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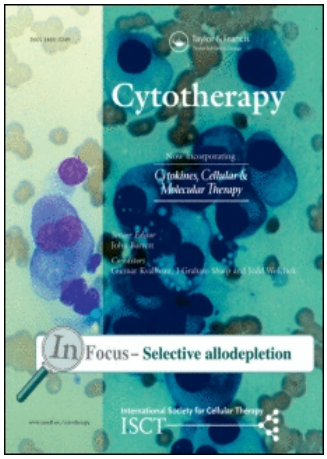

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Generating neuron-like cells from BM-derived mesenchymal stromal cells *in vitro*

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Background

The multipotency of stromal cells has been studied extensively. It has been reported that mesenchymal stromal cells (MSC) are capable of differentiating into cells of multilineage. Different methods and reagents have been used to induce the differentiation of MSC. We investigated the efficacy of different growth factors in inducing MSC differentiation into neurons.

Methods

MSC from human BM were isolated and cultured in media supplemented with 10% FBS. These cells were identified and later induced to differentiate into neuron-like cells using different neurotrophic factors. Three different growth factors were used, either alone or in combination: brain-derived neurotrophic factor, epidermal growth factor and neural growth factor.

Results

After 10 days of culture, MSC showed neuron-like morphologic changes. Immunostaining showed that these cells expressed markers for neurons (growth-associated protein-43, neuron-specific nuclear protein and neurofilament 200 kDa) and expression of these markers suggested the transition of immature stages to more mature stages of neuron-like cells.

Discussion

Our results show that BM-derived MSC can differentiate not only into target cells of mesodermal origin but also neuron-like cells of ectodermal origin. The findings show that a combination of growth factors is more effective in inducing MSC into neuron-like cells.

Keywords

adipogenic differentiation, chondrogenic differentiation, mesenchymal stromal cells, neuronal differentiation, osteogenic differentiation, transdifferentiation.

Introduction

Stromal cells are thought to be the major source of growth factors for development of hematopoietic stem cells, and interact closely with hematopoietic stem cells within the BM microenvironment [1]. Lately, these stromal cells, and a subset also known as mesenchymal stem cells, have been studied extensively for their ability to differentiate into target cells. It has been reported that mesenchymal stromal cells (MSC) are capable of differentiating into adipocytes, osteocytes and chondrocytes [2–9]. MSC have also been shown to differentiate into myocardiocytes [10–12] and neuron-like cells [13,14].

Different methods have been used to coax stromal cells to differentiate into neuron-like cells, such as using chemical reagents β-mercaptoethanol (BME), DMSO and butylated hydroxyanisole (BHA), growth factors and 5-Aza-C [15]. In this study, we investigated the efficacy of different neutrotrophic factors in generating neuron-like cells from BM-derived MSC, alone and in combination.

Methods

Sampling

BM aspirate, obtained from patients with informed consent, was used in this study. The samples were obtained in

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accordance with the protocol approved by the Ethics and Research Committee of The National University of Malaysia.

BM preparation and cell culture

BM aspirate was diluted with equal amounts of PBS prior to centrifugation with Ficoll-Pague (Amersham Biosciences, Uppsala, Sweden). The mononuclear cells were washed and suspended in DMEM-LG (Sigma-Aldrich, St Louis, MO, USA) before the viability of the cells was determined. The percentage of viable cells was 99.7%. The cells were seeded at a density of 1×10^5 cells/mL in DMEM supplemented with 10% FBS, 0.05 U/mL penicillin and 0.05 μg/mL streptomycin (Gibco, Grand Island, New York, NY, USA), at 37°C in 5% humidified CO_2. After 3 days, the suspension of cells was discarded and the adherent cells were expanded. Once the cells reached confluency, they were detached using 0.25% trypsin–EDTA (Gibco) and replated at a ratio of 1:4.

Characterization of BM-derived adherent cells

By morphology

Cells were stained with Wright–Giemsa stain for better observation of the cell morphology.

By cytochemical staining

The DMEM-derived adherent cells were analyzed *in situ* for the following cytochemical markers: periodic acid-Schiff (PAS), α-naphthyl acetate esterase (NSE), Sudan Black B (SBB), naphthol AS-D chloroacetate esterase (NASDA) and alkaline phosphatase (ALP). In all cases, analyzes as well as the selection of positive and negative controls were performed according to the manufacturer's guidelines (Sigma).

By immunophenotyping

To detect surface Ag, aliquots of adherent cells (1×10^5 cells/mL) were washed twice with PBS after detachment with 0.25% trypsin–EDTA. The cell pellets were diluted with PBS and incubated with FITC- or PE-conjugated MAb for 30 min in the dark. The Ab used were CD13, CD29, CD34, CD44, CD45, CD73, CD90, CD105, CD117, CD147 and HLA-DR (Becton Dickinson, San Diego, CA, USA). After incubation, the cells were washed again with PBS before being rediluted with sheath fluid and subjected to flow cytometric analysis using FACScan and CellQuest software (Becton Dickinson, USA).

Differentiation assay

Differentiation into adipocytes

To induce adipogenesis, cells were cultured in adipogenic induction medium comprising DMEM supplemented with 1.0 μm dexamethasone, 0.2 mm indomethacin, 0.01 mg/mL insulin, 0.5 mm 3-isobutyl-l-methyl-xanthine (Sigma), 10.0% FBS, 0.05 U/mL penicillin and 0.05 μg/mL streptomycin (Gibco). The medium was changed every 3 days for 2–3 weeks. Oil Red O (Sigma) was used as a histologic stain to visualize the presence of lipid droplets. RT-PCR was used to analyze the expression of adipogenesis-specific genes.

Differentiation into chondrocytes

To induce chondrogenesis, 3-D cell cultures were maintained in a chemically defined basal medium consisting of DMEM supplemented with 50 μg/mL ascorbate-2-phosphate, 1.0 mm sodium pyruvate, 40 μg/mL proline, 10 ng/mL transforming growth factor-β3, 6.25 μg/mL human insulin, 6.25 μg/mL transferrin, 6.25 μg/mL bovine insulin, 6.25 μg/mL selenous acid, 1.25 μg/mL linoleic acid, 5.35 μg/mL BSA (Sigma), 0.05 U/mL penicillin and 0.05 μg/mL streptomycin (Gibco). The 3-D chondrogenic culture utilized 1.0×10^6 MSC/pellet. MSC were suspended in 1 mL chondrogenic medium. The pellets were incubated in a humidified atmosphere at 37°C with 5% CO_2, with medium changes every 3–4 days. After every medium change, the pellet was agitated to ensure that the pellet was free floating and spheroid. Chondrogenic pellets were harvested after 5 weeks in culture. To assess chondrogenesis, Alcian Blue-PAS (Sigma) was used to stain cartilage matrix, and RT-PCR was used to analyze chondrogenesis-specific genes.

