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DISTINCT DIFFERENCE OF AZIDO SUGAR METABOLIC RATE BETWEEN NEURAL STEM CELLS AND FIBROBLASTS AND ITS APPLICATION FOR DECONTAMINATION OF CHEMICALLY INDUCED NEURAL STEM CELLS

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Experimental Section

Chemicals and Materials

NE-4C and 3T3 cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Dulbecco's modified Eagle's medium (DMEM), modified Eagle's medium (MEM), DMEM/F12+GlutaMAX^{TM}, Ac_4ManNAz, Dynabeads Biotin Binder (2.8 μm diameter) and SYBR Green, Non-essential Amino Acids, Glutamax, anti-Nestin APC-conjugated monoclonal antibody, anti-GFAP Alexa Flour 488-conjugated monoclonal antibody, Accutase, N-2 supplement, and B-27 supplement were purchased from Thermo Fisher Scientific (Shanghai, China). Fetal bovine serum (FBS), 0.25% trypsin/EDTA, 0.05% trypsin/EDTA, dibenzocyclooctyne-PEG4-biotin conjugate (DBCO-PEG4-biotin), bovine serum albumin (BSA), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), ManNAc, ROX-Reference Dye, DAPI, A-83-01, thiazovivin, purmorphamine, valproic acid, penicillin-Streptomycin solution, and fibronectin from human plasma were obtained from Sigma-Aldrich (Shanghai, China). Dylight 488-Avidin was brought from Boster Biological Technology (Wuhan, China). DiI, poly-L-lysine, poly-D-lysine and L-glutamine were brought from Sangon Biotech (Shanghai, China). RNA prep Pure Cell/Bacteria Kit, TIAN Script RT Kit, and Hot Master Taq DNA polymerase were obtained from TIANGEN Biotech Co., Ltd (Beijing, China). Custom synthesized primers and dNTPs were obtained from TaKaRa Biotechnology Co., Ltd (Dalian, China). Complete NeuroCult^{TM} differentiation medium was obtained from STEM CELL Technologies Inc.

Cells culture

3T3 fibroblasts were maintained in DMEM and supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin in a 5% CO2 humidified incubator at 37°C. NSCs cell line NE-4C was maintained in MEM and supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM Glutamax and 1× non-essential Amino Acids. The culture dishes were previously incubated with 15 ug/mL of poly-L-lysine for at least two hours.

Chemical induction of 3T3 fibroblasts

Induction assays were carried out according to previous studies with minimal modifications. Briefly, 3T3 fibroblasts (1×10^5/well) were seeded onto fibronectin-coated 12-well plates in standard NSC medium (DMEM/F12 supplemented with 1× N-2 supplement, 1× B-27 Supplement, 1% penicillin/streptomycin, 2 mM L-glutamine, 20 ng/ml EGF, and 20 ng/ml bFGF) containing 0.5 μM A-83-01, 0.5 μM Thiazovivin, 0.5 μM Purmorphamine, 0.5 mM VPA. After 10 days of induction, ciNSCs were used for further experiments. The medium was changed every 2 days.

Differentiation of NE-4C cells

For differentiation, NE-4C cells were gently detached with Accutase and seeded on PDL-coated 6 well dishes (1×10^5/well) containing fresh complete NeuroCult^{TM} differentiation medium. Then the NE-4C cells were cultured for in vitro differentiation into neural lineage cells. If the medium becomes acidic (turns yellow), perform a half-medium change. Seven days after being plated, the cells were used for further experiments.

Fluorescence microscopy and Flow cytometry

1×10^6 cells were seeded on a 35-mm petri dish. After 24 hours, cells were incubated with 50 μM Ac_4ManNAz in the culture medium for 8 hours, then cells were detached with 0.05% trypsin/EDTA and transferred to conical bottom centrifuge tubes (1×10^6 cells/sample). After washing with labeling buffer I (PBS containing 1% FBS and 1% BSA, pH=7.4), cells were incubated with 50 μM DBCO-PEG4-biotin in labeling buffer I for 1 hour at room temperature. Then the cells were washed with cold labeling buffer II (PBS containing 1% BSA, pH=7.4) and incubated with 2.5 μg/mL Dylight 488-Avidin for another 30 min at 4°C in the dark. After three washes with cold PBS, the fluorescence of the

labeled cells was visualized by laser confocal fluorescent microscope (Olympus FV1200) and flow cytometry (BD FACS Calibur).

