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Original Article

Suppression of IGF1R in Melanoma Cells by an Adenovirus-Mediated One-Step Knockdown System

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Abnormal activation of the IGF1R signaling pathway accelerates melanoma development and metastases. RNAi systems with complex cloning procedures and unsatisfactory efficiency in suppressing gene expression have become the technical difficulties that hinder their utility when studying gene knockdown. Here we established a simplified adenovirus-mediated gene knockdown system by which a single adenoviral vector carries multiple siRNA fragments that can effectively suppress IGF1R expression in melanoma cells. We first generated the adenovirus that simultaneously expresses three human or mouse siRNAs targeting IGF1R (AdRIGF1R-OK). qRT-PCR and immunofluorescence staining revealed that IGF1R expression was significantly decreased in the melanoma cells that were infected with AdRIGF1R-OK. Bioluminescence imaging showed that the size of the tumor formed by the xenografts infected with AdRIGF1R-OK was significantly smaller than that of the controls. Annexin V-FITC flow cytometry assay, immunofluorescence staining for cleaved caspase-3, and Hoechst staining showed that more cells underwent apoptosis after infection with AdRIGF1R-OK. Luciferase reporter assay, crystal violet cell viability assay, and cell-cycle analysis showed that the proliferation of melanoma cells infected with AdRIGF1R-OK was significantly decreased compared to the controls. This study demonstrates that the OK system is effective in silencing gene expression, with promising potential to treat melanoma and other diseases.

INTRODUCTION

Melanoma is one of the most lethal cutaneous malignancies, with mortality as high as 80%.[1] Therapeutic approaches such as surgical treatment, radiotherapy, or traditional chemotherapy on treating middle and advanced melanoma are still unsatisfactory. With progress in molecular cell biology, gene therapy has been used to treat cancers.[2] The insulin-like growth factor (IGF) signaling pathway is considered an effective therapeutic target for treating melanoma.[3] The IGF signaling pathway is activated in melanoma,[4] promoting tumor transformation and malignant cell survival via the phosphatidylinositol 3-kinase (PI3K)-AKT/PKB pathway and the Ras-mitogen-activated protein kinase (MAPK) pathway. IGF1R is the key receptor in the IGF signaling pathway. Inhibition of IGF1R function using small-molecule inhibitors or structural suppressor proteins suppresses melanoma growth[5] and promotes melanoma cells apoptosis.[4,6,7] However, IGF1R is widely distributed on the surfaces of many cells throughout the body, controlling tissue growth and survival.[8,9] Inhibition of its function through systemic administration of drugs may cause certain side effects.

Progress has been making in perturbing gene expression, including RNAi and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9.[10] RNAi is a technique that aims to reduce or eliminate gene expression through sequence-specific or post-transcriptional gene silencing by microRNA (miRNA) or exogenous small interfering RNA (siRNA). RNAi has become a valuable and powerful tool to analyze gene functions in *in vitro* and *in vivo* studies,[11,12] because it is known to be precise, stable, and efficient in suppressing gene expression. It also offers opportunities for developing novel and effective therapeutics for human diseases.[13] Progress has been making in improving the efficiency of RNAi in inhibiting gene expression, including delivery of a combination of vectors carrying different siRNA sequences in each vector. Multiple rounds of transfections or infections of the plasmid vectors or virus to the cells consume both time and finances. This elicits our attempt to develop an innovative technique by which we can block gene expression using one vector containing multiple siRNAs.

Adenovirus has long been used as a tool for gene therapy due to its ability to affect both dividing and non-dividing cells without integrating into the host cell genome.[14] Adenovirus can carry a large fragment of the gene of interest, and infect cells with higher efficiency, compared to the other expression viral systems, such as retrovirus, lentivirus, rabies virus, and baculovirus. Adenovirus can infect cells both *in vivo* and *ex vivo* and can drive gene or siRNA

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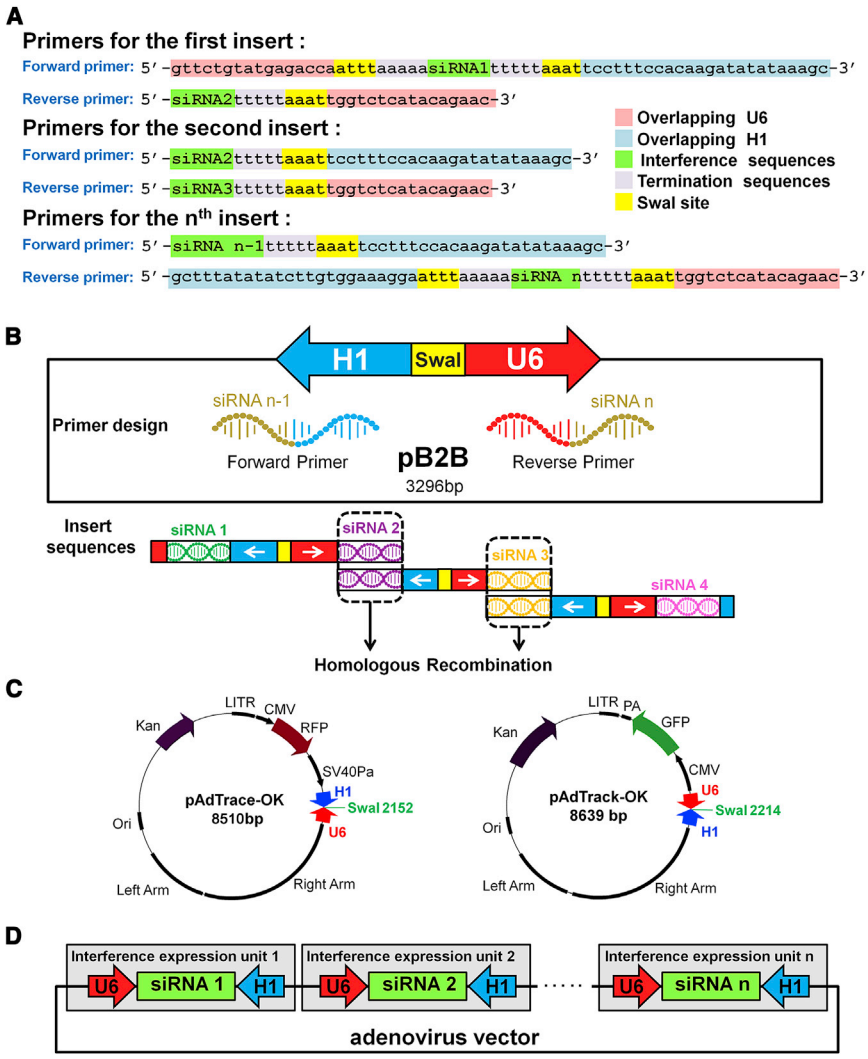

Figure 1. Design of the OK System

(A) Primer design. (B) Template vector pB2B for siRNA insert amplification. (C) Plasmid profiles of adenovirus shuttle vectors in the OK system (RFP or GFP). (D) Schematic representation of a tandem siRNA targeting adenovirus vector.

expression for about 4 weeks *in vivo* stably and efficiently.[15] Adenovirus has good biosafety; thus, it has been used to treat diseases such as cystic fibrosis[16] and hereditary retinal dystrophies.[17] Adenovirus-mediated gene therapy is also widely used in cancer treatment. Most melanoma lesions are on the body surface, making it convenient for application of adenovirus. In this case, using adenovirus to silence endogenous IGF1R expression can be an ideal therapeutic strategy for treating melanoma.

