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Differentiation of neurosphere-derived rat neural stem cells into oligodendrocyte-like cells by repressing PDGF-α and Olig2 with triiodothyronine

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ABSTRACT

One of the approaches for treating demyelination diseases is cytotherapy, and adult stem cells are potential sources. In this investigation, we tried to increase the yield of oligodendrocyte-like cells (OLCs) by inducing neural stem cells generated from BMSCs-derived neurospheres, which were used for deriving the neural stem cells (NSCs). The latter were induced into OLCs by heregulin, PDGF-AA, bFGF and triiodothyronine (T3). The BMSCs, NS, NSCs and OLCs were characterized by using immunocytochemistry for fibronectin, CD44, CD90, CD45, Oct-4, O4, Olig2, O1 and MBP markers. PDGF receptor α (PDGFR-α), Olig2 and MOG expression were evaluated by RT-PCR. The BMSCs expressed CD44, CD90, CD106 and Oct-4; the NSCs were immunoreactive to nestin and neurofilament 68. Incubation of the NSCs for 4 days with heregulin, PDGF-AA and bFGF resulted in their induction into oligodendrocyte progenitor-like cells (OPLCs), which immunoreacted to O4, Olig2 and O1, while Olig2 and PDGFR-α were detected by RT-PCR. Replacing heregulin, PDGF-AA and bFGF with T3 for 6 days resulted in repression of O4, O1, Olig2 and PDGFR-α. The OLCs were co-cultured with motoneurons resulted in induction of MOG and MBP, which were expressed in functional OLCs. The latter can be generated from BMSCs-derive NS with high yield.

1. Introduction

Oligodendrocytes form a myelin sheath around the axons in order to support the rapid nerve conduction in the central nervous system (CNS) (Baumann and Pham-Dinh, 2001). While myelin loss from intact axons is a prominent feature of a wide range of CNS disorders (Bjartmar et al., 1999; Cervos-Navarro and Lafuente, 1991), remyelination was reported to restore saltatory conduction in intact axons (Zhao et al., 2005). The treatment of demyelination is particularly amenable to cell replacement strategies, for example, glial-committed progenitor cells (Keirstead, 2005), Schwann cells (Razavi et al., 2012), neural stem cells (Cheng et al., 2012) and human embryonic stem cell-derived oligodendrocyte progenitors (Kang et al., 2007) were transplanted into animals and they remyelinated the demyelinated axons.

Generally, remyelination and subsequent restoration of neuronal function can be achieved by either promoting endogenous repair mechanisms or providing an exogenous source of myelinating cells via transplantation (Kocsis, 1999). On the one hand, the transplant should differentiate into myelinogenic cells and have genetic stability, migratory ability and immunocompatibility, also, it should also be safe (not to be tumorogenic) and ethically acceptable (Zhang et al., 2009). On the other hand, some investigators used bone marrow stromal cells (BMSCs) as an autologous source for transplantation. Their accessibility, growth properties (Sanchez-Ramos et al., 2000) and induction into glial cells (Lamoury et al., 2006) made them a feasible candidate for cell therapy of demyelinating diseases (Chopp and Li, 2007).

Sanchez-Ramos (2002) revealed that the differentiation of BMSCs into oligodendrocytes was difficult to achieve, however, Hermann et al. (2004) reported the yield of oligodendrocyte

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differentiation from BMSCs to be approximately 27%. In a previous study, BMSCs were transdifferentiated into OLCs using dimethyl sulfoxide and retinoic acid at the preinduction, and PDGF, bFGF, heregulin and T3 at the induction stages, however, the protocol showed low yield (Kaka et al., 2012), besides, the in vitro functionality was not evaluated. Combination of other factors such as bFGF, PDGF-AA, Sonic hedgehog (Shh), neurotrophic factor 3 (NT-3), N-2 (supplement) and T3 were used for the induction (Monaco et al., 2012). Shh was used by Sundberg et al. (2011) combined with PDGF-AA, IGF-1, EGF, basic FGF and CNTF, in addition to RA, T3, human laminin and ascorbic acid as a new modality for generating oligodendrocytes from ESCs. Neri et al. (2010) used FGF2, PDGF-AA, NT3 and (T3), as combined inducers for NSCs. Most of the above investigations used PDGF, bFGF and T3 in the cocktail of the inducers. PDGF and bFGF were reported to have proliferative activity (Baas et al., 1997). An in vivo study suggested that T3 promoted the differentiation of progenitors into oligodendrocytes (Franco et al., 2008).

In this study, we attempted to use an alternative strategy for generating high yield of OLCs by using autologously prepared bone marrow stromal cells. Neurospheres derived from BMSC induction into OLCs can interact with the differentiated neurons, resulting in mature OLCs capable of expressing functionality markers such as MBP and MOG; and downregulating PDGFR-α and Olig2 following T3 induction.

2. Materials and methods

2.1. BMSC extraction, culturing and characterization

Sprague-Dawley female rats (200–250 g: Razi Institute, Tehran, Iran) were housed in a temperature and humidity controlled room, they were maintained on a 12-h light/dark cycle at 18–25 °C. Food and water were available ad libitum throughout the experiment. The study protocol conformed to the Declaration of Helsinki and was approved by the Ethical Committee supervising procedures on experimental animals at Tarbiat Modares University, Faculty of Medical School. The rats were sacrificed, the femurs and the tibias were separated and the bone marrow was aspirated using a syringe needle (18 G) containing 3–5 ml of Dulbecco's modified Eagles medium (DMEM: stem cell technology company, Tehran, Iran) supplemented with 10% fetal bovine serum (FBS: GIBCO, Eggenstein, Germany) and 0.25% trypsin/1 mM EDTA (GIBCO, Eggenstein, Germany). The whole bone marrow cells (BMSCs) were plated on 75 cm^2 plastic flasks (Nunc, Roskilde, Denmark) in DMEM/F12 (stem cell technology company, Tehran, Iran) supplemented with 10% FBS, 100 U/ml penicillin/100 mg/ml streptomycin and L-glutamine (2 mM/ml). The flasks were incubated in a humidified incubator with 5% CO_2 at 37 °C. After 24 h, the non-adherent cells were discarded, and the medium was changed daily until the BMSCs reached more than 70–80% confluency. They were harvested with 0.25% trypsin/1 mM EDTA (GIBCO, Eggenstein, Germany) for 5 min at 37 °C in order to obtain a single cell suspension, then they were re-plated (5000 cells/cm^2) for four passages. They were plated on gelatin-coated flasks (Nunc, Roskilde, Denmark) or on 6-well plates containing gelatin-coated glass cover slips and were immunostained for fibronectin (FN), CD44, CD45 and CD90. The stemness at the 4th passage was checked by evaluating Oct-4 gene expression using the RT-PCR technique (Abdanipour et al., 2011). The viability was estimated by the trypan blue exclusion method.

