 doi:10.20944/preprints202008.0028.v1

Combination of Mesenchymal Stem Cell-delivered Oncolytic Virus with Prodrug Activation Increases Efficacy and Safety of Cancer Therapy

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 doi:10.20944/preprints202008.0028.v1

List of Abbreviations:

Ad-5: Adenovirus type 5

CRAd: Conditionally replicative adenovirus

FBS: Fetal bovine serum

GDEPT: Gene-directed enzyme-prodrug therapy

i.p.: Intraperitoneal

MSC: Mesenchymal stem cell

NAb: Neutralized antibody

NTR: Nitroreductase

s.c.: Subcutaneous

SPE: Solid-phase extraction

TSA: Trichostatin A

qTOF: Quadrupole-time-of-flight

 doi:10.20944/preprints202008.0028.v1

Abstract

Although oncolytic viruses are currently being evaluated for cancer treatment in clinical trials, systemic administration is hindered by many factors that prevent them from reaching the tumor cells. When administered systemically, mesenchymal stem cells (MSCs) target tumors, and therefore constitute good cell carriers for oncolytic viruses.

Methods: MSCs were primed with trichostatin A under hypoxia, which upregulated the expression of CXCR4, a chemokine receptor involved in tumor tropism, and coxsackievirus and adenovirus receptor that plays an important role in adenoviral infection. After priming, MSCs were loaded with conditionally replicative adenovirus that exhibits limited proliferation in cells with a functional p53 pathway and encodes *Escherichia coli* nitroreductase (NTR) enzymes (CRAdNTR) for targeting tumor cells.

Results: Primed MSCs increased tumor tropism and susceptibility to adenoviral infection, and successfully protected CRAdNTR from neutralization by anti-Adenovirus antibodies both *in vitro* and *in vivo*, and specifically targeted p53-deficient colorectal tumors when infused intravenously. Analyses of deproteinized tissues by UPLC-MS/QTOF revealed that these MSCs converted the co-administered prodrug CB1954 into cytotoxic metabolites, such as 4-hydroxylamine and 2-amine, inducing oncolysis and tumor growth inhibition without being toxic for the host vital organs.

Conclusion: This study shows that the combination of oncolytic viruses delivered by MSCs with the activation of prodrugs is a new cancer treatment strategy that provides

 doi:10.20944/preprints202008.0028.v1

a new approach for the development of oncolytic viral therapy for various cancers.

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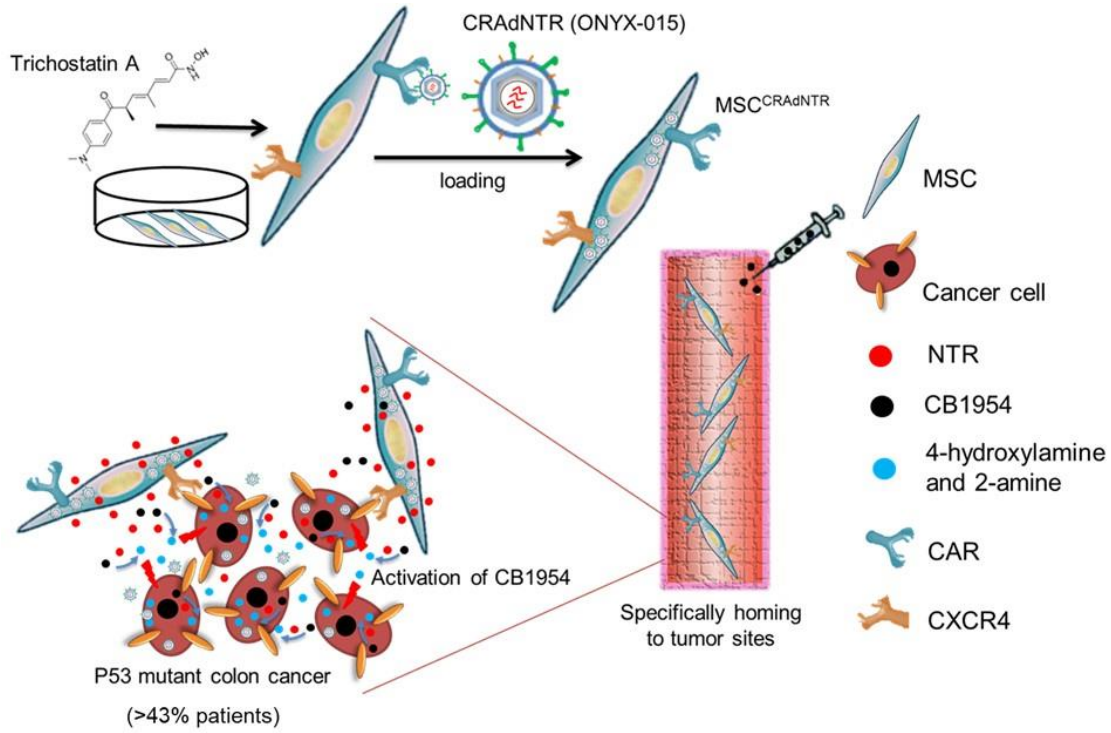
Graphical abstract:



Trichostatin A-primed MSCs increase tumor tropism and susceptibility to adenoviral infection, protect CRAdNTR from neutralization, and induce oncolysis of p53-deficient tumors by CB1954 activation without being toxic for the host.

Keywords: Oncolytic virus; Mesenchymal stem cell; Prodrug activation; P53 mutant tumor; Colorectal cancer

Running title: *Oncolytic virotherapy with MSC-delivery and prodrug activation*

 doi:10.20944/preprints202008.0028.v1

Introduction:

Colorectal cancer is a major global health problem owing to its high prevalence and mortality rates, and it is the fourth most common cancer worldwide, including many parts of the western world [1]. The most prevalent problem in cancer therapy is the regrowth and metastasis of malignant cells after standard treatment with surgery, radiation, and/or chemotherapy. Colorectal cancer recurrence can occur either locally or at a distant site; therefore, metastatic colorectal cancer remains difficult to treat effectively.

Oncolytic virotherapy is an emerging treatment modality with great therapeutic potential that uses replication-selective viruses to replicate specifically in tumors to destroy tumor cells by cytolysis [2]. Once the tumor cells are destroyed, viral progenies are released, allowing them to continuously hijack the neighboring tumor cells. This property is either inherent or genetically engineered [3]. Human adenovirus serotype 5 (Ad5) is one of the most widely studied engineered oncolytic viruses in preclinical studies and clinical trials [4]. When adenoviruses target host cells, coxsackievirus and adenovirus receptor (CAR) plays an important role in the infection process [5, 6]. Conditionally replicative adenovirus (CRAd) has recently been used in preclinical studies and clinical trials to treat a broad range of cancers [7]. ONYX-015, an adenovirus made conditionally replicative by E1B-55k gene deletion [8, 9], is a

 doi:10.20944/preprints202008.0028.v1

potential candidate for treatment of p53-deficient cancers, with minimum replication in cells with a functional p53 pathway [10]. The safety of CRAd for cancer therapy has been verified in early phase clinical trials [11]. However, its antitumor efficacy has not been well documented, since there are many systemic barriers that prevent it from reaching tumor cells when administered systemically [12].

Multipotent stromal cells (MSCs) possessing self-renewal and multipotent capacities are inherently tumor-homing [13-15], and can be isolated, expanded, and transduced, making them viable candidates for cell carriers to deliver oncolytic viruses [16]. The migration of MSCs is induced by several chemokine/chemokine receptor-mediated signaling pathways, in which the chemokine receptor CXCR4 plays a crucial role with regard to tumor tropism, as it allows dynamically interacting with the host by responding to stromal cell-derived factor-1 (SDF-1) α secreted by tumors [17]. Previous studies have also shown that MSCs expanded under hypoxic conditions (referred to as hypoxic MSCs) exhibit greater proliferation, reduced senescence, increased stemness, and increased homing to tumor sites via upregulation of CXCR4 [16, 18, 19]. Moreover, hypoxic MSCs are immunologically privileged, and could be allo-transplanted, without causing severe adverse effects [20].

Gene-directed enzyme-prodrug therapy (GDEPT), also known as suicide gene therapy, is based on the delivery of a foreign gene that encodes a prodrug-activating

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enzyme, followed by systemic administration of a nontoxic prodrug that is subsequently converted into a potent cell-killing drug [21]. The concept of using a bacterial nitroreductase (NTR) enzyme to react with its substrate prodrug 5-(azaridin-1-yl)-2,4-dinitrobenzamide (CB1954), and produce potent DNA cross-linking intermediates that can induce apoptosis in both dividing and non-dividing cells, is a promising and emerging GDEPT strategy that has reached clinical trials [22, 23]. In the current study, we first improved the susceptibility of MSCs to CRAds infection, and further increased MSC tumor tropism. We then combined MSC-delivered CRAds and prodrug activation to kill colorectal cancer cells both *in vitro* and *in vivo*. Through this combinational approach, we are able to increase both efficacy and safety of cancer therapy.

