

## Original Article



# Human Umbilical Cord Mesenchymal Stem Cells Differentiated into Neuron-Like Cells via Laminin and Schwann Cells

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## Abstract

**Background:** Human umbilical cord mesenchymal stem cells (hUMSCs) have been considered to repair damaged tissues and cells. This study aims to investigate the differentiation efficiency affected by Schwann cells (SCs) and laminin and also compare them to other strategies using chemicals or growth factors.

**Methods:** SCs and hUMSCs were separated from dorsal root ganglion of rats and newborn human umbilical cords (hUCs), respectively, and then cultured. The marker expressions of mesenchymal stem cells (MSCs), hematopoietic and endothelial for hUMSCs were confirmed by flow cytometry. The hUMSCs were cultured in four groups: 1) control, 2) co-culture with SCs (C), 3) laminin (L), and 4) co-culture with SCs treated by laminin (CL). The expression of protein and gene-related differentiation NSE, MAP2 and  $\beta$ -tubulin were examined by real-time polymerase chain reaction (PCR) and immunocytochemistry after 12 days.

**Results:** The flow cytometry analysis revealed high expression of mesenchymal and low expression of hematopoietic and endothelial markers, where the SCs expressed S100 at a high level ( $97.4\% \pm 2.25$ ). The expression of NSE, MAP2 and  $\beta$ -tubulin increased significantly in the C, L and CL groups compared to the control group ( $P < 0.001$ ), where the CL group had the highest expression among the groups [ $7.59 \pm 0.126$ ,  $7.87 \pm 0.191$ ,  $6.36 \pm 0.420$ , respectively, ( $P < 0.01$ )]. Also, the expression of neural proteins was significantly increased in tested groups in comparison to the control group.

**Conclusion:** Combined laminin and SCs co-culturing with hUMSCs could be the most effective strategy for neural differentiation.

**Keywords:** Differentiation, Human umbilical cord mesenchymal stem cells, Laminin, Neuron, Schwann cells

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## Introduction

Human umbilical cord mesenchymal stem cells (hUMSCs) have been considered as a promising stem cell source for regeneration of damaged cells and tissues. Typically, mesenchymal stem cells (MSCs) isolated from human umbilical cord (hUC), consisting of a young population of stem cells with the ability to differentiate into various cell lineages including neurons.<sup>1-3</sup> In addition, they are obtained through a non-invasive procedure and readily cultured, making them a superior candidate for cell transplantation.<sup>4-7</sup>

Laminin is an extracellular matrix (ECM) molecule that provide an adhesive substrate for anchorage-dependent cells, affecting cell and tissue behavior through interaction with a wide variety of cell differentiation processes.<sup>8</sup> Recently, various chemical stimulators and neurotrophic factors in combination with laminin have been used to induce neural morphology and neurite-like extensions in human MSCs.<sup>9</sup> Recently, the mechanical properties in ECM-MSCs have become an area of interest.<sup>10</sup> Moreover,

ECM plays a major role in the cellular environment that regulates crucial functions such as survival, proliferation, and cell motility by interaction with integrin. Thus, it is known that ECM-integrin interactions can activate a number of signaling pathways similar to those stimulated by cytokines and growth factors.<sup>11</sup>

Schwann cells (SCs) are known for their reliance on neuron-derived signals during maturity and development.<sup>12</sup> SCs extension, including myelination, requires interaction with both axon and ECM. Hence, they have been shown to enhance regeneration of axons after being grafted into both peripheral and central nervous systems – a process that depends on secreted cell adhesion molecules, ECM, and neurotrophic factors.<sup>13-15</sup> Additionally, they secrete various cytokines that play crucial roles in migration, survival, apoptosis, neural differentiation and synaptic plasticity.<sup>16</sup>

Taking into account these considerations, in this study, we explored hUMSCs differentiation using both SCs and

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laminin based on the hypothesis that these two substances could effectively induce *in-vitro* differentiation toward a neuronal phenotype. The confirmation of this hypothesis would be a cornerstone for the utility of co-grafts for hUMSCs, SCs, and laminin, when cell therapy techniques are required to treat nervous system disorders. The differentiation-related neurons markers (NSE, MAP2 and  $\beta$ -tubulin) were also assessed quantitatively by both real-time immunocytochemistry (ICC) and polymerase chain reaction (PCR).

