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TITLE:

Identifying Cell Surface Markers of Primary Neural Stem and Progenitor Cells by Metabolic Labeling of Sialoglycan

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KEYWORDS:

Metabolic glycan labeling, sialic acids, bioorthogonal reaction, neural stem cells, progenitor cells, surface markers, neural-endothelial cell co-culture

SUMMARY:

Presented here is a protocol that combines an *in vitro* neural-endothelial co-culture system and metabolic incorporation of sialoglycan with bioorthogonal functional groups to expand primary neural stem and progenitor cells and label their surface sialoglycoproteins for imaging or mass-spectrometry analysis of cell surface markers.

ABSTRACT:

Neural stem cells have the potential to produce different types of neural cells through self-renewal and differentiation, and they are the cellular basis for the complex structures and functions of the brain. Neural stem and progenitor cells (NSPCs) are located in specialized niches *in vivo* and can be isolated and expanded *in vitro;* furthermore, they may serve as an important resource for cell transplantation in repairing brain damage. However, NSPCs are heterogeneous and not clearly defined or purified due to a lack of specific cell surface markers at the molecular level. The protocol presented, which has been previously reported, combines a neural-endothelial co-culture system with a metabolic glycan labeling method to identify the surface sialoglycoproteome of primary NSPCs. The NSPC-endothelial co-culture system allows expansion

of primary NSPCs *in vitro*, generating a sufficient number of NSPCs for sialoglycan labeling using an unnatural sialic acid metabolic reporter with bioorthogonal functional groups. By comparing the sialoglycoproteome from an endothelial co-culture expanded NSPCs and differentiated neural culture, we identify a list of membrane proteins that are enriched in NSPCs. In detail, the protocol involves: 1) set-up of an NSPC-endothelial co-culture and NSPC differentiating culture; 2) labeling with azidosugar per-O-acetylated N-azidoacetylmannosamine (Ac_4ManNAz); and 3) biotin conjugation to modified sialoglycan for imaging after fixation of neural culture or protein extraction from neural culture for mass spectrometry analysis. Then, the NSPC-enriched surface marker candidates are selected by comparative analysis of mass spectrometry data from both the expanded NSPC and differentiated neural cultures. This protocol is highly sensitive in its identification of membrane proteins of low abundance in the starting materials, and it can be applied to marker discovery in other systems with appropriate modifications.

INTRODUCTION:

Neural stem cells are defined as a multipotent cell population that can self-renew to maintain a stem cell pool and differentiate into neurons and glia. They are the majority cell type in the nervous system and may offer great therapeutic potential in regenerative medicine through cell transplantation into diseased and injured brains[1,2]. As development proceeds, the neural stem cell population becomes heterogenous[3,4], and the proportion of neural stem cells in the brain gradually decreases[5]. Generally speaking, neural stem cells and other neural progenitor cells, collectively called neural stem and progenitor cells (NSPCs), are located in the germinal zones, ventricular zone, and subventricular zone in mice[6]. In the embryonic brain, neural stem cells generate neurons directly or indirectly through intermediate progenitor cells (IPCs), and in some species through outer subventricular zone progenitors (oRGs)[7,8]. The specific molecular signature, morphology, location in the stem cell niche, and differentiation potential all determine the role of each subtype in brain organogenesis and clinical applications[9]. However, the currently available cell surface markers cannot unequivocally discriminate and purify different subtypes of NSPCs, limiting the understanding and utilization of these subtypes.

The identification of primary NSPCs surface markers is limited by three major hurdles. The first one is the limited cell number of NSPCs in the tissue, making it hard to prepare cell surface protein samples for common mass spectrometry analysis. The second limitation is the difficulty producing pure cell subtypes for generating subtype-specific membrane protein data. Finally, the third challenge is the low ratio of cell surface proteins in whole cell proteins, which hampers their detection sensitivities by mass spectrometry analysis.

To overcome these problems, we developed a chemoproteomic approach to selectively enrich and identify cell surface proteins in primary NSPCs by metabolic labeling the sialoglycoproteins[10]. To generate a sufficient number of NSPCs, we took advantage of an established protocol to expand and maintain primary embryonic NSPCs in undifferentiated states *in vitro*, by co-culturing NSPCs with mouse brain endothelial cell lines using a permeable permeable support matrix insert (*e.g.,* transwell) system[11]. In contrast, NPSCs cultured alone without endothelial cells generate differentiated progeny[11,12]. Thus, proteins samples from these two culture systems can be comparatively analyzed to identify proteins that are differentially expressed in NSPCs and

differentiated neurons. As most cell surface proteins are modified by sialic acid[13], unnatural sialic acid precursor analog N-azidoacetylmannosamine-tetraacylated (Ac_4ManNAz) was used to hijack the intrinsic metabolic pathway so that endogenous, newly synthesized sialoglycans are labeled with azido groups, generating a chemical handle[14]. Through azido-alkyne-mediated bioorthogonal reactions, which conjugate biotin to sialoglycans, cell surface proteins can be visualized and enriched for proteomic identification through a streptavidin-coupled fluorophore or matrix[14].

