Methods of Mesenchymal Stem Cell Homing to the Blood–Brain Barrier: Methods and Protocols

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Chapter 6

Methods of Mesenchymal Stem Cell Homing to the Blood–Brain Barrier

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Abstract

Mesenchymal stem/stromal cells (MSC) are multipotent cells that can be isolated from adult and fetal tissues. In vitro, MSCs show functional plasticity by differentiating into specialized cells of all germ layers. MSCs are of relevant to medicine and have been proposed for several disorders. MSCs can be transplanted across allogeneic barriers as "off the shelf" cells. This chapter focuses on methods to deliver MSCs to the brain because neurological pathology such as damage due to stroke can lead to debilitating mental and physical problems. In general, neurological diseases are difficult to treat, partly due to the challenge in getting drugs across the blood–brain barrier (BBB). MSCs as well as other stem cells can cross the BBB. The described method begins to develop procedures, leading to efficient delivery of drugs to the brain. Here describe how MSCs can be propagated from bone marrow aspirates and their utility in delivering small RNA to the brain. The chapter discusses the issue to enhance efficient delivery of MSCs to the brain.

Key words Mesenchymal stem cell, Bone marrow, Blood–brain barrier, Noncoding RNA, Drug delivery

1 Introduction

The application of stem cells (SC) for neurological damage caused by events such as a stroke or hypoxic-ischemia (HI) are well studied [1, 2]. Systematic research studies have been done to determine if SCs can form functional neurons. Among the studied SCs are mesenchymal SC (MSC), which can be induced to generate cells of all germ layers, including neural cells [3]. MSC are obtained from adult and fetal tissues. These include but are not limited to cord blood, fetal liver, bone marrow (BM), and adipose tissue [4]. Once removed from in vivo sources, the MSCs are expanded in vitro, and characterized to ensure consistency with the literature.

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There is no clear answer on the method of using MSCs to reverse brain damage. The question that remains is whether the MSCs should be injected directly to the injured site versus other routes such as intravenous injection [5–7]. Direct engraftment of the MSC could be an advantage since the cells will be implanted directly to the damaged site. This approach might be more efficient if the process does not require an invasive procedure, which could lead to damage to the site of engraftment, including to the blood brain barrier (BBB) [5]. Systemic delivery of MSCs is safe but may be less efficient to reach the brain as compared to direct implantation of MSCs [8]. One of the problems of indirect delivery is the lung effect, indicating that intravenous injection of MSCs may be trapped in the lungs [6, 9]. This would limit the number of MSCs that could have the potential to reach the brain. As an alternative, intranasal application of cells provides a noninvasive method to deliver SC directly into the central nervous system (CNS) [10].

Experimental trials with MSCs have indicated improvement in neural outcomes, without evidence of cell replacement [10, 11]. This suggested that the MSCs may be acting through mediators to correct the pathological problems. The secretome of MSCs could be soluble factors as well as microvesicles such as exosomes [12, 13]. The identification of the MSC-derived secretome is a complex process since the particular factor could be different within the complex microenvironment in vivo. The identification of how MSCs secrete distinct secretome is thus crucial. Answers to these questions could continue to be sought as drug delivery of MSCs continue in experimental studies. An additional layer of complexity arises when autologous therapies are being designed as age and disease state might alter the secretome: it is well known that age alters the secretome of MSC and that it can even become harmful, making it necessary to think about cell quality [14].

The BBB is a major physical barrier that MSC must overcome [8]. This highly selective, semipermeable membrane is made up of interacting CNS endothelial cells which are held together by tight junctions [9]. Under normal conditions, the BBB prevents most cell types from entering into the brain, however there have been no detailed studies conducted regarding how much the BBB is an active barrier for MSC in treating the brain. This limits interaction with the CNS that might benefit neural functions. The efficient protection by the BBB could be damaged by injury such as stroke, brain tumor, and aging [15–17]. During pathological conditions, the tight connection of the BBB is disrupted and would allow cells to cross into the neural tissues. Among the cells that may get into the brain are stem cells such as MSCs. However, it is important to be able to control the modulation of the BBB in order to allow a greater number of MSCs or their secreted secretome to reach the desired site in the brain. Mannitol, which has been used in clinical

practice to reverse intracranial pressure, could be used to temporarily get MSCs into the brain [18].