For competition experiment with natural sugar ManNAc, cells were first incubated with 50 μM Ac_4ManNAz in the culture medium for 8 hours. Then cells were treated with different concentrations of ManNAc for another 3 days.

Magnetic-activated cell sorting (MACS)

For 3T3/NE-4C: 1×10^6 cells were seeded on a 35-mm petri dish. After 24 hours, cells were treated with 50 μM Ac_4ManNAz in the culture medium for 8 hours, then cells were detached with 0.05% trypsin/EDTA and transferred to conical bottom centrifuge tubes (1×10^6 cells/sample). After washing with labeling buffer I, cells were incubated with 50 μM DBCO-PEG4-biotin in labeling buffer I for 1 hour at room temperature. Then, the cells were washed with cold labeling buffer I and incubated with Dynabeads Biotin Binder for another 30 min. Finally, cells were isolated by NdFeB magnet (N_{42} 40×40×20 mm).

For ciNSCs: we firstly allowed 3T3 fibroblasts to be fully induced in standard NSC medium containing ATPV for 10 days. The next day, induced 3T3 fibroblasts continue to be cultured in standard NSC medium containing ATPV and 15% knockout serum replacement for another two days. Then, cells were treated with 50 μM Ac_4ManNAz in the culture medium. Subsequently, the MACS experiment was conducted with 50 μM DBCO-Biotin for 1 hour and the binding time of 30 min.

Gene expression analysis

Total RNA was extracted from cultured cells using RNA prep Pure Cell/Bacteria Kit and cDNA synthesis was carried out using TIANScript RT Kit. Quantitative PCR was performed on an ABI 2700 thermocycler (Applied Biosystems, USA) at the following thermal cycles: denaturation at 98°C for 3 min, followed by 50 cycles of 95°C for 10 s, 55°C for 20 s, 65°C for 30 s. The system contains 0.05 U/μL HotMaster Taq DNA Polymerase, 1× HotMaster Taq DNA buffer, 0.25 mM dNTP, 1× SYBR Green, 1× ROX-Reference Dye, 0.2 μM Forward Primer, 0.2 μM Reverse Primer. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method for relative quantification[1]. The expression level of each gene was calculated by normalizing it with the glyceraldehydes3-phosphate dehydrogenase (GAPDH) gene. Each experiment was performed in triplicate. The primers used in the experiment are shown in Table S1.

Immunofluorescence analysis

Cells were fixed in 4% paraformaldehyde solution for 15 minutes at room temperature, washed with 1× PBS for three times, and pretreated with 1× PBS containing 0.1% bovine serum albumin (BSA), 0.3% Triton X-100, and 10% mice serum for 40 minutes at room temperature. Then, cells were incubated with mouse anti-Nestin APC-conjugated monoclonal antibody (1:10) overnight at 4°C. Cells were washed with PBS for three times and then resuspended in PBS for FACS analysis. For NE-4C cells differentiation, cells were stained with anti-GFAP Alexa Flour 488-conjugated monoclonal antibody (1:50) at room temperature for 2 hours and DAPI for 10 min.

References

[1] K. J. Livak, T. D. Schmittgen, *Methods* 2001, **25**, 402-408.

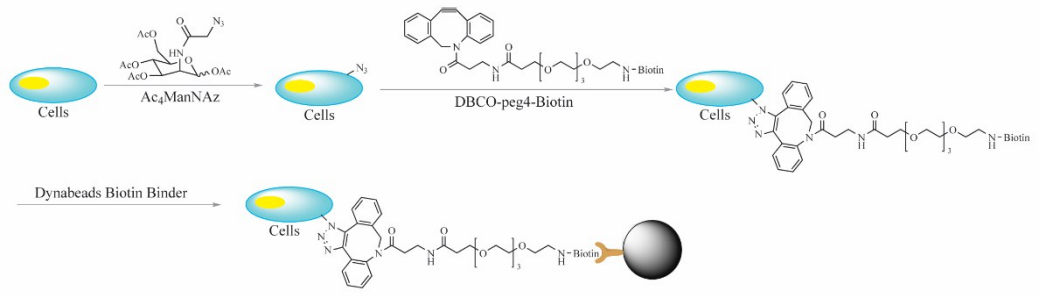

Figure S1. Schematic representation of the reaction between azido sugar labeled cells with DBCO-Biotin and magnetic beads.

