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Integrin α5β1 Mediated Cellular Reorganization in Human Mesenchymal Stem Cells During Neuronal Differentiation

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Abstract. *Background/Aim: Mesenchymal stem cells (MSCs) have been widely used for yielding neurons in culture to study nervous system pathologies and develop regenerative approaches. In this study, cellular rearrangements of human MSCs related to the expression of the fibronectin common receptor integrin α5β1 and its cell surface localization during neuronal differentiation, were examined. Materials and Methods: Proliferation kinetics of neuronal induced hMSCs (hMd-Neurons) were quantified by BrdU assay, and hMd-Neurons were immunostained for neuronal marker expression. Additionally, cDNA and protein samples were collected at different time points for integrin α5β1 expression analysis. Results: Endogenous integrin α5β1 expression was significantly upregulated by day 6 and maintained until day 12. Cell surface localization of α5β1 integrin was increased by day 6; the integrin was internalized into the cytosol by day 12. Conclusion: Integrin dynamics around day 6 of differentiation might be involved in neuronal differentiation and maturation or specification of hMd-Neurons.*

Mesenchymal stem cells (MSCs) are one of the most promising stem cell type due to their availability and relatively simple requirements for *in vitro* expansion and genetic manipulation (1, 2). Multipotential differentiation of MSCs has been detailed by Friedenstein and coworkers in the mid-1970's and modified by other groups in the 1990's (3-6). Due to their general multipotential differentiation capacity and relative ease of isolation from numerous tissues, MSCs have been considered in tissue engineering and therapeutic applications (7, 8). The plasticity and self renewal ability of MSCs offer a huge potential for clinical tissue regeneration (9, 10). MSCs have been isolated from a number of tissues, including foetal blood, cord blood, bone-marrow, and amniotic fluid (11, 12). One of the most studied and accessible source of MSCs is the bone marrow (BM). Human bone marrow derived MSCs can be prolonged in an undifferentiated state *in vitro*, but have the ability to generate a functional stroma, to support hematopoiesis, or to differentiate along osteogenic, chondrogenic, and adipogenic lineages under certain conditions (13). Additionally, MSCs can also transdifferentiate into neurons. This ability of MSCs facilitates their use in neural tissue devolopment and repair.

Neural differentiation of MSCs *in vitro* was reported from two simultaneous studies by Sanchez-Ramos *et al*. and Woodbury *et al*. in 2000 (14, 15). These two studies were the pioneers for establishing MSC-derived neuronal cells. Afterwards, a number of protocols were reported for developing an optimized method for neuronal induction of MSCs (16-25).

Human MSCs express a large number of different cell surface proteins, including various integrins, growth factor receptors (bFGFR, PDGFR, EGFR, TGFβIR/IIR), chemokine receptors (interleukins, CC and CXC receptors) and cell adhesion molecules (VCAM-1, ICAM-1/', ALCAM-1, L-selectin, CD105, CD44). Moreover, hMSCs produce a vast array of matrix molecules including fibronectin, collogens, laminin, and proteoglycans (26).

As a first significant finding, in 2001, Gronthos and his colleagues examined the mechanisms mediating the growth of hMSCs on different extracellular matrix (ECM) components. In summary, they found that hMSCs show a higher colony-forming efficiency when seeded onto collagen type IV, fibronectin, vitronectin, and laminin coated surfaces

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in comparison with collagen type I and III (27). This may be caused by their functional origin in the BM, stage of commitment, and unique integrin expression. Moreover, this study revealed that β1 integrin seems to have a regulatory function in the *in vitro* differentiation of hMSCs into osteoblasts. Furthermore, plenty of data have been provided by studies on the involvement of integrins during *in vitro* osteogenic, chondrogenic, and adipogenic differentiation of hMSCs (13, 28, 29). Consequently, it is a promising area of research to study spesific patterns of integrin expression and utilizations in hMSCs. Understanding the underlying mechanisms of hMSC differentiation in relation tointegrin dynamics may aid in the development of novel therapeutic and tissue engineering approaches.

