

# Intracranial Delivery of Synthetic mRNA Using Common Transfection Reagent

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# Abstract

**Background:** Owing to messenger RNA's unique biological advantages, it has received increasing attention to be used as a therapeutic gene carrier, known as mRNA-based gene therapy. It is critical to precisely deliver specific mRNA into targeted part of the body to achieve effective treatment.

**Methods:** In the present study, a mouse model of intracranial delivery of synthetic mRNA was established using commonly used transfection reagent. Synthetic luciferase mRNAs were wrapped with two different transfection reagents and microinjected into the brain at the fixed point. The expression status of delivered mRNA was monitored by a small animal imaging system. The possible reagent-induced biological toxicity was evaluated by behavioral and blood biochemical measurements. Synthetic modified TRAIL mRNA was also used as an example of therapeutic application.

**Results:** This model demonstrated that synthetic mRNA could be successfully delivered into the brain with commonly used transfection reagents without measurable toxicity. The expression of exogenous mRNA persisted in a reasonable period following intracranial injection.

**Conclusions:** This mouse model of intracranial delivery of synthetic mRNA can be applied in preclinical studies of mRNA-based gene therapy.

## Background

DNA-based gene delivery has been widely used in most preclinical and clinical studies of gene therapy, in which plasmids and viral vectors are commonly utilized as target gene carriers. However, the use of DNA-based gene delivery has been limited by its drawbacks, especially the possibility of insertional mutagenesis and targeted cells restricted on the dividing cells[1]. It is critical to explore a safe and efficient gene delivery system for gene therapy. Owing to the unique biological nature of message RNA (mRNA), it has received a great attention to be used as a therapeutic gene carrier, known as mRNA-based gene therapy. The mRNA can be translated into therapeutic proteins or peptides in the cytoplasm without entering the nuclei, so that it is able to work on both static and dividing cells without the risk of insertional mutagenesis in the host cells[2]. The main concern about mRNA's therapeutic application is its relative short life time inside the cell. In recent years, along with the discoveries of 5'mRNA anti-reverse cap analogues (ARCA), poly(A) tails and the insertion of additional untranslated regions, foreign mRNA's stability and translation efficiency can be significantly enhanced in host cells[3, 4]. In addition, it is easy to be transfected into target cells, because the construct size of synthetic mRNA is much smaller than that of corresponding DNA plasmid. The novel modified mRNA constructs have become more attractive alternatives to the most commonly used DNA-based gene carriers.

Glioblastoma multiforme (GBM) is the most common and deadly malignant primary tumor of the brain. Despite the progressive development of surgical techniques and adjuvant therapies, the therapeutic outcomes of GBM treatment remain unsatisfactory for decades. In addition to GBM's biological features, such as complex cellular composition, diffuse invasiveness and capacity to escape conventional

therapies, the existence of blood-brain barrier (BBB) and brain-tumor cell barrier (BTB) in the brain further increases the intractability of GBM[5]. Therefore, under certain circumstances, intracranial injection of synthetic mRNA may be an ideal choice for the treatment of gliomas. In the present study, *in vitro* synthesized luciferase-mRNA (Luc-mRNA) was used as the target gene that was wrapped with commonly used transfection reagents. Synthetic Luc-mRNA was microinjected into mouse brain at the fixed point. The expression status of delivered mRNA was monitored and the possible reagent-induced biological toxicity was evaluated. Meanwhile, synthetic TRAIL-mRNA was used as an example of therapeutic application in a mouse xenografted tumor model. This mouse model of intracranial delivery of synthetic mRNA could provide additional option for mRNA-based gene therapy.

## Methods

### *In vitro* synthesis of Luc-mRNA and TRAIL-mRNA

Luc-mRNA and TRAIL-mRNAs were synthesized *in vitro* as previously described [6]. Briefly, the human 5'UTR with Kozak sequence and 3'UTR sequence were commercially synthesized by Integrated DNA Technologies (Coralville, Iowa) and sub-cloned into pcDNA3.3. The DNA templates of human TRAIL and luciferase were obtained from our previously constructed expression vectors through restriction enzyme digestion. MEGAscript T7 kit (Ambion) was used to synthesize mRNAs, whereas m7GpppG was replaced with ARCA cap analog (New England Biolabs) and cytidine and uridine were replaced with 5-methylcytidine triphosphate and pseudouridine triphosphate (TriLink Biotechnologies) respectively. Reactions were sustained for 5 h at 37°C followed by DNase treatment. Then, the reactions were treated with Antarctic Phosphatase (New England Biolabs) for 2 h at 37°C to remove residual 5'-triphosphates. The synthesized mRNAs were purified with Ambion MEGAclean spin columns (Ambion) and quantitated with Nanodrop (Thermo Scientific).

### Cells and animals

293T cells stored by the laboratory, They were cultured in DMEM with 10% FCS, 2 mM L-glutamine and 1% penicillin-streptomycin solution (all from Invitrogen, Carlsbad, CA, USA) and incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Human glioblastoma cell line (DBTRG) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) to be used as target cells in the xenografted tumor model. DBTRG cells were maintained as suggested by ATCC and pre-labeled with luciferase using pGL4.51[luc2/CMV/Neo] vector (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol.

C57BL/6J and nude mice (female, 4–6 weeks of age) were purchased from the Model Animal Research Center at Nanjing University (Nanjing, China) and housed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocols of the present study were approved by the Animal Care Committee at Hubei University of Medicine (Shiyan, China).

### Intracranial injection of synthetic mRNA in mice

The C57BL/6J mice were anesthetized by intraperitoneal injection of 1% sodium pentobarbital. A sagittal incision (1.0 to 1.5 cm) was made on the scalp, and the calvarium was exposed by blunt dissection. A tiny parietal hole was created on the sagittal suture of skull. The microinjector was positioned at the right caudate nucleus (2 mm forward and 6 mm right side the anterior fontanelle) and vertically punctured 3 mm. The Luc-mRNA or TRAIL-mRNA solution (20 µl) was injected at a rate of 2 µl/min through the microinjector. The TransIT-mRNA kit-mediated mRNA solution was composed of 1 µl of synthetic mRNA (1 or 2 µg/µl Luc-mRNA or 1 µg/µl TRAIL-mRNA), 15 µl of Opti-MEM, 2 µl of Boost reagent and 2 µl of TransIT-mRNA. The *in vivo*-jetPEI kit-mediated mRNA solution was composed of 1 µl of synthetic mRNA (1 µg/µl Luc-mRNA), 0.12 µl or 0.16 µl of *in vivo*-jetPEI reagent (N/P ratio 6 or 8), 2 µl of 10% glucose solution, 0.88 or 0.84 µl DEPC water. After injection, the microinjector was kept in place for 5 minutes.

