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Research article

Effects of Short Term Hypoxia-Preconditioning on Glial Phenotype Induction of Human Mesenchymal Stem Cells

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Abstract: Development of successful clinical treatments for peripheral nerve injury is limited due to the complications behind neural physiology. Human mesenchymal stem cells (hMSCs) have the ability to directly promote tissue repair and protect cells at the injury site. Studies have shown that hMSCs can be transplanted to improve nerve regeneration. Hypoxic culture condition has been proven to maintain the stemness of hMSCs for later differentiation. In this study, we investigated the effects of low oxygen (O_2) (2% and 5% O_2) pre-treatment and initial seeding density (500, 1000, and 2000 cells/cm^2) on glial protein expression during glial differentiation of hMSCs. Results showed that the secretion of glial proteins was tunable by modifying the seeding density. Moreover, glial induction of hMSCs, characterized by the glial fibrillary acidic protein (GFAP) and S100β expressing phenotype, were enhanced by short-term hypoxia pretreatment. The significantly increased gene expression, including GFAP (10 folds in 2% O_2, 25 folds in 5% O_2), 2',3'-Cyclic Nucleotide 3' Phosphodiesterase (CNP) (600 folds in 2% O_2, 800 folds in 5% O_2), and neural growth factor receptor (NGFR) (4 folds in 5% O_2), indicated that low oxygen, especially 5% O_2 pretreated hMSCs had an improved potential for peripheral nerve regeneration.

Keywords: mesenchymal stem cells; hypoxia; glial phenotype induction

1. Introduction

A variety of trauma can lead to peripheral nerve injury. According to the statistical report from U.S. Department of Transportation, 2,313,000 people were injured from car accidents in 2013 [1]. In addition, 3,701,652 people were injured from sports and recreational activities in 2014 [2]. Peripheral nerve injury induces endoneurial hypoxia which is attributed to fibrosis, metabolic failure, and persistent vascular dysfunction. After injury, a set of inflammatory activities were initiated to promote the newly recruited glial cells to form supporting networks for axon regeneration [3–5]. However, complete recovery from self-regeneration is fairly infrequent. The key to surgical repair of the transected nerve is to bridge the lesion with either autologous nerve graft [6], or synthetic nerve graft [7] for the local axon to rejoin. Advanced technologies such as a drug loaded hydrogel [8], decellularized tissue scaffold [9], and cell transplantation [10] have been incorporated into conduit design for nerve repair. Since the Büngner bands formation is critical to initiate the regeneration of axons during Wallerian degeneration, transplanting autologous Schwann cells to assist this process can be promising. However, robust harvesting and expanding Schwann cells *in vitro* can be challenging [11]. With the development of stem cell based technology, using stem cell to repair damaged neural tissues as an alternative of autologous Schwann cells has become more applicable [12].

Human mesenchymal stem cells (hMSCs) are stem cells primarily harvested from bone marrow and fat tissue. hMSCs are easy to obtain from adults and can be expanded rapidly *in vitro* to reach a desired number for effective therapy [13]. Besides their multi-lineage differentiation ability, hMSCs have the ability to directly promote tissue repair and protect cells at the injury site [14–16]. Moreover, hMSCs have immunoregulatory properties, which can modulate the immune system and reduce the extensive inflammatory response. Clinical studies suggest that transplantation of allogenic hMSCs does not lead to any immune rejection [17,18]. With proper induction using specific growth factors, hMSCs can be induced to express glial proteins [19] or neuronal proteins [20] and show morphological similarities. hMSCs can improve function of damaged nerves, which has been shown to be promising by transplanting hMSCs to the injury site [21]. Although the detailed mechanism of how hMSCs participate in neurological functions has not yet been fully discovered, it was suggested that the neurological regeneration function of hMSCs is dependent on the paracrine interaction with neighboring cells, including direct neuroprotective effect of the hMSCs and secretion of neurotrophic factors [22,23].

The bone marrow niche where hMSCs reside has physiological low oxygen (O_2) partial pressure varies from 1% to 7% [24]. Mimicking the physiological condition for hMSCs before differentiation can inhibit senescence and maintain stem cell multi-potency [25]. Due to the ectodermal origin of glial cells, inducing hMSCs (mesodermal) into glial protein expressing cells requires superior differentiation potency, which means the stemness of hMSCs needs to be maintained at a high level. To achieve this,

using hypoxic culture along with early passage of hMSCs will be necessary [26]. Besides the effect of maintaining the multi-potency of hMSCs, hypoxia pre-treatment can also promote cell survival and help hMSCs migrate to the target site where regeneration takes place [27]. Since endoneurial hypoxia takes place after injury, transforming this pathological condition into a promotional effect for hMSC therapy would be a significant advantage.

Cell-cell interaction plays a vital role in hMSC differentiation. For example, it has been reported that high seeding density is crucial for adipose-derived hMSCs to self-assemble into vascular structures [28]. Similarly, high cell seeding density promotes osteogenic differentiation of hMSCs, but has no effect on chondrogenic differentiation [29]. However, few studies were done to optimize the cell seeding density when inducing hMSCs into glial phenotype expressing cells. Previous studies on neural progenitor cells (NPC) have found that glial fibrillary acidic protein (GFAP) expression is enhanced by increasing the seeding density of NPCs [30]. Another study found that the expression of 2',3'-cyclic nucleotide 3' phosphodiesterase (CNP) can be reduced by increasing the seeding density of NPCs [31]. Thus, finding an optimized seeding density of hMSCs can be a good starting point for controllable glial cell phenotype modification for neural tissue engineering applications.

The objective of this study was to investigate the effect of short term low oxygen pre-treatment and initial seeding density on glial induction of hMSCs. Hypoxia conditions mimicking the residential microenvironment of bone marrow-derived hMSCs (2% O_2 and 5% O_2) were adapted as pretreatment conditions before glial induction. Initial seeding density of 500, 1,000, and 2,000 cells/cm ²were investigated. The expression of GFAP and S100β (Calcium-binding protein B) at both transcriptional and translational levels, regarding different experiment groups, were characterized.

