Magnetic-Tagged Stem Cells

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Neural Stem Cells Harvested from Live Brains by Antibody-Conjugated Magnetic Nanoparticles**

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Generation of induced pluripotent stem (iPS) cells from fibroblasts[1] has given a powerful tool for the development of stem-cell based therapy.[2] However, the use of both embryonic stem (ES) cells and iPS cells gives rise to considerably safety and ethical issues, including genetic instability and risks of immune rejection.[3] On the other hand, the use of adult neural stem/progenitor cells,[4] though not as proliferative and expandable as ES and iPS cells, can bring significant amelioration in disease models including multiple sclerosis,[5] Parkinson's diseases,[6] Huntington's disease,[7] and spinal cord injury.[8] It is noted that the generation of the neural phenotypes from a single fertilized cell has been well recognized for decades. Neural stem cells (NSCs) are multipotent cells and are able to self-replicate, which may lead to the development of new neurological disorder therapies if these cells can be extracted, modified, and re-applied to the same subject. These remarkable characteristics of NSCs have therefore attracted scientists, raising the hope of novel neural stem cell therapies and regenerative medicine.

However, isolating NSCs from adult subjects is technologically a great challenge both regarding the risks of brain surgery and the uncertainty in their location (or even their presence) for their extraction. In particular, the existence of neural stem/progenitor cells in the adult central nervous system (CNS) has always remained questionable.[9] It has been proposed that ependymal cells, the single-layer multiciliated cells lining the sub-ventricular zone (SVZ) are the "source" of adult NSCs. They are claimed to be the first layer of cells of this discrete region in a direct contact with cerebrospinal fluid (CSF).[6] Coskun et al. published their results on CD133 based selection, in vivo identification and analysis of ependymal cells, which supported this postulation.[10] However, they also suggested that such $CD133^+$ neural stem ependymal cells are very quiescent and limited to the ependyma.

In the present study, we have attempted to employ a magnetic-nanoparticle (MNP) based extraction system for the collection of $CD133^+$ ependymal cells from adult mammals and assessed the simplicity of this technique and the activity of the extracted $CD133^+$ ependymal cells for differentiation. It is noted that the clinical use of MNPs as contrast agents was approved by the US Food and Drug Administration (FDA) several years ago.[11] A myriad of utilities with MNPs, such as blood purification, hyperthermia therapy, and drug delivery, have also been recently established.[11,12] It is thus envisaged that the magnetic nanoparticles when functionalized with a high affinity for the ependymal cells can liberate them from the surface lining of SVZ as a non-invasive extraction technique for brain tissue. As a result, we have developed new magnetic iron oxide nanoparticles in silica covered with anti-CD133 antibodies to enhance the specificity for the stem-cell extraction. We have injected these anti-CD133 antibody conjugated MNPs particles (Ab-MNPs) into the SVZ of a rat brain by a micro-syringe through a preformed hole on its skull by micro-surgery. It is revealed that the ependymal cells on the superficial linings can indeed be made accessible by the coated magnetic particles with ultra-specificity. The attached cells when subjected to magnetic agitation under an external spinning magnetic field can be released from the endothelial lining before their extraction from the suspension in CSF either by a syringe or by attachment on a neodymium magnet probe. All the subjects have remained alive and apparently healthy despite receiving the above treatments. We have also found that the extracted cells are active NSCs, which can be differentiated into neurone cells in culture medium. Thus, we believe that this technique may have significant implications in isolating individual patients' own neural stem cells for tailor-made treatments of their specific neurological problems in future stem cell therapy.

The synthesis of MNPs with a narrow particle size distribution was performed (see Experimental) and typical

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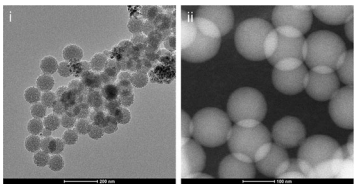


Figure 1. Transmission electron micrographs of typical MNPs in silica at two different magnifications, the particles are approximately 100 nm in diameter. Scale bars: i) 200 nm, ii) 100 nm

images of them are shown in Figure 1 and the particle size distribution is described in Figure S1 of the Supporting Information. The magnetic susceptibility of these particles was measured and optimized. The magnetic susceptibility curves of the functional MNPs were probed by using a superconducting quantum interference device (SQUID) with and without an external magnetic weak field of 100 Oe (Figure S1a). A high saturation magnetization value of about 30 emu g^{-1} was retained for the silica-coated MNPs compared to 70–80 emu g^{-1} for the naked iron oxide nanoparticles. Non-ferromagnetic or slightly anti-ferromagnetic coatings, such as antibodies and fluorescent tags, could further reduce the susceptibility value of silica-coated MNPs. More details about the magnetic measurements are given in the Supporting Information.