2.2. Osteogenic and adipogenic differentiation:

The in vitro differentiation of the BMSCs into osteogenic and lipogenic phenotypes using induction media was carried out according to Abdanipour and Tiraihi (2012). The bone ossicles were stained with Alizarin red stain after 3 weeks of culturing. The adipogenic differentiation was done by incubating the cells in the differentiation medium for 3 weeks and then staining them with Oil red stain.

2.3. Neurosphere formation

The BMSCs were dissociated with 0.25% trypsin/1 mM EDTA and plated on plastic flasks at a density of 5000 cells/cm^2 in neurosphere formation medium (DMEM/F12 medium supplemented with 2% B27 (Invitrogen, Eggenstein, Germany), 20 ng/ml basic fibroblast growth factor (bFGF: Sigma-Aldrich, Steinheim, Germany), 20 ng/ml epidermal growth factor (EGF: Sigma-Aldrich, Steinheim, Germany), 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO, Eggenstein, Germany)). After 2 days, neurosphere-like structures were observed, which were cultured for 7 days.

2.4. Neural stem cell generation

Seven days after plating, the neurospheres were harvested with 0.25% trypsin/1 mM EDTA and plated on poly-L-lysine-coated coverslips in 6-well culture plates in DMEM/F12 medium supplemented with 5% FBS, 10 ng/ml EGF, 10 ng/ml bFGF, 1% B27, 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were maintained for 5 days, fixed and immunostained for nestin (NT), neurofilament (NF) 68, NF200 and GFAP. Neuro D was evaluated by the RT-PCR technique (unpublished results).

2.5. Induction

The cells were induced by incubating them in DMEM/F12 medium containing the platelet derived growth factor (PDGF-AA: 5 ng/ml), bFGF (10 ng/ml) and heregulin (HRG: 200 ng/ml) (bFGF, PDGF-HRG; all purchased from Sigma-Aldrich, Steinheim, Germany) for 2 days resulting in oligodendrocyte progenitor-like cells (OPLCs) differentiation. This was followed by induction with different concentrations of T3 for 2 days in order to differentiate them into oligodendrocyte-like cells (OLCs). The finding of the optimal concentration of T3 was obtaining by using a dose response study (T3: 0, 5, 12.5,25, 50, 100 and 200 ng/ml; Sigma-Aldrich, Steinheim, Germany). The cells were immunostained for Olig2, O4, O1 and MBP markers, while PDGFR-α, Olig2 and MOG markers were evaluated by RT-PCR (Kaka et al., 2012).

2.6. Co-culture

The co-culturing of the OLCs was done in order to evaluate their capacity to ensheath the axons in vitro, we co-cultured the OLCs with motoneuron-like cells (MNLCs: transdifferentiated from adipocyte derived stem cells) prepared according to Abdanipour and Tiraihi (2012). Briefly, the MNLCs were transdifferentiated from adipocyte derived stem cells using B27 (2%), bFGF (20 ng) and Shh (1 μg) for 2 days, then they (10^4 cells/ml) were incubated in neurobasal medium (GIBCO, Eggenstein, Germany) at 37 °C with 5% CO_2 for 2 days. The OLCs were plated (10^4 cells/ml) on cover slips in 6-well plates with 1 mL medium comprised of PDGF, bFGF, heregulin and T3 for 6 days. The MNLCs and the OLCs were co-cultured in a serum-free medium for 7 days. The MNLCs were prelabeled with DiI (Sigma-Aldrich, Steinheim, Germany), while the OLCs were prelabeled with hochest 33342 (Sigma-Aldrich, Steinheim, Germany). Both were photographed with inverted microscope Olympus 1X71 (Olympus, Tokyo, Japan), fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), immunostained by anti-myelin basic protein (MBP) and then photographed.

2.7. Immunostaining

The cultured cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min. Prior to staining, they were permeated with 0.3% PBS-Triton X-100 (Sigma-Aldrich, Steinheim, Germany) for 5 min. and incubated with blocking solution (5% fetal bovine serum) for 30 min. Immunostaining was done on the BMSCs, NSCs and OLCs with these specific markers: mouse anti-FN monoclonal antibody (1:100; Abcam, Cambridge, UK), for the BMSCs; mouse anti-CD106 monoclonal antibody (1:200; Abcam, Cambridge, UK), for the mesenchymal stem cells; mouse anti-CD90 monoclonal antibody (1:200; Abcam, Cambridge, UK), for the BMSCs; rabbit anti-CD45 polyclonal antibody (1:100: Abcam, Cambridge, UK) for the mesenchymal stem cells; mouse anti-NT monoclonal antibody (1:50; Abcam, Cambridge, UK), for the neuroprogenitor cells; mouse anti-NF68 monoclonal antibody (1:50; Abcam, Cambridge, UK), for the neuroprogenitor cells; rat anti-NF200 monoclonal antibody (1:100; Abcam, Cambridge, UK), for the neurons; mouse anti-GFAP monoclonal antibody (1:100; Abcam, Cambridge, UK), for the asrtrocyte cells; mouse anti-O1 monoclonal antibody (1:100; EMD Millipore, Darmstadt, Germany), a specific marker for the oligodendrocytes; mouse anti-O4 monoclonal antibody (1:100; antibodies-online GmbH, Aachen, Germany), for the immature oligodendrocytes; mouse anti-Olig2 monoclonal antibody (1:100; EMD Millipore, Darmstadt, Germany) for the immature oligodendrocytes, and mouse anti-MBP monoclonal antibody (1:1000; EMD Millipore, Darmstadt, Germany), for the mature oligodendrocyte. The cells were incubated with the secondary antibody rabbit anti-mouse polyclonal secondary antibody conjugated with FITC (1:100; Abcam, Cambridge, UK), goat anti-rabbit polyclonal secondary antibody conjugated with FITC (1:100; EMD Millipore, Darmstadt, Germany) or goat anti-mouse polyclonal secondary antibody conjugated with FITC (1:100; Abcam, Cambridge, UK) for 2 h at room temperature and were counterstained with ethidium bromide (1:10,000; Sigma-Aldrich, Steinheim, Germany) for 1 min. The percentage of the immunoreactive cells were estimated by dividing the immunostained cells with a specific marker to the total number of cells (500 cells were counted) multiplied by 100.

2.8. RT-PCR

The BMSCs at the end of the 4th passage, the rat neonate brain cells (controls), the NSCs and the OLCs were evaluated for the expression of Oct-4, Olig2, PDGFR-α and MOG genes using RNX Plus kit (Fermentas, Vilnius, Lithuania). Two μg of the total RNA from each sample were treated with DNase I (Fermentas, Vilnius, Lithuania). The purity and the integrity of the extracted RNA were evaluated by optical density measurements and electrophoresis on 2% agarose gel. One microgram of the extracted RNA was converted to cDNA using the first strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). Fifty nanogram of cDNA were added to the PCR reaction for 35 cycles with denaturing at 95 °C for 45 seconds, annealing at 58 °C for 45 s, and elongating at 72 °C for 30 s. GAPDH was used as an internal control.

