ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2020, **56**, 2344

Received 2nd December 2019,
Accepted 20th January 2020

DOI: 10.1039/c9cc09362a

rsc.li/chemcomm

The distinct difference in 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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In our report, we found a distinct difference in azido sugar metabolic rate between neural stem cells and fibroblasts, which can be used for selective removal of fibroblasts from neural stem cell mixtures. Chemically induced neural stem cells (ciNSCs) serve as a highly valuable source of NSCs. Incompletely induced fibroblasts could interfere with ciNSC differentiation and become tumorigenic. Herein, we applied our method for the decontamination of ciNSCs and it exhibited excellent selectivity for ciNSCs. The results demonstrate that the ciNSC population can be efficiently purified to 98.1%. As far as we know, this is the highest purity obtained so far. We envision that, in the future, our method could be used as a safe, effective, and chemically-defined tool for decontaminating ciNSCs in both fundamental research and clinical stem cell therapy.

Neural stem cells (NSCs), which are capable of self-renewing and differentiating into various types of neuron and glia, have captured great interest as a valuable resource for regenerating the nervous system and repairing nerve damage.[1] However, the application of NSCs is severally limited by their rare existence in adult brain tissues (0.1–1%) and relatively low isolation yield.[2] The isolation of NSCs requires dissection of the tissue of the fetal or adult brain and dissociation of cells for *in vitro* cell culture.[3] Obviously, it is hardly practical to obtain NSCs from human brain tissues.

Alternative sources for the generation of NSCs include embryonic stem cell (ESC)[4] and induced pluripotent stem cell (iPSC)[5] differentiation. However, the clinical application of ESCs is hindered by ethical problems and immune rejection.[6] Also, undifferentiated iPS cells can cause teratoma formation *in vivo*.[7] Recently, it was found that fibroblasts can be transformed into NSCs without undergoing an intermediate pluripotent state by the exogenous expression of transcription factors[8] or by chemical compounds.[9] The exogenous expression of transcription factors increases the potential risks of unexpected genetic modifications. Chemically induced NSCs (ciNSCs) by the direct reprogramming of fibroblasts provide a practical way to obtain NSCs and avoid the risks of unexpected genetic modifications.[10] However, the incompletely induced fibroblasts would interfere with ciNSC differentiation.[11a] Some fibroblasts may survive and become tumorigenic which poses serious safety issues.[11b] Also, the higher proliferation rate of fibroblasts than ciNSCs makes long-term cultivation nearly impossible,[12a] which poses a problem of tumor formation following *in vivo* engraftment, or of subsequently dominating the *in vitro* cell population.[12b] Therefore, there is a pressing demand for a reliable, effective, and safe chemical tool that eliminates any fibroblast from neural cell mixtures.

A few biological strategies for selective recognition and decontamination have been reported: their self-renewal ability or biomarkers, such as colony or sphere formation, marker proteins,[13] and specific fluorescent probes.[14] However, these methods require extended time duration or rely on sophisticated fluorescence-activated cell sorting (FACS). In other words, these recognition methods retain FACS's limitations. Magnetic bead-based cell sorting or magnetic-activated cell sorting (MACS) can enrich targeted cells in parallel,[15] which is much cheaper and it is easier to achieve fast isolation.[16] MACS is more suitable for cell-based therapies as large scale separation is usually required.

Compared to the recognition of NSCs with specific proteins on the cell surface or fluorescent probes, recognition and decontamination of NSCs by using their unique metabolic characteristics can cause much less influence on cellular activity and biology. Like stem cells, NSCs have a lower rate of turnover for glycoprotein biosynthesis;[17] furthermore, more sialylated glycans in NSCs are carried by glycolipids than glycoproteins.[18] As a result, the quiescent phenotype and lower glycoprotein levels might contribute to lower incorporation of azido sugar.[19] In order to achieve negative labeling, we will keep the azido group only in fibroblasts cells and not in NSCs after an appropriate incubation time, which gives the possibility of eliminating azido sugar labeled cells with simple MACS.

