Original Article:

DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS THROUGH THE NATURAL MATRIX TO NEUROSPHERES FOR CHOLINERGIC-LIKE CELLS.

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ABSTRACT

This study aimed to differentiate human mesenchymal stem cells (hMSCs) from the human umbilical cord in cholinergic-like cells using a natural matrix. The isolation of hMSCs from Wharton's jelly (WJ) was carried out using "explant" and mononuclear cells by density gradient. hMSCs were plated in a natural functional biopolymer matrix for the production of neurospheres. Neural precursor cells were subjected to a standard cholinergic differentiation protocol. Dissociated neurospheres, neural precursor cells, and cholinergic-like cells were characterized by immunocytochemistry. The RT-PCR was performed. hMSCs were CD73+, CD90+, CD105+, CD34- and CD45- and demonstrated the trilineage differentiation. Neurospheres and their isolated cells were nestin-positive, and also expressed *NESTIN*, *MAP2*, *ßIII-TUBULIN*, *GFAP* genes. Neural precursor cells that were differentiated in cholinergic-like cells expressed ßIII-TUBULIN protein and choline acetyltransferase enzyme. hMSCs on the natural matrix were capable of differentiating hMSC into neurospheres, obtaining neural precursor cells without growth factors or gene transfection before cholinergic differentiation.

Keywords: Mesenchymal Stem Cells; Wharton's Jelly; Based Biopolymer; Cholinergic; Nestin; Differentiation Capacity.

INTRODUCTION

The World Health Organization (WHO) has included Alzheimer's Disease (AD) among the major health problems, making it essential to develop diagnostic strategies and treatments for this disease. According to the WHO, about 35.6 million people had the disease in 2010, and this number might reach 65.7 million in 2030 and 115.4 million in 2050. According to a report issued in 2012 by the WHO, and The International Association for Alzheimer's Disease, the number of people with dementia in the world can be compared to the spread of HIV/AIDS in the 1980s. The report presents this comparison to warn the world about the importance of alternative treatments for AD (Cai and Xiao, 2016; Mukai et al., 2016).

In this context, cell therapies have become one of the research targets that may contribute to the treatment of neurodegenerative diseases, such as AD. Among cell types, the mesenchymal stem cells (MSC) emerge as an option of cells that are easy to obtain and present pluripotency such as cholinergic-like cells to prospect functional repair of nervous tissue. It seems relevant to emphasize that MSC could be easily obtained from adipose tissue and from the umbilical cord, both discharged materials that require the approval of Ethics Committees.

On the other hand, to obtain neural precursor cells (including neural stem cells and neural progenitor cells), which rise to neurons, it is necessary to establish protocols for developed neurospheres (Lee et al., 2015). When the neurospheres are obtained from mesenchymal stem cells, the authors need to use various growth factors and/or gene transfection for the induction of neural precursor cells. According to Mukai et al., 2016, several neural differentiation protocols are reported, and the culture of neurospheres is widely described in the literature because it is a crucial way to expand the production of neural stem cells (NSCs). The vast majority of articles use the formation of neurospheres for further neural differentiation, or preclinical application. The differentiations performed are basically for oligodendrocytes, glial cells, and neural cells in general (Huang and Mucke, 2012; Lu et al., 2015; Tsai et al., 2015; Yang et al., 2015; Zhang et al., 2011).

The need for adhesive properties for cell anchorage of the mesenchymal stem cells requires interaction with the culture flask substrate in the cell culture as well as the matrix with the cells in the tissue, in vivo; both conditions there are cell-matrix relations.

The matrices could act as epigenetic factors for stemness of cell fate to select the cellular type of cells, not necessarily acting only as scaffold. In this study, a natural functional biopolymer matrix named NFBX was used. Its characteristics allow those aggregated MSCs to express grouped nestin-positive cells, like spheres. After that, the spheres were demonstrated to have the majority of nestin-positive cells. Nestin is a protein present in neural precursor cells. There is still some limitation to expose the origin of the biopolymer for patent protection reasons. Some description of this matrix is presented in the results.

This research project aims to evaluate the possibility of differentiation of MSCs from the human umbilical cord in nestin-positive neural precursor cells (NPCN+) through the NFBX into cholinergic “like” cells. Following the establishment of the differentiation protocol, NPCN+ and cholinergic-like cells derived from the mesenchymal stem cells derived from Wharton Jelly (MSC-WJ) and umbilical cord blood (MSC-UCB), could be evaluated in preclinical models of neurodegenerative disease therapy, for example in AD (Carvalho et al., 2015; Chen et al., 2015; Monteiro et al., 2010).

MATERIALS AND METHODS

Umbilical Cord Stem Cells - Ethical Donors

The Research Ethics Committee of Pequeno Príncipe Faculty approved this study, which was numbered 2.432.717. Then, the human umbilical cords (HUCs) were collected after the donor/responsible person had signed the informed consent form. Donor/responsible people do not have the identities revealed, and all methods were performed with the agreement of the Research Ethics Committee of Pequeno Príncipe Faculty. The collections were completed at term, shortly after the placenta was discharged. Four HUC samples were collected and used in this study in triplicate for stem cell isolation from Wharton Jelly, and two of these cords were also used for both Wharton jelly and human umbilical cord blood (HUCB) mesenchymal stem cell isolation.

Isolation of Stem Cells from Wharton Jelly

Explant Method: HUCs were washed with Phosphate Buffer Saline (PBS) with 3% antibiotic (300 IU/mL penicillin, 0.3 mg/mL streptomycin), the longitudinal intersection was performed, and the vein and arteries were removed. The tissue was fragmented into 2mm x 2mm pieces, which were plated onto culture plates containing DMEM/Ham-F12 (Sigma Aldrich, USA) supplemented with 10% Fetal bovine serum (FBS) and 1% antibiotic (100 IU/mL penicillin, 0.1 mg/mL streptomycin) and incubated at 37°C and 5% CO2. The new replacement of the medium was performed after five days of culture. After that, the medium was replaced every 72 hours up to 85% of confluence (Cho et al., 2012).

Isolation of Stem Cells from Human Umbilical Cord Blood

Density Gradient Method: Human umbilical cord blood (HUCB) was collected through the open syringe system containing heparin, and processed within 24 hours. Mononuclear cells were isolated using the Ficoll-Paque PLUS® (GE Healthcare Life Sciences, USA) density gradient method. In this method, three parts of blood were added to one part of Ficoll-Paque PLUS® in the tube; then, the tube was centrifuged at 22°C, 1200 rpm for 30 minutes, after that, the phase containing the mononuclear cells was removed. Then these cells were washed in PBS containing 2% FBS at 1/1 ratio and centrifuged at 1200 rpm for 10 minutes. The pellet cells were resuspended in 4 mL DMEM/Ham-F12 culture medium with 20% FBS. Subsequently, $1x10^7$cells/mL were plated in 25 cm^2 culture flasks and incubated at 37ºC and 5% of CO2 (Sibov et al., 2012; Tondreau et al., 2005).

Mesenchymal Stem Cells Characterization

The characterization of MSCs was performed according to the International Society of Cell Therapy. For the immunophenotypic characterization of MSCs, the markers used were CD105, CD73, CD90, CD29, CD34, CD45, and 7-AAD, the last one was used for cell viability. Cells were subjected to trypsinization and suspended in PBS at the concentration of $1x10^6$ cells/ml. From this suspension, 200µL were placed in correctly identified tubes, and corresponding antibodies were added and incubated for 20 min. After that, 400 µL of PBS was added to the tubes and centrifuged for 5 min/1200 rpm. The supernatant was discarded, and the pellet suspended in 400µL PBS, followed by homogenization and 10µL 7-AAD added. Finally, the samples were processed in a flow cytometer (FACS Calibur; Becton Dickinson, USA) and analyzed using Infinicyt Flow Cytometry software, Version 1.6.0 (Histograms are shown in Figure S1, Supplementary Material) (Dominici et al., 2006). The gating strategy was carried out excluding non-viable cells (positive for the 7-AAD marker) and comparing each CD marker with isotype control. Thus, all marking that overlapped the isotypic control was considered negative for the analyzed marker, any marking that did not overlap the isotypic control, or to its left, was considered positive for the analyzed marker.

To verify the multipotent characteristic of the isolated MSCs, adipogenic, osteogenic, and chondrogenic differentiation were performed. Commercial kits were used in these differentiations: StemPro® Adipogenesis Differentiation Kit, StemPro® Osteogenesis Differentiation Kit, and StemPro® Chondrogenesis Differentiation Kit, respectively. After differentiation, the samples were subjected to their specific staining: Osteogenic - Alizarin Red (Sigma Aldrich, USA), Adipogenic - Oil Red O (Sigma Aldrich, USA), and Chondrogenic - Alcian Blue (Sigma Aldrich, USA).

Production of neural precursor cells

The neurospheres and neural precursor cells were both nestin-positive as demonstrated by immunocytochemistry methods and produced using the natural functional biopolymer matrix named NFBX* as a substrate. Neurospheres production on NFBX required a density

plating of $2x10^4$ cells/mL to develop neurospheres. Neurospheres and neural precursor cells were represented in the graphical abstract (Supplementary Material).

*Ongoing Patent, designated by a capital letter symbol: NFBX.

In this paper, the NFBX is briefly explained for the understanding of the interaction of cells with the matrix based on their morphology that permits the attachment of the selection of cells committed to being neural precursors. These facts require further studies on the mechanisms of mechanic-physicochemical processes to determine the geometry of the attached neurospheres.

