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Fluorescent Nissl Stain for Monitoring Neuronal Differentiation in Human Adipose-Derived Stem Cells

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ABSTRACT

Nissl stain has been widely used to identify mature neuronal cells in neuroscience and Nissl substance reflects the high protein synthesis capacity of neuronal cells. This study explored whether Nissl stain can be developed as a standard approach to monitor neuronal differentiation of human adipose-derived stem cells (hADSCs). hADSCs were induced with neural culture media supplemented with growth factors, including human basic fibroblast growth factor (bFGF), epidermal growth factor(EGF), and ciliary neurotrophic factor (CNTF). Immunofluorescent staining was performed to visualize the expression of nestin (neural progenitor marker), β-□-Tubulin (early marker of neurons) and microtubule-associated protein 2 (MAP2, marker of mature neuronal cells). Neuron Trace Fluorescent Nissl Stain was applied to stain the Nissl substance at different time points during induction. After 7 days of neuronal induction, cells started to lose the characteristic shape of mesenchymal stem cells and expressed high levels of nestin. Low levels of Nissl substance were detected within these nestin positive cells. Only a small percentage of cells expressed MAP2 and showed positive fluorescent Nissl stain. From day 14 to day 21 after the initiation of neuronal induction, cells developed typical neuronal cell morphology with axons and dendrite-like shapes. In these cells, the expression of both MAP2 and β-□-Tubulin, as well as positive fluorescent Nissl stain, were observed. Our results indicate that the use of Neuron Trace Fluorescent Nissl Stain is a more sensitive, faster, easier and accurate approach compared with immunocytochemistry for observing the progression of neuronal differentiation in hADSCs

Keywords : Nissl stain; neuronal differentiation; hADSCs

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent cells that can self-renew, proliferate, and differentiate into a variety of cell types [2, 4, 5, 19]. They are a relatively accessible source of stem cells, either from bone marrow or peripheral blood, with which there is extensive clinical applications. For example, musculoskeletal diseases, ischmia, central nervous system, autoimmune diseases, liver diseases, heart diseases, etc [13]. In addition to their potency and accessibility, these cells do not elicit graft versus host (GVH) disease because they are less immunologically sensitive[11,16]. More importantly, autologous MSCs are available directly from the patient, eliminating the need for immunological matching. Since they have already been reported to differentiate into neuron-like cells with soluble differentiation chemical stimuli or specific culture supplements, they may represent the best suitable cell source for autologous stem cell–based therapies.

Adipose-derived stem cells (ADSCs) may represent a valid alternative to bone marrow-derived mesenchymal stem cells (BMSCs) due to their pluripotency and ability to differentiate into mesenchymal and non-mesenchymal lineages. Moreover, they are readily accessible and can proliferate quickly in vitro, with lower senescence ratios than BMSCs [1]. Additionally, the number of cells obtained by liposuction aspirates is usually sufficient for clinical application, avoiding further manipulation[9].Regarding their neuronal differentiation potential, some researchers have recently demonstrated that

AMSCs display the same neuronal potential as BMSCs in vitro[8,14].

Current methods for identifying neural differentiation in hADSCs rely on morphological changes and immunoreactivity for neural specific markers, such as neuron specific enolase (NSE), s found in mature neurons and cells of neuronal origin; neuronal nuclei (NeuN), which has been widely used to identify neurons in tissue culture and in sections and to measure the neuron/glia ratio in brain regions; microtubule-associated protein 2 (MAP2), that are enriched in dendrites, implicating a role in determining and stabilizing dendritic shape during neuron development. However, neuronal cell differentiation at the immunocytochemical level is not well understood. What is more, the aforementioned neuronal markers are not specific for neurons. Current neuronal markers are also found in other cells and tissues, therefore it is necessary to find a unique neuron marker. Furthermore, a neuron in the central nervous system (CNS) is defined not only by its polarity in cytology and specific protein, but more importantly by its physiological condition [7].The process of stem cell differentiation into neuronal cells is a long and complex process, so the capability to monitor, accurately and in detail, individual cell characteristics and physiological changes during neuronal differentiation in not only AMSCs, but all sorts of stem cells is of great utility.