2. Materials and Methods

2.1. Pre-treat hMSCs in physiologically low O_2

Bone marrow–derived hMSCs were provided by Texas A & M University Health Sciences Center. The cells were expanded following our published methods [32,33]. Passage 3 hMSCs were prepared for glial phenotype induction and cultured in basal α-Minimum Essential Medium (MEM) (Life Technology, Rockville, MD) supplemented with 1% L-glutamine, 1% Pen/Strep (Life Technology), and 20% Fetal Bovine Serum (FBS). Initial seeding density of 500, 1,000, and 2,000 cells/cm^2 were used for seeding density optimization. Before glial induction, hMSCs were cultured under different O_2 tension: 2%, 5%, and 20% (control group). The O_2 level was controlled by utilizing the low O_2 culture chamber (BioSpherix, Lacona, NY). Low O_2 treatment was performed during the pre-differentiation phase, where hMSCs were treated with β-mercaptoethnol (Thermo Fisher Scientific, Waltham, MA) for 24 hours, followed by a 72-hour treatment with all-trans-retinoic acid (Sigma-Alderich, St Louis, MO).

2.2. Glial phenotype induction of hMSCs

Glial induction was performed according to the method described by Brohlin M, 2009 [19]. Glial induction medium was made with 5 ng/mL platelet derived growth factor-AA (Sigma-Alderich), 10 ng/mL basic fibroblast growth factor (Thermo Fisher Scientific), 5.7 μg/mL Froskolin (Abcam, Cambridge, MA), and 126 ng/mL glial growth factor-2 (Reprokine, Israel) supplied in α-MEM. The glial induction medium was added after low oxygen pre-treatment, and all culture plates were moved to the incubator under 20% O_2. The induction medium was replaced every 72 hours. Three samples were prepared for each condition.

2.3. Cell proliferation assay

The cell proliferation under different culture conditions was evaluated by using XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) cell proliferation assay kit (ATCC, Manasass, VA) at day 3 and day 7 during the induction process. Cells were incubated in activated XTT solution for 4 hours as directed by the kit manual. Results were obtained using VersaMax ELISA microplate reader (Molecular Device, Sunnyvale, CA). hMSCs cultured with non-induction medium and under normoxia was used as undifferentiated control. Three independent samples were tested under each experimental condition.

2.4. Immunofluorescent staining and quantification

Glial specific proteins S100β (Abcam) and GFAP (Cell Signaling Technology, Danvers, MA) primary antibodies were selected to identify the glial phenotype of differentiated hMSCs as they were widely used as indicators of glial cells by other groups. With the completion of glial induction, cells were fixed in 3.7% formaldehyde in phosphate buffered saline (PBS) solution (Thermo Fisher Scientific) for 30 minutes. Fixed cells were permeabilized in PBS (Fisher Scientific) with 0.2% Triton X-100 (Thermo Fisher Scientific) for another 30 minutes followed by 1 hour of blocking in PBS with 1% bovine serum albumin (Thermo Fisher Scientific). Cells were then incubated with primary antibody overnight at 4 ℃ followed by Alexa fluor 488 secondary antibody (Life Technology) for 40 minutes. DAPI (Thermo Fisher Scientific) was used to stain cell nuclei. The samples were viewed using an Olympus BX51 fluorescent microscope. ImageJ was used to perform fluorescent intensity analysis in which threshold was set to highlight the positively stained area of single cell followed by integrated intensity measurement. This method was adopted from Jensen EC. 2013 [34]. Blind analysis was performed by three persons on samples from triplicate experiments.

2.5. Quantitative RT-PCR analysis on expression level of glial and stem cell genes

Table 1. Primer Design of Glial and Stem Cell Proteins.

Gene Name	*Gene ID*	*Forward*	*Reverse*
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	5'-ACAGTTGCCATGTAGACC	5'-TTTTTGGTTGAGCACAGG
Glial fibrillary acidic protein	GFAP	5'-CTGCTCAATGTCAAGCTG	5'-GCTGGTTTCTCGAATCTG
Calcium-binding protein B	S100B	5'-ACCAATATTCTGGAAGGGAG	5'-CCTCTAAGAAATGGGAAAGC
2',3'-Cyclic Nucleotide 3' Phosphodiesterase	CNP	5'-ACCTACTTTGGAAAGAGACC	5'-TTAACACATCTTGTTGAGCG
Nerve Growth Factor Receptor	NGFR	5'-AACCTCATCCCTGTCTATTG	5'-CCTCTTGAAGGCTATGTAGG
Octamer-binding transcription factor 4	POU5F1	5'-GATCACCCTGGGATATACAC	5'-GCTTTGCATATCTCCTGAAG
SRY (Sex Determining Region Y)-Box 2	SOX2	5'-ATAATAACAATCATCGGCGG	5'-AAAAAGAGAGGCAAACTG

Reverse transcriptional quantitative polymerase chain reaction (RT-qPCR) was used to determine the amount of mRNA production of glial specific proteins. RNA was extracted using RNeasy® extraction kit (Qiagen, Germany). Extracted RNA was reverse transcribed into cDNA using reverse transcription kit (Life Technology) and quantified by StepOnePlus™ real-time PCR system (Applied Biosystems, Waltham, MA) via SYBR® Green Real Time PCR Master Mixes (Life Technology). GAPDH gene was used as endogenous control for this analysis. Customized KiCqStart® SYBR® Green Primers (Sigma-Alderich) were used and the sequences were listed in Table 1. RNA extraction was performed on three independent samples from each group, and each qPCR amplification was triplicated.