Cholinergic like cells differentiation protocol

After the neurospheres production, the culture medium (DMEM/Ham-F12, 100 UI/mL penicillin; 0,1 mg/mL streptomycin) was supplemented with nerve growth factor (NGF - Peprotech®, USA), epidermal growth factor (EGF - Peprotech®, USA), basic fibroblast growth factor (bFGF - Peprotech®, USA) and B27 (Gibco® BRL, Life Technologies, Inc., Grand Island, NY) for 11 days (Table S1 - Supplementary Material). The final concentration was maintained for four days, after which phenotypic characterization tests were performed (Tondreau et al., 2005).

Immunocytochesmistry

The cells were fixed with 4% paraformaldehyde (Sigma®, USA) for 20 minutes, washed with PBS and made permeable with a PBS solution containing 3% Triton X-100 (Sigma®, USA) and 1% BSA for 5 minutes at room temperature, being washed three times with PBS after this period. The cells were then incubated overnight at 4°C with the primary antibodies. The primary antibody used to characterize NPCN + was the anti-nestin monoclonal antibody (Sigma Aldrich, USA). After differentiation in cholinergic "like" cells, the primary antibody used was anti-ßIII-TUBULIN (Sigma Aldrich, USA) and anti-ChAT (Merck Millipore, USA).

After 24 hours incubated with the primary antibodies, the cells were washed with PBS and incubated at room temperature with the secondary antibody FITC-IgG (Sigma Aldrich, USA). The undifferentiated control was obtained using MSC cultured only with standard medium (DMEM/Ham-F12, 100 UI/mL penicillin; 0,1 mg/mL streptomycin and 10% FBS). The reading was performed in an inverted fluorescence microscope (Axio Vert A1, Car Zeiss, Oberkochen, Germany) (Devetten and Armitage, 2007; Naghdi et al., 2009).

Scanning Electron Microscopy

For the Scanning Electron Microscopy (SEM) analysis, the MSC-WG was differentiated in neurospheres and cholinergic-like cells. Then, the cells were washed with 0.1 M sodium cacodylate solution (PH 8,5), followed by fixation in Karnovski solution for 1 hour and a half, dehydrated using alcohol solution 30%, 50%, 70%, 90%, 100%. The samples were subjected to the critical point (CPD-Balzers union/ Baltec, Germany) and metalized with gold (CPD- Balzers union/ Baltec, Germany). Then, cells were observed under scanning electron microscope (Vega-3LMU, Tescan, Brno, Kahoutovice-Czech Republic).

Qualitative reverse transcription-polymerase chain reaction (RT-PCR)

The RNA was extracted using the RNeasy Mini Kit (Quiagen®), as indicated by the manufacturer. The complementary strand of DNA (cDNA) was generated precisely following the manufacturer's instructions, using the High Capacity cDNA (Thermo Fisher) kit. The RT-PCR was performed using a 20µL system, containing the cDNA pattern and Master Mix Promega®. The primers used to amplify *MAP2* (Forward:

CATACAGGGAGGATGAAGAGGG and Reverse: GGTGGAGAAGGAGGCAGATTAG), *GFAP* (Forward: ATCAGCCGATGCGAAGGG and Reverse: TAGACGCTGATCCGCTCCAG), *ßIII-TUBULIN* (Forward: ATTGAGTCGCTGGAGGAGGAGA and Reverse: GGTAGTCGTTGGCTTCGTGCTT) and *NESTIN* (Forward: AACAGCGACGGAGGTCTCTA and Reverse: TTCTCTTGTCCCGCAGACTT) genes (SIGMA®). The samples were prepared in biological replicates, using *ß-ACTIN* control (Forward: CTGGGACGACATGGAGAAAA and Reverse: AAGGAAGGCTGGAAGAGTGC). Agarose gel was used, and the results were visualized on Ultra Violet transilluminator.

Atomic Force Microscopy Analysis

Morphological analysis of the matrix was carried out using a TT-AFM instrument (AFM Workshop, USA) in vibrating (tapping) mode with 512 × 512 lines. Representative images were examined using ACT-20 cantilevers (AppNano-USA) with a resonant frequency of approximately 353 kHz. Images were analyzed using Gwyddion software 2.4, and the roughness value (Ra) for multiple areas of 1.5 x 1.5 μm (n=15) examined is shown, Ra was expressed as mean ± SD.

RESULTS

Flow Cytometry Analysis

In the flow cytometry test for MSC-WJ, an average of 91.27% of the mesenchymal stem cell characteristics was obtained, demonstrated by the expression for CD73, CD90, and CD105 markers. Out of these cells, 17.65% were 7AAD positive cells, i.e., non-viable cells. For the MSC-UCB derived sample, an average of 91.30% of the mesenchymal stem cell characteristics and 10.43% of non-viable cells were obtained and labeled 7AAD. When hematopoietic markers (CD34 and CD45) were considered, no expressive marking was observed for either of them, WJ-MSC or MSC-UCB. The gating strategy was achieved by excluding the cells that were marked 7-AAD (non-viable cells) and comparing the marking of each antibody with the isotype control. For the histograms and average of each protein expression, see Figure S1 in Supplementary Material.

Trilineage differentiation test

Samples of MSC-WJ and MSC-UCB were successfully differentiated in adipogenic, chondrogenic, and osteogenic cells, known as trilineage differentiation test. A control test was performed using MSCs cultured only with standard medium (DMEM/Ham-F12, 100 UI/mL penicillin; 0,1 mg/mL streptomycin and 10% FBS) without differentiation stimulation, and these controls did not show specific staining (Figure 1).

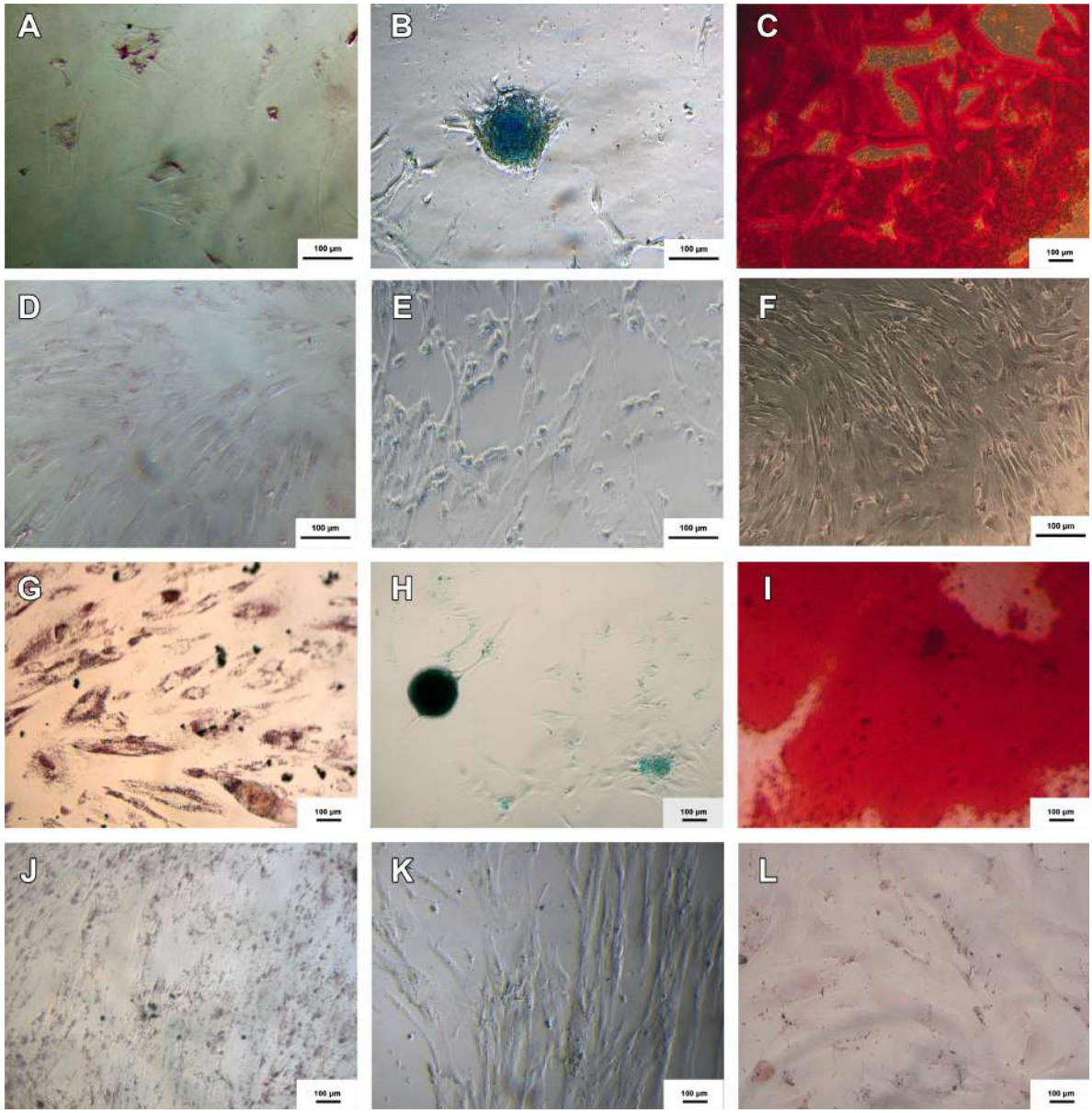

Figure 1. Adipogenic, chondrogenic and osteogenic differentiations of MSC-WJ and MSC-UCB. (A), (B) and (C) represent the adipogenic, chondrogenic and osteogenic differentiations of MSC-WJ respectively; And (D), (E), and (F) represent the negative controls, respectively; (G), (H) and (I) represent the adipogenic, chondrogenic and osteogenic differentiations of MDC-UCB respectively; And (J), (K) and (L) the negative controls, respectively. Adipogenic, chondrogenic and osteogenic differentiations were stained with Oil Red O, Alcian Blue and Alizarin Red respectively (Inversion optical microscope, x100).