## Materials and Methods

### Isolation of hUMSCs

All experiments have been performed with approval of the Iran University of Medical Sciences (IUMS) ethical committee. After obtaining parental consent, the babies were evaluated for HIV and hepatitis, and then three fresh hUC samples were collected from full-terms births with a cesarean delivery, placed in Hank's balanced salt solution and moved to the laboratory. The hUC specimens were washed off using sterile PBS supplemented with 1% penicillin-streptomycin (P/S) (Gibco, Waltham, USA) and diced into 3–5 cm pieces. hUC vessels were removed and the jelly-like tissue was cut to small pieces, transferred to T25 cell culture flask with DMEM-F12 with 10% FBS (medium) (Gibco, Waltham, USA). To feed the cells, fresh media were added every 2–3 days and the first mesenchymal cells colony, tissue species were extracted after 10–20 days.

### Isolation of Schwann Cells

To obtain SCs, four newborn rats were separated and anesthetized with chloroform. The spine of each rat was removed and the spinal cord separated from the dorsal root ganglion. The dorsal root ganglia were washed with sterile PBS and 1% P/S to remove blood clots and then cut to small pieces and transferred to 15 mL tube with collagenase type I (Gibco, Waltham, USA) to perform enzymatic digestion that separated SCs from other cells. After 1 hour of incubation at 37°C with 5% CO<sub>2</sub>, trypsin EDTA 0.25% (Gibco, Waltham, USA) was added carefully and incubated at 37°C with 5% CO<sub>2</sub> for two minutes for SCs isolation. Fresh medium was added to halt enzyme reaction. To separate the cells, the suspension was centrifuged for 5 minutes at 1200 rpm. The supernatant was discarded, cell pellet was re-suspended in fresh medium and transferred into the T25 flask.

### Cell Culture and Harvest

The hUMSCs and SCs cells were cultured in medium incubated at 37°C with 5% CO<sub>2</sub>. To feed the cells, media were changed every 2–3 days. When flasks reached 70%–80% confluency, the cells were harvested from the flask using trypsin EDTA 0.25% (Gibco, Waltham, USA), and enzymatic digestion was ceased immediately by addition

of fresh media. The detached cells were then centrifuged at 1200 rpm for 5 minutes to separate the media. The cell suspensions were given a ratio of 1: 3 passages.

### Surface Markers Analysis

hUMSCs were evaluated for surface markers to confirm MSCs. First, cells in the third passage were detached by trypsin-EDTA 0.25% and centrifuged at 1200 rpm for 5 minutes. The cells were re-suspended in PBS with a concentration of 10<sup>5</sup> cells per antibody. Afterward, the cells were incubated with monoclonal antibodies against CD34-PE, CD45-FITC, CD73-PE, CD105-FITC and CD90-FITC (Abcam, Cambridge, UK) for 20 minutes at ambient temperature in the dark. Isotype antibodies conjugated to PE or FITC were used for cell surface staining at the end. The cells were fixed with 1% paraformaldehyde solution (PFA) and analyzed by Flomax software.

### Feeder Cells Culture

SCs were cultured, proliferated at third passages and inactivated using mitomycin-C as previously described. To provide a reciprocal and indirect co-culture system, SCs were dissociated by trypsin-EDTA 0.25%, and seeded on insert with 0.4  $\mu$ m pores (Greiner, Kremsmünster, Austria) using DMEM-F12 supplemented with 5% FBS. To feed the cells, half of the medium was replaced by fresh medium supplemented with 5% FBS every two to three days.

### Precoating of Culture Substrates

For the dose-response adhesion assay, 6-well flat-bottomed plates were precoated with laminin-L2020 (Sigma-Aldrich, St. Louis, USA) at a concentration of 1–2  $\mu$ g/cm<sup>2</sup> and incubated for 3 hours at 37°C with CO<sub>2</sub> gas prior to cell seeding.