2.6. Statistical analysis

Results from experiments were expressed as means ± standard deviation. T-test function of Excel (Microsoft, Redmond, WA) was used for comparisons between groups, and statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Proliferation of hMSCs under physiologically low O2 tension

To assess the status of the hMSCs during the glial induction process, cell proliferation were evaluated by XTT assay at day 3 and day 7. At day 3, the 2,000 group (2,000 cells/cm ²seeding density) displayed the highest proliferation rate (55% increase compared to non-induction control, as shown in Figure 1A.); however, it didn't increase much from day 3 to day 7. On the contrary, the 500 group (500 cells/cm^2) and 1,000 group (1,000 cells/cm^2) showed dramatically increased cell proliferation rate from day 3 to day 7. When evaluating hMSCs proliferation under different O_2 tensions at seeding density of 2,000 cells/cm^2, the 2% O_2 group had the highest proliferation rate both at day 3 and day 7 as shown in Figure 1B. Note that the proliferation rate of the 2% O_2 group is higher (139%) than the undifferentiated control. Both 5% O_2 group and 20% O_2 group have a slight increase in proliferation from day 3 to day 7.

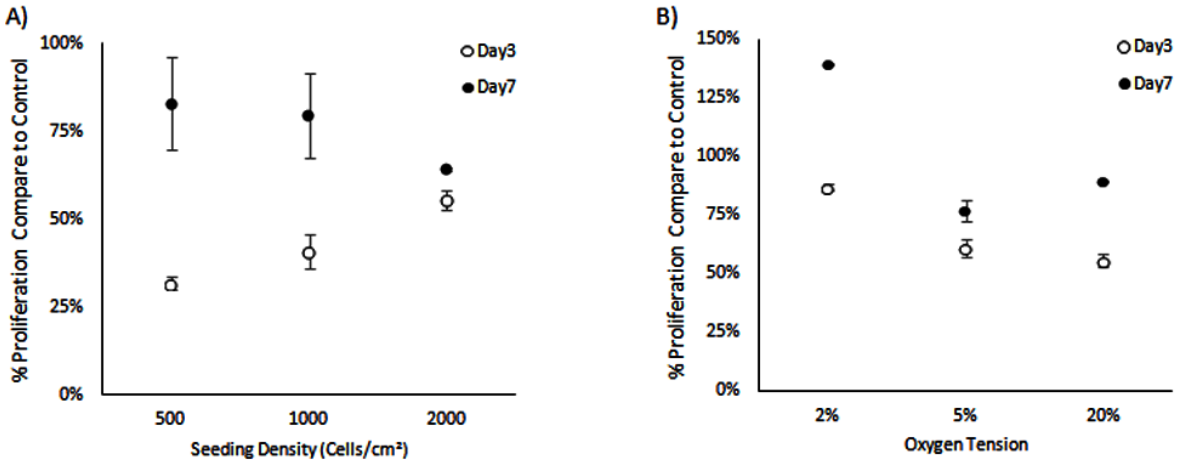

Figure 1. XTT proliferation assay of glial induced hMSCs. (A) Proliferation of cells in groups with different initial seeding density. Note that proliferation of lower initial cell seeding density groups started with lower proliferation then increased dramatically after 7 days. (B) Proliferation of cells in groups with different O_2 concentration. Note that 2% O_2 group has higher proliferation after 7 days compared to the 20 % O_2 control group.

3.2. Expression of GFAP and S100β protein during glial phenotype induction

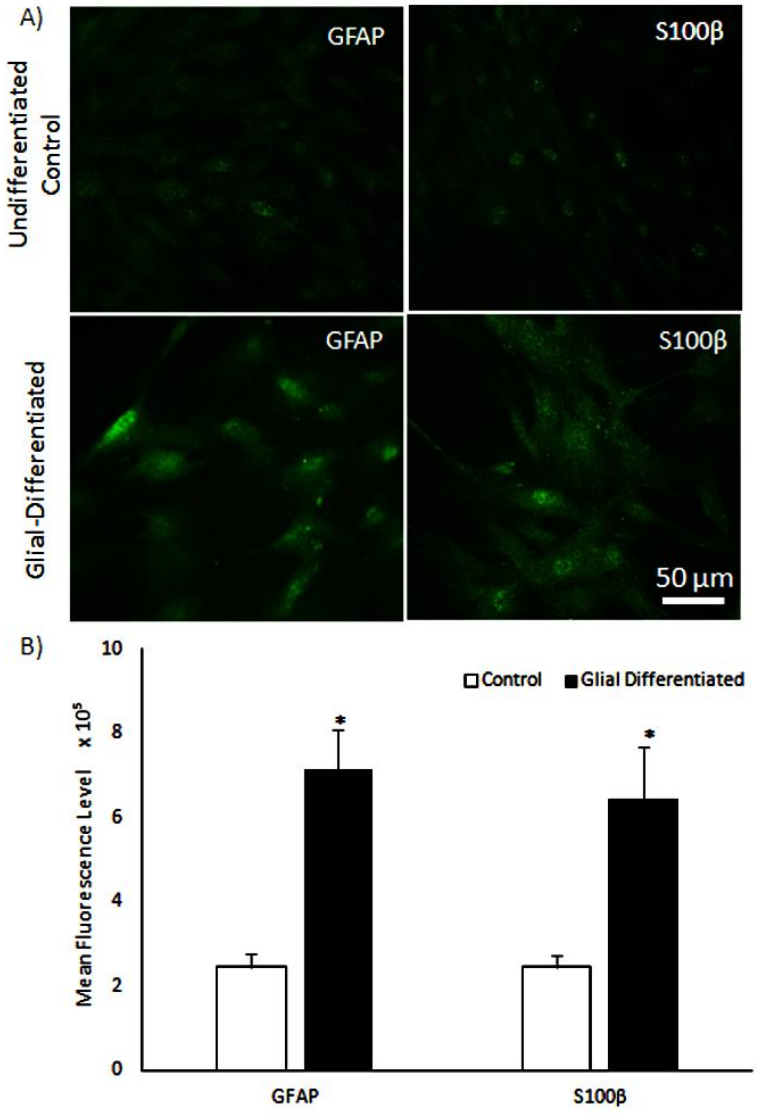

Figure 2. Expression of GFAP and S100β on untreated hMSCs and glial induced hMSCs (2000 cells/cm ² 20% O_2). (A) Immunofluorescent staining of GFAP and S100β in undifferentiated control and glial differentiated group. (B) Quantitative fluorescent analysis of GFAP and S100β. Significant differences were found between undifferentiated and glial differentiated hMSCs on both GFAP and S100β expression. * $p < 0.01$.