Materials and Methods

Cell culture

Human primary MSCs from bone marrow were harvested with the approval of Institutional Review Board and prepared as described previously [24]. The cells were seeded at 50 cells/cm^2 and grown in α-minimal essential medium (α-MEM; Gibco, Gaithersburg, MD), supplemented with 16.6 % fetal bovine serum (FBS; Gibco), 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate and 25 μg/mL

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amphotericin B (Antibiotic Antimycotic Solution, Sigma-Aldrich, St. Louis, MO). For the hypoxic culture, MSCs were maintained as previously described [19]. The MSCs were cultured in a gas mixture composed of 94 % N_2, 5 % CO_2 and 1 % O_2, which was maintained using an incubator with two air sensors: one for CO_2 and the other for O_2. The O_2 concentration was achieved and maintained via the delivery of N_2 from a tank containing pure N_2. IF the percentage of O_2 exceeded the desired level, N_2 gas was automatically injected into the system, to displace the excess O_2. The passage number of MSCs used in this study was no more than five. MSCs were treated with 100 ng/mL Ttichostatin A (TSA, Sigma-Aldrich, St. Louis, MO) for 24 h given a substitution name as primed MSCs, applied in subsequent study. Human colorectal cancer cell line HT29, p53-mutant cancer cells [25], was obtained from the American type culture collection (ATCC, Rockville, MD) and grown in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% FBS.

Flow cytometry

To confirm the MSCs phenotype and to analyze the effect on the surface expression level of CXCR4 and CAR after TSA treatment, the MSCs were plated at 2×10^5 per 10-cm dish recovered for 24 h. The MSCs were treated with 100 ng/mL TSA for 24 h. After 24 h, the primed MSCs were washed with phosphate-buffered saline (PBS) and

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were harvested with Accutase™ (StemCell Technologies Inc., Vancouver, Canada) and incubated with control rabbit IgG (eBioscience, San Diego, CA), or polyclonal antibodies against human CXCR4 (1:50, Novus Biologicals, Littleton, CO) and CX3CR1 (1:100, Abcam, Cambridge, UK), CAR (1:100, Merck KGaA, Darmstadt, Germany), CD29 (1:50, Ancell, MN), CD31 (1:50, Ancell, MN), CD34 (1:50, Ancell, MN), CD44 (1:50, BD pharmogen), CD45 (eBioscience, 1:20), CD105 (1:50, Ancell), at 4 °C for 1 h. After the PBS wash, the cells were incubated with PE-conjugated, goat anti-rabbit IgG, at 4 °C for 30 min. The samples were then analyzed on a Becton Dickinson FACSCalibur flow cytometer and data analysis was performed using BD CellQuest Pro software version 4.0.2.

In vitro migration assay

HT29 human colorectal cancer cell line was plated onto 24-well plates at $1x10^5$ cells per well in DMEM supplemented with 10% FBS for 24 h before the migration assay was performed. Hypoxic MSCs were treated with 100 ng/mL TSA for 24 h, then MSCs and hypoxic primed MSCs were seeded onto the 8 μm pore-size cell culture inserts (Corning, NY), at 5000 cells per well, in DMEM supplemented with 10% FBS, and allowed to adhere for 1 h at 37°C. The inserts were then added to the lower chambers with or without HT29 and incubated at 37 °C, with 5 % CO_2. After 14 h of

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migration, the medium was removed and cells that remained attached to the upper surface of the insert filters were removed, using cotton swabs. The cells that had migrated to the opposite side of the filters were stained with Fluoroshield™ with DAPI (Sigma-Aldrich, St. Louis, MO) and counted under a fluorescent microscope at 100× magnification. The average number of migrating cells per field was assessed by counting six random fields per filter.

Adenovirus infection

Hypoxic primed MSCs were trypsinized and count the cell numbers, then infected with CRAdNTR (PS1217H6), kindly gifted by Dr. Ming-Jen Chen of Mackay Memorial Hospital, at a multiplication of infection (MOI) of 1000 vp/cell, in serum free α-MEM, for 1.5 h at 37 °C, and then an equal volumes of growth medium (10%FBS) was added. After 24 h of infection, the medium was replaced with fresh culture medium, containing 10 % FBS. CRAdNTR loaded-MSCs were indicated to $MSC^{CRAdNTR}$. For investigation of adenoviral infection rate, CRAdEGFP was used in the same method to MSCs.

Combination cytotoxicity assay

To determine the cytotoxic effect of CB1954 in combination with MSCs-delivered CRAdNTR, HT29 cells were plated in a 24-well plate at 2×10^6 cells per well for 24 h. The hypoxic primed MSCs, $MSC^{CRAdNTR}$ for 48 h in a ratio of 1 : 10 and 2×10^8 CRAdNTR (MOI=1000) were direct co-cultured with HT29 cells in presence of 50 µl high titer human serum diluted in 2 mL DMEM per well of a 24-well plate. The mixed culture cells were incubated at 37 °C for 2 days. Then, cells were treated with

 doi:10.20944/preprints202008.0028.v1

10μM CB1954 for 2 days, before the determination of cell viability using the tetrazolium-based colorimetric assay (MTT) and crystal violet staining. At different times during the culture, the medium was removed, the cells were washed once with PBS and 900 μL of medium and 100 μL of MTT solution (5 mg/mL) was added into each well and incubated at 37 °C for 4 h. At the end of incubation, the medium was aspirated and 200 μl of DMSO was added to dissolve the dye. The absorbance at O.D.570 was measured using Tecan microplate reader (Infinite M1000, Tecan). The results were shown as the mean of three independent replicates ± SD. The control wells without cells were used to account for the colorant retained by the culture plates. For crystal violet staining, the cells were fixed with 10 % buffered formalin for 5 min and stained with 1 % crystal violet, at room temperature for 20 min. Images of the plates were then captured with a scanner. The MTT assay and the crystal violet were performed in parallel.

Genomic DNA extraction

The excision of tissue and organ (20 mg) from mouse including tumor, heart, liver, lung and kidney were quickly minced and ground with micropestles. For adherent cells, the cells were trypsinized before harvesting. The cells were then centrifuged for 30 sec at 13000 ×g, to discard the supernatant. The EasyPure Genomic DNA mini kit (Bioman, Taiwan) was used to extract genomic DNA. Aliquots of 600 μL cell lysis solution were added to the extracted sample. For tissue extraction, proteinase K (4mg/mL, Sigma-Aldrich, St. Louis, MO) was added to the lysate and mixed by inverting 25 times and incubated at 55 °C for 3 h to overnight, until tissue particles have dissolved. For lysing the cells, pipetting up and down was made until no visible cell clumps remain, then 3 μl of 20 mg/mL RNase A was added to the nuclear lysate and the sample was mixed by inverting the tubes 25 times and incubated for 15-30

 doi:10.20944/preprints202008.0028.v1

min at 37 °C, followed by adding 200 µl of Protein Precipitation solution to RNase-treated samples and vortexing vigorously at high speed for 20 sec and centrifugeing for 3 min at 13000 ×g. The precipitated proteins will form a tight white pellet and the supernatant containing the DNA was transfered carefully to a clean 1.5 mL-tube containing 600µl of room temperature isopropanol (Sigma-Aldrich, St. Louis, MO) and the solution was gently mixed by inversion until white threadlike strands of DNA forming a visible mass then the solution was centrifuged for 1 min at 13000xg at room temperature. Then, using 600 µL 70 % ethanol (Sigma-Aldrich, St. Louis, MO) and interval several times to wash DNA. Finally, the DNA was rehydrated in the DNA rehydration solution by incubating at 65 °C for 1h and the genomic DNA concentration was subsequently determined at OD 260/280 and OD 260/230. The genomic DNA was stored at 4 °C

PCR

Expression of the adenoviral E1A gene was confirmed by PCR using forward E1A primer and as internal control (primer sequence are list on Table S1). The amplification was carried out in a total volume of 25 µL containing 500 ng genomic DNA, 0.4 µM of each primer, 200 µM dNTP (Takara Biochemicals, Otsu, Japan) 1.5 mM $MgCl_2$ (Roche), 1U FastStart Taq polymerase (Roche) and 1× PCR reaction buffer (Roche). The entire mixture of 25 µL was subjected to 35 cycles of 1 min denaturation at 95°C, 1 min to allow annealing at 63°C, and 2 min of extension at 72°C. During the last cycle, the extension time was increased by 7 min. During the last cycle, the extension time was increased by 7 min. Amplified products were analyzed by 1.5% agarose gel electrophoresis.