Here, we perform staining of SDS-PAGE gel analysis of the surface sialoglycoproteome from NSPCs expanded in an endothelial co-culture and differentiated non-co-culture system. We also selectively purify surface sialoglycoproteome in the two culture systems for proteomic comparison. Our protocol, compared to the traditional centrifugation-based cell surface purification protocols[15], increases extraction efficacy by reducing the surface protein extraction procedures through specific tag conjugation and affinity purification. Meanwhile, it increases the extraction purity of cell surface proteins based on the premise that sialylation happens mostly at the cell surface proteins. Although endothelial factors cannot completely block differentiation of expanded NSPCs, the comparative study between a co-culture and differentiated culture provides a convenient method to pinpoint stem cell-enriched surface proteins without the need to analyze proteins from NPCs purified by FACS[16]. We believe this approach can be applied to studies of surface proteins in other systems with the appropriate modifications.

PROTOCOL:

All animal protocols used in this study were approved by the IACUC (Institutional Animal Care and Use Committee) of Tsinghua University and performed in accordance with guidelines of the IACUC. The laboratory animal facility at Tsinghua University has been accredited by the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International). For staging of embryos, mid-day of the vaginal plug identified was calculated as embryonic day 0.5 (E0.5).

NOTE: All cells are cultured in the cell incubator under conditions of 37 °C and 5% CO_2.

1. Preparation of Mouse Endothelial Culture in Permeable Support Inserts

NOTE: BEND3 cells are maintained according to manufacturer's instructions.

1.1. Prepare BEND3 cell medium (BM) by adding 50 mL of FBS and 5 mL of penicillin-streptomycin into 500 mL of DMEM and mix well.

1.2. Aspirate the medium from the dish and wash the BEND3 cells culture with 1 mL of PBS once. Add 1 mL of 0.25% Trypsin-EDTA into the cells and incubate the cells for 4 min at 37 °C.

1.3. Add 1 mL of BM into the cells to neutralize Trypsin-EDTA and pipette up and down gently to completely dissociate the cells. Transfer the cell suspension into a new 15 mL conical tube and pellet by centrifugation at room temperature (RT) for 5 min at 400 x g.

1.4. Aspirate the supernatant from the tube and resuspend the cells with 9 mL of fresh BM, then add 1 mL of cell suspension into one permeable support insert. Add another 2 mL of fresh BM per well at the bottom chamber of the matrix. Continue to culture the cells for one day.

2. Preparation of Mouse Primary Cortical NSPCs Culture

2.1. Preparation of culture plate, papain digestion medium, and cortical adherent culture medium (AM)

2.1.1. Coat 6-well plates with poly-L-lysine (PLL) by adding 1 mL of PLL solution per well into 6-well plates. Then, incubate the plates at RT for 30 min.

2.1.2. Transfer the PLL solution into a 15 mL conical tube. Wash the plates 3 times with double distilled water. Airdry the plates and put them aside until use.

2.1.3. Prepare the papain digestion medium by adding 50 U of papain, 50 µL of L-glutamine, and 50 µL of 100 mg/mL acetyl-L-cysteine into 5 mL of DMEM. Mix the medium briefly and warm it to 37 °C for 30 min for enzyme activation.

2.1.4. Prepare the cortical cell adherent culture medium (AM): add 500 µL of L-glutamine, 500 µL of sodium pyruvate, 500 µL of 100 mg/mL N-acetyl-L-Cysteine, 500 µL of N2, 1 mL of B27, and 5 µL of 100 µg/mL bFGF into 50 mL of DMEM. Mix the medium well and warm it to 37 °C before use.

2.2. Preparation of primary cerebral cortical cells and subsequent plating

2.2.1. Sacrifice an E10.5 timed pregnant mouse by cervical dislocation.

NOTE: At E10.5, a majority of cells are proliferating NSPCs in the cerebral cortex, giving rise to large clones of progeny *in vitro*.

2.2.2. Sterilize the abdomen by 75% ethanol. Use fine scissors and micro-serrated forceps to open the abdomen by cutting the skin and underlying muscle along the right side of the middle line. Remove the uterus from the abdominal cavity gently with serrated forceps and cut it out from the abdominal cavity with fine scissors.

2.2.3. Wash the uterus with 40 mL of pre-chilled HBSS in 10 cm Petri dish. Then, transfer the uterus into a new 10 cm Petri dish and wash it again with 40 mL of pre-chilled HBSS.

2.2.4. Transfer the uterus into a new 10 cm Petri dish with 40 mL of pre-chilled HBSS. Remove the embryos from the uterus and amniotic membrane, then cut the heads of the embryos off from the trunks with Jewelers microforceps.

2.2.5. Wash the heads with 40 mL of pre-chilled HBSS and transfer the heads to a new 10 cm Petri dish with 40 mL of pre-chilled HBSS. Use Jewelers microforceps to peel away skin and cartilage covering the brains, then cut the cerebral cortices off and collect them in a 15 mL conical tube with pre-chilled HBSS.

2.2.6. Pellet the cortices by centrifugation for 3 min at 4 °C and 300 x g. Aspirate the supernatant from the tube, then add activated papain digestion the medium and 15 µL of 4 mg/mL DNase I into the tissue pellet.

2.2.7. Resuspend the tissue pellet briefly by gentle vortexing. Incubate the tissue at 37 °C for 30 min. During this time, loosen the tissue by brief vortexing every 10 min.

NOTE: At the end of the digestion, there should be no visible tissue pieces in the tube.

2.2.8. Pellet the cortical cells by centrifugation for 10 min at 4 °C and 450 x g. Aspirate the supernatant from the tube and wash the cell pellet with pre-chilled DMEM. Repeat this step once.

NOTE: During the digestion and washing, take caution not to pipette the tissues and cell pellet roughly to avoid damaging the cells with a strong shearing force.