In the present study, we aimed to design a simplified and versatile interfering adenovirus system called the one-step knockdown (OK) method, by which a single adenovirus vector carries multiple siRNA sequences to suppress melanoma cell growth. To achieve this, we have introduced the Gibson Assembly method to engineer the adenovirus vectors pAdTrace-OK and pAdTrack-OK, based on AdEasy adenovirus vectors.[18] We generated adenovirus vectors that contain multiple siRNA fragments by PCR amplifications using the back-to-back U6-H1 promoter vector pB2B as a template. Using the OK system, we constructed adenoviruses that contain multiple siRNA sequences targeting human IGF1R (AdRhIGF1R-OK) and mouse IGF1R (AdRmIGF1R-OK), respectively. Infection of these adenoviruses to the human and mouse melanoma cells showed effective silencing of endogenous IGF1R expression, with decreased proliferation and migration but enhanced apoptosis of these cells *in vitro*. In addition, knockdown of IGF1R by AdRhIGF1R-OK or AdRhIGF1R-OK significantly reduced the growth of the xenograft of human and mouse melanoma cells transplanted to the nude mice. This study demonstrated that the OK system is a simplified and effective tool for gene silencing in research or in clinical therapeutics.

RESULTS

Design and Construction of the Adenovirus-Mediated OK Interfering System

We designed an OK system by constructing an adenovirus vector that carries multiple siRNA inserts. The primers for the first and the last inserts were designed as the following sequence: the U6 promoter, the SwaI cutting site, the stop codon for the U6 promoter, the siRNA1 sequence, the stop codon for the H1 promoter, the SwaI cutting site, and the H1 promoter (Figure 1A). The other siRNA sequences are mainly composed of four parts, including either the U6 or the H1 promoter at the 3′ end, the SwaI cutting site, the stop codon, and the siRNA forward or reverse primer.

The pB2B vector was used as the template vector to amplify the inserts carrying the siRNA sequence (Figure 1B). After PCR reaction, the insert was amplified, with a back-to-back U6-H1 promoter fragment in the middle of the insert and one siRNA sequence in the upstream and the other one in the downstream of the U6-H1 promoter (Figure 1B). The downstream siRNA sequence is the same as the upstream sequence of the next insert. This enables the performance of Gibson Assembly, which is a commonly used molecular cloning method allowing insertion of multiple DNA fragments into a vector with a single and isothermal reaction.[19]

We also constructed two adenovirus shuttle vectors, pAdTrace-OK and pAdTrack-OK, based on the AdEasy adenovirus vectors and our previously designed pSOK vector.[20] pAdTrace-OK and pAdTrack-OK

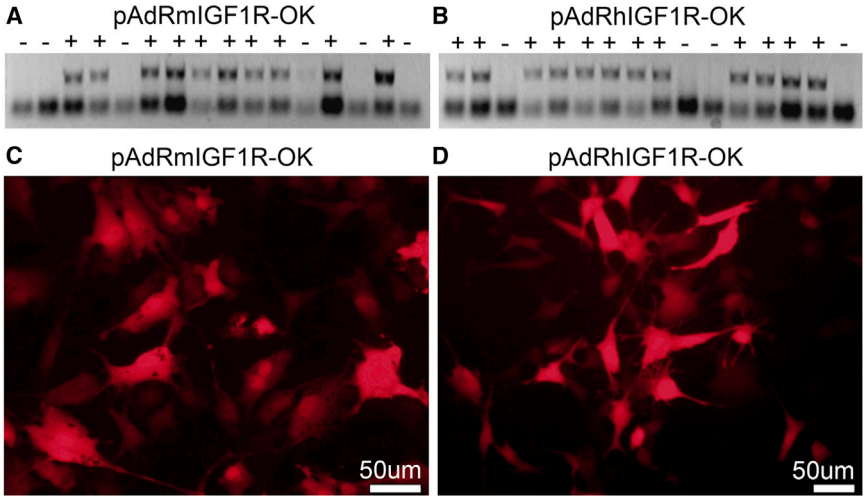

Figure 2. Construction of AdRmIGF1R-OK and AdRhIGF1R-OK

(A and B) PCR screening results show the positive clones carrying pAdTrace-OK vector containing the mouse (A) or human (B) IGF1R siRNA inserts. + indicates a positive clone, and – indicates a negative clone. Upper panels represent PCR bands, and lower panels represent primer dimers. (C and D) Red fluorescence shows the successful infection of AdRmIGF1R-OK or AdRhIGF1R-OK into mouse (C) or human melanoma (D) cell lines, respectively.

contain both U6 and H1 promoters with opposing orientation, driving duplex expression of the siRNA. The SwaI site between U6 and H1 promoters serves as the cutting site, allowing linearization of the vector into the blunt end for Gibson Assembly reaction. The main difference between pAdTrace-OK and pAdTrack-OK is that one carries the red fluorescent protein (RFP) expression sequence and the other carries the GFP expression sequence, respectively (Figure 1C).

In this case, all insert fragments and the linearized vector could be ligated together in one Gibson Assembly reaction in a short time (Figure S1). One adenovirus vector carries multiple siRNA fragments, with each siRNA fragment flanking with one U6 promoter and one H1 promoter.

Construction of AdRmIGF1R-OK and AdRhIGF1R-OK

The two inserts carrying different siRNA sequences targeting human or mouse IGF1R were prepared by PCR with the pB2B template plasmid and primers (Figure S1A). The PCR products were ligated to the pAdTrace-OK vector, which was linearized by the SwaI restriction enzyme. Each assembly reaction contained 200 ng of each insert and 50 ng of the SwaI-linearized pAdTrace-OK vector (Figure S1A). The positive recombinants were screened by colony PCR[20] using the forward and reverse primers that bind the DNA sequence flanking the siRNA sites, with a positive screening rate of around 60% (Figures 2A and 2B). The positive clones identified from colony PCR screening were confirmed by PCR using the plasmid as the template extracted from the colonies (Figures S1B and S1C). DNA sequencing results showed that the three siRNAs were successfully cloned into the pAdTrace-OK vector (see Supplemental Information).