Integrin α5β1 is a transmembrane receptor that binds to fibronectin (Fn) through its extracellular domain and its cytoplasmic tail interacts with intracellular actin filaments (30-33). Among other members of the integrin family receptors, α5β1 is a fibronectin specific integrin that can be found in different adhesion structures and has been implicated in the control of differentiation of various cell types, such as precursor osteogenic cells (34-37). Integrin α5β1 is also known as the "fibronectin common receptor" due to its specific binding property on this core ECM protein. Functionally, α5β1 interaction requires both the traditional integrin-binding sequence (RGD) as well as the synergy sequence (PHSRN), whereas most other RGD dependent integrins do not require PHSRN (38). Besides, this receptor-ligand pair (integrin α5β1- Fn) is functionally very important because it mediates fibronectin fibril formation and governs ECM assembly, which is vital to cell function *in vivo*. The interaction between α5β1 and Fn is fundamental for vertebrate development, since lack of α5β1 or Fn results in early embryonic lethality (39-41). Since Fn is believed to be the most common fibronectin receptor and a significant component of the framework of hMSCs, integrin α5β1 has been used to investigate hMSC-integrin engagement. Martino *et al*. demonstrated that α5β1 has an important role in the control of MSC osteogenic differentiation (42).

Considering previously suggested cellular dynamics regulated by integrin α5β1, in this study, involvement of integrin α5β1 in neuronal differentiation of hMSCs was examined based on a differentiation protocol we previously reported (43). The results showed that cytokine based induction of hMSCs into hMd-Neurons requires integrin α5β1 surface localization at specific time points (around day 5), while translocation into the cytosol was observed during further maturation. Considering the integrin α5β1 expression profile of hMd-Neurons, these findings suggest that modification of the hMSC neuronal induction environment may contribute to neuronal differentiation and maturation or specification of hMd-Neurons.

Materials and Methods

Culture of hMSCs. To analyse neuronal differentiation of hMSCs, a commercial hMSC cell line (UE7T-13 cells, no RBRC-RCB2161; RIKEN, Wako, Japan) was used. hMSCs were maintained in DMEM (GIBCO, New York, NY, USA) containing 10% FBS+1% Penicillin/Streptomycin and cells were incubated at 37˚C, in an atmosphere containing 5% CO_2. Cultured hMSCs were subcultured at 80-90% confluency.

Flow cytometry analysis. To confirm that isolated cells from human BM were hMSCs at passage 3, cells were analyzed using immunotyping. For this, flow cytometry was performed using a FACS system (BD Influx Cell Sorter with Bioprotect IV Safety Cabinet). The data were analysed with FlowJo (Tree Star) software (FlowJo LLC, Ashland, OR, USA) and the forward and side scatter profile was used to gate out debris and dead cells. Immunophenotyping of hMSC was performed with antibodies against the following antigens: CD44 (ab27285; Abcam, Cambridge, MA, USA), CD73 (ab157335; Abcam), CD90 (ab11155; Abcam), CD105 (ab53321; Abcam), CD34 (ab18227; Abcam), CD45 (ab134202; Abcam), and their isotype controls (IgG1 FITC, IgG2a PE, IgG1 PE) (Abcam).

Neuronal differentiation. hMSCs were seeded in culture dishes prior to neural induction. Cell frequency was set at 3,000 cells/cm^2. The culture was maintained in DMEM containing 10% MSC-FBS and 1% Penicillin/Streptomycin; the cells were incubated at 37˚C in 5% CO_2 for 24 h. Previously defined neuronal induction media, composed of 20 ng/ml hEGF (human epidermal growth factor), 40 ng/ml bFGF (basic fibroblast growth factor), 10 ng/ml FGF-8 (fibroblast growth factor-8), 10 ng/ml human BDNF (human brain-derived neurotrophic factor), 40 ng/ml NGF, 0.125 mM dbcAMP (dibutyryl cyclic AMP), 0.5 mM IBMX (3-isobutyl-1-methylxanthine), 2 mM L-Glutamine in Neurobasal medium, and B27 supplement in the absence of serum was used for neural differentiation (43). Then, cells were refreshed with neural induction media every 48 h for 12 days. Samples were collected for western blotting and RT-PCR.