Before further processing these particles their cytotoxicity was first assessed; lactate dehydrogenase (LDH) cytotoxicity assay was used. As shown in the Supporting Information (Figure S2), the percentage of cell death in P0 rat ventricle after 24-hour treatment with the silica-coated MNPs in the concentration range of 200–5000 μg mL^{-1} was comparable with the control indicating that these MNPs were not toxic. To assess the specificity of the Ab-MNPs, primary astrocytes ($CD133^+$) and SH-SY5Y ($CD133^-$) were employed for an in vitro test and Ab-MNPs were first fluorescently labeled with FITC (fluorescein isothiocyanate). By merging the differential interference contrast (DIC) and confocal images the distribution of Ab-MNPs in a cell solution was monitored. It is shown in Figure 2 that the Ab-MNPs can favorably bind

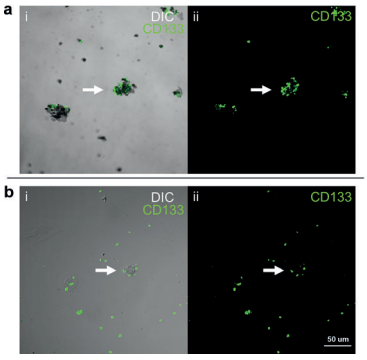


Figure 2. Selective isolation of $CD133^+$ astrocytes with FITC-conjugated Ab-MNPs. a), b) The FITC-conjugated Ab-MNPs were incubated with the cells for 6 h. A merge of differential interference contrast and confocal images show that the anti-CD133-conjugated MNPs bind strongly to the $CD133^+$ astrocytes (a; white arrow) while few Ab-MNPs distribute evenly in $CD133^-$ SH-SY5Y cells culture solution (b). Scale bar: 50 μm. i) confocal images; ii) differential interference contrast images.

to the cell surface of the $CD133^+$ astrocytes. With a magnetic field applied to the cell culture, the $CD133^+$ cells tagged with Ab-MNPs could also be effectively driven in solution. While for the $CD133^-$ SH-SY5Y cell culture Ab-MNPs were evenly distributed in the solution and the cells were immobile on the application of a magnetic field. In addition, the diffusion coefficient of the Ab-MNPs was also assessed (Supporting Information). The Ab-MNPs were shown to recognize and extract $CD133^+$ NSCs in vitro by magnetic means (Figure S3).

An in vivo extraction of NSCs was then performed in adult SD rats. The duration of incubation and magnetic agitation was found to be critical for an efficient extraction of NSCs from the SVZ region. Of the different time points (0 h, 1 h, 3 h, 6 h and 24 h), extensive binding of the Ab-MNPs to the cells could be observed at the 6 h incubation time (Figure S4a–e). It is envisaged that selective binding and removal of labels/cells from the endothelial linings of SVZ is rather dynamic in a rat body. An induction time was clearly required for the diffusion and binding process but a significant lower yield was obtained at the end of 24 h incubation (Figure S4e). The animals were also placed under a weak external spinning magnetic field generated by magnetic stirrer plate. As shown in Figure 3 a–e, 15 min spinning was found to be the optimum time for the maximum detachment of the NSCs with no severe damage to the tissues. Interestingly, a high concentration of Ab-MNPs could be clearly observed on the lining of SVZ with 0 to 10 min agitation. But the particle concentration on the lining was found to progressively decrease presumably due to their levitation and entering to the CSF fluid phase. In contrast, without the magnetic agitation the particle concentration on the lining was not much altered, implying that magnetic agitation is an essential step to liberate the surface assessable magnetic labeled cells into the fluid. Note that the same animals were subject to this repeated magnetic surgery but all of them, after the treatments, were found alive and apparently healthy.