We then transfected the pAdTrace-OK plasmid containing three siRNAs into 293 cells to generate the AdRmIGF1R-OK and AdRhIGF1R-OK adenoviruses, which were then added to B16F10 mouse melanoma cells and A375 human melanoma cells, respectively. The strong fluorescence indicates successful infection of the adenovirus into the melanoma cells (Figures 2C and 2D).

We next examined whether the siRNAs expressed by adenovirus can knock down gene expression efficiently. We infected mouse and human melanoma cells with AdRmIGF1R-OK and AdRhIGF1R-OK, respectively. qRT-PCR results showed that the expression of IGF1R mRNA was significantly decreased in both melanoma cell lines infected with AdRmIGF1R-OK or AdRhIGF1R-OK compared to controls that were infected with adenovirus expressing only RFP (AdRFP) (Figures 3A and 3B). Immunofluorescent staining with an antibody against IGF1R showed that the expression of IGF1R protein was decreased in both melanoma cell lines infected with AdRmIGF1R-OK or AdRhIGF1R-OK compared to controls that were infected with AdRFP (Figures 3C and 3D). These results suggest that the adenovirus-mediated OK system can effectively target and suppress IGF1R expression in both human and mouse melanoma cells.

AdRmIGF1R-OK and AdRhIGF1R-OK Inhibit Melanoma Xenograft Tumor Growth

To examine whether the OK system-generated AdRmIGF1R-OK or AdRhIGF1R-OK adenoviruses can suppress tumor growth, firefly luciferase-tagged B16F10 and A375 cells were first infected with AdRIGF1R-OK (mouse or human [m/h]) or AdRFP (control) and then subcutaneously injected into the dorsal back skin of the athymic nude mice. Seven days after injection, the xenografts were collected. Tumor growth was examined using Xenogen bioluminescence imaging 14 days after cell injection (Figure 4A). Quantitative analysis of the Xenogen imaging data revealed that the xenografts infected with AdRmIGF1R-OK or AdRhIGF1R-OK showed significantly lower luciferase activity when compared to those of the control groups (Figure 4B). qRT-PCR results showed that the expression of IGF1R mRNA was significantly decreased in xenografts that were infected with AdRmIGF1R-OK or AdRhIGF1R-OK compared to controls that were infected with AdRFP (Figure 4C). The xenografts that were infected with AdRIGF1R-OK (m/h) formed a significantly smaller tumor compared to the control groups that were infected with AdRFP (Figures 4D and 4E). These studies confirmed that the OK system-generated adenoviruses containing multiple siRNAs can effectively block melanoma growth by inhibiting IGF1R expression.

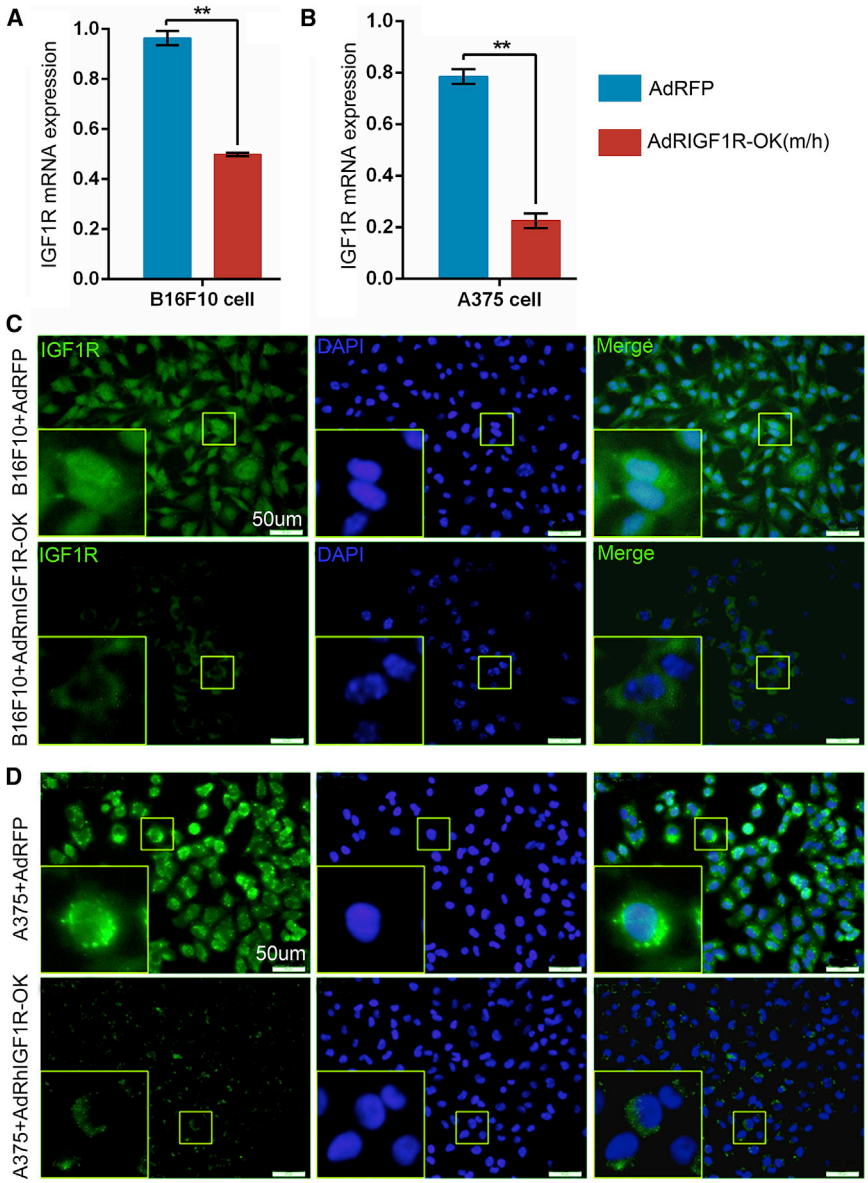

Figure 3. Efficiency Test of AdRmIGF1R-OK and AdRhIGF1R-OK

(A and B) qRT-PCR results show that the mRNA level of IGF1R is significantly decreased in mouse or human melanoma cells after AdRmIGF1R-OK (A) or AdRhIGF1R-OK (B) treatment, respectively, compared to controls that are infected with adenovirus-mediated expression of red fluorescence protein, the control group (AdRFP). (C and D) Immunofluorescence shows that IGF1R expression is decreased in mouse or human melanoma cells that are treated by AdRmIGF1R-OK (C) or AdRhIGF1R-OK (D), respectively. Each assay was done in biological triplicate. **$p < 0.01$.

