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Original Article

In Vitro Differentiation of Human Wharton's Jelly–Derived Mesenchymal Stem Cells into Auditory Hair Cells and Neurons

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OBJECTIVE: We attempted to induce mesenchymal stem cells (MSCs) from human Wharton's jelly (WJ) to differentiate into neuronal progenitor cells, neurons, and auditory hair cells.

MATERIALS and METHODS: MSCs were isolated from WJ from human umbilical cords and cultured in medium containing epidermal growth factor and basic fibroblast growth factor. Differentiation into hair cells and neurons was induced using a neurobasal medium containing glial cell-derived neurotrophic factor, brain-derived neurotrophic factor, and neurotrophic factor 3. Fluorescence-activated cell sorting (FACS), immunocytochemistry, and reverse transcription polymerase chain reaction were performed to characterize the differentiated auditory hair cells and neurons.

RESULTS: MSCs isolated from human WJ were confirmed by FACS. Double immunocytochemistry confirmed the expression of the hair cell markers myosin VIIA and TRPA1 and the functional marker C-terminal binding protein 2. Differentiation into neurons was revealed using neurofilament and βIII-tubulin markers. Gene expression of neuronal lineage-specific markers confirmed the neuronal differentiation state.

CONCLUSION: MSCs from human WJ can be successfully induced to differentiate into auditory hair cells and neurons *in vitro*.

KEYWORDS: Wharton's jelly, mesenchymal stem cells, auditory hair cells

INTRODUCTION

Mesenchymal stem cells (MSCs) have promising therapeutic potential in cell transplantation due to their multipotent ability, and can differentiate into mesenchymal lineages, including osteoblasts, adipocytes, and chondroblasts *in vitro*. MSCs are easy to obtain, highly proliferative, and not immunoreactive. Adult MSCs have been isolated from bone marrow, adipose tissue, and lung tissue [1-3]. Because of the non-invasiveness and therapeutic potential of MSCs in cell transplantation, various MSC sources have been investigated. The collection of MSCs from the placenta and its derivatives, such as the amniotic membrane, amniotic fluid, chorionic villi, umbilical cord blood, and Wharton's jelly (WJ), has been demonstrated [4-8]. However, the quantity of MSCs obtained from umbilical cord blood has been found to be very low and to have poor proliferation potential [9]. WJ is the primitive connective tissue of the umbilical cord, which surrounds the two umbilical arteries and one umbilical vein that is enclosed by simple amniotic epithelium. WJ-derived MSCs have properties similar to those of bone marrow-derived MSCs but are considered a more primitive population than the latter.

Studies have indicated that human WJ-derived MSCs can be differentiated into chondrogenic, osteogenic, adipogenic, myogenic, and neurogenic lineages [8-12]. To the best of our knowledge, no studies have demonstrated the *in vitro* differentiation of WJ-derived MSCs into auditory hair cells. Therefore, the purpose of this study was to isolate and characterize MSCs derived from WJ and to determine their capacity to undergo differentiation into neuronal and auditory hair cells *in vitro*.

MATERIALS and METHODS

Isolation and Culture of MSCs from Human WJ

This study was approved by the local institutional review board (KC10TISI0144). Human umbilical cords were obtained from healthy donors with their written informed consent. Each umbilical cord unit was manipulated under sterile conditions, cut into sections of about 5 cm, and washed with Hanks' balanced salt solution (Gibco; Grand Island, NY, USA) to remove blood. After the vein and arteries were removed to avoid endothelial cell contamination, WJ was cut into 2–5-mm sections and incubated with 0.25% trypsin

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(Gibco) with 10 mL of EDTA (Gibco) at 37°C in a water bath. After 1 h, 10 mL of 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY, USA) was added to inhibit enzymatic activation. The mixture was then pipetted 20 times. A 100 μm strainer was placed in a 50 mL centrifuge tube, and the pellet solution was filtered and centrifuged at 1000 rpm at 4°C. The cells were then seeded in 100 mm^2 culture plates and grown in culture medium with epidermal growth factor (EGF) in a humidified atmosphere containing 5% CO_2 at 37°C for 5 days. The cells were subcultured until the third passage.