Immunofluorescence staining. The medium was aspirated off and cells were permeablized with prewarmed TZN buffer (10 mM pH 7.5 Tris-HCl, 0.5% Nondet P40, 0.2 mM $ZnCl_2$) at RT for 15 min by mixing on a rocking shaker at very low speed (when staining membrane bounded integrins, TZN treatment was not applied). Cells were then fixed with 4% paraformaldehyde in phosphate buffer saline (PBS; Sigma, St. Louis, MO, USA) and incubated at RT for 15 min. After washing with PBS, cells were blocked with a solution containing 1% normal goat serum (Sigma), 3% BSA (Sigma) 0.3% sodium azide (Sigma), and 0.1% Triton X in PBS (PBS-Tx) to prevent nonspesific binding.. After discarding the blocking solution, cells were treated with primary antibodies: Anti-Integrin α5β1 (Chemicon, Merck KGaA, Darmstadt, Germany; 20 μg/ml), Anti-Actin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 10 μg/ml), Anti-Neurofilament (NF-H) (Chemicon; 10 μg/ml), Anti-NSE, (Chemicon, 10 μg/ml), Anti-NeuN (Chemicon, 50 μg/ml), in PBS containing 3% BSA (Sigma), 0.3% sodium azide (Sigma), 1% Tween-20 and 3% normal human serum (NHS, Biochrom, Ltd., Cambridge, UK) and incubated at 4˚C overnight. Normal human serum was applied

onto the negative control wells instead of primary antibodies and after antibody exposure both wells were washed with PBS for three times. Cells were then incubated at RT for 3 h with 1:200 diluted goat-anti Mouse (GAM) IgG Alexa Fluor 488 (Abcam; ab150113), 1:200 diluted goat anti rabbit (GAR) IgG Alexa Fluor 488 (Abcam; ab150077), 1:200 diluted donkey anti-goat (DAG) IgG Alexa Fluor 568 (Abcam; ab175474), 1:500 diluted goat anti-rabbit (GAR) IgG Alexa Fluor 594 (Abcam; ab150088) and 1:100 diluted goat anti-chicken (GAC) IgG Alexa Fluor 633 (A-21103) secondary antibodies. For nuclear staining, cells were treated with 1:15,000X DAPI solution and incubated at RT for 3 min. They were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). During neuronal differentiation, cell images for each specific time point were obtained under a fluorescent microscope (Zeiss LSM780 Confocal Microscope).

BrdU cell proliferation assay. hMSCs were seeded as triplicates in 96-well plates (3×10^3 cells/cm^2) in 100 μl of medium. After 24 h, hMSCs were induced towards neural differentiation. Then, 4 h and 24 h after the induction, BrdU assay (Cell proliferation ELISA, BrdU, ROCHE, Branchburg, NJ, USA) was performed according to the manufacturer's instructions. Absorbance was measured at 370 nm using an ELISA reader (SpectraMax).

Cell death analysis. Active caspase-3 is a downstream marker of apoptosis. Neuronal-induced hMSCs were evaluated for caspase-3 activation to analyse cell death during neuronal differentiation in culture. Accordingly, hMSCs were seeded into 35 mm petri dishes (3,000 cells/cm^2). After 24 h, the culture medium was replaced with induction medium. At days 2, 6, and 12 cells were collected and caspase-3 activity (caspase-3 assay kit, BD Pharmingen, San Jose, CA, USA) was measured according to the manufacturer's instructions. In positive control cells, apoptosis was induced by treatment with camptothecin (Sigma). Caspase-3 activity was inhibited by Z-DEVD-FMK (Sigma) treatment.

Nuclear diameter measurements. The diameter changes in nuclei of both neuronal induced or uninduced hMSCs at day 12 were calculated by Zen Blue Software (Zeiss Inverted Microscope with Hoffman Modulation). A region of interest (ROI) on 10 different images was determined for each time point. A total of 200 cells/group were investigated on DAPI stained cell nuclei for determination of changes in diameter *via* Violin plot. Welch's *t*-test was used for comparisons.