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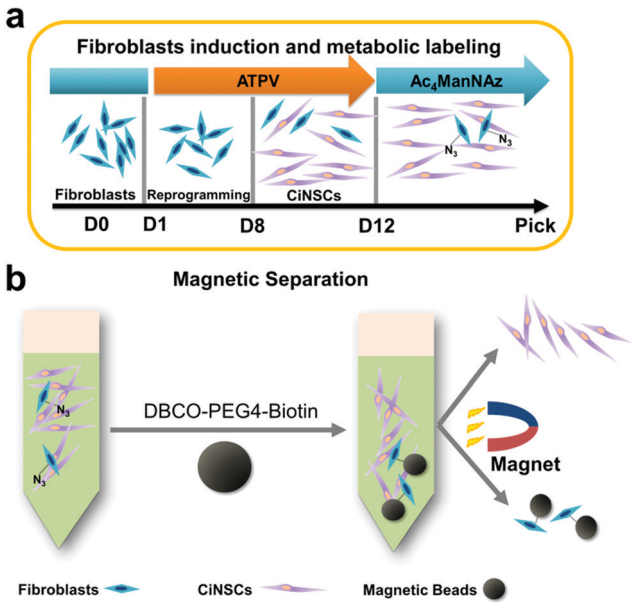
† Electronic supplementary information (ESI) available: Experimental details and Fig. S1–S9 and Table S1 are represented. See DOI: 10.1039/c9cc09362a



Scheme 1 (a) Chemical induction of NSCs from fibroblasts and metabolic labeling of a mixed population. (b) Decontamination of ciNSCs by click chemistry and magnetic beads.

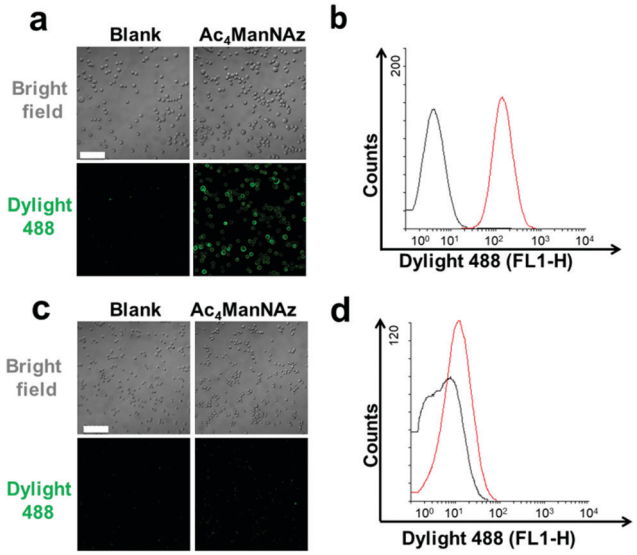


Fig. 1 Metabolic labeling of 3T3 fibroblasts and NE-4C NSCs with 50 μM azido sugar for 8 hours. (a and c) Fluorescence microscopy images of 3T3 fibroblasts and NE-4C NSCs, where the scale bar is 120 μm; (b and d) flow cytometry histogram of blank cells (black) and metabolically labeled cells (red).

Our strategy is superior in the following ways: (1) incapability of metabolic incorporation of azido sugar as a fundamental phenotype of NSCs that ensures the robustness of the purification; (2) less perturbation to NSCs as no antibody or dye is involved; (3) magnetic assisted cell sorting ensures rapid decontamination of ciNSCs.

Here, we propose a method for metabolic labeling of neural stem cell mixtures with azido sugars, which can be used as a chemical tool for selectively eliminating proliferative fibroblasts from ciNSCs. The selectivity takes advantage of the distinct difference in azido sugar metabolic rate between NSCs and fibroblasts. The decontamination route of ciNSCs is shown in Scheme 1. We hypothesized that Ac_4ManNAz will be metabolically processed only in fibroblasts and not or little in ciNSCs after an appropriate incubation time, leading to a selective reaction with subsequent dibenzocyclooctyne–PEG4–biotin conjugate (DBCO–biotin) through copper-free click chemistry (Fig. S1, ESI†). The magnetic beads that bear the biotin recognition component were added later to bind the labelled cells for the decontamination of ciNSCs.

