# Peritumoral administration of IFNβ up-regulated mesenchymal stem cells inhibits tumor growth in an orthotopic, immunocompetent rat glioma model

**Supplementary Materials:**

**Supplementary Methods**

**Supplementary Table 1. Imaging Sequences and Acquisition Parameters of in vitro MRI**

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**Supplementary Fig.S1**

**Supplementary Fig.S2**

**Supplementary Fig.S3**

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## Supplementary Methods

### 1. Lentiviral vector construction

The rat IFNβ and FTH cDNA was chemically synthesized by Gene-Chem Co., Ltd. (Shanghai, China) according to the gene sequences in GenBank (accession number of IFNβ: NM_019127; accession number of FTH: NM_012848). The IFNβ was connected to the FTH cDNA by a 'self-cleaving' T2A sequence for post-translational splicing. IFNβ-T2A-FTH was amplified by polymerase chain reaction (PCR) with the primers forward, AgeI 5'-GAGGATCCCCGGGTACCGGTCGCCACCATGACCACCGCGTCTCCCTCGCAAG -3' and reverse, AgeI 5'- CACACATTCCACAGGCTAGTCAGTTCTGGAAGTTTCTATTAAG -3'. Then, this cDNA was subcloned into the lentiviral expression vector encoding enhanced green fluorescent protein (eGFP), pLV-Ubi-MCS-SV40-eGFP-IRES-puromycin (GV367; Gene-Chem) using In-Fusion cloning to yield the recombinant vector pLV-Ubi-IFNβ-T2A-FTH-SV40-eGFP-IRES-Puromycin (pLV-IFNβ-T2A-FTH-eGFP). The derived construct, pLV-IFNβ-T2A-FTH-eGFP, was checked for appropriate insertion and the absence of undesirable mutations and flanking sequences by PCR analysis and DNA sequencing. The recombinant lentiviruses encoding IFNβ and FTH genes (LV-IFNβ-T2A-FTH-eGFP) were generated by human embryonic kidney 293 T packaging cells being co-transfected with pLV-IFNβ-T2A-FTH-eGFP and the packaging vector pHelper 1.0 and the envelope vector pHelper 2.0 using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After transfection for 48 h, lentiviruses containing supernatant were collected, purified by centrifugation, filtered, and stored at -80 °C. Lentiviral titer was determined by assessing eGFP expression in 293T cells using a

FACSVerse flow cytometer (Becton Dickinson, Mountain View, CA). Titers were expressed as the number of transduction units per milliliter.

**2. Transduction of mesenchymal stem cells (MSCs)**

MSCs were cultured and expanded at 37 °C under an atmosphere of 5% $CO_2$ in standard Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS; Cyagen) and 1% glutamine (GIBCO), supplemented with 1% penicillin/streptomycin (GIBCO). MSCs that had undergone two to five passages were used in the following experiments. For cell transduction, the cultured MSCs were dissociated into single cells by 0.25% trypsin-EDTA (GIBCO). Then, MSCs were transduced with the LV-IFNβ-T2A-FTH-eGFP at a multiplicity of infection (MOI) of 10 for 12 h to obtain IFNβ and FTH overexpressed MSCs (IFNβ-FTH-MSCs) and were then selected with 2 ug/ml puromycin for 7 d. The transduction efficiency before and after puromycin selection was quantitatively evaluated by assessing the expression of eGFP using a FACSVerse flow cytometer. MSCs transduced with lentivirus encoding eGFP alone were used as a positive control group (eGFP-MSCs), and wild-type MSCs served as a normal control group (WT-MSCs), respectively.

**3. Quantitative PCR (qPCR)**

For qPCR, total RNA was extracted from $1 \times 10^6$ cells using TRIzol (Life Technologies), according to the manufacturer's protocol. After the concentration of total RNA was measured, cDNA was synthesized using PrimeScrpt RT Master Mix Kit (Takara Bio, Shiga, Japan). Primers were designed with Primer Premier 5.0 software (Molecular Biology Insights Inc., Cascade, CO, USA) and the sequences of primers were as follows: IFNβ forward primer: 5' GAATGGAAGGCTCAACCTCA

and reverse primer: 5' ACCCAAGTCAATCTTTCCTC; FTH forward primer: 5' TGAGCCCTTTGCAACTTC and reverse primer: 5' CCCGGTCAAAATAACAAGAC; β-actin-forward primer: 5' AGGGAAATCGTGCGTGACAT and reverse primer: 5' GAACCGCTCATTGCCGATAG. qPCR was performed in a 20 μL reaction mixture containing primers, SYBR Green qPCR SuperMix (Life Technologies) reagent, and 2 μL cDNA sample using a PRISM 7500 Sequence Detection System (Applied Biosystems; Foster City, CA, USA). All data were analyzed by the $2^{-\Delta\Delta CT}$ method, and target mRNA levels were normalized to those of GAPDH, which served as an internal control. This experiment was performed in triplicate.

