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Original Article

Construction and evaluation of tissue distribution of oncolytic herpes simplex virus type 2 expressing firefly gene

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Abstract: Objective: Oncolytic virus is a new and safe therapeutic strategy for cancer treatment. In our previous study, we constructed a novel oncolytic herpes simplex virus type 2 (oHSV2). oHSV2 has now entered into clinical trials for the treatment of melanoma and other solid tumors (NCT03866525). Intratumoral injection is the administration route of oncolytic virus. There is no report on intravenous administration of oHSV2. Methods: To investigate possible intravenous administration of oHSV2, we constructed a novel oncolytic virus expressing firefly gene termed oHSV2-Fluc and evaluated the tissue distribution of oHSV2-Fluc in vivo. Results: oHSV2-Fluc could be detected in intraperitoneal and subcutaneous xenografts in mice. The intraperitoneal and subcutaneous xenograft mice with oHSV2-Fluc injected intravenously or subcutaneously showed wide biodistribution of oHSV2-Fluc. We further confirmed that oHSV2-Fluc injected intravenously or intratumorally could target the tumor in vivo. Conclusion: The intravenous injection of oHSV2 is a possible administration route in a preclinical animal model.

Keywords: Oncolytic herpes simplex virus type 2, tissue distribution

Introduction

Cancer endangers human health all over the world [1]. At present, the common methods of cancer treatment are surgery, chemotherapy, and radiation therapy [2-4]. With the rapid development of biological technology, oncolytic virus has shown promise in the field of tumor treatment [5]. Oncolytic virus can induce local and systemic anti-tumor immune responses [6, 7]. Currently, some oncolytic viruses have been studied, such as herpes simplex virus [8], adenovirus [9], and reovirus [10]. Herpes simplex virus (HSV) with a linear double-stranded DNA is a member of the α subfamily of the herpes virus family. HSV can be divided into HSV-1 and HSV-2 [11]. In our previous studies, oncolytic herpes simplex virus type 2 (oHSV2) expressing human granulocyte macrophage colony stimulating factor, has shown therapeutic efficacy in many tumor models [12], and now it has entered phase II clinical trials (NCT03866525).

Intratumoral administration of oncolytic virus is a common administration route [13]. In our previous study, we evaluated the anti-tumor effect and biodistribution of oHSV2 following intratumoral administration [14, 15]. oHSV2 is a safe and effective therapeutic agent when delivered directly to tumors. Intravenous administration of oncolytic virus has been studied. Andrew et al., reported a phase I trial of oncolytic virus PV701 in patients with advanced solid cancer [16]. Myers et al., reported a preclinical pharmacology and toxicology study of intravenous oncolytic virus MV-NIS [17]. Intravenous administration of HSV-1 has been reported [18]. Wong et al., reported that intravenous delivery of an oncolytic herpes virus expressing IL-12 may achieve effective infection and oncolysis at dis-

tant tumor sites [19]. There has not been a report about intravenous administration of HSV-2. In this study, based on oHSV2, we constructed a new oncolytic herpes simplex virus type 2 expressing firefly gene (oHSV2-Fluc). The biodistribution of oHSV2-Fluc was evaluated in a graft/xenograft mouse model. The results indicated that oHSV2-Fluc could be detected in intraperitoneal and subcutaneous graft/xenograft mice tissues and tumor sites. We further confirmed that oHSV2-Fluc, injected intravenously or intratumorally could target the tumor.

Materials and methods

Ethics statement

All animal experiments were conducted under protocol approved by the Hubei Province, PR China, Biological Studies Animal Care and Use Committee (HBUT No. 2018010).

Cell lines

The Vero (African green monkey kidney), CT-26 (Murine colorectal cancer), MC38 (Murine Carcinoma-38), A375 (Human malignant melanoma cell line), Hep2 (Human Epithelioma-2) were purchased from the American Type Culture Collection (ATCC). Cells were cultured in DMEM medium with 10% fetal bovine serum, at 37°C and 5% CO_2 in a CO_2 incubator.

Mice and reagents

BALB/c mice, nude mice and C57, which were 6-7 weeks years old, were purchased from Wuhan Food and Drug Safety Evaluation Center, and raised in our laboratory animal laboratory.

Virus construction

oHSV2-Fluc was constructed using the following steps. First, the oHSV2-GFP DNA was extracted. The plasmid pHG52-34.5 expressing firefly luciferase was constructed. Second, the constructed plasmid pHG52-34.5 and oHSV2-GFP DNA were transferred into Vero cells, which involved homologous recombination. Then the new oncolytic virus (oHSV2-Fluc) with the gene expressing firefly luciferase can be identified by selection of phage spots twice, that do not express GFP green fluorescence. The primers used for sub-cloning hGM-CSF, GFP and firefly luciferase gene are as follows: hGM-CSF Forward primer: ATGTGGCTGCAGAGCCTG. hGM-CSF Reverse primer: CCCAATCCTCCCCCTTGCTG. GFP Forward primer: ATGGTGAGCAAGGGCGAG. GFP Reverse primer: CCCAATCCTCCCCCTTGCTG. Firefly luciferase Forward primer: ATGGAAGACGCCAAAAACATTAAGAA. Firefly luciferase Reverse primer: CCCAATCCTCCCCCTTGCTG.