### Differentiation Process

hUMSCs were dissociated by Trypsin-EDTA 0.25% after 3 passages, seeded into 6-well plates and cultured into a fresh medium. They were divided into four groups: (1) control, (2) laminin (L), (3) co-culture with SCs (C), and (4) laminin and co-culture with SC cells (CL). To initiate the differentiation process, they were cultured in DMEM-F12 with 5% FBS for 12 days. In order to make the co-culture system, inserts with SCs were placed on 6-well plates.

### Cell Analysis

After 12 days, the differentiated cells were rinsed with PBS and fixed in 4% PFA for 10 minutes at room temperature. To permeabilize the cells, they were washed 3 times with PBS and incubated in 0.1% Triton X-100 (Sigma, Missouri, United States) for 10 minutes, followed by another incubation in blocking solution including PBS with 5% goat serum for 45 minutes at ambient temperature, to block non-specific binding sites. To identify the neural markers, the cells were incubated with anti-NSE, anti-

MAP2 and anti- $\beta$  tubulin antibodies (Abcam, Cambridge, United Kingdom) at 4°C overnight, without washing. The cells were also washed with PBS and incubated with Goat Anti-Rabbit FITC (Abcam, Cambridge, United Kingdom) in PBS with 1% PBS for 1 hour at ambient temperature. They were washed again with PBS and fixed with 4% PFA for 5 minutes to crosslink antigen/antibody complexes. Finally, to analyze cells markers, they were washed 3 times with PBS, the cells' nuclei were counter-stained with DAPI (4', 6-diamidino-2-phenylindole) (Sigma, Missouri, United States) for 3 minutes and analyzed using a fluorescent microscope (Olympus, IX 71).

**Gene Expression Analysis**

Isolation of RNA was done using the RNeasy mini kit (Qiagen, Hilden, Germany) according to its manual's instructions, and its purity and concentration were determined by spectrophotometry (thermo fisher one plus, life science). cDNA synthesis was achieved using QuantiNova Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manual's instructions. cDNA concentration and purity were both measured using the spectrophotometric method. Gene expression was evaluated by real-time PCR using primers MAP2

(forward 5 CTTTTGCTTGCTCGGGATT 3 –reverse 5 GGGTCACTAAACTGCCACCT 3), NSE (forward 5 GGAGGTGGGTGCTCTAAAGG 3 –reverse 5 TCCCCATCTACCCCACTCAG 3) and  $\beta$ -tubulin (forward TGAGGCCTCCTCTCACAAGT 3 –reverse 5 TGCAGGCAGTCACAATTCTC 3), and combined forward primer (0.5  $\mu$ L), reverse primer (0.5  $\mu$ L), cDNA template (1  $\mu$ L), SYBER Green (5  $\mu$ L) [Takara, Japan] and RNase free water (3  $\mu$ L). The PCR parameters were set as follows: 10 minutes at 95°C (polymerase activation), 40 cycles at 95°C for 15 seconds and 60°C for 30 seconds by a Rotor-gene Q thermocycler (Qiagen) using  $\Delta\Delta$ Ct method.