### **Establishment of xenografted tumor model**

Since IVIS Spectrum System is preferentially sensitive to bioluminescence, luciferase gene-transfected DBTRG (DBTRG-Luc) cells were used for the xenograft test. A total of  $3 \times 10^5$  DBTRG-Luc cells were implanted into the right frontal lobe of nude mice. This xenografted tumor model was used to test the therapeutic effect of synthetic TRAIL-mRNA through intracranial injection. Seven days after *in situ* implantation of DBTRG-Luc cells, 20 µl of cock tail solution (containing 1 µg TRAIL-mRNA) was intracranially injected to each mouse. The bioluminescence was determined at day 0, 14, 28 and 60 using IVIS spectrum system. The body weight was recorded at day 0, 14, 28 and 60. At last, statistical analysis of survival rate of mice was performed at day 75.

### **Determination of tumor size by MRI**

In order to detect the tumor formation of DBTRG-Luc cells, 75 days after *in situ* implantation of DBTRG-Luc cells, the mice were anesthetized by intraperitoneal injection of 1% sodium pentobarbital, and the tumors size were detected by MRI (General Electric, GE).

### **Immunohistochemistry**

The tumors were isolated from the brain tissues. One section per sample was deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). Additional sections were immunostained using the automated immunohistochemistry (IHC) and *in situ* hybridization staining system Bond RX (Leica Biosystems). Sections were stained for proliferating cells using rabbit anti-Ki67 antibody (1:50, Abcam).

### **Determination of blood biochemical indexes in exogenous mRNA-injected mice**

To assess the toxicity of luciferase mRNA and Mirus reagents, routine blood examinations were performed for both control and Luc-mRNA injected mice. Blood was collected by cardiac puncture in EDTA-K2 blood collection tubes at the day of sacrifice. The white blood cells (WBC), hemoglobin (HGB), aspartate transaminase (AST), alanine transaminase (ALT), creatinine (Cr) and C-reactive protein (CRP) were determined using Mindray BC-6900 Vet animal automatic hematology analyzer. The blood cells of

the mice were analyzed by using Mindray BC-5300 Vet animal automatic hematology analyzer (Mindray, China).

### **Behavioral evaluation of mice injected with synthetic mRNA**

According to previous literature reports, behavioral test has been used for the evaluation of drug-induced potential biological toxicity in animals [7, 8]. In the present study, open field test and Y maze test were performed to determine the possible transfection reagent-induced neurotoxicity. These two behavioral tests were conducted in C57BL/6J mice on day 14 post-inoculation of Luc-mRNA wrapped with Mirus TransIT-mRNA or Mirus TransIT-mRNA alone. For the open field test, each mouse was placed in the center of a darkened white box (50 × 50 × 38 cm) and monitored using an infrared video tracking system (Ethovision XT 9.0, Noldus Information Technology) for 10 min. A 30-by-30-cm square in the center of the box was defined as the “zone,” and the peripheral arena was defined as the “residual”. The distance traveled and time spent in the zone and residual were recorded for further analysis.

For the Y maze test, the Y-maze was fabricated from gray plastic and consisted of three arms (21 cm long, 15.5 cm high, 7 cm wide at the bottom, and 10 cm wide at the top) with an angle of 120°. Visual cues were placed outside each arm, and the apparatus was illuminated at 10 lux. Each mouse was placed at the end of one arm and allowed to freely explore the maze for 10 minutes. An arm entry was defined as all four paws of the mouse being in the arm, and the sequence of arm entries was monitored with a video camera and counted manually. An alternation was defined as successive entries into the three arms on overlapping triplet sets.

### **Flow cytometry analysis the apoptosis of synthetic TRAIL-mRNA transfection into DBTRG-Luc cells**

Briefly,  $1 \times 10^6$  DBTRG-Luc cells were seeded in six-well plate and incubated for 24 h to resume exponential growth. Synthetic TRAIL-mRNA transfection into DBTRG-Luc cells and incubated for additional 24 h, then the cells were harvested and washed with PBS. The extent of apoptosis was measured through AnnexinV-FITC apoptosis detection kit (Beyotime, China), and analyzed by flow cytometry software (Beckman, USA). The upper right part represents apoptotic cells undergoing secondary necrosis at the last stage or dead cells (annexin-V and PI double positive), and the lower right part represents the early stage apoptotic cell population (annexin-V positive and PI negative).

### **Viability assay of DBTRG-Luc glioma cells**

The cell viability was detected by real-time assessment by the xCELLigence cell analyzer (RTCA, Roche, USA) as previously described [6]. A volume of 100 µl of DBTRG-Luc cell suspension ( $5 \times 10^3$  cells) was seeded in E-Plate 16. All cells were allowed to settle at the bottom of the wells at room temperature (RT) for 15 min, and then incubated at 37°C and 5% CO<sub>2</sub>. The impedance signals were recorded every 5 min for the first 6 h. After 6 h of base line measurement, 0.1 µg synthetic TRAIL-mRNA mixture was added into each well. The impedance signals were recorded using the same time intervals until the end of the experiment (up to 72 h). Cell index (CI) value was defined as relative change in measured impedance to

background impedance and represented cell status, which is directly proportional to the quantity, size, and attachment forces of the cells.