AdRmIGF1R-OK and AdRhIGF1R-OK Induce Apoptosis but Inhibit Proliferation of Mouse and Human Melanoma Cells

To test how adenovirus-mediated suppression of IGF1R expression inhibits tumor growth, we first examined cell apoptosis after B16F10 and A375 cells were infected with AdRmIGF1R-OK and AdRhIGF1R-OK, respectively. Flow cytometry results revealed that the proportion of Annexin V and phosphatidylinositol (PI)-positive late apoptotic cells was significantly increased in the AdRmIGF1R-OK- and AdRhIGF1R-OK-treated B16F10 cells (10.4%) and A375 cells (38.4%), respectively, compared to the control groups that were treated with AdRFP (6.84% and 7.08%, $p < 0.0001$) (Figures 5A and 5B). Caspase-3 is a key effector in the apoptosis protease cascade.[21,22] Immunofluorescence staining showed that the expression of cleaved Caspase-3 was increased in B16F10 and A375 cells that were treated with AdRIGF1R-OK compared to those that were treated with AdRFP (Figures 6C and 6D). We also checked cell apoptosis by Hoechst 33258 staining (Figure 6E). Quantitative analysis revealed that the percentage of apoptotic cells was significantly increased in AdRIGF1R-OK-treated B16F10 and A375 cells ($p < 0.0001$) (Figure 6F) 7 days after the adenovirus infection compared to the control group. Altogether, these results suggest that inhibition of IGF1R expression promotes apoptosis of B16F10 and A375 cells.

To examine whether the smaller tumor formation in AdRIGF1R-OK (m/h)-treated groups is due to cell migration, we performed the cell scratch assay *in vitro*. Phase-contrast microscopy images and statistical analysis revealed that cells migrated and closed the wound more quickly in the AdRIGF1R-OK (m/h)-treated groups than in the control groups (Figures 5A–5D). We also tested the invasion ability of the cells that were treated with AdRIGF1R-OK (m/h) using a transwell assay. Crystal violet staining showed that the number of invaded cells was significantly decreased in the AdRIGF1R-OK (m/h)-treated groups compared to the control groups (Figures 5E and 5F). These results suggest that smaller tumor formation is not due to migration or invasion of the AdRIGF1R-OK (m/h)-treated cells to other locations in nude mice.

We also tested the melanoma cell viability after AdRIGF1R-OK treatment. Luciferase reporter assay revealed the luciferase activity was significantly decreased in the AdRIGF1R-OK (m/h)-treated group compared to the control groups (Figure 7A), indicating that the cell numbers were significantly decreased in the AdRIGF1R-OK (m/h)-treated group. Crystal violet staining showed that cell proliferation was significantly inhibited in the AdRIGF1R-OK (m/h)-treated group compared to the control

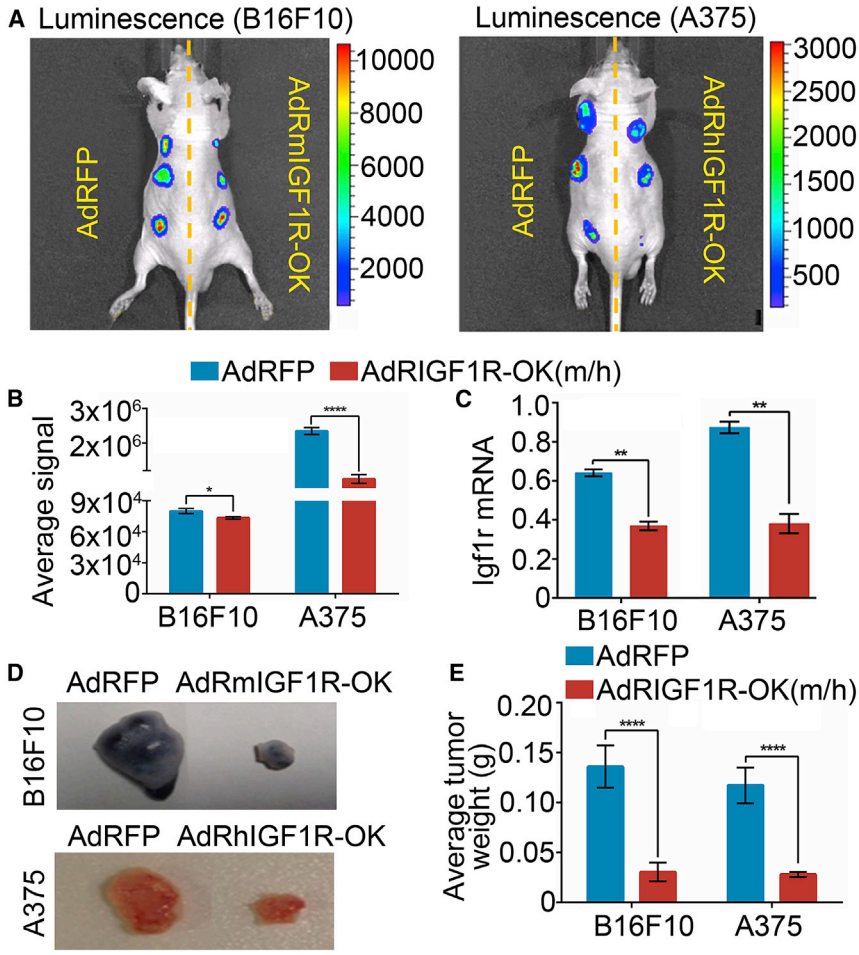

Figure 4. Inhibition of IGF1R by AdRIGF1R-OK (m/h) Effectively Inhibits Tumor Growth *In Vivo*

(A) Xenogen bioluminescence imaging of xenograft tumor growth. The animals were imaged at 3, 7, 10, and 14 days after cell injection. Representative images at day 14 are shown. (B) Average signals for each group at different time points were calculated using the Xenogen Living Image analysis software. (C) qRT-PCR results show that the mRNA level of IGF1R is significantly decreased in both mouse and human melanoma cells after AdRIGF1R-OK (m/h) treatment compared to controls that are infected with AdGFP. (D) Representative images show tumor formation 14 days after the infected melanoma cells were transplanted to the nude mice. (E) Average tumor weight for each group. Each experiment was done in biological triplicate. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

groups (no adenovirus-added group or AdRFP-treated group) (Figures 7B and 7C). We also performed cell-cycle analysis by using flow cytometry of AdRmIGF1R-OK-treated B16F10 cells and AdRhIGF1R-OK-treated A375 cells, respectively. The number of cells arrested in the G1 phase was significantly increased in AdRIGF1R-OK-treated cells, whereas the number of cells in the S/M phase was significantly decreased in AdRIGF1R-OK-treated melanoma cells compared to controls that were treated by AdRFP (Figures 7D and 7E). These results suggest that AdRIGF1R-OK inhibits melanoma cell proliferation by arresting cell-cycle progression.