Ac_4ManNAz is a well-known cell-labeling agent with close structural similarity to the native ManNAc, which can be easily introduced into the sialic acid biosynthesis pathway. It will be readily deacetylated by cytosolic esterase and metabolically converted to the corresponding *N*-azidoacetyl sialic acid, and subsequently incorporated into sialoglycoconjugates.[20] Ac_4ManNAz as an azido sugar can be easily incorporated into glycoproteins *via* cellular synthesis machinery and azido groups will be displayed on the cell surface, which gives a chemical handle for imaging or MACS. The azido group rapidly reacts with strained alkynes under physiological conditions, which does not require a toxic metal catalyst.[21] We firstly studied the metabolic incorporation difference between NE-4C NSCs and 3T3 fibroblasts at 8 hours of incubation. The azido groups were reacted with DBCO–biotin and Dylight-488 avidin and visualized by using fluorescence microscopy and flow cytometry. As expected, we found that 3T3 fibroblasts were almost completely labeled with azido groups (Fig. 1a and b), while NE-4C NSCs cells nearly failed to be labeled (Fig. 1c and d). These results collectively demonstrate that Ac_4ManNAz is an excellent substrate for the decontamination of NSCs and exhibited excellent selectivity for NSCs.

To evaluate the effect of incubation time on selectivity, we adjusted the time of metabolic labeling from 2 to 12 hours. The percentages of positive cells (labeled cells) were analyzed by flow cytometry. As shown in Fig. S2 (ESI†), 3T3 fibroblasts were fully labeled even at 2 hours, while the positive population in the NE-4C cells increased very slowly from 2 to 12 hours. The percentage of labeled 3T3 cells reached more than 99% at 8 h but only 3.46% of NE-4C cells were labeled. Short time (4 h) resulted in lower incorporation of Ac_4ManNAz in 3T3 fibroblasts (98.62%), whereas longer incubation time (12 h) resulted in relatively more incorporation of Ac_4ManNAz in NE-4C cells (8.36%), leading to more target cells being removed along with the 3T3 fibroblasts. Incorporation of azido sugar in NSCs would result in a small percentage loss but will not affect the purity of the isolated NSCs.

Next, we investigated the ability of metabolic labeling to recognize NSCs from cell mixtures. A mixture of NE-4C cells and fibroblasts was treated with azido sugar (50 μM) for 8 h. NE-4C cells were completely stained by 5 μM DiI before mixing (Fig. S3, ESI†). As shown in Fig. 2a and b, NE-4C cells and 3T3 cells were mixed at 1 : 9 ratio. Three distinguishable populations could be observed under fluorescence microscopy, Dylight-488 positive cells (green), DiI positive cells (red), and Dylight-488/DiI double positive cells (orange), respectively. Only a few cells were