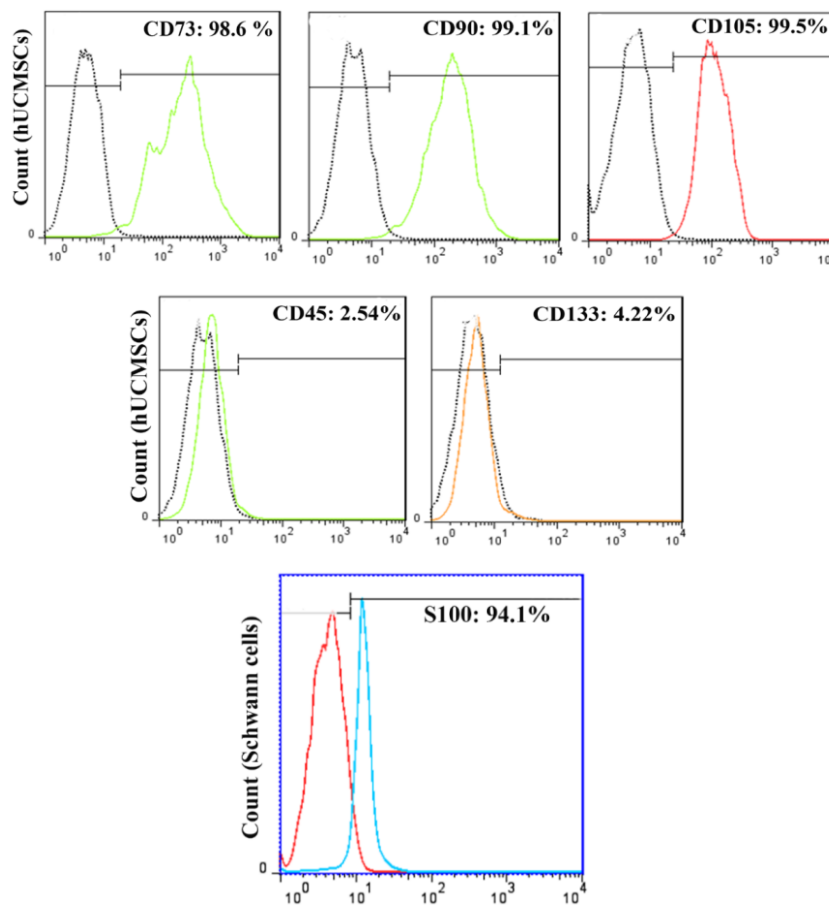
**Statistical Analysis**

Mean  $\pm$  standard deviation was calculated for each group and between-group comparisons were made using one-way ANOVA.

**Results**

**Cell Morphology and Surface Markers**

The SCs with tri-polar and multipolar shapes attached to the bottom of the dish in less than 24 hours. However, they proliferated quickly in the next days. Immunophenotypical evaluation indicated that the S100 marker (Schwann cell



**Figure 1.** Characteristics of hUMSCs and SCs, Flow Cytometric Analysis of Surface-Markers Expression. hUMSCs after passage 3 were labelled with FITC or PE antibodies. They were positive for CD73 (a), CD90 (b), CD105 (c), and negative for CD45 (d) and CD33 (e). SCs (after passage 3) were positive for S100 (f) surface-marker.

marker) was expressed in SCs at  $97.4\% \pm 2.25$ . Figure 1 shows the hUCMSCs in third passage under phase contrast microscope. Morphologically, hUCMSCs were differentiated into fibroblast-like cells with long and short process (Figure 2). However, immunophenotyping analysis indicated expression of high levels of positive markers (CD73, CD90 & CD105) and low levels of negative markers (CD45 & CD 133) in hUMSCs, which were  $(98.6\% \pm 1.31, 99.1 \pm 1.57)$  and  $99.5 \pm 2.68$ , and  $(2.54\% \pm 1.26, 4.22\% \pm 1.01)$ , respectively. Moreover, CD45 as a hematopoietic marker was expressed at  $2.54\% \pm 1.26$  and the CD 133 as an endothelial at  $4.22\% \pm 1.01$  (Figure 1).

### Gene Expression Analysis

The expression of neural markers such as  $\beta$ -tubulin, NSE and MAP2 was measured by Real-time PCR (Figure 3). We observed different levels of marker expression across the four groups of, control, L, C and LC. The  $\beta$ -tubulin marker was 1,  $2.67 \pm 0.149$ ,  $4.92 \pm 0.164$  and  $6.36 \pm 0.420$  for the control, L, C and CL groups, respectively, with a significant difference between the three groups and the control group ( $P=0.0001$ ). The NSE marker was expressed in the control, L, C and CL groups at 1,  $1.62 \pm 0.060$ ,  $3.64 \pm 0.138$  and  $7.59 \pm 0.126$ , respectively, showing a significant difference between the three groups and the control group ( $P=0.003$ ). The level of expression for the MAP2 marker was 1,  $1.84 \pm 0.112$ ,  $4.04 \pm 0.145$  and  $7.87 \pm 0.191$  for the control, L, C and CL groups, respectively, with a significant difference between the three groups and the control group ( $P=0.0001$ ).