Reverse transcriptase PCR (RT-PCR). RNA samples of hMSCs were extracted by RNeasy kit (Qiagen, Venlo, the Netherlands). 0.5 μg of total RNA was reverse transcribed to obtain cDNA by Quantitect Reverse Transcription kit (Qiagen). A cDNA library was obtained after 35 cycles of amplification (PCR core kit, Qiagen). Primer pairs (Forward; Fw and Reverse; Rv) used in the experiments were as follows: Integrin α5 (Fw: 5'-AGCCTGTGGA GTACAAGTCC-3' and Rv: 5'-AAGTAGGAGGCC ATCTGTTC-3') generating 584 bp fragment; Integrin β1 (Fw: 5'-AGCAGGGCCA AATTGTGGGT-3' and Rv: 5'-CCACCAAGT TTCCCATCTCC-3') generating 756 bp fragment. A human GAPDH primer pair (Fw: 5'-GTCAGTGGTGGACCTGACCT-3', Rv: 5'-TGCTGTAGCCAAATTCGTTG-3') generating a 245 bp fragment was used as a positive control.

Western blotting. hMSCs were seeded into $1.0\text{-}1.5\times10^5$ cells/well into glass bottom 6-well plates and incubated at 37˚C in 5% CO_2 for 24 h. Protein lysates were obtained using RIPA lysis buffer (Thermo Fischer Scientific, Waltham, MA, USA; #89900). Equal amounts of protein samples were run on SDS-PAGE; subsequently gels were transferred by using the iBlot-2 Dry Blotting (Thermo Fisher Scientific) system. After the blocking step, the membrane was probed with primary antibodies: anti-Integrin α5β1 (Chemicon, Mouse Human 0.25 μg/ml, monoclonal) and anti-Actin (Santa Cruz, Rabbit Human 1 μg/ml, polyclonal). After labelling with primary antibody, the membrane was washed with Tris Buffered Saline with Tween-20 (TBST) and treated with Horse radish peroxidase (HRP)-labeled goat-anti mouse or goat anti rabbit secondary antibody (GAM-HRP, GAR-HRP, Chemicon) for 1 h at RT. After applying the ECL substrate (Bio-Rad, Hercules, CA, USA), the ChemiDoc MP Imaging System (Bio-Rad) was used and protein bands were chemiluminescently detected.

Statistics. Statistical analsyses were performed by unpaired Student's *t*-test for comparisons between 2 groups. One way ANOVA and a post-hoc test (Bonferroni) were applied when comparing three or more groups. Differences were considered statistically significant at $p<0.001$ (*) and $p<0.0001$ (**). Data are shown as mean±standard error (SE).

Results

Human mesenchymal stem cells (hMSCs) differentiated into viable neurons in vitro. As for neuronal induction, a bone marrow derived hMSC cell line (UE7T-13 cells, no RBRC-RCB2161; RIKEN) was used in experimental settings as previously described (43). Firstly, hMSC cells were immunophenotyped and more than 99% of the cells were positive for the mesenchymal stem cell markers CD44, CD73, CD90 and CD105, while negative for hematopoietic lineage markers CD34 and CD45 (Figure 1A). Afterwards, hMSCs were iduced towards neuronal differantiation in the presence of certain cytokine combinations for 12 days and showed neurite outgrowths in culture (Figure 1B). Neuronal induced hMSCs were then examined for neuron spesific enolase (NSE) and neurofilament (NF) neuronal protein expression. Both neuronal proteins were expressed in differentiated hMSCs (Figure 1C). The data were correlated with our previous findings in hMd-Neurons (43). To investigate apoptosis, caspase-3 activity was measured in neuronal induced cells, as caspase-3 is involved in the downstream signaling of the apoptotic cascade. Assay results showed that induced hMSCs did not show any increase in caspase-3 activity. Conversely, camptotechin (CAM)- treated positive control cells displayed significant caspase-3 activation when compared to uninduced or induced hMSCs or apoptosis inhibited hMSCs ($p<0.0001$) (Figure 1D). These results indicate that the viability of neuronal induced hMSCs was maintained while the cells underwent neural differentiation and/or maturation.