Immunofluorescent staining of GFAP and S100β were done after 3 days of culture in glial induction medium under the 20% O_2 condition. hMSCs cultured under the same condition in normal culture medium were used as control. There was weak expression of GFAP and S100β in the undifferentiated control, but both GFAP and S100β proteins were gathered around the nuclei, as shown

in Figure 2A. In the differentiated group, the expression of GFAP and S100β was higher in intensity, and both GFAP and S100β expression are more spread into cytoplasm than undifferentiated control. Quantitative fluorescent intensity analysis demonstrated that GFAP and S100β expression in glial differentiated group was approximately 3 folds higher than non-differentiated group ($p < 0.01$), as shown in Figure 2B.

3.3. Influence of initial seeding density on glial induction of hMSCs

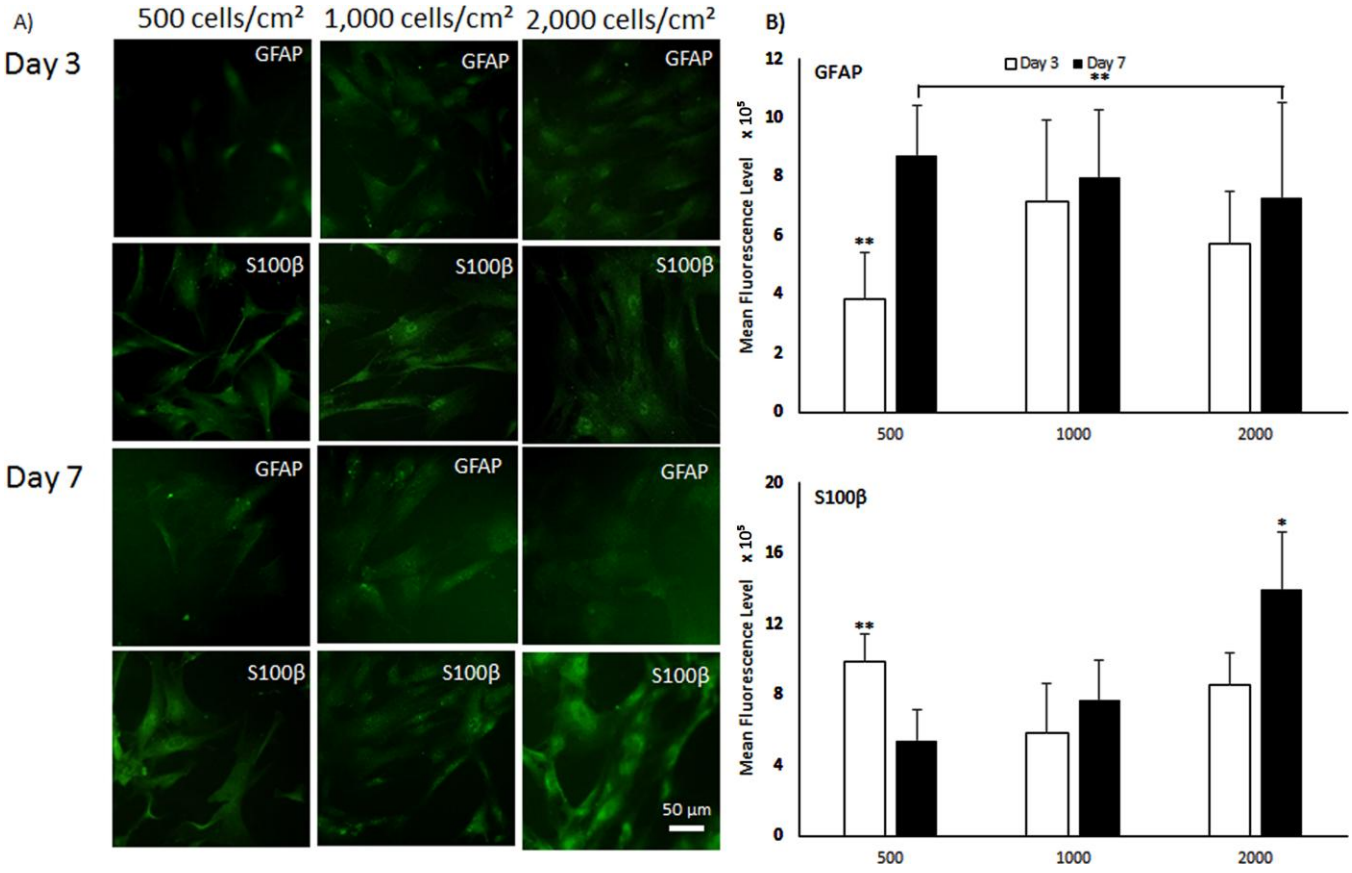

Figure 3. Glial protein expression of glial induced hMSCs with different cell seeding densities from day 3 to day 7. (A) Immunofluorescent staining of GFAP and S100β at day 3 and day 7. (B) Quantitative fluorescent analysis of GFAP and S100β. Note that S100β expression in 2000 cells/cm ²group was significantly higher than all other groups at day 7. * $p < 0.05$, ** $p < 0.01$.