The present study evaluated the use of Nissl stain as a new tool for the characterization of the neuronal differentiation process. Nissl bodies are large granular structures consisting of clumps of endoplasmic reticulum in the cytoplasm of neurons. Nissl staining is a standard histological method for visualizing neurons in the brain and spinal cord. Various fluorescent or chromophoric "Nissl stains" have been used to stain the Nissl substance in tissue samples, thereby identifying neuronal cells. Nevertheless, in the neuronal differentiation of hADSCs, Nissl stain has not been applied to the monitoring of the process. The objective of our investigation is to explore whether Nissl stain is suitable for developement as a simple and reliable method for monitoring and labelling the neuronal differentiation of hADSCs. We performed Nissl staining together with other neuronal marker labeling within the same cell during differentiation. Our results indicate that Nissl staining can be a new marker for monitoring hADSC neuronal differentiation and physiological status with the potential clinical utility for monitoring stem cell therapy in nervous system diseases.

MATERIALS AND METHODS

Isolation and culture of hADSCs

Samples of human adipose tissue (10ml) were collected during hip liposuction surgery from 5 donors (20-45 years old) after informed signed consent and according to the requirements of the Hospital Ethics Committee. Liposuction tissues were washed three times with sterile phosphate-buffered saline (PBS), and treated with an equal volume of 0.1% collagenase type I for 30min at 37° C with intermittent shaking. The floating adipocytes and liquid supernatant were separated from the stromal vascular fraction by centrifugation (1200 rpm for 5 min). The cellular pellet was resuspended and washed twice in PBS (1000 rpm for 10 min). The cells were then resuspended in H-DMEM medium supplemented with 10% FBS, 100U/ml penicillin, and 100μg/ml streptomycin. The suspension was plated in tissue culture flasks at 2×106 cells/cm2 and incubated in a humidified atmosphere of 5% CO2 at 37° C, After 72 hours, non-adherent cells were removed. When 70–80% confluent, adherent cells were trypsinized, harvested and expanded in larger flasks.

Flow cytometric analysis

hADSCs were characterized by cellular surface marker proteins of CD13, CD34, CD44, CD45, CD71, CD90 and CD106. Passage four cells were collected were washed in PBS (pH=7.4) by centrifugation at 1000 rpm for 10min, adjusted at 1×106 cells/ml. Monoclonal CD antibodies were added and the cells incubated for 30 min in the dark and then washed again with PBS at 1000 rpm for 10 min. Specific fluorescent labeling was analyzed with a FACS AriII flow cytometer (BD). 10,000 events were counted and analyzed with the Diva software program of the same company.

Osteogenic and adipogenic differentiation of hADSCs

Osteogenic and adipogenic differentiation of hADSCs was induced according to a protocol published by Bertani et al [3]. For osteogenic differentiation, 1.0×105 cells were seeded into each 35 mm dish and cultured for 28 days with 100 nM dexamethasone, 50 μM ascorbic acid and 10 mM β-glycerophosphate. Osteoblast differentiation was evaluated by Von Kossa staining according to Sheehan's method [23]. To achieve adipogenic differentiation, cells were grown in the presence of 1 μM dexamethasone, 0.2 mM indomethacin, 10 μg/ml insulin and 0.5 mM 3-isobutyl-1-methylxanthine. After 3 weeks of culture, cells were fixed with 4% paraformaldehyde, covered with 3 mg/ml Oil Red O (Sigma, USA) dissolved in 60% isopropanol for 10 min and the excess dye was washed out with water.

