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Supplemental Information

Intranasal Oncolytic Virotherapy with CXCR4-Enhanced Stem Cells Extends Survival in Mouse Model of Glioma

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Supplemental Figures and Legends:

Figure S1

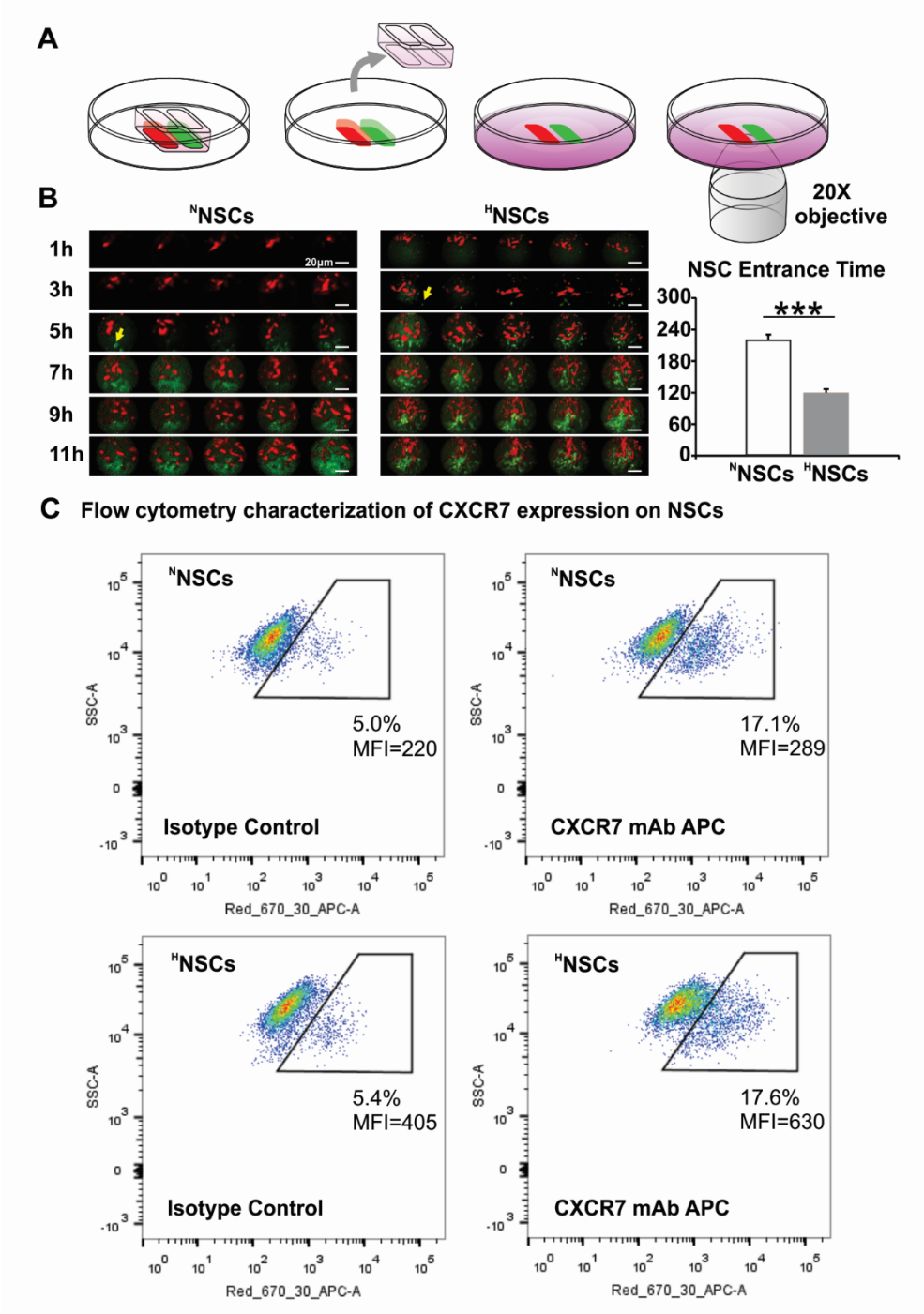

Figure S1. A. Schematic of VivaView^{TM} live imaging 2D migration assay using co-culture of U87cherry (red cells) and GFP+ NSCs (green cells) in an iBidi^{TM} silicone chamber. B. Montage of time series of cell behaviors at the U87cherry cell patch border shows an earlier emergence of GFP+ NSCs across the cell patch gap (yellow arrows indicate the earliest GFP+ NSCs at the U87cherry cell patch border; scale bars, 20μm). Significantly shortened time lapse (faster NSC tumor tropism) was observed for all cells cultured under hypoxia (n=6 fields of view, ***p< 0.001, Student's t test, error bars=SEM). C, Flow cytometry analysis of the other SDF1 binding receptor, CXCR7, on the surface of NSCs indicates minimal differences between ^{N}NSCs vs. ^{H}NSCs.