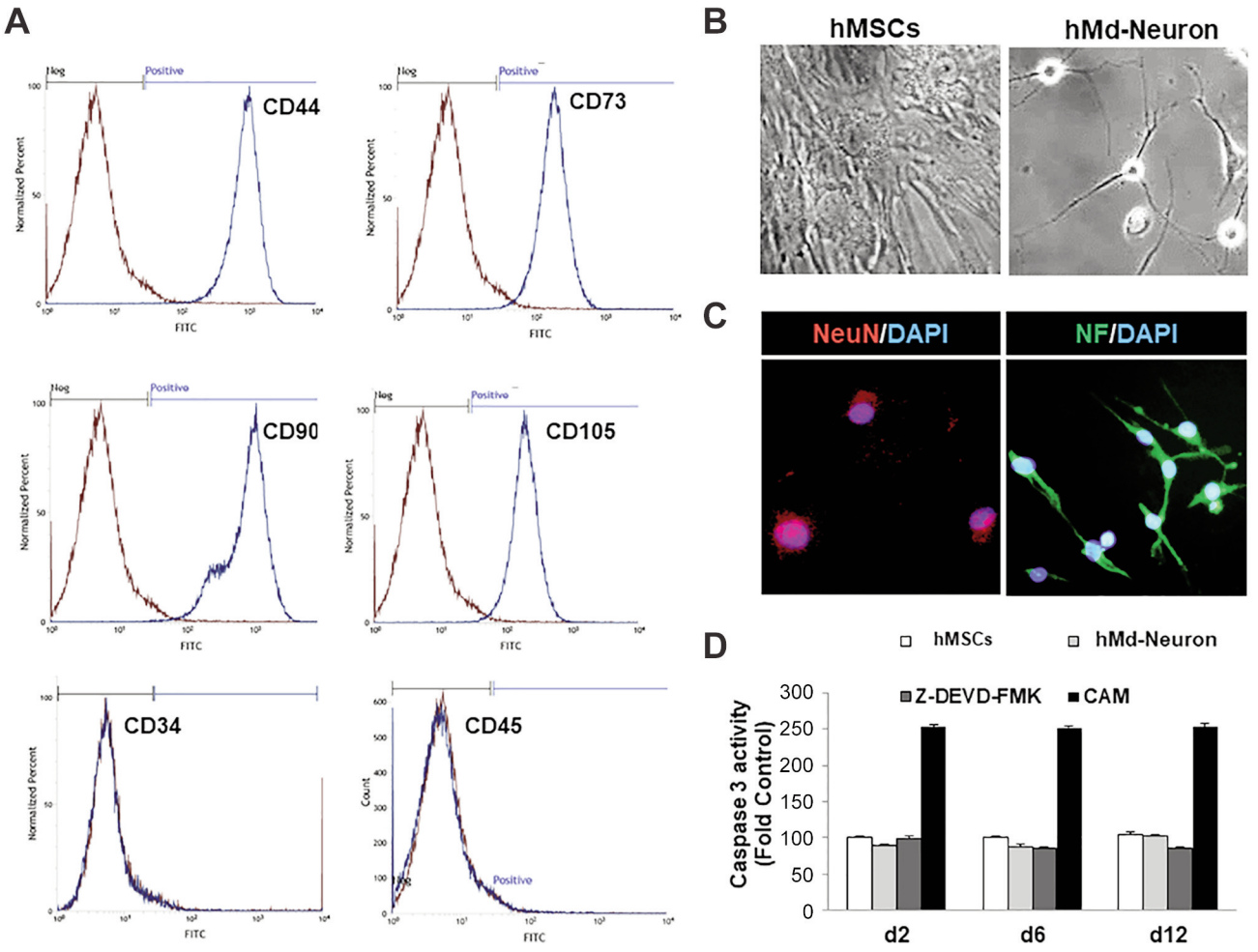

Figure 1. *Derivation of neurons from hMSCs (hMd-Neurons). A) hMSC immunophenotyping by flow cytometry shows that cells are positive for MSC markers CD44, CD73, CD90, and CD105 while lacking hematopoietic lineage markers CD34 and CD45. B) Bright field images of hMd-Neurons and uninduced hMSCs (40×). C) Immunoflourescence images of NeuN and Neurofilament (NF) in hMd-Neurons at day 12 (40×). D) Caspase 3 assay for induced, uninduced hMSCs as well as CAM treated (apoptosis inducer; positive control) and Z-DEVD-FMK treated (apoptosis inhibitor; negative control) hMSCs at days 2-12. CAM treated hMSCs show significant caspase 3 activity. Data represent mean±SE; p<0.0001.*