Live animal bioluminescence imaging

Tumor cells were injected subcutaneously into the right flanks of female BALB/c nude mice or intraperitoneally into the abdominal cavities of female BALB/c nude mice to induce tumor growth. An IVIS Lumina Series III Optical *In Vivo* Imaging System (PerkinElmer, MA, USA) was used for fluorescence analysis.

mRNA extraction and real-time PCR

Total tissue RNA was extracted from nude mice using TRIzol reagent (Invitrogen, CA, USA). Following RNA extraction, genomic DNA was digested, then the RNA was reverse transcribed to cDNA using Roche reverse transcriptase, and cDNA amplification was performed with the Roche SYBR Green I real-time PCR assay. The primers used are as follows: Forward primer: CATTACCTACGCCGAGTA. Reverse primer: TTTGTATTCAGCCCATAGC.

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software (IBM, USA) for Windows. Differences were assessed using one-way ANOVA. Statistical significance was defined as $P<0.05$, and all values are expressed as the mean ± standard deviation of three independent experiments.

Results

Construction of oncolytic HSV2-Fluc

In our previous study, we constructed an oncolytic herpes simplex virus type 2 (oHSV2) with ICP34.5, ICP47 deletion, and hGM-CSF insertion in a HG52 strain. In this study, based on oHSV2, we constructed a novel oncolytic virus with firefly luciferase gene expression (oHSV2-Fluc). The construction of recombinant oncolytic viruse, oHSV2-Fluc, is depicted in (**Figure 1A**). The hGM-CSF (682 bp), GFP (986 bp), and