Figure S2

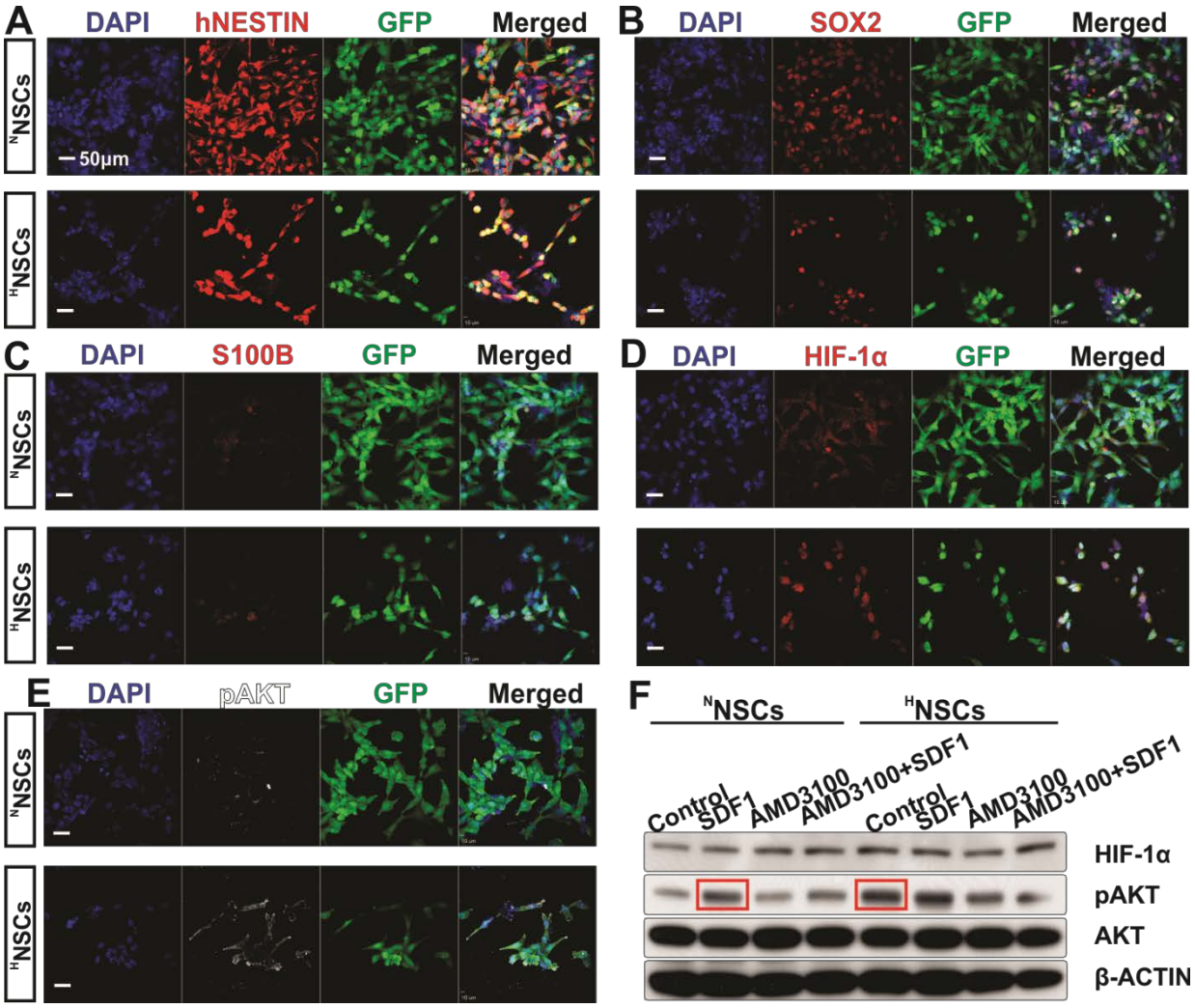

Figure S2. The analysis of the effect of hypoxia on phenotype of NSCs. A, hNESTIN staining (red) indicates no loss of hNESTIN in the cytoplasm of ^{H}NSCs. However, cells show the distinct migratory spindle cellular morphology in comparison to the non-directional morphologies of ^{N}NSCs. B, Reduced SOX2 expression in ^{H}NSCs in comparison to ^{N}NSCs; C, terminal astroglial differentiation marker S100B indicates no astrocyte differentiation under either conditions; D, HIF1α demonstrates striking nuclear accumulation in ^{H}NSCs when compared with the distribution patterns of ^{N}NSCs. Conversely, in E, phosphorylated AKT is more prominently distributed along the