After amplification, the products were run on 2% agarose gel and visualized using ethidium bromide under UV light. Each experiment was repeated at least 3 times in order to ensure reproducibility. The primer sequences (forward and backward), the size of the product and the PCR condition were as follows: the expression of rat Oct-4 gene (a marker for BMSCs stemness) was evaluated using forward primer Oct-4 (5′-AAGCTGCTGAAACAGAAGAGG-3′ and backward primer 5′-ACACGGTTCTCAATGCTAGTC-3′: 225 bp, gene accession number: NM_001009178, annealing at 62 °C). The internal control was rat GAPDH (5≠CCACAACTC TTCCATTCTC-3ánd 5′-CCAAGATTCACGGTAGATAC-3′, forward and backward primers, respectively: 200 bp, gene accession number: NM_017008, annealing at 62 °C). The expression of rat oligodendrocyte transcription factor 2 (Olig2), a marker for immature oligodendrocytes was evaluated (using 5≠GACGACATTATGGGCTTTGATGG-3ánd 5≠GTTTCTGCCTGAACAGTCCAC-3′, forward and backward primers, respectively: 170 bp, gene accession number: NM_022668.2, annealing at 62 °C). The expression of rat PDGFR-α gene, (a marker for immature oligodendrocyte) was performed (using 5′-CTAATTCACATTCGGAAGGTTG-3′ and 5′-GGA CGATGGGCGACTAGAC-3′, forward and backward primers, respectively: 175 bp, gene accession number: NM_63837.1, annealing at 57 °C). The expression of rat myelin oligodendrocyte glycoprotein (MOG), (a marker for mature oligodendrocyte) was evaluated (using 5≠GAGCTCATTATGGCCTTTCATGG-3ánd 5≠GTTCCTGCGTGAACAGTCCAC-3; forward and backward primers, respectively: 180 bp, gene accession number: NM_022668, annealing at 59 °C). The expression of rat Neuro D (a neuroprogenitor marker) was accessed (using 5≠CAG ATGATGGCACAAAGGGTAG-3ánd 5≠GACCGAGAGCATCGCATATTG-3; forward and backward primers, respectively: 210 bp, gene accession number NM_001105729.3, annealing at 59 °C) (Abdanipour and Tiraihi, 2012).

2.9. Statistical analysis

The analysis was done using SPSS software, all of the data in this study were compared by one way analysis of variance (ANOVA), while the difference between the groups was examined by Turkey's test.

3. Results

3.1. Isolation and characterization of BMSCs

The morphology of the BMSCs showed spindle shape after the 4th passage. The cells were induced into lipogenic and osteogenic differentiation in order to confirm their mesodermal lineage. The lipogenic phenotype showed many cells with multilocular fat cells, which was demonstrated by Oil red O stain, while the osteogenic differentiation was confirmed using Alizarin stain, where small ossicles could be seen (Fig. 1a and b, respectively). The isolated cells were immunoreactive to FN, CD44 and CD90 (mesenchymal cell markers) but not to CD45 (a hematopoietic cell marker) (see Fig. 1c, d, f and e, respectively). The viability of BMSCs was 98.18 ± 0.94% (mean ± SEM). However, the viability of the cells harvested from the neurospheres and the NSCs were significantly lower than that of the BMSCs ($P < 0.05$) (see Fig. 2a). The percentages of the BMSCs immeunoreactive to FN, CD90 and CD106 were 94.32 ± 0.45%, 95.48 ± 0.24% and 97.16 ± 0.82%, respectively.

3.2. Neurosphere formation

The cultured BMSCs in the suspension medium aggregated forming neurospheres, sometimes the neurospheres attached to each other forming larger fused neurospheres.

3.3. Neural stem cell generation

The NSCs, following harvesting the neurospheres, showed polyhedral shape with few cells with short extensions, they were immunostained with NT (neural stem cell marker) and NF-68 (neuroprogenitor cells marker), but not with NF-200 (neuronal marker) and GFAP (astrocyte cell marker) (See Fig. 3a, b, c and d, respectively). The mean percentages of the NSCs immunoreactive to FN, NT, NF-68, NF-200, GFAP, O1, O4 and Olig2 were 4.14 ± 0.86%, 18 ± 0.26%, 16.04 ± 2.16%, 10.78 ± 1.04%,