2.2.9. Aspirate the supernatant from the tube then add 1.5 mL of pre-chilled HBSS into the tube. Dissociate the cortical cell pellet into single cells with gentle pipetting. Count the cell number with a hemocytometer.

2.2.10. Add 2 mL of AM and 2 x 10^4 cortical cells per well into 6-well plates. Incubate the plate at 37 °C and 5% CO_2 for 3 h to let cells attach to the plate.

3. Set-up of Neural-endothelial Co-culture and Ac_4ManNAz Labeling System

3.1. One day after plating BEND3 cells in the inserts, gently aspirate the medium in the bottom chamber first, then the inserts. Wash the enface of the inserts 3 times with pre-warmed DMEM. Wash the outer surface of the inserts by rinsing with pre-warmed DMEM.

3.2. Add 1 mL of pre-warmed AM into one insert, then transfer the inserts into the wells with primary cortical cells. Incubate the co-culture at 37 °C and 5% CO_2 for 12 h.

3.3. Dissolve Ac_4ManNAz in DMSO to achieve a stock concentration of 200 mM. 12 h after setting up the neural-endothelial co-culture, add 1 µL of Ac_4ManNAz stock per bottom chamber and 0.5 µL of stock per insert into the co-culture. Shake the plates immediately and gently to mix the medium well. For the control cells, add equal volume of DMSO.

3.4. Culture the cells for another 5 days at 37 °C and 5% CO_2. Prepare the AM with 10x bFGF as refeeding medium (RM). During this time, add 100 µL of RM per insert and 200 µL of RM per bottom chamber to refeed the endothelial and neural cells every other day. During the refeeding,

do not supply Ac_4ManNAz or DMSO into the culture.

4. Immunofluorescent Staining of Sialoglycoproteins in Expanded Primary NSPCs and Differentiated Neurons

4.1. Prepare BTTAA-$CuSO_4$ complex 1 30x stock containing 1.5 mM $CuSO_4$ and 9 mM BTTAA in double-distilled water. Prepare freshly biotin-conjugated buffer 1 containing 50 µM biotin-alkyne, 2.5 mM sodium ascorbate, and 1x BTTAA-$CuSO_4$ complex in PBS.

4.2. Remove the inserts from the co-culture plates. Aspirate the culture medium from the bottom wells and wash the neural cells once with pre-warmed PBS.

4.3. Aspirate the PBS from the wells. Add 1 mL of pre-chilled 4% paraformaldehyde PBS solution per well into the cells and fix the cells at RT for 10 min. Then, wash the cells 3 times with pre-chilled PBS.

4.4. Aspirate PBS from the wells and add 1 mL of freshly prepared biotin-conjugated buffer 1 per well into the cells. Incubate the cells at RT for 10 min.

4.5. Aspirate the reaction buffer from the wells. Wash the cells 3 times with PBS. Prepare the staining buffer containing 1% FBS and 1 µg/mL Alexa Fluor 647-streptavidin. Add 1 mL of staining buffer per well into the cells and incubate the cells at RT for 30 min.

4.6. Aspirate the staining buffer from the wells and washed cells 3 times with pre-chilled PBS. Prepare the blocking buffer containing 5% BSA and 0.3% non-ionic detergent-100 in PBS. Add 1 mL of blocking buffer per well into the cells and incubate at RT for 10 min.

4.7. Prepare a primary antibody solution by diluting the anti-nestin and anti-β-tubulin III antibodies together into the blocking buffer at ratios of 1:20 and 1:1000, respectively. Remove the blocking buffer from the wells and add 1 mL of primary antibody solution per well into the cells. Incubate the cells at 4 °C overnight.

4.8. Remove the primary antibody solution from the wells. Wash the cells 3 times with pre-chilled PBS. Prepare a secondary antibody solution by diluting Alexa Fluor 488 goat anti-mouse IgG1, Alexa Fluor 546 goat anti-mouse IgG2b, and DAPI together into blocking buffer at a dilution of 1: 1000. Aspirate the PBS from the wells and add 1 mL secondary antibody solution per well into cells. Incubate the cells at RT for 2 h.

4.9. Aspirate the antibody solution from the wells and wash the cells 3 times with pre-chilled PBS. Afterwards, the cells are ready for image capture.

5. Purification of Sialoglycoproteins from Expanded Primary NSPCs and Differentiated Neurons

5.1. Prepare BTTAA-$CuSO_4$ complex 2 15x stock containing 1.5 mM $CuSO_4$ and 3 mM BTTAA in

double distilled water. Prepare protein resuspension buffer A containing 4% SDS and 10 mM EDTA in double distilled water; protein resuspension buffer B containing 150 mM NaCl, 50 mM triethanolamine, and 1% polyoxyethylene oleyl ether (e.g., Brij97) in double distilled water with pH 7.4. Before use, mix buffer A:buffer B = 1:8 (vol/vol) to prepare the full protein resuspension buffer. Prepare protein washing buffer 1 containing 2% SDS in PBS; protein washing buffer 2 containing 8 M urea in 250 mM ammonium bicarbonate (ABC); and protein washing buffer 3 containing 2.5 M NaCl in PBS.

5.2. Remove the inserts from the co-culture plates. Aspirate the culture medium from the bottom wells and wash the neural cells once with pre-chilled PBS.

