus armed with mda-7/IL-24 gene. Clin. Cancer Res. 14, 2450–2457.
Ma, L., Liu, J., Shen, J., Liu, L., Wu, J., Li, W., Luo, J., Chen, Q., Qian, C., 2010. Expression of miR-122 mediated by adenoviral vector induces apoptosis and cell cycle arrest of cancer cells. Cancer Biol. Ther. 9, 554–561.
Malzkorn, B., Wolter, M., Liesenberg, F., Grzendowski, M., Stuhler, K., Meyer, H.E., Reifenberger, G., 2010. Identification and functional characterization of microRNAs involved in the malignant progression of gliomas. Brain Pathol. 20, 539–550.
McCutcheon, I.E., Friend, K.E., Gerdes, T.M., Zhang, B.M., Wildrick, D.M., Fuller, G.N., 2000. Intracranial injection of human meningioma cells in athymic mice: an orthotopic model for meningioma growth. J. Neurosurg. 92, 306–314.
Nan, Y., Han, L., Zhang, A., Wang, G., Jia, Z., Yang, Y., Yue, X., Pu, P., Zhong, Y., Kang, C., 2010. MiRNA-451 plays a role as tumor suppressor in human glioma cells. Brain Res. 1359, 14–21.
Post, D.E., Khuri, F.R., Simons, J.W., Van Meir, E.G., 2003. Replicative oncolytic adenoviruses in multimodal cancer regimens. Hum. Gene Ther. 14, 933–946.
Rao, S.A., Santosh, V., Somasundaram, K., 2010. Genome-wide expression profiling identifies deregulated miRNAs in malignant astrocytoma. Mod. Pathol. 23, 1404–1417.
Reardon, D.A., Galanis, E., DeGroot, J.F., Cloughesy, T.F., Wefel, J.S., Lamborn, K.R., Lassman, A.B., Gilbert, M.R., Sampson, J.H., Wick, W., Chamberlain, M.C., Macdonald, D.R., Mehta, M.P., Vogelbaum, M.A., Chang, S.M., Van den Bent, M.J., Wen, P.Y., 2011. Clinical trial end points for high-grade glioma: the evolving landscape. Neuro Oncol. 13, 353–361.
Sempere, L.F., Freemantle, S., Pitha-Rowe, I., Moss, E., Dmitrovsky, E., Ambros, V., 2004. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol. 5, R13.
Silber, J., Lim, D.A., Petritsch, C., Persson, A.I., Maunakea, A.K., Yu, M., Vandenberg, S. R., Ginzinger, D.G., James, C.D., Costello, J.F., Bergers, G., Weiss, W.A., Alvarez-Buylla, A., Hodgson, J.G., 2008. miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. BMC Med. 6, 14.
Skalsky, R.L., Cullen, B.R., 2011. Reduced expression of brain-enriched microRNAs in glioblastomas permits targeted regulation of a cell death gene. PLoS One 6, e24248.
Small, E.M., Sutherland, L.B., Rajagopalan, K.N., Wang, S., Olson, E.N., 2010. MicroRNA-218 regulates vascular patterning by modulation of Slit-Robo signaling. Circ. Res. 107, 1336–1344.
Smirnova, L., Grafe, A., Seiler, A., Schumacher, S., Nitsch, R., Wulczyn, F.G., 2005. Regulation of miRNA expression during neural cell specification. Eur. J. Neurosci. 21, 1469–1477.
Su, C.Q., Sham, J., Xue, H.B., Wang, X.H., Chua, D., Cui, Z.F., Peng, L.H., Li, L.F., Jiang, L. H., Wu, M.C., Qian, Q.J., 2004. Potent antitumoral efficacy of a novel replicative adenovirus CNHK300 targeting telomerase-positive cancer cells. J. Cancer Res. Clin. Oncol. 130, 591–603.
Wu, N., Zhao, X., Liu, M., Liu, H., Yao, W., Zhang, Y., Cao, S., Lin, X., 2011. Role of microRNA-26b in glioma development and its mediated regulation on EphA2. PLoS One 6, e16264.
Xia, H., Qi, Y., Ng, S.S., Chen, X., Li, D., Chen, S., Ge, R., Jiang, S., Li, G., Chen, Y., He, M. L., Kung, H.F., Lai, L., Lin, M.C., 2009. microRNA-146b inhibits glioma cell migration and invasion by targeting MMPs. Brain Res. 1269, 158–165.
Xu, C., Sun, Y., Wang, Y., Yan, Y., Shi, Z., Chen, L., Lin, H., Lu, S., Zhu, M., Su, C., Li, Z., 2012. CEA promoter-regulated oncolytic adenovirus-mediated Hsp70 expression in immune gene therapy for pancreatic cancer. Cancer Lett. 319, 154–163.
Ylosmaki, E., Hakkarainen, T., Hemminki, A., Visakorpi, T., Andino, R., Saksela, K., 2008. Generation of a conditionally replicating adenovirus based on targeted destruction of E1A mRNA by a cell type-specific MicroRNA. J. Virol. 82, 11009–11015.
Yoo, A.S., Sun, A.X., Li, L., Shcheglovitov, A., Portmann, T., Li, Y., Lee-Messer, C., Dolmetsch, R.E., Tsien, R.W., Crabtree, G.R., 2011. MicroRNA-mediated conversion of human fibroblasts to neurons. Nature 476, 228–231.
Yue, X., Wang, P., Xu, J., Zhu, Y., Sun, G., Pang, Q., Tao, R., 2012. MicroRNA-205 functions as a tumor suppressor in human glioblastoma cells by targeting VEGF-A. Oncol. Rep. 27, 1200–1206.
Zhang, W., Cai, R., Luo, J., Wang, Y., Cui, Q., Wei, X., Zhang, H., Qian, C., 2007. The oncolytic adenovirus targeting to TERT and RB pathway induced specific and potent anti-tumor efficacy in vitro and in vivo for hepatocellular carcinoma. Cancer Biol. Ther. 6, 1726–1732.
Zheng, F., Liao, Y.J., Cai, M.Y., Liu, Y.H., Liu, T.H., Chen, S.P., Bian, X.W., Guan, X.Y., Lin, M.C., Zeng, Y.X., Kung, H.F., Xie, D., 2012. The putative tumour suppressor microRNA-124 modulates hepatocellular carcinoma cell aggressiveness by repressing ROCK2 and EZH2. Gut 61, 278–289.