After showing that hMSCs can be successfully induced to express glial proteins, the influence of different seeding densities on glial protein expression after the induction was investigated. Initial seeding densities of 500, 1,000, and 2,000 cells/cm^2 were employed before the pre-treatment process. Cells in all cultures were positively stained for GFAP and S100β (Figure 3A). Quantitative fluorescent analysis showed that significant differences were found between 500 and 1,000 group ($p < 0.01$) at day 3,

and between 500 and 2,000 group ($p < 0.05$) at day 7 for GFAP expression. For S100β expression, significant differences were found between 500 and 1,000 group ($p < 0.01$) at day 3, and 2,000 group is significantly higher ($p < 0.05$) at day 7 (Figure 3B).

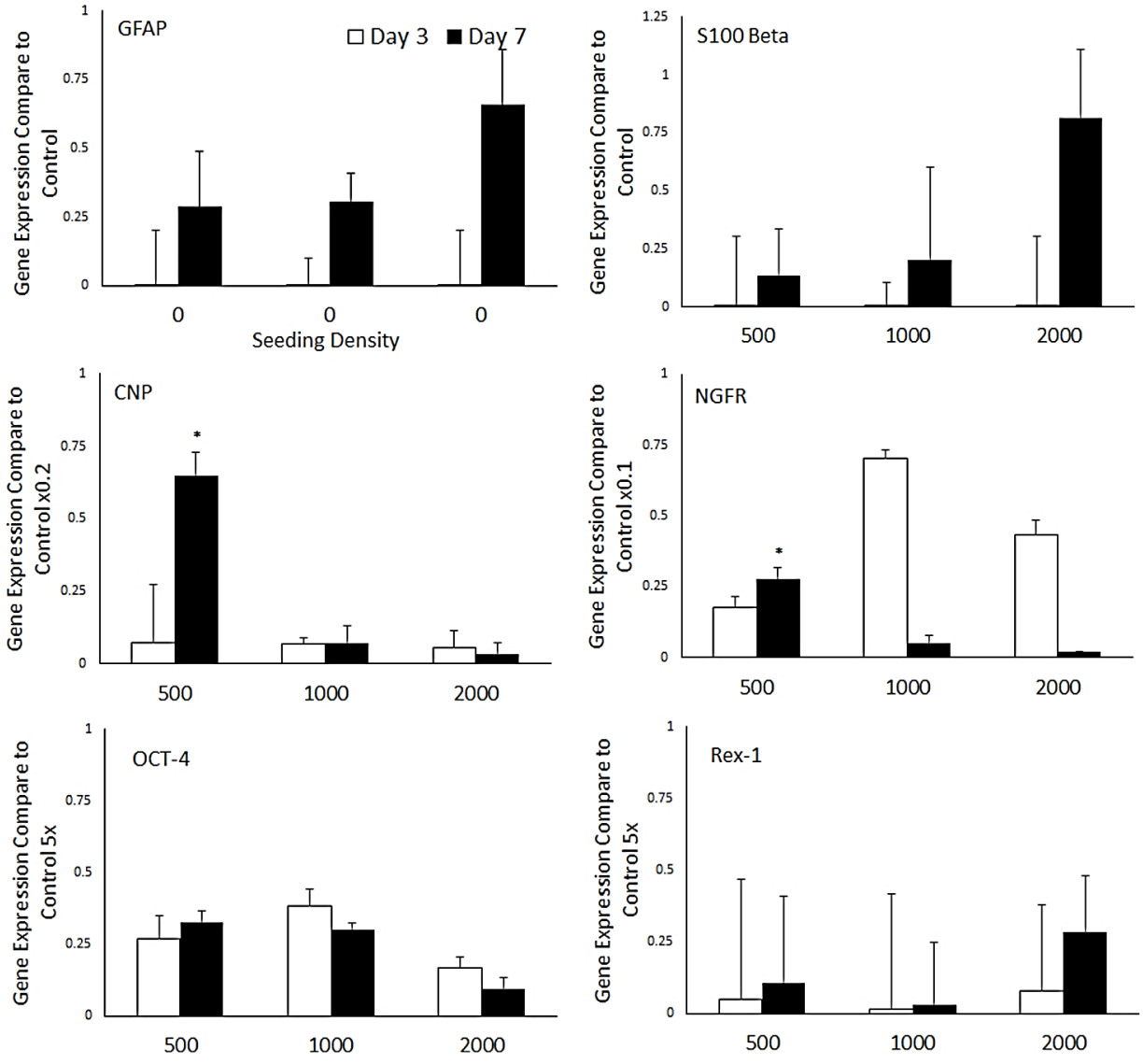

Figure 4. Quantitative RT-PCR analysis on glial phenotype induced hMSCs with different seeding densities from day 3 to day 7. At day 3, the mRNA production of GFAP, S100β, and CNP levels were low while the NGFR level was high in all groups. At day 7, the expression level of GFAP and S100β, CNP and NGFR had significantly increased. * $p < 0.01$.

The expression of glial and stemness genes was further quantified using RT-qPCR. At day 3, the mRNA production of GFAP, S100β, and CNP levels were very low; however, the neural growth factor receptor (NGFR) mRNA production level was high in all groups: with 2.5 fold increase in 500 group, 7.5

folds increase in 1,000 group, and 5 folds increase in 2,000 group. Furthermore, NGFR production in the 1,000 group was significantly higher than the other two groups. The stem cell gene Oct-4 had the lowest expression in the 2,000 group at day 3, while another stemness gene protein Rex-1 expression was at minimum low level. At day 7, the expression level of GFAP and S100β significantly increased. Moreover, the expression level of CNP in the 500 group was higher than other groups. The expression level of NGFR was also higher in the 500 group at day 7. From day 3 to day 7, Expression of stem cell gene Oct-4 was slightly lower in the 1,000 and 2,000 groups, and slightly increased in the 500 group from day 3 to day 7. The expression of Rex-1 was slightly increased in the 500 group and 2,000 group (Figure 4).

