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Transient Differentiation of Adult Human Bone Marrow Cells into Neuron-like Cells in Culture: Development of Morphological and Biochemical Traits Is Mediated by Different Molecular Mechanisms

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Abstract

Studies on rodent bone marrow stromal cells (MSCs) have revealed a capacity, for at least a portion of cells, to express neuron-like traits after differentiation in culture. Little, however, is known about the ability of human MSCs in this regard. We show here that incubation with certain differentiation cocktails, particularly those that include reagents that increase cellular cAMP levels, produces a rapid (1–4 h) and transient (24–48 h) transformation of nearly all hMSCs into neuron-like cells displaying a complex network of processes using phase or scanning electron microscopic optics. In addition, differentiated human (h) MSCs express increased quantities of neuron-[β-tubulin III, neurofilament (NF), neuronal-specific enolase (NSE)] and glial- [glial fibrillary acidic protein (GFAP)] specific proteins and mRNAs, which are also expressed in low levels in undifferentiated MSCs. In contrast, the mesenchymal marker, fibronectin, which is highly expressed in the undifferentiated state, is reduced following differentiation. These biochemical changes, but not the acquisition of a neuron-like appearance, are partially inhibited by incubation of hMSCs with protein (cycloheximide) and mRNA (actinomycin D) synthesis inhibitors with differentiating reagents. Only incubation with 100 ng/ml colchicine, which disrupts the microtubular cytoskeleton, prevents the conversion of hMSCs into neuron-like cells. These results demonstrate that hMSCs acquire the morphological appearance and the biochemical makeup typical of neurons by independently regulated mechanisms.

INTRODUCTION

Finding a reliable and abundant source of tissue for the replacement of missing neurons in neurodegenerative disease has been a worthwhile but elusive goal. Ethical, moral, and legal issues have surrounded the use of embryonic stem cells or fetal tissues for these purposes. Therefore, the isolation of stem cells from adult tissues, particularly those that are relatively easily accessed, such as bone marrow, have been the subject of recent scrutiny and considerable controversy. In particular, bone marrow stromal cells (MSCs), which normally give rise to a variety of mesenchymal derivatives, such as osteoblasts, adipocytes, myoblasts, and

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chondrocytes (1), have been studied for their ability to differentiate into cells with neural characteristics. Indeed, in animal studies, peripheral injection of these cells (2-7) or transplantation into the blastocyst (8) or directly into the brain (8-10) results in the expression of central nervous system (CNS) markers in donor cells. Similarly, in human postmortem studies, Y-chromosome-labeled neural and glial cells have been identified in the brains of females with bone marrow transplants from male donors (11). Taken together, these findings suggest that donor MSCs can give rise to CNS derivatives. However, a number of recent studies have opposed that position, reporting little evidence for the appearance of labeled bone marrow-derived cells in host chimeric brains (12,13). This picture was further complicated by the discovery of spontaneous cell fusion of donor marrow with host cells as a mechanism for the change of phenotype in some cells (14-16).

Much of the disagreement over the multipotentiality of MSCs in vivo has been attributed to differences in the source of cells and experimental protocols. Similarly, in culture, MSCs can behave in a variable fashion. Undisputed, however, is the fact that murine and rodent MSCs can be converted into process-bearing cells resembling neurons and express a variety of CNS proteins when they are incubated in media containing particular reagents (17-19) or grown in co-culture with certain tissues (20). However, little is understood about the underlying transcriptional, translational, or mechanical mechanisms mediating these morphological and biochemical phenotypic changes, nor the degree to which human (h) MSCs share this plasticity.

Therefore, in the present study, we sought to determine whether hMSCs can indeed forsake their mesenchymal heritage to acquire morphological and biochemical traits of a neuronal phenotype when they are treated with the appropriate differentiation cues in culture, and, if so, the stability of those phenotypic changes. Moreover, we explored the underlying mechanisms mediating these changes, determining whether they require transcriptional, translational, or merely mechanical modifications of the cell. These findings have previously appeared in abstract form (21,22).

METHODS

Tissue recovery

Human bone marrow cells were isolated from volunteers undergoing spinal surgery (using a protocol approved by the Jefferson University Institutional Review Board). All patients were <60 years of age, had no past history of alcohol abuse or steroid use and were undergoing surgery as a result of traumatic injury. A 5- to 6-ml volume of bone marrow was harvested from the iliac crest into a 10-ml syringe containing 1 ml of heparin (10,000 units/ml). Marrow suspension was spun (600 × *g*; 6 min) to sediment blood cells from marrow.