Production of neural precursor cells

After two weeks of WJ-MSC seeded on NFBX, the formation of the neurospheres could be observed. On the other hand, the formation of neurospheres from the MSC-UCB cells could be observed only after a longer period, about three to four weeks, as shown in Figure 2.

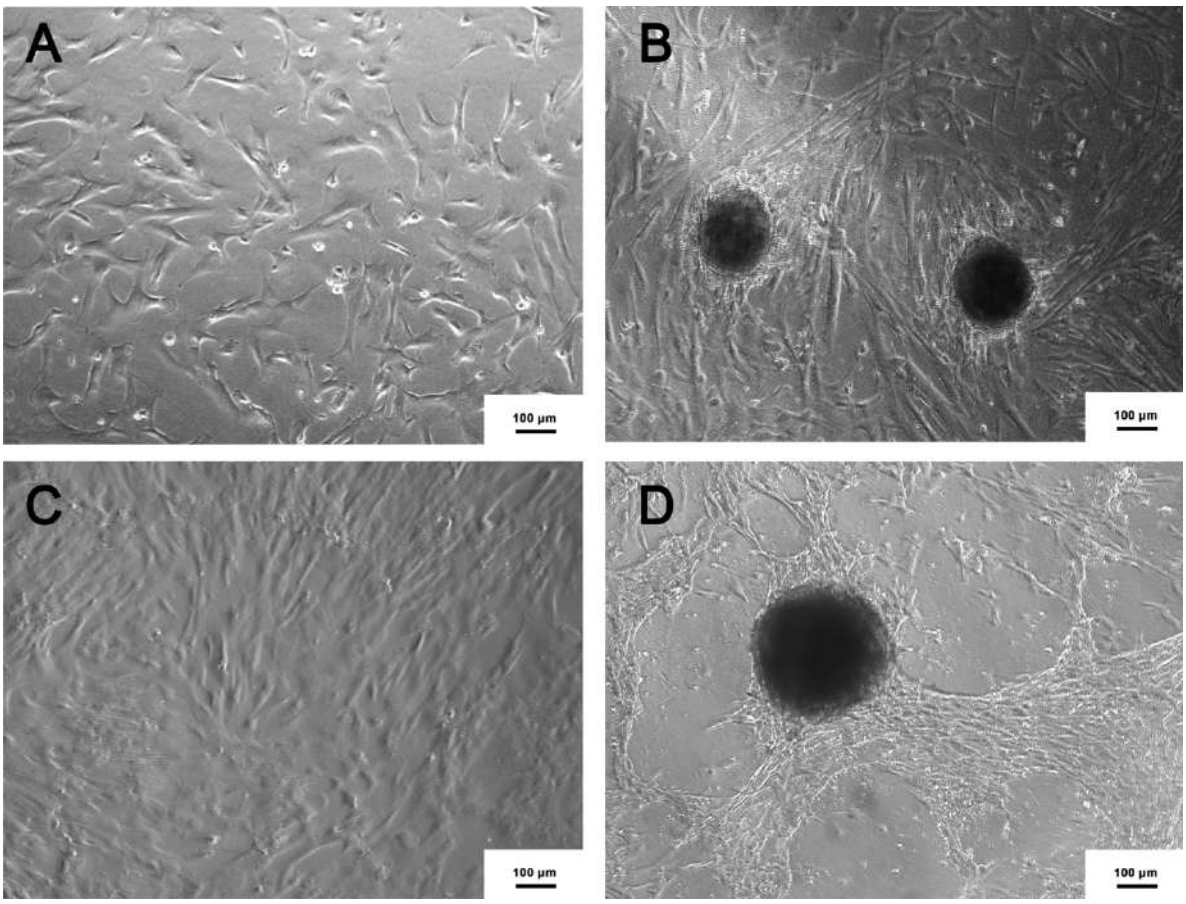

Figure 2. Neurospheres (NPCN+). (A) MSC-UCB (B) Neurospheres derived from MSC-UCB. (C) MSC-WJ (D) Neurospheres derived from MSC-WJ (Inversion optical microscope, x100).

Neurospheres and Neural Precursor Cells

The neurospheres produced were subjected to the immunocytochemistry protocol to verify the presence of the nestin protein. Nestin is considered a marker of neural precursor cells (Suzuki et al., 2010). As shown in Figure 3, the neurospheres produced from MSC-WJ and MSC-UCB presented nestin expression and, therefore, were characterized as nestin-positive neuronal precursor cells.

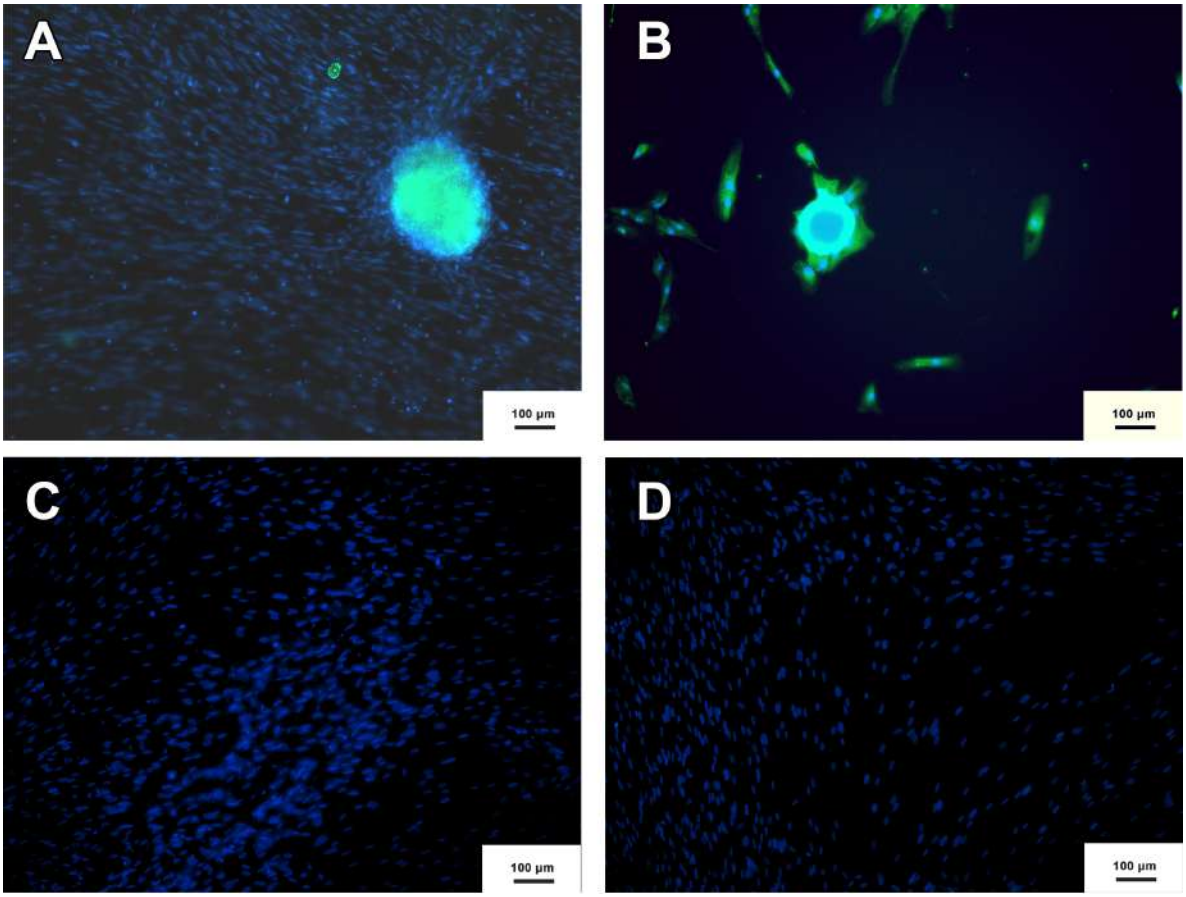

Figure 3. Neurosphere immunocytochemistry. (A) Neurosphere derived from MSC-WJ (B) Neurosphere derived from MSC-UCB. (C) and (D) represent the negative controls of both MSC-WJ and MSC-UCB respectively. It means, neither MSC-WJ nor MSC-UCB presented Nestin protein before being seeded in the NFBX. NESTIN protein is shown in green color by FITC as a secondary antibody, and Hoechst was used for labeling the nuclei of the cells represented by the blue color (Inverted fluorescence microscope x100 (Inverted fluorescence microscope Axio Vert A1, Carl Zeiss, Oberkochen, Germany).

Scanning Electron Microscopy

The neuronal precursor cell from MSC-WJ and MSC-WJ neurospheres was observed using Scanning Electron Microscopy (SEM) (Vega-3LMU, Tescan, Brno, Kahoutovice-Czech Republic), presented in Figure 4. As shown in Figure 4 (A), MSC-WJ starts growing, reaches the confluency, and becomes the organizer in neurospheres (B). Anchor cells could be observed in Figure 4 (B) casting projections that are grouping structures. Finally, Figure 4 (C) shown the cells that are grouped and participate in the neurospheres formation, and (D) the unique neurosphere formed could be observed.