Differentiation into osteoblasts

To induce osteogenesis, osteogenic induction medium was prepared by supplementing DMEM with 10% FBS, 50 μg/mL ascorbate-2-phosphate, 10 mm β-glycerophosphate, 100 nm dexamethasone (Sigma), 0.05 U/mL penicillin and 0.05 μg/mL streptomycin (Gibco). MSC were plated at a density of 2.4×10^4 cells/cm^2 of plastic culture flasks and incubated in osteogenic induction medium in a humidified atmosphere at 37°C with 5% CO_2. The medium was changed every 3 days continuously for 2–3 weeks. Alizarin Red S (Sigma) was used to stain matrix mineralization associated with osteoblasts, and RT-PCR

was used to analyze expression of osteogenesis-specific genes.

Neuronal induction

To induce neuronal differentiation, the adherent MSC was exposed to a cocktail of induction agents. Briefly, prior to treatment with induction medium, the cells from passage 2 were pre-treated with DMEM containing 0.001% beta-mercaptoethanol (BME; Sigma), 0.05 U/mL penicillin, 0.05 μg/mL streptomycin, 10% FBS (Gibco), 10 ng/mL epidermal growth factor (EGF) and 10 ng/mL fibroblast growth factor-beta (FGFβ) (Chemicon, Temecula, CA, USA) [16] for 1 week. Then, to initiate neuronal differentiation, cells were induced in induction medium containing different growth factors. However, the basal medium for all the induction medium was the same, i.e. N2 (10 000 mg/L human transferrin Holo, 500 mg/L insulin recombinant, 0.63 mg/L progesterone, 1611 mg/L putrescine and 0.52 mg/L selenite; Gibco), in DMEM supplemented with 0.001% BME, 20 μg/mL insulin, 0.5 μM retinoid acid (Sigma), 0.05 U/mL penicillin and 0.05 μg/mL streptomycin (Gibco). The cells were grown in these media for 21 days. The combinations of growth factors and concentrations used are shown in Table 1.

Immunocytochemistry

Using a streptavidin-biotin peroxidase system, we could use surface markers and proteins to identify certain cells. Cells were fixed with 4% paraformaldehyde (Sigma) at 4°C for 15 min and washed twice in distilled water before permeabilization in 0.5% Triton-X100 (Pharmacia Biotech, Uppsala, Sweden) for 15 min. Non-specific binding was blocked using blocking reagent. The cells were then incubated for 2 h at room temperature with anti-neuron-specific nuclear protein (NeuN) (dilution 1:100), anti-neurofilament 200 kDa (NF-H) (dilution 1:200) or anti-growth-associated protein-43 (GAP-43) (dilution 1:200) (Chemicon). After washing, cells were incubated with secondary Ab containing biotinylated goat anti-rat IgG and goat anti-rabbit IgG for 15 min. After several washes in PBS, cells were incubated with horseradish peroxidase (HRP)-coupled streptavidin for 10 min. DAB served as chromogen.

RT-PCR

Total RNA from cells was isolated using TriReagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. The RT-PCR process was carried out using a BcaBest RNA PCR Kit (Takara, Shiga, Japan) according to the manufacturer's protocol. To ensure equal distribution of the target, a master mix containing all components except specific primers (Table 2) was generated and then aliquoted to each reaction tube. PCR was performed following 30 cycles of amplification (94°C, 15 seconds; 58°C, 45 seconds; 72°C, 30 seconds) using an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) and then the PCR reaction was resolved on a 1.2% agarose gel. The band was observed under UV light and photographed.

Results

Isolation and expansion of MSC

Adherent cells grown on culture flasks displayed typical morphologies of undifferentiated MSC. Figure 1 shows

Table 1. Growth factors and concentration used in the neuronal induction media

Induction media	Growth factors added	Concentration of the growth factors added
1	Neuron growth factor (NGF) (modified from ref. 30)	10.0 ng/mL
2	Brain-derived neurotrophic factor (BDNF) (modified from ref. 32)	10.0 ng/mL
3	Epidermal growth factor (EGF) (modified from ref. 32)	10.0 ng/mL
4	Epidermal growth factor (EGF)	10.0 ng/mL
	Brain-derived neurotrophic factor (BDNF)	10.0 ng/mL
5	Neuron growth factor (NGF)	10.0 ng/mL
	Brain-derived neurotrophic factor (BDNF)	10.0 ng/mL
6	Neuron growth factor (NGF)	10.0 ng/mL
	Epidermal growth factor (EGF)	10.0 ng/mL
	Brain-derived neurotrophic factor (BDNF)	10.0 ng/mL

Table 2. Sequences of RT-PCR primers for analysis of differentiation-specific gene expression

Gene	Primer sequences	Size (bp)
Adipose-specific genes		
PPARγ2	Sense 5′- GCT GTT ATG GGT GAA ACT CTG-3′	351
	Antisense 5′-ATA AGG TGG AGA TGC AGG CTC-3′	
LPL	Sense 5′- GAG ATT TCT CTG TAT GGC ACC -3′	276
	Antisense 5′- CTG CAA ATG AGA CAC TTT CTC -3′	
Cartilage-specific gene		
Type II collagen	Sense 5′- CTG GCA AAG ATG GTG AGA CAG GTG -3′	294
	Antisense 5′- GAC CAT CAG TGC CAG GAG TGC -3′	
Bone-specific genes		
Osteocalcin	Sense 5′- ATG AGA GCC CTC ACA CTC CTC -3′	294
	Antisense 5′- GCC GTA GAA GCG CCG ATA GGC -3′	
Osteopontin	Sense 5′-CAC CTG TGC CAT ACC AGT TAA AC -3′	220
	Antisense 5′-ATC CAT GTG GTC ATG GCT TT -3′	
ALP	Sense 5′-GTA CTG GCG AGA CCA AGC G -3′	200
	Antisense 5′-AGG GGA ACT TGT CCA TCT CC -3′	
Neuron-specific genes		
NF-H	Sense 5′-GTGGTGGAGAAGTCTGAGAA-3′	1192
	Antisense 5′-CTTTGACTTTCACCTCCTGGG-3′	
Neuro-progenitor genes		
Nestin	Sense 5′-GGCAGCGTTGGAACAGAGGTTGGA-3′	496
	Antisense 5′-CTCTAAACTGGAGTGGTCAGGGCT-3′	
Endogenous control for cDNA		
β-actin	Sense 5′- GGC ACC CAG CAC AAT GAA GA -3′	629
	Antisense 5′- GGC ACG AAG GCT CAT CAT TC -3′	

that long spindle-shaped adherent cells colonized the whole surface, while some flatten morphology was also observed. Further chemical staining showed that the adherent cells stained positively towards NSE and PAS (Figure 2). The results obtained were similar to the characteristics of MSC.