The method by which mannitol has been used to release excess fluids from the brain may apply to allow MSCs to enter the brain. In fact, there is support to test the effectiveness of mannitol as a potential modulator of the BBB for the effective use of systemic MSC therapy [18] (*see* **Note 1**). The clinical application of mannitol has been used to ease the intracranial pressure, which could occur by the blockage of veins that drain blood from the brain, or an increase in volume of blood or brain tissue [19]. The osmolarity of mannitol releases the pressure through a temporary increase in the size of the tight junctions in the BBB via contraction of the endothelial cells [20]. This property of mannitol has been proposed to allow the MSCs to get into the brain after the junctions have been temporarily opened. This opening into the brain is believed to be able to allow for the efficient crossing of MSCs and/or their secretome to the desired site in the brain [19, 20]]. Experimental studies are needed to determine how the treatment may get to the desired site within the brain for targeted outcome. Research studies include methods to identify how inflammatory mediators and their receptors could aid in the precision method to get injected cells into the intended target. The dosing and timing of mannitol will need to be further studied to ensure that there is no damage caused by the opening of the tight junctions as prolonged use of mannitol can lead to swelling of the brain.

The method to isolate large numbers of MSCs has been established in the majority of academic/clinical centers. However, methods to get MSCs in the brain remain an unmet need. More importantly, if methods could be established to get MSCs into the brain, these stem cells could be used as drug delivery vehicle. The instruction part of this chapter will describe how a representative drug, noncoding RNA, can be delivered within MSCs for target in the brain. Additionally, the chapter will include instruction on experimental methods to label MSCs for tracking both in vitro and in vivo.

Several studies have attempted to track MSCs in animal models, some yielding confounding results [2, 6–8]. In all cases, intravenously injected MSCs are initially trapped in the lungs where they can secrete various factors for a systemic response [7]. Over time, many of the MSCs migrate from the lung to various tissues, including the brain, bone marrow, liver, spleen, kidney, and pancreas [7]. However, different groups have reported varying degrees and efficiencies of engraftment of transplanted MSCs. These differences can be explained, perhaps, by varied efficiency of MSC from distinct sources (bone marrow, adipose, placenta) to migrate, the cell culture conditions, and the number of times the MSCs were passaged [9, 10]. Further, the delivery method itself can influence

the MSCs' homing ability, with direct injection permitting—or increasing efficiency of—homing to target organs not efficiently reached by intravenous injection [11]. As such, cells administered via intranasal administration are able to migrate and to reside in the CNS [21–23]. Migration initiates in the nasal mucosa through the cribriform plate following the olfactory neural pathway and other nasal routes (e.g., trigeminal and perivascular routes) [23]. Thus, the described methods can be used to improve the in vivo tracking of transplanted MSCs in a time-dependent manner.

2 Methods

2.1 Culturing Bone Marrow-Derived MSCs

1. Add 10 mL of MSC media to vacuum-gas plasma treated tissue culture Falcon 3003 petri dishes.
2. Add 1–2 mL of bone marrow aspirate to the dish. Gently swirl the plates mix the aspirate within the tissue culture media.
3. Incubate at 37 °C in 5% CO_2 for 3 days.
4. Remove nonadherent cells and slowly transfer into a 50-mL tissue culture tube containing 25 mL of Ficoll Hypaque 1.077 g/mL (*see* **Notes 2–4**).
5. Immediately after removing the nonadherent cells, add 5 mL of MSC media to the petri dish. The media should be replaced as quickly as possible to avoid the dish to become overly dry.
6. Separate the red blood cells and neutrophils placed on top of the Ficoll Hypaque by centrifuging for 30 min at 500 × *g* at room temperature. To aid in a clear separation, place the brake on the centrifuge in the "off" position.

   After centrifugation if you notice a cloudy upper layer, this could be due to remaining mononuclear cells in the upper layer. To ensure recovery of all mononuclear cells, continue to centrifuge for ~5 min. If needed, you may prolong the centrifugation time. You should be cautious at this point since you may centrifuge the desired middle layer containing the mononuclear fraction within the Ficoll Hypaque layer.
7. Immediately after centrifugation, aspirate the top layer, which contain media without interrupting the middle layer, which contains the desired mononuclear cells.
8. Immediately use a Pasteur pipette to carefully remove the mononuclear layer and transfer to a tube containing any tissue media with 2% FCS (*see* **Note 5**).
9. Wash the cells by centrifuging at 500 × *g*, room temperature for 20 min. If you notice a cloudy upper layer, this will likely indicate that the mononuclear cells have not been pelleted. To recover all of your cells, recentrifuge for a longer time, ~5 min,

but this could require a longer time until the upper layer is clear.