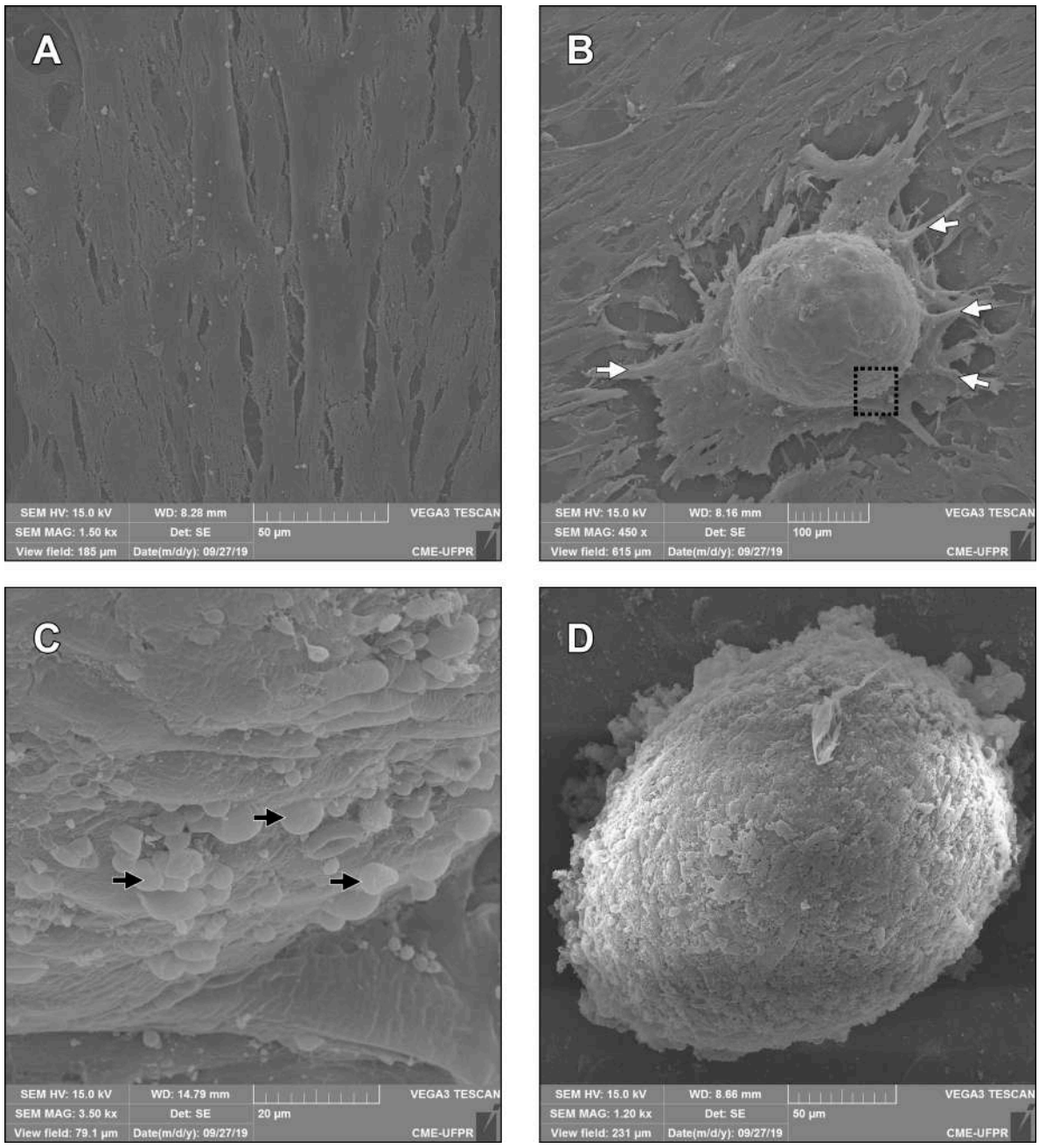

Figure 4. Neurospheres Scanning Electron Microscopy. (A) MSC-WJ reached confluency; (B) Neurosphere began to be formed by the MSC-WJ, these cells initiated pseudopod projection for grouping and anchoring and began spherical organization (white arrows); (C) Cells that migrated and took part in the neurospheres formation (black arrows); (D) neurospheres formed by the MSC-WJ organization. SEM: Vega-3LMU, Tescan, Brno, Kahoutovice-Czech Republic.

Cholinergic Differentiation

At the beginning of the cholinergic differentiation protocol, morphological differences were observed in some cells, which showed bipolar characteristics as demonstrated in Figure 5 A. During the differentiation, a decrease was noticed in the number of MSC and an increase in the number of morphologically altered cells as identified in the cholinergic-like cells, as shown in Figures 5 B-D. Regarding this morphology, similar results were obtained by other study (Adib et al., 2014). Controls that

were performed without using induction factors did not present any morphological differences, as demonstrated in Figures 5 E and F.

Neurospheres derived from MSC-WJ, and MSC-UCB presented different morphology when subjected to the cholinergic differentiation protocol. The differentiated cells were characterized by immunocytochemistry to verify the presence of two proteins, one that demonstrated the neuronal characteristic of the cell, ßIII-TUBULIN, and another that demonstrated the cholinergic property of the cell, the enzyme choline acetyltransferase. The differentiated cells from both sources (MSC-WJ and MSC-UCB) showed the expression of both proteins, as presented in Figures 6 and 7. Negative controls were performed with neurospheres without differentiation induction, which showed the labeling for ßIII-TUBULIN, but did not show a label for the enzyme choline acetyltransferase.

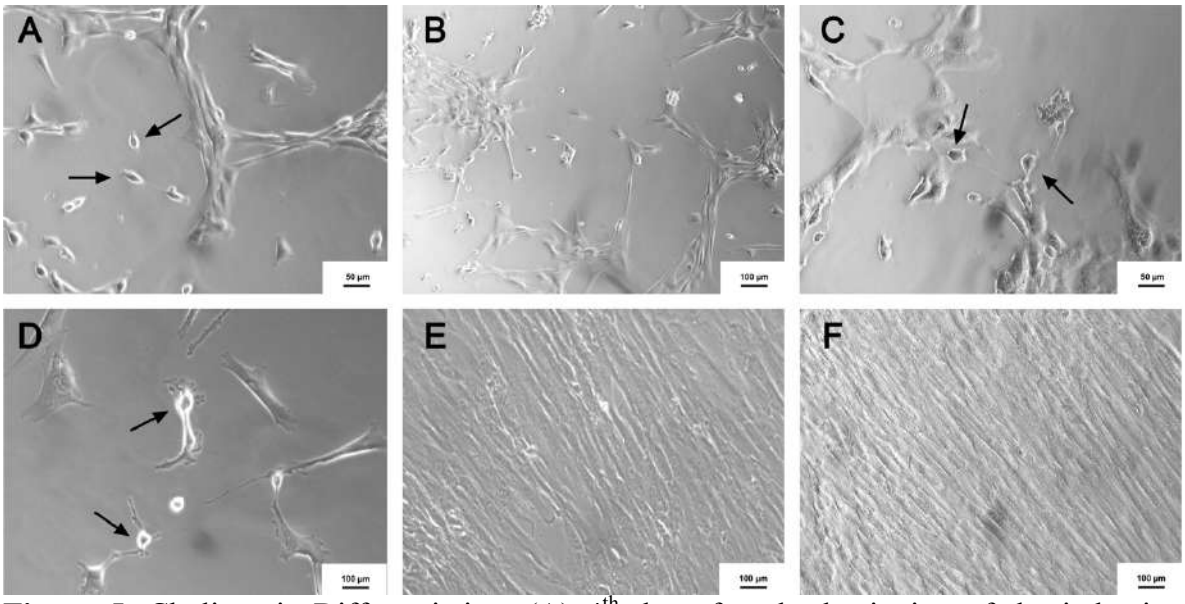

Figure 5. Cholinergic Differentiation. (A) 4th day after the beginning of the induction (x100); (B) 8th day after induction (x100); (C) 10th day after induction (x100); (D) 14th day after induction (x200); (E) Control (MSC) without the use of induction (x100); (F) Precursor without induction (x100). Black arrows represent the formation of the cholinergic-like cells. (Image obtained using an inverted microscope x100, Axio Vert A1, Carl Zeiss, Oberkochen, Germany).