cytoplasm membrane, suggesting an activated state of CXCR4 receptor and downstream signaling (e.g. AKT phosphorylation). F, HIF1a is increased in hypoxia treated NSCs; Western Blotting confirms the activation of pAKT by either SDF-1 or hypoxia pre-treatment (red boxes), and functional blockade of CXCR4 receptor activities by the CXCR4 receptor antagonist AMD3100. Scale bars, 50μm.

Figure S3

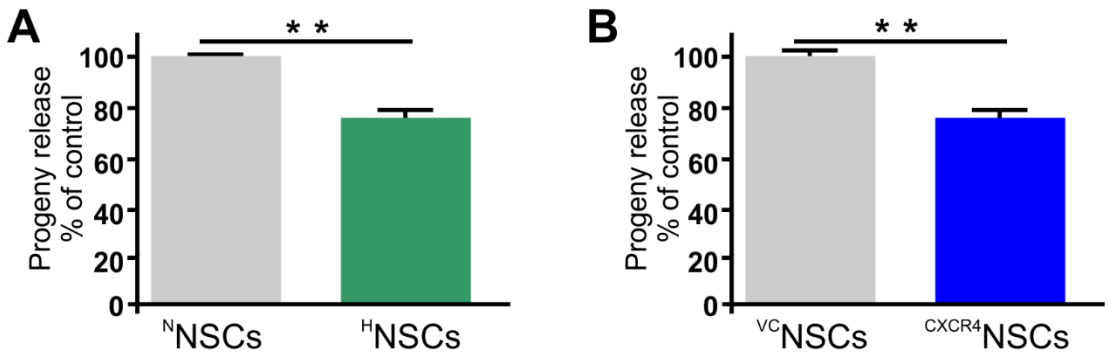

Figure S3. Evaluation of the viral replication in NSCs. A, Comparison of progeny release of NSCs cultured under normoxic and hypoxic conditions (n=3 , $^{**}p<0.01$, Student's t-test). B, Comparison of progeny release between ^{VC}NSCs or ^{CXCR4}NSCs (n=3,$^{**}p<0.01$, Student's t- test, error bars=SEM).