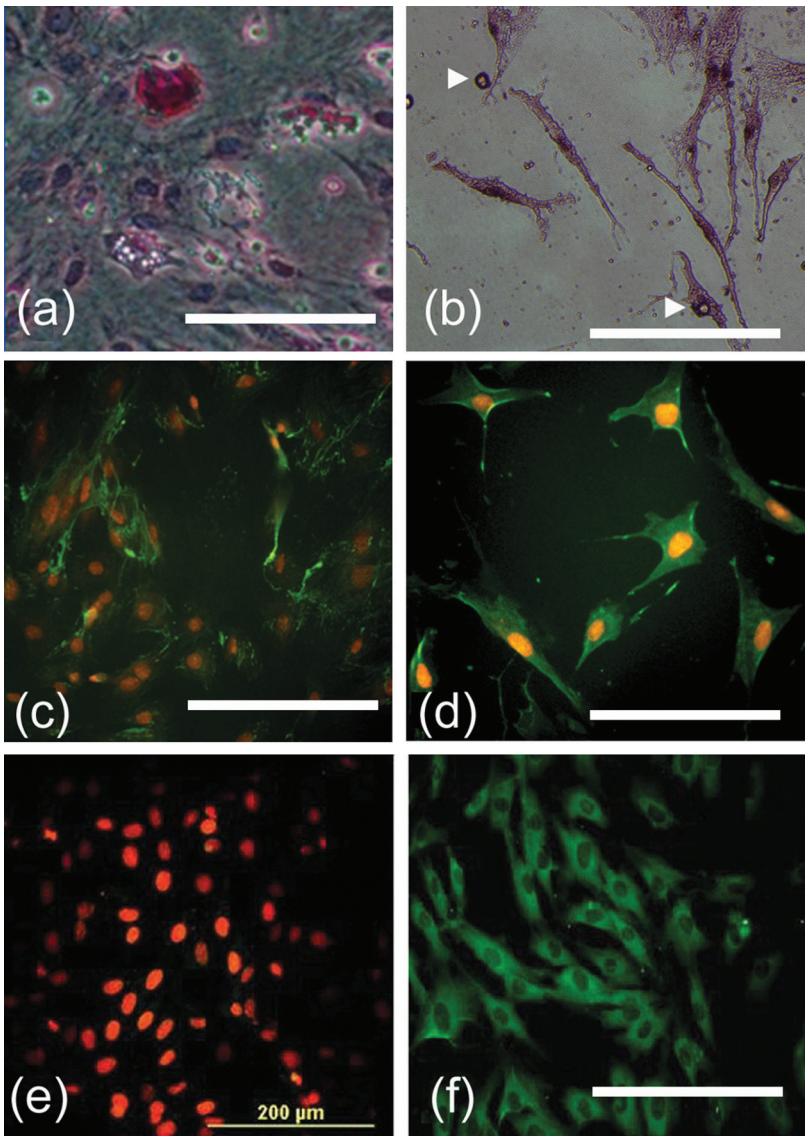

Fig. 1. The characterization of the cultured bone marrow stromal cells (BMSCs) using mesenchymal differentiation (a and b); and the immunostaining against several markers, where the primary antibodies against the markers were labeled with BMSCs, incubated with secondary antibody conjugated with FITC and counterstained with ethidium bromide (c–f). (a) The lipogenic differentiation of the BMSCs following incubation with lipogenic differentiation medium, and staining with Oil red O stain. (b) Osteogenic differentiation of the BMSCs following incubation with osteogenic differentiation medium and staining with Alizarin stain, arrowhead represents the calcified ossicle. (c) immnostaining of the BMSCs with anti-fibronectin primary antibody. Accordingly, d, e and f represent anti-CD44, anti-CD45 and anti-CD90, respectively. (Scale bar = 200 μm, a, c, d, e and f; scale bar = 300, b).

$34.49 \pm 1.47\%$, $81.74 \pm 2.08\%$, $79.54 \pm 0.46\%$ and $80.19 \pm 0.42\%$, respectively (See Fig. 2b). The immunostaining of the NSCs with NT and NF-68 was significantly higher than that of the other markers ($P < 0.05$).

3.4. Differentiation of NSCs into oligodendrocytes

The neural stem cells were induced with PDGF, bFGF and heregulin into OPLCs, the latter were differentiated into OLCs using T3. Fig. 2c shows a histogram of the percentages of the viable cells at the induction stage of NSCs into oligodendrocyte phenotype for evaluating T3 dose response using untreated group, groups treated with 5, 12.5, 25, 50, 100 and 200 ng/mL (T0, T5, T12.5, 25, T50, T100 and T200 ng/mL, respectively). The highest viability of the T3-treated group at the induction stage was at the dose 25 ng/mL (considered the optimal induction dose), which was not significantly different from that of the 5 and 12.5 ng/mL T3-treated groups ($P > 0.05$), but the viability was significantly low at 50, 100 and 200 ng/mL concentrations ($P < 0.05$). This may indicate that they 50, 100 and 200 ng/mL exerted toxic effect on the (OPLCs).

The result of the RT-PCR shows that Oct-4 (pluripotency marker) was expressed in the undifferentiated BMSCs, neonate brain and NSCs, but not in the OLCs. The Neuro D (early neural marker) was not expressed in the undifferentiated BMSCs and the OLCs, but was expressed in the neonate brain and the NSCs (See Fig. 4).

3.5. Maturation of oligodendrocytes

Fig. 5 presents Olig2, PDGFR-α and MOG expressions obtained by RT-PCR. Olig2 and PDGFR-α were expressed in immature OLCs following the replacement of the growth factor with T3. While MOG was expressed in mature oligodendrocytes. Olig2 and PDGFR-α were downregulated when the OLCs were co-cultured with MNLCs. The immunostaining of the oligodendrocyte-lineage markers including Olig2, O4, O1 and MBP (myelin basic protein, expressed in differentiated oligodendrocytes) is presented in Fig. 6.

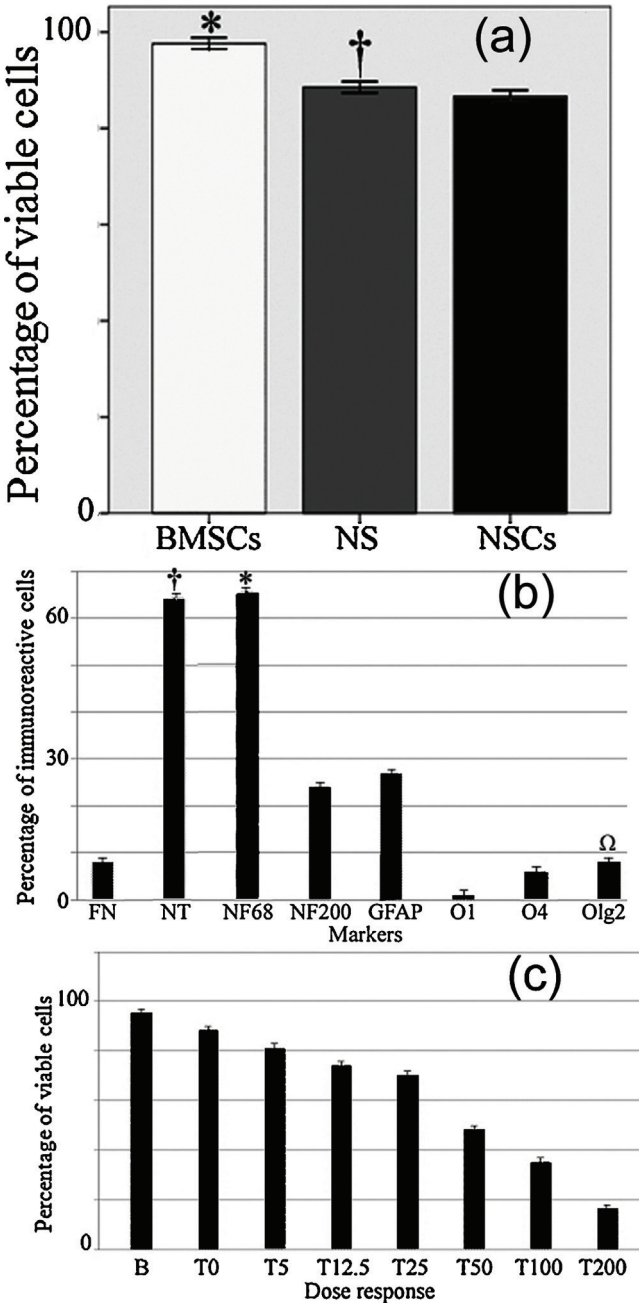

Fig. 2. The viability, percentage of immunoreactive neural stem cells (NSCs) to different markers and dose response of the induced NSCs (with platelet derived growth factor, basic fibroblast growth factor and heregulin into oligodendrocyte progenitor-like cells and later into oligodendrocyte-like cells by triiodothyronine). (a) a histogram shows the mean percentages of viable cells in untreated bone marrow stromal cells (BMSCs: white column), harvested cells from neurospheres (NSs: gray column) and neural stem cells (NSCs: black column). "*" indicates that the viability in the BMSCs is significantly higher than those of NS and NSCs. "†" indicates no significant difference with the NSCs. (b) shows a histogram of the mean percentages of immunoreactive NSCs to the different markers: fibronectin (FN), nestin (NT), neurofilament 68 (NF68), NF200, glial fibrillary acidic protein (GFAP), O1, O4 and Olig2. "*" indicates the highest level of expression. "†" indicates no significant difference between nestin and NF68. "Ω" indicates no significant difference between O4 and Olig2. (c) shows a histogram of the dose-response of the oligodendrocyte progenitor-like cells to T3, and the mean percentages of the viable cells at the induction stage in a dose response of triiodothyronine (T0, T5, T12.5, 25, T50, T100, and T200; 0, 5, 12.5, 25, 50, 100 and 200 ng/mL, respectively). The bone marrow stromal cells (B) are the control group. "*" indicates significantly high viability.