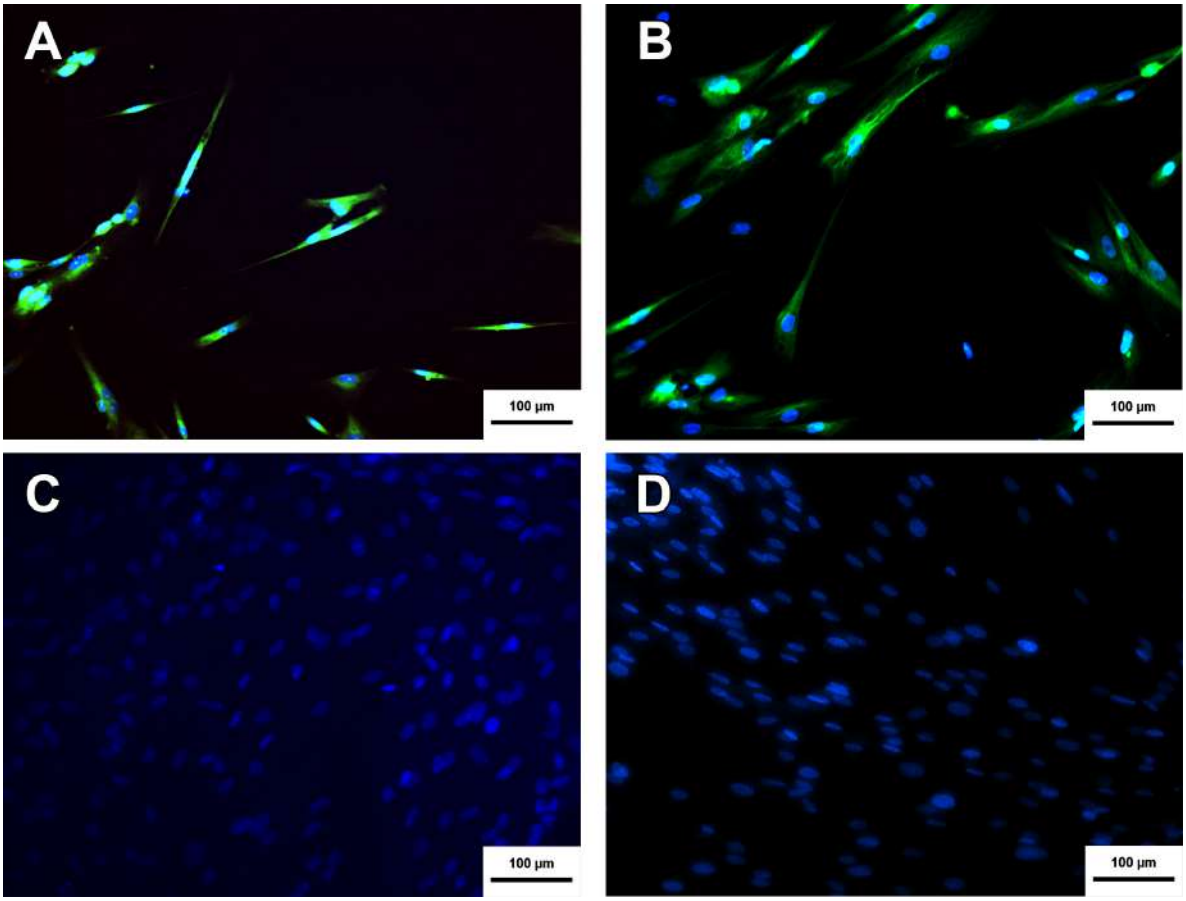

Figure 6. Cholinergic differentiation immunocytochemistry of NPCN+ cells derived from MSC-WJ. (A) MSC-WJ labeling using the anti-ChAT antibody (FITC). (B) MSC-WJ labeling using the anti-ßIII-TUBULIN antibody (FITC). (C) and (D) represent the negative controls (MSC-WJ not differentiated) for both markers anti-ChAT and anti-ßIII-TUBULIN, respectively Hoechst was used for labeling the nuclei (Image obtained using an inverted fluorescence microscope x100, Axio Vert A1, Carl Zeiss, Oberkochen, Germany).

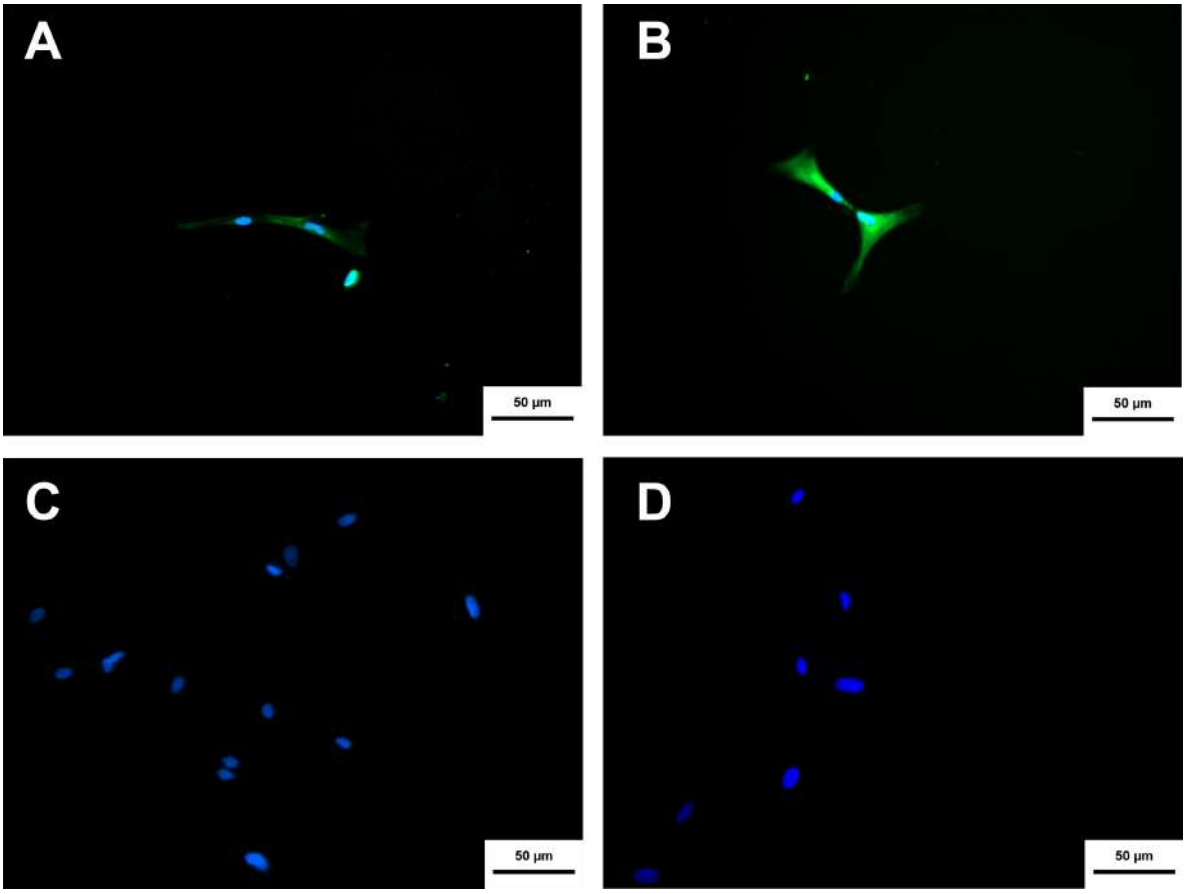

Figure 7. Cholinergic differentiation immunocytochemistry of NPCN + cells derived from MSC-UCB. (A) MSC-UCB labeling using the anti-ChAT antibody (FITC). (B) MSC-UCB labeling using the anti-ßIII-TUBULIN antibody (FITC). (C) and (D) represent the negative controls (MSC-UCB not differentiated) for both markers anti-ChAT and anti-ßIII-TUBULIN, respectively Hoechst was used for labeling the nuclei. (Inverted fluorescence microscope with increase of x100, Axio Vert A1, Carl Zeiss, Oberkochen, Germany).

Qualitative reverse transcription–polymerase chain reaction (RT-PCR)

In this study, the RT-PCR technique was used to evaluate the presence of molecular markers from neural precursor cells. We evaluated the expression of *GFAP*, *MAP2*, *NESTIN*, and *ßIII-TUBULIN* genes that are not dissociated neurospheres (NS), cultured neuronal precursor cells (NP), and MSC-WJ (MSC). The *ß-ACTIN* gene was evaluated as a control gene expression. Cells from the neurospheres (NS) showed expression for all the neural genes tested, and the cultured neural precursor cells (NP) also expressed all genes, however, with weaker expressions for *ßIII-TUBULIN*, and *GFAP*, as demonstrated in Figure 8. The MSC-WJ (MSC), which was not cultured on the NFBX matrix, presented expression only for the *NESTIN* gene. Thus, these results confirm the efficacy of the induction protocol to neural precursor cells using the NFBX.

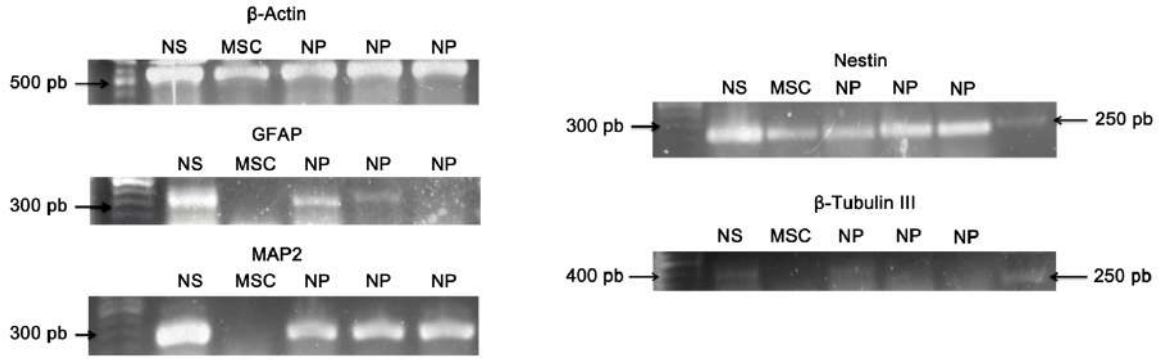

Figure 8. RT-PCR for markers of precursor neural cells. The *ß-ACTIN* gene was used as a technique control, and expression was observed for all samples. The *GFAP* gene was expressed in NS and NP's samples. The *MAP2* gene was expressed in NS and NP's gene. The *NESTIN* gene was expressed for all samples, and the *ßIII-TUBULIN* was observed in NS and NP's samples. Abbreviations: Not dissociated neurospheres: NS; MSC-WJ: MSC; Neural Precursor cells: NP.

Atomic Force Microscopy Analysis

Atomic Force demonstrated the roughness value, Ra= 14.28 ± 3.28 nm as shown in Figure 9.