**4. ELISA**

For ELISA, $1 \times 10^6$ cells were cultured in 25 $cm^2$ flasks. After 72 h culturing, supernatants were collected and stored in -80 °C until used for measurement of IFNβ secretion using the ELISA kit (Elabscience Biotechnology Co., Ltd, Wuhan, China). The ELISA plate was pre-coated with an antibody specific to rat IFNβ. 100μL samples were added to the ELISA plate and then incubated for 2 h at room temperature. Then a biotinylated detecting antibody specific for rat IFNβ (Elabscience) was added, and detection was accomplished by using avidin-HRP (Elabscience) and substrate (1x TMB) (Sigma). Absorbance was read at 450 nm using a microplate reader (Bio-Rad). All samples for ELISA were examined in triplicate.

**5. Western blot analysis**

For Western blot analysis, $1 \times 10^6$ cells were lysed in radioimmunoprecipitation assay (RIPA; Thermo Fisher Scientific Inc., Rockford, IL) buffer containing a protease inhibitor cocktail (Thermo Fisher Scientific). Total protein concentration was quantified with the bicinchoninic acid (BCA)

protein assay kit (Life Technologies), and 25 μg of total proteins were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer onto polyvinylidene difluoride (PVDF) membranes. After incubation with blocking Tris-buffered saline Tween-20 containing 5% fat-free dry milk for 1 h at room temperature, the protein bands were probed with different primary antibodies including FTH (rabbit anti-FTH, 1:1000; Cell Signaling Technology, Boston, USA) and GAPDH antibodies (mouse anti-GAPDH, 1:10000; Kang Chen, Shanghai, China), followed by the corresponding horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit or anti-mouse IgG, 1:20000; southern biotech, Birmingham, USA), then were detected by the Immobilon Western Chemiluminescent Detection Reagents and chemiluminescence system (Millipore, Temecula, CA, USA). The expressions of FTH protein were normalized to that of GAPDH. This experiment was performed in triplicate.

**6. Prussian blue (PB) staining**

For PB staining, $1 \times 10^5$ IFNβ-FTH-MSCs and $1 \times 10^5$ WT-MSCs were suspended in each well of 24-well plates containing StemPro MSC SFM medium with or without incubation of ferric citrate (FC, 350 μM; Sigma) for 72 h, respectively. Then, the cells were fixed and incubated with Prussian blue solution containing 10% hydrochloride acid and 10% potassium ferrocyanide (II) trihydrate for 30 min at 37 °C.

**7. Transmission electron microscope (TEM)**

For TEM, $1 \times 10^6$ IFNβ-FTH-MSCs and $1 \times 10^6$ WT-MSCs treated with 350 μM FC were centrifuged into a solid cell pellet and fixed in 2.5% glutaraldehyde solution at 4 °C overnight, then fixed with 1% OsO4 for 1 h. After cells were dehydrated and embedded in artificial resin, ultrathin

50-nm sections were cut and double-stained with 2% uranyl acetate and 2% lead citrate for 15 min, respectively. Sections were imaged using TEM (JEM-2000EX; JEOL, Tokyo, Japan) at 60 to 80 kV.

**8. In vitro MRI and atomic absorption spectrometry**

For in vitro MRI, $1 \times 10^6$ IFNβ-FTH-MSCs, $1 \times 10^6$ eGFP-MSCs were grown in culture medium in the presence or absence of 350 μM FC for 72 h. $1 \times 10^6$ WT-MSCs were used as control. The cells were suspended in 200 μL 2% agarose solution, then in vitro MRI was performed on a clinical 3.0 T MR unit (Ingenia; Philips Medical Systems, the Netherlands) with an 8-channel SENSE orthogonal coil. Turbo spin echo T1-weighted images, Turbo spin echo T2-weighted images, and fast field echo T2*-weighted images were obtained to observe the iron accumulation mediated by the expression of the FTH gene. T2 maps were obtained using single-section multi spin-echo sequences to acquired T2 relaxation times. The imaging sequences and acquisition parameters are listed in Supplementary Table 1. After MRI, the cells were suspended in 1 M HCl solution to cause the thorough release and dissolution of iron. Then the iron concentration per cell was quantified by atomic absorption spectrometry (Z-200, Hitachi).