Neuronal differentiation of hADSCs

hADSCs were trypsinized and subcultured on sterile glass coverslips in 35 mm dishes at a number of 5.0×103 cells per dish. To induce neural differentiation, hADSCs were preincubated for 24 h with stage I media, consisting of H-DMEM supplemented with 1% FCS, 10 ng/ml basic fibroblast growth factor (bFGF) and 10 ng/ml epidermal growth factor human (EGF). Next, the cells were incubated for 7~21 days with stage II media, composed of H-DMEM supplemented with 20 ng/ml bFGF, 20 ng/ml EGF, 20 ng/ml brain derived neurophic factor (BDNF) and 6mg/ml all-trans-retinoic acid (RA) (all from Sigma, USA). hADSCs cultured in basic medium and neurons were used as negative and positive control groups, respectively.

Nissl staining and immunofluorescence

After neuronal differentiation, the cells were stained with 200 μl of Neuro Trace (Invitrogen, USA) and incubated for 2 h. After staining, the cells were washed for 10 min in PBS plus 0.1% TritonX-100 (Sigma, USA) and then in PBS for 2 h at room temperature. For immunofluorescence staining, the cells were fixed with 4% formaldehyde (Solarbio, China) for at least 1 h at room temperature, and then the coverslips were incubated with PBS plus 0.1% TritonX-100 for 10 min at room temperature. Subsequently, the coverslips were washed twice in PBS and incubated overnight in rabbit anti-MAP2 antibody (1:200) and anti-β-III-Tubulin (0.4 μg/ml). After washing with PBS, the cells were incubated for 2 h at room temperature with a fluorescein-conjugated sheep anti-rabbit IgG antibody (1:500) (all from Sigma-Aldrich, USA). Finally, the converslips were washed for 5 min with PBS. Hoechst33258 (Sigma-Aldrich, USA) was used to stain the nuclei. Slides were visualized with fluorescent microscope (Leica, Germany). At least 10 images were randomly chosen from each staining, and the number of positive cells were counted and analyzed.

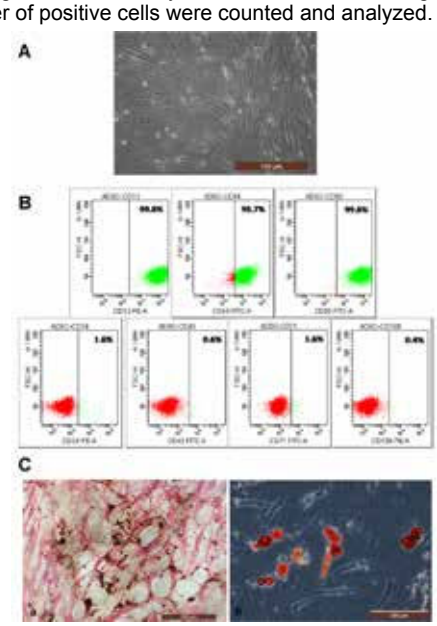


Figure 1: Morphology and characterization of hAMSCs. (A) Phase-contrast photomicrograph of hAMSCs. In untreated culture, hAMSCs showed a spindle-shaped, fibroblastic morphology. Bar=100 μm. (B) Flow cytometric analysis of surface adhesion molecules on hAMSCs. hAMSCs were labelled with monoclonal antibodies specific for molecules indicated in each flow cytometric histogram: the cells were positive for CD13, CD44 and CD90 but negative for CD34, CD45, CD71 and CD106. (C) Analysis of (a) osteogenic and (b) adipogenic differentiation of hAMSCs. (a) Von Kossa staining after 28 days of osteogenic induction. (b) Oil Red O staining showing lipid droplets after 21 days of adipogenic induction. Bar=200 μm.

Statistical analysis

The results are presented as the mean ± standard deviation (SD) and were evaluated using the Student's t-test. $P < 0.05$ is considered statistically significant.