### Cell Analysis

The *in-vitro* neural differentiation of hUMSCs was assessed by immunocytochemistry. The neural proteins as stem cell markers consisted of  $\beta$ -tubulin, NSE and MAP2 in four groups of control, L, C and CL.  $\beta$ -tubulin was expressed in the three groups of L, C and CL, at  $24\% \pm 2\%$ ,  $40\% \pm 2\%$  and  $70\% \pm 2\%$ , respectively, with a significant difference between these three groups and the control group ( $P=0.0001$ ). Also, the expression of NSE as the mature neural marker was also detected in the three studied groups at  $28\% \pm 2\%$ ,  $42\% \pm 2\%$  and  $73\% \pm 5\%$  for L, C and CL, respectively, with a significant difference between these groups and the control group ( $P=0.0002$ ). Moreover, the expression of MAP2 in L, C and CL was  $22\% \pm 2\%$ ,  $45\% \pm 2\%$  and  $68\% \pm 3\%$  respectively, indicating a significant difference between these groups and the control group ( $P=0.00008$ ).

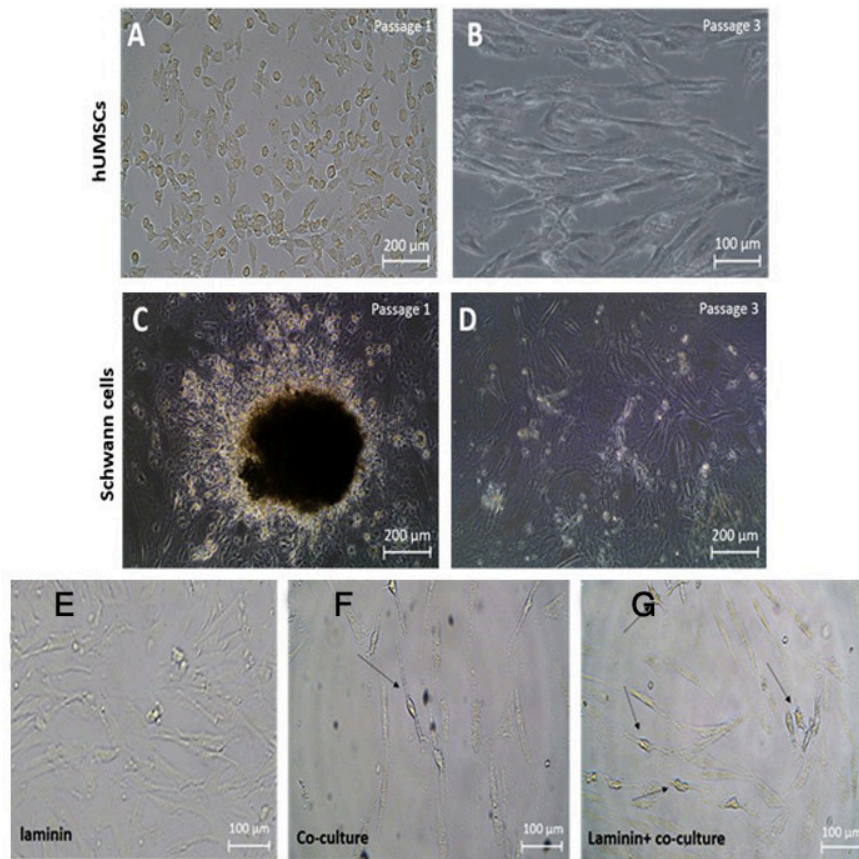
### Discussion

The hUC and placenta are considered to be primitive, non-invasive and major sources of MSCs that have increasingly gained attention due to the absence of moral or ethical concerns.<sup>17</sup> Based on the standard definition, MSCs and their clonal cells attached to plastic can differentiate into chondrogenic, osteogenic, and adipogenic lineages *in*