### **Immunoblotting analysis of TRAIL-induced apoptosis-related proteins expression**

Immunoblotting analysis was used to detect the cellular expression of TRAIL induced apoptosis-related proteins (caspase-3, Bcl-2 and Bax) in DBTRG-Luc cells. Briefly, DBTRG-Luc cells were washed with PBS three times and collected with the cell lysis RIPA buffer (Cowin Bio, China). Cell lysates were incubated on ice for 30 min. Protein concentration was determined using BCA protein assay reagents (Beyotime) according to the manufacturer's protocol. Equal amounts of protein (50 µg/sample) were loaded on each lane and separated by electrophoresis in 12% SDS polyacrylamide gel and electrotransferred to nitrocellulose membranes. The membrane was put in blocking buffer for 1h at RT followed by overnight incubation at 4°C with appropriate primary TRAIL antibody (1:1000, CST, USA), caspase-3 antibody (1:1000, CST, USA), Bcl-2 antibody (1:1000, CST, USA) and GAPDH antibody (1:4000, Proteintech, China). The blots were rinsed with TBST three times, incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) for 60 min, and detected by chemiluminescence using ECL Hyperfilm (Bio-rad, USA).

## **Results**

### **Expression verification of synthesized Luc-mRNA and TRAIL-mRNA**

The expression of Luc-mRNA and TRAIL-mRNA was verified in 293T cells. After transfection of 293T cells with Luc-mRNA, the bioluminescence was detected at 12 h, 24 h and 48 h using IVIS Spectrum System. The luciferase expression maintained at least 48 h (Fig.1A). To verify the expression of TRAIL-mRNA, TRAIL-mRNA was transfected into 293T cells and detected by western blot. As shown in Fig.1B, the expression of TRAIL was highly upregulated by TRAIL-mRNA transfection.

### **Intracranial expression of synthetic mRNA delivered with common transfection reagents**

Two commercially available transfection reagents, in vivo-jetPEI from Polypus Transfection and TransIT-mRNA from Mirus, were used for the intracranial mRNA delivery. As shown in Fig. 2, the intracranial injected synthetic Luc-mRNA was successfully expressed either with jetPEI or with TransIT-mRNA. The peak bioluminescence signals of Luc-mRNA injected with these two reagents appeared in 12 h after intracranial injection (Fig. 2A-B). However, the bioluminescence intensity produced by Luc-mRNA delivered with TransIT-mRNA was significantly higher than that produced by Luc-mRNA delivered with jetPEI at every time points. As shown in Fig. 2C, the duration of luciferase expression was much longer in Luc-mRNA injection with TransIT-mRNA (60 hours) than that in Luc-mRNA injection with jetPEI (36 hours).

### **The evaluation of biochemical toxicity of intracranially injected Luc-mRNA with TransIT-mRNA**

After injecting 1 µg mRNA mixture, all mice remained healthy without any toxic symptoms or behavioral abnormalities. There were no significant differences between TransIT-mRNA injection and controls on all determined blood biochemical parameters, including HGB, WBC, ALT, AST, Cr and CRP (Fig. 3A-3F). No

significant transfection reagent-related neurovirulence was detected by both Y maze (Fig. 4A-E) tests and open field (Fig. 4F-J). The results of blood test and behavioral test indicated that this transfection reagent is safe to be used as a wrapping material of synthetic mRNA for intracranial injection.

### **The effects of synthetic TRAIL-mRNA on DBTRG-Luc glioma cells**

The effect of synthetic TRAIL-mRNA on the viability of DBTRG-Luc glioma cells was determined using RTCA, and TRAIL-induced apoptosis was detected by flow cytometric analysis. As shown in Fig. 5A, the mean apoptotic population of normal DBTRG-Luc cells was 4.38% + 4.7%, however, the apoptotic population of DBTRG-Luc cells transfected with 1 µg TRAIL-mRNA was 11.01% + 17.96%. RTCA results indicated that synthetic TRAIL-mRNA significantly inhibited the viability of DBTRG-luc cells (Fig. 5B). Figure. 5C showed the results of immunoblotting analysis of apoptosis-related proteins in DBTRG-Luc cells after transfection with synthetic TRAIL-mRNA for 24 h. DBTRG-Luc cells expressed similar amount of total caspase-3. However, the cleaved form of caspase-3 and Bax were obviously upregulated by the treatment of synthetic TRAIL-mRNA transfection. Bcl-2 was down regulated by synthetic TRAIL-mRNA transfection.

### **Therapeutic application of synthetic TRAIL-mRNA through intracranial injection in orthotopic glioma mouse model**

A DBTRG-Luc cell-derived xenografted glioma mouse model was used to test the therapeutic application of synthetic TRAIL-mRNA through intracranial injection. As shown in Fig. 6A, the intensity of luminescence in DBTRG-Luc cells pretreated with synthetic TRAIL-mRNA for 6 h was not different from DBTRG-Luc cells without synthetic TRAIL-mRNA pretreatment. DBTRG-Luc cell-derived and TRAIL-mRNA-pretreated DBTRG-Luc cell-derived xenografted glioma mouse models were used to further evaluate the effect of intracranial injection of synthetic TRAIL-mRNA. The tumor growth was significantly inhibited by both TRAIL-mRNA pretreatment and TRAIL-mRNA intracranial injection (Fig. 6B-C). Fig. 6D and 6E showed the changes of body weight and animal survival rate under different conditions. At the end point, tumor size was detected with brain MRI scan and the measurement of isolated tumors (Fig. 6F). The inhibitory effect of injected TRAIL-mRNA on tumor cell proliferation was also confirmed by immunohistochemical staining of tumor tissues (Fig. 6G).

## **Discussion**

Because mRNA possesses high positive charge and high hydrophilicity, it is usually delivered into cells as a complex with transfection reagent. TransIT-mRNA and jetPEI are commonly used for mRNA transfection in most *in vitro* studies. It is essential to verify the safety of these reagents *in vivo* application and their effects on the expression of associated genes after intracranial injection. In the present study, luciferase mRNA was synthesized *in vitro* and its expression was verified in 293T cells. In order to study the safety of TransIT-mRNA and jetPEI *in vivo* application and their effects on the expression of Luc-mRNA after intracranial injection, we injected Luc-mRNA wrapped with TransIT-mRNA or jetPEI into the brain of C57BL/6J mice. Considering the volume safety of intracranial injection, we compared the transfection

efficiency of 20  $\mu$ l system containing 1  $\mu$ g Luc-mRNA and 30  $\mu$ l system containing 2  $\mu$ g Luc-mRNA. After intracranial injection of Luc-mRNA, the longest time, in which Luc-mRNA-produced bioluminescence can be detected by IVIS, was 72 h for 20  $\mu$ l system and 48 h for 30  $\mu$ l system respectively. The poor performance of 30  $\mu$ l system might be due to the large volume leading to an incomplete injection into the brain. So, 20  $\mu$ l system containing 1  $\mu$ g Luc-mRNA was used for right caudate nucleus injection in the related *in vivo* experiments. In addition, according to *in vivo* jetPEI manufacturer's instruction, a 5  $\mu$ l system containing 1  $\mu$ g Luc-mRNA at N/P ratio 6 was injected into the right caudate nucleus. The longest detectable time of Luc-mRNA-produced bioluminescence was 36 hours. When the N/P ratio was increased to 8, the bioluminescence intensity was significantly higher than that at N/P ratio 6, but the longest detectable time retained 36 hours.