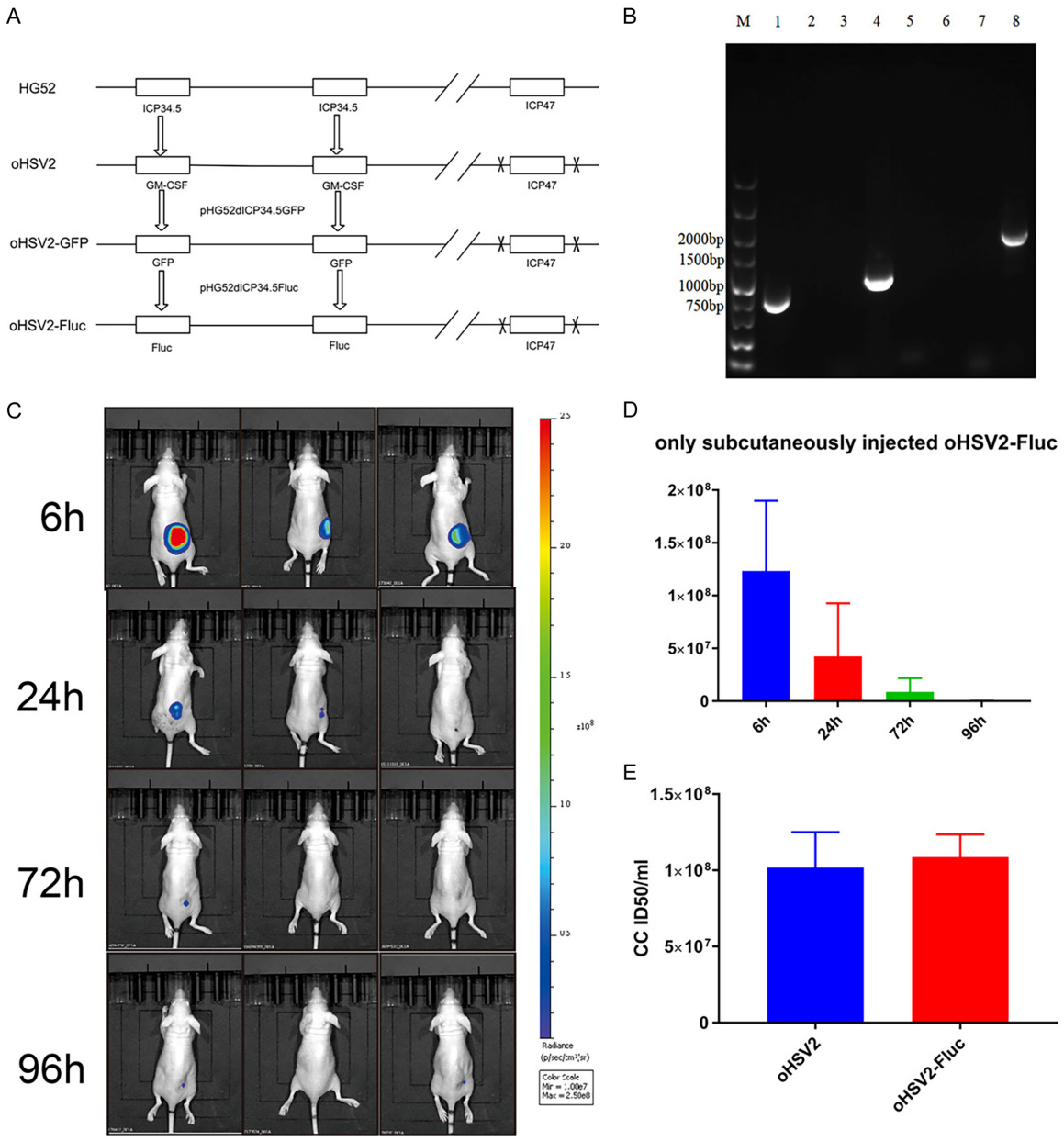


Figure 1. Construction of oncolytic HSV2-Fluc. A. Schematic diagrams of virus of HG52, oHSV2, oHSV2-GFP, and oHSV2-Fluc. B. PCR analysis of hGM-CSF (lanes 1-3), GFP (lanes 4-6) and Firefly luciferase gene (lanes 7, 8) in oHSV2, oHSV2-GFP, and oHSV2-Fluc respectively. M: DNA marker; Lane 1: oHSV2 as template, Lane 2, 5, 7: Negative control, Lane 3, 6, 8: oHSV2-Fluc as template, Lane 4: oHSV2-GFP as template. C. Fluorescence analysis of oHSV2-Fluc in BALB/c nude mice at 6 h, 24 h, 72 h, and 96 h after oHSV2-Fluc injection subcutaneously. D. oHSV2-Fluc by the quantitation of average fluorescence signal intensity. E. Virus titers of oHSV2-Fluc and oHSV2.

Firefly luciferase (1900 bp) genes could be detected in oHSV2, oHSV2-GFP, and oHSV2-Fluc respectively (**Figure 1B**). In order to evaluate whether the virus was correctly constructed, 100 ul of virus were injected subcutaneously into nude mice, and the signals (at 6 h, 24 h, 72 h and 96 h) were observed by IVIS Lumina Series III Optical *In Vivo* Imaging System. Results indicated that the fluorescence signal could be detected until 72 h after subcutaneous injection into nude mice (**Figure 1C**, **1D**). We further investigated the virus titer of oHSV2-Fluc and compared it with oHSV2. The results indicated that virus titers of oHSV2-Fluc and oHSV2 had no significant differences (**Figure 1E**).

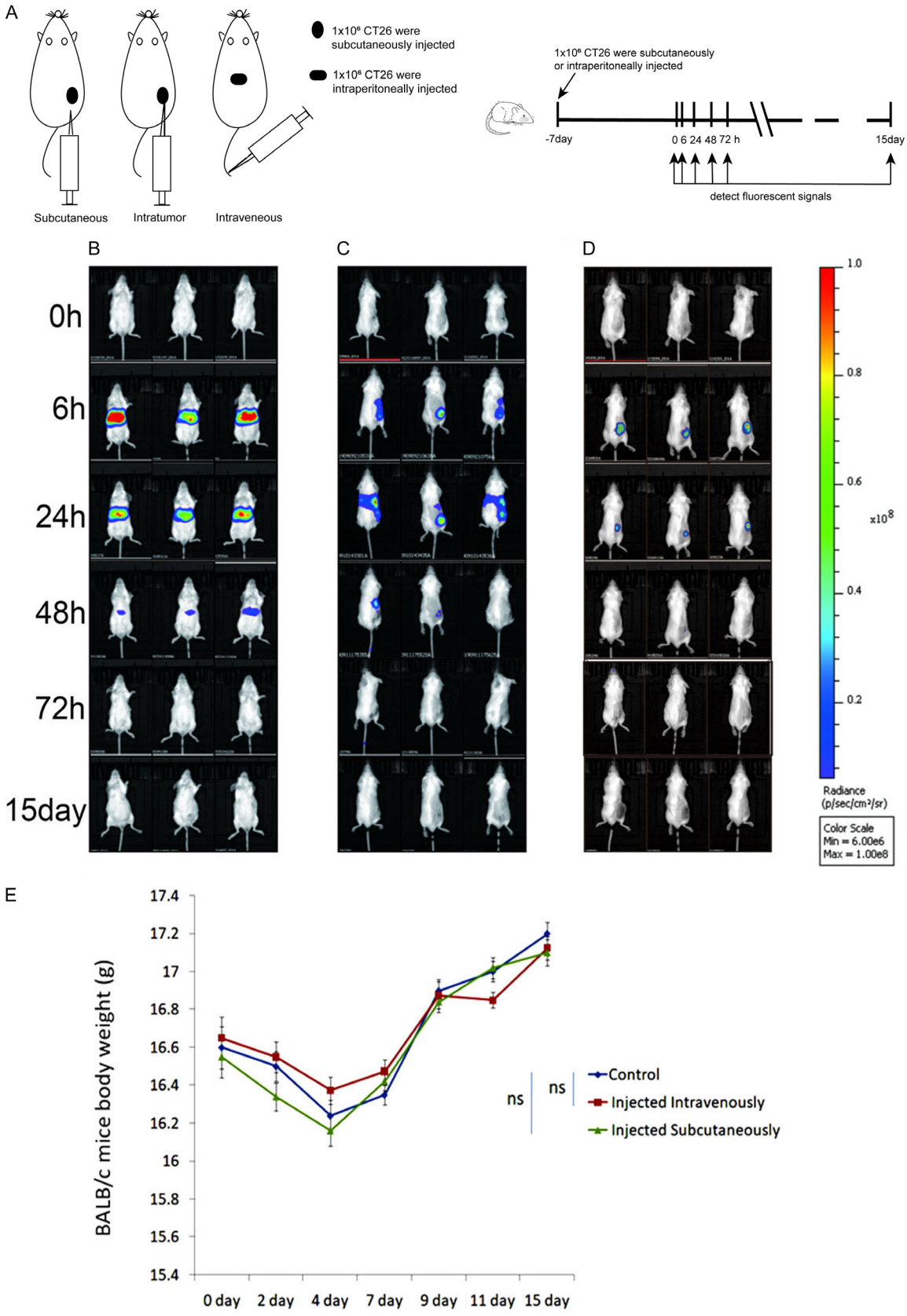


Figure 2. Evaluation of tissue distribution of oncolytic virus expression firefly luciferase gene in a BALB/c model. A. Schematic diagram of the animal experiment. B. CT-26 cells were injected intraperitoneally into BALB/c mice, then

oHSV2-Fluc was injected intravenously into the tail vein. C. CT-26 cells were injected subcutaneously into the right flanks of female BALB/c mice. oHSV2-Fluc was directly injected intratumorally. D. CT-26 cells were injected subcutaneously into the right flanks of female BALB/c mice and, oHSV2-Fluc was injected subcutaneously. E. Average body weights of BALB/c mice.