DISCUSSION

Though melanoma is not the most common type of skin cancer, it has the highest mortality because it often spreads and has a low responsiveness to traditional radiotherapy and chemotherapy. Studies have shown that inhibition of IGF1R maybe one of the therapeutic strategies for treating melanoma. Here, we designed a novel RNAi system, named the OK system, by which multiple siRNA inserts can be ligated to a linearized adenoviral vector in one step through Gibson Assembly. We constructed two adenoviral vectors that contain three siRNA sequences targeting human IGF1R (AdRhIGF1R-OK) or mouse IGF1R (AdRmIGF1R-OK), respectively. We then generated adenoviruses that were applied to the human or mouse melanoma cells. The results demonstrated that inhibition of IGF1R by the OK system successfully blocked melanoma cell proliferation and migration and promoted cell apoptosis *in vitro*. By transplanting the human or mouse melanoma cells infected with mouse or human AdRIGF1R-OK, respectively, into nude mice, we showed that the xenografts formed smaller tumors compared to the controls. These studies indicate that our newly designed OK system is a simple and useful tool to knock down gene expression.

RNAi is one of the most common means of gene therapy. It is an indispensable tool for loss-of-function studies due to its stable and reversible ability to silence gene expression. Efforts have been taken to identify effective RNAi fragments using conventional and machine learning-based approaches. Such studies have advanced the technique used in gene knockout study, but it usually takes time to construct a mixture of functional and non-functional sequences into different plasmid vectors that then require time-consuming validation. Consequently, single-gene studies still apply on screening a pool of siRNA candidates, with each siRNA introduced into the cells to examine the effect on gene silencing. Here, we designed two new adenoviral vectors, pAdTrace-OK and pAdTrack-OK, based on AdEasy adenovirus vectors, that allow us to perform gene interference by visualizing two types of fluorescence.

Adenovirus has long been a popular viral vector for gene therapy due to its ability to affect both replicating and non-replicating cells with high efficiency. We previously succeeded in designing a retroviral interference expression system with similar strategies.[20] Using our

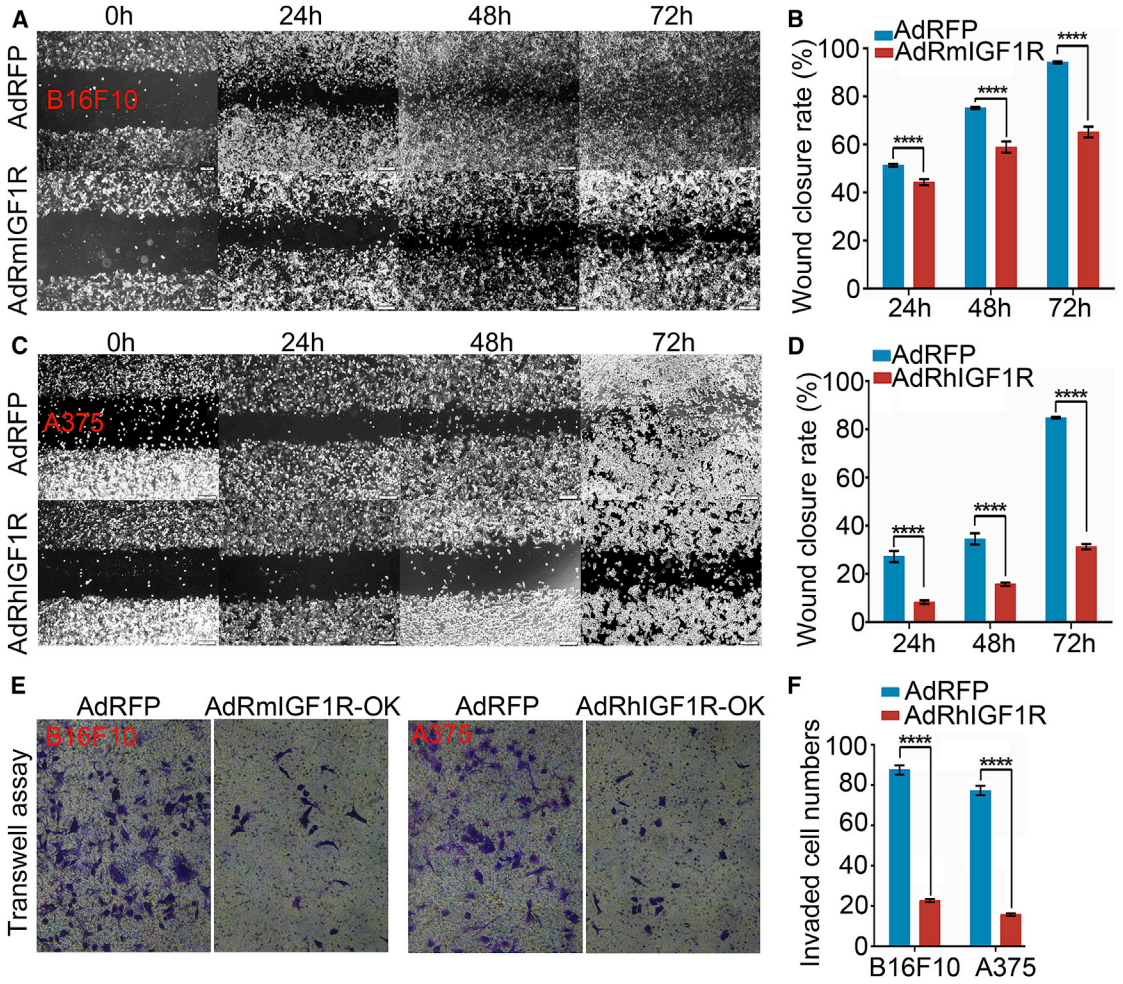

Figure 5. Inhibition of IGF1R in Melanoma Cells Inhibits Cell Migration

(A and B) Scratch assay (A) and statistical analysis (B) show that the wound closure ability of the melanoma cells is significantly decreased in the AdRmIGF1R-OK-treated groups compared to the control group. (C and D) Scratch assay (C) and statistical analysis (D) show that the wound closure ability of the melanoma cells is significantly decreased in the AdRhIGF1R-OK-treated group compared to the control group. The wounding gaps were recorded at 0, 24, 48, and 72 h after infection with AdRIGF1R-OK (m/h). Each assay was done in biological triplicate. (E and F) Crystal violet staining (E) and statistical analysis (F) showed that the number of the invaded cells was significantly decreased in the AdRIGF1R-OK (m/h)-treated groups compared to the control groups. ****$p < 0.0001$.