3.4. Effect of hypoxia pretreatment on glial induction of hMSCs

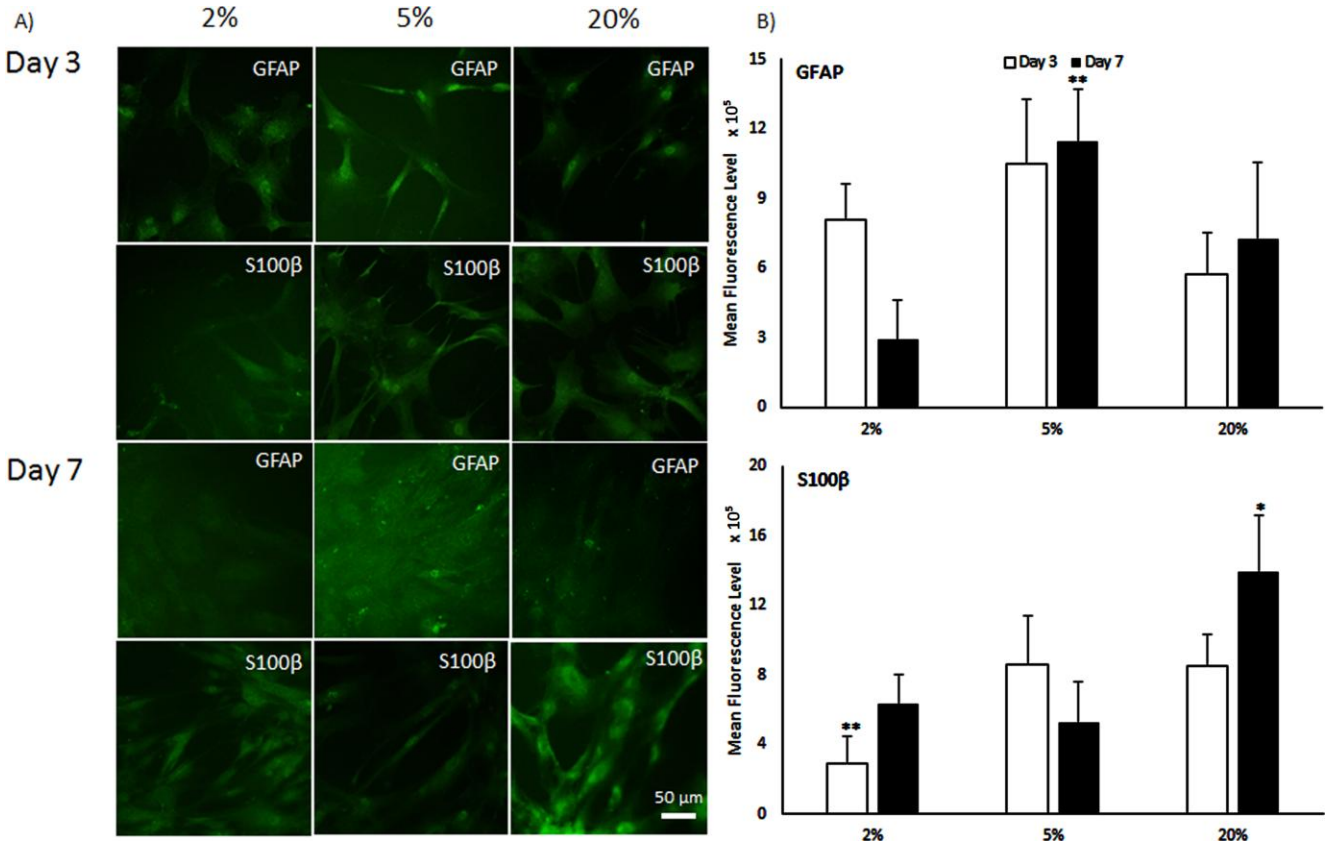

Figure 5. Glial protein expression of glial induced hMSCs pre-conditioned with different O_2 from day 3 to day 7. (A) Immunofluorescent staining of GFAP and S100β at day 3 and day 7. (B) Quantitative fluorescent analysis of GFAP and S100β. Note that GFAP expression was significantly lowered by 2% O_2 but increased by 5% O_2 group; at day 7, while S100β expression was lowered by both 2% and 5% O_2 group. * $p < 0.05$, ** $p < 0.01$.