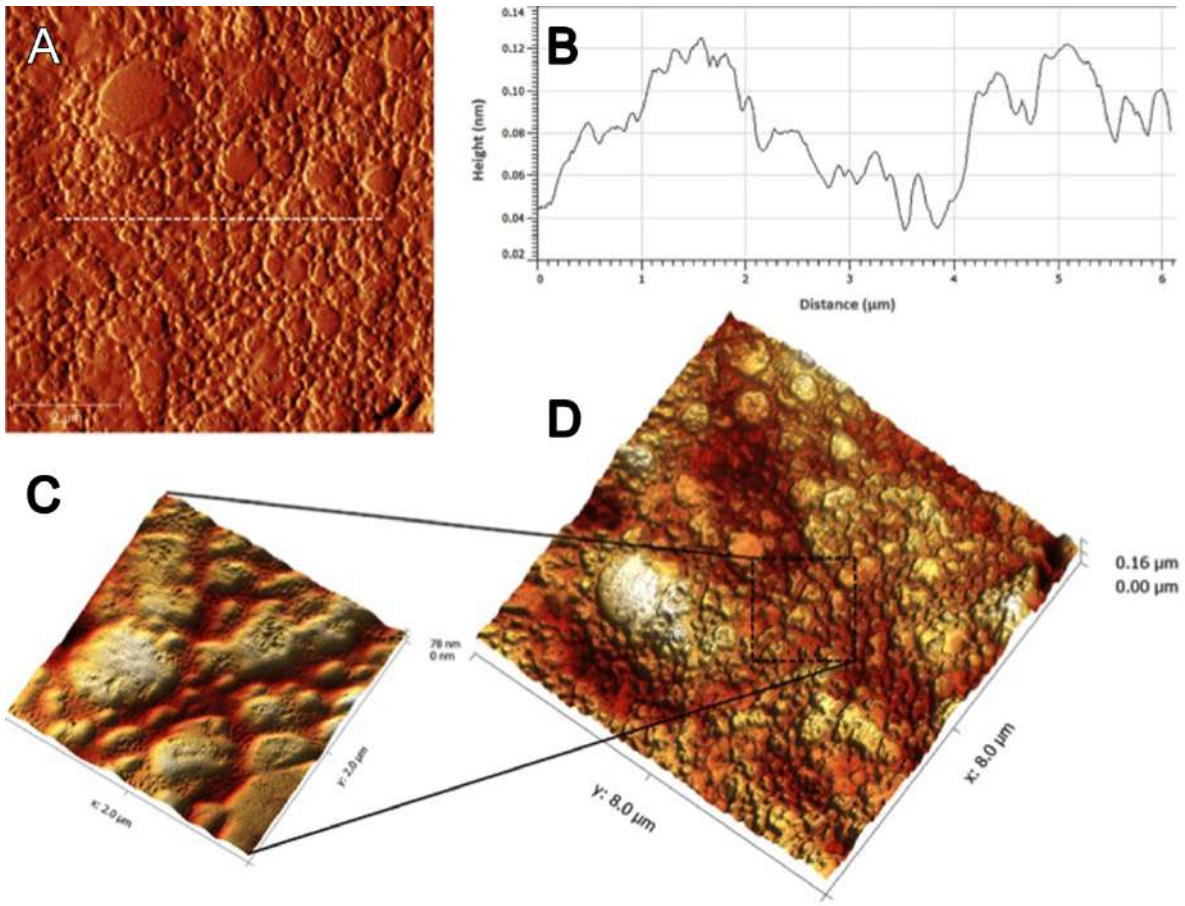

Figure 9. Atomic Force Microscopy images of the NFBX surface. (A) AFM height image; B) Cross-section height profile (taken along the white line in the image A) and C – D) 3D AFM topographic images. Ra= 14.28 ± 3.28 nm.

DISCUSSION

The umbilical cord is an essential cellular source, particularly of stem cells, mainly because it is a disposal material in hospitals and constitutes an important potential source for therapeutic applications. Both hematopoietic cells, used in transplants for the treatment of onco-hematologic diseases, for example, and MSCs can be isolated from this material, making it a significant highlight in research in recent years (Devetten and Armitage, 2007).

In this study, the isolation of umbilical cord MSC from both Wharton jelly (WJ) and umbilical cord blood (UCB) was successfully performed considering the adaptation of the techniques (Cho et al., 2012; Karimi et al., 2012; Sibov et al., 2012; Tondreau et al., 2005; Zhang et al., 2011). MSC-WJ isolated by employing the explant technique was satisfactory (Cho et al., 2012). The isolation of MSC-UCB was performed by density gradient where fast cell adhesion could be observed, but the confluence took longer, 30 to 60 days on average, unlike another study that could observe confluent cells in 20 days of culture (Tondreau et al., 2005). A possible explanation for the confluence of the cells isolated by those authors to have the fastest confluence is the plating performed in a large concentration of cells, whereas other authors reported using $1x10^7/10^8$ cells per cm^2 in $25cm^2$ culture flasks, this study uses $4x10^6$ cells per cm2 in $25cm^2$ culture flasks (Sibov et al., 2012).

MSCs' characterization was performed through flow cytometry and observation of cell differentiation in three distinct cell types, according to the International Society of Cell Therapy. According to the results described, MSC-UCB and MSC-WJ presented expression for the pre-established markers (CD73+, CD90+, CD105+, CD34- and CD45-). Also, the cells had the characteristic of adherence to the plastic substrate and fibroblast-like morphology. Cellular plasticity was demonstrated through chondrogenic, osteogenic, adipogenic, and neurogenic differentiation (Harichandan and Bühring, 2011; Jin et al., 2013; Lu et al., 2015; Maslova et al., 2015; Sarugaser et al., 2005; Sibov et al., 2012).

Authors who developed a cellular automata model and concluded that cell death, while resulting in a decrease in growth rate and final size of neurospheres, increases the differentiation potential of neural precursor cells, similar that was currently observed on a kinetic curve of growing cell culture (Sipahi and Zupanc, 2018). Moreover, it is known that cell architecture is related to changes in the cytoskeleton, and its biophysical state can regulate cell metabolism (Von Erlach et al., 2018). The used of NFBX as a substrate for the MSC cultivation could provide a cell interaction and related metabolism changes and consequently, MSC differentiation. Future studies are being carried out to give a proteomic and genomic explanation about how MSC can form neurospheres and differentiate into neural precursors cells when seeded in NFBX. The relationship between matrix and cell fate was discussed by authors and pointed out that geometric conditions interacting with and cellular cytoskeletal protein associated with microdomains of plasma membrane lipid rafts seem to regulate unknown signals (Von Erlach et al., 2018). In the used matrix, it was observed that, due to the atomic force (Figure 9), the roughness value could be responsible for the low adhesion and selection of the nestin-positive mesenchymal stem cells for the differentiation in their cellular fate by aggregating the neural precursor cells in neurospheres. The culture of neurospheres is widely described in the literature because it is a crucial way to expand the production of NSCs, and several neural differentiation protocols are reported (Mukai et al., 2016).

A study aimed to determine whether a stem cell subpopulation present in rat adult adipose tissue can be directly expanded in NSCs. One of their changes was to reduce the expense and time required to derive and expand NSCs. The sources used were rat adipose tissue and rat embryonic neural stem cells. The protocol to form neurospheres was composed of neurobasal medium supplemented with Glutamax; B-27; N2; bFGF and EGF. This protocol was able to form neurospheres with characteristics of neural stem cells, and

these cells expressed NESTIN, SOX2, TUJI, and GFAP proteins. The ideal plated density to form neurospheres using rat adipose tissue was between $1{,}0x10^7$ and $8{,}0x10^7$ cells/mL. This amount is much higher than the one used in this study, which was only $2x10^4$ cells/mL. Although the sources of formation were different, in the present study, it does not use growth factors, and the number of cells necessary to produce neurospheres was lower, which made the study more economical (Petersen et al., 2018). In general, the authors demonstrated, as well as this study, the potential of neurosphere formation from non-neural and non-embryonic cellular sources (Petersen et al., 2018).

When considering neurospheres obtained from umbilical cord-derived MSCs, more specifically from Wharton jelly, the majority of authors are confronted with the need to use growth factors and/or gene transfection for the induction of neural precursor cells (Zhang et al., 2011). Some protocols used neurobasal medium and growth factors such as EGF, N2, bFGF, and B27 for the induction of neurosphere production (Zhang et al., 2011, 2010). A study, for example, used DEMEM-F12 medium with EGF and bFGF supplementation only (Mukai et al., 2016). In contrast, the present study used conventional media (DMEM/Ham-F12, 100 IU/mL penicillin, 0.1 mg/mL streptomycin and 10% FBS) and no supplementation in the culture medium for the production of neurospheres from undifferentiated MSCs, regardless of MSC-WJ or MSC-UCB, only the NFBX plating was conducted. The NFBX method made this study more economical, practical, and without the drawbacks of inductions. Also, this method of cultivation provides a cleaner environment for interferers with considering future therapeutic applications, following the Good Manufacturing Practice (GMP). The neurospheres obtained from plated MSC-WJ and MSC-UCB in NFBX were characterized by immunocytochemistry for the nestin protein, and the presence of this protein was demonstrated by immunofluorescence (Lu et al., 2015; Mukai et al., 2016; Zhang et al., 2011). Nestin is an intermediate filament protein present mainly in neural precursor cells. However, some authors suggest that it is also present in some adult neurons, mainly in regeneration areas, thus indicating a marker of NSCs and NPCs, which is with differentiation and proliferation capacity (Park et al., 2010; Suzuki et al., 2010). Besides, SEM results shown that NFBX provides a cell organization, originating from the neurospheres. The monolayer-forming by MSC-WJ organize themselves through anchor cells, and cells migrate to the formation of neurospheres.