Figure S4

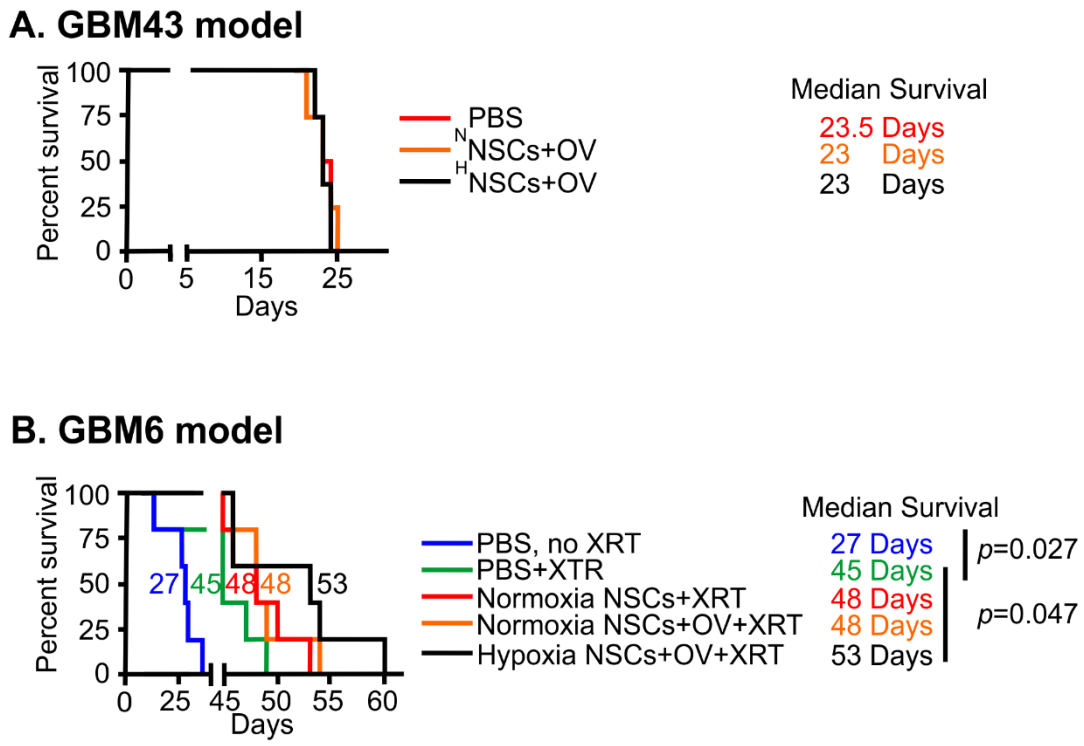

Figure S4. The effect of intranasally delivered OV loaded ^{H}NSCs on survival of mice bearing GBM43 and GBM6 xenografts. A, Without XRT treatment, neither OV loaded ^{N}NSC nor ^{H}NSC intranasal treatment regimens provides therapeutic survival benefits over PBS intranasal treatment in mice bearing GBM43 intracranial xenografts; B, the improvement in survival of mice (bearing GBM6 intracranial xenografts) treated with XRT and subsequently with OV loaded ^{H}NSCs replicates the effect achieved in patient derived GBM43 xenograft model (see Fig 6). Log-rank test has been performed to determine statistical difference between groups (n=8 mice/group for GBM43 and n=5 mice/group for GBM6 model).

Supplemental Methods:

Western blotting

The proteins were extracted using M-PER reagent (Thermo Fisher, Waltham, MA). Equal amounts of proteins were loaded into 4-20% gradient gels (Bio-Rad, Hercules, CA) and transferred to a PVDF membrane (Millipore, Billerica, MA). The primary antibodies used were against human NESTIN (hNESTIN), SOX2 (Millipore, Billerica, MA), β-ACTIN, βIII TUBULIN (Cell Signaling Technologies, Boston, MA), and the secondary antibodies used were anti-rabbit, or anti-mouse IgGs conjugated with infrared dyes for multiplex quantitative Western Blot. Stained membranes were imaged on a Li-COR Odyssey scanner and the data quantified using the Image Studio Lite software (Li-COR Biosciences, Lincoln, Nebraska).

Flow cytometry

To analyze NSC differentiation profiles and CXCR4/CXCR7 expression levels, cells were collected by trypsinization, and resuspended in flow buffer (1% bovine serum albumin [BSA] in sterile PBS) at 200k cells/200µl/well. Cells were then stained with APC-labeled antibodies against CXCR4, CXCR7 or isotype control IgG. NSCs were analyzed using a LSRII 4-12 analyzer and the FACS Diva™ software (BD, Franklin Lakes, NJ, USA). The flow cytometry data were processed using the FlowJo™ software (FlowJo LLC, Ashland, OR, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from NSCs using RNeasy plus kit (Qiagen, Boston, MA). One microgram RNA was reverse-transcribed using iScript cDNA conversion kit (Bio-Rad, Hercules, CA). qRT-PCR was conducted using SYBR green kit (Bio-Rad, Hercules, CA, USA) using primers indicated in Supplementary Table 1. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method for relative quantification, and all sample values were normalized to the *GAPDH* expression value.