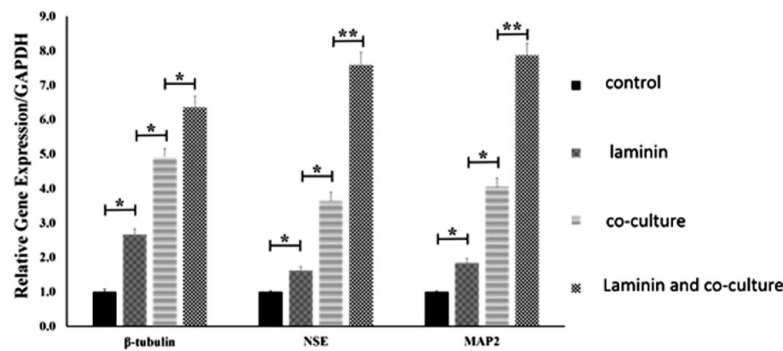
*vitro*, and express CD markers such as CD73, CD90, as well as CD105 markers. However, isolated hUMSCs have been reported to vary in self-renewal potential and their potency. Hence, when applied for clinical purposes, they often lead to variable or even conflicting results.<sup>18</sup>

Various studies have used chemical inducers for differentiating MSCs into neural cells. However, chemical induction may cause cellular stresses that result in physical contraction of cells into neuron-like morphology.<sup>19,20</sup> Biological substances such as growth factors (NGF, PDGF, BDNF and bFGF) can also induce stable neural differentiation of MSCs.<sup>21-25</sup> Nonetheless, usage of entire growth factors as an inducer is fairly expensive and complicated. Cell co-culture systems can overcome these aforementioned obstacles; they can create a cross link between two cells and make a dynamic niche excels compared to exogenous use of entire cytokines.<sup>26</sup> Jing et al showed that the co-culture of MSCs with hematopoietic stem cells can drastically increase the level of hematopoietic cell proliferation compared to the state where soluble cytokines are used exogenously.<sup>27</sup> Our results also confirm that SCs co-culture can significantly increase the proliferative process ( $P < 0.05$ ), as shown in Figure 2.

The destiny of stem cells differentiation is regulated by different signaling keys in the stem cell niche, including various cell type, ECM and soluble factors.<sup>28</sup> SCs can secrete different neurotrophic factors, such as FGF, NFG, BDNF and GDNF, which make it possible to induce differentiation of MSCs into neural cells when they are co-cultured with SCs.<sup>12</sup> Recently, a number of studies have demonstrated that BDNF combined with NGF treatment increased the proportion of differentiated neurons, and induced the development of more neurons than BDNF or NGF alone. Liu et al reported the roles of NGF, BDNF, and combined effects of BDNF and NGF in differentiation of neural stem cells. Their results demonstrated that BDNF combined with NGF augmented the effect on neuronal cell differentiation. Although an increased dose of NGF or BDNF alone did not alter the neuronal differentiation rate, the differentiation proportion was highly enhanced when they were combined.<sup>29</sup> In previous studies, it has been indicated that NGF and BDNF are secreted by SCs.<sup>30,31</sup> The present study illustrated that both NGF and BDNF secreted by SCs can effectively trigger hUMSCs differentiation into neural cells. Nonetheless, many studies have indicated that MSCs differentiation into neural like cells has been achieved by co-culturing with SCs. Zurita et al used bone marrow MSCs co-cultured with SCs, and demonstrated the expression of neural markers such as Nestin and MAP2 as immature and mature neural markers that increased during 2 weeks of differentiation.<sup>32</sup> On the other hand, Liao et al evaluated the expression of Nestin as an immature neural marker on adipocyte stem cells through co-culturing with SCs. They reported that the level of expression of Nestin increased by approximately 12 folds within 2 weeks of