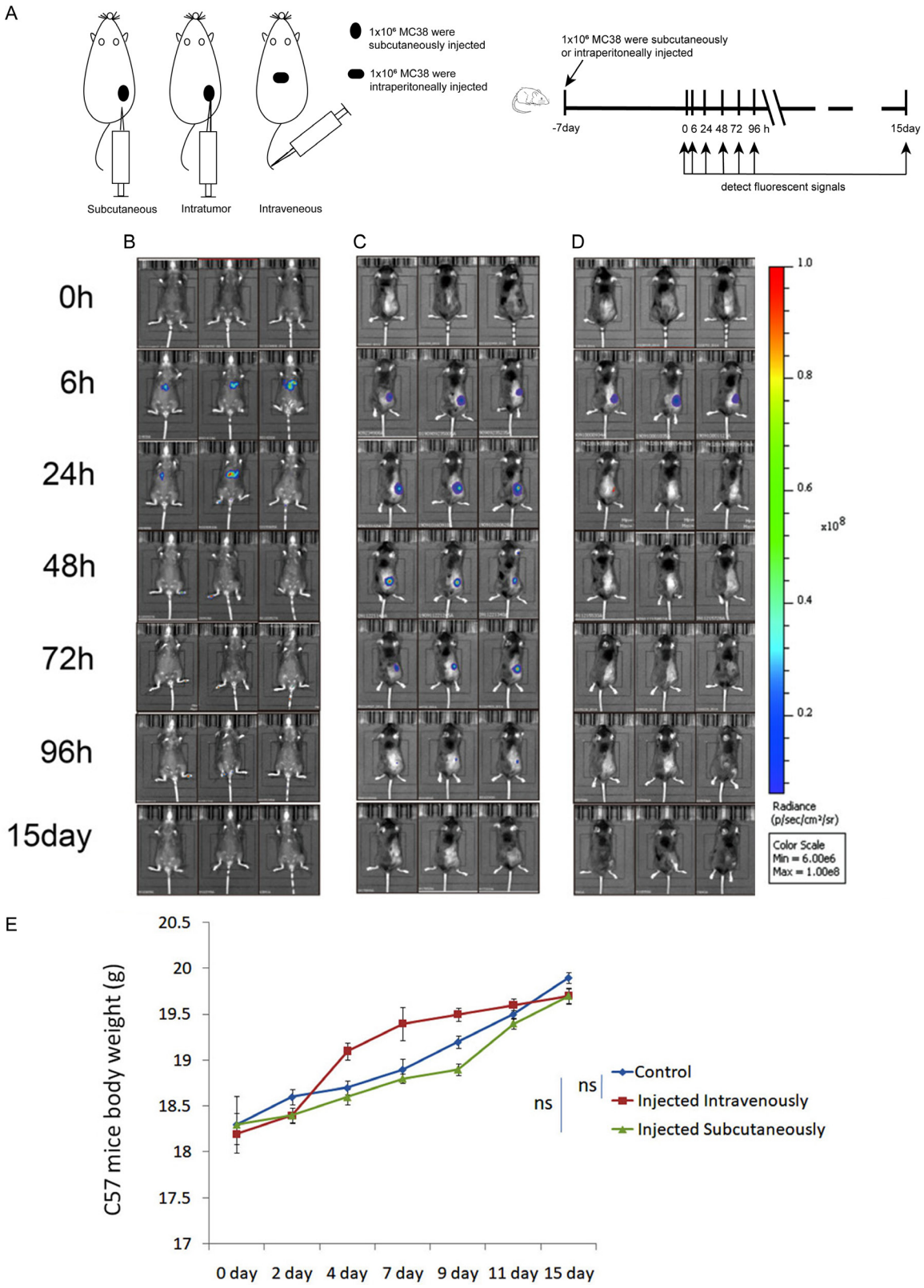


Figure 3. Evaluation of tissue distribution of oncolytic virus expression firefly luciferase gene in C57 model. A. Schematic diagram of the animal experiment. B. MC38 cells were injected intraperitoneally into C57 mice, then oHSV2-Fluc was injected intravenously into the tail vein. C. MC38 cells were injected subcutaneously into the right flanks of C57 mouse. oHSV2-Fluc was injected intratumorally. D. MC38 cells were injected subcutaneously into the right flanks of female C57 mouse, and oHSV2-Fluc were injected subcutaneously. E. Average body weights of C57 mice.