Neural induction led to cellular reorganization of hMSCs. Considering neuro-developmental processes, newborn neurons from stem cells are expected to loose their highly proliferative ability and reach a post-mitotic phase. Thus, to study conversion of hMSCs into neuronal cells, the proliferation ability of neuronal induced hMSCs within 48 h was analysed As observed, hMSCs gain neuronal cell morphology by day 1, stabilize in number by day 2, then maintain the neuronal cell percentages during the following 12 days in culture. Considering the timing of morphological changes observed in culture BrdU assay was applied 4, 24, and 48 h after neuronal induction. The assay results confirmed that the proliferation rate was significantly decreased in neuronal induced hMSCs after 48 h (p<0.001) (Figure 2A). In addition to that, the cell bodies and nuclei of the neural induced hMSCs were 50% reduced (p<0.0001 compared to control) (Figure 2B and C).

Integrin α5β1 localization differs during neuronal differentiation of hMSCs. Integrin receptor expression was analysed for its contribution to cell motility during the differentiation of hMSCs (44-46). The mRNA and protein levels of integrin α5β1 at day 12 of hMSC-neural induction were evaluated. Integrin α5 subunit transcripts (mRNAs) were significantly increased at day 12 of hMd-Neurons, as compared to the uninduced hMSCs. Conversely, integrin β1 levels were similar in both hMSCs and hMd-Neurons at day 12 of the neuronal induction (Figure 3A). Endogenous