5.3. Aspirate the PBS from the wells and add 200 µL of pre-chilled RIPA buffer per well into the plates. Incubate the plates on ice for 5 min. Collect the protein lysis into 1.5 mL tubes. Pellet the cell debris by centrifugation for 10 min at 4 °C and 12000 x g.

5.4. Transfer the supernatant into new 1.5 mL tubes. Determine protein concentration with the BCA kit according to the manufacturer's instructions. Adjust the protein concentration to 1 mg/mL.

5.5. Add 100 µM alkyne-biotin, 2.5 mM sodium ascorbate, and 1x BTTAA-$CuSO_4$ complex 2 to 1 mL of protein lysis and mix the solution well. Incubate the mix at RT for 1 h.

5.6. Transfer the reaction solution into 20 mL of pre-chilled methanol in a 50 mL conical tube. Mix well and incubate at -30 °C overnight to precipitate the proteins.

5.7. Pellet the protein precipitates by centrifugation for 15 min at 4 °C and 4500 x g. Wash the protein pellet twice with 20 mL of pre-chilled methanol. Aspirate the supernatant from the tube. Resuspend the protein pellet with 4 mL of protein resuspension buffer and transfer the protein resuspension into a new 15 mL conical tube.

5.8. Take 50 µL of streptavidin beads and wash them 3 times with PBS. Add the washed beads into the protein resuspension. Incubate the solution at RT for 3 h on a vertical rotator at a rotation speed of 20 rpm.

5.9. Wash the beads sequentially with protein washing buffer 1, protein washing buffer 2, and protein washing buffer 3, at concentrations of 0.5 M ABC, 0.25 M ABC, 0.05 M ABC, respectively.

5.10. After washing, resuspend the beads with 20 µL of PBS and transfer the beads into a new 1.5 mL tube. Add 20 µL of 2x protein loading buffer into the beads and treat at 95 °C for 10 min. The protein samples should then be subjected to SDS-PAGE and stained with Coomassie brilliant blue R-250 according to the manufacturer's instructions. Cut the proteins in gel as indicated by Coomassie brilliant blue R-250 for mass spectrometry analysis.

REPRESENTATIVE RESULTS:

The whole procedure for *in vitro* expansion and metabolic labeling of primary embryonic NSPCs takes 6 days (**Figure 1A**). Quality of the BEND3 cell line and freshly isolated primary NSPCs are key to a successful experiment. BEND3 cells are the source of soluble factors that stimulate self-renewal and proliferation of NSPCs. It should be ensured that the BEND3 cells are free of any contamination and divide actively with minimal cell death before co-culturing with neural cells. The primary NSPCs must be carefully prepared to avoid excess damage during dissociation. Damaged NSPCs may still grow and differentiate; however, they are not able to respond to endothelial stimuli well to maintain stemness and expand. Extra caution should be taken to be aseptic during cell culturing, as the protocol does not suggest addition of antibiotics to the primary culture medium.

Successful endothelial co-culture will lead NSPCs to form large, sheet-like clones. Such featured clone shapes become evident at day 4 and are very typical at day 6. Within the clones, the cells maintain close contact with each other. Immunostaining with antibodies against the NSPC marker nestin and the neuronal marker β-tubulin III should reveal that in the clone, most of the cells are nestin^{+} NSPCs and very few are β-tubulin III^{+} neuronal cells. In contrast, the percentage of nestin+ cells and β-tubulin III^{+} neuronal cells in clone formed in non-co-culture system are nearly the same (**Figure 1B, 1D,** and **1E**).

The chemical reporter, Ac_4ManNAz, is a metabolic analog and can be incorporated into the intrinsic protein sialylation pathway. High doses of Ac_4ManNAz are toxic to cells. For each specific type of cell, the labeling concentration of Ac_4ManNAz should be pre-tested to achieve the highest labeling efficiency without significant cytotoxicity. Here, the optimized labeling concentration of Ac_4ManNAz for primary NSPC is 100 μM. Combinatory evaluation of cell death indicated by cellular and nuclei morphology suggests this labeling concentration does not cause obvious cytotoxic effects and is able to efficiently label NSPCs (**Figure 1C** and **1D**). The clonal morphology, self-renewal, and differentiation potential of NSPCs in both the endothelial co-culture and non-co-culture system are not affected (**Figure 1C, 1D,** and **1E**).

The successful labeling of NSPCs by Ac_4ManNAz can be examined after conjugating biotin to a culture mediated by an bioorthogonal reaction between azide and alkyne. Every cell in the Ac_4ManNAz-labeled culture is stained and visualized with Alexa Fluor 647-streptavidin. No cell is positive for Alexa Fluor 647-streptavidin staining in the DMSO control group. In addition, protein samples prepared from the Ac_4ManNAz-labeled culture by biotin conjugation and streptavidin beads purification show strong Coomassie brilliant blue staining signal in SDS-PAGE gels. Meanwhile, there were only staining background and nonspecific binding signals in the lanes loaded with protein samples from the DMSO control group. This also indicates the efficient labeling of NSPCs by Ac_4ManNAz (**Figure 1F**).