The results showed that the NSCs following induction with PDGF, bFGF and heregulin, expressed Olig2 and O4, while fewer cells expressed O1 and MBP. The latter showed higher level of expression one week following the co-culturing of the OLCs with the MNLCs, where the immunoreactivity to MBP was noticed at the points of contact of the OLCs with the motoneurons (Fig. 7).

4. Discussion

In this study, we observed NSC differentiating into OLCs derived from neurospheres generated from BMSCs. The OLCs expressed MBP and MOG, markers of oligodendrocyte maturity and functionality, upon coculturing with motoneurons. The results show the optimum expression of O1, O4 and Olig2 at the dose of 25 ng/mL (T3) with a yield of 82%, while in our previous study it was 60% at the same dose (Kaka et al., 2012).

This study shows that the percentage of the BMSCs at the 4th passage was 96, and they were immunoreactive to fibronectin, a marker of BMSC. They also expressed Oct-4, a stemness marker, as well as CD44, CD106 and CD90, but not CD45. These findings are consistent with previous ones (Mohammad-Gharibani et al., 2009). Our results show that Neuro D expression was not detected in the undifferentiated BMSCs (Yeu et al., 2004), however, others reported its expression at very low levels in the BMSCs (Woodbury et al., 2002), it may be caused by the spontaneous differentiation of the BMSCs into neuron-like cells (Ni et al., 2010). The NSCs were induced by PDGF, bFGF and HRG, followed by different concentrations of T3 (Dose response), and the optimal dose was 25 ng/mL. At higher doses of T3, the viability declined, which may be due to toxicity, and is consistent with other investigations (Cowan et al., 1997). The percentage of the induced OLCs was 82%, which is higher than that of other investigations with different induction protocols (Kaka et al., 2012; Kang et al., 2007).

While T3 is involved in the functional maturation of postmitotic oligodendrocytes, as indicated by switching on myelin/oligodendrocyte glycoprotein gene (MOG) (Baas et al., 1997), its deficiency causes changes in the distribution of the oligodendrocyte/myelin markers during oligodendroglial differentiation in vitro (Kaka et al., 2012). Moreover, our results show that T3 at the dose of 25 ng/mL is more effective than at that of 10 ng/mL (Kaka et al., 2012). The high yield in this investigation could be related to the dose dependent mode of action of T3, which is consistent with previous findings (Almazan et al., 1985; Fritsche et al., 2005). However, other investigators reported a yield of 21% at a dose of 40 ng/ml, where the oligodendrocytes were differentiated from fetal derived NSCs (Neri et al., 2010), whereas oligodendrocytes derived from human embryonic stem cells resulted in a yield of 81% at a dose of 30 ng/ml (Kang et al., 2007). Despite using adult stem cells as the source of the OLCs in this study, the yield is comparable to pluripotent stem cells.

Our RT-PCR results show that the differentiating OLCs expressed PDGFR-α and Olig2, while the untreated BMSCs and NSCs did not express these markers, consistently, Olig2 was identified in the oligodendrocyte precursors (Ligon et al., 2006). The differentiated oligodendrocytes expressed MBP and MOG, which are important markers of maturity and functionality (Lin et al., 2006). Some of the myelin protein genes, such as MBP and PLP, contain thyroid hormone response elements and are directly regulated by thyroid hormone receptors (Thompson and Potter, 2000). This may explain the requirement of T3 for the differentiation and maturation of OLCs as the last inducer in the induction process (Kaka et al., 2012). It was mentioned that oligodendrocyte progenitor cell proliferation and differentiation were regulated by T3, and that the subsequent maturation was characterized by MBP expression, which was independently regulated by T3 (Baas et al., 1997). Besides,

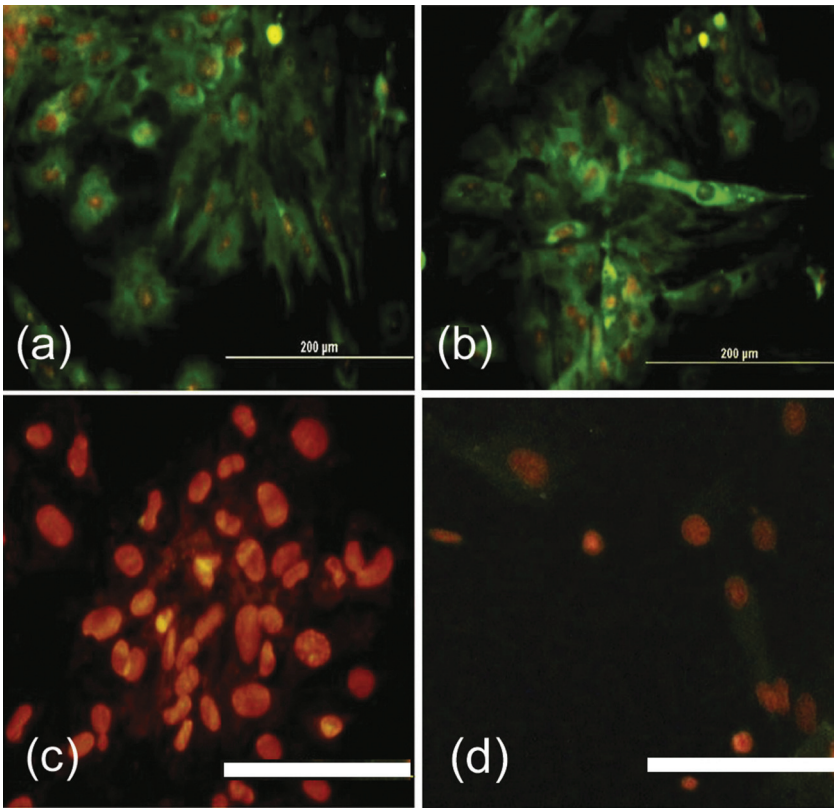

Fig. 3. The characterization of the cultured neural stem cells (NSCs) using immunostaining against several markers, where the NSCs was labeled with the primary antibodies against the markers, incubated with secondary antibody conjugated with FITC and counterstained with ethidium bromide. (a) Immnostaining of the NSCs with anti-nestin primary antibody. Accordingly, b, c and d represent anti-neurofilament 68, anti-neurofilament 200 and anti-glial fibrillar acidic protein, respectively (Scale bar = 100 μm, c and d).