**9. Cell viability analysis**

Cell viability was evaluated by the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay according to the manufacturer's instruction. In brief, IFNβ-FTH-MSCs, eGFP-MSCs and WT-MSCs were seeded in 96-well plates at $5 \times 10^3$ per well with 200 μL culture media, respectively. 24 h later, 10 μL CCK-8 solution was added to incubate for another 4 h. Then, the absorbance at 450 nm was recorded on a microplate reader (SpectraMaxM5; Molecular Devices, CA, USA). Cell viability was expressed as the relative percentage to untreated MSCs.

**10. Cell apoptosis analysis**

Analysis of apoptotic cells was performed by using Annexin V/Propidium Iodide (PI) double staining method. In brief, $5 \times 10^5$ IFNβ-FTH-MSCs, eGFP-MSCs and WT-MSCs were harvested separately and washed thoroughly with ice-cold PBS, then resuspended in 200 μL of Annexin-binding buffer solution. 10 μL of Annexin V-APC and 5 μL PI were added to the cell suspension and incubated for 15 min in the dark. Cells were analyzed using a flow cytometer (FACSVerse, Becton Dickinson) to measure the apoptosis rate.

**11. Reactive oxygen species (ROS) assay**

The ROS assay kit (Genmed, Shanghai, China) was used to measure the production of intracellular ROS in the presence or absence of iron supplementation. In brief, IFNβ-FTH-MSCs, eGFP-MSCs and untreated MSCs were treated with or without 350 μM FC for 72 h; the cells were then harvested and resuspended in 1000 μL of diluent buffer containing 1 mM dihydroethidium (DHE). Following incubation at 37°C for 20 min, the cells were centrifuged, suspended, and examined on a FACSVerse flow cytometer (Becton Dickinson). An increased level of intracellular ROS was labeled by a rightward peak in the phycoerythrin (PE) fluorescent wave. The ROS production results were recorded as the geometric mean fluorescence (Geo-means).

**12. Cell phenotype analysis**

The phenotype of IFNβ-FTH-MSCs was characterized by FACS analysis of cell surface markers. In brief, IFNβ-FTH-MSCs were labeled with PE, V450 and APC-conjugated monoclonal antibodies against CD90, CD29, and CD45, respectively, for 30 min (1:10; BD Biosciences, San Jose, CA). Cell surface marker expression was then evaluated using a FACSVerse cytometer (Becton

Dickinson).

**13. Cell differential potential analysis**

IFNβ-FTH-MSCs, eGFP-MSCs and WT-MSCs were cultured using two different mesenchymal differentiation kits (Cyagen) for 2–3 weeks to induce osteogenic and adipogenic differentiation, and then were detected by Alizarin red and Oil Red O staining respectively.

**14. Transwell migration assay**

The transwell migration assay was used to assess the effect of transduction on the ability of MSCs to migrate toward glioma cells. F98 glioma cells were purchased from Cyagen Bioscience Technology Co. (Guangzhou, China). The cells were cultured and expanded at 37 °C under an atmosphere of 5% CO2 in high-glucose DMEM (HG-DMEM, GIBCO) containing 10% FBS (Cyagen, Guangzhou, China), supplemented with 1% penicillin/streptomycin (GIBCO). In brief, $1\times10^6$ F98 cells were incubated in serum-free media for 48 h, 600 μl of resultant conditioned media were then collected as the chemoattractant and placed in the bottom chambers of the transwell plate (Corning Costar, NY, USA) with 8-um pore filters. $2\times10^4$ IFNβ-FTH-MSCs, eGFP-MSCs and WT-MSCs were respectively suspended in 200 μl serum-free medium containing 0.1% bovine serum albumin (BSA, Sigma) and seeded into the top chambers. After incubation for 8 h at 37°C, non-migratory cells on the upper side of the filters were removed carefully by swabbing with a cotton swab. Cells that migrated to the lower surface were fixed and stained with 0.1% Crystal Violet Staining Solution (Bigtime, Shanghai, China) for 15 min. The number of migrated cells was counted under a light microscope in five high-power random fields (×40) (Nikon, Tokyo, Japan) per well, and the mean values were calculated. $2\times10^4$ WT-MSCs with the serum-free medium as stimulants were used as

negative controls.