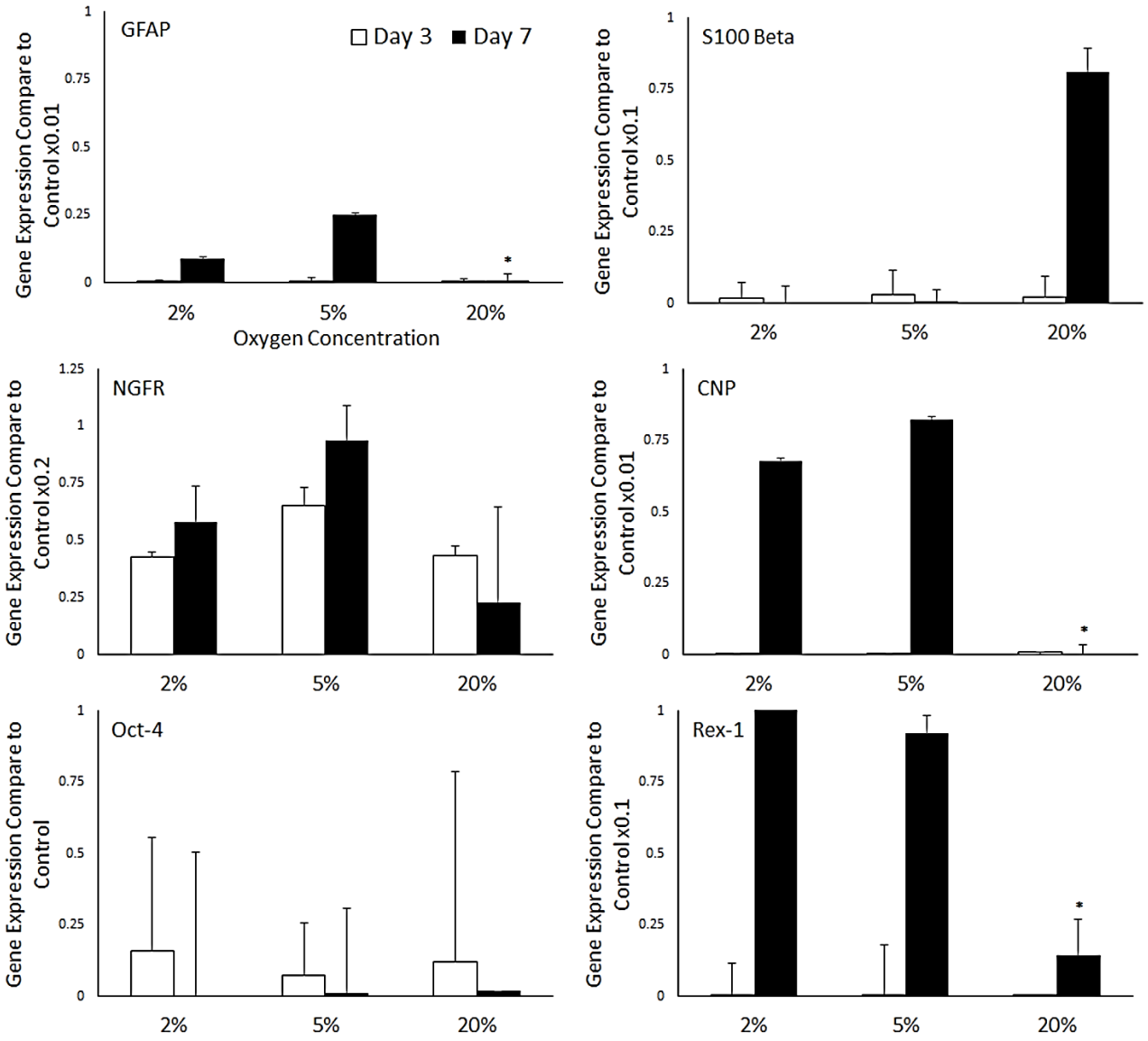

Figure 6. Quantitative RT-PCR analysis on glial induced hMSCs pre-conditioned with different O_2 tensions from day 3 to day 7. Note that the expression of GFAP, S100β, and CNP was kept at lower level at day 3. At day 7, GFAP, CNP, and Rex-1 expression increased in 2% and 5% O_2 groups compared to 20% group. * $p < 0.01$.

To mimic the native growth environment of bone marrow derived hMSCs, 2% and 5% O_2 tension were used to pre-treat hMSCs before glial induction. Immunofluorescent staining and quantification in Figure 5 indicated that the expressions of GFAP and S100β were kept at lower levels. For GFAP expression, significant differences were found between 5% and 20% O_2 groups ($p < 0.05$) at day 3, and 2% O_2 group was significantly lower in expression ($p < 0.01$) at day 7. For S100β expression, 2% O_2 group was significantly lower ($p < 0.01$) at day 3, and 20% O_2 group showed significantly higher expression

($p < 0.01$) at day 7. Quantitative RT-PCR results were consistent with the observed increase with GFAP and S100β expression from the immunofluorescent staining in all groups. The expression of CNP was dramatically higher in 2% O_2 (600 folds higher) and 5% O_2 (800 folds higher) groups than 20% group. NGFR expression was the highest in the 5% O_2 group for both day 3 and day 7 compared to other groups. The 2% O_2 group had lower NGFR expression than the 5% O_2 group, but higher than the 20% O_2 group. The stem cell gene Rex-1 expression was higher in 2% and 5% O_2 groups at day 7 compared to the 20% O_2 group. However, the Oct-4 expression was dramatically decreased after 7 days for all groups (Figure 6).

4. Discussion

It has been confirmed that hMSCs can be transplanted to accelerate the regeneration of peripheral nerve after injury *in vivo* [35]. However, the debates about whether or not the glial induction of hMSCs is necessary to achieve neurological regeneration (full-functional recovery) remains unsolved. It has been reported that transplantation of undifferentiated hMSCs had success to accelerate regeneration in rat sciatic nerve. Nevertheless, it was indicated that potential differentiation process was triggered by the local microenvironment after transplantation [23]. Another study demonstrated that undifferentiated hMSCs had impaired regeneration results while glial differentiated hMSCs had comparable results with Schwann cells [21]. These results indicated that differentiation of hMSCs may promote peripheral nerve regeneration more efficiently than undifferentiated hMSCs. It is critical to understand how the glial induction of hMSCs is affected by different cell culture conditions. Here we conducted a preliminary study to assess the ability of hMSCs to express glial proteins as the result of a glial induction protocol. Since there were none well-establish protocols being published by any group, we have tried to tune the culture condition of the hMSCs to optimize the adapted protocol. In this study, the oxygen concentration was tuned to examine its effect on the glial induction of hMSCs. Physiologically low O_2 is a factor that has drastic influence on cell survival and regeneration effectiveness; moreover, low O_2 culture can be used to better mimic the native microenvironment of peripheral nerves after injury [4]. Studying how hypoxia pre-treatment affects glial phenotype expression of hMSCs can help to provide better understanding of hMSCs based peripheral nerve regeneration therapy. Beside the peripheral glial marker expression, we also attempted to explore central nervous glial marker expression to investigate the potential of tuning hMSCs for central nervous system repair. Beyond hypoxic preconditioning, hMSC differentiation is also affected by cell seeding density as shown in studies of osteogenic, adipogenic and chondrogenic lineage differentiation [28,29]. Seeding density also has critical effect on the differentiation cascade of NPCs [30]. Examining how seeding density affects the amount of different glial protein expression is valuable to customize the property of glial phenotype expressing hMSCs for different applications.