Identification of Human WJ-Derived MSCs by Fluorescence-Activated Cell Sorting

Cell surface markers specific to human MSCs, namely, CD34, CD45, CD73, CD90, CD146, CD103, CD105, and HLA-DR, were used to confirm whether the cells obtained from human WJ were MSCs. After three subcultures, human WJ-derived mononuclear cells were collected, counted, and aliquoted into a test tube at a dilution of 1×10^6 cells/mL. The samples were washed three times in buffer containing 0.2% BSA, 0.1% NaN_3, and 0.5 mM EDTA. The samples were treated with antibodies against CD34, CD45, CD73, CD90, CD146, CD103, CD105, and HLA-DR (all from BD Biosciences, San Jose, CA) conjugated with fluorescein isothiocyanate and phycoerythrin for 1 h. The samples were again washed with the buffer solution three times and analyzed by flow cytometry (FACSCalibur; Becton Dickson, San Diego, CA, USA).

Differentiation into Neuronal Progenitor Cells

To differentiate the human WJ-derived MSCs into neuronal progenitor cells, MSCs were cultured in a medium containing 20 ng/mL of EGF (Invitrogen) and 10 ng/mL of basic fibroblast growth factor (bFGF; Invitrogen) for 14 days. In addition, 10 ng/mL of bFGF was added to the culture medium thrice at 3-day intervals (Table 1).

Differentiation into Hair Cells and Neurons

Differentiation into hair cells and neurons was induced using a neurobasal medium containing glial cell-derived neurotrophic factor (GDNF; Invitrogen), brain-derived neurotrophic factor (BDNF; Invitrogen), and neurotrophic factor 3 (NT-3; Invitrogen) (Table 1).

Immunocytochemical Analysis of Differentiation into Neuronal Progenitor Cells, Hair Cells, and Neurons

Human WJ-derived MSCs were cultured in a 4-well chambered slide (Nalgene Nunc International Rochester; NY, USA).

Immunocytochemical analysis was performed after the induction of differentiation into neuronal progenitor cells, hair cells, and neurons. Bromo-2′-deoxyuridine (BrdU) is a marker for proliferating cells to confirm specific differential proliferation. First, the cells were labeled with the BrdU Labeling and Detection Kit (Roche; Indianapolis, IN, USA) and washed three times with PBS containing 3% bovine serum albumin (BSA; Bovogen Biologicals Pty. Ltd.; Essendon, Australia). Thereafter, the cells were fixed using 4% paraformaldehyde for 20 min and treated with 0.5% Triton X-100 (Promega Corporation, Madison, WI). The samples were treated with anti-BrdU working solution as a primary antibody for 12 h and washed thrice with PBS containing 3% BSA. Next, the samples were treated with anti-mouse-Ig-fluorescein working solution (1:10) as a secondary antibody for 1 h at room temperature. The samples were then blocked with 5% normal goat serum and double stained using specific cellular markers, including the glial cell markers glial fibrillary acidic protein (GFAP; Abcam, Cambridge, UK), S100 (Abcam), and myelin basic protein (MBP); neuronal cell markers βIII-tubulin (Abcam), neurofilament (NF; Abcam), and microtubule-associated protein 2 (MAP2, Abcam); neuronal progenitor cell marker nestin (Abcam); and hair cell markers myosin VIIA (Abcam) and transient receptor potential cation channel, subfamily A, member 1, (TRPA1; Abcam). All specimens subjected to double staining were processed overnight and rinsed with PBS containing 3% BSA. The secondary antibody, Alexa 555, was allowed to react with the samples at room temperature at a ratio of 1:100. For nuclear staining, DAPI-conjugated mounting medium (VECTASHIELD; Burlingame, CA, USA) was used for mounting. The expression was confirmed on the basis of the number of cells expressing the marker as determined by fluorescence microscopy (Olympus; Tokyo, Japan).