Tissue culture

The marrow cells were then transferred to a tissue culture flask containing Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum (FBS) and penicillin/ streptomycin. Adherent cells were refed every 3 days and expanded in monolayers for up to 3 weeks at 37°C at 5% CO_2. Cells were used before undergoing passage 3 and while still displaying an undifferentiated fibroblast-like phenotype. Cells from the stromal cell line hMPC 32F were maintained in the same media described above. Adherent cells were re-fed every 3 days and expanded in monolayers for up to 3 weeks at 37°C at 5% CO_2. Glial beds were generated from postnatal day 1 mouse as described previously (23). After reaching confluence, MSCs were either directly seeded onto glial beds or conditioned medium (CM) was collected and fed to undifferentiated hMSCs or previously differentiated hMSCs as detailed in Table 1. Cultures of embryonic day-13 caudate neurons were established as described previously (24). Primary

human neural progenitors (HNPs) (derived from 19- to 21-week fetuses) were purchased from Clon-express, Inc. (Gaithersburg, MD) and maintained in culture as described in ref. 25.

Immunocytochemistry

Fixed cultures were processed using polyclonal antibodies to tyrosine hydroxylase (TH; Protos), glial fibrillary acidic protein (GFAP; Sigma), neuronal-specific enolase (NSE; Polysciences), neurofilament (NF; Sigma), fibronectin (FN; Polysciences), and mouse monoclonal antibodies to β-tubulin III (β-Tub III; Sigma). Secondary antibodies (donkey anti-rb-FITC; donkey anti-mouse-FITC) were purchased from Jackson Immunoresearch. All antibodies were used at a 1:100 dilution and staining analyzed on a Nikon-Scanalytics Image System.

Scanning electron microscopy

Cells were rinsed three times with 0.1 M cacodylate buffer, pH 7.2, and fixed overnight in cacodylate-buffered 2.5% glutaraldehyde at 4°C. The specimens were post-fixed in 1% OsO_4 for 1.5 h, dehydrated with a series of ethanols, dried in a critical point dryer (VG Microtech, East Grinstead, UK), mounted onto aluminum stubs, sputter coated with gold, and viewed under a scanning electron microscope (JEOL 840, Peabody, MA).

Western blotting

Cultures of undifferentiated and differentiated hMSCs were rinsed, harvested, pooled, and homogenized using a Dounce homogenizer in 0.2 ml of lysis buffer containing 1% (wt/wt) NP-40, 0.5% (wt/vol) sodium deoxycholate, 10 mM phosphate-buffered saline (PBS), pH 7.2, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 0.5% (wt/vol) sodium vanadate, 0.15 M NaCl, 50 mM sodium fluoride, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethyl sulfonyl sulfate (PMSF), 2 μg/ml leupeptin, and 5 mg/ml aprotinin. The cells were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected and the pellet discarded. Samples containing 25–50 μg of protein were analyzed by 8% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to an ImmunoBlot PVDF membrane (Bio-Rad). The membranes were blocked with Blotto A (Santa Cruz) and then incubated for 1 h in primary antibody [from sources described above at the following dilutions: NSE 1:1000; GFAP 1:1000; β-Tub III 1:500; FN 1:5000; NF 1:2000; and for β-actin 1:500 (from Santa Cruz)] followed by horseradish peroxidase conjugated rabbit anti-mouse or goat anti-rabbit IgG (1:1000, Santa Cruz). The target protein bands were detected by electrochemical luminescence (ECL) (Amersham).

Quantitative RT-PCR

The real-time PCR experiment was performed according to the modified instruction manual of the kit Cells-to cDNA™ II (Ambion) and the protocol of SYBR Green PCR Master Mix and RT-PCR (Applied Biosystem). Briefly, cells cultured at 2×10^5/dish were harvested and cell number determined. Cells were diluted (5×10^5) and incubated with lysis buffer (75°C for 10 min.). DNase was added into each tube with 0.06 U/μl of final concentration. Genomic DNA was digested by incubating cell lysate at 37°C for 45–60 min. In the pilot experiment, the RT reaction was conducted by incubating 5–10 μl of cell lysate, 4 μl of dNTP mix, 2 μl of 16 μl random decamers, and 5–10 μl of nuclease-free water (70°C for 3 min) followed by the addition of 2 μl of 10 RT buffer, 1 μl of M-MLV RTase, and 1 μl of RNase inhibitor supplied in the kit at 42°C for 1 h. The PCR reaction for each target gene and the internal control gene β-actin was performed according to the manual. By comparing the intensity of each PCR band resolved by an agarose gel electrophoresis, a suitable cell number ($2–5 \times 10^5$) for further cDNA preparation was chosen.

For further analysis of the expression of genes, the real-time PCR was performed with the agents supplied in the ABI Prism7700 Sequence Detection System kit at the conditions (50°C for 2 min, 95°C for 10 min, 95°C 15 sec, and 60°C 1 min, total 40 cycles). Serial titration was conducted with different concentrations of each pair of primers (1 nM to 100 nM) and different volumes of the template of each gene and of β-actin gene (1 nM to 50 nM). The best concentrations, which gave the same efficiency in the real-time PCR for each target gene and internal control gene β-actin, was determined. Six duplicate samples were run in each real-time PCR reaction to calculated mean value of C_T for each amplified gene. The results were analyzed with the Sequence Detection System software 1.7 and computed according to Comparative C_T Method for relative quantitation of gene expression (Applied Biosystems).