Short term hypoxia exposure of hMSCs is known to maintain stem cell progenicity, improve cell proliferation, and increase extracellular matrix secretion [36]. To develop an efficient therapy for peripheral nerve regeneration, it is crucial to monitor how the hMSC glial phenotypical proteins would change when the cells are pre-conditioned under the hypoxia condition. During developmental stage, GFAP is expressed in neural progenitor cells differentiating towards astrocytes. When the progenitor cells are differentiated, they express high level of S100β, corresponding to the phenotype change from progenitor cells to mature astrocytes [37]. Studies have shown that GFAP expression was enhanced in astrocytes after hypoxic injury, and the elevated expression of GFAP could promote the tissue regeneration [38,39]. In addition, GFAP is essential for Schwann cell proliferation and lack of GFAP expression will cause delayed peripheral nerve regeneration [40]. In this study, we first verified that the GFAP and S100β protein level could be upregulated by 3-day induction under this protocol (Figure 2). The 2% and 5% O_2 tensions were used before the hMSC induction to mimic the native bone marrow microenvironment, and also the pathological endoneurial hypoxia condition. low O_2 (both 2% and 5% O_2) pretreatment significantly increased GFAP expression after 7 days of induction, compared to normoxia (20% O_2) pretreatment (Figure 6). However, S100β expression was lowered by hypoxia (Figure 5), suggesting that hypoxic condition did not promote the differentiation of GFAP-expressing cells into mature astrocytes [37]. Other than these two markers, CNP and NGFR (p75NTR) are also important glial markers reflecting the functional phenotype of differentiated glial cells. CNP is an important myelination associated structure protein during neural tissue regeneration in the central nervous system [41]. NGFR is the receptor of neural growth factor, which can increase the pro-healing capacity of NGF [42]. In addition, the NGFR is critical for motor function recovery during the remyelination process of the regenerated axons [43]. Elevation of CNP and NGFR are enhanced by hypoxia, suggesting that hypoxia could potentially assist in peripheral nerve fiber regeneration, and may be capable to benefit hMSC based central nervous system repair. It has been reported that hypoxia culture preserves the progenicity of hMSCs, which can be reflected by the expression level change of Oct-4 and Rex-1. Study has shown that continuous hypoxia (3% O_2) culture of hMSCs resulted that the expression of Rex-1 (self-renewal associated gene) was not increased, as Oct-4 was significantly increased [44]. In our study, we found that non-continuous hypoxia treatment could increase the Rex-1 expression but not the Oct-4 expression (Figure 6). This discovery can be possibly related to the mechanism of how hMSCs respond to different duration of oxidative stress [45]. On the other hand, maintaining the glial phenotype of differentiated hMSCs can be challenging due to suppression of S100β after hypoxia exposure, prohibiting the differentiation of stem cells. Nonetheless, this study suggested that the hypoxia preconditioning promotes hMSCs to demonstrate peripheral glial cell phenotype instead of astrocyte phenotypes, which could potentially take advantage of post-injury endoneurial hypoxia as an enhancement of hMSC transplantation to assist peripheral nerve regeneration. The effect of hMSCs on peripheral nerve regeneration needs to be further examined by seeding dorsal root ganglia (DRG) cells *in vitro* or using an animal peripheral nerve injury model *in vivo*. A modeling system of 3-D nerve structure similar to the one Phillips, J. B. described [46] can be used to test that how DRG migration is

changed under hypoxia and if this change can be reversed by introducing hMSCs as a therapeutic cue for DRG regeneration. In conclusion, the hypoxia preconditioning was proved to create the suitable microenvironment for peripheral glial phenotype induction of hMSCs.

Cell-cell interaction plays a vital part on proliferation and differentiation of most stem cell types especially in neurogenic differentiation [47]. We studied how the initial seeding density difference could affect expression of glial proteins such as GFAP and S100β in glial-induced hMSCs (Figure 3). Higher GFAP and S100β expression was observed at day 7 in 1,000 and 2,000 groups (Figure 4). A similar trend was observed by Ashton et al., who discovered that GFAP expression was proportional to NPC seeding density [30], suggesting that the communication between the differentiating hMSCs (similar to NPCs) is important for GFAP transcription. CNP expression was dramatically increased at day 7 in the 500 group from our results (Figure 4), and the Wernicke group had observed similar activity where CNP activity was the highest when initial seeding density was the lowest [31]. This result suggested that under current induction protocol, oligodendrocyte induction of hMSCs was favorable under low seeding density, which enhances the potential of oligodendrocyte-like cells for central nervous system applications. In conclusion, different glial protein expression was tunable by modifying initial seeding density, which enabled the potential of creating customizable hMSCs based regeneration therapy for both peripheral and central nervous system injuries.

5. Conclusions

hMSCs have been extensively used in regeneration therapy designs. For peripheral nerve regeneration, direct hMSCs transplantation has been examined as a therapeutic application. Our study demonstrated that the glial induction of hMSCs was enhanced by hypoxia pre-treatment, with mRNA expression profile changed towards peripheral glial phenotypes. Higher initial seeding density was required for hMSCs to express peripheral glial proteins *in vitro,* while lower initial seeding density promotes oligodendrocyte phenotype induction. These results suggested that optimization of the glial induction culture conditions of hMSCs led to the control of different glial phenotype induction. In summary, this study paved the preliminary understanding of how hMSCs respond to different microenvironment, which was critical to design better hMSC based neural regeneration therapy.

Acknowledgements

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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