Table 1. Media for inducing differentiation into neurospheres and neurons

Neurosphere medium (Total volume, 50 mL)	Neuron medium (Total volume, 50 mL)
L-Glutamine 2 mM	L-Glutamine 2 mM
B-27 supplement 1 mL	B-27 supplement 1 mL
DMEM:F12 (1:1)	Neurobasal medium
bFGF 20 ng/mL	BDNF, GDNF, and NT3 (10 ng/mL each)
EGF 10 ng/mL	1% Penicillin–streptomycin
1% Antibiotic–antimycotic	

DMEM: Dulbecco's Modified Eagle's medium; bFGF: basic fibroblast growth factor; EGF: epidermal growth factor; BDNF: brain-derived neurotrophic factor; GDNF: glia-derived neurotrophic factor; NT3: neurotrophic factor 3

Reverse Transcription Polymerase Chain Reaction

The MSCs that were subcultured thrice were cultured in a 100-mm dish at a concentration of 1×10^6 cells/well. Thereafter, differentiation into neuronal progenitor cells, hair cells, and neurons was induced. Cells were collected after 10 days. RNA was extracted and reverse transcription PCR (RT-PCR) was performed. We attempted to examine whether the number of cells differentiating into neuronal progenitor cells, hair cells, and neurons increased over time. RNA was isolated from cultured cells using the Axygen RNA miniprep kit (Axygen Scientific; Union City, CA, USA). The isolated RNA was subject to reverse transcription at 4°C for 60 min and at 95°C for 5 min using the RT Premix Kit (Intron Biotechnology; Sungnam, Korea). Then, cDNA was synthesized, quantified, and equalized at a concentration of 100 ng. Using a PCR premix (Bioneer; Daejeon, Korea) and primers for various genes (Table 2), the DNA was denatured at 94°C for 5 min, annealed for 30 s at the annealing temperature of each primer, and extended at 72°C for 5 min. As a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase was used. The expression was confirmed by 2% agarose gel electrophoresis and gel imaging (Image Analysis System; Bio-Rad, Hercules, CA, USA).

Statistical Analysis

All results have been reported in terms of mean±standard error of the mean values. Statistical analysis was performed with an independent sample t-test comparison of the two experimental groups by using Statistical Package for the Social Sciences version 15.0, (SPSS Inc.; Chicago, IL, USA). Null hypotheses of no difference were rejected if p values were less than 0.01. All experiments results were minimally calculated by individual triplicates.

Table 2. Reverse transcription PCR conditions

Gene name		Sequence (5′-3′)	Annealing Temp (°C)	Product size (bp)
S100	F	ctttaaatgcgttcctcatc	51	156
	R	ttctgatggagttgctttt		
Nestin	F	aacaggaccaagagacattg	56	211
	R	tttactgcctctacgctctc		
GAPDH	F	ctactggcgctgcaaaggctgt	54	357
	R	gccatgaggtccaccaccctgt		
βIII-Tubulin	F	aagttttggagagggaaatc	54.5	188
	R	agggaggtagagttggaag		
BMP4	F	agtgaaaactctgcttttcg	53	217
	R	ccagtctcgtgtccagtagt		
BMP7	F	caacgtcatcctgaagaaat	50.9	217
	R	atatgctgctcatgttttct		
GFAP	F	tttctaaaggcctcttcctt	56	192
	R	ctgggtacatttgtgtgtg		
Myosin VIIA	F	tacatcgacatccacttcaa	54.5	154
	R	tctgatcctcactcataccc		
NF	F	caagaagagggaagccac	55	258
	R	ttcatctgctgggctcaag		
TRPA1	F	ctccatctggcagcaaagaa	56.5	210
	R	tgcagtgttcccgtcttcat		
MAP2	F	atttatcaggggagagtggt	53	177
	R	cctgatttgtcaccagagat		

GAPDH: glyceraldehyde-3-phophate dehydrogenase; BMP4: bone morphology protein 4; GFAP: glial fibrillary acidic protein; NF: neurofilament; TRPA1: transient receptor potential cation channel; subfamily A, member 1, MAP2: microtubule-associated protein 3

RESULTS

Isolation and Primary Culture of Human WJ

After the cells from WJ were cultured for 5 days, mononuclear cells attached to the plate surface were obtained. As the number of subcultures increased, the shape of cells became elongated, and they changed into fibroblast-like cells. When MSCs differentiated into neurosphere-forming cells, the cells became spherical and further differentiated into neurons (Figure 1, 2).