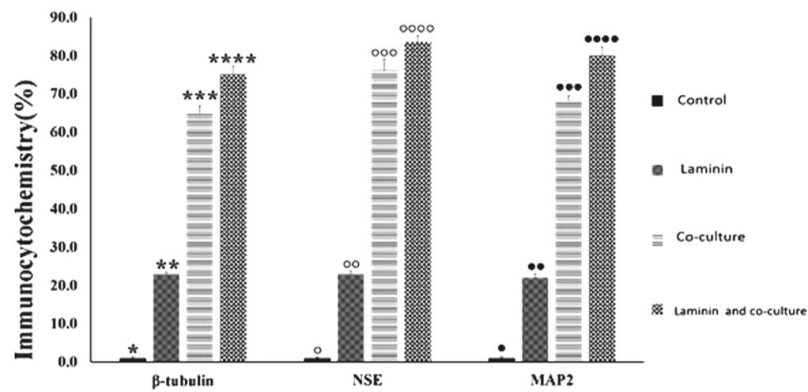
**Figure 2.** Cell Morphology. The appearance of differentiated hUMSCs (A) and (B) colony formation after first and third passages. Schwann cells (C) and (D) after first and third passages. In the first passage, cells are migrating out from a piece of dorsal root ganglion (white arrow). After third passage, cells filled the entire flask. Differentiation of hUMSCs into neuron-like cells in laminin (E), co-culture with SCs (F), and co-culture with SCs+laminin (G) groups after 12 days of induction. The hUMSCs show morphological changes in the differentiation pathway (F and G, black and white arrows).



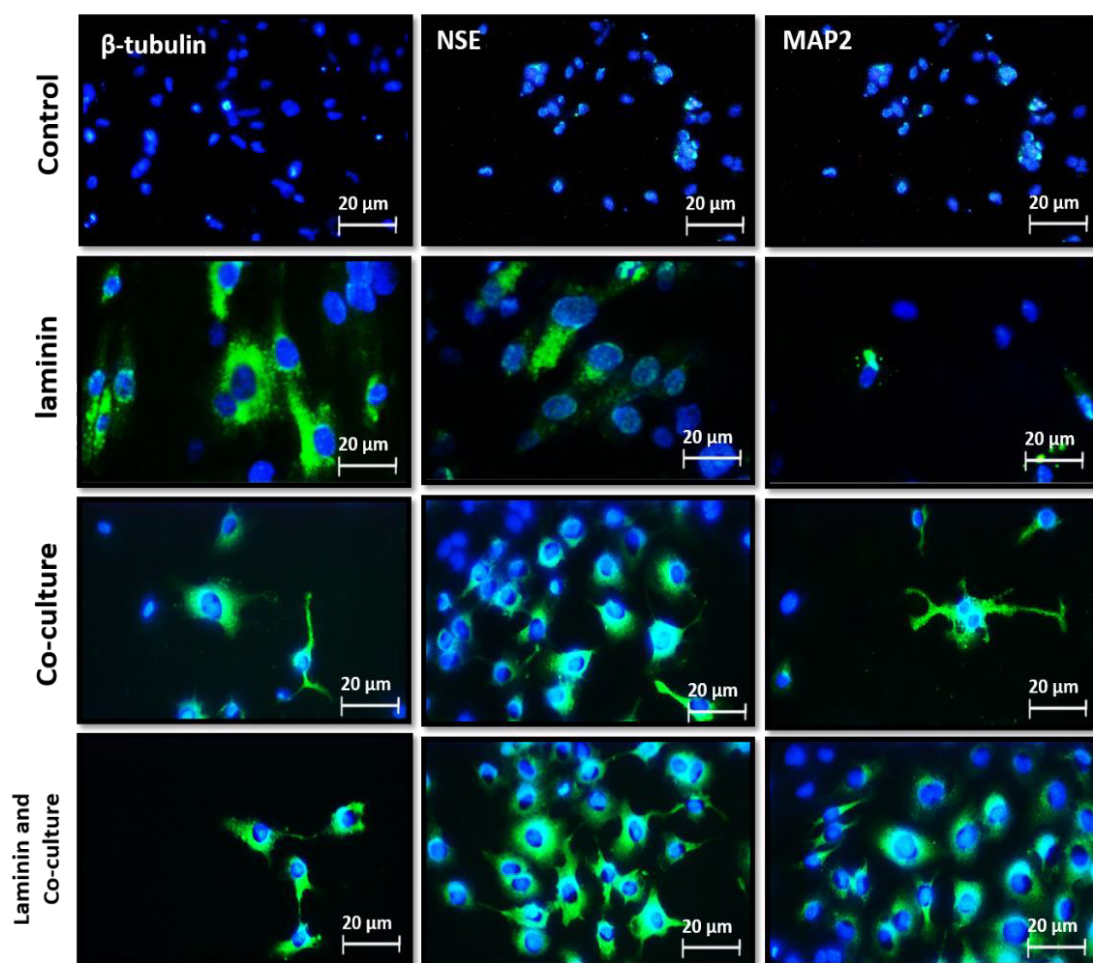
**Figure 3.** RT-PCR Analysis of Isolated Cells after Differentiation Under Influence of Laminin and Co-culturing with SCs for 12 Days. Results showed that hUMSCs had a greater potential to differentiate into neuron-like cells. We observed different levels of expression across the control, L, C and CL groups. The expressed neural markers such as  $\beta$ -tubulin, NSE and MAP2 increased significantly in the tested groups compared to control (lack of expression). The CL group expressed neural markers significantly more than the other groups ( $P < 0.05$ ).