OLCs showed the same pattern of expression with switching on the gene in mature oligodendrocytes (Crang et al., 2004), which was also dependent on T3 (Baas et al., 1997). The replacement of the inducers with T3 caused switching off PDGFR-α gene, which plays a certain role in progenitor proliferation. Moreover, PGGFR-α was reported to be expressed in early oligodendrocyte lineage (Ellison and de Vellis, 1994), and was essential for the proliferative activity of PDGF (Baron et al., 2002). Baas et al. (1997) reported that the presence of PDGF delayed differentiation of the progenitors. The treatment of oligodendrocyte progenitor cells with T3 induced their differentiation and postmitotic maturation (Baas et al., 1997). The termination of PDGFR-alpha expression following the change in the inducer may explain the progression of oligodendrocyte cells into

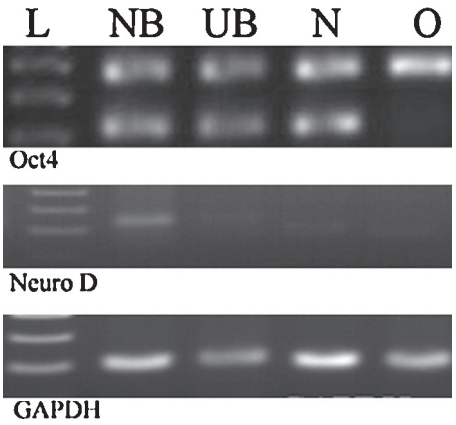

Fig. 4. Electrophorograms of the expression profile of stemness and early neural differentiation genes in the rat neonate brain (NB: as positive control), undifferentiated bone marrow stromal cells (BMSCs: UB; as negative cell control), neural stem cells (N) and oligodendrocyte-like cells (O). L is the DNA ladder. GAPDH is an internal control. The stemness gene (Oct-4) was expressed in NB, UB and N but not in O. Neuro D (early neural differentiation gene) was downregulated in all except in NB.

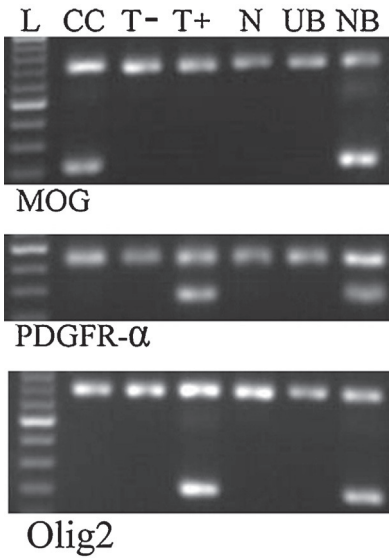

Fig. 5. Electrophorograms of the expression profile of several oligodendroglial genes in the rat neonate brain (NB: as positive control), undifferentiated bone marrow stromal cells (BMSCs: UB) and neural stem cells (NSCs: N). T− is oligodendrocyte progenitor-like cells (NSCs induced with the platelet derived growth factor (PDGF), the basic fibroblast growth factor (bFGF) and heregulin (H)), they were induced by triiodothyronine into oligodendrocyte-like cells (T+). CC means oligodendrocytes-like cells co-cultured with motoneuron-like cells. L is the DNA ladder.

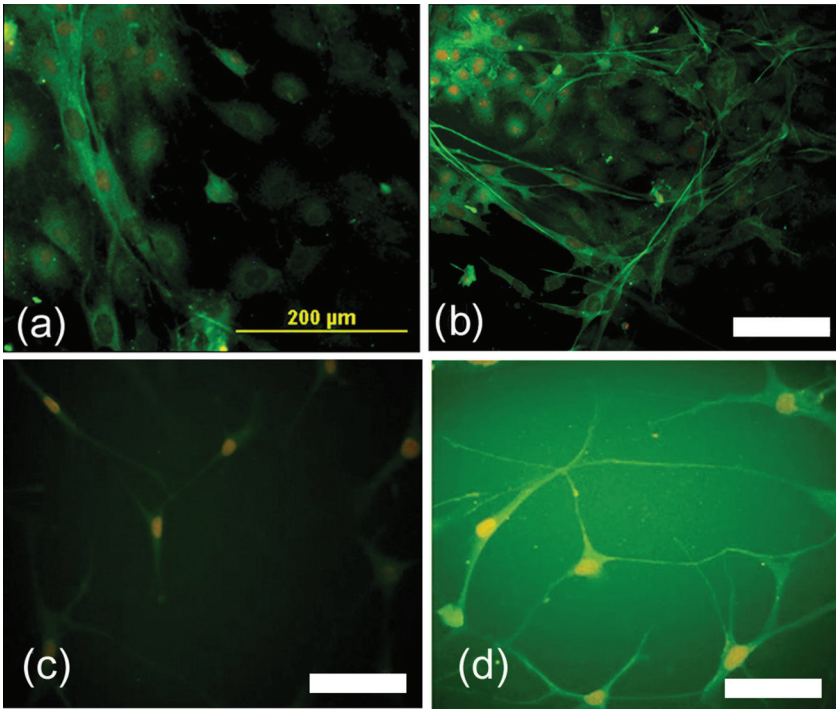

Fig. 6. The characterization of the cultured oligodendrocyte-like cells (OLCs) using immunostaining against several markers, where the OLCs were labeled with primary antibodies against the markers and then were incubated with secondary antibody conjugated with FITC and counterstained with ethidium bromide. (a) Immnostaining of OLCs with anti-Olig2 primary antibody. Accordingly, b, c and d represent anti-O4, anti-O1 and anti-myelin basic protein (MBP), respectively. (Scale bar = 240 μm; b and c; 120 μm; d).

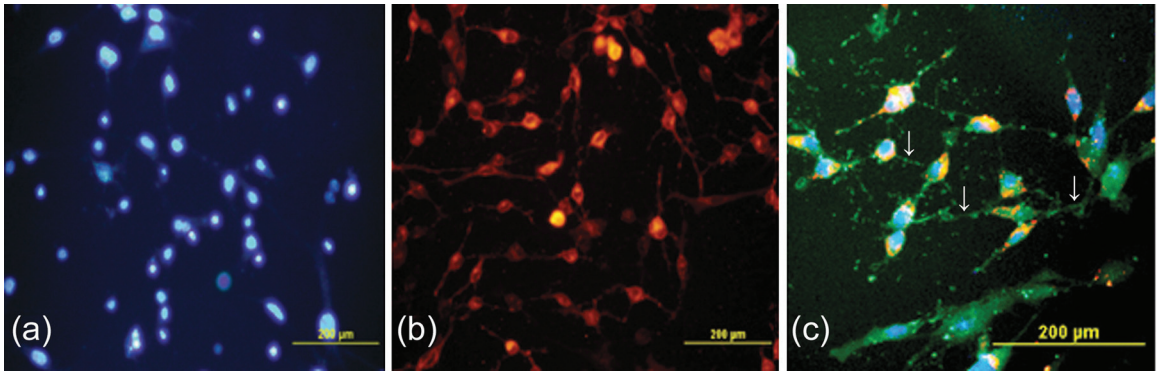

Fig. 7. Photomicrographs of co-culturing of oligodendrocyte like-cells with motoneurons. After 1 week, the immature oligodendrocytes developed into MBP-immunoreactive cells. (a) The oligodendrocyte progenitor-like cells were cultured for 7 days and stained with Hoechst (blue), (b) motor neurons incubated in neurobasal media and stained with DiI (red). (c) Co-cultured motor neurons and oligodendrocytes in serum-free medium. The cells were fixed and immunostained by myelin-basic protein (MBP). The sites of contact between the oligodendrocytes and the motoneurons are immunostained with MBP (arrow) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