Identification of Human WJ-Derived MSCs by Fluorescence-Activated Cell Sorting

To confirm whether human WJ-derived cells were MSCs, cell surface-specific markers were examined. Fluorescence-activated cell sorting (FACS) showed the cells to be negative for the hematopoietic stem cell markers CD34 and CD45 and positive for the MSC surface

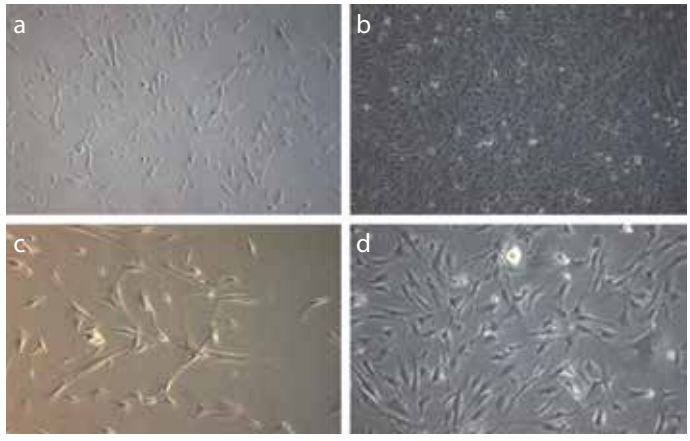

Figure 1. a-d. Bright-field images (a, b) showing primary MSCs derived from Wharton's jelly after primary culture for 5 days (a: 100×, b: 200×). Bright-field images (c, d) showing neurosphere-forming cells differentiating into neurospheres over 15 days