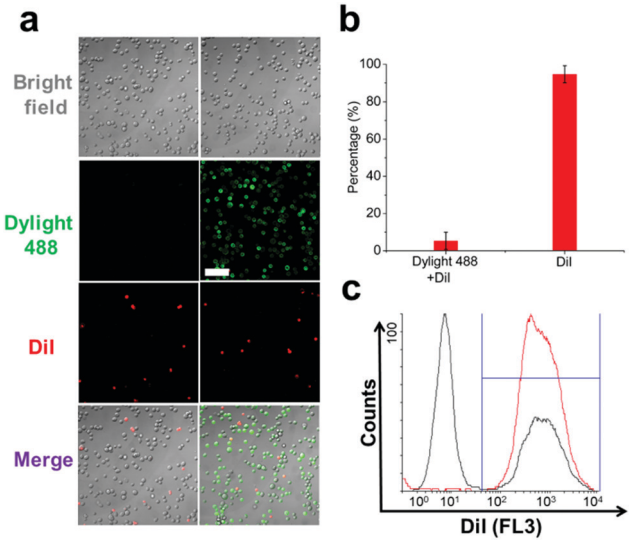


Fig. 2 (a) Fluorescence microscopy images of the mixed-cell population treated with 50 μM Ac_4ManNAz for 8 hours. NE-4C cells were stained by DiI before co-culturing. NE-4C cells and 3T3 cells were mixed at a 1 : 9 ratio. The scale bar is 120 μm. (b) Percentages of DiI-only positive cells and both Dylight 488 and DiI positive cells in mixed-cell populations of 1 : 9 ratio. (c) Flow cytometry histogram of mixed cells (black) and leftover cells (red). NE-4C cells and 3T3 cells were mixed at a 1 : 1 ratio.

double positive for Dylight 488 and DiI, demonstrating the incorporation of azido sugar in a few NSCs. Of the DiI-positive cells, about 95% of cells were DiI single positive, while around 5% of cells were double positive for Dylight 488 and DiI (Fig. 2b). These results were in good agreement with the incorporation of Ac_4ManNAz in the separated NE-4C cells and 3T3 fibroblasts, further suggesting that their metabolic levels are independent of each other in a heterogeneous population.

Finally, to evaluate the capability of decontaminating NE-4C cells from cell mixtures, we replaced Dylight-488 avidin with magnetic beads (dynabeads). Dynabeads have good biocompatibility.[22] In particular, dynabeads are only attached to the fibroblasts. Thus, dynabeads would not have any significant impact on the cell viability of leftover NSCs. Likewise, we stained the NE-4C cells with DiI and tracked the DiI signal by using flow cytometry and fluorescent microscopy, which were used to testify the two populations of cells in which dynabeads were captured and not captured. We increased the percentage of NE-4C to 50% for the quantitative determination of the isolation efficiency by flow cytometry. 1×10^7 dynabeads (Fig. S4, ESI†) were used to separate NE-4C cells from 1×10^6 mixed cells (NE-4C : 3T3 = 1 : 1). As we expected, the leftover cells that were not captured by the dynabeads were almost all stained with DiI and only a few percent of the isolated cells were stained with DiI under fluorescence microscopy (Fig. S5, ESI†). The overlap might arise from the uniformity of the cell growth rates or metabolic rates of ManNAz among a few NE-4C cells as well as 3T3 fibroblasts. More importantly, the percentage of DiI-positive cells in the leftover cells is 97.4% (Fig. 2c). These results indicated that NE-4C NSCs can be effectively purified by magnetic isolation without any cellular surface makers involved.

To elucidate the impact of the addition of the Ac_4ManNAz on NSCs, we performed more experiments. Firstly, NSCs have very low metabolic incorporation of Ac_4ManNAz (Fig. 1c), which would lead to much less influence on the metabolic pathway. The nonspecific labeling of Ac_4ManNAz from the acetyl groups *via* non-enzymatic cysteine *S*-glycosylation was observed at much higher sugar concentrations and in cell lysate.[23] Secondly, the influence of natural sugar *N*-acetylmannosamine (ManNAc) on incorporated Ac_4ManNAz was investigated by a competition experiment in 3T3 fibroblasts.[24] A significant decrease of the fluorescence signal occurred upon the addition of ManNAc (Fig. S6, ESI†), indicating that ManNAc can compete with Ac_4ManNAz in the same biosynthetic pathway and the natural metabolic pathway worked well after the incorporation of Ac_4ManNAz. Finally, there are no significant differences between NSCs with or without Ac_4ManNAz incorporation in terms of the morphology and GFAP (a biomarker of astrocytes) fluorescence staining (Fig. S7, ESI†). The differentiation of NSCs was not altered by Ac_4ManNAz.