The results of comparative experiment clearly indicate that TransIT-mRNA is much better than jetPEI in terms of assisting intracranial Luc-mRNA delivery. TransIT-mRNA is preferred to be used for mRNA delivery through intracranial injection. However, long-term safety is an important prerequisite for it to be routinely used in mRNA-based gene therapy studies. The test results of blood biochemical parameters showed relatively stable internal environment after intracranial injection of TransIT-mRNA, indicating that TransIT-mRNA solution was harmless to the metabolism of tested subjects. Two behavioral methods were used to test the potential neurotoxicity of TransIT-mRNA. Fourteen days after the intracranial injection, the results of open field test and Y-maze test demonstrated that the behaviors of tested mice were not significantly affected by intracranial injection of TransIT-mRNA reagent. Taken together, TransIT-mRNA reagent-assisted synthetic mRNA delivery is safe for *in vivo* intracranial administration.

The major reasons that limit the effectiveness of conventional therapies for GBM include tumors' exceptional anatomy location and the existence of BBB. Intracranial injection of synthetic mRNA is able to directly deliver specific anticancer genes to the tumor. TRAIL (TNF-related apoptosis-inducing ligand) is an anticancer gene. Its protein product can specifically kill cancer cells without harming normal cells [9–13]. Owing to its tumor cell-specific killing effect, TRAIL has been widely used in preclinical and clinical studies [12, 14–23]. In the current study, we used synthetic TRAIL-mRNA as an example to verify whether this method of intracranial injection can be applied for the treatment of GBM. First, the tumor cell killing effect of synthetic TRAIL-mRNA was verified *in vitro* with DBTRG-Luc cells. Then, the *in vivo* antitumor effect of intracranially injected TRAIL-mRNA was investigated with DBTRG-Luc cell-derived xenografted glioma mouse model. The antitumor effect of intracranially injected TRAIL-mRNA was comparable to that of pretreatment of DBTRG-Luc cells with TRAIL-mRNA, and the combination of pretreatment and intracranial injection of TRAIL-mRNA showed the best antitumor effect. This result shows that the method of synthetic mRNA intracranial delivery using common transfection reagent is suitable for the experimental studies of intracranial tumors.

In summary, the *in vitro* synthetic luciferase mRNAs were highly expressed in the mouse brain through intracranial injection, in which the injected mRNAs were wrapped with commonly used transfection reagents. Mouse behavioral and blood biochemical measurements verified the biological safety of related reagent's intracranial application for mRNA transfection. Using synthetic TRAIL-mRNA as an experimental

therapeutic example, its intracranial delivery with common transfection reagents significantly inhibited the tumor growth in DBTRG cell-derived xenografted glioma mouse model. This study provides an intracranial synthetic mRNA delivery model for preclinical studies of mRNA-based gene therapy.

## Abbreviations

mRNA: messenger Ribonucleic Acid

GBM: Glioblastoma multiforme

BBB: blood-brain barrier

BTB: brain-tumor barrier

ARCA: anti-reverse cap analogues

PCR: Polymerase Chain Reaction

TRAIL: tumor necrosis factor-related apoptosis inducing ligand

IVIS: in vivo imaging system

MRI: magnetic resonance imaging

## Declarations

### Acknowledgements

Not applicable.

### Authors' contributions

JL, XJT and LJD contributed to the conception. HP, XRG and JJH performed all experiments and constructed the manuscript. HP analyzed interpreted all the data, XRG and XJT supported the experimental techniques. LJD and BW helped write and critically reviewed the manuscript and provided intellectual input, LZ, RF, DKW, FLC, HC, HG, JXX and XHZ conceived the study, and participated in coordination, and helped in drafting the manuscript. JL, XJT and LJD supervised all studies. All authors read and approved the final manuscript.

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## Ethical statement

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the Hubei University of Medicine prior to the initiation of any studies.

## Conflicts of Interest

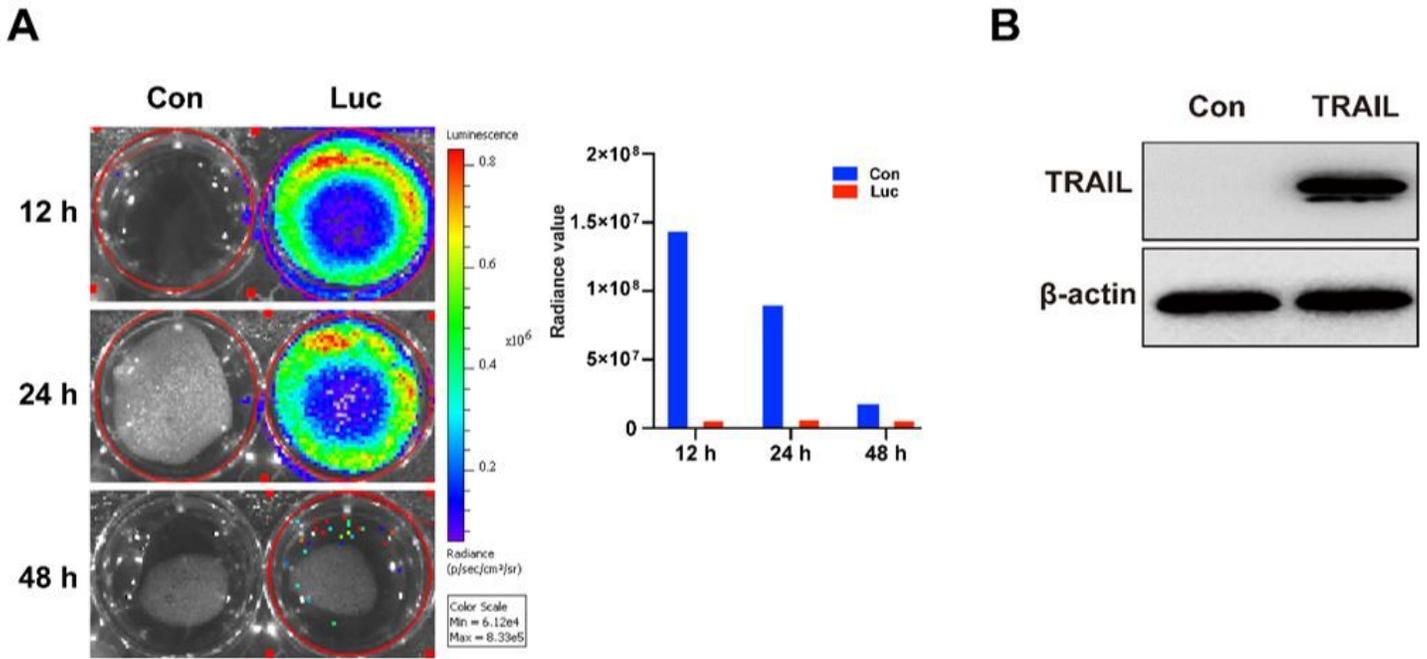
The authors declare no potential conflicts of interest