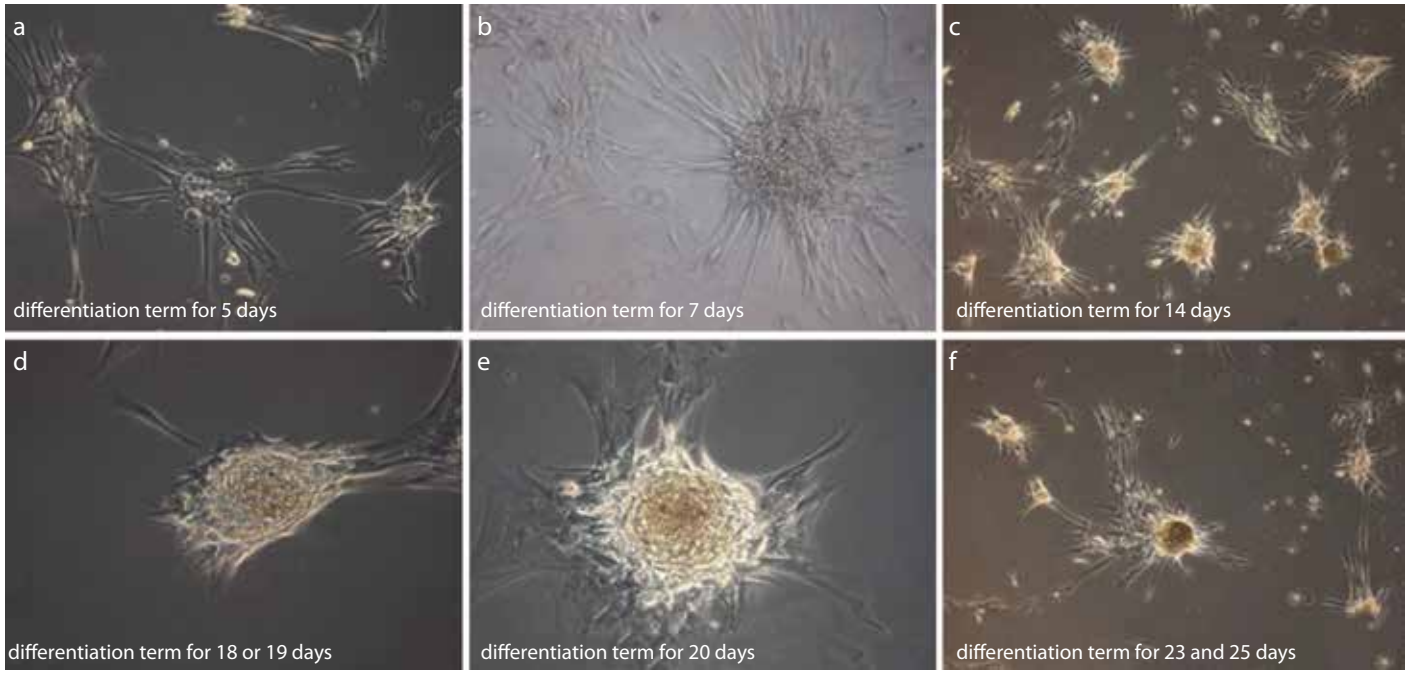

Figure 2. a-f. Neurosphere induction. MSCs derived from human Wharton's jelly could be converted into spheres, and these cells finally differentiated into neurospheres at 25 days (average, 25–30 days). Neurosphere induction cultures were carried out for 5, 7, 14, 18, 19, 20, and 23–25 days to confirm changes in neurosphere formation

markers CD73, CD90, and CD146. Almost 100% of the cells were negative for the HLA-DR surface marker. Therefore, the cells are not expected to pose any immunological problems related to the human histocompatibility complex (Figure 3).

Differentiation of Human WJ-Derived MSCs into Hair Cells and Neurons

The human WJ-derived MSCs were cultured for three passages and induced to differentiate into auditory hair cells and neurons. To confirm whether these cells could be induced to differentiate into neurons and auditory hair cells, the cells were assessed for the expression of markers specific to hair cells and neurons using double immunocytochemistry.

Neurons have axons and dendrites and can be identified by the staining of neuron-specific markers MAP2, NF, and βIII-tubulin. The MSCs were positive for MAP2, NF, and βIII-tubulin as well as for the neurogenic progenitor cell marker nestin and glial cell markers S100, GFAP, and BrdU.

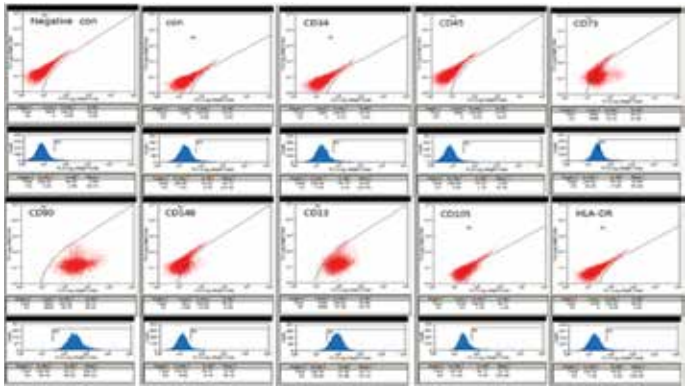

Figure 3. FACS analysis shows MSCs derived from Wharton's jelly. CD34 and CD45 are hematopoietic stromal cell markers, and HLA-DR is a human leukocyte antigen. The remaining markers are mesenchymal stromal cell-positive markers

Auditory hair cells have elongated cell bodies with cilia. Double staining revealed the staining of the hair cell-specific markers TRPA1, myosin VIIA, and BrdU on day 14. The cells were positive for the hair cell markers myosin VIIA and TRPA1 as well as for the functional marker C-terminal binding protein (CtBP or ribbon marker). They were also positive for the neuronal markers NF, βIII-tubulin, and MAP-2 and for the glial cell markers GFAP and S100 (Figure 4, 5).