We further applied our method to ciNSCs. MEFs can be switched to the NSC fates on day 8 by using ATPV *in vitro*.[10] We observed that ATPV-treated 3T3 fibroblasts underwent a morphological change from fibroblasts to NSCs (Fig. 3b). NSC-specific gene (Nestin, Sox2, Sox1) expression was significantly upregulated in ciNSCs (Fig. 3c). In particular, Nestin, a class VI intermediate filament protein, can be utilized as a preponderant marker to identify NSCs,[13,25] which was additionally confirmed in NE-4C cells by immunostaining and qRT-PCR, demonstrating that NSCs have a robust expression of Nestin

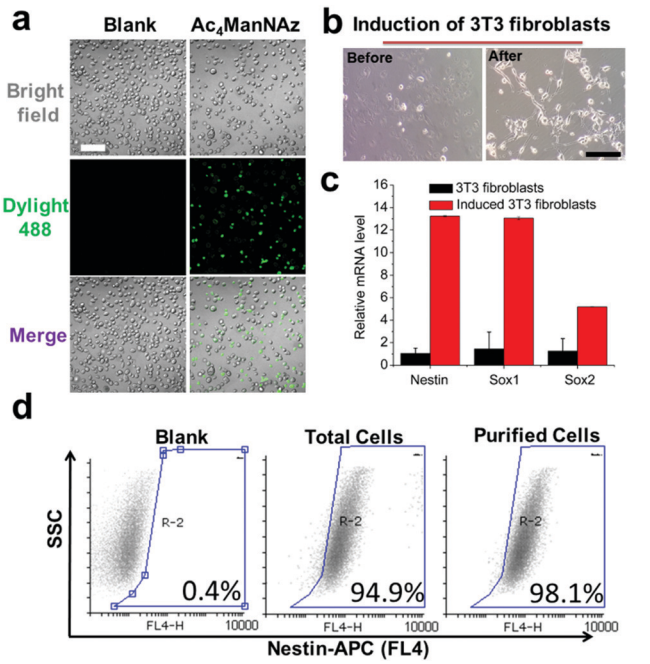


Fig. 3 (a) Fluorescence microscopy images of induced 3T3 fibroblasts at day 8. (b) Morphology of induced fibroblasts at day one (before) and eight (after). (c) mRNA levels of Nestin, Sox1, and Sox2 in 3T3 fibroblasts and induced fibroblasts. (d) Immunostaining of Nestin in total cells and purified cells. Cells were treated with 50 μM Ac_4ManNAz for 8 hours on day 12.

compared with 3T3 fibroblasts (Fig. S8, ESI†). These data demonstrated that 3T3 fibroblasts have been directly reprogrammed into NSC fates. We further studied the incorporation of Ac_4ManNAz in induced 3T3 fibroblasts on day 8. As shown in Fig. 3a, two distinguishable populations can be observed under fluorescence microscopy. A subpopulation of cells has almost no fluorescence. These results suggested that ciNSCs are similar to NSCs in marker expression, morphology, and metabolic property.

For clinical application, it is important to ensure the purity of decontaminated ciNSCs. 3T3 fibroblasts were fully induced by ATPV for 12 days before purification. Then, the cells were treated with azido sugar. The MACS experiment was conducted subsequently. The immunostaining of Nestin was performed to evaluate the purity of the resulting ciNSCs. In Fig. 3d, a high percentage of ciNSC population (94.9%) was observed before MACS, indicating high reprogramming efficiency.[10] After MACS, the percentage of Nestin-positive cells was further elevated to around 98.1%. As far as we know, this is the highest purity obtained so far.[26] There was no significant difference in the decontaminating efficiency from 8 h to 16 h, which indicated the robustness of the discrimination ability of metabolic labeling (Fig. S9, ESI†).

In summary, the quiescent metabolic characteristics of NSCs provided a safe, rapid and efficient chemically-defined tool for decontamination of ciNSCs. At present, selective elimination based on the fundamental phenotype of NSCs has not been reported. By adjusting the time of metabolic labeling, our method may also be useful for purification of other types of neural stem cell mixtures to further improve their safety profiles. Together, this work represents a practical route for purification of NSCs for clinical stem cell therapy and translational biological research.

We thank the National Natural Science Foundation of China (Grant No. 21675108) and the Fundamental Research Funds for Central Universities (Grant No. GK201901003, GK201903027) for financial support.

Conflicts of interest

There are no conflicts to declare.

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