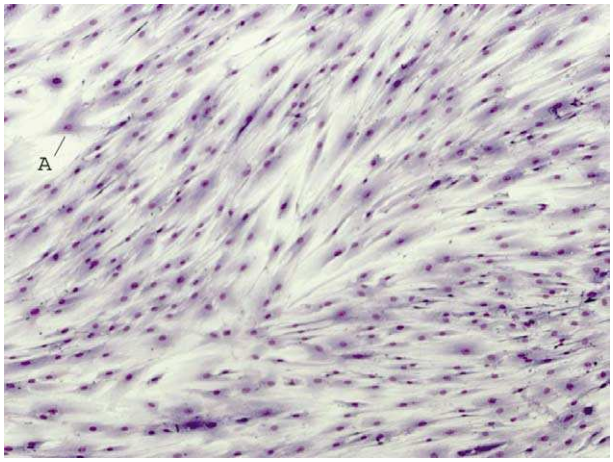

***Figure 1**. The long and spindle-shaped cells divided actively and colonized the whole surface of the plastic cell culture flask, while some larger, flatten morphology (A) was also observed (× 40).*

Immunophenotyping on MSC

Flow cytometry was used to determine the phenotype of MSC. Cell-surface markers of MSC from passage 3 were analyzed. The results showed that the cells expressed at least 80% of the putative markers for MSC, such as CD13, CD29, CD44, CD73, CD90, CD105 and CD147, but lacked expression of CD34, CD45, CD117 and HLA-DR (Figure 3). The negative expression of CD34 and CD45 showed that the cells were free from hematopoietic stem cells as well as mature hematopoietic cells. We needed to obtain a homogeneous population for the next experiment, regarding the transdifferentiation capability of MSC, to show that no contaminant was present and that the transdifferentiation was done by MSC alone.

Differentiation into adipocytes, chondrocytes and osteocytes

After 3–5 days of incubation in adipogenic medium, long and spindle-shaped MSC were observed to change into large polygonal cells. Small refractive vesicles believed to be lipids were seen in the cytoplasm, and these vesicles

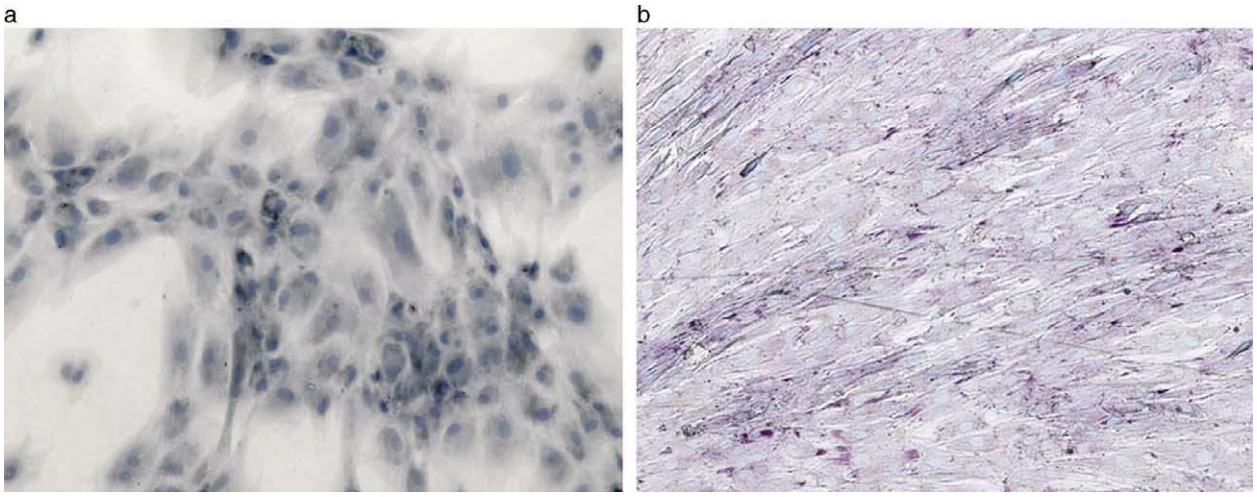

Figure 2. *Cytochemical stain of DMEM-derived adherent cells. The adherent cells stained positively with NSE (a) and PAS (b) (× 100).*

became enlarged and fused together to form bigger lipid droplets (Figure 4a). At the end of the incubation period, almost 90% of the confluent cells had lipid droplets. The lipid was found to stain positively with Oil Red O (Figure 4b). For RT profiling, RNA was extracted from another adipogenic culture from the same sample of MSC. The results showed that the mRNA of PPAR-γ2 and LPL were expressed by the MSC after the induction, whereas uninduced cells did not express either adipogenesis-specific mRNA (Figure 4c).

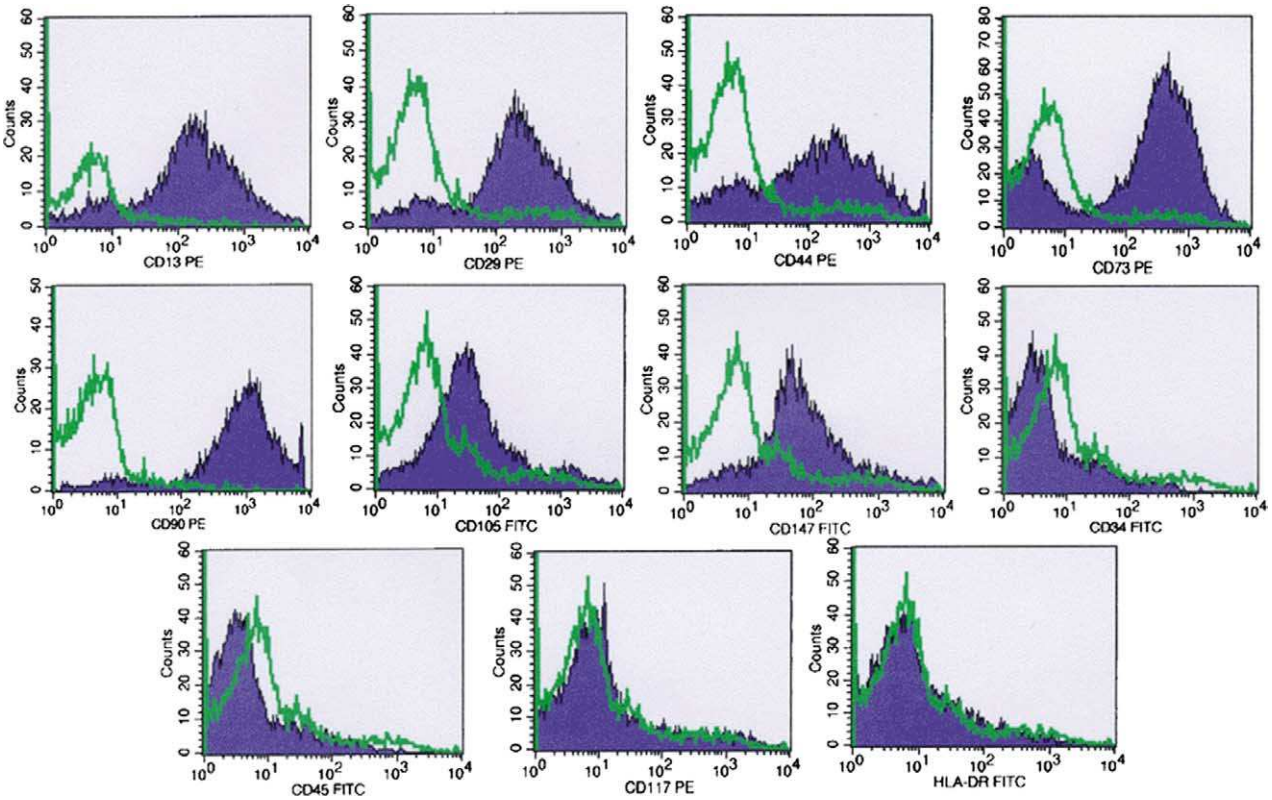

Figure 3. *Flow cytometric analysis showed that, at passage 3, the cell population was rather homogeneous, with 80% expression of the putative MSC markers CD13, CD29, CD44, CD73, CD90, CD105 and CD147. However, this population of cells did not express CD34, CD45, CD117 and HLA-DR, suggesting that the cells were free from contamination by hematopoietic cells.*