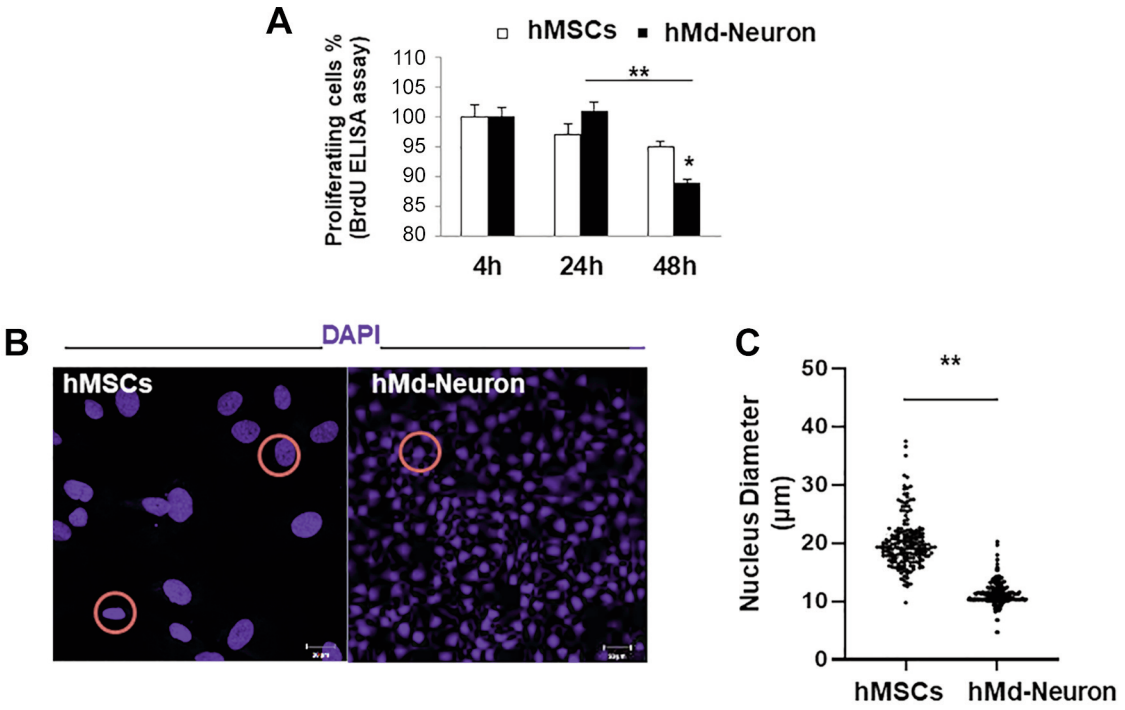

Figure 2. *Cellular rearrangements during hMSC differentiation into neurons. A) BrdU assay showing that hMSC proliferation decreases by neuronal differentiation within 48 h after neuronal induction. B) Florescent images of DAPI staining in uninduced hMSCs and hMd-Neurons (40×). Data represents mean±SE. C) Violin plot indicating changes in nucleus diameter in hMSCs when reaching day 12 of neuronal differentiation. Each dot represents a single cell. Plot width is proportional to cell frequency at a given diameter. p<0.001 and p<0.0001 using Welch's t-test.*

expression of integrin α5β1 was significantly increased in hMd-Neurons by day 6 of neuronal induction and variation in α5β1 integrin expression was detected during neuronal differentiation (Figure 3B). The change in expression led us to further examine the localization of integrin α5β1 during differentiation into hMd-Neurons. At the early stages of differentiation, by day 2, integrin α5β1 was localized at both the membrane and cytoplasm (Figure 3C). At day 6, integrin α5β1 was mostly localized at the membrane, seen as a bright circular staining (Figure 3D), and by day 12, it was internalized into the cytoplasm (Figure 3E). Quantification analysis also indicated that integrin α5β1 was localized at the cell surface of hMd-Neurons at day 6, compared to earlier (day 2) or later (day 12) phases of differentiation (Figure 3F). Additionally, at day 12, α5β1 was mostly internalized in hMd-Neurons as shown by the cytoplasmic specific staining and image analysis (Figure 3E and F).

Discussion

This study showed that neuronal induction leads to cellular reorganization of hMSCs. This dynamic change of hMSCs involves regulation of proliferation in response to differentiation inducers. One of the integrin family receptors, integrin α5β1, increased at day 6 compared to day 2 of cytokine-mediated neural differentiation and levels were maintained at day 12. To understand the role of this integrin, we stained hMd-Neurons to study the localization of integrin α5β1 and our results showed that it was localized mainly at the cell membrane at day 6, and relocalized in the cytosol at day 12. According to these findings, intermediate stages of differentiation (in this case, around day 6) is possibly a critical time point for differentiation/maturation. Cells require integrins on their membrane to transmit extracellular signals in order to enhance their interaction with surrounding cells and the extracellular matrix (ECM), and to maintain survival, growth and/or differentiation. Considering neural development in adult neurogenesis, more neurite extensions give rise to more connections and survival, which is most likely through a process related to integrins. The findings of this study also suggest that integrin kinetics lead to more neurite extensions around day 6 of hMd-neurons' differentiation. Additionally, previous reports have shown that integrin α5β1 binding to fibronectin (Fn) guides ECM assembly, and the lack of this interaction may result in lethality during vertebrate development. Therefore, integrin α5β1 and Fn engagement has been a promising area to focus on in order to optimize succesfull differentiation protocols; the crucial role of integrin α5β1 binding to Fn was also reported for mesodermal