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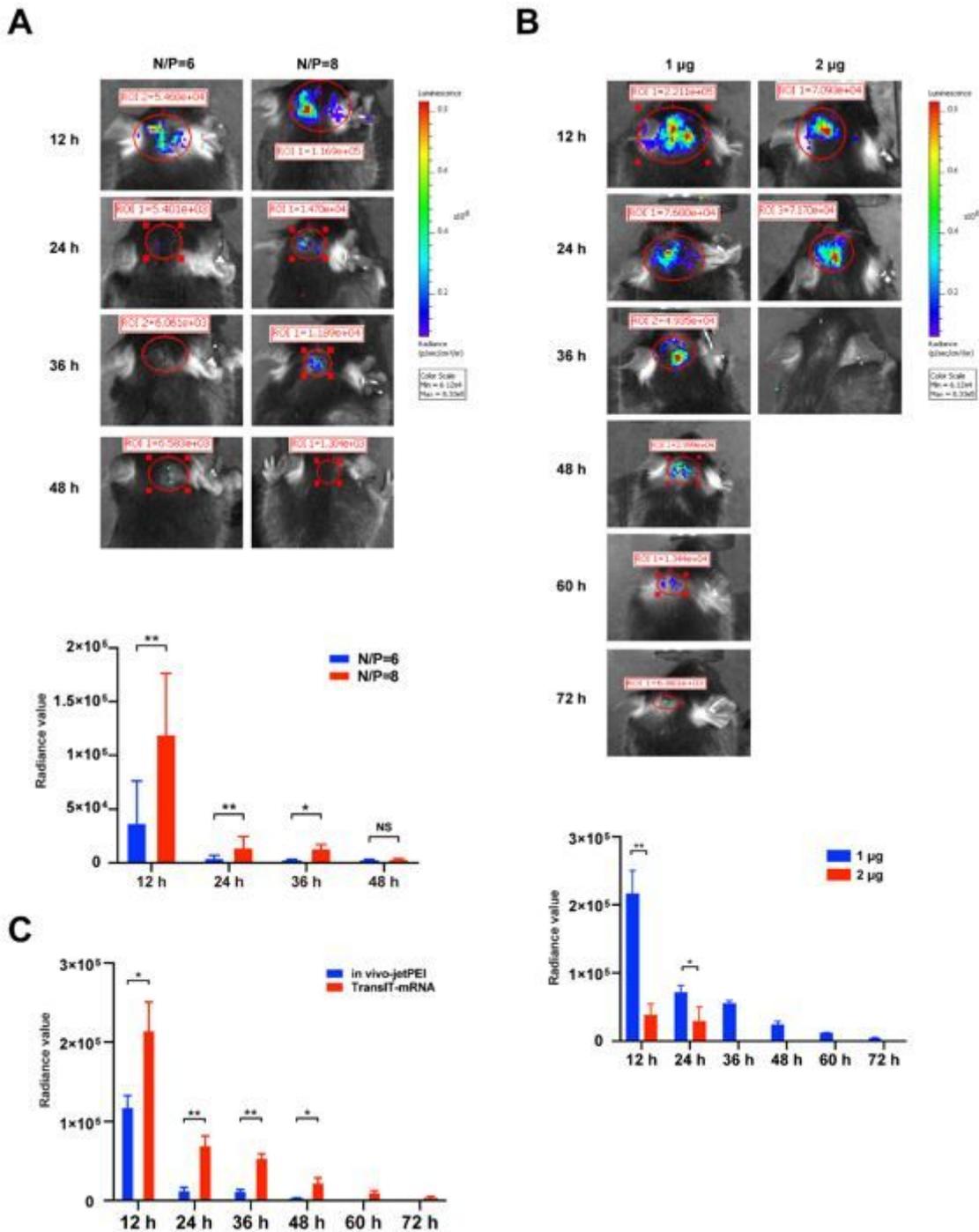
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## Figures