Quantitative real-time PCR

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The method of quantitative real-time RT-PCR was performed as described [26].Total RNA (2 μg) of each sample reversely transcribed in 20 μl using 0.5 μg of oligo (dT) (Invitrogen, Carlsbad, CA) and 200U Superscript III RT (Invitrogen, Carlsbad, CA). Amplification was carried out in a total volume of 20 μL, with SYBR Green PCR MasterMix (Applied Biosystems, Foster City, CA), the cDNA and 500 nM of each primer. All of Primer sequences are list on Table 1. The reaction conditions were one cycle at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing at 56 °C for 15 sec, and extension at 72 °C for 40 sec. Standard curves (cycle threshold values versus template concentration) were prepared for each target gene and for the endogenous reference (GAPDH) in each sample. The quantification of the unknown samples were performed by the ABI Applied Biosystems (Foster City, CA) with StepOne software v2.0.

Human xenograft HT29 colorectal cancer model

All experimental procedures involving animals were reviewed and approved by the animal center committee of China Medical University. To establish peritoneal colorectal cancer mice models, eight-week-old male athymic Balb/c mice were s.c. injected with 1×10^6 HT29 colon carcinoma cells. After 14 days, the mice were randomly divided into three groups of 9 mice each and i.v. injected once with 1×10^6 control primed MSCs (MSC), CRAdNTR-loaded primed MSCs ($MSC^{CRAdNTR}$), or 1×10^9 CRAdNTR suspended in the 1 : 10 diluted NAbs-containing human serum (50 μL serum in 500 μL PBS). Two days later, all mice were i.p. administrated with 25 mg/kg CB1954 (Sigma-Aldrich, St. Louis, MO) for 5 consecutive days per week for 2 cycles. After 14 days of last cycle of CB1954 administration, all mice were given third cycles of 25 mg/kg CB1954. The mice were sacrificed for harvest of tumor and vital organ tissues 7 days later. Tumors from all groups of mice were measured every

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week with calipers after treatment. The volume was estimated by the formula: volume = width^2 × length/2.

Immunohistochemistry

Resected xenograft tumor fixed in formalin, paraffin-embedded and sectioned at 5 μm thickness. Paraffin-embedded xenograft tumor sections were deparaffinized, rehydrated and antigen retrieved by sodium citrate buffer pH 6.0 at 100°C for 30 min. Endogenous peroxidase activity in tissue was blocked with 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO).Residual enzymatic activity was removed by washing in PBS, and non-specific staining was blocked with Ultra V block (Thermo Scientific, Fremont, CA) for 10 min and incubated with primary antibodies against adenovirus type 5 (1:800, Ad-5,rabbit polyclonal antibody, ab6982, Abcam, Cambridge, MA) and NTR was detected using polyclonal sheep anti-NTR antibody, was kindly provided by Dr. Chen, MJ, of Mackay Memorial Hospital, Taipei, at 4°C overnight, , washed extensively with PBS. After that, for primary Ad-5 antibody, the sections were incubated at room temperature with secondary polymeric antibody following the manufacturer's instruction (Super SensitiveTM IHC Detection Systems Kit, BioGenex, Milan, Italy). For primary NTR antibody, reacted with a HRP conjugated secondary rabbit anti-sheep antibody (1:5000, 31480, Thermo Scientific Pierce, US) at room temperature for 1 h. followed by diaminobenidine (DAB) staining (LSAB kit, Dako, Carpinteria, CA). Finally, counterstaining was performed with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO) and observed with a microscope.

UPLC/MS-QTOF

Sample preparation

For samples containing FBS, 2 v methanol was added and the samples were held

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at -20°C to precipitate proteins. Then, centrifuged at 5000 rpm for 15 min and transferred the supernatant to clear tubes. For tissue sample, to extracted the metabolites the 100 mg tissue, including heart, liver, lung, kidney and xenograft tumor, were ground micropesles followed by adding 500 mL of 50% acetonitrile (ACN, JT-baker), then, homogenized by using microtube homogenizer (BeadBug™, Benchmark, Edison, NJ), the homogenous samples were centrifuged at 13000 rpm for 10 min, harvested the supernatant and transferred to clear tubes. To remove matrix effect, samples were cleaned-up by off-line solid-phase extraction (SPE) using Oasis HLB™ Cartridge 1 cc (30 mg) (Waters, Wexford, Ireland). The SPE cartridges were conditioned with 1.0 mL methanol followed by 1.0 mL water Milli-Q and the samples were loaded in the cartridge. Then, cartridges were washed with 1 mL of 5% (v/v) methanol in water Milli-Q. The analytes were eluted by 1 mL absolute methanol. The elution was under a stream of nitrogen. The extracted sample was reconstituted with 100 µl absolute methanol and transferred to an injection vial or store at -20°C.

Chromatographic System

The chromatographic system consisted of Waters ACQUITY UPLC were equipped with a spectrophotometric with Waters Synapt G1 high definition mass spectrometer. CB1954 and metabolite separation was performed at 30.0 °C on an analytical ACQUITY UPLC BEH C18 (100 mm × 2.1 mm I.D.) with a particle size of 1.7µM (Waters). The compounds of interest were analyzed by electrospray ionization-quadrupole time-of-flight mass spectrometry (ESI-QTOF MS). The SYNAPT G1 mass spectrometer was used in V mode and operated in electrospray ionization positive mode with a capillary voltage of 3.0 kV, the sampling cone at 40 V and the extraction cone at 4.0 V. The scan time was 0.2 s covering the range of 50 to 320 Da mass range. The source temperature was 80 °C and the desolvation

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temperature was set at 250 °C. Nitrogen gas was used as the nebulization gas at a flow rate of 500 L/h. The trap collision energy was set at 6.0 V while the transfer optics collision energy was set to 4.0 V. The MassLynx software (Waters) was used to pilot the ACQUITY UPLC instrument and to process the data (i.e., Plotting of chromatograms) throughout the method validation and sample analysis.

Mobile phase solutions

The mobile phase is composed of solution A (2% Acetonitrile/H2O + 0.1% Formic acid) and B (100%Acetonitrile + 0.1% Formic acid). Both solutions were degassed by separating with helium. The injection volume was 10 µL. The mobile phase was delivered at 1.0 mL/min. The gradient program conditions are reported in Table S2.

Statistical analysis

All values are expressed as mean ± SD. Analysis of variance (ANOVA) and student's t-test were used for statistical comparisons in groups greater than and equal to two, respectively (GraphPad Prism 5.0; GraphPad Software, CA). A value of $p < 0.05$ is considered to be statistically significant.

Results

Trichostatin A (TSA)-primed MSCs have a normal MSC surface phenotype

TSA is known to upregulate chemokine receptors, such as CXCR4 [27], and increase CAR expression on bladder cancer cells [28] although its effects on MSCs are unknown. We first demonstrated that TSA at a concentration greater than 150 ng/mL induced cytotoxicity in hypoxic MSCs, as evidenced by crystal violet staining (Supplementary Figure S1A) and the MTT assay (Supplementary Figure S1B).

 doi:10.20944/preprints202008.0028.v1

Therefore, we used a concentration of 100 ng/mL to induce primed MSCs for 24 h. TSA-primed MSCs (referred to as primed MSCs) highly expressed CD29, CD44, and CD105, which are the most commonly reported positive surface markers of MSCs, but did not express the most frequently reported negative surface markers, such as CD31, CD34, and CD45 (Supplementary Figure S2). These results suggest that MSCs primed with TSA have a normal MSC surface phenotype.

TSA enhances MSC tumor tropism

Since only a small portion of MSCs reach the tumor sites after systemic administration [29, 30], optimization of MSC tumor tropism is important for using MSCs to deliver oncolytic virus in the context of various tumor models. CXCR4 is a chemokine receptor responsible for MSC tumor tropism [31]. A previous study showed that histone deacetylase inhibitors (HDACi) increase CXCR4 expression in hematopoietic stem/progenitor cells [32]. We therefore asked whether TSA could increase CXCR4 expression on MSCs. Quantitative RT-PCR and flow cytometry revealed that primed MSCs had significantly upregulated CXCR4 mRNA (Figure 1A) and surface protein levels (Figure 1B). In addition, primed MSCs also showed improved migration ability and cancer cell tropism, e.g., when employing the HT29 human colorectal cancer cell line (Figures 1C, D). These data suggest that TSA treatment can be used to upregulate CXCR4 expression, as well as to enhance tumor tropism in MSCs.