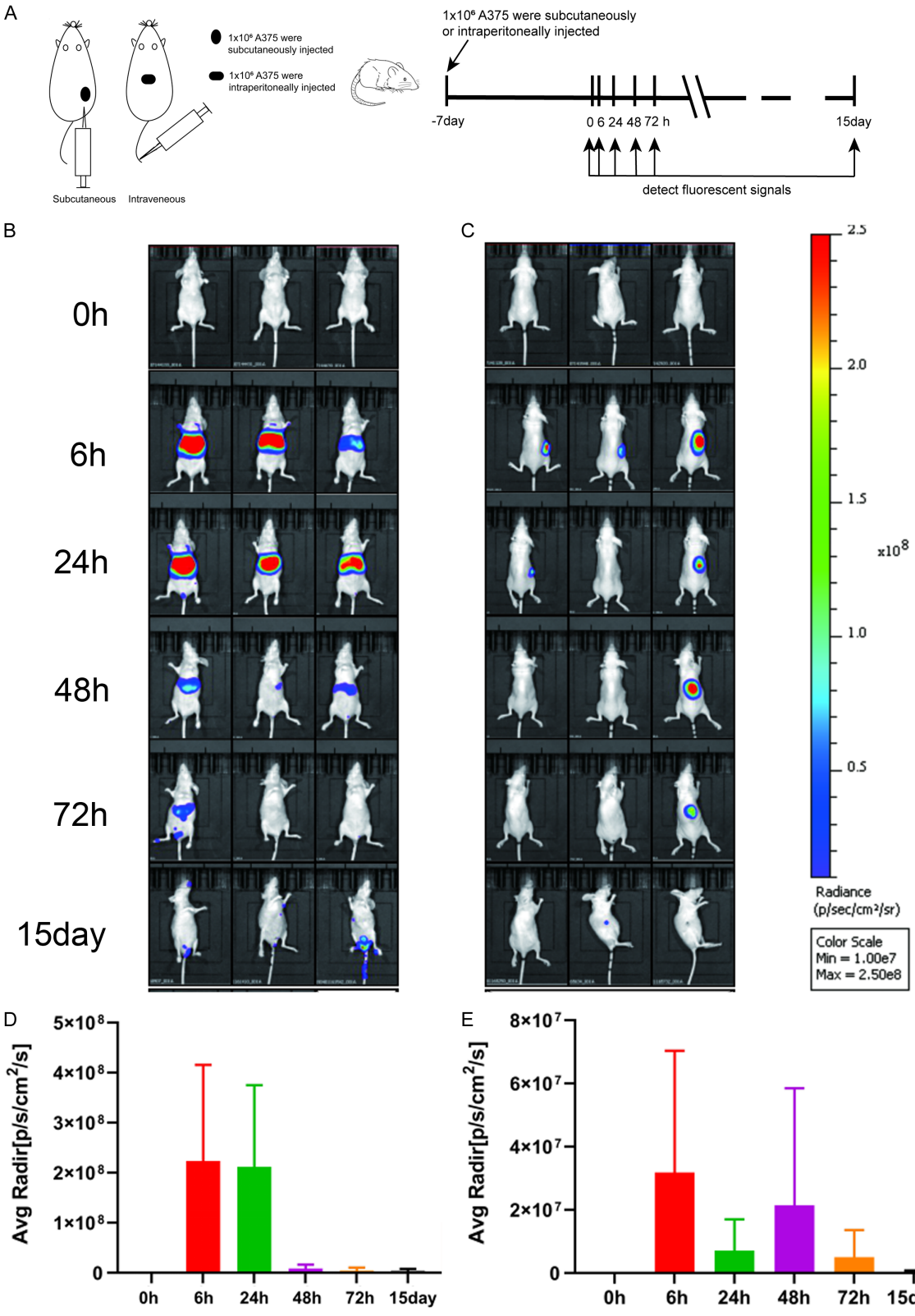
Evaluation of tissue distribution of oHSV2-Fluc in graft mouse model

In the in vivo evaluation of tissue distribution of oHSV2-Fluc, an intraperitoneal and a subcutaneous graft mouse model were used. In the intraperitoneal graft mouse model, CT-26 cells (**Figure 2A**) or MC38 (**Figure 3A**) were injected intraperitoneally into the abdominal cavities of female BALB/c or C57 mouse to induce tumors. Subsequently, oHSV2-Fluc was injected intravenously. The results indicated that oHSV2-Fluc could be detected in the abdominal cavity of BALB/c mice until 48 h after administration (**Figure 2B**) and in C57 mice until 24 h after administration (**Figure 3B**). In the subcutaneous graft mouse model, CT-26 or MC38 cells were injected subcutaneously into the right flanks of female BALB/c or C57 mice to induce tumors. Subsequently, oHSV2-Fluc was injected intratumorally or subcutaneously. In BALB/c mouse model, oHSV2-Fluc could be detected in tumor until 48 h after injection intratumorally (**Figure 2C**) and 24 h after injection subcutaneously (**Figure 2D**). In the C57 mouse model, oHSV2-Fluc could be detected in tumor until 96 h after injection intratumorally (**Figure 3C**) and 6 h after injection subcutaneously (**Figure 3D**).

In CT-26 bearing mouse, the tissues including heart, kidney, lung, spleen, liver, and tumor from subcutaneous graft mouse and intraperitoneal graft mouse at day 15 after administration were collected and SYBR Green I Real-time PCR was used to analyzed the biodistribution of oHSV2-Fluc. The results indicated that oHSV2-Fluc DNA could not be detected in tissues and tumor sites from subcutaneous graft mice and intraperitoneal graft mice. We further investigated the toxicity of oHSV2-Fluc in healthy BALB/c and C57 mice. The BALB/c mice and C57 mice were injected subcutaneously or intraneneously with oHSV2-Fluc. The body weight of BALB/c mice and C57 mice were investigated and the results indicated that there were no treatment-related effects on body weight for BALB/c or C57 mice (**Figures 2E** and **3E**).