Gene Expression Analysis Using RT-PCR

The differentiation of the WJ-derived MSCs into auditory hair cells and neurons was analyzed by RT-PCR. The neuronal progenitor cell marker nestin, inner ear developmental markers (bone morphogenetic proteins 4 and 7), hair cell markers (myosin VIIa and TRPA), and neuronal markers (βIII-tubulin, MAP-2, GFAP, MBP, and S100) were expressed. Pre/post-synaptic markers, namely, Ca^{2+} and Na^{+} channel markers, CtBP, and glutamate vesicular transporter (VGLUT), were also expressed (Figure 6).

DISCUSSION

Cell therapy based on MSCs isolated from various sources is the mainstay of regenerative medicine. In the near future, MSCs are expected to occupy the leading position in the treatment of various disorders, such as degenerative diseases, cancers, and auditory and retinal diseases, as well as for repairing tissue damage, e.g., spinal cord injuries. Bone marrow MSCs have been used to differentiate into various target cells for treatment at the cellular level. However, the highly invasive and painful harvesting process and low MSC yield are the obstacles hindering the widespread application of such MSCs. Although there have been considerable developments in stem cell therapy applications, certain limitations persist because of various surgical and technical risks [13]. Since the introduction of stem cell therapy as an alternative approach for cochlear implants, the treatment of hearing loss using auditory hair cells differentiated from MSCs has been reported in animal models in different studies [14-16].

No effective methods to isolate MSCs from WJ have been established till date. Wang et al. [8] separated vessels from the umbilical cord,

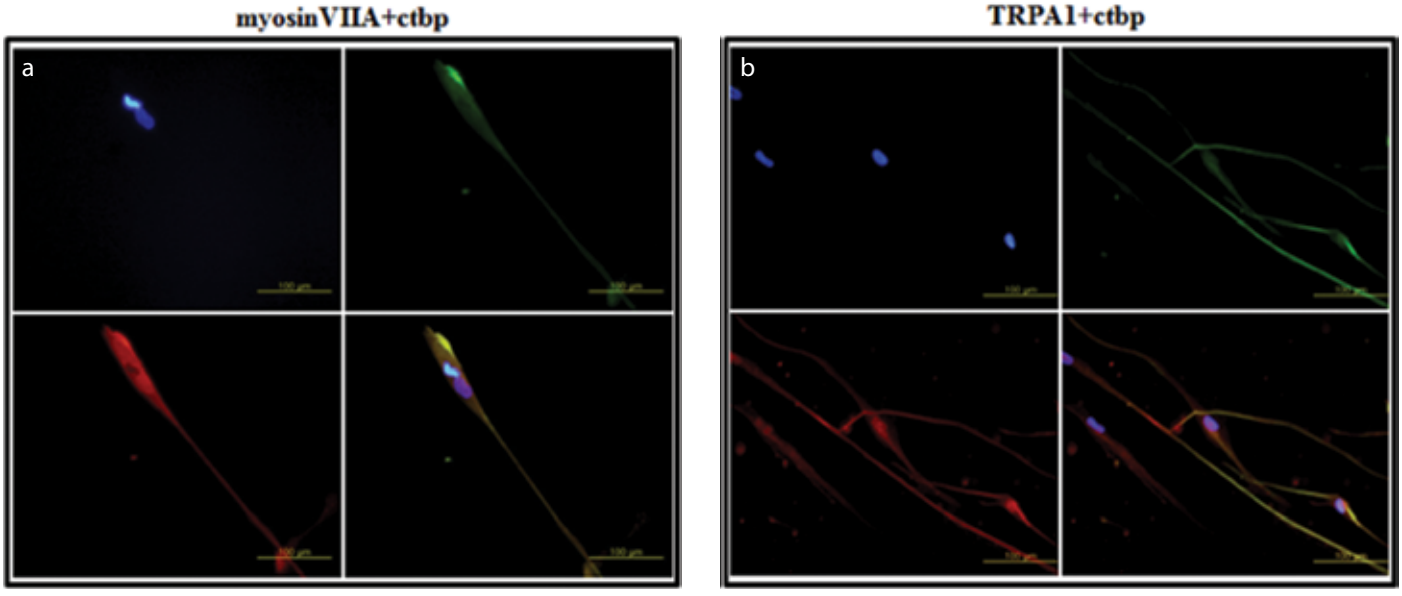

Figure 4. a, b. Double-labeled immunocytochemistry shows hair cell-positive markers. Nuclei counterstained with DAPI (blue), stained with Ca2+ (green) and with (a) myosin VIIA (red), or (b) TRPA1 (red). (400×, n=3, bars; 100 µm)