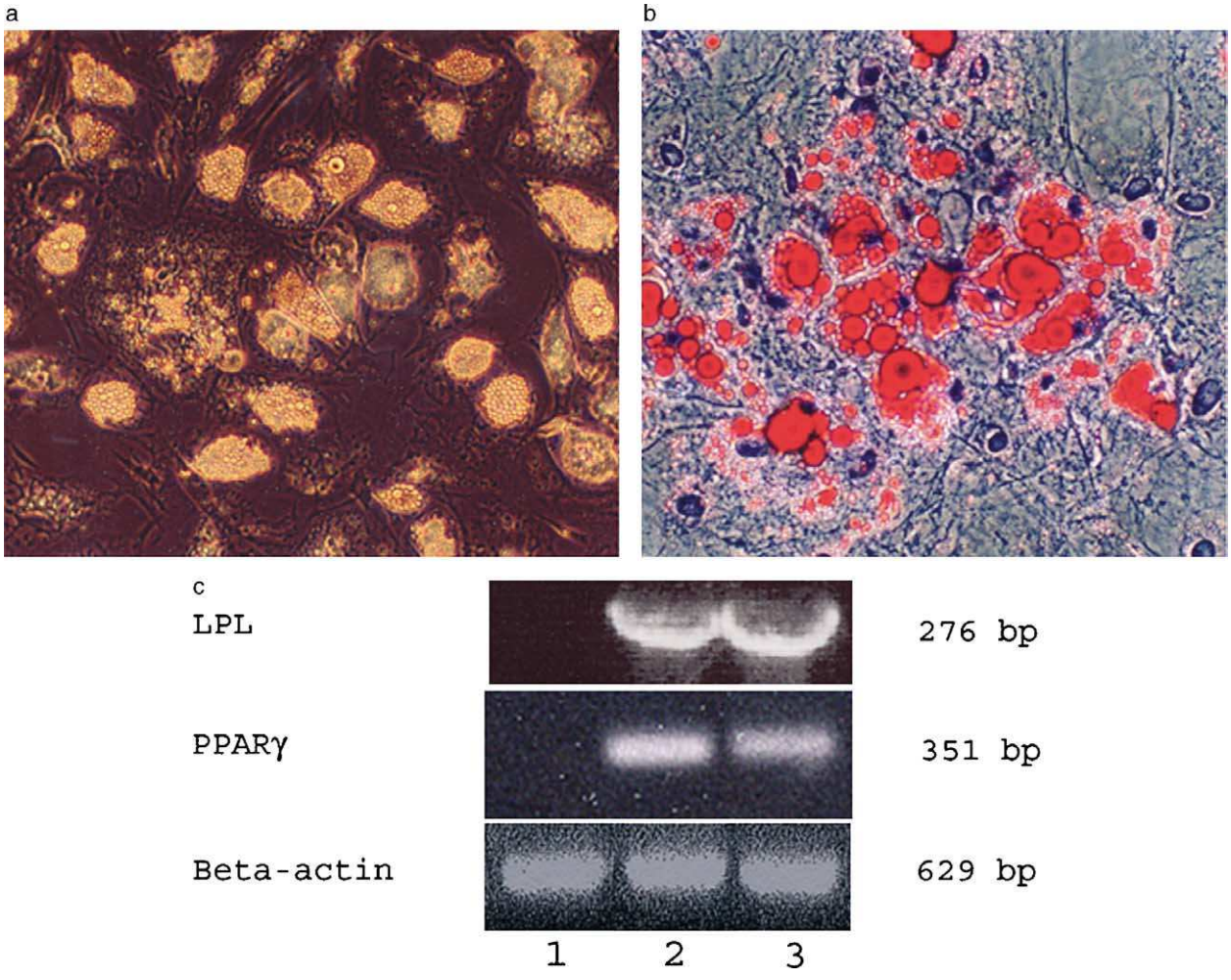

Figure 4. *Adipogenic induction. (a) Incubation of MSC in adipogenic medium (× 40). Most cells had small refractive vesicles, believed to be lipid droplets in the cytoplasm, and some of the vesicles fused together to form bigger droplets at the end of incubation. (b) MSC stained positively with Oil Red O after 3 weeks of incubation in adipogenic medium (× 400). (c) RT-PCR results showed the expression of mRNA-specific genes for adipogenesis, LPL and PPARγ2. Uninduced MSC (lane 1) did not express mRNA for LPL and PPARγ2, but when they were induced chemically the MSC (lane 2) showed the expression of these mRNA. Liposuction aspirate was used as a positive control (lane 3). β-actin served as an internal control. Fibroblasts were used as a negative control (not shown).*

After 4–5 weeks of incubation in chondrogenic medium, the pellet was fixed and stained with Alcian Blue-PAS (Figure 5a, b). Chondrocytes were seen occupying the lacunars and were separated by the matrix. Some of the lacunars were found to contain more than two chondrocytes, known as an isogenous group (Figure 5b). When observed under high magnification, the territorial matrix containing highly negatively charged and low negative charge glycosaminoglycans stained with Alcian Blue, whereas the interterritorial matrix containing low negatively charged glycosaminoglycans and collagen stained pink with PAS. RT-PCR result showed that the chondrogenic pellet expressed collagen type I whereas uninduced MSC did not express the respective mRNA (Figure 5c).