FIGURE AND TABLE LEGENDS:

Figure 1: Identification of cell surface markers for primary NSPCs assisted by endothelial co-culture system and metabolic sialoglycan labeling. (A) Schematic of the workflow for the protocol. This figure has been modified from Bai *et al.*[10]. The BEND3 cells are seeded into matrix inserts on D0. The preparation of primary cortical NSPCs and set up of co-culture system are

performed on D1. Metabolic labeling of culture lasts from D2 to D6. Culture refeeding is carried on D3 and D5. (B) The immunofluorescent images for clones formed by primary NSPCs after 5-day culture with or without endothelial cells. Scale bar indicates 20 μm. (C) Bright-field images for clones formed by primary NSPCs after a 5-day culture with Ac_4ManNAz or DMSO. The nuclei were counterstained by DAPI. The scale bar indicates 20 μm. The error bar indicates SEM (n.s. = not significant). (D) The immunofluorescent images for NSPC formed clones in the endothelial co-culture with Ac_4ManNAz or DMSO. Dashed circle demarcates a single neural clone. The scale bar indicates 50 μm. (E) Quantification of NSPCs and differentiated neurons in clones formed by NSPCs in endothelial co-culture and non-co-culture system with Ac_4ManNAz labeling or DMSO control. The error bar indicates SEM (***$p < 0.0005$; n.s. = not significant). (F) Coomassie brilliant blue staining of proteins purified by streptavidin beads from neural cells labeled with Ac_4ManNAz or DMSO in endothelial co-culture and nonco-culture system. The 55 kD band in control labelling groups represents nonspecific binding proteins. (B, C, E and F) corresponding to this protocol have been adapted from Bai *et al.*[10].

DISCUSSION:

Surface markers are commonly used to label and purify specific cell types *in vitro* and *in vivo*[17,18]. Discovery of surface markers contributes greatly to regenerative medicine and stem cell researches by providing molecular tools to selectively enrich a stem cell population from normal or pathological tissues and culture dishes, offering a purified cell resource for clinical use or study of biological properties. However, progress in developing surface markers for neural stem cell research has been slow due to the difficulty in isolating stem cells from primary tissues. The protocol described here is based on a simplified *in vitro* platform. By comparing primary NSPCs expanded by an endothelial co-culture to a differentiating neural culture, proteins differentially expressed in expanded NSPCs are highlighted and allow for further identification. Our protocol also provides an alternative strategy to purify cell surface proteins by hijacking the intrinsic metabolic pathway to label sialoglycan with bioorthogonal groups. Compared with traditional protocols for purifying cell surface proteins, the advantages of this protocol are underpinned by two specific features: 1) the prevalence of sialylation on cell surface proteins ensures maximal coverage of the cell surface proteome, and 2) the reaction specificity between the bioorthogonal group and its ligands grants purity of the acquired surface proteome. Thus, our protocol results in a more sensitive proteomic analysis in the case of less starting materials. We have demonstrated the feasibility of this protocol in primary NSPCs surface markers. With the appropriate modifications on expanding stem cells *in vitro*, this chemoproteomic approach can be compatible with identifying surface markers of other stem cell types.

Preparation of primary cortical neural progenitor cells and endothelial cells are critical steps of the protocol. First, when digesting embryonic cortical tissues, the digestion time, amount of enzyme, and strength of handling must be carefully controlled. Excessive digestion and mechanical shearing forces will damage the integrity of plasma membrane and cell surface receptors that mediate signal transduction for cell survival and growth, and they will also disturb the responsiveness of NSPCs to the stimulation of endothelial cells and their self-renewal ability. To achieve proper digestion, experimenters must activate the papain fully and stop the digestion as soon as the tissue blocks disappear. Second, BEND3 cells must be maintained in a healthy state

to support the secretion. It is recomennded to use BEND3 cell batches with fewer passages and passage the cells before they reach 100% confluence. This will prevent cell cycle arrest and senescence caused by DNA damage accumulated during passaging or by overcrowded contact between cells.

High throughput sequencing technology boosts the identification of cell surface markers through analyzing RNA expression, especially for cell types including tissue stem cells, which are often present *in vivo* in amounts too small to perform proteome analysis by mass spectrometry. Even though RNA-seq analysis can identify genes specifically expressed in NSPCs, it may not truly reflect protein expression levels, because RNA expression is not always consistent with protein expression[19]. In addition, non-protein biomolecules that can work as surface markers are not able to be detected by transcriptomic studies. For example, oligosaccharide Lewis X is a well-known surface maker widely used to label human embryonic stem cells and NSPCs, even though it can be associated with multiple proteins[20]. Therefore, direct mass spectrometry analysis is still not substitutable, and the development of methods that can make mass spectrometry analysis more feasible and convenient is of great interest for future studies.

In addition to sialylation, other types of post-translational protein modifications play an important role in regulating functions of modified proteins. These modifications affect protein properties such as the conformation, half-life, and subcellular localization[21,22]. Several protein modifications have cell type specificity[23,24,25]. With the growing contents of the chemical toolbox, more modification types are amenable to metabolic labelling with chemical reporters[26]. Hence, the chemical approach described here can be used for studying other differences in protein modification between stem cells and differentiated cells, illustrating the molecular mechanisms behind maintenance of stem cell properties and differentiation regulation.

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Figure 1C and 1D are reproduced from Bai *et al*.[10] with permission from the Royal Society of Chemistry. We thank Yi Hao in X. C.'s lab for figure editing. This work is supported by the National Natural Science Foundation of China (No. 91753206 to Q. S. and X. C., No. 31371093 to Q. S., and Nos. 21425204 and 21672013 to X. C.).

DISCLOSURES:

The authors have nothing to disclosure.