As a result, the 6 h incubation with 15 min magnetic agitation times were chosen as the treatment conditions for further experiments. The detached magnetically labeled cells were then extracted from the SVZ region by a micro-syringe (Figure 4 a,b). The cells of interests (Figure 4 b) were isolated from the extracted-cell mixture by magnetic means by exploiting the magnetic properties of the Ab-MNP-conjugated NSCs. Magnetic agitation was again shown to be essential for the detachment of the $CD133^+$ cells (Figure 4 c). The number of the $CD133^+$ cells for each extraction was around 221.67 ± 79.89 cells/μL. After sorting by a conventional magnetic separation, $CD133^+$ NSCs were concentrated for further processing (see Figure S5). In addition, the magnetic $CD133^+$ cells could also be attracted by a neodymium magnetic probe which can be utilized as a tool for the selective cell extraction (see Figure S5c). Once the probe was inserted into the SVZ of the subjects, the magnetic Ab-MNP-conjugated NSCs were quickly tagged on the probe. Consequently, the magnetic microsurgery process was found to be simple and safe to implement for the animal subjects.

The CD133 spheres were observed in adult rat $CD133^+$ cell culture 6 days after seeding (Figure 4 d) and their average diameter exceeds 100 μm at day 9 (Figure 4 e) whereas no

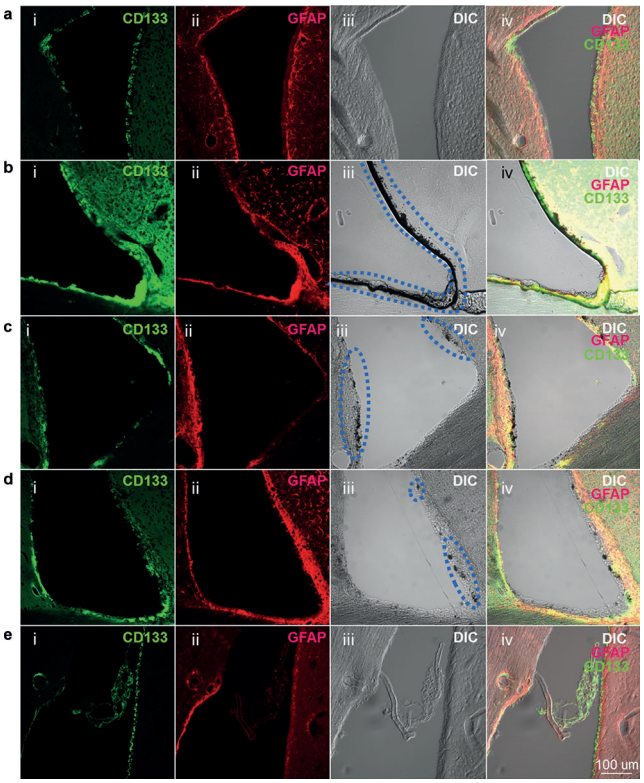


Figure 3. A study of time events for "magnetic agitation" for efficient extraction of NSCs from SVZ. a) without administration of Ab-MNPs; b) 0 min; c) 5 min; d) 10 min; e) 15 min with corresponding images captured from the SVZ linings. The Ab-MNPs (2000 $\mu g\,mL^{-1}$ in 5 μL of PBS) were administrated into SVZ of the adult subjects and incubated for 6 h. Most Ab-MNPs are coated along the walls of the ventricular zone without the application of magnetic agitation (b). However, under the magnetic spinning, the Ab-MNPs labeled cells are agitated and broke away from the SVZ linings (c–e). The longer the magnetic agitation is applied, the lower the concentration of labeled cells is observed. The dashed circles indicate the localization of the nanoparticles. Scale bar: a–e, 100 μm.