Evaluation of tissue distribution of oHSV2-Fluc in xenograft mouse model

In the vivo evaluation of tissue distribution of oHSV2-Fluc, an intraperitoneal and a subcutaneous xenograft mouse model were used (**Figures 4A**, **5A**). In the intraperitoneal xenograft mouse model, A375 or Hep2 cells were injected intraperitoneally into the abdominal cavities of female BALB/c nude mice to induce tumors. Subsequently, oHSV2-Fluc was injected intravenously. In the A375 cells group, oHSV2-Fluc could be detected in the abdominal cavity until 72 h after administration. Interestingly, oHSV2 could be detected in the tail vein and in the abdominal cavity at 15 days after administration (**Figure 4B**, **4D**). In the Hep2 cells group, oHSV2-Fluc could be detected in the abdominal cavity until 48 h after administration. Interestingly, oHSV2-Fluc could be detected again in the abdominal cavity at 21 days after administration (**Figure 5B**, **5D**). In the subcutaneous xenograft mouse model, A375 or Hep2 cells were injected subcutaneously into the right flanks of female BALB/c nude mice to induce tumors. Subsequently, oHSV2-Fluc was injected subcutaneously. In the A375 cells group, oHSV2-Fluc could be detected in the tumor at 15 days after administration (**Figure 4C**, **4E**). In the Hep2 cell group, oHSV2-Fluc could be detected in the tumor until 24 h after administration. Interestingly, oHSV2-Fluc could be detected again at 21 days after administration (**Figure 5C**, **5E**). In A375 cell-bearing mice and Hep2 cell-bearing mice, the tissues including heart, kidney, lung, spleen, liver and tumor from subcutaneous xenograft mice and intraperitoneal xenograft mice were collected separately and SYBR Green I Real-time PCR was used to analyze the oHSV2-Fluc. Results indicated that oHSV2-Fluc DNA could be detected in tumors, heart, liver, spleen, lung and kidney (**Figures 4F**, **5F**). We further investigated the toxicity of oHSV2-Fluc in healthy BALB/c nude mice. The BALB/c nude mice were injected subcutaneously or intraveneously with oHSV2-Fluc. The body weight of BALB/c nude mice was investigated and the results indicated that there were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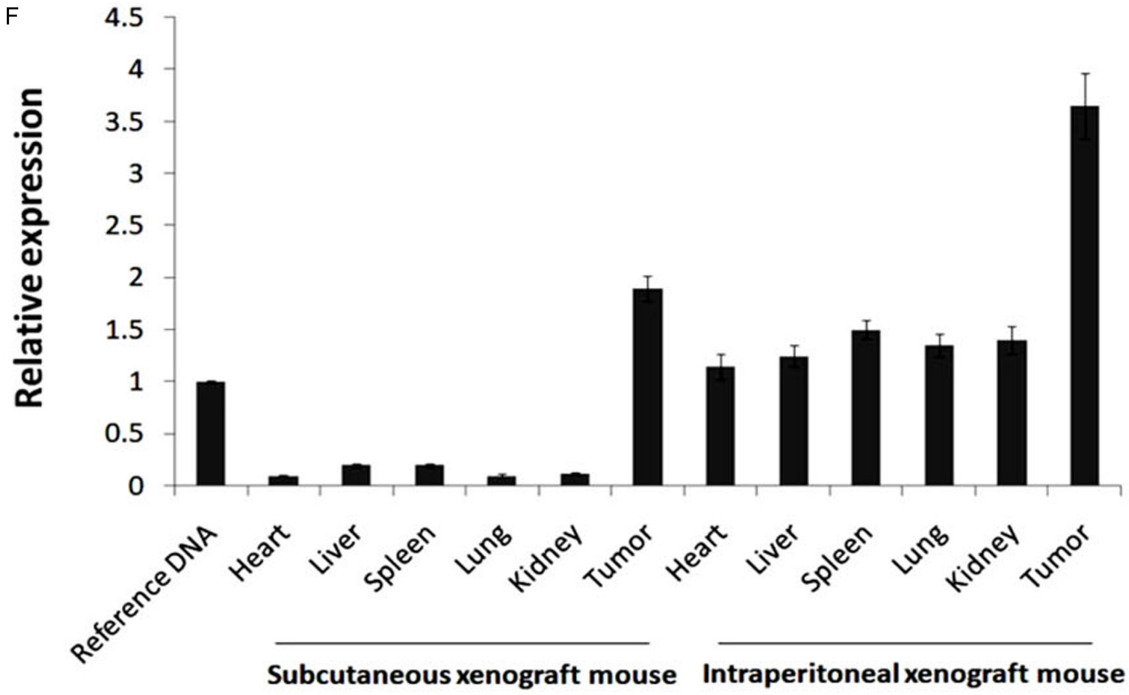