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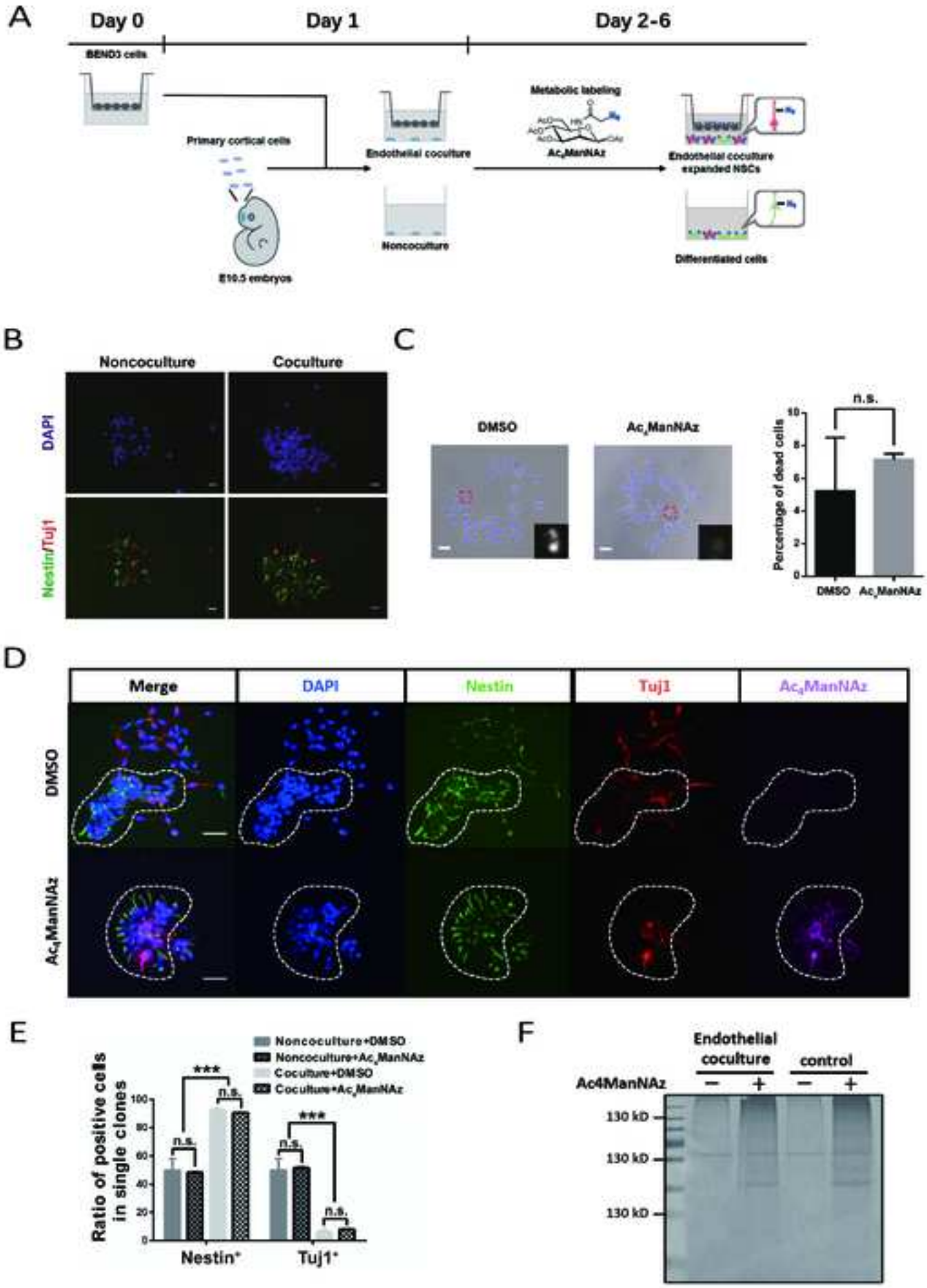

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
BEND3	ATCC	CRL-229	
DMEM	Gibco	11960044	
L-glutamine	Gibco	25030081	1%
Sodium pyruvate	Sigma	P5280	1%
N2 supplement	Gibco	17502048	1 to 100
N-acetyl-L-cysteine	Sigma	A7250	1 mM
Papain	Worthington	LS003726	10 U/mL
B27 supplement	Gibco	17504044	1 to 50
Poly-L-lysine	Sigma	P4707	
basic Fibroblast growth factor	Gibco	PHG0261	10 ng/mL
Penicillin-Streptomycin	Gibco	15140122	1%
Fetal bovine serum	Gibco	10099141	10%
HBSS	Gibco	14175095	
Tripsin-EDTA, 0.25%	Gibco	25200056	
DPBS	Gibco	14190094	
Transwell	Corning	3450	
Paraformaldehyde	Sigma	158127	4%
Sucrose	Sangon	A100335	
DAPI	Gibco	62248	
RIPA buffer	Thermo Scientific	89900	
SDS-PAGE loading buffer 2X	Solarbio	P1018	
6-well plate	Corning	3335	
Tris-Glycine protein gel	invitrogen	xp00100box	
mouse monoclonal anti-Nestin	Developmental Study Hybridoma Bank	Rat-401	1 to 20
mouse monoclonal anti-beta-tubulin III	Sigma	T8860	1 to 1000
Alexa Fluor 488 goat anti-mouse IgG1	invitrogen	A-21121	1 to 1000
Alexa Fluor 546 goat anti-mouse IgG2b	invitrogen	A-21143	1 to 1000
Albumin Bovine V	Amresco	0332	
Triton X-100	Amresco	0694	
BCA assay kit	Thermo Scientific	23225	
dimethyl sulfoxide	Sigma	D2650	
Brij97	Aladdin	B129088	