RESULTS

hADSC characterization

hADSCs were isolated from abdominal fat and cultured in vitro. Undifferentiated hADSCs appeared as a monolayer with spindle morphology as revealed by phase contrast microscopy. As the cells approached near confluence, they assumed a more spindle-shaped, fibroblastic morphology (Fig.1A). Flow cytometry analysis of the hADSCs

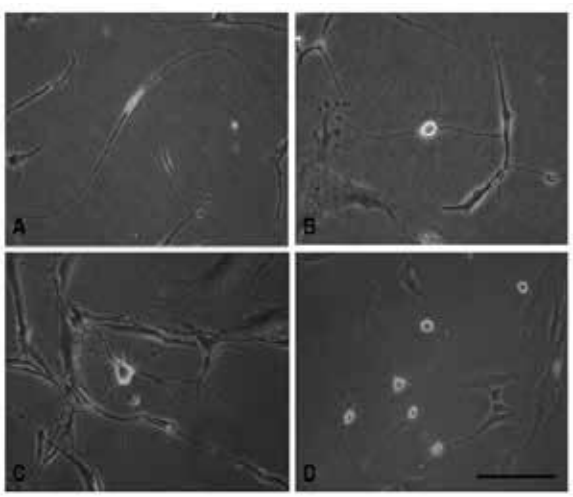


Figure 2: Cell morphology change after neural induction for 6 hours. Morphologic changes were observed and recorded after 6 hours with phase contrast microscope. Neural-like cells derived from AMSCs displaying bipolar (A), tripolar (B), multipolar (C) and wreath-like chains (D). Magnifications: 100X.

demonstrated that the cells were positive for CD13, CD44, CD90 and negative for CD34, CD45, CD71 and CD106 (Fig. 1B), as consistent with other reports [11,3,18]. To verify that isolated hAMSCs were multipotent, their ability to differentiate into osteocytes and adipocytes was analysed using the protocols published by Bertani et al [3]. After 28 days in osteogenic medium and 21 days in adipogenic medium, the hADSCs differentiated into osteocytes and adipocytes as attested by an increase of calcium salts or Oil Red O staining, respectively (Fig.1C (a) and (b)).

Cell morphological change after neural induction

In standard culture conditions, confluent hADSCs appeared as spindle-shaped and did not express neuronal markers (Fig.1A). After 6 hours of induction, the cells displayed small, spherical, and refractive cell bodies, and developed long bipolar branching. The number of cells with this morphology increased progressively over the induction period, forming secondary and tertiary branches with growth cone-like terminal expansions (Fig.2).

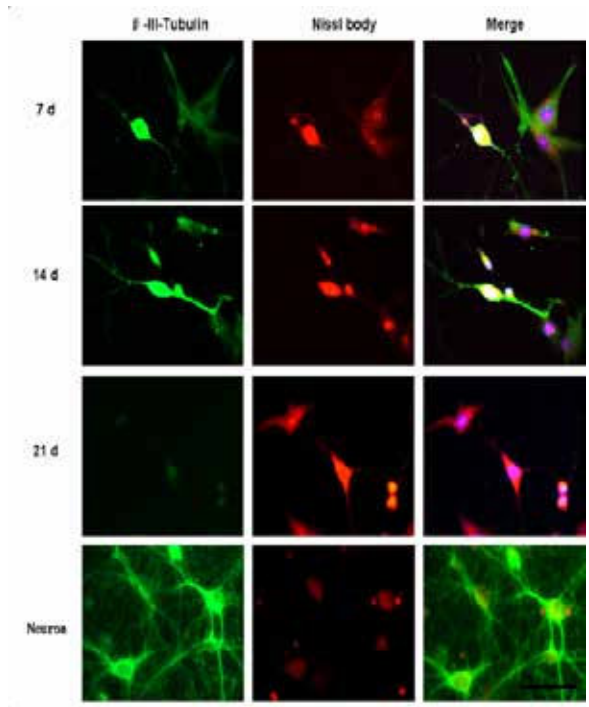


Figure 3: β-III-Tubulin expression and Nissl staining of hAMSCs after induced with bFGF, EGF, CNTF and RA for 7, 14 and 21 days. Nuclei were counterstained with Hoechst 33258. Photographs were obtained with fluorescence microscopy. Neurons were used as the positive controls. Bar=50 μm.