For osteogenic induction, the long spindle-shaped cells started to divide rapidly and become confluent in the plastic culture flasks after 4 days in induction medium. Some crystals were seen deposited sparsely on the cells (Figure 6a). The number of crystals increased and became very crowded towards the end of the incubation, and this caused difficulty in identifying the morphology of the cells. The whole monolayer cells appeared orange red when stained with Alizarin Red S at pH 4.0, and some black-blue lake with sharp edges was observed when the culture was counterstained with hematoxylin (Figure 6b, c). In the

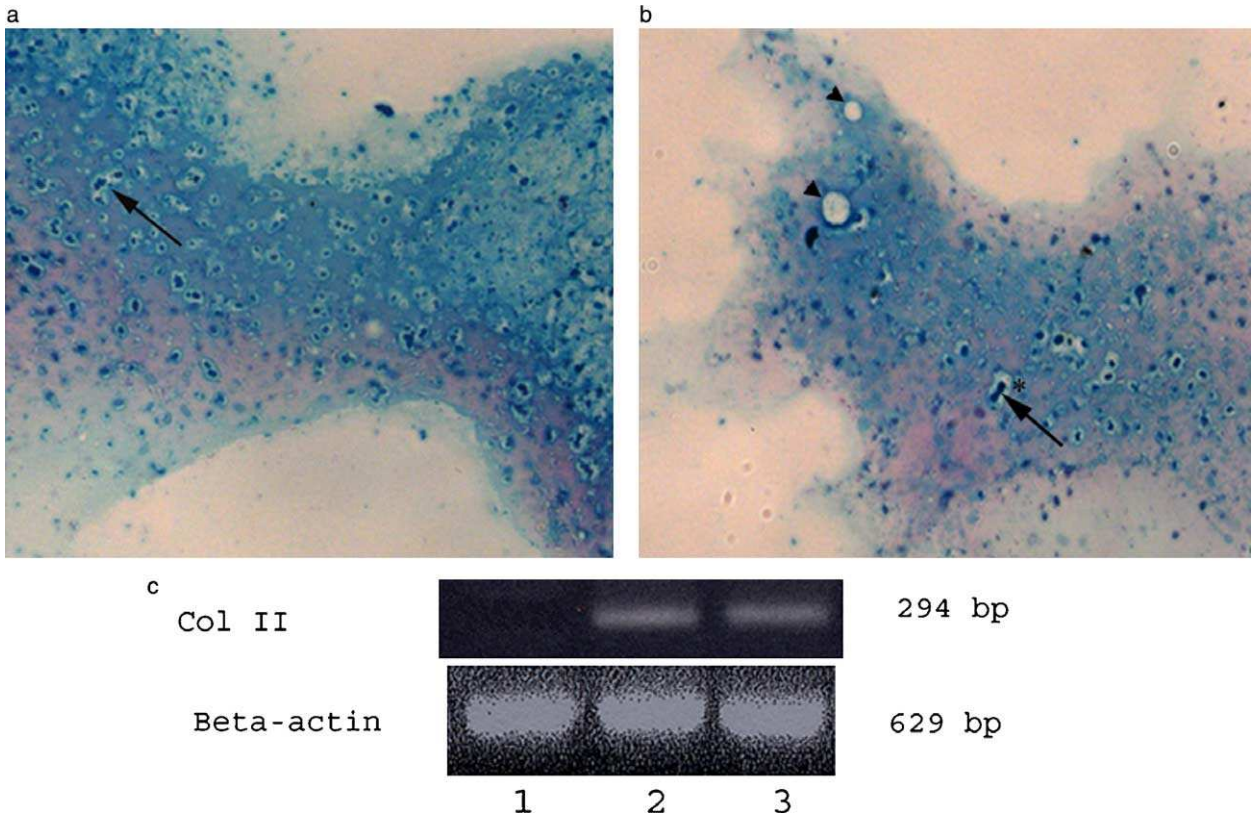

Figure 5. *Chondrogenic induction. (a, b) Alcian Blue-PAS stain on the MSC pellet after a 5-week incubation in chondrogenic medium (× 400). The chondrocytes (arrow) were seen occupying the lacunars surrounded by territorial matrix (stained blue). The interterritorial matrix was stained pink. Some of the lacunars contained two or more chondrocytes, believed to be an isogenous group (arrow *). The cavities in the pellet were mainly artifacts formed during sample preparation for staining (marked by arrow head). (c) The RT-PCR result showed the expression of mRNA of collagen type II. Uninduced MSC (lane 1) did not express collagen type II mRNA but, when they were induced chemically, the MSC (lane 2) showed expression of collagen type II mRNA. Cartilage was used as a positive control (Lane 3). β-actin served as internal control.*

meantime, the uninduced MSC culture proliferated much slower than the osteogenic-induced culture and no crystals were seen. The uninduced cells stained negatively with Alizarin Red S (Figure 6d). The RT-PCR results showed that the induced cells expressed higher alkaline phosphatase and lower osteocalcin and osteopontin compared with uninduced cells (Figure 6e).

Transdifferentiation into neuron-like cells

The ability of BM MSC to transdifferentiate into neuron-like cells was determined by induction in media supplemented with different growth factors. Prior to induction, MSC was cultured in proliferation medium consisting of EGF and FGFβ for about 10 days. After culture in induction media for 21 days, the morphologies of the cells were observed. From all the media used, medium supplemented with EGF, NGF and BDNF managed to induce MSC to produce multi-branches (arrow) and secondary branches. Induced cells in this medium seemed to be forming networks with each other (arrow *) (Figure 7). This induction experiment was conducted on three different samples and all gave similar results (data not shown). A summary of the effects of other growth factors used alone or in combination is shown in Table 3.

Therefore, we used this induction medium further to characterize the differentiation stages of MSC transdifferentiation into neuron cells. The differentiation status was verified based on the expression of neuronal markers by neuronal-induced MSC. Typical neuronal markers, such as GAP-43, NF-H and NeuN, were used to identify the differentiated cells by immunocytochemistry. RT-PCR was used to detect the NF-H mRNA level in induced cells at different differentiation stages.