CuSO4	Sigma	209198	
alkyne-biotin	Click Chemistry Tools	TA105	
BTTAA	Click Chemistry Tools	1236	
Ac4ManNAz	Click Chemistry Tools	1084	100 μM
9AzSia	synthesized in lab		
sodium ascorbate	Sigma	A4034	
Methanol	Sigma	34860	
EDTA	Sangon	A100322	
NaCl	Sangon	A100241	
SDS	Sangon	A100227	
Alexa Flour 647-conjugated streptavidin	invitrogen	S21374	1 to 1000
Triethanolamine	Sigma	V900257	
Dynabeads M-280 Streptavidin	invitrogen	60210	
ammonium bicarbonate	Sigma	9830	
Coomassie Brilliant Blue R-250	Thermo Scientific	20278	
Isoflurane	RWD Life Science Co.	970-00026-00	
DNase I	Sigma	DN25	12 μg/mL
urea	Sigma	U5378	

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Author(s): Qing-Ran Bai, Lu Dong, Xing Chen, Qin Shen.

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Title:	Professor
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Dear Dr. DSouza,

Thank you very much for handling our submission! The listed below is the point-by-point response to editorial comments and reviewers' comments.

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank the editor. We have corrected all the typos in the revised manuscript.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Thank the editor's reminding. Here is the link to the website where the reusing policy is explicated stated, http://www.rsc.org/journals-books-databases/journal-authors-reviewers/licences-copyright-permissions/#reuse-permission-requests. The figures refer to the published reference has been cited correctly.

3. Please spell out each abbreviation the first time it is used.

We have added the full name for each abbreviation where they were used first time.

4. Please use centrifugal force (x g) for centrifuge speeds.

We have confirmed that all the description about centrifuge speed was in unit of centrifugal force. We used rpm as unit only at the step 5.8 for rotation speed of affinity purification incubation. We have added the description in this step.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from

your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: ATCC, Triton X-100, etc.

Thank the editor for pointing this out. We have rewritten the related part with generic terms.

Triton X-100 is a widely used non-ionic detergent. In order to make sure this is a generic term, we referred the published protocols by JOVE and found "Triton X-100" is directly used in the text.

6. Line 116: Please remove the weblink which contains commercial information.

We have removed the links.

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:
Approximate volumes for all buffers, gradients, and stock solutions to be set up should be given.

Thank the editor for reminding. We have added the details corresponding to the following listed points in the revised manuscript.

1.1: What container is used?
1.2: Aspirate what medium, and from where? Please specify.
1.4: What are the culturing conditions?
2.2.2: Please describe how to remove the uterus and specify all surgical instruments used.
2.2.3: What is used to cut?
2.2.5: Please specify the volume of pre-chilled HBSS.

8. 2.2.6, 3.3: Please break up into two steps.

Thank the editor. We have separate step 2.2.6 into two steps. Step 3.3 describes to add Ac_4ManNAz or DMSO into culture systems. We think it is not proper to separate it.

9. 2.2.9: Please spell out AM.

AM appeared for first time at step 2.1 and represents adherent culture medium.

10. 5.10: Please write the text in the imperative tense.

We have rewritten the text.

11. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We have combined the short steps.

12. Please include single-line spaces between all paragraphs, headings, steps, etc.

We have added spaces accordingly.

13. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the essential steps for video preparation.

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Thank the editor for the instruction. We have highlighted the appropriate parts.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have highlighted the appropriate parts in the revised manuscript.

16. Figure 1: Please define the error bars in panel C in the figure legend. Please include a space between numbers and their units in panel D (130 kD, 55 kD, 25 kD).

Thank the editor for pointing this out. We have defined error bar as SEM for panel C and

add the space in panel D.

17. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Thant the editor for the instruction. We have revised the discussion part according to the instruction listed above.

Reviewers' comments:

Reviewer #1:
Minor Concerns:
A couple of places the authors need to provide clarifications, which are labeled on the manuscript (Please see the attached file from Reviewer 1).

We are very grateful for the reviewer's suggestions!

When seeding BEND3 cells into the transwell inserts for coculture, we chose to use the cells when the cell confluence in 10 cm dishes reachs 90% and then split the cells into the transwells at the ratio of 1:9. We found in this way the cell state of BEND3 and the cell density of plating is reproducible. We have added this step into the text.

According to the workflow shown by Fig. 1A, the labelling of culture began on D2 and ended on D6. The refeeding should be carried on D3 and D5. We have clarified the procedure in figure legend. Ac4ManNAz or DMSO are not needed to be supplied when refeeding and we have clarified it in the corresponding part of revised manuscript. The 55 kD band in old Fig1 D indicated nonspecific binding proteins. We have clarified this in the new figure legend.

Reviewer #2:

The authors present a useful protocol for the isolation of proteins from neural stem and progenitor cells. The protocol is detailed enough to enable reproducibility. However, the mass spec analysis was not discussed in detail. It is clear that for anyone using the present protocol and proceeding to mass spec analysis it will be necessary to obtain resources and protocols to go all the way to identify the neural stem cell proteome. Nevertheless, the labeling protocol described here is helpful but requires some improvement at certain places.