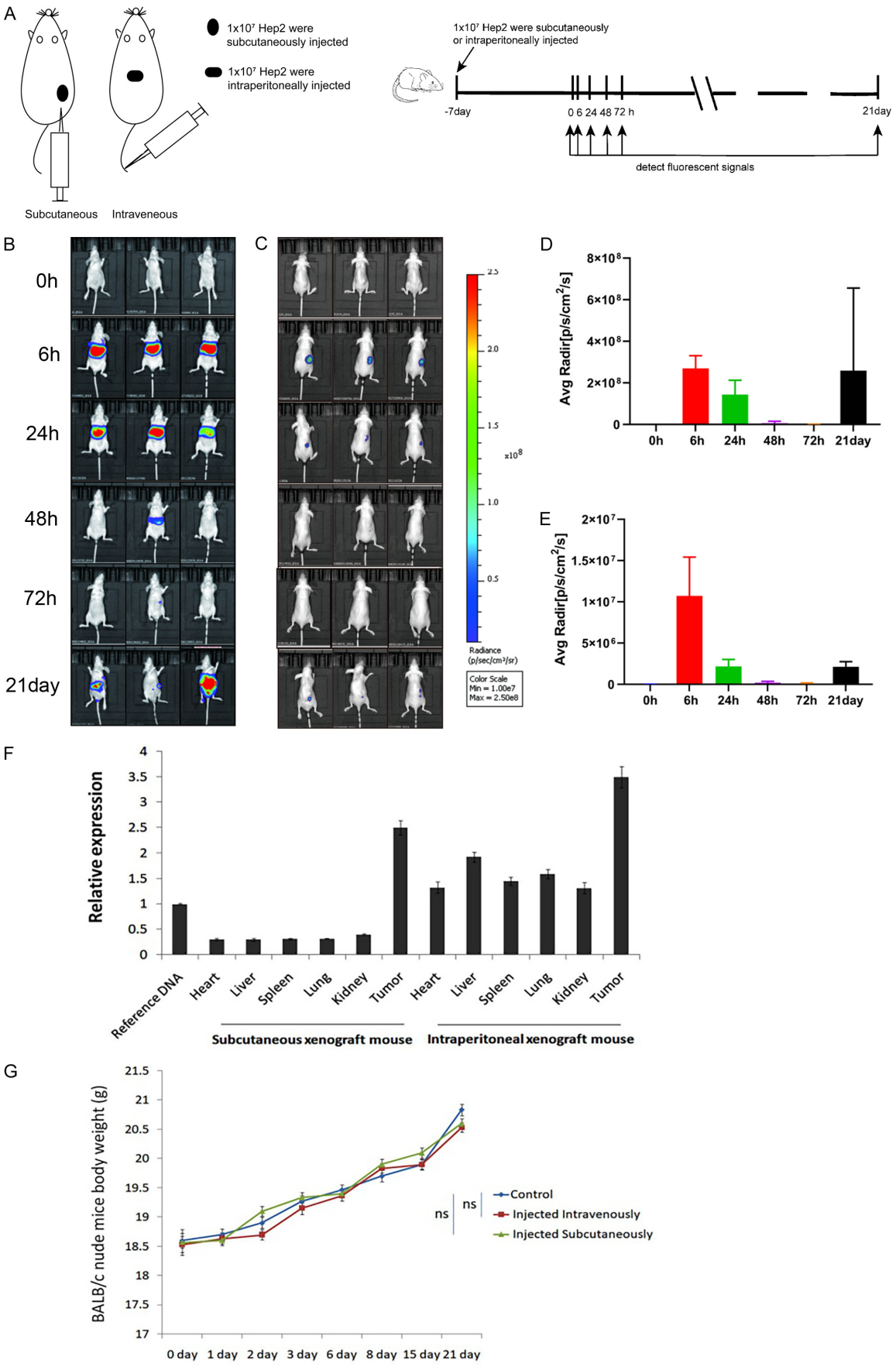
Figure 4. Evaluation of tissue distribution of oncolytic virus expression firefly luciferase gene in BALB/c nude mice in the A375 tumor model. A. Schematic diagram of the animal experiments. B. A375 cells were injected intraperitoneally into female BALB/c nude mice, then oHSV2-Fluc was injected intravenously into the tail vein. C. A375 cells were injected subcutaneously into the right flanks of female BALB/c nude mice, and oHSV2-Fluc was injected subcutaneously. D, E. Fluorescence signal quantitation diagram. F. Biodistribution of oHSV2-Fluc in subcutaneous xenograft mice and intraperitoneal xenograft mice by SYBR Green I Real-time PCR.

no treatment-related effects on body weight for BALB/c nude mice (**Figure 5G**). In Hep2 bearing mice, immunohistochemical stains were used to detect oHSV2-Fluc in tumor. Results indicated that oHSV2-Fluc could be detected at day 21 after injection intraperitoneally or subcutaneously (**Figure 5H-K**).

Discussion

Oncolytic viruses are emerging as important agents in cancer treatment [20]. Oncolytic HSV selectively replicates within tumors and directly destroys tumor cells by virus-induced cell lysis [21]. In our previous study, we constructed a novel oncolytic herpes simplex virus oHSV2 (with deletions in ICP34.5 and ICP47 and expression of human GM-CSF). Following the completion of a preclinical study, oHSV2 has now entered into clinical trials for the treatment of melanoma and other solid tumors (NCT03-866525).