**15. Intracranial orthotopic glioma model**

To establish the intracranial orthotopic glioma model, rats were anesthetized by with 3% isoflurane gas delivered by air at a rate of 3 liters/minute flowing over the nose/ head, and $5\times10^5$ F98 cells were stereotaxically injected into the left striatum over 5 min according to the following coordinates: 1 mm anterior and 3 mm lateral to the bregma, at 5 mm depth from the pial surface using a Hamilton 30-G needle connected to a 10-uL syringe (Hamilton Company, Reno, NV). Establishment of the intracranial gliomas was confirmed by in vivo MRI four days after inoculation of tumor cells.

**16. Cell delivery**

Before intracranial cell delivery, $2\times10^6$ IFNβ-FTH-MSCs were grown in FC supplemented medium for 72 h and then suspended in 5 μL PBS. The cell suspension was injected at a constant rate of 0.5 μL/min using a Hamilton 30-G needle connected to a 10-uL syringe (Hamilton Company, Reno, NV). After injection, the needle was kept in situ for an additional 15 min and then slowly withdrawn.

**17. Ex vivo ELISA**

Animals were sacrificed through anesthetic overdose, rat brains were then rapidly removed, and the ipsilateral cerebral hemisphere into which cell transplantation was performed was dissected on ice immediately. Then, the samples were homogenized in 1 g/mL homogenate buffer and centrifuged with a speed of 5000 rpm for 5 min at 4°C. Supernatants were collected for IFNβ secretion measurement with the ELISA kit (Elabscience Biotechnology Co., Ltd, Wuhan, China) according to the manufacturer's protocols. All samples were examined in triplicate.

**18. Immunohistochemical and immunofluorescence staining**

For immunohistochemical staining, brain sections were incubated with primary antibodies against CD8 (mouse anti-CD8, 1:200; Bio-Rad) and Batf3 (rabbit anti-Batf3, 1:200; Abcam, Cambridge, UK) respectively, followed by the corresponding secondary goat anti-mouse (1:200; Abcam) or anti-rabbit antibody (1:200; Abcam). After diaminobenzidine staining, slides were counterstained with hematoxylin for cell nuclei. Negative controls were established by omitting the primary antibody incubation. For immunofluorescence staining, brain sections were incubated with primary antibodies against CD8 (mouse anti-FTH, 1:200; Bio-Rad) and Batf3 (rabbit anti-Batf3, 1:200; Abcam, Cambridge, UK), then the corresponding Alexa 594-conjugated secondary antibody (goat anti-mouse,1:200; Invitrogen) and Alexa 647-conjugated secondary antibody (goat anti-rabbit, 1:200; Invitrogen) were added to the sections. Cell nuclei were counterstained with DAPI, and sections were observed using a confocal microscope (LSM-880; Zeiss, Jena, Germany).

**Supplementary Table 1. Imaging sequences and acquisition parameters of in vitro MRI**

| Sequence | TR/TE (msec) | Flip angle | Slice thickness/ gap (mm) | FOV ($mm^2$) | Acquisition matrix | Reconstruction matrix | NSA |
|---|---|---|---|---|---|---|---|
| **TSE T1WI** | 500/15 | 90˚ | 2/0 | 80×80 | 228 × 290 | 528×528 | 3 |
| **TSE T2WI** | 2000/100 | 90˚ | 2/0 | 80×80 | 268 × 267 | 512×512 | 3 |
| **FFE T2*WI** | 300/11.5 | 20˚ | 1/0 | 80×80 | 268 × 296 | 512×512 | 4 |
| **T2-map** | 2000/4×20 | 90˚ | 2 | 80×80 | 160 × 266 | 512×512 | 1 |

Abbreviations: TSE T1WI: turbo spin echo T1-weighted imaging; TSE T2WI: turbo spin echo T2-weighted imaging; FFE T2*WI: fast field echo T2*-weighted imaging; TR: repetition time; TE: echo time; FOV: field of view; NSA: number of signal average.

**Supplementary Table 2. Imaging sequences and acquisition parameters of in vivo MRI**

| Sequence | TR/TE (msec) | Flip angle | Slice thickness/ gap (mm) | FOV ($mm^2$) | Acquisition matrix | Reconstruction matrix | NSA |
|---|---|---|---|---|---|---|---|
| **TSE T2WI** | 1600/60 | 90˚ | 1/0 | 50×50 | 240×237 | 512×512 | 4 |
| **FFE T2*WI** | 500/22 | 30˚ | 1/0 | 60×60 | 300×300 | 768×768 | 3 |

Abbreviations: TSE T2WI: turbo spin echo T2-weighted imaging; FFE T2*WI: fast field echo T2*-weighted imaging; TR: repetition time; TE: echo time; FOV: field of view; NSA: number of signal average.