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TSA enhances CAR expression and adenovirus infection rates

TSA has been reported to increase CAR expression on bladder cancer cells [28]. We then asked whether TSA had a similar effect on MSCs. Both quantitative RT-PCR (Figure 2A) and flow cytometry (Figure 2B) showed that surface expression of CAR was significantly upregulated in MSCs after TSA treatment. When evaluating the viral loading capacity of MSCs via CRAdEGFP infection followed by analysis of EGFP fluorescence intensities, we found that primed MSCs also featured increased viral loading (Figure 2C). These data suggest that TSA treatment increases CAR expression, as well as the susceptibility to adenoviral infection of MSCs.

Combination of MSC-delivered CRAdNTR with a prodrug induces cytotoxicity in colorectal cancer cells in the presence of NAb *in vitro*

Since preexisting neutralizing antibodies (NAb) in the circulation represent a major obstacle of oncolytic viral therapy upon clinical application, we performed all subsequent experiments in the presence of NAb to mimic conditions reflecting those in clinical practice. To reduce the cytotoxic effects of oncolytic viral therapy on normal host tissues or cells, we investigated the cytotoxic effects on HT29 cells, a p53-deficient cell line, through the combination of prodrug activation and MSC-delivered CRAdNTR both *in vitro* and *in vivo*. The corresponding experimental procedures are shown in Figure 3A. The data revealed that CRAdNTR alone, blocked by NAb, did not cause cytotoxicity in HT29 cells either with or without CB1954 treatment, suggesting that CRAdNTR did not infect HT29 cells, and that CB1954 was not activated. Interestingly, when primed MSCs loaded with CRAdNTR ($MSC^{CRAdNTR}$) were added to HT29 cells, cytotoxicity was only observed in the presence of CB1954, as evidenced by a significant decrease in crystal violet staining (Figure 3B) and the

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MTT assay (Figure 3C). Notably, co-culture of primed MSCs did not induce any decrease in crystal violet staining or the MTT assay, both in the absence or presence of CB1954, suggesting that primed MSCs alone did not induce cytotoxicity in HT29 cells (Figure 3B, C). Furthermore, quantitative RT-PCR for adenoviral E1A confirmed that CRAdNTR, carried by $MSC^{CRAdNTR}$ cells, was delivered into HT29 cells in the presence of NAb (Figure 3D). These results suggest that $MSCs^{CRAdNTR}$ successfully protected CRAdNTR from NAb neutralization, and could deliver the viruses to colorectal cancer cells. Moreover, the combination of MSC delivery and prodrug CB1954 activation potentially enhanced the *in vitro* cancer-killing efficacy of CRAdNTR in the presence of NAb.

MSC-delivered CRAdNTR combined with a prodrug reduces colorectal cancer growth in the presence of NAb *in vivo*

To determine the *in vivo* cancer-killing effect of CRAdNTR via the combination of MSC delivery and prodrug CB1954 activation in the presence of NAb, the experimental design shown in Figure 4A was employed, where CRAdNTR alone, $MSCs^{CRAdNTR}$, and primed MSCs were intravenously injected into tumor-bearing immunodeficient mice. The mice also received CB1954 via i.p. injection every five consecutive days per week at a dosage of 25 mg per kilogram for two cycles (weeks). Tumor growth curves revealed that tumor growth in mice receiving $MSCs^{CRAdNTR}$ was significantly abrogated compared to the other two groups that received CRAdNTR or primed MSCs (Figure 4B). In addition, to investigate the durability of activated oncolytic adenovirus after inoculation *in vivo*, CB1954 injection was delayed by about 20 days after the second cycle of CB1954 treatment before the third cycle was initiated. Delayed CB1954 injection also significantly inhibited the increase in tumor volume in the $MSC^{CRAdNTR}$ group at approximately day 50, while the tumors in the

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other two groups continued to grow. These results suggest that oncolytic adenovirus is a powerful agent that possesses a long-lasting replication activity to induce the cytolytic effect, which might enhance its therapeutic efficacy.

To examine whether oncolytic adenoviruses were successfully transported into solid tumor sites via MSC delivery, xenografted tumors were excised for genomic DNA extraction, followed by analysis of adenoviral gene expression. As expected, the adenoviral E1A gene was detected the tumor grafts of mice receiving MSCs^{CRAdNTR} (n = 5), but not in tumor grafts of mice receiving CRAdNTR or primed MSCs (Figure 4C). Immunohistochemistry further showed that tumors treated with MSCs^{CRAdNTR}, but not those treated with CRAdNTR or primed MSCs, were positive for NTR and Ad5, the adenoviral capsid protein (Figure 4D). Furthermore, hematoxylin and eosin (H&E) staining revealed a significant increase in necrotic areas in MSC^{CRAdNTR}-treated tumor sections compared to those of mice that received CRAdNTR or primed MSCs (Figure 4E), suggesting that the combination of CB1954 activation with MSC-delivered CRAdNTR effectively lysed tumors, and generated areas containing lytic cells. These results suggest that MSCs act as carriers to successfully protect CRAdNTR from NAb-mediated neutralization, and transfer the virus to the tumor site, where it replicates and lyses tumor cells. The combination of the gene suicide system NTR/CB1954 caused cytolytic effects to reduce colorectal cancer growth *in vivo*.

MSC-delivered CRAdNTR combined with a prodrug induces cytotoxicity in colorectal cancer cells *in vitro* via the generation of cytotoxic metabolites

Escherichia coli NTR has been described to convert CB1954 into cytotoxic metabolites, like 2-hydroxylamine, 4-hydroxylamine, 2-amine, and 4-amine [33]. To confirm this, metabolites present in the extracellular medium of *in vitro* cultures were

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deproteinized, concentrated, and analyzed by UPLC-MS/QTOF. The mass spectra gave prominent parent molecular ions at the expected m/z values: CB1954 ($[M + H]^+$ = 253.057), 2-hydroxylamine and 4-hydroxylamine ($[M + Na]^+$ = 261.0599), and 2-amine and 4-amine, ($[M + Na]^+$ = 245.065). The metabolites of interest were identified by comparing the retention times with those of the standards, which are the products of CB1954 (retention time: 4.7 min) reacted with exogenous *Escherichia coli* NTR enzyme. The metabolites hydroxylamine and amine had retention times of 5.1 and 3.1 min, respectively, as illustrated by the extracted-ion chromatogram in Figure 5. These results indicated that the metabolites from HT29 cells treated with $MSCs^{CRAdNTR}$, as well as with CB1954, were detected at retention times of 5.1 and 3.1 min. Furthermore, we could show that the conversion of CB1954 was incomplete. However, metabolites were not found in HT29 cells treated with CRAdNTR or primed MSCs in combination with CB1954. These data suggest that the *in vitro* cytotoxic effects on colorectal cancer cells were caused by the production of cytotoxic metabolites from the prodrug CB1954.

Cytotoxic metabolites from CB1954 specifically exist in the tumor area rather than in other vital organs

We further demonstrated that toxic metabolites were only detected in tumors of mice receiving $MSCs^{CRAdNTR}$, but not in tumors of mice receiving CRAdNTR or primed MSCs in combination with CB1954 treatment (Figure 6A). Notably, the safety issues regarding the use of oncolytic viruses for cancer treatment are well recognized [34]. Although CRAdNTR carried by MSCs was successfully delivered to tumor sites, it was uncertain whether it would also be transferred to vital organs, causing unexpected toxicity. It was revealed that the cytotoxic metabolites, hydroxylamine and amine, which had single peak profiles, were detectable only in the tumors of

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MSC^{CRAdNTR}-treated mice, but not in other organs, such as the hearts, livers, lungs, and kidneys of these mice (Figure 6A). Metabolites of CB1954 were also not detected in solid tumors and vital organs of mice receiving CRAdNTR and primed MSCs (Figure 6A). Similarly, the adenoviral E1A gene was specifically detected in xenografted tumors, but not in other organs of mice that had received MSCs^{CRAdNTR} (Figure 6B), which expressed an internal control, mRPL13a, a murine housekeeping gene (Figure 6C). These results provide evidence in support of the safety of our therapeutic strategy, without causing toxicity to vital organs, since MSCs^{CRAdNTR} specifically homed to tumor sites, while the tumor lysis effect was caused by cytotoxic metabolites generated from the prodrug CB1954.

Discussion

In the current study, TSA not only increased the CXCR4 level and migration ability of primed MSCs towards cancer cells without affecting MSC viability and phenotype, but also resulted in elevated CAR receptor expression on primed MSCs to promote the adenovirus infection rate, thereby enhancing the capacity of CRAdNTR loading. We further demonstrated that an oncolytic adenovirus combined with a prodrug is a powerful tool in cancer therapy, with the ability to convert CB1954 into potentially cytotoxic metabolites by reacting with the NTR enzyme of CRAdNTR, causing tumor lysis and reduction of colorectal cancer growth, while side effects are minimized by avoiding toxicity for vital organs.