The same protocol for the production of neurospheres derived from MSC-WJ was used to produce neurospheres derived from the MSC-UCB; however, the time required to form neurospheres from the last source was longer. The reason may be that umbilical cord blood cells are mostly used for hematopoietic purposes, and in this work, difficulties were still found in establishing the mesenchymal cell culture of this source. Some authors explain that this difficulty is due to the few precursors of MSCs circulating in the umbilical cord blood, most of which reside in the adult organism (Kern et al., 2006).

Differentiated neural precursor cells in cholinergic-like cells could be observed in all sources of MSCs. The immunophenotypic labeling for both the choline acetyltransferase protein produced by cholinergic cells and the ßIII-TUBULIN protein, which is a protein involved in the formation of microtubules in neurogenesis also was observed in the two sources of neurospheres. Because of this result, the growth factors used in this protocol were seen to be useful for the formation of cells with neuronal and cholinergic characteristics, corroborating results obtained by other authors (Adib et al., 2014). Those authors stated that the medium used for cholinergic differentiation, which is characterized by the gradual decrease of EGF and bFGF and the gradual increase of NGF, acted as the main factor of cholinergic differentiation, and allowed the survival of the cell trying to resemble the real conditions of a living organism. We can also point out that the formation of neurospheres from a biopolymer matrix without the addition of growth factors and/or gene transfection, contributed to the success of the experiment (Adib et al., 2014).

The genes used to characterize genotypically the neurospheres are extensively described in the literature, in various MSC sources; *NESTIN* as a neural precursor cell marker, *MAP2* as well *GFAP* are considered glial markers of mature neurons and *ßIII-TUBULIN* is specific for neuron cytoskeleton (Balasubramanian et al., 2013; Birenboim et al., 2013; Chung et al., 2014; Ivanov et al., 2016; Materne et al., 2015; Zhang et al., 2011). The expression of the *NESTIN* gene in MSC-WJ, which was not cultured on the NFBX matrix, means that these cells did not induce the formation of neuronal precursor cells, confirming results obtained by another study, in which MSC-WJ presented expression of the nestin gene in the undifferentiated form (Balasubramanian et al., 2013). Such expression increased when cells were induced to form neurospheres (Balasubramanian et al., 2013; He et al., 2009). According to this study, MSC-WJ demonstrated higher efficiency in neural precursor differentiation when compared to other sources described, such as adipose tissue and bone marrow. Considering the expression of *NESTIN*, *MAP2*, *GFAP*, and *ßIII-TUBULIN* genes on neurospheres and on cultured neural precursors cells derived from MSC-WJ, the results obtained in this study followed the gene pathways found by another study (Mukai et al., 2016).

It is important to emphasize that this study does not aim to demonstrate how this natural matrix determined the formation of neurospheres. Nevertheless, the NFBX was compared to other studies, still unpublished, with seeded hMSC on polystyrene, cellulose, and chitosan matrix. The NFBX was not the aim of this study, but the tool to obtain the neurospheres.

Some questions remain to be investigated to understand the development of these neurospheres from the MSCs contact with this matrix. First, to provide more details of the morphologies and functions emerging from their properties and cell interactions, and how the precise gene expression patterns from a heterogeneous population of MSCs could select precisely the cells that would be committed to being precursors of neurons. Second, to investigate how cells communicate and make decisions during spheroid formation, and how shape and morphology emerge by mechanic-physicochemical processes to determine their geometry.

CONCLUSION

The main aim was attended: to produce neurospheres using mesenchymal stem cells, and differentiate the neurospheres in cholinergic-like cells. Other studies utilize growth factor or transfection to turn mesenchymal stem cells into neurospheres; in this study, the natural matrix plays this role. Thus, it is possible to differentiate mesenchymal stem cells in cholinergic-like cells without the natural matrix. However, this method has the advantage of not using products or methods that induce the risk factors associated with cell transformation. These neurospheres were developed without growth factors and/or gene transfection, ideal for translation proceedings, only seeding the undifferentiated MSCs on the matrix coating in the flask substrate. This study suggests a potential use of the neural precursor cells, and neuron-like cells obtained from this protocol to preclinical models, and then translate to neurodegenerative diseases, for example in the preclinical model of Alzheimer Disease (in press).

This study has two significant limitations: the absence of gene expression and functional characterization of cholinergic cells. The first limitation was because the material used was only two umbilical cords. Second, when neural precursor cells were differentiated into cholinergic cells, the concentration of these cells decreased and was not sufficient to obtain an abundance of mRNA for this method as well as characterization of the function. Therefore, it was decided to indirectly prove the cellular phenotype of cholinergic cells with the demonstration of three proteins: NESTIN, ChAT, and ßIII-

TUBULIN, although there is a risk of the dichotomy between mRNA consequent gene expression and protein levels.

Further studies are needed to understand the mechanistic functionalities of NFBX to promote the aggregated cells in neurospheres.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author Contributions

Conceptualization, Katherine Athayde Teixeira de Carvalho; Formal analysis, Priscila Elias Ferreira Stricker, Daiany de Souza and Katherine Athayde Teixeira de Carvalho; Investigation, Priscila Elias Ferreira Stricker and Daiany de Souza; Methodology, Priscila Elias Ferreira Stricker, Daiany de Souza, Ana Carolina Irioda, Bassam Felipe Mogharbel, Celia Regina Cavichiolo Franco, José Roberto de Souza Almeida Leite, Alyne Rodrigues de Araújo and Katherine Athayde Teixeira de Carvalho; Project administration, Katherine Athayde Teixeira de Carvalho; Resources, Felipe Azevedo Borges, Rondinelli Donizetti Herculano, Carlos Frederico de Oliveira Graeff and Katherine Athayde Teixeira de Carvalho; Supervision, Katherine Athayde Teixeira de Carvalho; Writing – original draft, Priscila Elias Ferreira Stricker; Writing – review & editing, Katherine Athayde Teixeira de Carvalho.

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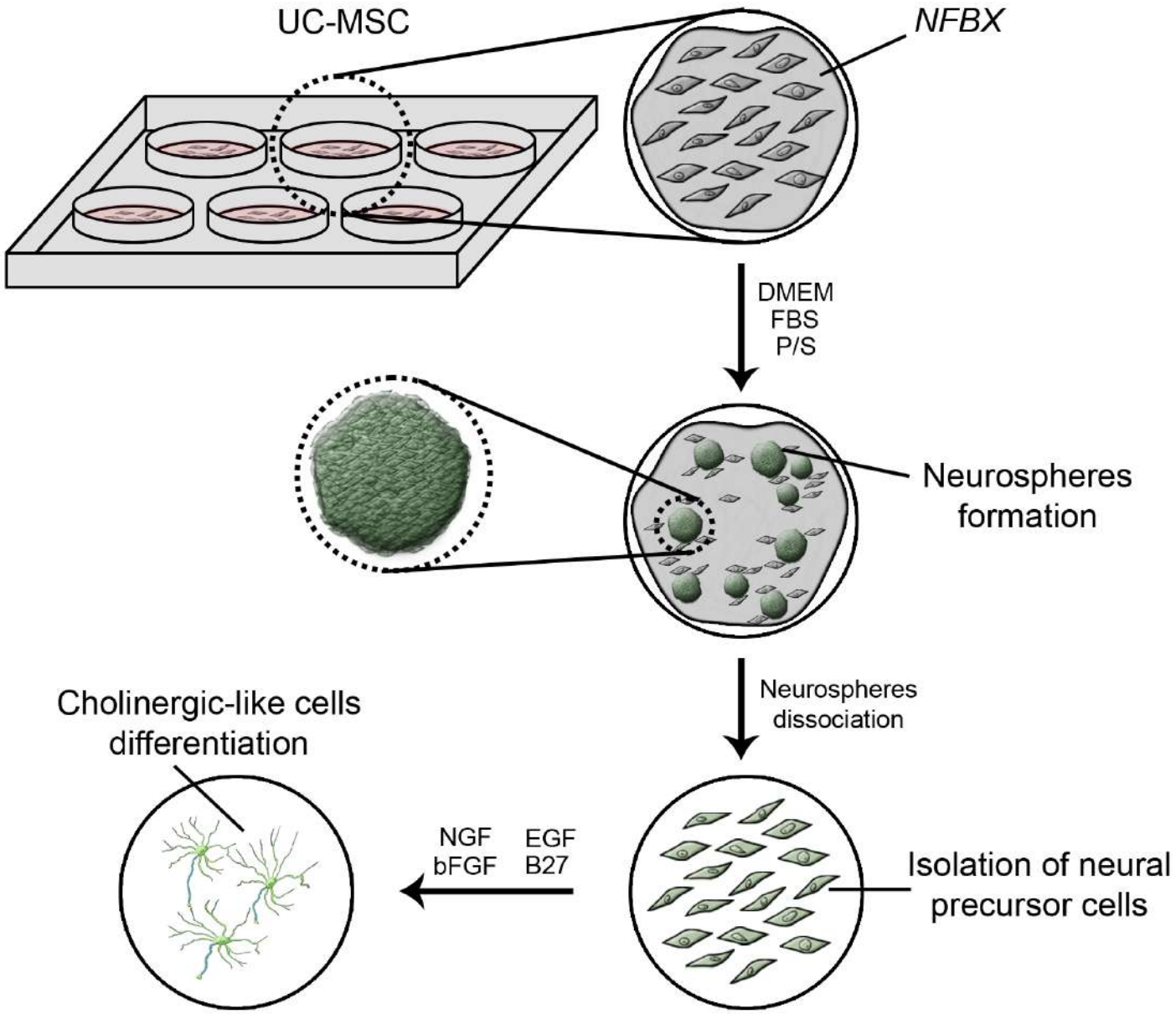
GRAPHICAL ABSTRACT