Nissl stain and neuronal marker expression of hADSCs after induction

To investigate whether a Nissl-type staining would be a more adequate approach to monitor the

progress of neuronal differentiation, we compared Nissl staining with immunochemistry staining for β-III-Tubulin and MAP2, both neuron specific markers (Fig. 3-5). The expression of β-III-Tubulin has been suggested to be one of the earliest markers to signal neuronal commitment in primitive neuroepithelium. Microtubule-associated protein 2 (MAP2), that are enriched in dendrites, implicating a role in determining and stabilizing dendritic shape during neuron development. Also found exclusively in nerve cells are the most well studied MAP2, being found mostly in dendrites.

After 6 hours of neuronal induction, the cells lost the characteristic morphology of mesenchymal stem cells, nevertheless, morphological changes may appear as coincidental, having no relationship with a neuronal phenotype (Fig.2). We therefore attempted to correlate these results with neuronal marker expression after induction. Neuronal cells are classically characterized by the expression of cytoskeletal protein β-III-Tubulin and MAP2. As an early marker of a neuronal phenotype, expression of β- III -Tubulin was increased after induction for 7 days (Fig.3 and 4). The maximum percentage of cells expressing β- III-Tubulin was 94.3±1.4% (Fig.6). When induced for 21 days, the percentage of β- III -Tubulin expression decreased to 34.1±2.1%. To examine the subcellular distribution of Nissl bodies in induced ADSCs, we immunostained cells with a Nissl body staining. When induced for 7 days, the percentage of Nissl substance expression was 89.1±2.1% and over 90% of cells were positive for Nissl staining when induced for 21 days (Fig.3-5). The appearance of nissl bodies is associated with mature neurons. To confirm this, we combined β- III -Tubulin with Nissl body immunostaining and found that β- III -Tubulin clusters colocalized with Nissl substance (Fig. 3and 4). This suggested that the variation trend of β- III -Tubulin expression and Nissl body formation are associated processes of mature neural-like cells.

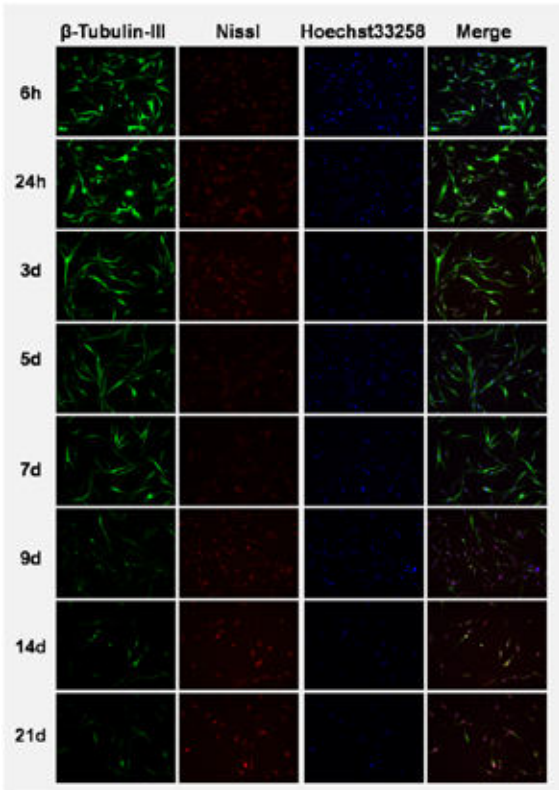


Figure 4: A continuous monitor process of β-III-Tubulin expression and Nissl staining of hAMSCs. Nuclei were counterstained with Hoechst 33258. Photographs were obtained with fluorescence microscopy. Bar=50 μm.

After 14 days of neuronal induction, the cells started to express low levels of MAP2. Over 60% of cells were MAP2 positive after 21 days of induction (Fig.5-6) and these cells exhibited typical neuronal cell morphology with axons and dendrite-like shapes. For this population of cells, both the expression of MAP2 and positive fluorescent Nissl stain were observed. The formation of Nissl bodies further suggested that these induced cells have the characteristics of neurons. There was no significant increase in β- III -Tubulin, MAP2, and Nissl stain positive cells in the negative control group.