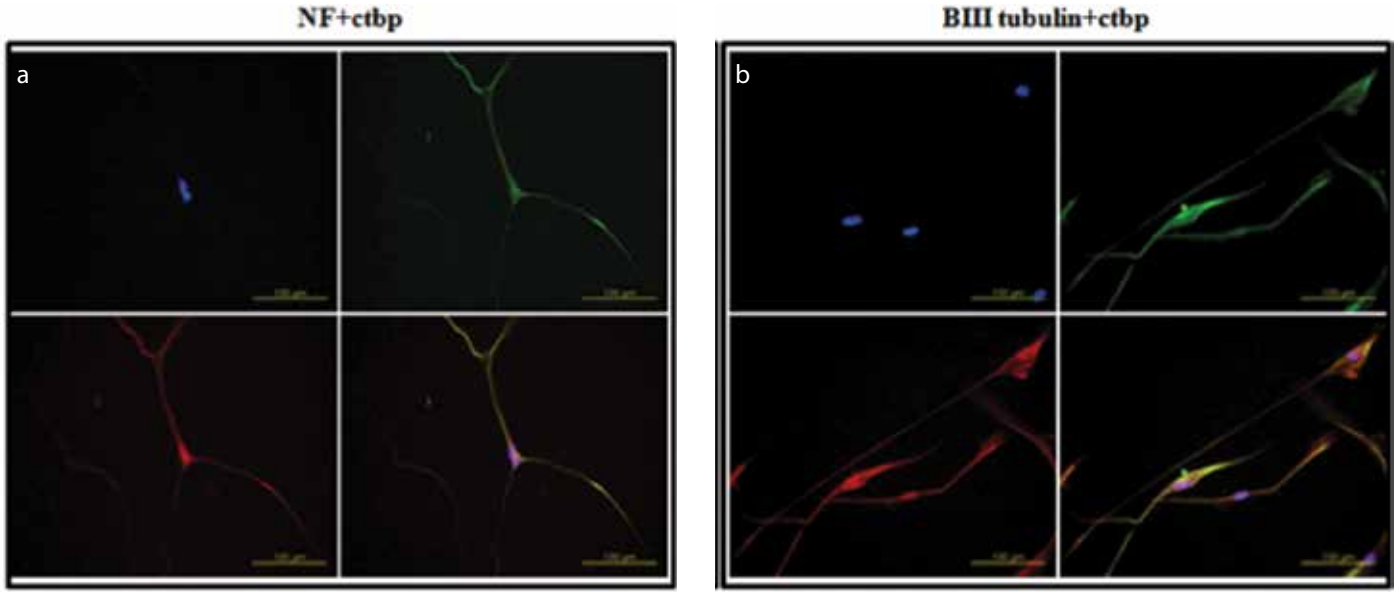

Figure 5. a, b. Double-labeled immunocytochemistry shows neuronal markers. Nuclei counterstained with DAPI (blue), stained with CtBP (green) and with NF (red)(a), stained with βIII-tubulin (red) (b) (400×, n=3, bars; 100 μm)