Since NTR/CB1954 holds the potential for use in cancer gene therapy, 4-hydroxylamine is the major cytotoxic metabolite, and 2-amine is the main bystander metabolite with better diffusion ability in cellular multilayers [33]. According to our newly obtained data, metabolites could be successfully generated from CB1954 based on the detection of its m/z ratio. However, 2-hydroxylamine and 4-hydroxylamine, as

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well as 2-amine and 4-amine are isomers with the same molecular formulas and weights, but with different chemical structures and functional groups. Therefore, even if we try to distinguish them by tandem mass spectrometry, they cannot be well distinguished (data not shown).

Since these metabolites are very rare, making it difficult to purchase a standard for each metabolite from commercial suppliers, it may not be possible to directly determine which peak belongs to which isomer by simply comparing the retention time with that of the standard. To determine which isoform of each metabolite is produced from CB1954 in colorectal cancer, it will be necessary to elute and determine the retention times of the metabolites by UPLC/MS-QTOF, followed by purification and analysis of their configuration by nuclear magnetic resonance (NMR) spectroscopy.

Because MSCs have been shown to possess the ability to home to metastatic cancers [35], our strategy of combining MSCs carrying oncolytic adenovirus with GDEPT holds potential and provides superiority with regard to cancer gene therapy. Recent studies have demonstrated the potential of oncolytic viruses carrying genes encoding immune-modulating molecules to combine immune checkpoint blockades for cancer therapy [36, 37] thus, combination of TSA-primed MSCs with GDEPT is a promising approach for combining immune checkpoint blockades for treatment of metastatic cancers.

Acknowledgments

This work was supported by the “Drug Development Center, China Medical University" from The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in

 doi:10.20944/preprints202008.0028.v1

Taiwan.

Grant support: This work was financially supported by Minister of Science and Technology (MOST 106-2321-B-039 -003). The funding sources had no involvement in study design, in the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the article for publication.

Competing Interests

The authors have declared that no competing interest exists.

 doi:10.20944/preprints202008.0028.v1

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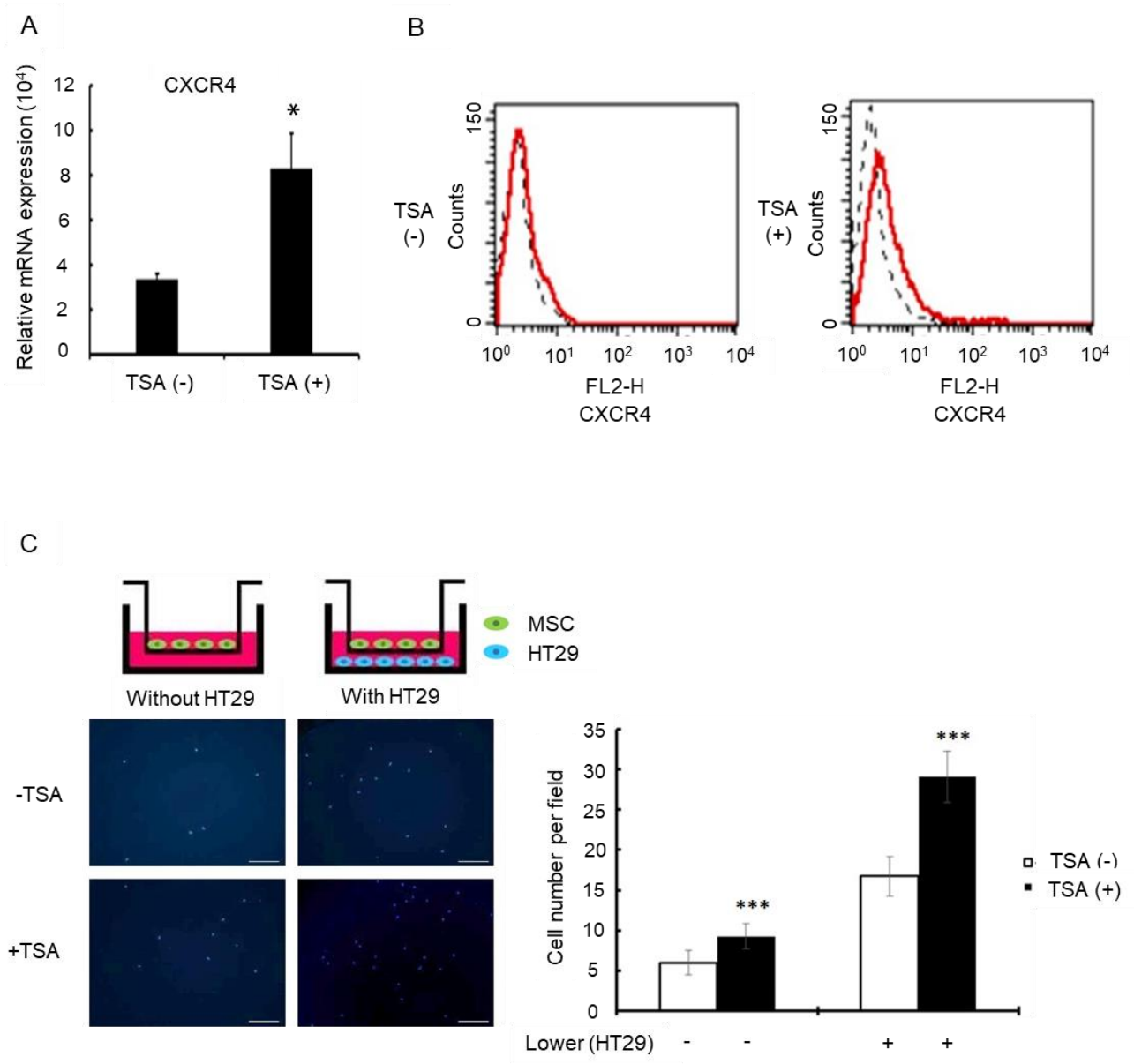

Figure 1. TSA enhances the expression of CXCR4 and the capacity for migration to tumor. MSCs treated without or with 100 ng/ml of TSA treatment for 24 h were analyzed for the expression of CXCR4 using (A) quantitative RT-PCR and (B) flow cytometry. The results of quantitative RT-PCR are presented as mean ± SD of three independent experiments. (C, D) *In vitro* migration abilities of MSCs treated without or with TSA in the absence of presence of HT29 were determined using transwell migration wells. (C) The representative photomicrographs showing DAPI-stained cells that migrated to the opposite side of the filter. (D) The average numbers of migrating cells per field were assessed by counting four random fields per filter. The quantification results are shown as mean ± SD. Asterisks indicate significant differences Asterisks indicate significant differences as determined by the Student's *t* test (*p<0.05, ***P<0.005 versus MSCs untreated with TSA). Scale bar, 200 μm