**Figure 1**

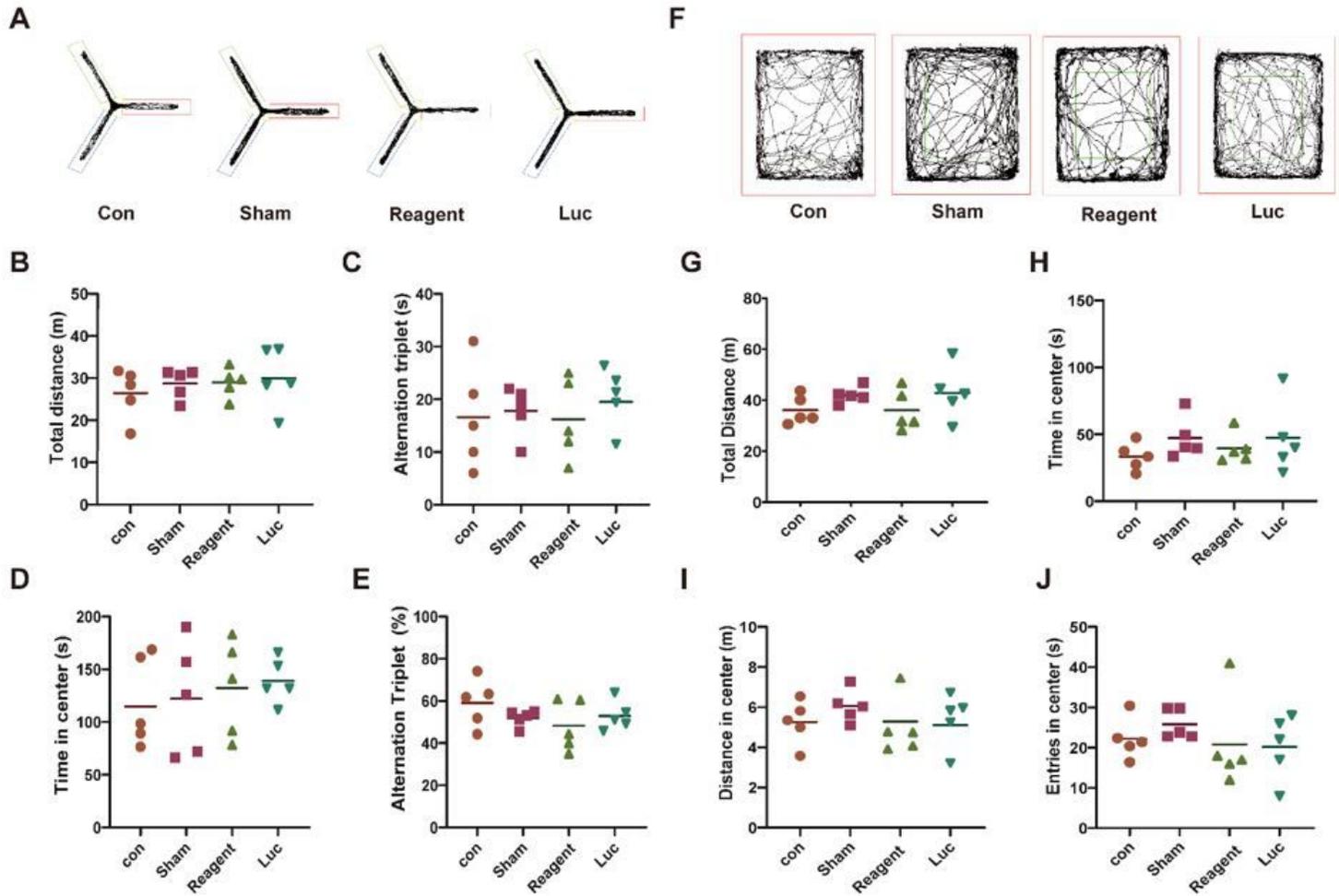
Synthetic Luc-mRNA and TRAIL mRNA transfection into 293T cells. A Bioluminescence was measured at 12 h, 24 h and 48 h after Luc-mRNA transfection using a small animal image system. Radiance value represents the expression of transfected Luc-mRNA in 293T cells. B The expression of transfected TRAIL-mRNA in 293T cells was detected using western blot at 12 h after TRAIL-mRNA transfection.



**Figure 2**

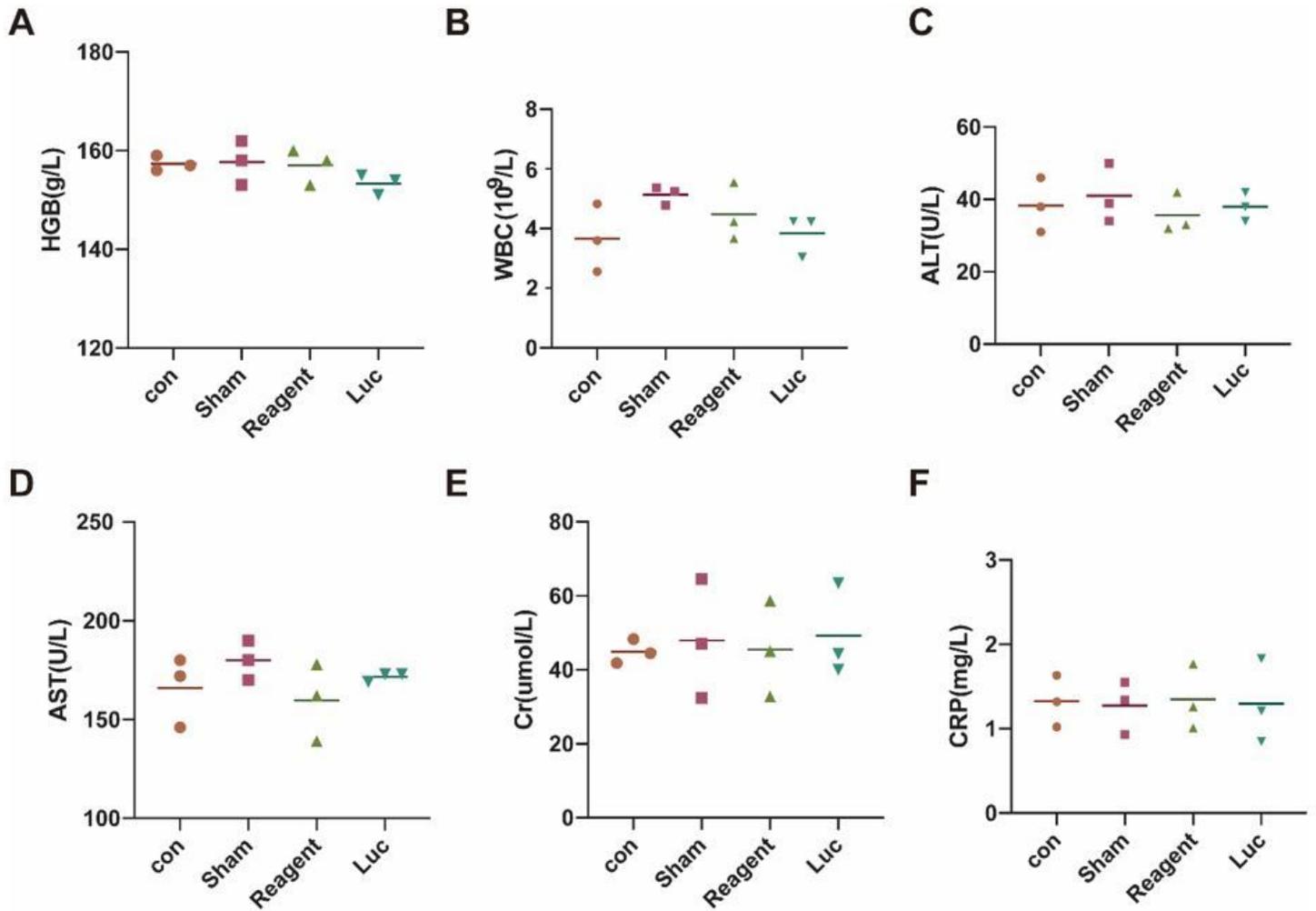
Intracranial expression of synthetic mRNA delivered with common transfection reagents. A The intracranial expression of in vivo-jetPEI-mediated intracranial injection of synthetic Luc-mRNA was detected using an IVIS with N/P=6 and N/P=8 at 12 h, 24 h, 36 h and 48 h. The upper panel shows the representative images at each time point, and lower panel represents the summary. B The intracranial expression of TransIT-mRNA-mediated intracranial injection of synthetic Luc-mRNA was detected using an IVIS with 1 µg system and 2 µg system at 12 h, 24 h, 36 h, 48 h, 60 h and 72 h. The upper panel shows the representative images at each time point, and lower panel represents the summary. C The transfection