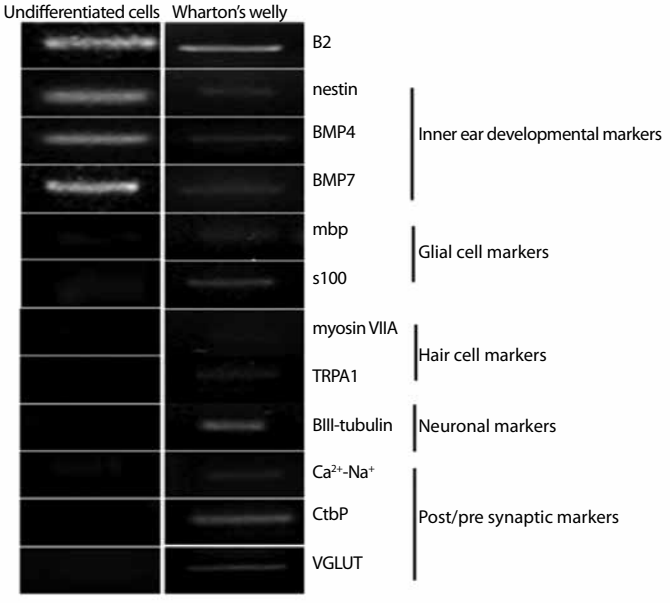

Figure 6. RT-PCR analysis showing gene expression of neuronal lineage-specific markers

scraped off WJ with a scalpel, and treated the tissue with an enzymatic solution. Others dissected umbilical cord segments into very small pieces instead of using enzymatic treatment to minimize cell damage [17]. In this study, WJ was separated by stripping off vessels from the umbilical cord and cutting WJ into small pieces, followed by treatment with enzymatic solution to obtain homogenous cell populations.

The proliferative potential of WJ-derived MSCs has been reported to be higher than that of chorionic plate-derived MSCs or amniotic membrane-derived MSCs [10, 11]. In the present study, the yields of WJ MSCs and differentiated neurogenic progenitor cells were less than 10% higher than those of chorion-derived MSCs (data not shown). In an earlier study, we demonstrated that umbilical cord blood-derived MSCs can be differentiated into neurons and auditory hair cells *in vitro* [18]. Choi et al. [19] reported that intravenous transplantation of umbilical cord blood-derived MSCs can enhance hearing thresholds and increase the number of spiral ganglion neurons in a deaf animal model. In the present study, we showed that MSCs can be isolated from human WJ and can be differentiated into auditory hair cells and neurons. As WJ-derived MSCs have a higher expression of neuronal genes, such as nestin and GFAP, WJ-derived MSCs can be induced to differentiate into neuronal subpopulations, unlike bone marrow-derived MSCs [20].

Fluorescence-activated cell sorting (FACS) showed WJ-MSCs to be negative for hematopoietic stem cell markers CD34 and CD45 and for the cell surface marker HLA-DR, which implied no inherent immunological problems related to HLA. This underscores the potential for the use of WJ-derived MSCs in stem cell therapy. These findings are consistent with those of earlier studies suggesting that WJ-derived MSCs have the weakest expression of MHC II genes and the strongest immunomodulatory and immunosuppressive potential among human bone marrow, adipose tissue, WJ, and the placenta [21].

The CtBP family includes four proteins, namely, CtBP1, CtBP3/BARS, CtBP2, and RIBEYE. RIBEYE is expressed in ribbon synapses, which are found in neurons in retinal photoreceptor and bipolar cells and cochlear hair cells. In this study, RIBEYE was expressed in cochlear hair cells differentiated from WJ-derived MSCs. Thus, these cells can differentiate into auditory hair cells; moreover, BrdU staining demonstrated their proliferation potential.

The use of WJ as a source of hematopoietic cells is advantageous because it is obtained by a simple and painless procedure. Furthermore, "afterbirth" is considered medical waste, thereby precluding ethical issues for its use in regenerative medicine. Here we demonstrated that WJ-derived MSCs could differentiate into neuronal progenitor cells, which in turn, differentiated into auditory hair cells and neurons. Our current findings highlight the applicability of WJ-derived MSCs in regenerative medicine.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Seoul St. Mary's Hospital (IRB No. KC10TISI0144).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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Author Contributions: Concept - M.Y.C., K.H.P.; Design - M.Y.C., K.H.P.; Supervision - M.Y.C., K.H.P.; Resources - K.K., M.Y.C.; Materials - K.K.; Data Collection and/or Processing - M.Y.C., K.H.P.; Analysis and/or Interpretation - M.Y.C., K.H.P.; Literature Search - K.K.; Writing Manuscript - K.K.; Critical Review - M.Y.C., K.H.P.

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