The percentage of positive β- III -Tubulin/Nissl stain and MAP2/Nissl stain is shown in the table of Fig 6. Interestingly, there was no significant difference between the percentage of positive cells for both β- III -Tubulin/Nissl and MAP2/Nissl. The ratios for double positive cells were all over 0.8, even when the percentage of β- III -Tubulin or MAP2 positive cells was low. This suggests that Nissl body formation is a common feature of different neuronal differentiation stages.

DISCUSSION

The immunoreactivity of neural specific markers, such as β-III-Tubulin, NSE, NeuN, MAP2 is the most common method employed to detect the neural differentiation of stem cells. However, current neural markers are also found in other cells and tissue. For example, NSE, although found in mature neurons, is also used as a tumor marker in lung cancer patients [17]. Additionally, nestin has been reported to exist in undifferentiated mensenchymal stem cells [18,20], making its specificity poor for use as a differentiation detection marker. Moreover, the process of stem cell functional differentiation is highly complicated and the mere expression of neural proteins may not reflect a change of cell physiology. Therefore, it is necessary to explore the use of new neuronal markers with greater specificity in order to effectively monitor cell change characteristics during mesenchymal stem cell differentiation into functional neuron-like cells.

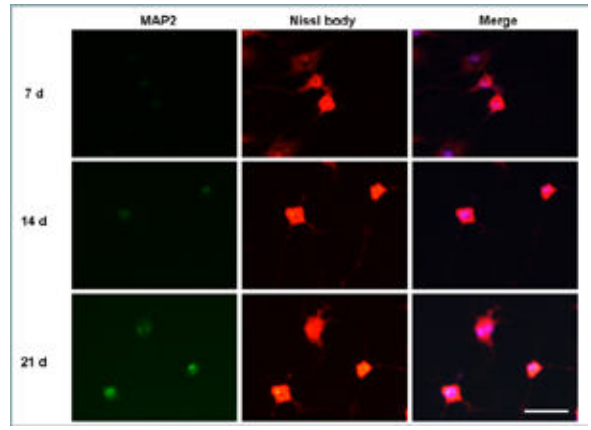
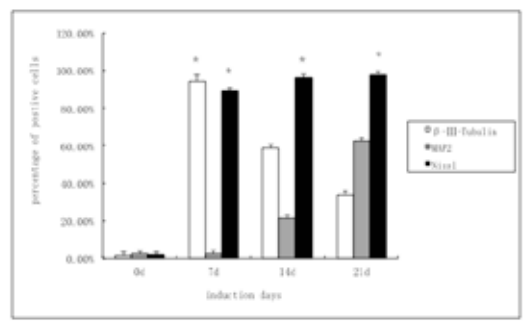


Figure 5: MAP2 expression and Nissl staining of hAMSCs after induced with bFGF, EGF, CNTF and RA for 7, 14 and 21 days. Nuclei were counterstained with Hoechst 33258. Photographs were obtained with fluorescence microscopy. Bar=50μm.



	0 d	7 d	14 d	21 d
β-III-Tubulin /Nissl	1.1±1.3%	87.5±1.7%	47.8±2.1%	27.3±1.7%
MAP2/Nissl	0.3±1.1%	1.1±1.6%	16.7±1.8%	57.8±2.1%

Figure 6: MAP2 expression and Nissl staining of hAMSCs after Percentages of positive neuronal markers β- III-Tubulin, MAP2 and Nissl body hAMSCs after neuronal induction. The x-axis shows induction time, and y-axis shows pencentage of hAMSCs positive for Nissl stain, and express MAP2 and β- III-Tubulin. Numerical value is shown in the table below. After 7 days of induction, the expression of β- III-Tubulin and Nissl staining increased significantly. After induction for 14 days, percentage of MAP2 increased while β- III-Tubulin were getting lower compared with that at day 7. Positive percentage of MPA2 had been enhanced significantly when the cells were induced for 21 days, while β- III-Tubulin continued decreasing. There is no significant difference between positive percentages of Nissl staining during the whole neuronal differentiation. * P<0.05.