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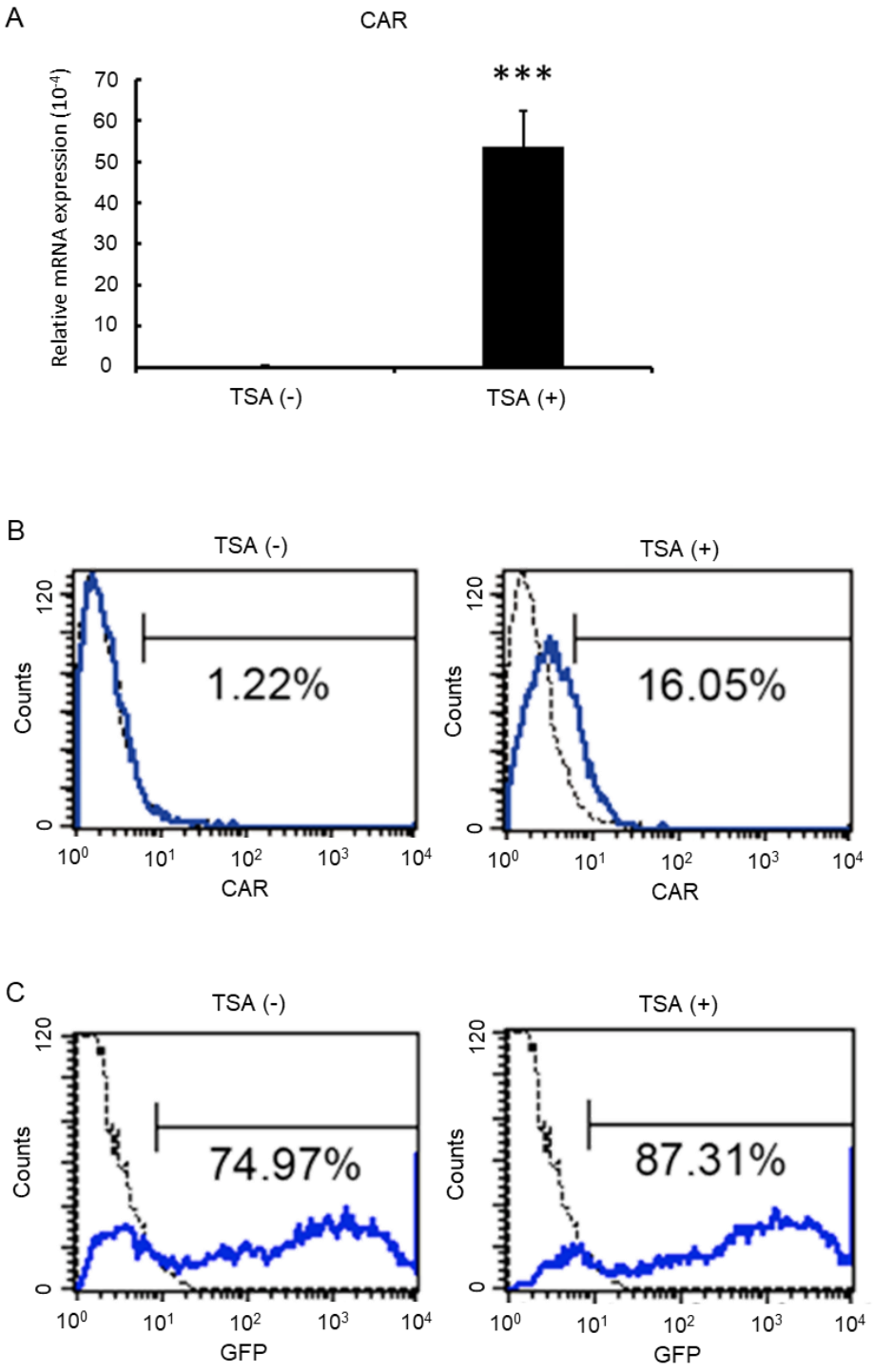

Figure 2. TSA enhances CAR expression and adenoviral infection rate. MSCs treated without or with 100 ng/ml of TSA treatment for 24 h were analyzed for the expression of coxsackievirus and adenovirus receptor (CAR) using (A) quantitative RT-PCR and (B) flow cytometry. The results of quantitative RT-PCR are presented as mean ± SD of three independent experiments. (C) These cells were also subjected to comparison of adenoviral infection rates using CRAdEGFP to infect these cells and analyzing the GFP expression by

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flow cytometry. Asterisk indicates a significant difference as determined by the Student's *t* test (***p<0.005 versus MSCs untreated with TSA).

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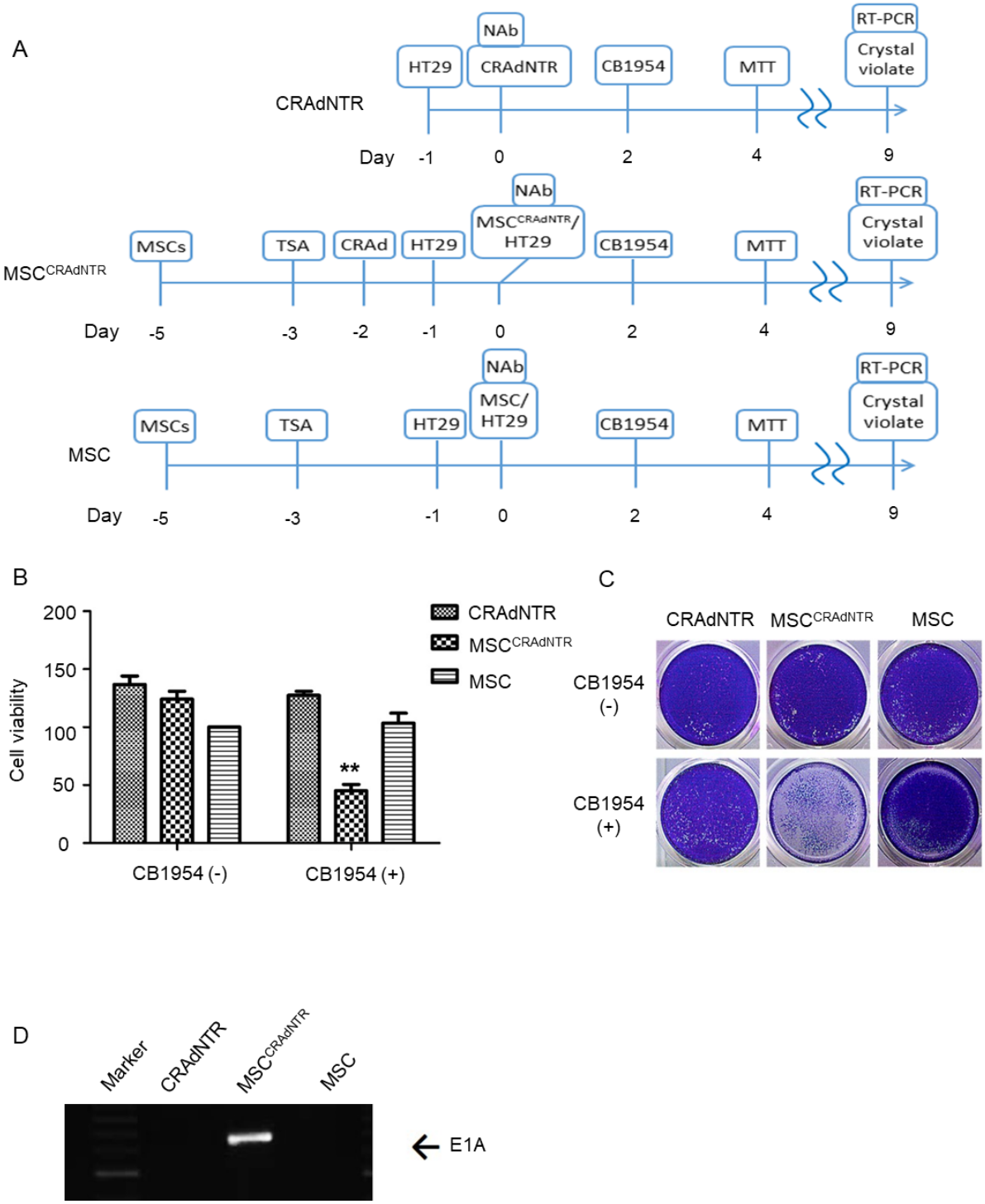

Figure 3. Combination of MSCs-delivered CRAdNTR with prodrug induces cytotoxicity in colorectal cancer cells in the presence of NAb *in vitro*. Flow chart of the *in vitro* study design. (B-D) CRAdNTR, MSCs pretreated with 100 ng/ml TSA for 24 h, followed by loading with CRAdNTR ($MSC^{CRAdNTR}$), or MSCs pretreated with TSA alone (MSC) were seeded into wells pre-seeded with HT29. The cell cultures were treated with anti-Adv neutralization antibodies (NAb) and CB1954 at indicated time. Aliquot of cells

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were subjected to (B) MTT assays, (C) crystal violet staining, and (D) RT-PCR detection of the adenoviral E1A gene (675bp) at indicated time. Asterisk indicates a significant difference as determined by One Way ANOVA (**$p<0.01$ versus other groups).