OK system, which we established in the present study, we can design multiple siRNA sequences for one gene and clone them into one vector in one step. This newly designed method has at least three advantages compared to other gene editing systems such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or CRISPR. First, it saves time, effort, and resources when constructing the plasmid, because it can be generated in one step based on Gibson Assembly. Second, it allows us to effectively silence one or multiple genes at the transcription level using a single plasmid construct. Third, it improves the possibility and efficiency of silencing a gene, because one plasmid vector contains multiple siRNA sequences. In addition, adenovirus-mediated expression of the OK system can infect cells efficiently without integrating to the host genome and with better biosafety than other gene silencing systems for clinical applications. Moreover, with the high infectivity and transient expression of the adenovirus to the cells, one can quickly check the genotype or phenotype without needing to establish an immortalized cell line for basic research.

Our newly designed siRNA-based OK system has a strong gene silencing effect, which was demonstrated by its strong effect on silencing IGF1R expression in melanoma cells. Our result showed that both AdRmIGF1R-OK and AdRhIGF1R-OK constructs have strong gene silencing ability when they are infected into mouse and human melanoma cells, respectively. Studies have shown that inhibition of IGF1R can be one of the strategies for gene therapy for melanoma.[23] Consistent with these studies, our study confirmed that knockdown of IGF1R using our newly designed OK system

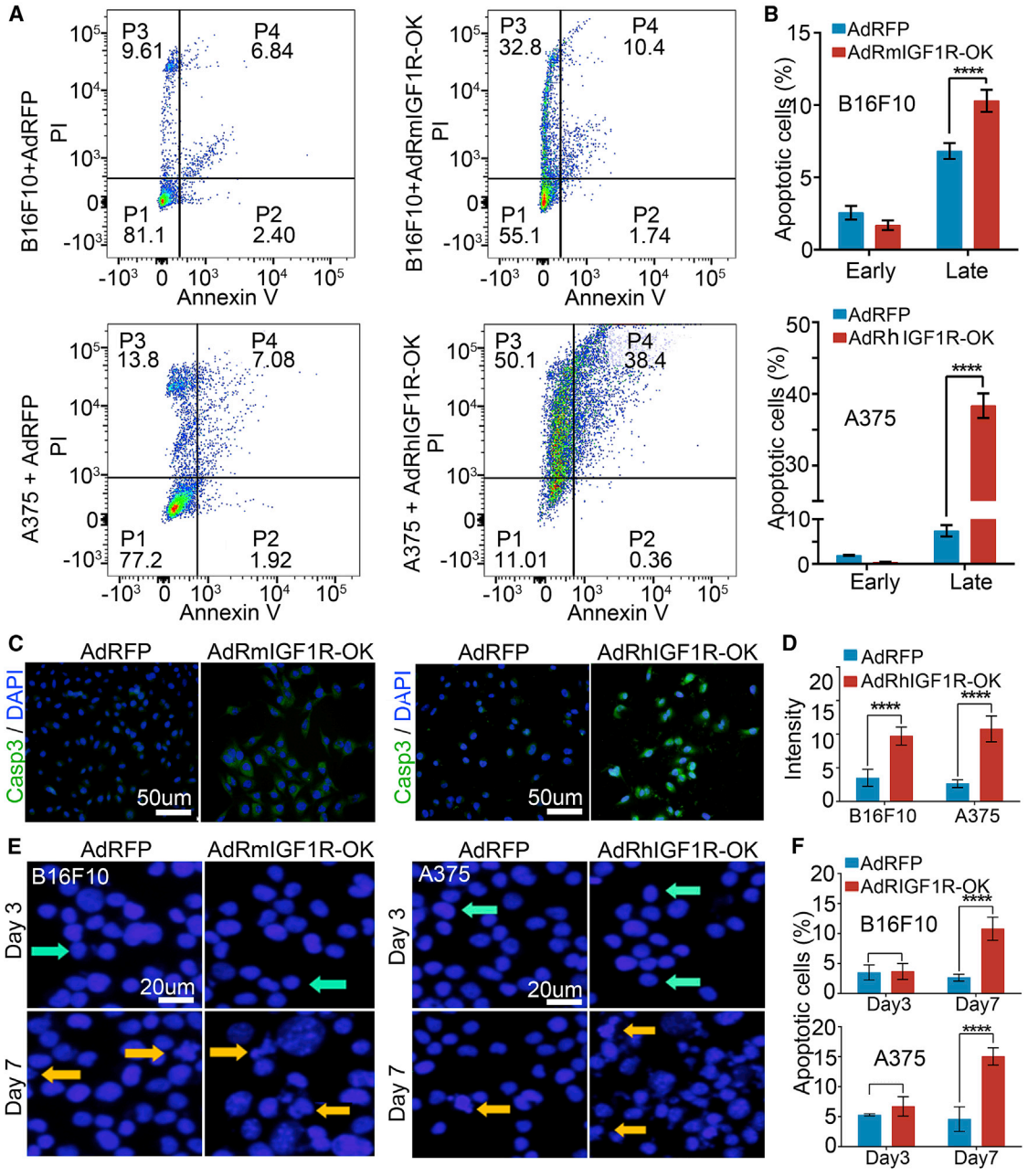

Figure 6. Inhibition of IGF1R Induces Apoptosis of Mouse and Human Melanoma Cell

(A and B) Annexin-V apoptosis assay (A) and statistical analysis (B) reveals that there are significantly more late apoptotic cells in the AdRmIGF1R-OK- and AdRhIGF1R-OK-treated groups compared to the control groups. Each assay was done in triplicate. (C and D) Immunofluorescence staining (C) and statistical analysis (D) showed that cleaved caspase-3 was increased in its expression in the AdRmIGF1R-OK- and AdRhIGF1R-OK-treated groups compared to the control groups. (E and F) Hoechst 33258 staining (E) and statistical analysis (F) revealed that there are significantly more apoptotic cells in AdRmIGF1R-OK- and AdRhIGF1R-OK-treated groups compared to the control groups. Green arrows, live cells; yellow arrows, apoptotic cells. Each assay was done in biological triplicate. ****$p < 0.0001$.

suppresses melanoma growth both *in vivo* and *in vitro*. In addition, we showed that knockdown of IGF1R in melanoma cells results in decreased cell proliferation but increased melanoma cell apoptosis. Previous study showed enhanced cell proliferation during early differentiation of mesenchymal stem cells to neural progenitor-like cells after IGF1 overexpression.[24] IGF also acts as a key regulator in inhibiting cell apoptosis by controlling Bcl2 family proteins, caspases, and signaling of death-inducing receptors.[25] It promotes resistance to