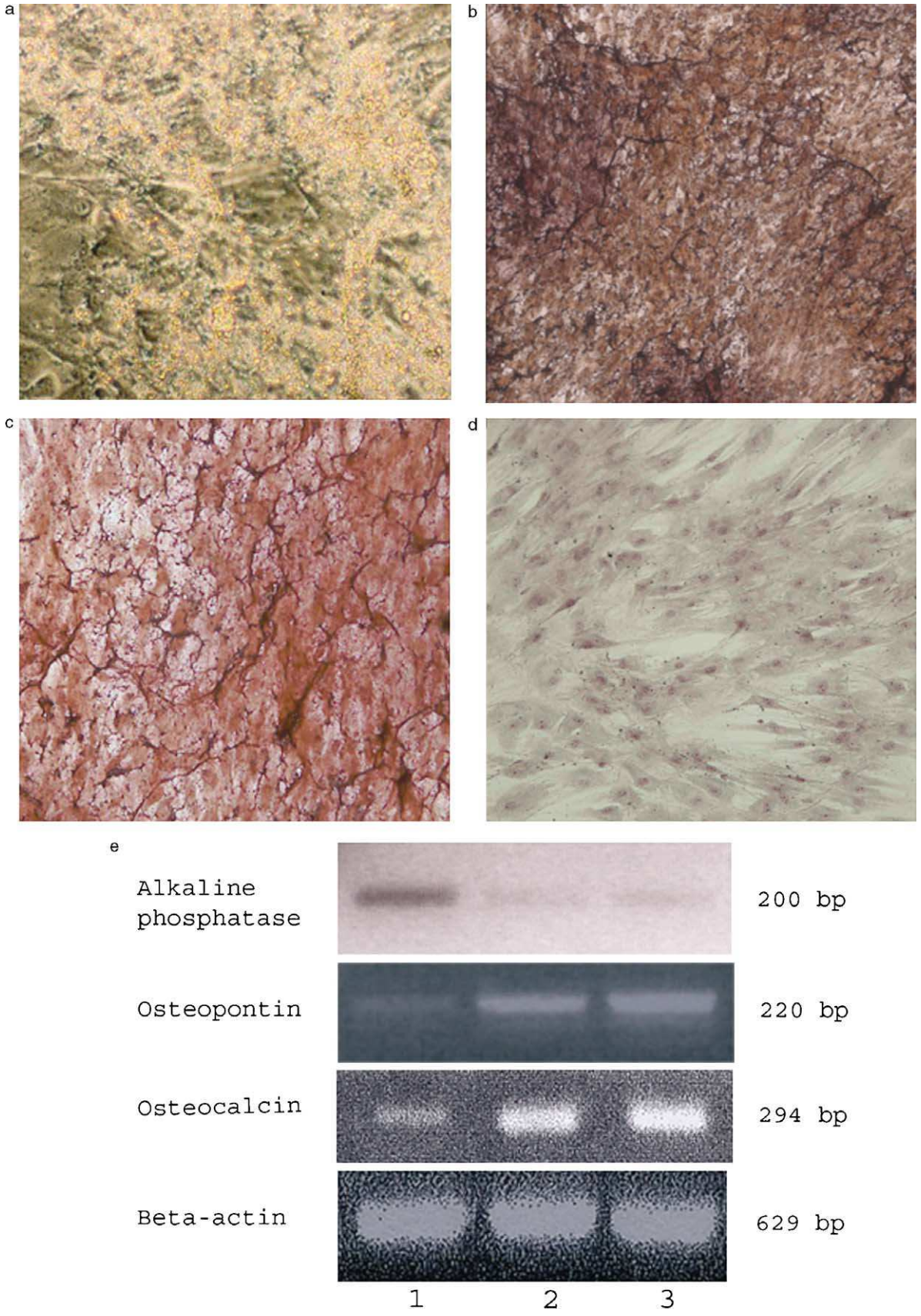

Figure 6 (*Continued*)

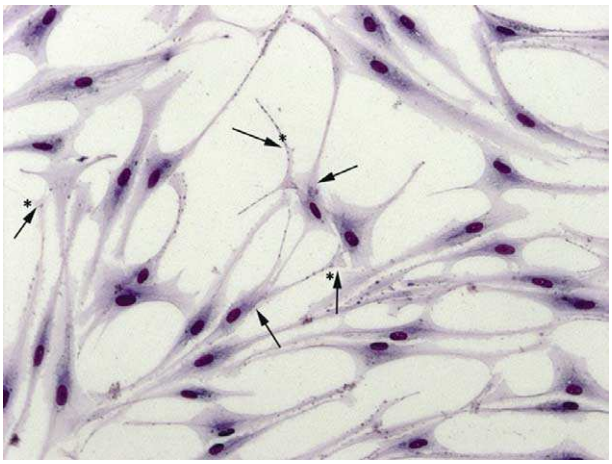

Figure *7. Typical morphologies of neural lineage displayed by neuronal induced-MSC cells. Neuron-like cells with multi-branches (arrow) and bipolar neuron-like cells with further branches or secondary branches (arrow *) at the end of the processes (× 100, Wright–Giemsa stain).*

The expression of GAP-43 was detected after 10 days in neuronal induction, and examination on days 21 and 28 showed that the GAP-43 expression was decreasing (Figure 8a–c). Meanwhile, the expression of NF-H and NeuN demonstrated different findings from GAP-43. The longer the cells were in the induction medium, the better the expression of NF-H and NeuN on differentiated cells (Figure 8d–f, g–i, respectively). Cells were also analyzed by RT-PCR, as shown in Figure 9, following different differentiation stages; at days 14, 21 and 28, all were positive for NF-H. Moreover, the expression of NF-H visibly increased in the later stages of differentiation, at day 28 day (lane 3). A summary of these results is shown in Table 4.

Discussion

MSC were characterized by their ability to proliferate in culture, with a tendency to adhere to the plastic culture flask, and by the presence of a consistent set of protein markers or surface Ag determinants. The results in this study show that MSC can be readily isolated from mononuclear cells of BM aspirate. The initial culture of the mononuclear cells generated a layer of heterogeneous, adherent cells, with at least two different morphologies. This process of generating adherent cells took 3–7 days. Cells with fibroblastic morphology, also described as spindle shape, dominated the flask and were observed to proliferate faster and became confluent after another 3–5 days in culture. After a few passages in culture, a rather homogeneous population of fibroblast-like cells was obtained. The fibroblast-like cells might be MSC because MSC has the ability to adhere to plastic culture flask and also display fibroblastic morphology. However, further characterization is necessary for MSC identification.

As well as observing the morphology to determine the type of cells, the expression of cell-surface proteins is often used in the characterization of different cell types. In this study, flow cytometry was used to determine the expression of cell-surface markers on MSC. The analysis showed that the population of isolated cells was uniformly positive for CD10, CD13, CD29, CD44, CD73, CD90, CD105 and CD147 (Figure 3). Previous researchers have demonstrated that these surface markers are markers used for MSC identification [17–20]. The absence of certain surface markers also helps to characterize MSC. Notable was the lack of expression of the hematopoietic lineage markers of CD34 and CD45, indicating that the cultured cells were free from cells of hematopoietic lineage.

Although no specific surface molecule has been found that would unequivocally identify MSC, the MSC population could still be determined by studying a list of surface molecules and by eliminating the expression of known negative markers, such as markers for hematopoietic lineage. However, there are now putative markers that readily identify MSC, i.e. SH2 and SH3 Ab, originally

Figure 6*. Osteogenic induction. (a) Morphologic observation of osteogenic-induced MSC. Crystals were seen deposited on most of the surface of monolayer cells at the end of the incubation period. The over-crowded crystals made the morphologies of the cells difficult to be distinguished (× 400). (b) The crystals suspected to be hydroxyapatite formed a blue-black lake with sharp edges when stained with hematoxylin. Meanwhile, the amorphous calcium phosphate formed by the action of alkaline phosphatase on the matrix was stained orange red at pH 4.0 with Alizarin Red S. Both alkaline phosphate and matrix were produced by osteoblasts (× 400). (c) Alizarin Red S staining on osteogenic-induced MSC at smaller magnification (× 40). (d) Alizarin Red S staining on uninduced cells showed negative results. (e) RT-PCR results to determine the expression of mRNA-specific genes for osteogenesis. Osteogenic-induced MSC (lane 1), uninduced MSC (lane 2) and bone chips as a positive control (lane 3). The osteocalcin, osteopontin and alkaline phosphatase did not appear to be good osteogenesis markers, as both uninduced MSC and osteogenic-induced MSC samples showed these mRNA expressions. Fibroblasts were used as a negative control (not shown).*