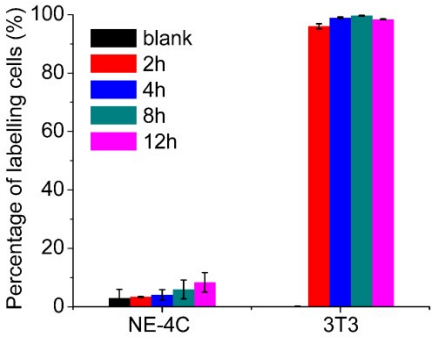

Figure S2. Effect of incubation time on the discrimination ability of 3T3 fibroblasts and NE-4C cells. Cells were respectively treated with 50 μM Ac_4ManNAz for 2, 4, 8, and 12 hours. The percentage of fluorescent cells was analyzed by flow cytometry. Cells that did not treat with Ac_4ManNAz were used as control.

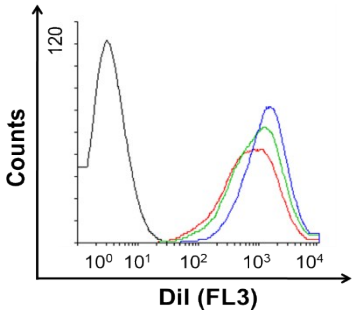

Figure S3. Flow cytometry analysis of NE-4C cells stained by DiI at different concentrations. 1×10^6 NE-4C cells were stained by 5 μM (red), 10 μM (green), 20 μM (blue) DiI. Cells that not stained by DiI were used as control (black).

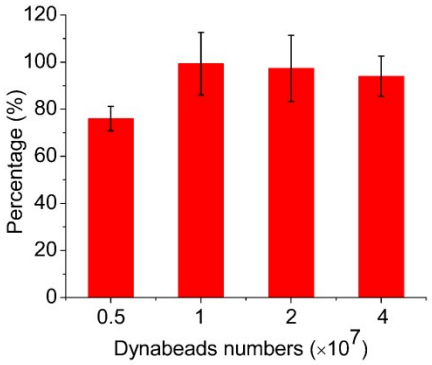

Figure S4. Optimization of Dynabeads numbers when isolated 1×10^6 3T3 fibroblasts. 3T3 fibroblasts were previously treated with 50 μM Ac_4ManNAz for 8 hours.

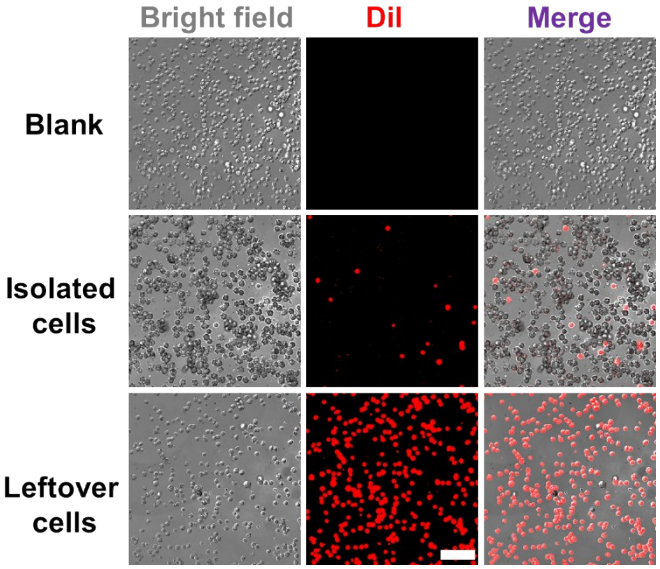

Figure S5. MACS assay of mixed-cells population and validation of isolated cells and leftover cells by fluorescence microscopy images. The percentage of NE-4C cells in cell mixtures is 50%. NE-4C cells were stained by DiI before co-culturing. The scale bar is 120 μm.