In our previous study, a preclinical safety evaluation of oHSV2 was carried out. The biodistribution assays of oHSV2 in tumor-bearing mice and tumor-bearing nude mice were investigated. In tumor-bearing BALB/c mice, the BALB/c mice were subcutaneously injected with CT-26 cells. Results indicated that oHSV2 could be detected at the injection site and the inguinal lymph nodes, and oHSV2 was completely cleared in mice on day 28. In this study, tumor-bearing BALB/c and C57 mice were used to investigate biodistribution of oHSV2-Fluc. Findings showed no widespread distribution of oHSV2-Fluc in mice during the experiment. Additionally, oHSV2-Fluc was completely cleared in mice on day 15. The injection of oHSV2-Fluc did not affect body weight for BALB/c or C57 mice. In tumor-bearing BALB/c nude mice, our previous study indicated that oHSV2 DNA was widely distributed in tumor and other tissues, including heart, spleen, kidney, liver and lung. The abundance of oHSV2 DNA in these tissues was much lower than that in the tumor. In this study, oHSV2-Fluc was injected subcutaneously or intravenously into BALB/c nude mice. As a result, oHSV2-Fluc DNA could be detected in intraperitoneal and subcutaneous xenograft mice tissues and tumor sites. The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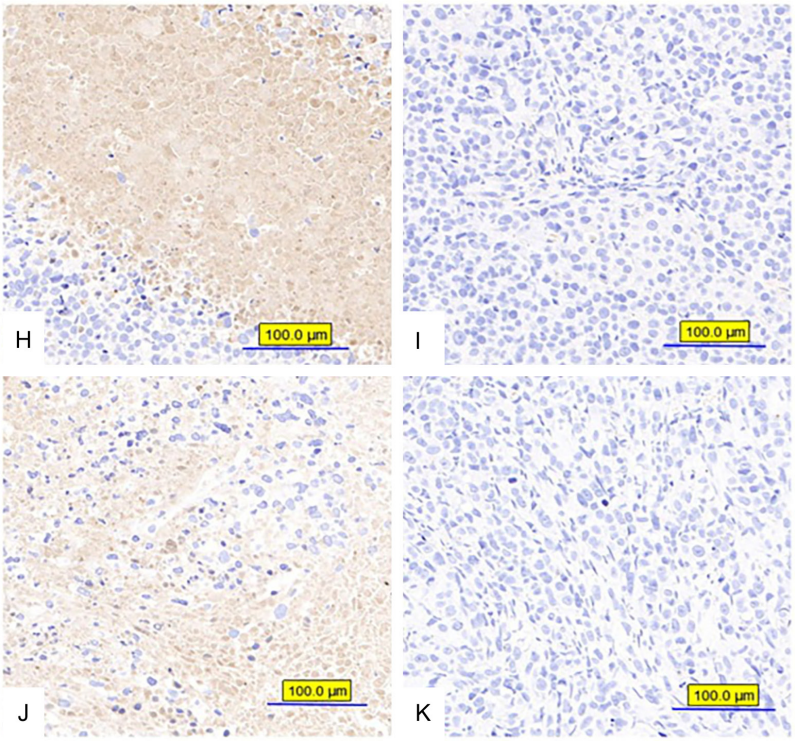

Figure 5. Evaluation of tissue distribution of oncolytic virus expression firefly gene in BALB/c nude mice in Hep2 tumor model. A. Schematic diagram of the animal experiment. B. Hep2 cells were injected intraperitoneally into female BALB/c nude mice, then oHSV2-Fluc was injected intravenously into the tail vein. C. Hep2 cells were injected subcutaneously into the right flanks of female BALB/c nude mice. D. oHSV2-Fluc was injected around tumor. E. Fluorescence signal quantitation diagram. F. Biodistribution of oHSV2-Fluc in subcutaneous xenograft mice and intraperitoneal xenograft mice by SYBR Green I Real-time PCR. G. Average body weights of female BALB/c nude mice. H. Immunohistochemical analysis of oHSV2-Fluc (gD protein) in tumor from intraperitoneal xenograft mice with oHSV2-Fluc injected intravenously into the tail vein. I. Immunohistochemical analysis of oHSV2-Fluc (gD protein) in tumor from intraperitoneal xenograft mice without oHSV2-Fluc injection intravenously into tail vein. J. Immunohistochemical analysis of oHSV2-Fluc (gD protein) in tumor from subcutaneous xenograft mice with oHSV2-Fluc injected subcutaneously. K. Immunohistochemical analysis of oHSV2-Fluc (gD protein) in tumor from subcutaneous xenograft mice without oHSV2-Fluc injected subcutaneously.

abundance of oHSV2-Fluc in tumor was much higher than that in the tissues. Interestingly, the intraperitoneal xenograft mice with oHSV2-Fluc injected intravenously showed a wide biodistribution of oHSV2-Fluc. The live animal bioluminescence imaging, SYBR Green I Real-time, and immunohistochemical stains were used to confirm that oHSV2-Fluc could be detected in tumor at 21 days after administration. This is the first evidence that HSV2 injected intravenously can target the tumor in vivo. We also investigated the toxicity of oHSV2-Fluc injected intravenously. Results indicated that oHSV2-Fluc injected intravenously did not affect on body weight of BALB/c nude mice.

In summary, the newly constructed oncolytic virus oHSV2-Fluc can be detected in intraperitoneal and subcutaneous graft/xenografts in mice. The intraperitoneal and subcutaneous xenografted mice with oHSV2-Fluc injected intravenously or subcutaneously showed a wide biodistribution of oHSV2-Fluc. We further confirmed that oHSV2-Fluc injected intravenously or intratumorally could target the tumor in vivo. Our study provides a new way to expand the use of oHSV2 administration.

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Disclosure of conflict of interest

None.

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