Table 3. Effects of different growth factors towards inducing transdifferentiation of MSC to neurons. MSC was induced in neuronal media for 14–21 days. (−, no protein expression; + / −, slight protein expression; +, protein expression; + + +, high protein expression)

		Immunocytochemistry			RT-PCR	
Induction media	Growth factors added	GAP-43 (day 14)	NF-H (day 21)	NeuN (day 21)	NF-H (day 21)	Nestin (day 21)
1	No growth factor	−	+ / −	−	+ / −	+
2	NGF	−	−	−	−	−
3	BDNF	+	+	+	+	−
4	EGF	−	+ +	−	+ +	+ +
5	EGF & BDNF	+	+ +	+	+	−
6	NGF & BDNF	−	−	−	−	−
7	EGF, NGF & BDNF	+ +	+ + +	+	+ + +	−

developed by Haynesworth *et al.* [21], that recognize CD105 and CD73, respectively. Many investigators have explored CD105 as an important antigenic determinant in the identification of MSC [7,22–24]. Pittenger *et al.* [17] have reported that CD29, CD44 and CD90 are also important determinants.

The differentiation assay showed that MSC could be differentiated into adipocytes, osteogenic lineage cells and chondrocytes based on Oil Red O, Alizarin Red and Alcian Blue-PAS staining, respectively. The differentiated adipocytes exhibited the expression of LPL and PPARγ2 mRNA, whereas the chondrocytes exhibited collagen

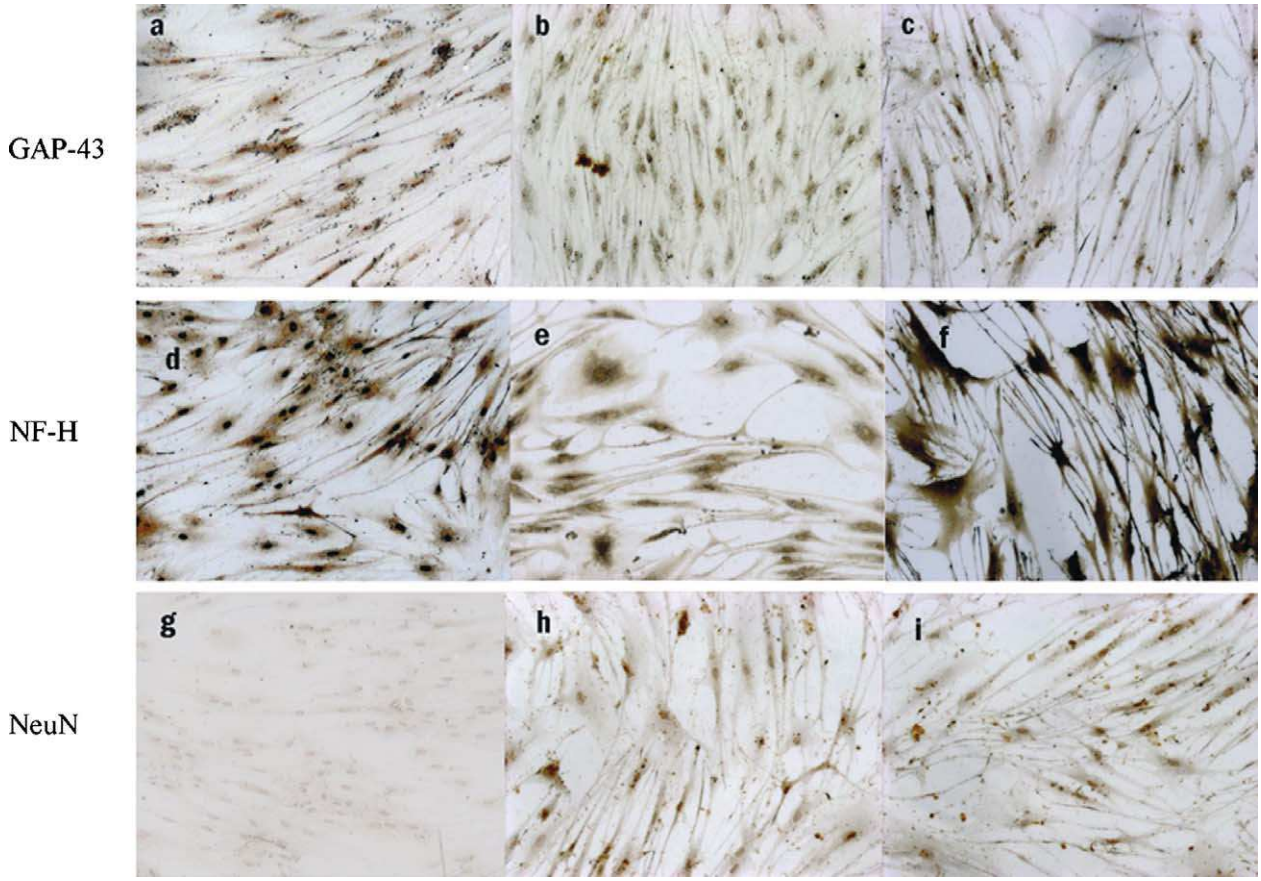

Figure 8. *Expression of neuronal protein on differentiated cells after different duration. (a–c) The expression of GAP-43 (a) 10 days, (b) 21 days and (c) 28 days after induction. (d–f) Expression of NF-H (d) 10 days, (e) 21 days and (f) 28 days after induction (f). (g–i) Expression of NeuN (g) 10 days, (b) 21 days and 28 days after induction (i) (× 100).*

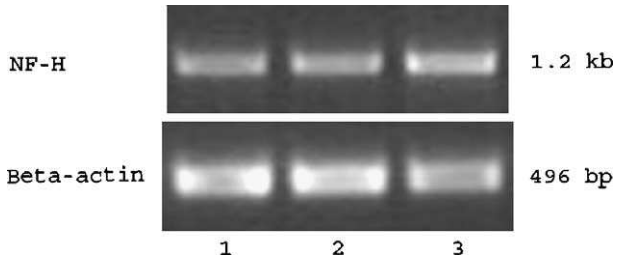

Figure 9. *RT-PCR results of NF-H level in induced cells at different differentiation stages, 14 days (lane 1), 21 days (lane 2) and 28 days (lane 3), on a 1.0% agarose gel. MSC was induced in induction media supplemented with EGF, NGF and BDNF. β-actin served as an internal control.*

type II mRNA expression. Meanwhile ALP, osteocalcin and osteopontin seemed to be unsuitable markers for osteogenesis as the uninduced MSC did have these expressions. The upregulation of alkaline phosphatase, and downregulation of osteocalcin and osteopontin mRNA, expression in induced cells compared with uninduced cells indicated that the osteogenic induction medium had an essential role in the regulation of the transcription of these specific mRNA. The high expression of alkaline phosphatase and low expression of osteocalcin and osteopontin also showed that the induced cells might be differentiating into late osteoblasts or pre-osteocytes at the time when total RNA was extracted from the osteogenic culture.