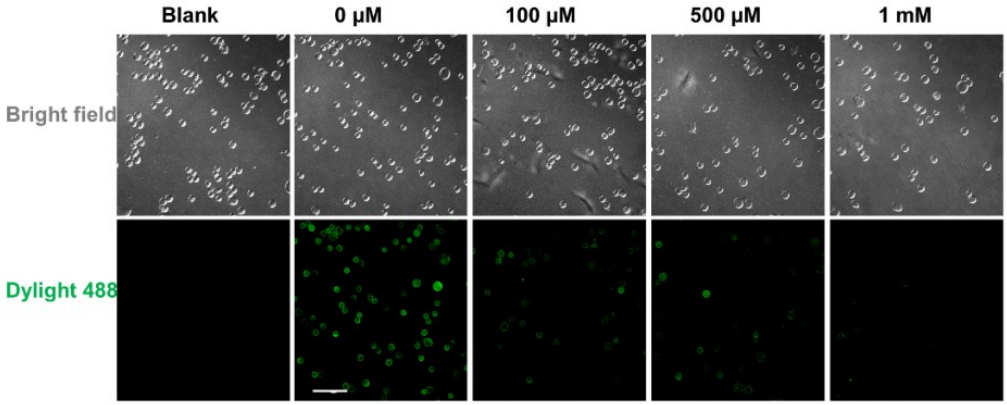

Figure S6. Influence of the ManNAc concentration after 72h incubation on incorporated Ac_4ManNAz. 3T3 fibroblasts were previously incubated with Ac_4ManNAz for 8 h. The scale bar is 120μm. 3T3 fibroblasts without incorporation of Ac_4ManNAz were used as blank.