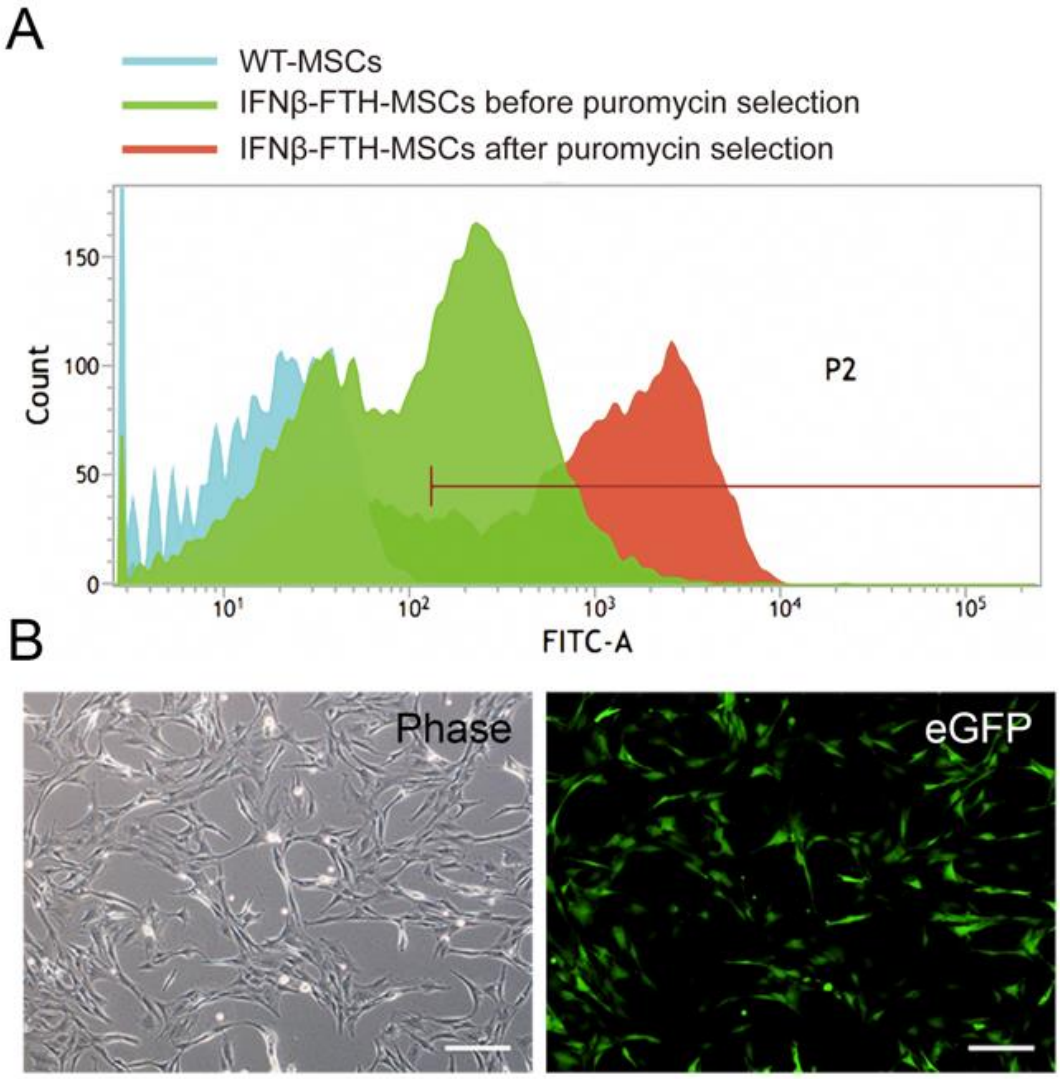
A
WT-MSCs
IFNβ-FTH-MSCs before puromycin selection
IFNβ-FTH-MSCs after puromycin selection
Count
150
100
50
0
P2
$10^1$
$10^2$
$10^3$
$10^4$
$10^5$
FITC-A
B
Phase
eGFP

**Figure S1.** Transduction efficiency. (A) FACS analysis of eGFP expression in IFNβ-FTH-MSCs with (red population) or without puromycin selection (green population). (B) Representative fluorescence micrographs show the eGFP expression in IFNβ-FTH-MSCs. Bar=200 μm.