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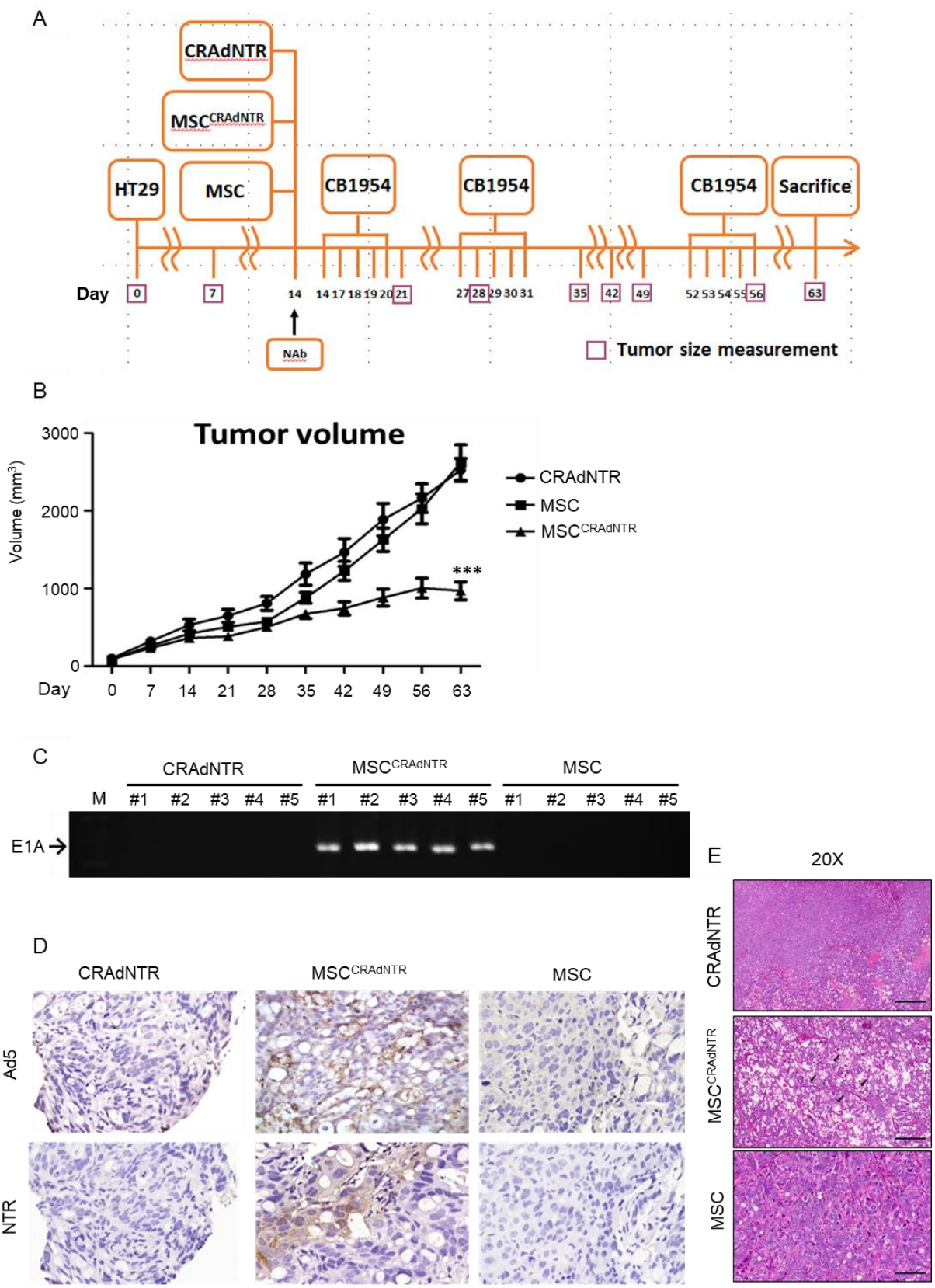

Figure 4. MSCs-delivered CRAdNTR combined with prodrug reduces colorectal cancer growth in the presence of NAb *in vivo.* (A) Flow chart of the animal study design.

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(B-E) CRAdNTR, MSCs pretreated with 100 ng/ml TSA for 24 h, followed by loading with CRAdNTR ($MSC^{CRAdNTR}$), or MSCs pretreated with TSA alone (MSC) were i.v. co-infused with anti-Adv neutralization antibodies (NAb) into mice bearing HT29 tumors established 14 days ago. The mice were i.p. injected with CB1954 at indicated times. (A) Tumor volumes were measured every week, and the mice were sacrificed and tumors were harvested for (C) RT-PCR detection of the adenoviral E1A gene (675bp), (D) IHC detection of Ad5, capsid proteins of adenovirus type 5, and NTR in serial sections. and (E) H&E staining for histological analysis. Scale bar, 50 μm. Arrows indicate tumor lysis areas. Asterisk indicates a significant difference as determined by One Way ANOVA (**$p<0.01$ versus other groups.) Scale bar: 50 μm in (D); 100 μm in (E).

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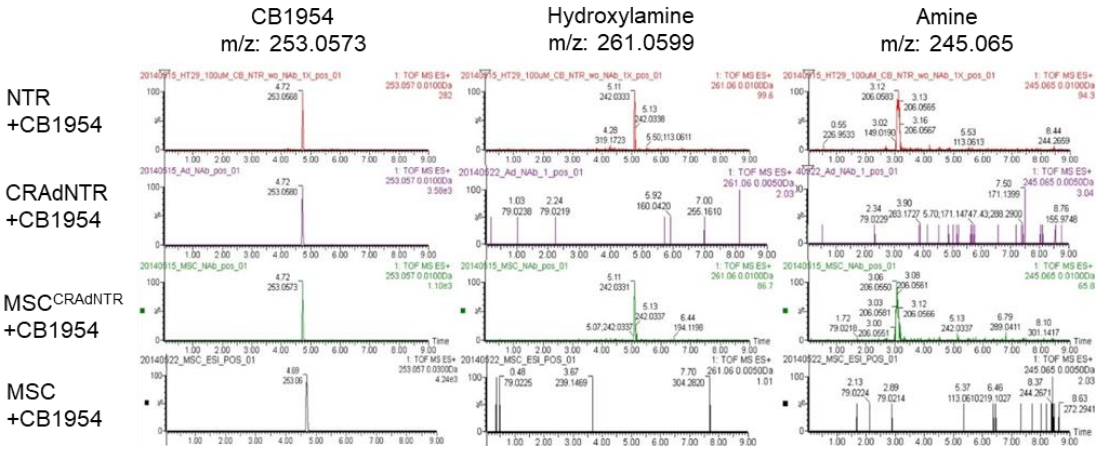

Figure 5. Detection of cytotoxic metabolites of CB1954 in the conditioned medium of HT29 cells. Flow chart of the *in vitro* study design is the same as Figure 3A. The conditioned medium of HT29 cells treated with CRAdNTR, MSC^{CRAdNTR} or MSC alone, followed by treatment with anti-Adv neutralization antibodies (NAb) and CB1954, were analyzed by UPLC/MS-QTOF. NTR+CB1954 solution referred to as standard metabolites of the reaction of CB1954 with exogenous NTR is showed in upper panel. CB1954 were protonated molecule $[M+H]^+$ at m/z 253.0573. However, hydroxylamine and anime have been ionized by the addition of a sodium cation $[M+Na]^+$ at m/z 261.0599 and 245.0645, respectively. CB1954, hydroxylamine and amine exhibited peaks at retention times 4.7, 5.1 and 3.1 mins in the extracted ion chromatogram. The results show that only the conditioned medium from CB1954 treated to MSC^{CRAdNTR} which co-cultured with HT29 cells exhibited peaks of hydroxylamine and amine.

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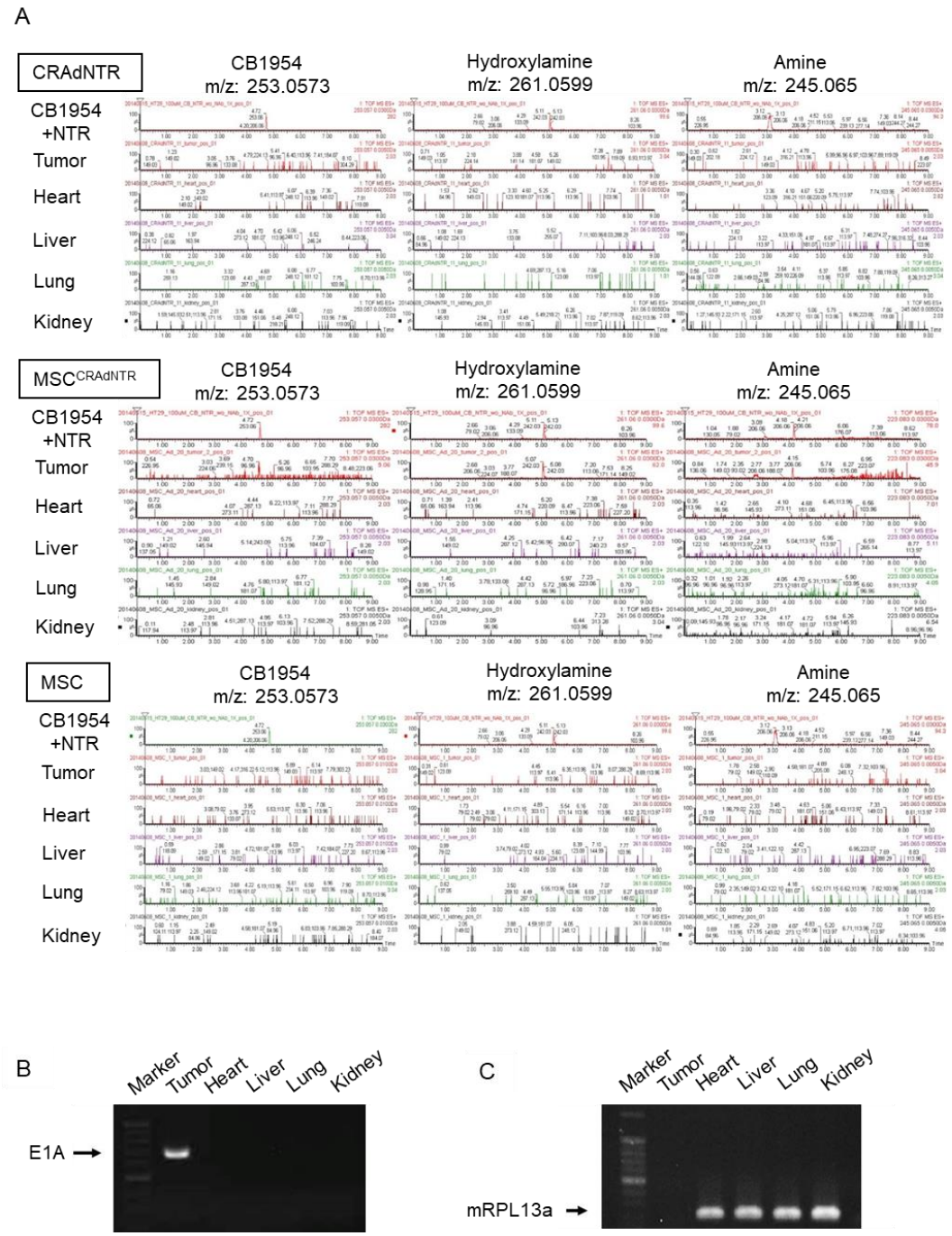