10. Immediately aspirate the media and disassociate the pellet by forcefully tapping the pellet with your fingers. This should not be delayed since the pellet, if remained in the tube for a prolonged period, will compromise the cell viability. Add fresh media with 2% FCS and then centrifuge for 15 min (Wash step).
11. Add MSC media to the pellet and then resuspend.
    Rule of thumb: Add 5 mL of the suspension cells/original petri dish. Specifically, if you began with ten petri dishes and the contents of the ten dishes were added to one 50 mL tube then you should add 50 of media.
12. Mix the cells and distribute the cell suspension equally into the dishes. In this described method, you should add 5 mL cell suspension/dish.
13. Reincubate the culture dishes. At weekly intervals, replace 50% of the media (*see* **Note 6**).
14. At 80–90% confluence, trypsinize the adherent cells and split at a ratio of 1:3 to 1:6 (*see* **Note 7**).

2.2 Preparation of MSCs

This step is needed if further research is conducted with a particular population of MSCs. Also, if the experiment needs to track the MSCs such as for in vivo transplantation, the MSCs should be labeled with a fluorescence dye (see below) or the use of a vector containing a fluorescence reporter gene.

2.2.1 Cell Sorting

1. Wash MSCs with 1× PBS.
2. Add 2 mL of 0.05% trypsin to cells in T75 Flask and incubate at 37 °C for 4 min (*see* **Note 8**).
3. Transfer trypsinized cells to media containing 5–10% FCS (*see* **Notes 9–11**).
4. Pellet the cells by centrifuging at 500 × *g* for 10 min. Aspirate the supernatant and resuspend in 10 mL of 1× PBS.
5. Repeat **step 4** (*see* **Note 12**).
6. Label MSCs with the desired fluorescence-tagged antibody. For example, if you need the population expressing leptin receptor, able accordingly. Incubate with the antibody for 30 min; wash once by filling the tube with PBS and then centrifuge.
7. Resuspend cells in 10 mL of sorting buffer.
8. Centrifuge at 500 × *g* for 5 min.
9. Aspirate the supernatant and resuspend the pellet in 2 mL of sorting buffer.

10. Determine cell concentration and dilute with sorting buffer as needed (*see* **Note 13**).
11. Filter cells through a strainer cap to remove any clumps that may clog the instrument (*see* **Note 14**).
12. Use the tube consistent with the institutional cell sorter for collection. Add 2 mL of complete media to the collection tubes (*see* **Note 15**).

2.3 Cell Transfection

This section describes the method to take the MSCs and load noncoding RNA for delivery to the brain.

1. On the first day, seed MSCs to achieve 60–80% confluence for transfection (*see* **Notes 16** and **17**).
2. On day of transfection, dilute 9 μL of Lipofectamine RANiMAX Reagent in 150 μL of Opti-MEM medium per well.
3. Dilute 3 μL of miRNA (10 μM) in 150 μL of Opti-MEM medium per well.
4. Add 150 μL of diluted miRNA to 150 μL of diluted Lipofectamine RNAiMAX Reagent per well and incubate for 5 min.
5. Add 250 μL of miRNA–lipid complex into each well. In each well, this will contain 25 pmol of the RNA and 7.5 μl of Lipofectamine RNAiMAX.
6. Incubate the cells for 1–3 days at 37 °C. Take an aliquot of cells to ensure you have transfected your miRNA. This can be done by PCR for the miRNA. Alternatively, if you tag the miRNA with a fluorochrome, you can test your MSCs by flow cytometry.

2.4 In Vivo Injection

1. Resuspend ~10^6 anti-miRNA or fluorochrome-labeled transfected MSCs in 0.2 mL PBS.
2. Inject the entire volume in a mouse, intraorbitally or intravenously. If you are using human cells, you could use immune deficient mice. However, this is not necessary since human MSCs can survive in xenografts.