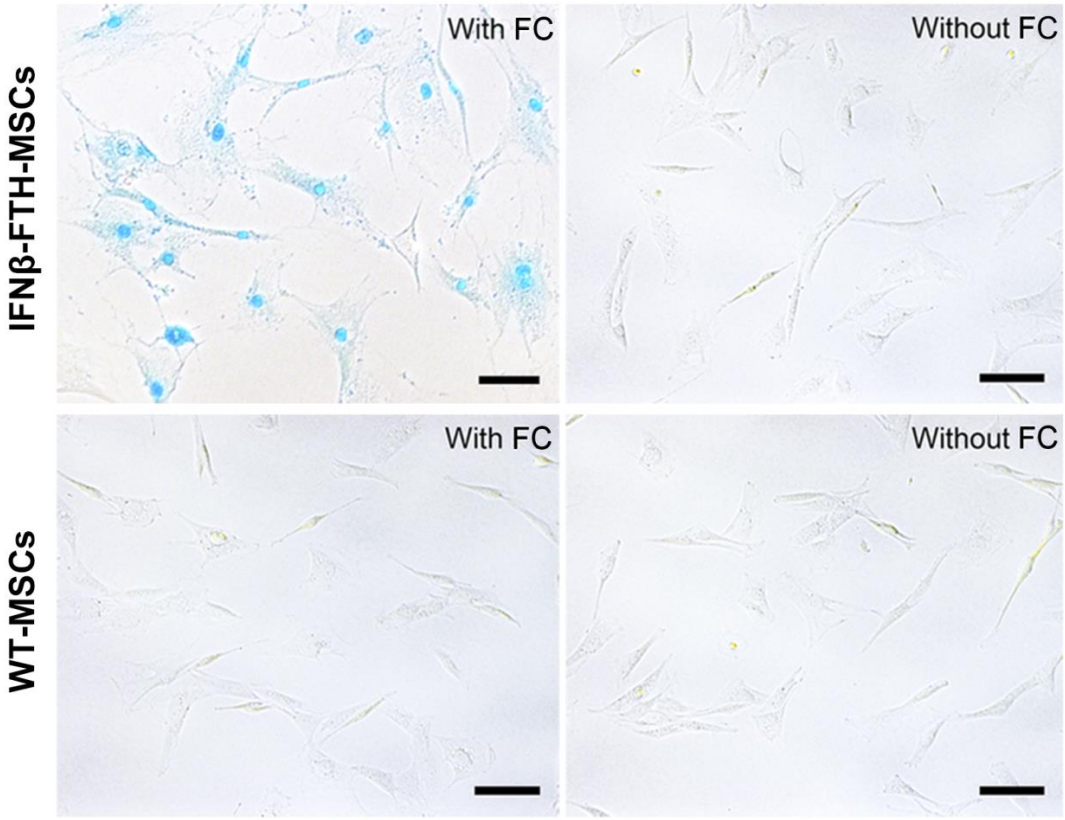


**Figure S2.** Prussian blue (PB) staining. Representative micrographs of cell PB staining showing abundant blue-stained particles inside IFNβ-FTH-MSCs with ferric citrate (FC) treatment, and negligible blue-stained particles in IFNβ-FTH-MSCs without FC treatment, or in WT-MSCs either with or without FC incubation. Bar = 100 μm.