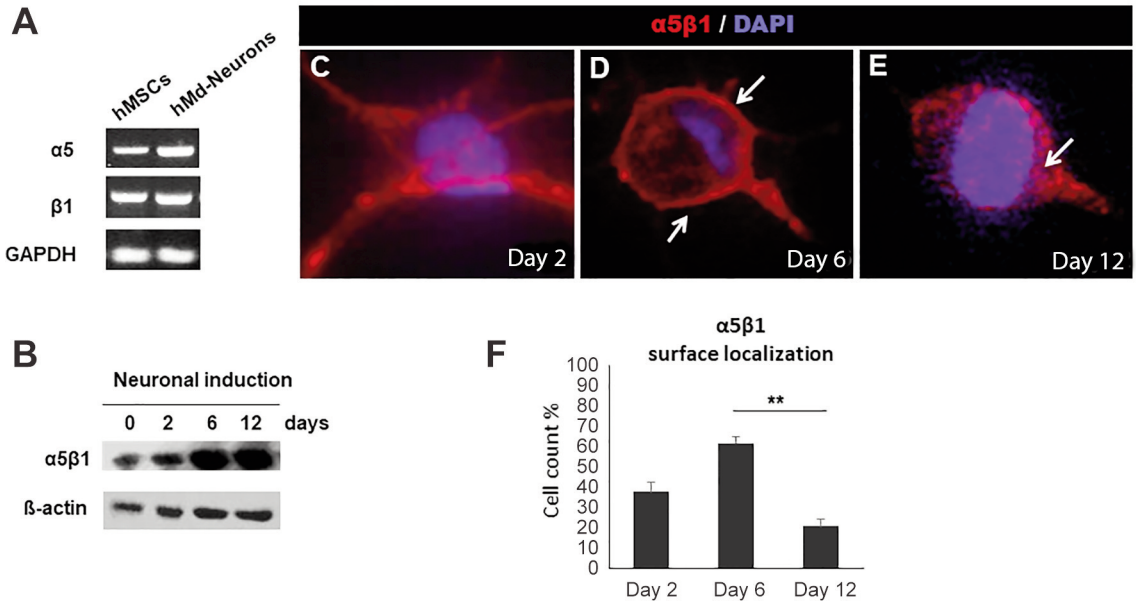

Figure 3. *Integrin α5β1 expression dynamics of hMd-Neurons. A) Transcripts of α5 and β1 in hMSCs and hMd-Neurons at day 12. B) Endogenous protein expression levels of integrin α5β1 and C-E) fluorescent images of integrin α5β1 localization during neuronal differentiation of hMSCs. F) Plots indicate percentage of integrin α5β1 cell surface localization among stained cells at days 2, 6, and 12 of differentiation. One hundred cells from three different regions were counted and data represent mean±SE, p<0.001.*

differentiation of hMSCs. Each induction protocol has its own molecular signature and determination of specific cell surface molecules and their ECM partners may alter the induction protocol. This may then guide maturation or spesification of differentiating cells. Our findings show that integrin α5β1 can guide transdifferentiation of hMSCs into neurons. Considering its expression profile and surface localization during neuronal differentiation, integrin α5β1 is most likely involved in rearrangement of hMSCs for neuronal maturation around day 6. Although our findings and observations with hMd-Neuron suggests day 6 as a specific time point for neuronal differentiation, this may vary depending on the hMSC donor. Taken together, Fn binding moieties of integrin α5β1 could be possible substrates guiding the fate of neuronal differentiation/maturation of hMSCs, since α5β1 is a fibronectin receptor and transmits signals from the extracellular environment to inside the cell. In the future, determination of the integrin dimers' involvement in hMd-Neuron differentiation may provide a well defined structure of neuronal maturation or specification. This can ultimately lead to building optimized tissue scaffolds for neuronal tissue engineering. In terms of the cytoskeletal mechanisms related to integrin dynamics, modulation of the actin cytoskeleton and the study of the signaling pathways involved, will increase understanding of the mechanism of neuronal differentiation from mesodermal lineage to neuroectodermal cells and the essential molecules involved in this process.

Taken together, this study revealed the importance of integrin α5β1 in the establishment of hMSC niche during *in vitro* differentiation of hMSCs into hMd-Neurons. The molecular mechanisms of this interaction can be further investigated considering integrin linked cellular processes required for hMd-Neuron maturation or specification. Accordingly, focusing on the involvement of different integrin heterodimers during hMd-Neuron differentiation may contribute more to the integrin dependent transdifferentiation of hMSCs.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

N.K.: Conception and design, financial support, provision of study material, assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript. U.K.: Assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript.

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