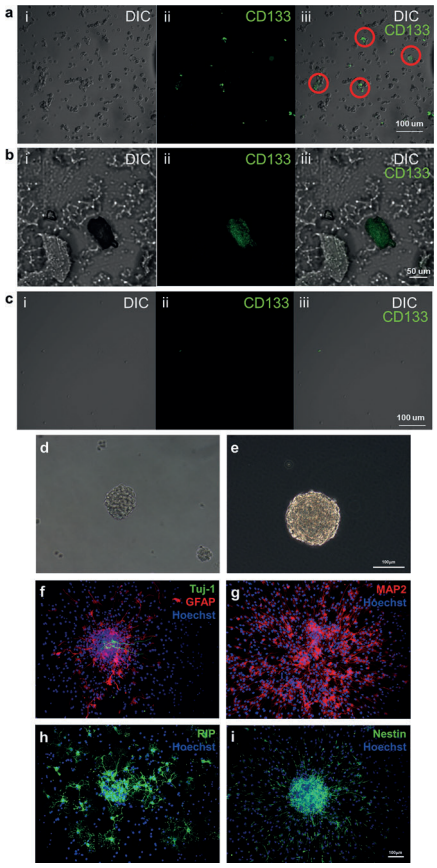
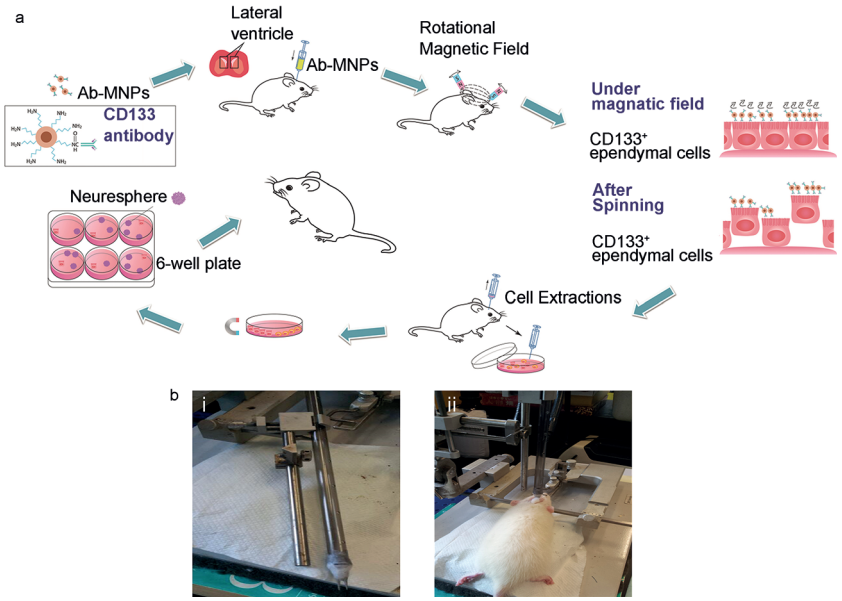


Figure 4. Generation of neurospheres from extracted cells. a) The detached cells with CD133 immunoreactivity obtained after magnetic separation by a syringe. The red rings indicate the presence of the Ab-MNPs tagged cells. b) Higher Magnification of (a). As seen in (b) the MNPs bind specifically around the target cells. c) Without the magnetic agitation, $CD133^+$ cells are hardly found in the extract. d) An enlarge image of an isolated adult rat lateral ventricle $CD133^+$ cell forming a neurosphere before its differentiation into different cell; e) $CD133^+$ cells generated neurospheres after 6 days in culture medium; Neurosphere reaching 100 μm in diameter after 9 days in culture. f, g, h, i) 5 days after being plated onto PDL-coated surface with different types of cells generated from the spheres, including: f–g) Tuj-1 + /$MAP2^+$ neurones; f) $GFAP^+$ astrocytes; h) RIP^+ oligodendrocytes and i) $nestin^+$ uncommitted progenitors. Scale bar: a,c–i) 100 μm; b) 50 μm.

neurospheres could be generated from the discarded extract (the remaining extract after the isolation of $CD133^+$ cells by magnetic separation). In this experiment, only cell clusters with diameter over 50 μm were counted as neurospheres. The extent of sphere formation was approximately $4.49 \pm 2.02\,\%$. Five days after differentiation induction, Tuj-1^+ and $MAP2^+$ neurones (Figure 4 f,g), $GFAP^+$ astrocytes (Figure 4 f), RIP^+ oligodendrocytes (Figure 4 h) and $nestin^+$ uncommitted progenitors (Figure 4 i) were clearly observed, demonstrating the multipotency of the $CD133^+$ spheres. Thus, the in vitro proliferation and multipotent differentiation capability of the adult rat ventricular $CD133^+$ NSCs undoubtedly indicate that the magnetically extracted cells are active for use and modification.

As a proof of the concept, we have demonstrated an efficient method for the single-step isolation of active $CD133^+$ stem cells using magnetic means through tethering antibodies on the tagged nanoparticles (see Scheme 1), which have successfully generated neurospheres in vitro. Neurones, oligodendrocytes, astrocytes, and $nestin^+$ uncommitted progenitors are also produced in further cell differentiations. Despite obtaining a relatively low extent of neurospheres (at present, ca. $4.49 \pm 2.02\,\%$) we believe that it is a first step to lead to the novel application to isolate multipotent neural stem cells/progenitor from adult brains without killing the animal or inflicting significant damage. The average diameter of the spheres of over 100 μm is observed on day 9. Upon induction of differentiation, the neural and glial cells quickly emerge from the neurospheres, while the $nestin^+$ uncommitted progenitors are still largely available. This evidence clearly suggests that adult $CD133^+$ neural stem cells magnetically extracted from surface linings of the lateral ventricle (LV) and SVZ with mild magnetic agitation are active and highly efficient in autologous stem-cell-based cell replacement therapy. Thus, the isolated stem cells/progenitors can be