efficiency comparison of the two transfection reagents. 1  $\mu\text{g}$  Luciferase mRNA with TransIT-mRNA or in vivo-jetPEI solution was intracranially injected. \* $p < 0.05$ ; \*\* $p < 0.01$ .



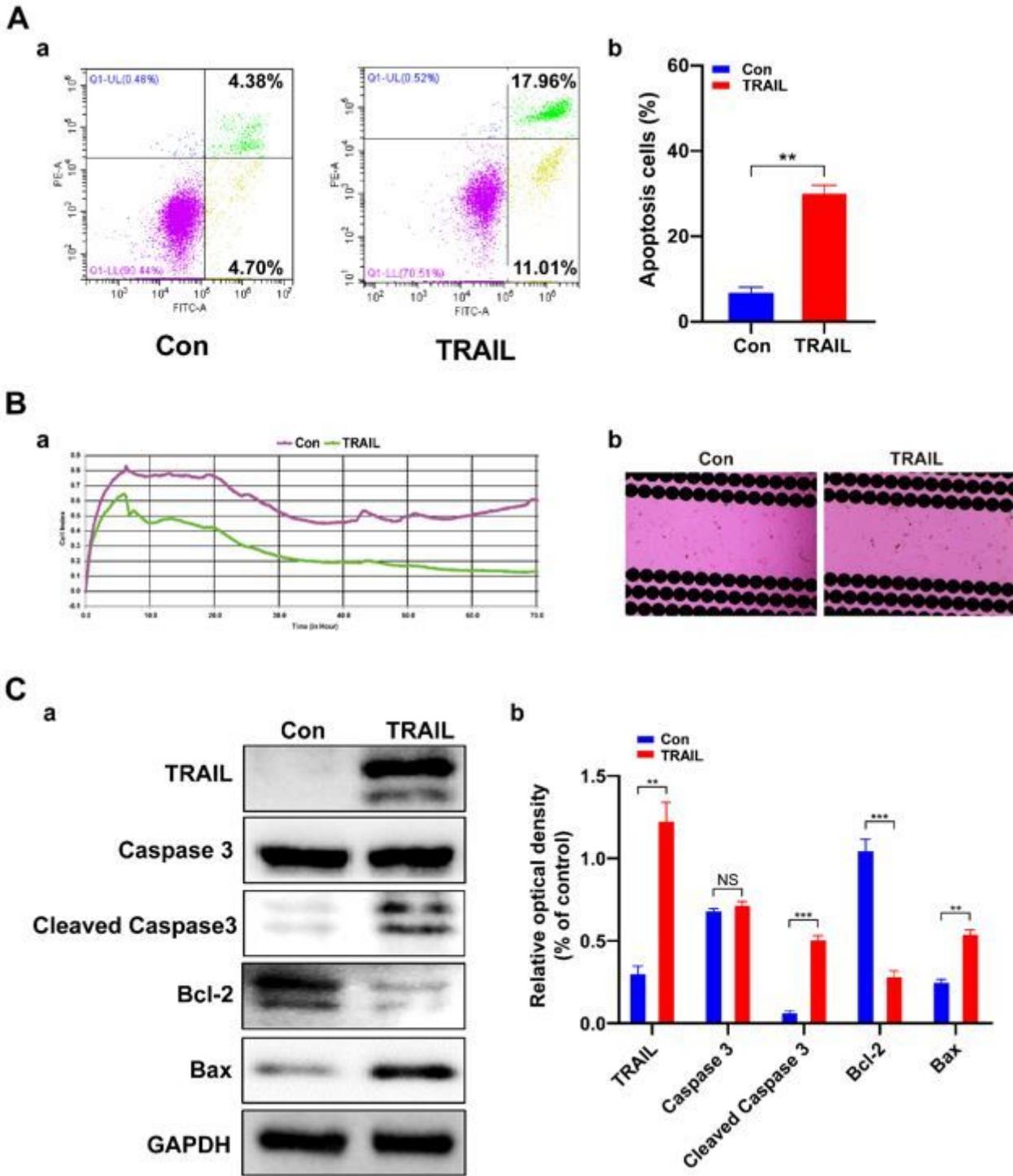
**Figure 3**

Behavior evaluation of mice receiving intracranial injection of synthetic Luc-mRNA. Four groups of 8-week-old C57 mice were intracranially injected with synthetic Luc-mRNA in TransIT-mRNA solution or TransIT-mRNA reagent alone. The behavior tests were performed 14 days after injection. A-E Y maze test, A Infrared video recorder monitored the activity trajectory of mice in Y maze within 8 minutes. The overall traveled distances in the Y maze were recorded. B Total distances of the mice traveled in the Y maze. C Alternation triplet times of the mice traveled in Y maze. D Total times of the mice traveled in the center of the Y maze. E The percentage of alternation triplet of the mice traveled in Y maze. F-J Open field test, F Infrared video recorder recorded the activity trajectory of mice in open field within 10 minutes. G Total distance of the mice traveled in the open field. H Total times of the mice traveled in the center of open field. I Total distance of the mice traveled in the center of open field. J Total times of the mice entered into the center of open field.