Track the homing of MSCs after sacrificing the mice, and then label for human protein using anti-human NUMA [4]. If you opted to use fluorescence-labeled MSCs, sections of tissue can be analyzed for cell fluorescence. While traditional fluorescent labeling may be beneficial for short-term experiments (e.g., DiR), long-term tracking requires fluorochromes that will not degrade during the experiment period. Qdots overcome this difficulty, with an ability to identify as few as 1000 labeled transplanted MSCs after greater than 30 days [1, 2]. Like DiR and Qdots, labeling with firefly luciferase allows for tracking transplanted MSCs in vivo, by imaging systems [3]. In addition to these staining methods, MSCs

can be labeled with contrast or trackers for identification by magnetic resonance imaging (MRI), positron emission tomography (PET), and single-photon emission CT (SPECT). However, the effect(s) of these labeling mechanisms on MSC multipotency and function have largely yet to be evaluated [5].

2.5 Intranasal Application

1. Hold the mouse via hand constrain and recline the mouse onto its back while the skull is immobilized.
2. Apply 6 μL of 100 U hyaluronidase solution per nostril twice to a final volume of 24 μL. The hyaluronidase solution is applied by nose drops placing 10 μL pipette tip carefully in the nostril allowing the drop to be inhaled. After the first application wait 2 min before administering the 2nd application [24] (*see* **Note 18**).
3. Place the mouse back into the cage and wait 30 min prior to administering the cells.
4. Resuspend 10^6 anti-miRNA or fluorochrome-labeled transfected MSC in 24 μL PBS.
5. Apply transfected MSC as described in **steps 1** and **2** without the hyaluronidase.

2.6 Summary

The method to expand MSCs seems to be established within the community of stem cell scientists. However, it is unclear if all sources of MSCs will show similar efficiency to home to the brain. This chapter provides the basis for more in-depth research studies. Figure 1 shows the initiating culture with BM aspirate following by phenotypic characterization. The expanded MSCs are sorted if needed and then injected into mice. The MSCs if loaded with a tracking dye can be imaged. Alternatively, sections of the brain can be used to identify the homing of human cells.

3 Notes

1. Studies are needed to optimize dosing and administration times, but the following was found in a study done by Gonzales-Portillo et al. and can be used as a reference for further study [10]. Mannitol (1.1 mol/L at 4 °C) is administered at the same injection site as the umbilical cord blood with injections taking place over 10 min. For adults, 1.5–2.0 g/kg of mannitol was administered while the dose for pediatrics was 0.25–1.0 g/kg.
2. This assumes that you have multiple culture plates from the same donor. If not, use a 15-mL conical polystyrene tube. In cases where there are multiple plates, pool the nonadherent cells from the same donor into the same tube of Ficoll