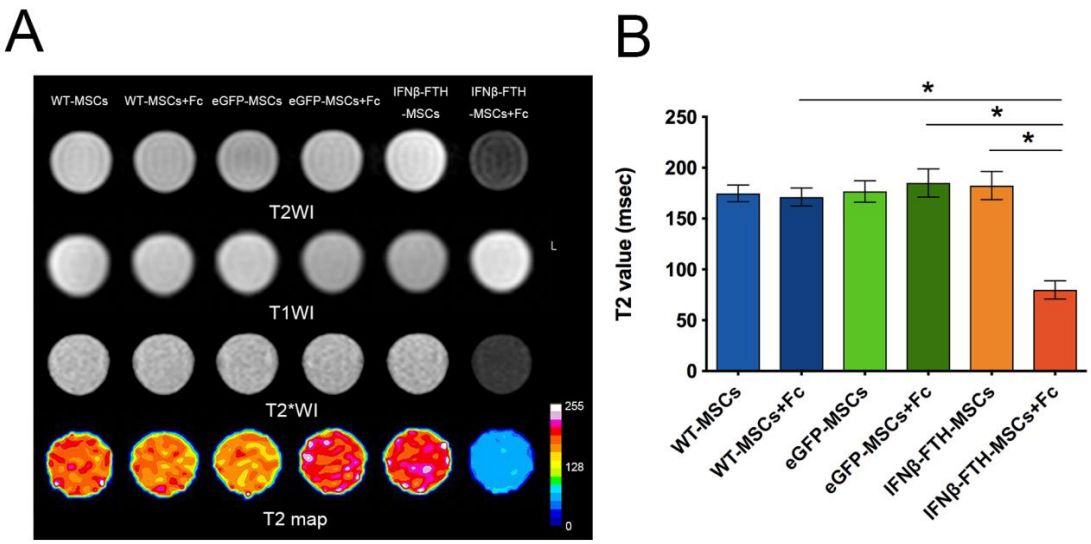


**Figure S3.** In vitro MRI. (A) In vitro MRI of IFNβ-FTH-MSCs treated with FC showed an obvious hypointense signal on T2W image, T2*W image and T2 map. The T2-map was pseudo-colorized with a color look-up table using ImageJ software. (B) Graph showing a significant reduction of T2 values in IFNβ-FTH-MSCs with FC treatment compared with other groups. $n = 3$. *$p < 0.05$.

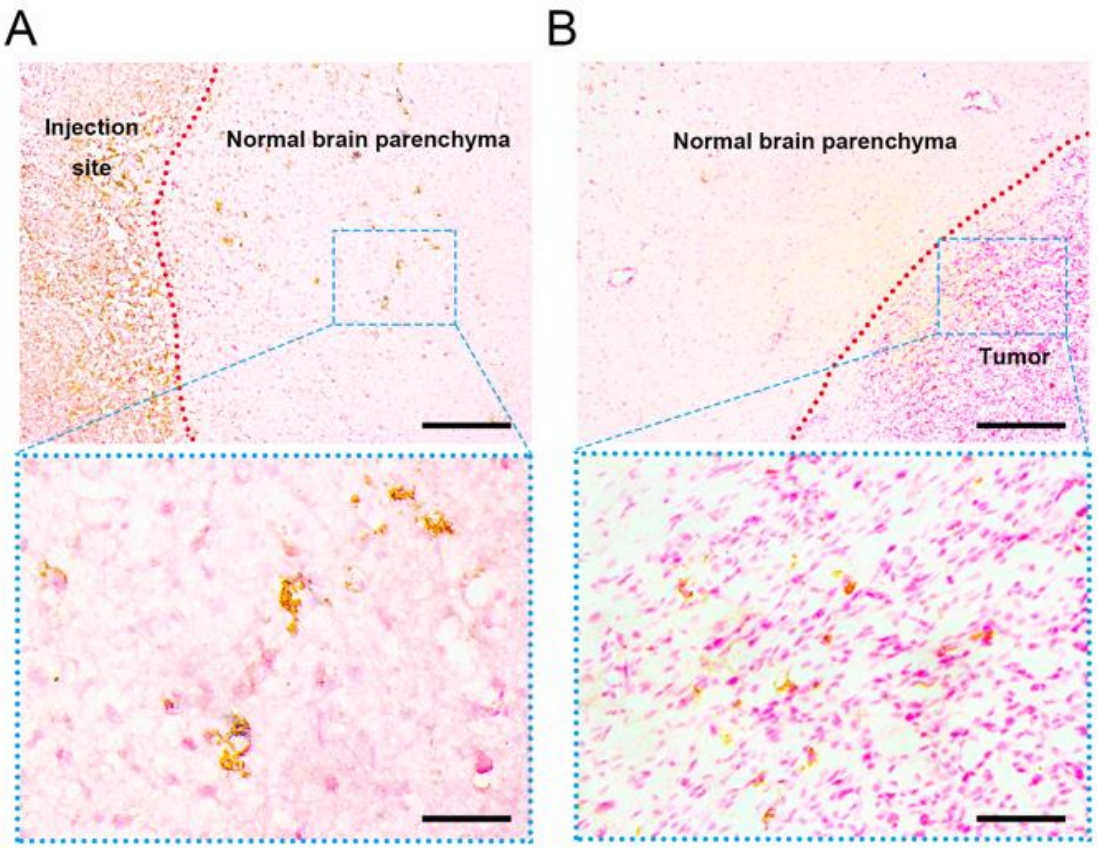


**Figure S4**. Representative micrographs of immunohistochemistry for FTH showing a small fraction of $FTH^+$ cells (brown) migrated from the injection site to the glioma (A) and infiltrated the tumor mass 6 days after intracerebral injection (B). Bar=200 μm (upper panel) and 50 μm (lower panel).