Figure 6. Cytotoxic metabolites of CB1954 were produced only in tumor of MSC^{CRAdNTR} treated mice. Flow chart of the animal study design is the same as Figure 4A. (A) Mice bearing HT29 tumors i.v. infused with CRAdNTR, MSC^{CRAdNTR} or MSC alone, followed by treatment with anti-Adv neutralization antibodies (NAb) and CB1954. The mice were sacrificed for harvest of tumor and vital organ tissues. (A) The extracts were analyzed by UPLC/MS-QTOF. NTR+CB1954 solution referred to as standard metabolites of the reaction of CB1954 with exogenous NTR is showed in upper panel. Retention times of CB1954 (4.7 min), hydroxylamine (5.1 min) and amine (3.1 min) are shown in the extracted

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ion chromatograms of “CB1954+NTR”. RNA was isolated and subjected to RT-PCR analysis for the expression of (B) adenoviral E1A gene (675bp) and (B) mRPL13a, a mouse housekeeping gene. “M” represented as marker.

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imer name	equence	oduct size p)
eal-time PCR		
AR	orward 5'-AAATTTACGCTTAGTCCCGAAGAC-3' everse 5'-CTGATATGTGCCAATATCTGACAG-3'	4
XCR4	orward 5'-CCTTATCCTGCCTGGTATTGTC-3' everse 5'-CACCCTTGCTTGATGATTTCC-3'	1
APDH	orward 5'-CTTCGATGATGCCGCAGTG-3' everse 5'-GGGCTCAGGTACTCCGAGG-3'	5
raditional PCR		
A	orward 5'-CTGCCACGGAGGTGTTATTACC-3' Revers -GTGGCAGGTAAGATCGATCACCTC-3'	5
RPL13a	orward 5'-GCCTCTGCAGTTTGCTTA CC-3' everse 5'- ATGTCAGAGCGGTTGCTT CT-3'	8

mRPL13a: mouse ribosomal protein large subunit 13a

Table S1. Primer sequences for real-time PCR and traditional PCR

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ime(min)	ow (mL/min)	olution A (%)	olution B (%)	radient Curve
	4	.9	1	
	4	.9	1	
	4	.0	.0	
	4	.0	.0	
01	4	.9	1	
	4	.9	1	

A: 2% Acetonitrile/H2O + 0.1% Formic acid

B: 100%Acetonitrile + 0.1% Formic acid

Table S2. The gradient elution program for UPLC/MS-qTOF

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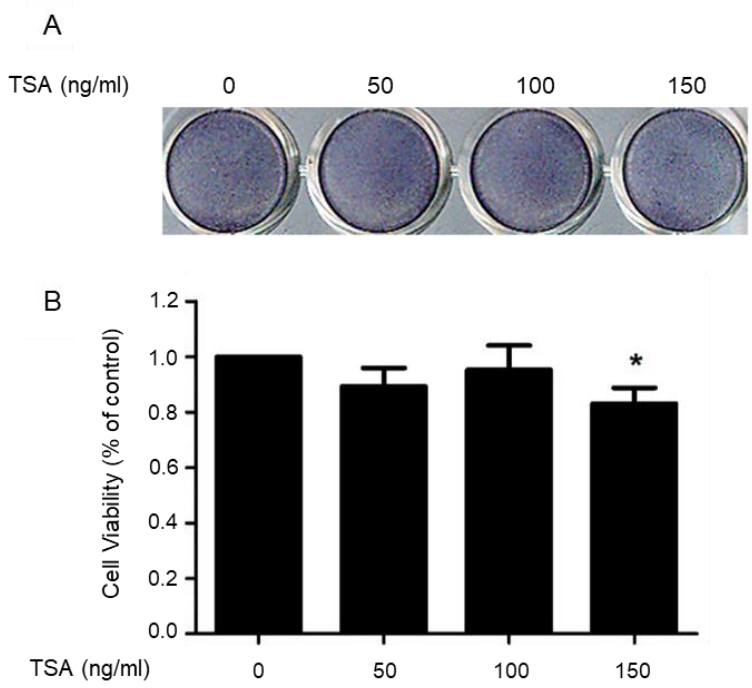

Figure S1. Identification of the optimal concentration of TSA.

MSCs were treated with TSA at the dose of 50, 100 and 150 ng/ml for 24 h. Cell viability was determined by the crystal violate staining (A) and MTT assay (B). The results are presented as mean ± SD of three independent experiments. Asterisk indicates a significant difference (*P < 0.05 versus MSCs untreated with TSA)

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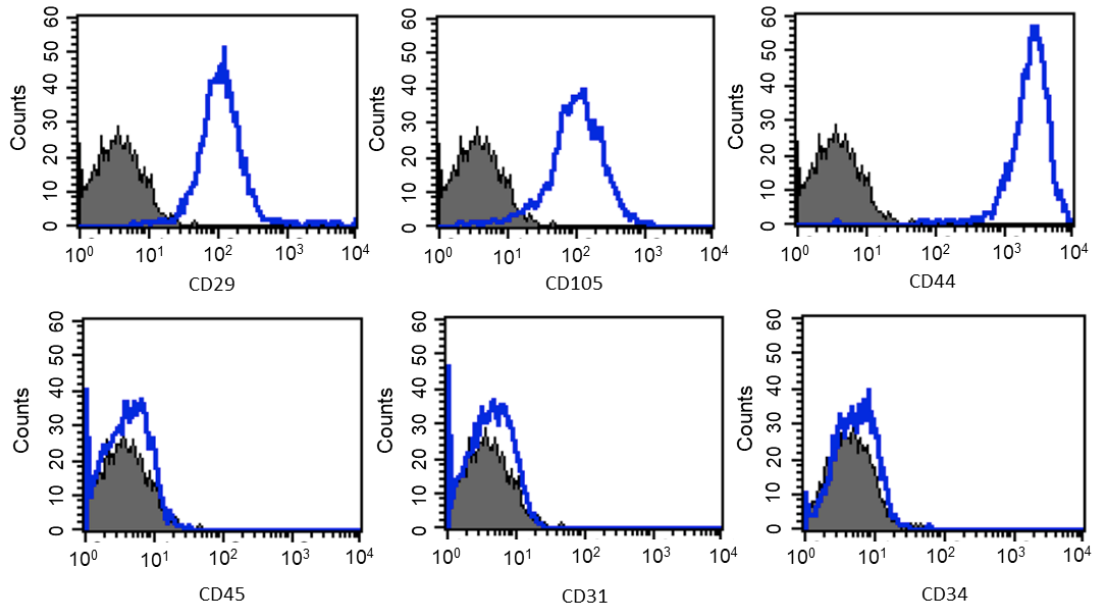

Figure S2. Cell surface profiles of MSCs after TSA treatment.

Flow cytometry for surface profiles of hypoxic MSCs treated with 100 ng/ml TSA for 24 h. The cells were found positive for CD29, CD44 and CD105, markers for mesenchymal stem cells and negative for CD31, CD34 and CD45.