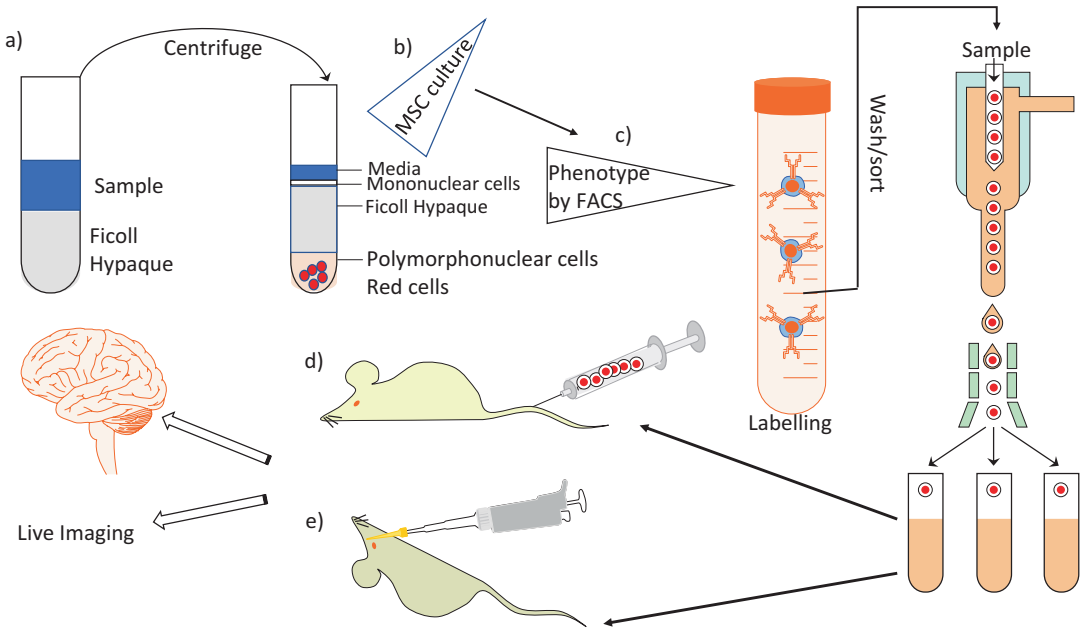

Fig. 1 Work flow with MSC to studies on brain homing. (**a**) 3-day nonadherent media from MSC cultures are subjected to Ficoll Hypaque gradient separation. (**b**) The MSCs are expanded and then characterized phenotypically. (**c**) MSCs are subjected to phenotypic characterization by FACs and if needed by sorting. (**d**) The expanded MSCs are injected intravenously into mice. (**e**) Alternatively, the MSCs are injected intranasally to maximize cell delivery to the brain. Tracking of the MSCs can be done by live imaging or by sectioning of the brain

Hypaque. As you transfer the nonadherent cells onto the Ficoll, try to remove most of the red blood cells from the culture dish. This is important because as the red cells begin to lyse this could become toxic to the MSCs.

3. Pipet the nonadherent cells with a 2-mL pipette or a glass 9" Pasteur pipette. Slowly add to the Ficoll Hypaque gradient by placing the tip of the pipette to the corner of the tube. Rapid addition to the Ficoll Hypaque could prevent a clean separation of the different fractions and this would result of the mononuclear cells.
4. Failure to remove the red blood cells will lead to the subsequent lysis during the period of expansion. If this occurs, the lysed material will be toxic to the adherent cells. To avoid this, use a 5 or 10 mL pipette to mix the nonadherent cells before removing them from the culture dish.
5. Ficoll Hypaque is toxic to the cells. Thus, immediately after centrifugation, remove the mononuclear cells. Since you are likely to pipette some of the Ficoll Hypaque, try to dilute this with sera-free media; otherwise it will be difficult to pellet the cells. The latter could remain within the Ficoll. As a rule of

thumb, you can use conical tubes: E.g., 15 mL tubes could contain ~7 mL of Ficoll and ~5–7 mL of cell suspension.

6. Avoid confluency of the MSC cultures as cell contact could lead to spontaneous differentiation.
7. After four passages the adherent cells should be asymmetrical and spindle shaped, and display the following phenotypes: $CD14^-$, $CD29^+$, $CD44^+$, $CD34^-$, $CD45^-$, $SH2^+$, prolyl-4-hydroxylase$^-$.
8. Deadhered cells should be floating with trypsin. If not, hit sides of flasks to dislocate the cells. If your cells are in petri dishes you can dislocate the cells by pipetting up and down.
9. If you are planning to study surface markers or if the cells are sensitive to trypsin, use Accutase or TrypLE to deadhere.
10. The volume of the media needed to transfer the trypsinized cells will depend on the volume of trypsin. The sera within the media served as a substrate for residual trypsin. However, if the trypsin is diluted sufficiently, it will become inactive.
11. Media containing diluted FCS is necessary to stop the trypsin activity. FCS will act as a substrate for any active diluted trypsin.
12. If residual FCS is believed to remain, another wash may be performed. However, avoid excess washing as each wash would result in cell loss.
13. If the cells form clumps, incubate with 5 mM EDTA for 10 min and then resuspend in sorting buffer.
14. Cell concentration used for sorting will depend on the number of final cells required. Also, the starting number of cells will depend on the expected frequency in your population. As a general rule, the cell concentration is $1–2 \times 10^6$ cells/mL, otherwise sorting time will be long. It is always better to start with a higher concentration of cells and then dilute as needed.
15. A small amount of bovine sera albumin or serum can result in healthier cells after sorting.
16. The following procedure is scaled for the use of 6-well plates. If using 24- or 96-well plates, adjust accordingly. Also, you may look online at the Invitrogen Lipofectamine RNAiMAX Reagent protocol.
17. If the reduction in viable cells is not an issue, you may also transfect by electroporation.
18. Hyaluronidase allows for invasion of the cells by breaking up the barrier of the nasopharyngeal mucosa. Diluted hyaluronidase should be aliquoted in small volumes to avoid freeze–thaw.

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