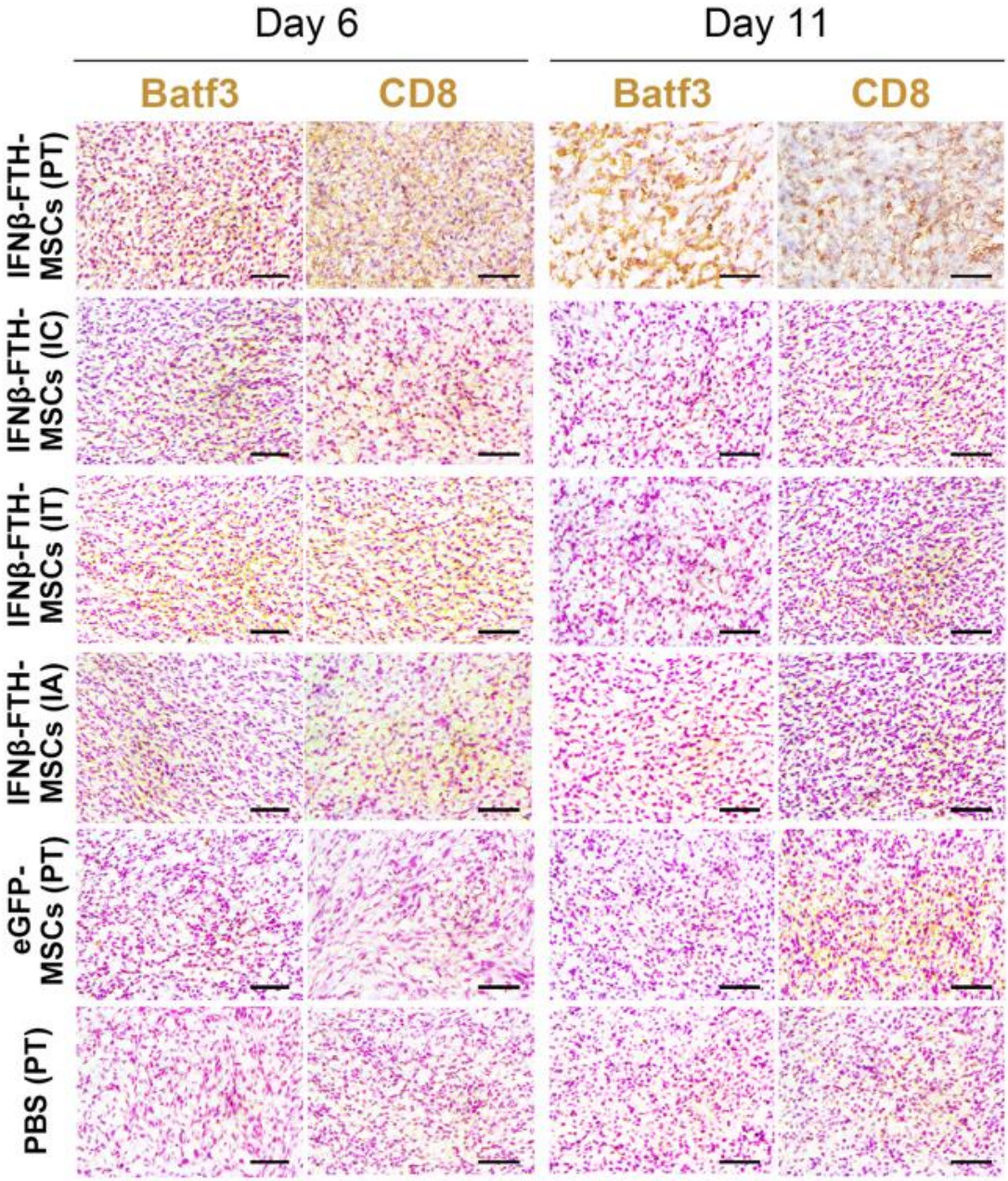


**Figure S5.** Representative micrographs of immunohistochemistry for Batf3 and CD8 show significantly enhanced Batf3$^+$ DCs and CD8$^+$ T cell infiltration (brown) within the tumor after peritumoral injection (PT) of IFNβ-FTH-MSCs compared to other groups on day 6 (6 days after injection) and day 11, while a moderately increased Batf3$^+$ DCs and CD8$^+$ T cell infiltration after

intracerebral (IC), intratumoral (IT) or intra-arterial injection (IA) of IFNβ-FTH-MSCs only on day 6.

Bar=50 μm.