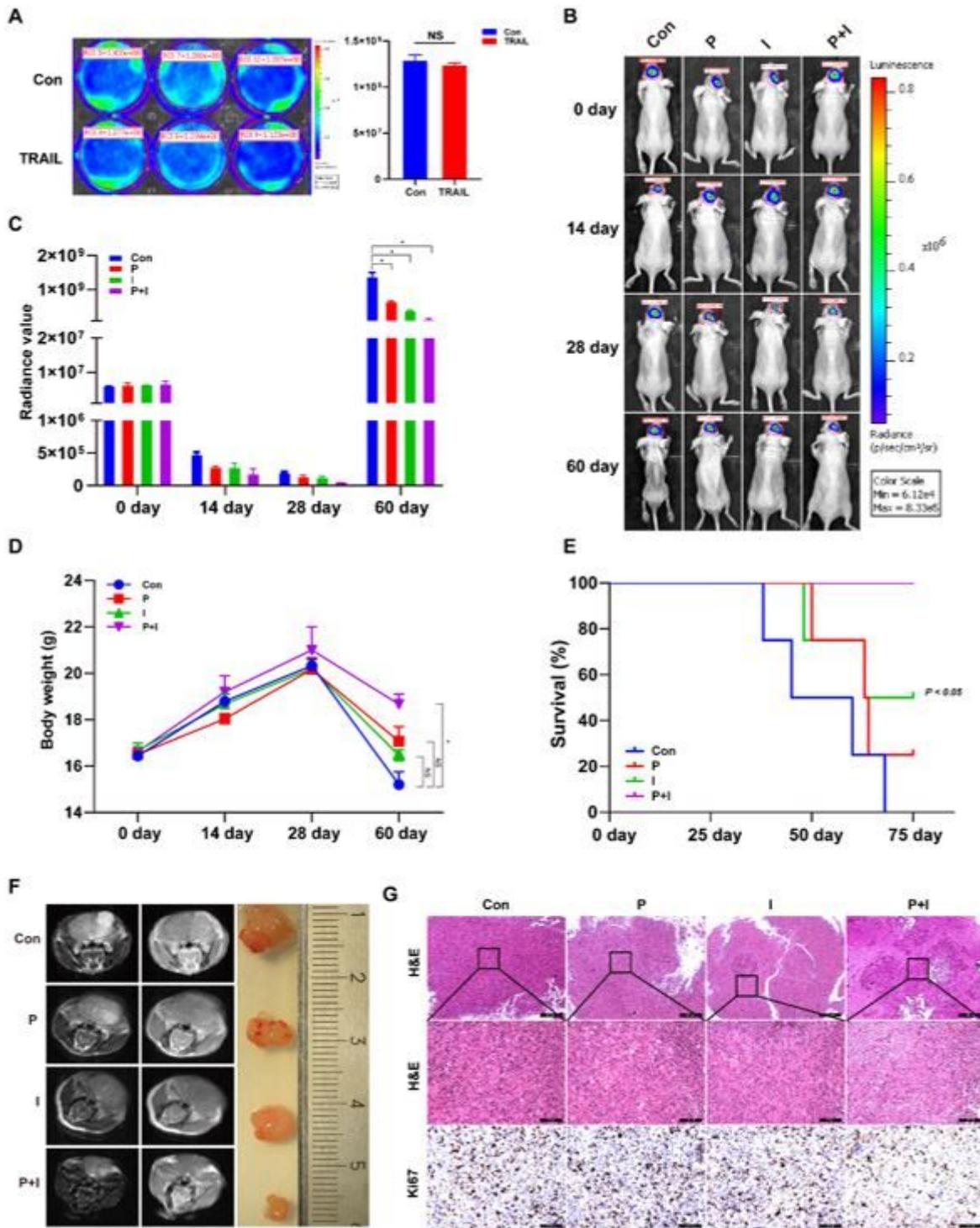
**Figure 4**

Safety evaluation of TransIT-mRNA in vivo by routine blood examination. A Hemoglobin (HGB) value in mouse serum. B White blood cells (WBC) in mouse blood. C Alanine aminotransferase value in mouse serum. D Aspartate aminotransferase value in mouse serum. E creatinine value in mouse serum. F C-reactive protein value in mouse serum.



**Figure 5**

The effects of synthetic TRAIL-mRNA on DBTRG-Luc glioma cells. A Flow cytometric analysis of synthetic TRAIL-mRNA induced apoptosis in DBTRG-Luc cells using annexinV-FITC/PI. B (a) Cell viability determined by RTCA at an interval of 5 min until the end of 72 h. (b) After the experiment, the cells stayed in the E-plate culture plate were observed under the microscope, 400 ×. C (a) The expression levels of apoptosis-related proteins in DBTRG-Luc cells determined by western blot at 24 h after TRAIL-mRNA transfection. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Figure 6**

Therapeutic application of synthetic TRAIL-mRNA through intracranial injection in orthotopic glioma mouse model. A The bioluminescent influence of pretreatment of DBTRG-Luc cells with synthetic TRAIL-mRNA transfection for 6 hours. B-C The IVIS detected image representatives of different groups (n=4) at day 0, 14, 28 and 60 and corresponding summary. D Mouse body weights measured at day 0, 14, 28 and 60. E Mouse survival rates. F Brain MRI scan images and isolated tumor samples. G Haematoxylin and eosin (H&E) and Ki67 staining of tumor tissues. Con: control; P: pretreatment with synthetic TRAIL-mRNA;

I: intracranial injection of synthetic TRAIL-mRNA; P + I: the combination of pretreatment and intracranial injection of synthetic TRAIL-mRNA. \*  $p < 0.05$ .