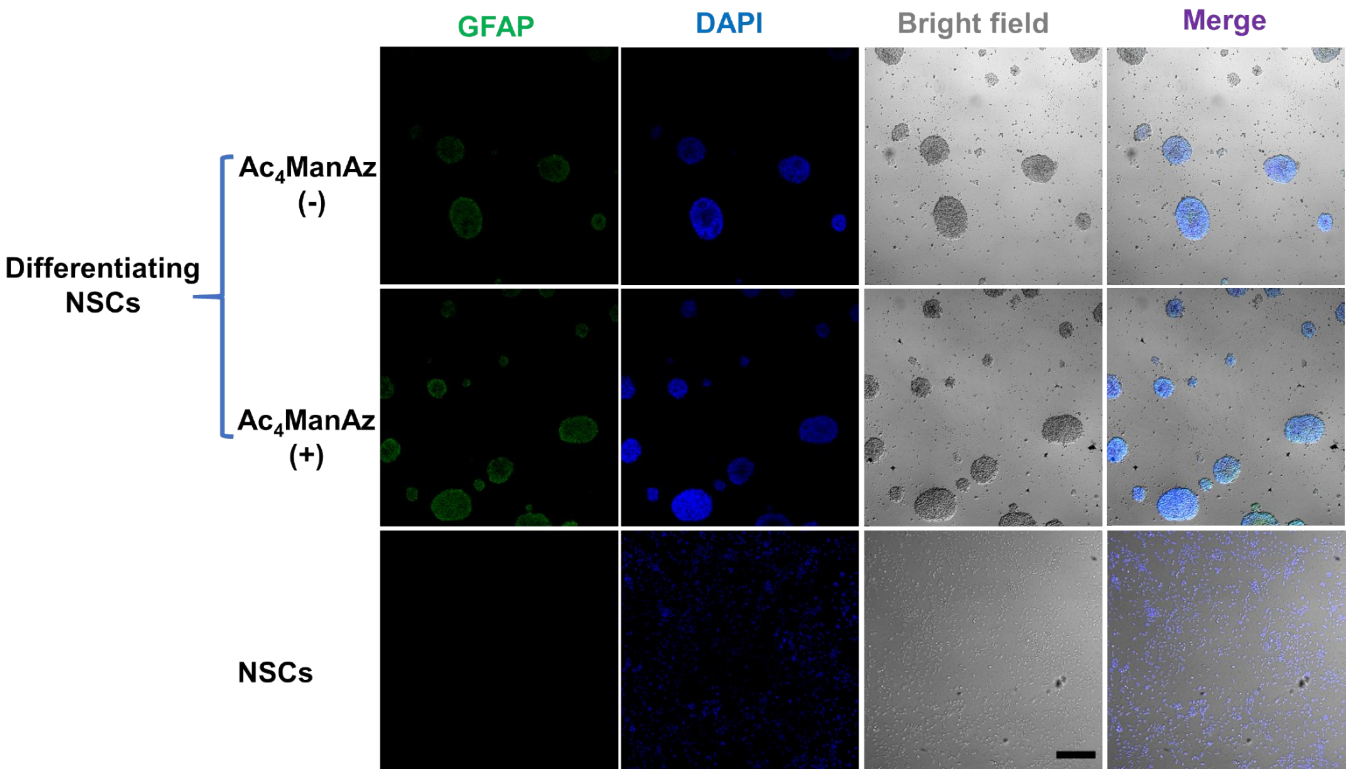

Figure S7. Differentiation of NSCs (NE-4C cells) without or with 8 hours incorporation of 50μM Ac_4ManNAz. Cells were stained with anti-GFAP antibody (green) and DAPI (blue). The scale bar is 200μm. NSCs without differentiation were used as blank (bottom row).

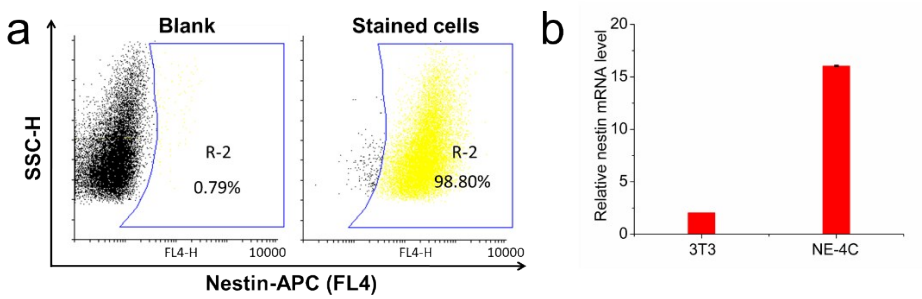

Figure S8. (a) Immunostaining of Nestin protein in NE-4C cells using a specific antibody. Cells that were not stained by the Nestin antibody were used as a negative control. (b) RT-PCR analysis of the mRNA levels of Nestin in 3T3 fibroblasts and NE-4C cells.

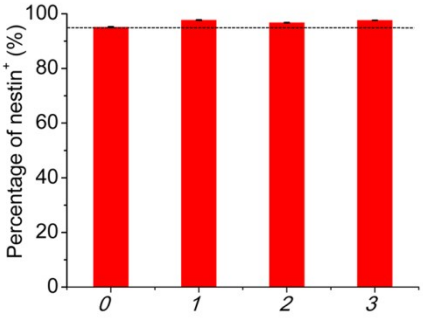

Figure S9. The percentage of $Nestin^+$ cells when cells were treated with 50 μM Ac_4ManNAz for different times. 0-3: non-isolated cells; leftover cells (8 hours); leftover cells (12 hours); leftover cells (16 hours).

Table S1. NSCs biomarkers primer sequences for mRNA expression analysis.

Gene	Primer sequence (5'-3')	
GAPDH	Forward	ACCACAGTCCATGCCATCAC
	Reverse	TCCACCACCCTGTTGCTGTA
Nestin	Forward	GAGAAGACATGAGGCAGATAAGTTA
	Reverse	GCCTGTGTTCTCCAGCTT GCT
Sox1	Forward	AACGGAGACTTCGAGCCGACAA
	Reverse	ACCACTTGCCAAAGAGGCCGAT
Sox2	Forward	AGCAACGGCAGCTACAGCATGA
	Reverse	CTGCGAGCTGGTCATGGAGTTGTA