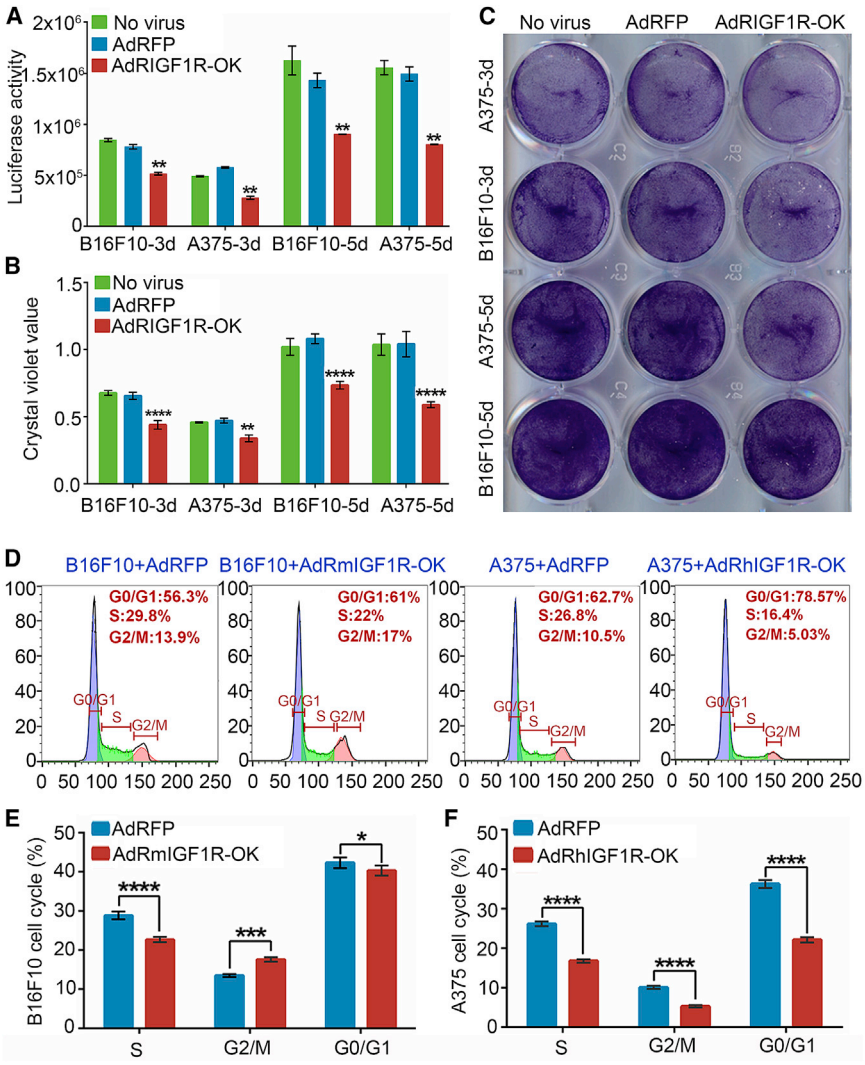

Figure 7. Inhibition of IGF1R Inhibits Proliferation of Mouse and Human Melanoma Cells

(A) Firefly luciferase assay shows that there is significantly less luciferase activity in melanoma cells infected with AdRmIGF1R-OK and AdRhIGF1R-OK for 3 and 5 days compared to those in the control groups. B16F10 and A375 cells infected with AdRFP were positive controls. (B and C) Crystal violet staining (B) and statistical analysis (C) reveal that there are fewer live cells in melanoma cells infected with AdRmIGF1R-OK and AdRhIGF1R-OK for 3 and 5 days compared to those in the control groups. Absorbance, 570 nm. Each assay was done in triplicate. (D–F) Cell-cycle analysis (D) shows that there are fewer cells are at the S phase of the cell cycle in AdRmIGF1R-OK- (E) and AdRhIGF1R-OK-infected (F) groups 72 h after treatment compared to control groups. Each assay was done in triplicate. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

apoptosis in melanoma cells.[26] The present study confirmed that inhibition of IGF1R using the OK system inhibits cell proliferation but promotes cell apoptosis. Although our study did not explore the downstream event of IGF1R during melanoma cell proliferation or apoptosis, the strong suppression effect of IGF1R expression by OK system-mediated gene knockdown provides new hope for future clinical application.

Pool-based siRNA screens require validation of the exact siRNA sequence that has the highest knockdown efficiency using one-by-one selection assays. Although our OK system containing multiple siRNA sequences has high efficiency in silencing gene expression, further experiment may be required to evaluate the efficiency of each siRNA sequence.

In summary, we designed a simplified and useful gene knockdown system that allows cloning of multiple siRNA sequences into one adenoviral vector and displays a strong gene silencing effect when the generated adenoviruses are introduced into mouse and human melanoma cells. This study not only establishes a novel gene silencing system but also provides a better way to target the IGF signaling pathway to treat melanoma. Thus, the OK system can be a valuable tool for gene silencing in research and an effective strategy for clinical treatment of melanoma or other diseases in clinics.

MATERIALS AND METHODS

Cell Culture and Chemicals

Human melanoma cell lines B16F10 and A375 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in complete DMEM containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 100 units of penicillin, and 100 μg of streptomycin at 37°C in 5% CO_2 as previously described.[20] Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). All procedures were in strict accordance with the Institutional Review Board of the Third Military Medical University.

Construction of the Adenovirus Shuttle Vectors pAdTrace-OK and pAdTrack-OK and the PCR Template Vector pB2B for Gibson Assembly Reactions

As depicted in Figure 1B, the adenovirus shuttle vectors pAdTrace-OK and pAdTrack-OK contain the opposing U6 and H1 promoters to drive siRNA duplex expression. The SwaI site is designed for linearization of the vector for Gibson Assembly. These vectors also confer blasticidin S resistance for generating stable mammalian cell lines. The pB2B vector was constructed based on our previously reported pMOLuc vector.[27] Briefly, the high-fidelity PCR-amplified U6 and H1 promoter fragments were subcloned into the EcoRI/HindIII sites

of pMOLuc in a back-to-back orientation and ligated at the MluI site (Figure 1C). Both U6 and H1 promoters contain a string of AAAAA preceding their transcription start sites, serving as the transcription termination signal for the reverse strand. The full-length sequences and maps of the vectors are shown in the Supplemental Information.

Gibson Assembly Reaction for Generating AdRmIGF1R-OK and AdRhIGF1R-OK Vectors

The Gibson Assembly reactions were performed using the Gibson Assembly Master Mix (New England Biolabs, Ipswich, MA) following the manufacturer's instructions. The overlapping inserts were prepared by PCR amplifications using the Phusion High-Fidelity PCR kit (New England Biolabs). For each Gibson Assembly reaction, 10 μL of each insert (20 ng/μL), 10 μL of linearized vector (5 ng/μL), and 30 μL of Gibson Assembly Master Mix were incubated at 50°C for 45 min. Three mouse or human IGF1R siRNAs were cloned into the shuttle vector, respectively. The production of the Gibson Assembly reaction was digested with SwaI restriction enzyme for 10 min and then was transformed into DH10B cells. After DNA sequencing, the successfully constructed vectors were transfected into HEK293 cells to generate the adenovirus. The infected cells were selected in blasticidin S (4 mg/mL) for 5–7 days. The stable pools of cells were kept in liquid nitrogen (LN2) for long-term storage. The resulting adenoviruses were designated as AdRmIGF1R-OK and AdRhIGF1R-OK, both of which also express GFP or RFP. Analogous AdRFP was used as the control.