mature oligodendrocyte (Ebrahimi-Barough et al., 2013), this finding was supported by other investigators (Buchet and Baron-Van Evercooren, 2009). On the other hand, Olig2 is a key bHLH transcription factor in the specification of oligodendrocytes (Lu et al., 2002; Zhou and Anderson, 2002). Cai et al. (2010) revealed that Olig2 expression was upregulated in the differentiating oligodendrocyte and downregulated in the differentiated ones (Cai et al., 2010). Recently, Mei et al. (2013) confirmed that the deletion of Olig2 gene in the immature oligodendrocytes enhanced their maturation and was associated with the myelination process. This is consistent with our finding, because the electrophorogram in Fig. 5 shows that the downregulation of olig2 is concurrent with the upregulation of MOG; the latter is a marker of a mature oligodendrocyte (Wolswijk, 2000).

On the other hand, the appearance of MOG in OLCs in this study, a reliable marker for fully differentiated myelin-forming oligodendrocytes, suggests that OLC maturation was achieved by the co-culture system and resulted in the expression of MBP and MOG. Other investigators showed that the capacity of oligodendrocyte progenitors to differentiate into oligodendrocytes was an intrinsic property of the lineage (Temple and Raff, 1986). In the absence of neurons, oligodendrocytes can clearly make a myelin-like membrane (Sarlieve et al., 1983) nevertheless, co-culturing with neurons increases myelin gene expression, such as PLP, MBP and MAG (Matsuda et al., 1997). The presence of MOG correlates with the late stages of maturation of the oligodendrocyte (Copray et al., 2006), which is consistent with our findings.

The conclusion of this investigation is that oligodendrocyte-like cells can be produced from NSCs generated from BMSCs-derived neurospheres. NSCs induced with heregulin, PDGF-AA, bFGF and followed by T3 produce OLCs, which upon their incubation with motoneurons result in their maturation and expression of MBP and MOG. The yield of OLCs is 82%.

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References

Abdanipour, A., Tiraihi, T., Mirnajafi-Zadeh, J., 2011. Improvement of the pilocarpine epilepsy model in rat using bone marrow stromal cell therapy. Neurol. Res. 33 (6), 625–632.

Abdanipour, A., Tiraihi, T., 2012. Induction of adipose-derived stem cell into motoneuron-like cells using selegiline as preinducer. Brain Res. 1440, 23–33.

Almazan, G., Honegger, P., Matthieu, J.M., Guentert-Lauber, B., 1985. Epidermal growth factor and bovine growth hormone stimulate differentiation and myelination of brain cell aggregates in culture. Brain Res. 353 (2), 257–264.

Baas, D., Bourbeau, D., Sarlième, L.L., Ittel, M.E., Dussault, J.H., Puymirat, J., 1997. Oligodendrocyte maturation and progenitor cell proliferation are independently regulated by thyroid hormone. Glia 19 (4), 324–332.

Baron, W., Shattil, S.J., ffrench-Constant, C., 2002. The oligodendrocyte precursor mitogen PDGF stimulates proliferation by activation of alpha(v)beta3 integrins. EMBO J. 21 (8), 1957–1966.

Buchet, D., Baron-Van Evercooren, A., 2009. In search of human oligodendroglia for myelin repair. Neurosci. Lett. 456 (3), 112–119.

Baumann, N., Pham-Dinh, D., 2001. Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol. Rev. 81 (2), 871–927.

Bjartmar, C., Yin, X., Trapp, B.D., 1999. Axonal pathology in myelin disorders. J. Neurocytol. 28, 383–395.

Cai, J., Zhu, Q., Zheng, K., Li, H., Qi, Y., Cao, Q., Qiu, M., 2010. Co-localization of Nkx6.2 andNkx2.2 homeodomain proteins in differentiated myelinating oligodendrocytes. Glia 58 (4), 458–468.

Cervos-Navarro, J., Lafuente, J.V., 1991. Traumatic brain injuries: structural changes. J. Neurol. Sci. 103 (Suppl), S3–S14.

Cheng, I., Mayle, R.E., Cox, C.A., Park, D.Y., Smith, R.L., Corcoran-Schwartz, I., Ponnusamy, K.E., Oshtory, R., Smuck, M.W., Mitra, R., Kharazi, A.I., Carragee, E.J., 2012. Functional assessment of the acute local and distal transplantation of human neural stem cells after spinal cord injury. Spine J. 12 (11), 1040–1044.

Chopp, M., Li, Y., 2007. Transplantation of bone marrow stromal cells for treatment of central nervous system diseases. Adv. Exp. Med. Biol. 585, 49–64.

Copray, S., Balasubramaniyan, V., Levenga, J., de Bruijn, J., Liem, R., Boddeke, E., 2006. Olig2 overexpression induces the in vitro differentiation of neural stem cells into mature oligodendrocytes. Stem Cells 24 (4), 1001–1010.

Cowan, W., Maxwell, J., Thomas, M., Zipursley, S.L., 1997. Molecular and cellular approaches to neural development. In: Anderson, D.J., Jan, Y.N. (Eds.), The Determination of the Neuronal Phenotype. Oxford University Press, Oxford, pp. 26–43.

Crang, A.J., Gilson, J.M., Li, W.W., Blakemore, W.F., 2004. The remyelinating potential and in vitro differentiation of MOG-expressing oligodendrocyte precursors isolated from the adult rat CNS. Eur. J. Neurosci. 20 (6), 1445–1460.

Ebrahimi-Barough, S., Kouchesfahani, H.M., Ai, J., Massumi, M., 2013. Differentiation of human endometrial stromal cells into oligodendrocyte progenitor cells (OPCs). J. Mol. Neurosci. 51 (2), 265–273.

Ellison, J.A., de Vellis, J., 1994. Platelet-derived growth factor receptor is expressed by cells in the early oligodendrocyte lineage. J. Neurosci. Res. 37 (1), 116–128.

Franco, P.G., Silvestroff, L., Soto, E.F., Pasquini, J.M., 2008. Thyroid hormones promote differentiation of oligodendrocyte progenitor cells and improve remyelination after cuprizone-induced demyelination. Exp. Neurol. 212 (2), 458–467.