Cytogenetics

Conventional cytogenetic analysis of $^{\mathbf{CXCR4}}$NSCs and ^{VC}NSCs was performed on unstimulated short-term cultures at the Cancer Cytogenetics laboratory at the University of Chicago. Slides for metaphase G-banding were prepared using standard techniques. Slides were scanned, metaphases were captured and analyzed using a CytoVision GSL-120 (Leica Microsystems, Switzerland) image analysis system.

Migration Assays

To compare the tumor tropism of ^{H}NSCs and ^{N}NSCs, we utilized a series of distinct *in vitro* migration assays. Firstly, transwell assay was performed. Patient-derived GBM43 cells and U87MG cherry cells were grown in serum-free Neurobasal medium and supplements for 72 h to cultivate neurospheres and adopt a glioma stem cell-like profile in a Med-Tek glass bottom cell culture dish (Med-Tek LLC, Miami, FL, USA; 200k cells). After 24 h of hypoxia or normoxia treatment, GFP-expressing NSCs were seeded in a cell culture insert (8µm pores; ThermoScientific, Billerica, MA, USA) at

200K/culture insert, and placed in the Med-Tek dish on top of the glioma neurospheres. Cells were fixed with 2% paraformaldehyde (PFA) after 24-48 h and mounted in Vectashield^{TM} medium with DAPI nuclear stain (Vector Labs, Burlingame, CA, USA). Confocal microscopy was performed to analyze the number of GFP^+ NSCs that penetrated the glioma tumorspheres (Fig. 1, GBM43 and U87MG mCherry cells). Secondly, we compared tropism of NSCs toward glioma cells via additional two strategies: 1), GBM43blue (blue fluorescent protein) or U87MG mCherry cells were grown as adherent monolayers in 10% FBS-containing DMEM medium, and were then coated with a layer of nutrient-enriched collagen matrix (Sigma-Aldrich, St. Louis, MO, USA), followed by seeding of GFP^+NSCs on top of the solidified collagen gel layer. After overnight (12h) incubation, cells were fixed in 2% PFA for subsequent analysis of the number of GFP^+ NSCs that migrated to glioma cells; 2). Lastly, to determine the roles of SDF-1 in the observed tumor tropism by NSCs, we utilized the 3D chemotaxis assay chambers from iBidi USA. Briefly, 6µl of NSCs (^{H}NSCs, ^{N}NSCs, or ^{CXCR4}NSCs) in nutrient enriched collagen gel were filled in the central fill ports and allowed to solidify over 30min, forming a uniform gel barrier separating the left and right reservoirs, which were then filled with SDF-1 (10ng/ml) containing reduced serum (1% FBS) DMEM on the left and containing control medium on the right. The migration of NSCs towards SDF-1 containing reservoir was monitored over 24 h using light microscopy. The average travel distances (pixels) of the 30 migrated cells were measured.

Time lapse microscopy of migrating stem cells

To compare the chemotaxic response of NSCs to glioma cells, we also utilized the ibidi dual chambers (ibidi USA Inc., Madison, Wisconsin, USA) to create neighboring

adherent cell patches and NSCs were allowed to migrate toward the glioma cells. VivaView™ incubator epifluorescence based live microscopy videos (Olympus of the Americas, Center Valley, PA, USA) were captured at 24 min intervals using the MetaMorph™ software (Molecular Devices LLC, Sunnyvale, CA, USA; Fig. S1A-C).

Immunocytochemistry and confocal microscopy

To confirm the findings of flow cytometry, Western Blot, and qPCR results, NSCs were seeded in glass bottom 96 wells at 100k cells / well density until adherence. The plates were then subjected to hypoxia or normoxia treatments for 24 h, and cells were then fixed in 2% PFA for 30 min, washed with PBS, permeabilized using 0.3% PBS-TritonX100 (not performed for CXCR4 and CXCR7 staining), blocked in 2% BSA with 2% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) and were subsequently incubated at 4ºC in primary antibodies (details see Supplementary Table 2), washed, and incubated for 1 hour with fluorophore conjugated secondary antibodies or isotope conjugated IgG.