Scheme 1. a) The in situ extraction of NSCs from an adult rat. A 6 h incubation time is required after injecting the MNPs into sub-ventricular zone of an adult brain; the magnetic labeled cells on the endothelial lines enter to cerebrospinal fluid when the subject is placed under an external spinning magnetic field for 15 min. The labeled cells can then be obtained either by using a micro-syringe or in contact with a typical approximately 6000G NdFeB magnetic probe needle (2 mm i.d., 10 mm in length) inserted into the region for 15 min. The extracted labeled cells are collected for further culture. The subject remains alive and apparently healthy despite receiving the above treatments. b) Photographic images showing the magnetic probe (i) and corresponding micro-surgery (ii) by inserting the probe into the head of a live adult rat.

expanded in vitro rapidly and transplanted back for regenerative treatment.

From a practical point of view, magnetic extraction of ependymal cells as NSCs from the surface linings of LV and SVZ appears to be a safe operation and a relative high concentration of the cells can also be obtained. These active extracted cells can be tailored-made or engineered in vitro to fit specific needs. Although a misregulation of iron metabolism that causes an accumulation of iron has been reported in patients with neurodegenerative disorder,[13] MNPs shows a low cytotoxicity and the amount needed for the extraction is generally very low ($< 0.19\ mg\,kg^{-1}$ for the extraction of $CD133^+$ cells in SVZ; typically $< 4\ mg\,kg^{-1}$ for contrast agents). Previous studies also demonstrated that MNPs can be rapidly metabolized by the endothelial cells in blood–brain barrier,[13,14] making the use of MNPs more acceptable for clinical practice. Thus, this technology though in an early stage of development, may have potential in biological and clinical applications, particularly in the area of regenerative medicinal treatment, from bench to bed.

Experimental Section

MNPs were synthesized by Massart's method[15] which based on the co-precipitation of iron(II) and iron(III) ion in 1:2 molar ratio. A solution of 1 M iron(III) chloride ($FeCl_3$; 4 mL) and 2 M iron(II) chloride ($FeCl_2$; 1 mL) were added to 0.7 M ammonium hydroxide (NH_4OH; 50 mL) under mechanical stirring. The solution was stirred for 1 h until the black iron oxide nanoparticles were formed. The supernatant solution was removed and sediment was washed with distilled water twice under external magnetic field and dried overnight at 70 °C. 3-aminopropyltriethoxy-silane (APTES) groups can be immobilized to the particle surface through -OH groups before *N*′-fluoresceyl thiourea is attached (Supporting Information). Alternatively, pre-conjugated *N*-1-(3-trimethyloxy-silylpropyl)-*N*′-fluoresceyl thiourea (FITC-APTES) can be prepared by adding 2.79 μL (3-Aminopropyl)triethoxysilane (APTES) to 1.11 μL 23 mM Fluorescein isothiocyanate (FITC) in ethanolic solutions and stirred in dark for 24 h. Silica coated MNPs were synthesized by water-in-oil (w/o) reverse micelle method.[16] MNPs (0.2846 g) were dispersed in distilled water (1.7 mL) and *n*-hexanol (8 mL). Then cyclohexane (38.5 mL) and Triton X-100 (9.4 mL) were added to the mixture under mechanical stirring to generate a microemulsion system. Then, TEOS (2 mL) was added to the solution under stirring. After 6 h, 28% ammonium hydroxide (5 mL) was added to the mixture. After 24 h FITC-APTES (1 mL) was added to the mixture and stirred in dark. After 24 h, the FITC-silica coated MNPs were formed and isolated by external magnetic field. The particles were washed with distilled water and ethanol repeatedly and re-suspended in distilled water (5 mL). The antibodies CD133 were covalently conjugated onto the silica coated magnetic nanoparticles by EDC/NHS chemistry.[17] Typically, fluorescent silica-coated iron oxide nanoparticles (160 μg) were incubated with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC; 100 μg), and *N*-hydroxysuccinimide (NHS; 100 μg) for 30 min. The mixture was incubated for 30 min. Then, antibodies CD133 (10 μL) were added to the mixture and incubated for 1 h at room temperature. Further detail of the experimental procedures, materials characterization, and measurements can be found in the Supporting Information.

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