We thank the reviewer for the positive comments. We sincerely apologize for the typographical and grammatical errors. We have proof read the manuscript and made corrections accordingly. The details of mass spec analysis are not included in this manuscript but can be found in our previous publications. We have added references in the text.

1. A rigorous proof read is required to correct typographical and grammatical errors. I point out the most critical ones below but the entire text should be proof read.

Thank the reviewer for pointing this out. We apologize for the errors and corrected them in the revised manuscript.

2. In the abstract lines 40-42 the authors make a strong statement about cell transplantation and brain repair. Thus far this is not possible and the statement is too strong. It is better to say … may offer… rather than …offering…. This point should also be addressed in the introduction lines 62-64.

We completely agree with the reviewer. We have revised this part.

3. Lines 103-104 the authors should avoid negative statements about FACS with the intention to better highlight their technique. FACS has been used by leading labs (Kriegstein, Walsh, Huttner to name just a few) to isolate embryonic progenitor cells from many species and human. It is simply not true that FACS is 'labor-consuming'. In contrast, FACS is way more efficient than the method described here. Thus the authors are advised to remove such negative statement.

Thank the reviewer for the advice. We have removed this statement.

4. Line 114. Correct the typo: transwell not tranwell

We have corrected it.

5. Line 168. Correct the typo: 2x10… not 2*10…

Thank the reviewer. We have correct it.

6. Line 202. Antibody dilution for anti-Nestin is 1:40, in the reagent list they state 1:20. Which dilution is the correct one?

Thank the reviewer for pointing this out. The dilution ratio for anti-Nestin antibody should be 1:20. We have corrected it in the revised text.

7. Line 203. Correct the typo: blocking buffer not block buffer

We have corrected it.

8.Line 230. Correct the sentence and remove After reaction,… It is obsolete.

We have removed this part.

Reviewer #3:

Thank the reviewer for critical comments and helpful advices.

it is essential that the authors include representative fluorescence images of both co-cultured and non-cocultured cells, so that the difference between them can be determined by the reader. FACS-mediated cell sorting could also probably assess the degree of differentiation in each culture type, but this would sacrifice the convenience of the FACS-free method described by the authors.

Using endothelial coculture system to expand NSPCs in vitro is first reported by our group in 2004. The secreted factors from endothelial cells stimulate NSPCs self-renewal. In the original paper, we have reported detailed comparison between the co-cultured and non-cocultured cells. In this revision, we have added a new panel in Figure 1 to show the neural clones formed by NSPCs in endothelial coculture have much more $Nestin^+$ NSPCs and

less Tuj1^{-} differentiating neuronal cells than differentiated NSPCs culture. This is in line with what we have shown in original Fig. 1B and 1C.

The cytotoxic and morphological effects of the MOE substrate on cells is also vital for determining the applicability of this method. Although the authors claim that optimization was done to determine the 100μM optimal Ac4ManNAz concentration and that such a concentration had no effects on the cells' morphology, self-renewal or differentiation potential, only a "ratio of positive cells" for each treatment case was included. Such a ratio provides inadequate evidence that the substrate has no effect on the differentiation or proliferative capabilities of the cells, and provides no evidence whatsoever about effects on morphology. Cell viability data and morphology images showing the negligible cytotoxicity at this concentration are essential for demonstrating the utility and applicability of this method to other systems.

We appreciate the reviewer's comments. We have added a new panel to show cell morphology and viability judged by nuclear morphology between Ac_4ManNAz labeling and control. The images demonstrate that 100 μM Ac_4ManNAz labeling does not affect cell morphology and viability significantly. In addition, we pointed out that the sensitivity and cytotoxicity of Ac_4ManNAz to different cell types could vary, so the labeling concentration needs to be optimized individually.

Finally, based on the SDS gel in Figure 1d, no clear difference between protein purified from NPSC-expanded cultures and differentiated cultures can be observed. In addition, there appears to be unspecific binding to the streptavidin bead resulting, raising the question of how well the Ac4ManNAz-based purification protocol can identify labelled glycoproteins. MS data or other proteomic studies are essential to demonstrate that the culturing method described produces detectable amounts of NPSC glycoprotein enrichment, and that such enrichment yields specific detectable quantities of glycoproteins. It is also unclear from the acknowledgements section whether figures 1c and 1d actually correspond to the studies described in this manuscript.

The reviewer is absolutely right about the proteins revealed in gel imaging. The Coomassie blue staining of SDS PAGE gel in Fig. 1D does not show the difference between NSPCs

with or without endothelial coculture. There could be two possible reasons. First, most abundant plasma membrane proteins are housekeeping proteins, like various transporters and channels. They are highly expressed by both NSPCs and differentiating cells. Because of their high abundance, they are very easy to be visualized after staining and corresponding to the major bands in gel. The cell type specific membrane proteins, however, are not as abundant as housekeeping membrane proteins. Second, purified membrane proteins are separated by SDS PAGE gel mainly based on their molecular weight. One protein band in gel could represent a bunch of membrane proteins with similar molecular weight. It may mask the cell type specific proteins if they have the similar size with housekeeping proteins. Therefore, the difference between the two groups is not easily discerned at this resolution. In our previous study, we show clearly mass-spec analysis could identify the difference in membrane proteins purified from NSPCs with or without endothelial coculture, providing quantitative evidence that our strategy can identify the membrane proteins which are specific or highly expressed in NSPCs rather than differentiating neurons.

Fig. 1C and 1D correspond to the study described here and we have rewritten this claim to make it clear.