Following the *in vivo* treatments, animals were sacrificed, perfused with PBS and 4% PFA via intra-cardiac puncture, and their brains were excised and flash frozen. Frozen tissue sections of 20 µm thickness were then probed with FITC conjugated anti-hexon antibody (Millipore, Billerica, MA, USA) or anti-SDF-1 rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Vectashield™ mounting medium with DAPI (Vector Labs, Burlingame, CA, USA) was used for nuclei counterstains. Confocal microscopy was performed at the University of Chicago Integrated Light Microscopy Facility using a 3i Marianas Yokogawa-type spinning disk confocal microscope with an

Evolve™ EM-CCD camera (Photometrics, Tucson, AZ) running SlideBook™ v5.5 software (Intelligent Imaging Innovations, Denver, CO). Whole slide scans were performed using a 3D Histech Pannoramic Scan whole slide scanner (Perkin Elmer, Waltham, MA) with a Zeiss AxioCam MRm CCD camera (fluorescence; Carl Zeiss Microscopy, Thornwood, NY) or Stingray F146C color camera (histology; Allied Vision Technologies, Stadtroda, Germany). Individual images were created with the 3D Histech Pannoramic Viewer™ software (Perkin Elmer, Waltham, MA). Digital images were then processed and analyzed for quantitative measurements using the Fiji™ Software. For SDF-1 and hexon quantitative comparison, 10-22 tissue sections sampled from 3 animal brains were analyzed for each experimental group.

Supplemental Tables

Table S1. List of Primers for qRT-PCR

Genes	Forward 5'-3'	Reverse 5'-3'
hNESTIN	ATC GCT CAG GTC CTG GAA GG	AAG CTG AGG GAA GTC TTG GAG
SOX2	GTT GTC AAG GCA GAG AAG AG	GAG AGA GGC AAA CTG GAA TC
MUSASHI	GAG ACT GAC GCG CCC CAG CC	CGC CTG GTC CAT GAA AGT GAC G
β III TUBULIN	CTC AGG GGC CTT TGG ACA TC	CAG GCA GTC GCA GTT TTC AC
GFAP	GGT ATC GGT CCA AGT TTG C	GCC TCT CCA AGG ACT CGT TC
β ACTIN	GGA CTT CGA GCA AGA GAT GG	AGC ACT GTG TTG GCG TAC AG

Table S2. List of Antibodies

Antibody	Species	Dilution	Vendor
hNESTIN	Rabbit	1:1000 for ICC, 1:5000 for Western Blotting	EMD Millipore
SDF-1	Rabbit	1:500 for ICC, 1:1000 for Western Blotting	Santa Cruz Biotechnology
SOX2	Rabbit	1:500 for ICC, 1:1000 for Western Blotting	EMD Millipore
β III TUBULIN	Rabbit	1:500 for ICC, 1:1000 for Western Blotting	Cell Signaling
S100B	Rabbit	1:200 for ICC, 1:1000 for Western Blotting	Santa Cruz Biotechnology
GFAP	Mouse	1:500 for ICC, 1:1000 for Western Blotting	Cell Signaling
β-ACTIN	Mouse	1:1000 for ICC, 1:1000 for Western Blotting	Santa Cruz Biotechnology
MUSASHI	Rabbit	1:250 for ICC, 1:500 for Western Blotting	EMD Millipore
HIF-1α	Goat	1:500 for ICC, 1:1000 for Western Blotting	Santa Cruz Biotechnology
AKT	Rabbit	1:500 for ICC, 1:1000 for Western Blotting	Cell Signaling
Phospho-AKT	Mouse	1:500 for ICC, 1:1000 for Western Blotting	Cell Signaling
Hexon-FITC	Mouse	1:200 for ICC	AbD Serotec
CXCR4-APC	Mouse	5µl / million cells for Flow Cytometry	BioLegend
CXCR7-APC	Mouse	5µl / million cells for Flow Cytometry	BioLegend
Isotype IgG2a	Mouse	5µl / million cells for Flow Cytometry	BioLegend

Author contributions:

MD, DY**:** Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing. DK: Collection and/or assembly of data, data analysis and interpretation. GL, MS, DAS, KCP, YH: Collection and/or assembly of data. LZ: Data analysis and interpretation. AUA: Data analysis and interpretation, manuscript

KSA: Provision of study material. MSL: Conception and design, financial support, data analysis and interpretation. IVB: Conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.