For a long time, adult stem cells have been thought to be developmentally committed in such a way that they appear restricted to produce specific cell lineages, namely those from where they reside. For example, MSC was thought to differentiate only into mesodermal cells, such as adipocytes, osteocytes and stromal supporting cells. However, recent findings are now challenging this belief [10–14,25,26]. Human BM MSC have been shown to differentiate into cells with neural morphologies (ectodermal cell) and hepatocytes (endodermal cell).

Our study has characterized the effects of growth factors in stimulating MSC transdifferentiation into neurons, defining the expression patterns of neural proteins and correlating expression with differentiation stages. Many studies have been conducted on embryonic stem cells and neural stem cells to study the differentiation into neurons [27–29]. These studies have reported that fetal or adult brain-derived neural stem cells and neuroprogenitors can be expanded with the mitogens EGF and FGFβ, and the differentiation to neurons occurred when FGFβ or EGF was withdrawn. We have exploited this observation in our study.

MSC was induced in neuronal induction medium supplemented with different growth factors, added either alone or in different combinations. The effects of the growth factors in inducing MSC transdifferentiation into neurons were first determined by the morphologic changes on the induced cells. From our observations, after about 21 days in neuronal induction media, morphologic changes could be observed in medium supplemented with EGF and BDNF (data not shown) as well as medium supplemented with EGF, NGF and BDNF. These morphologic changes, such as growth of dendrites and processes, have also been reported by other researchers [14,30,31]. Even though all media could stimulate MSC into neuron-like cells, EGF-, NGF- and BDNF-supplemented medium appeared to be able to induce cells with more advanced neural morphologies, such as multi-branches and secondary processes. Furthermore, neuron-like cells in EGF-, NGF- and BDNF-supplemented medium also appeared to form networks with neighboring cells.

Four different neural proteins, nestin, GAP-43, NeuN and NF-H, were used to study the expression of neural proteins by neuronal-induced MSC. These four proteins could be used to assess the correlation between the protein expression and type of neuron cells produced. Nestin identifies the most primitive neuroepithelium. Many

Table 4. Summary of the neuronal marker expression on differentiated cells, induced in neuronal medium with EGF, NGF and BDNF (−, no protein expression; +/−, slight protein expression; +, protein expression; +++, high protein expression)

	Neuronal marker (immunocytochemistry)			RT-PCR
Differentiation stages	GAP-43	NF-H	NeuN	NF-H
10 days/14 days	++	+	−	+
21 days	+	++	+	+
28 days	+	+++	+	++

studies have shown that undifferentiated MSC express nestin, suggesting that MSC are already committed for neural differentiation [31–33,35]. With differentiation, nestin progressively decreased, which is consistent with the production of more mature neurons [32]. In this study, isolated adherent cells also expressed nestin even before induction (Table 3).

Meanwhile, GAP-43 is expressed in almost all neurons during developmental axonal outgrowth, while NeuN is expressed later and NF-H is associated with mature neurons. After determining that EGF-, NGF- and BDNF-supplemented medium was the better medium for neuronal induction by morphologic observation, differentiation stages were studied and verified based on the expression of neuronal markers, such as GAP-43, NF-H, NeuN and nestin. Cultures were analyzed on days 10, 21 and 28. The cells cultured in this medium needed only 10 days for obvious morphologic changes. Other studies conducted on MSC have needed up to 2 weeks before morphologic changes could be observed [31,32].

The expression of markers seen in MSC is similar to the pattern of expression seen in developmental neurogenesis. Morphologic changes were observed after about 10 days in induction medium supplemented with EGF, NGF and BDNF. This time frame is compatible with the expression of early neural protein, GAP-43, which is related with axonal outgrowth. Expression of GAP-43 was undetectable in undifferentiated MSC. GAP-43 is expressed at an early stage of neural development. As the cultures matured (until day 28), the expression of GAP-43 decreased. Meanwhile, NF-H was observed even on day 10 and MSC before induction also expressed some NF-H. But later in the cultures NF-H expression increased, suggesting that the cultures were generating more mature neurons. NF-H is expressed exclusively by central and peripheral nervous system neurons and appears in more developed forms of neurons.

The expression of NeuN, which was not detectable before induction, was observed at day 21. NeuN expression is observed in most neuronal cells types throughout the nervous system of adult mice [36]. NeuN immunoreactivity is exclusively nuclear and perinuclear, and does not extend into the processes [37]. The immunocytochemical changes were consistent with the transition of immature neurons to mature neurons. The results obtained suggest that MSC behaves like neural stem cells, and differentiate into immature neuron-like cells and then into mature neuron-like cells *in vitro*.

These findings indicate that medium with EGF, NGF and BDNF is more effective in stimulating MSC transdifferentiation, and MSC transdifferentiation into neuron-like cells is similar to the differentiation pattern of neuron stem cells in neurogenesis. When EGF, NGF and BDNF are added together, there might be some synergistic effect that induces MSC into transdifferentiation. By comparing these results with the results obtained with EGF and BDNF, it is suggested that NGF may work later, when the MSC have been transdifferentiated into early stages of mature neurons. Further study needs to be carried out to confirm this observation.

Recent studies have suggested that the location at which a neurotrophin stimulates a cell is a crucial parameter in determining the signaling pathways activated and the ensuing biologic responses [38]. Campenot has demonstrated that NGF increases axon growth only at the location of stimulation, which is the axon, and signaling at the cell body did not help to increase axonal growth. The signaling events that occur within axons serve local functions, including axonal outgrowth and pathfindings, as well as stimulating endocytosis and transport of signaling endosomes [38]. This may explain the results obtained in the induction with EGF, BDNF and NGF. NGF in the medium stimulates axonal growth, and thus the morphology observed in this culture is different from the EGF and BDNF culture. Cells with more processes and longer axons were observed.

Other studies have also worked on determining the transdifferentiation potential of MSC. Woodbury *et al*. [35,39] have reported that, after exposure to β-mercaptoethanol (BME) for less than 3 h, changes in the cell morphology were apparent. However, the cell changes reported that occurred with 60–120 min appear unclear. A further study done by Safford *et al*. [40] showed that the induction method of Woodbury *et al.*, which contains DMSO, resulted in high rates of cell death. Bossolasco *et al*. [31] have demonstrated that a co-culture system with astrocytes is effective in inducing neuroglial differentiation of MSC and $CD90^+$ cells. In their experiments, they compared the ability of whole BM, mononuclear cells, MSC and $CD90^+$ cells to acquire neuronal fates *in vitro*. They found that only MSC and $CD90^+$ cells were able to transdifferentiate into neuron-like cells, and even before induction both cell types expressed neuroglial transcripts.

In conclusion, these results, along with those published by other researchers, suggest that adult MSC have an alternative developmental potential than previously appreciated. Perhaps it is not surprising that MSC can give rise to neural cells, as the marker for neural progenitors, nestin, is expressed in MSC before induction [31–33,35].

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