RNA Isolation and qPCR

Total RNA was isolated using TRIzol reagents (Invitrogen). cDNA templates were generated by reverse transcription reactions with hexamer and M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA). PCR primers were designed using the Primer3 program to amplify human IGF1R (product size, 185 bp) and mouse IGF1R (product size, 181 bp). Human IGF1R was 5′-ATGACATTCCTGGG CCAGTG-3′ and 5′-TAGCTTGGCCCCTCCATACT-3′, and mouse IGF1R was 5′-GTGTGGATCGCGATTTCTGC-3′ and 5′-TCTTCA TCGCCGCAGACTTT-3′. For qPCR analysis, SYBR Green-based qPCR analysis was carried out using the thermocycler Opticon II DNA Engine (Bio-Rad, CA) with a standard pUC19 plasmid. The qPCR reactions were done in triplicate.

Immunofluorescence Staining

Immunofluorescence staining was performed as previously described.[28,29] Briefly, after infection with AdRmIGF1R-OK, AdRhIGF1R-OK, or AdRFP for 72 hr, cells were fixed with methanol for 10 min, permeabilized with 1% nonyl phenoxypolyethoxylethanol-40 (NP-40) for 5 min, and blocked with 10% BSA. Then, the cells were incubated with antibody against IGF1R (sc-462, Santa Cruz Biotechnology) or cleaved caspase-3 (9661, Cell Signaling Technology) at 4°C for overnight. After washing, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Santa Cruz Biotechnology). Images were taken under a fluorescence microscope (Nikon Ti-S). Stains without primary antibodies, or with control immunoglobulin G (IgG), were used as negative controls.

Crystal Violet Cell Viability Assay

B16F10 and A375 cells were infected with AdRmIGF1R-OK, AdRhIGF1R-OK, or AdRFP (control). Cell cultures without infection of adenovirus were used as negative controls. At days 3 and 5 after infection, cells were washed with PBS and stained with 0.5% crystal violet and formalin solution at room temperature for 30 min. The cells were washed with tap water and air dried before imaging. For quantification, the cells were incubated with 100% acetic acid at room temperature for 20 min with shaking. The absorbance was set for 570–590 nm.

Cell-Cycle Analysis

B16F10 and A375 cells were seeded in 6-well plates and infected with AdRmIGF1R-OK, AdRhIGF1R-OK, or AdRFP. Three days after infection, cells were collected, fixed, and stained with propidium iodide for 5 min. Then, the cells were subjected to flow cytometry analysis using the BD FACSCalibur-HTS. The flow cytometry data were analyzed with FlowJo v.10.0 software. Each assay was done in triplicate.

Annexin V-FITC Flow Cytometry Assay

B16F10 and A375 cells were seeded in 6-well plates and infected with AdRmIGF1R-OK, AdRhIGF1R-OK, or AdRFP. Three days after infection, cells were dissociated with trypsinization, washed with PBS, and resuspended in Annexin V Binding Buffer at a density of 10^6 cells/mL. Then, the cells were stained with Annexin V-FITC (BD Pharmingen, San Jose, CA) for half an hour, followed by counterstaining with propidium iodide for 15 min at room temperature. After wash, the cells were subjected to flow cytometry analysis using the BD FACSCalibur-HTS. Data were analyzed using FlowJo v.10.0 software. Each assay was done in triplicate.

Hoechst 33258 Staining

B16F10 and A375 cells were infected with AdRmIGF1R-OK, AdRhIGF1R-OK, or AdRFP. Three days after infection, cells were fixed and stained with 1× Magic Solution (10× stock: 0.5% NP-40, 3.4% formaldehyde, and 10 μg/mL Hoechst 33258 in PBS). Apoptotic cells were examined under a fluorescence microscope. Each assay was done in triplicate. The average numbers of apoptotic cells were calculated by counting apparent apoptotic cells in at least ten random fields at 100× magnification for each assay.

Cell Migration Assay

Cell migration assay was performed as previously described.[30] B16F10 and A375 cells were infected with AdRmIGF1R-OK, AdRhIGF1R-OK, or AdRFP and replated in 6-well cell culture plates. When grown to 90% confluency, the cells were scratched with a sterile micro-pipette tip. The images were taken under bright-field microscopy at 0, 24, and 48 hr after cell scratch. Each assay was done in triplicate.

Luciferase Reporter Assay

Firefly luciferase reporter assay was performed as previously described.[31] B16F10 and A375 cells were infected with adenovirus-mediated firefly luminescence (AdFLuc). Immortalized cell lines

B16F10-Luc and A375-Luc were obtained by blasticidin S (4 mg/mL) screening. B16F10-Luc and A375-Luc cells were infected with AdRmIGF1R-OK, AdRhIGF1R-OK, or AdRFP. At days 3 and 5 after infection, cells were lysed and collected for examining the firefly luciferase activity assay using a Luciferase Assay Kit (Promega, Madison, WI). Each assay was performed in triplicate.

Xenograft of Human Melanoma Cells

The use and care of animals were approved by the Institutional Animal Care and Use Committee at the Third Military Medical University. All experimental procedures were carried out in accordance with the approved guidelines. B16F10-Luc and A375-Luc cells infected with AdRmIGF1R-OK, AdRhIGF1R-OK, or AdRFP were collected and resuspended at 10^7 cells/mL. 100 μL cells were subcutaneously injected into the dorsal back skin of athymic nude mice (4-week-old, male, 10^6 cells per injection). Tumor growth was monitored by whole-body bioluminescence imaging using the Xenogen IVIS 200 Imaging System at days 3, 7, 10, and 14 after injection. The mice were sacrificed at 14 days, and subcutaneous tumor masses were harvested for examination.

Statistical Analysis

The quantitative assays were performed in triplicate and/or repeated three times. Data were expressed as mean ± SD. Statistical significances were determined using Student's t test. A value of $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods and one figure and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.08.004.

AUTHOR CONTRIBUTIONS

F.D., H.X., and Z.Z. conceived and designed the experiments; H.X., F.D., J.L., A.W., H.Z., and X.L. performed the experiments; H.X. and F.D. analyzed the data; H.X. and F.D. contributed reagents, materials, and analysis tools; and F.D., H.X., and M.L. wrote the paper.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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