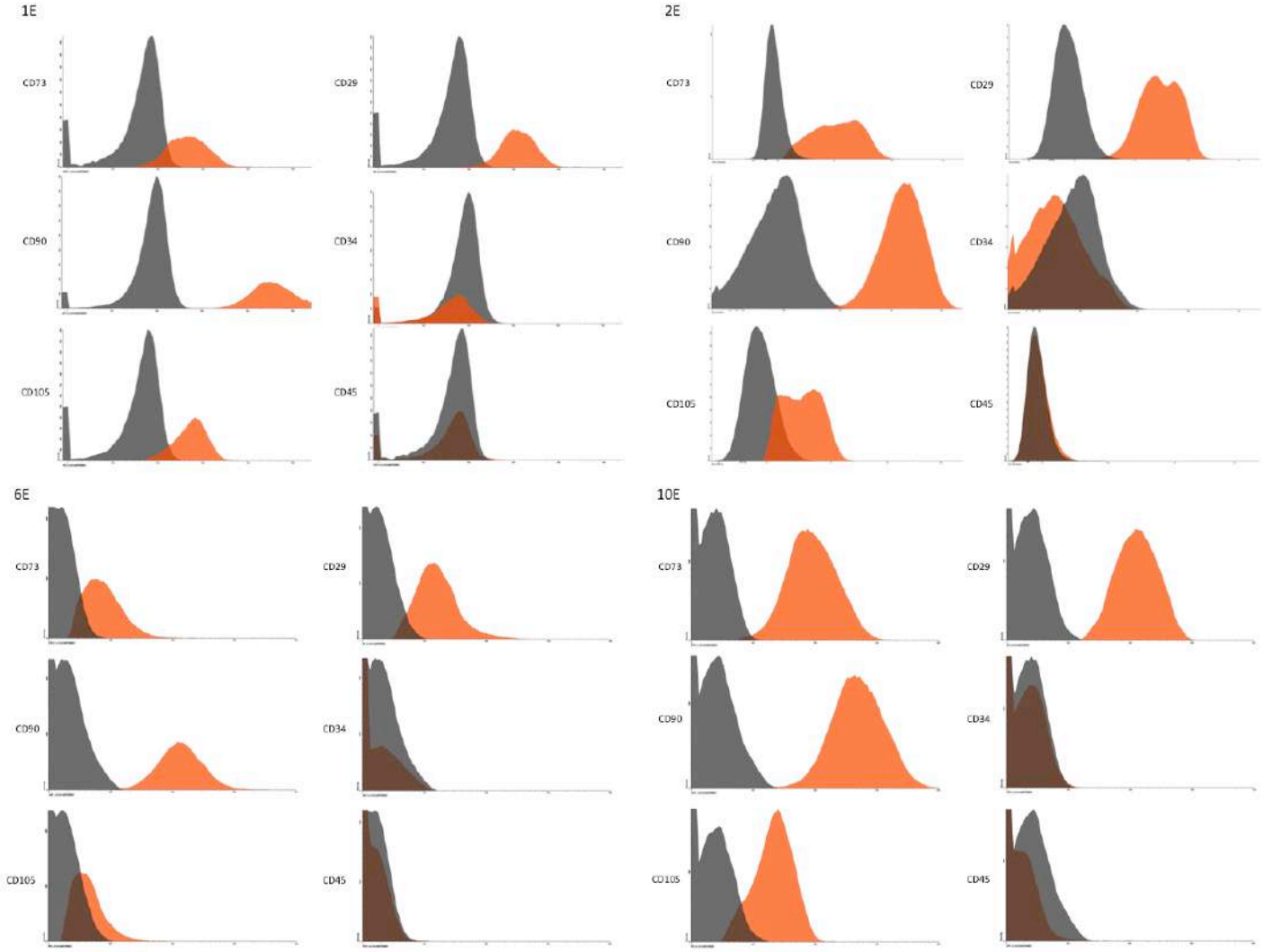
Graphical Abstract of Neurospheres until Cholinergic-like cells differentiation



UC-MSC were seeded on in NFBX to obtained neurospheres. Neurospheres were than mechanical dissociated to obtain the through the neurospheres the neural precursor cells. The neuronal precursor cells were induced to cholinergic-like cells through the addition of NGF, bFGF, EGF, and B27 in cultivation media. Abbreviations: UC-MSC: Umbilical cord mesenchymal stem cells; NFBX: Natural Functional Biopolymer Matrix; DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal Bovine Serum; P/S: penicillin and streptomycin, NGF: Nerve Growth Factor; EGF: Epidermal Growth Factor, bFGF: Basic Fibroblast Growth Factor, B27: neuronal supplementation.

SUPPLEMENTARY INFORMATION

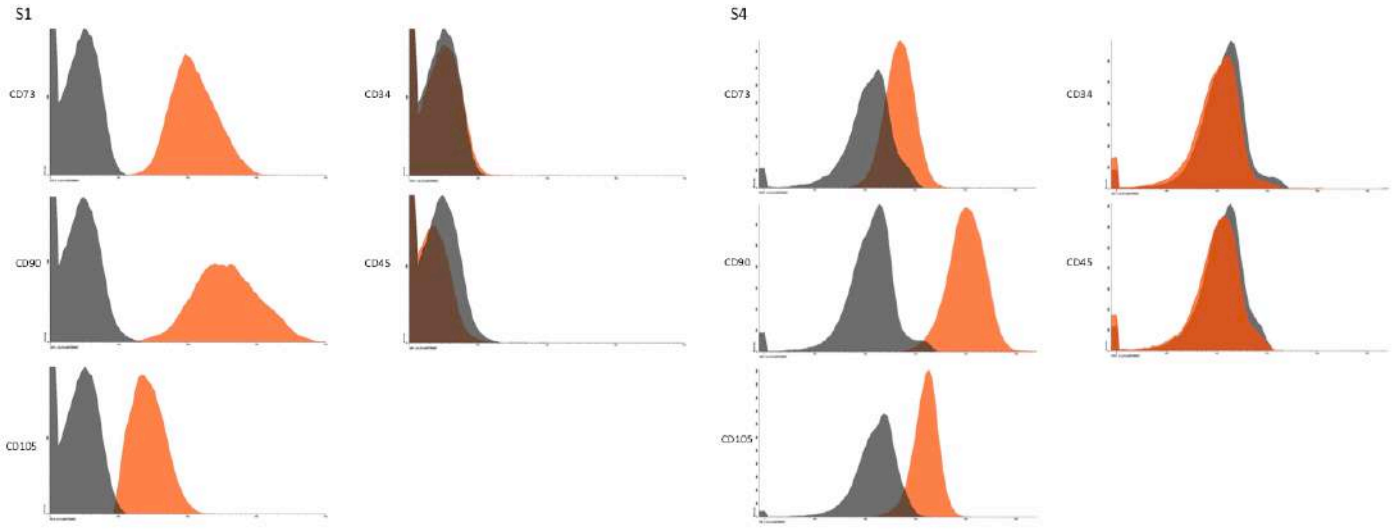
(A)



Surface marker expression of MSC-WJ. Note: SD – standard deviation. Referring to samples 1E, 2E, 6E and 10E.

Marker	CD73/CD105/CD90 (+)	CD73/CD29 (+)	CD34/CD45 (-)	7AAD (+)
1E	87.72	94.28	99.90	7.45
2E	91.75	89.61	99.97	15.67
6E	91.83	-	99.67	34.54
10E	93.76	-	99.99	12.95
Mean	91.27%	91.95%	99.88%	17.65%
SD	0.03	0.03	0.00	0.12

(B)



Surface markers expression of MSC-UCB. Note: SD – standard deviation. Referring to samples S1 and S4.

Marker	CD73/CD105/CD90 (+)	CD34/CD45 (-)	7AAD (+)
S1	90.26	99.75	11.47
S4	92.33	99.95	6.49
Mean	91.30%	99.85%	10.53%
SD	1.46	0.13	6.01

Figure S1. Flow Cytometry Histograms and table analysis. (A) Histograms for the samples 1E, 2E, 6E, and 10E from MSC-WJ, and the respective table showing the statistic values. (B) Histograms for the samples S1 and S4 from MSC-UCB, and the respective table showing the statistic values. The gray peaks represent the isotype controls determining the area in which the marker is negative. The orange peaks correspond to the sample marking with its respective immunostaining marker, which is positive when the right of the gray peak and negative when on top or the left of the gray peak. Flow Cytometer was performed in FACS Calibur; Becton Dickinson, USA, and the analysis were conducted using Infinicyt Flow Cytometry Version 1.6.0 software.

Day	NGF ng/mL	EGF ng/mL	bFGF ng/mL	B27 %
0	0	10	10	1
1	10	9	9	0.9
2	20	8	8	0.8
3	30	7	7	0.7
4	40	6	6	0.6
5	50	5	5	0.5
6	60	4	4	0.4
7	70	3	3	0.3
8	80	2	2	0.2
9	90	1	1	0.1
10	100	0	0	0

Table S1. Cholinergic differentiation protocol in accordance Adib et al.,2015. A different concentration of growth factors and B27 per mL of medium was required throughout the 11 days of the protocol.