Nissl bodies are structures peculiar to neurons and thus present an appealing observation target in neuronal differentiation. They are found in the cytoplasm of neurons and composed of rough endoplasmic reticulum and free polyribosomes [21]. The appearance of nissl bodies is associated with mature neurons. The Nissl staining method is based on the interaction of basic dyes such as cresyl violet, thionine, toluidin blue, methylene blue or anylin with the nucleic acid content of cells. These dyes can bind to the DNA content of the cell nuclei and also to RNA[22], which is highly concentrated in the rough endoplasmic reticulum and ribosomes (Nissl substance) within the cytoplasm. Since neurons are very active protein synthesizing cells, their cytoplasm contains a high concentration of rough endoplasmic reticulum[10,12]. Due to this unique characteristic of neurons, Nissl staining can specifically identify neurons. Furthermore, Nissl bodies show changes under various physiological conditions. For

example, in injured or regenerating neurons, Nissl substance redistributes within the cell body, providing a marker for the physiological state of the neuron[6].

To investigate whether a Nissl-type staining would be an adequate approach to identify MSC-derived neuron-like cells, we compared the NeuroTrace Fluorescence Nissl stain with immunostaining for β-III-Tubulin and MAP2, which are neuron-specific markers. During induction, expression of the early neuronal marker, β-III-Tubulin, increased (Fig.3-4) and while Nissl bodies began to form, Nissl substance was at a low level. A dramatic increase in Nissl body and MAP2 expression in the neuron-like cells suggested that the progenitor cells had become differentiated cells. MAP2 is a marker for neurons in the adult mammalian brain and appears only after neurogenesis begins within a given region and only slowly reaches adult levels. Thus, nerve cells must undergo an expression switch from β-III-Tubulin to MAP2. We co-immunostained for β- III -Tubulin and Nissl, and MAP2 and Nissl, and found that both β- III -Tubulin and MAP2 colocalized with Nissl staining. Furthermore, Nissl staining was positive throughout differentiation and there was no significant difference between the ratio of the percentage of positive cells for both β- III -Tubulin/ Nissl and MAP2/Nissl, indicating that Nissl staining is as sensitive as β-III-Tubulin and MAP2 immunostaining at identifying neural differentiation in hADSCs. The results suggest that Nissl staining is a feasible method for monitoring the neural differentiation of hADSCs. Furthermore, Nissl staining combined with other neuronal specific protein markers, could be a powerful method to identify stem cell neuronal differentiation.

Additionally, fluorescent Nissl stains are available in a wide spectrum of wavelengths, allowing for their combination with other immunofluorescence labeling. We combined Nissl staining simultaneously with nucleolus fluorescent labeling within the same cell. With regard to the Nissl method, the availability of fluorescent Nissl markers expands the usability of this approach for identifying lamination. Indeed, this approach is compatible with the use of several tissue preparations and combines well with multiple labeling techniques. Since Nissl bodies show changes under various physiological conditions, e.g. in injured or regenerating neurons, Nissl substance redistributes within the cell body, Nissl substance has the potential to be a useful marker for tracking the physiological state of cells during the neuronal differentiation of mesnechymal stem cells. This would make the identification of neuron-like cells for further cell function assays more straightforward. In our investigation, nissl body morphology seemed invariant during the entire differentiation process, indicating that the hADSCs did not undergo any pathological conditions during neuronal differentiation.

CONCLUSIONS

Our study indicates that NeuroTrace Fluorescent Nissl Stain is a sensitive and stable method for observing the progress of the neural differentiation of hADSCs. Fluorescent Nissl stain has the potential to be adopted as an effective method to monitor the progress of neural differentiation in hADSCs during therapeutic cell transplantation for acute and chronic neurodegenerative disease.

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