Fritsche, E., Cline, J.E., Nguyen, N.H., Scanlan, T.S., Abel, J., 2005. Polychlorinated biphenyls disturb differentiation of normal human neural progenitor cells: clue for involvement of thyroid hormone receptors. Environ. Health Perspect. 113 (7), 871–876.

Hermann, A., Gastl, R., Liebau, S., Popa, M.O., Fiedler, J., Boehm, B.O., Maisel, M., Lerche, H., Schwarz, J., Brenner, R., Storch, A., 2004. Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. J. Cell Sci. 117 (Pt 19), 4411–4422.

Kaka, G.R., Tiraihi, T., Delshad, A., Arabkheradmand, J., Kazemi, H., 2012. In vitro differentiation of bone marrow stromal cells into oligodendrocyte-like cells using triiodothyronine as inducer. Int. J. Neurosci. 122 (5), 237–247.

Kang, S.M., Cho, M.S., Seo, H., Yoon, C.J., Oh, S.K., Choi, Y.M., Kim, D.W., 2007. Efficient induction of oligodendrocytes from human embryonic stem cells. Stem Cells 25 (2), 419–424.

Keirstead, H.S., 2005. Stem cells for the treatment of myelin loss. Trends Neurosci. 28 (12), 677–683.

Kocsis, J.D., 1999. Restoration of function by glial cell transplantation into demyelinated spinal cord. J. Neurotrauma. 16 (8), 695–703.

Lamoury, F.M., Croitoru-Lamoury, J., Brew, B.J., 2006. Undifferentiated mouse mesenchymal stem cells spontaneously express neural and stem cell markers Oct-4 and Rex-1. Cytotherapy 8, 228–242.

Ligon, K.L., Kesari, S., Kitada, M., Sun, T., Arnett, H.A., Alberta, J.A., Anderson, D.J., Stiles, C.D., Rowitch, D.H., 2006. Development of NG2 neural progenitor cells requires Olig gene function. Proc. Natl. Acad. Sci. U. S. A. 103 (20), 7853–7858.

Lin, T., Xiang, Z., Cui, L., Stallcup, W., Reeves, S.A., 2006. New mouse oligodendrocyte precursor (mOP) cells for studies on oligodendrocyte maturation and function. J. Neurosci. Methods 157 (2), 187–194.

Lu, Q.R., Sun, T., Zhu, Z., Ma, N., Garcia, M., Stiles, C.D., Rowitch, D.H., 2002. Common developmental requirement for olig function indicates a motor neuron/oligodendrocyte connection. Cell 109 (1), 75–86.

Matsuda, Y., Koito, H., Yamamoto, H., 1997. Induction of myelin-associated glycoprotein expression through neuron–oligodendrocyte contact. Dev. Brain Res. 100, 110–116.

Mei, F., Wang, H., Liu, S., Niu, J., Wang, L., He, Y., Etxeberria, A., Chan, J.R., Xiao, L., 2013. Stage-specific deletion of olig2 conveys opposing functions on differentiation and maturation of oligodendrocytes. J. Neurosci. 33 (19), 8454–8462.

Mohammad-Gharibani, P., Tiraihi, T., Arabkheradmand, J., 2009. In vitro transdifferentiation of bone marrow stromal cells into GABAergic-like neurons. Iran Biomed. J. 13 (3), 137–143.

Monaco, M.C., Maric, D., Bandeian, A., Leibovitch, E., Yang, W., Major, E.O., 2012. Progenitor-derived oligodendrocyte culture system from human fetal brain. J. Vis. Exp. 20 (70).

Neri, M., Maderna, C., Ferrari, D., Cavazzin, C., Vescovi, A.L., Gritti, A., 2010. Robust generation of oligodendrocyte progenitors from human neural stem cells and engraftment in experimental demyelination models in mice. PLoS One 5 (4), e10145.

Ni, W.F., Yin, L.H., Lu, J., Xu, H.Z., Chi, Y.L., Wu, J.B., Zhang, N., 2010. In vitro neural differentiation of bone marrow stromal cells induced by cocultured olfactory ensheathing cells. Neurosci. Lett. 475 (2), 99–103.

Razavi, S., Ahmadi, N., Kazemi, M., Mardani, M., Esfandiari, E., 2012. Efficient transdifferentiation of human adipose-derived stem cells into Schwann-like cells: a promise for treatment of demyelinating diseases. Adv. Biomed. Res. 1, 12.

Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C., Stedeford, T., Willing, A., Freeman, T.B., Saporta, S., Janssen, W., Patel, N., Cooper, D.R., Sanberg, P.R., 2000. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp. Neurol. 164 (2), 247–256.

Sanchez-Ramos, J.R., 2002. Neural cells derived from adult bone marrow and umbilical cord blood. J. Neurosci. Res. 69, 880–893.

Sarlieve, L.L., Fabre, M., Susz, J., Matthieu, J.M., 1983. Investigations on myelination in vitro, iv. myelin-like or premyelin structures in cultures of dissociated brain cells from 14- to 15-day-old embryonic mice. J. Neurosci. Res. 10, 191–210.

Sundberg, M., Hyysalo, A., Skottman, H., Shin, S., Vemuri, M., Suuronen, R., Narkilahti, S., 2011. A xeno-free culturing protocol for pluripotent stem cell-derived oligodendrocyte precursor cell production. Regen. Med. 6 (4), 449–460.

Temple, S., Raff, M., 1986. Clonal analysis of oligodendrocyte development in culture: evidence for a developmental clock that counts cell divisions. Cell 44, 773–779.

Thompson, C.C., Potter, G.B., 2000. Thyroid hormone action in neural development. Cereb. Cortex 10 (10), 939–945.

Wolswijk, G., 2000. Oligodendrocyte survival, loss and birth in lesions of chronic-stage multiple sclerosis. Brain 123 (Pt 1), 105–115.

Woodbury, D., Reynolds, K., Black, I.B., 2002. Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J. Neurosci. Res. 69, 908–917.

Yeu, I.S., Lee, H.J., Yi, J.S., Yang, J.H., Lee, I.W., Lee, H.K., 2004. The survival and migration pattern of the bone marrow stromal cells after intra cerebral transplantation in rats. J. Korean Neurosurg. Soc. 36, 400–404.

Zhang, Y.H., Wang, H.F., Liu, W., Wei, B., Bing, L.J., Gao, Y.M., 2009. Insulin-producing cells derived from rat bone marrow and their autologous transplantation in the duodenal wall for treating diabetes. Anat. Rec. (Hoboken) 292 (5), 728–735.

Zhao, C., Fancy, S.P., Magy, L., Urwin, J.E., Franklin, R.J., 2005. Stem cells, progenitors andmyelin repair. J. Anat. 207 (3), 251–258.

Zhou, Q., Anderson, D.J., 2002. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell 109 (1), 61–73.