differentiation.<sup>12</sup> Our experiment was conducted on the basis of evaluating neural markers at different stages; thus, we demonstrated that co-culturing of SCs with hUMSCs significantly enhances the expression of neural markers (NSE,  $\beta$ -tubulin and MAP2), whether in mRNA or at protein level within 12 days of differentiation. We used laminin because it plays an important role as an essential

constitutive niche that regulates stem cells' fate, provides regulatory signals to support stem cell migration, self-renewal, proliferation and differentiation, as well as neural regeneration.<sup>33</sup> Moreover, we used laminin to precoat our culture plates to improve the neural differentiation process. Ma et al evaluated the effect of ECM molecules on neural differentiation of stem cells. They showed



**Figure 4.** Immunocytochemistry Analysis of Isolated Cells after Differentiation under Influence of Laminin and Co-culturing with SCs for 12 Days. The protein expression of  $\beta$ -tubulin, NSE and MAP2 were observed only in differentiated groups but they were absent in the control group ( $P < 0.05$ ).



**Figure 5.** Immunocytochemistry Staining of Differentiated Cells Under Influence of Laminin and Co-culture with SCs for Specific Neural Markers such as  $\beta$ -Tubulin, NSE and MAP2 after 12 Days. Nuclei were stained with DAPI. NSE as a mature neuronal marker was expressed more than MAP2 and  $\beta$ -tubulin in all groups, especially in the laminin co-culture group.

that laminin and laminin-rich matrigel significantly increased neural progenitors, neuronal generation and neurite outgrowth.<sup>34</sup> Mruthyunjaya et al, Kim et al, and Flanagan et al also demonstrated that laminin promoted neurite outgrowth, migration and differentiation of stem

cells.<sup>35-37</sup> However, in addition to enhanced adhesion and attachment, a coating protein laminin can also influence the final results of the assay and the coating conditions significantly affect cell viability.<sup>38,39</sup> Therefore, we investigated other approaches to use culture substrates

with less labor; hence, we used precoating laminin for cell adhesion and cellular signaling for neurogenesis.<sup>40</sup> We demonstrated that culture of hUMSCs with both laminin and SCs increased the expression of neural markers with different grades. In fact, we also observed that expression of  $\beta$ -tubulin as immature neural markers in CL group was significantly increased in contrast to other groups ( $P < 0.05$ ). Moreover, the quantity of MAP2 and NSE expression as general neural markers increased significantly in the CL group in comparison to the other groups. In conclusion, our study showed that co-culturing with SCs and laminin results in a significant improvement in neuronal differentiation of hUMSCs. Therefore, using both SCs and laminin can be considered as an effective and practical method to expedite the expression of neural markers without exerting stress on cells.

#### Authors' Contributions

FJ, MRK: participation in study design; project administration; analysis; manuscript drafting. MK: participation in study design; critical discussion; writing and editing. SAM, ARA: manuscript drafting and editing, critical discussion. FM: Isolation of Schwann cell, manuscript drafting and critical discussion. HRA: study design; critical discussion; writing, editing and responsible for overall supervision. All authors performed editing and approving the final version of this paper for submission

#### Conflict of Interest Disclosures

The authors declare that they have no conflict of interest.

#### Ethical Statement

All studies were performed in accordance with the Ethical guidelines set by the "animal care and use committee (ACUC), Iran University of Medical Sciences" (code: IR.IUMS.FMD.REC.1397.266).

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