RESULTS

We began our studies by comparing a number of different reagent cocktails, several published (20,26-28), others of our own devising, for their ability to differentiate human MSCs into cells that can express neuronal phenotypic traits in culture (Table 1). To do so, cells were grown in culture either as primary hMSCs harvested from patients as described in the Methods section or from a previously established hMSC cell line, hMPC 32F (29). In general, the cell line responded to differentiation cocktails in a fashion comparable to the primary cells. Although several differentiation protocols, including growth with glia or glial-derived factors, produced a low level of neuronal-like differentiation, the greatest change in cells was observed when primary hMSCs or hMPC 32F cells were incubated with cocktails containing agents that amplified cAMP levels, such as the adenylate cyclase activator forskolin or the phosphodiesterase inhibitor IBMX. Particularly effective was a cocktail adapted from our previous studies on dopamine (DA) differentiation (30,31). In addition to PKA activators, this cocktail also contained the protein kinase C (PKC) activator TPA (4β-12-0-tetradecanoylphorbol 13-acetate), which itself has no effect on the morphology of hMSCs, but in synergy with the other reagents, produced a rapid and marked transformation of nearly all cells from a typical mesenchymal appearance in the undifferentiated state to a phase-bright, process-bearing profile, more typical of neurons (Fig. 1A,B), after differentiation for 1–4 h. These dramatic morphological changes were similarly reflected at the scanning electron microscopic level (Fig. 1C,D) as cells shifted from a flattened, sheet-like morphology to bipolar or multipolar cells eliciting finely branched processes. Importantly, hMSCs did not differentiate into neuronal appearing cells using a sequential incubation protocol (DM + 100 ng/ml bFGF 7 days, 10 ng/ml FGF8 + 100 ng/ml SHH 7 days, 10 ng/ml BDNF 7 + days Glia 5d) recently described by Jiang et al. (32) to differentiate mouse multipotent adult progenitor cells (MAPC) that co-purify with bone marrow mesenchymal stem cells.

When the time course of differentiation was studied (Fig. 2), we found that primary neurons or cells from the MSC cell line converted into “neuron-like” cells, often within an hour of incubation with the differentiation cocktail. Primary hMSCs cells remained transformed for up to 48 h before spontaneously returning to the undifferentiated state (solid black line). Even the continual re-exposure of cultures every 24 or 48 h to the differentiation cocktail could not sustain their neuron-like appearance, and often resulted in the death of hMSCs, probably due to reagent-induced toxicity. Although many different reagent cocktails were tested (data not shown), only the subsequent incubation of differentiated cells with glial CM prolonged the appearance of neuron-like properties in hMSCs beyond 1–2 days. Even so, by 5 days, differentiated hMSCs grown in glial CM too began to die (Table 1). On the other hand, differentiation could be intentionally reversed by removal of the cocktail after 4 h and replacement with unsupplemented DM (Fig. 2, semi-dashed line). Following their dedifferentiation, few cells could be reinduced to express a neuron-like appearance upon re-exposure to the differentiation cocktail at 48 h. Although the cell line responded similarly to differentiation cues, the time course of dedifferentiation differed from primary cells. Thus,

with continual supplementation with the cocktail, nearly half of the hMPC 32F cells maintained a neuronal appearance even at 72 h as cells gradually reverted back to a stromal appearance (Fig. 2, solid line with square box). These cells also could not be reinduced if cocktail was removed early on and refed later (Fig. 2, dashed line).

We next wondered whether as hMSCs changed in their appearance, their complement of proteins also shifted from those characteristic of mesenchymal cells to those normally associated with the nervous system. Thus, 4 h after hMSCs were treated with TPA + IBMX + forskolin, cultures of differentiated hMSCs were fixed and stained and compared with undifferentiated hMSCs for the presence of the mesenchymal marker FN or the CNS markers β-tub III, NF, and NSE or GFAP. Although FN stained most intensely, CNS proteins were also observed at low levels in undifferentiated cells (Fig. 3A-E) as had been noted previously (20, 26,33). Likewise, differentiated neuron-like cells also expressed all markers, including low levels of FN (Fig. 3F-J). Because only pale background labeling was seen with preimmune serum, or when primary antibodies were eliminated (data not shown), this low-intensity staining is presumed to be specific. Further indicative of the specificity of staining is the fact that cultures containing neuronal progenitors stained for neuronal markers (Fig. 3K-M) but not GFAP or FN (data not shown), GFAP-stained astrocyte cultures (Fig. 3N) but not neurons or fibroblasts (data not shown), and fibroblast cultures stained for FN (Fig. 3O) but not other CNS markers (data not shown).

When neuronal-like hMSCs were examined for the appearance of other differentiated traits, such as the DA biosynthetic enzyme TH, we found no specific labeling of undifferentiated (Fig. 4A) or differentiated MSCs (Fig. 4B) despite the ability of the differentiation cocktail to induce de novo TH expression in brain-derived neurons (Fig. 4C) (34). Taken together, these results suggest that hMSCs can be differentiated to express discernible levels of general neuronal antigens but not neurotransmitter-related phenotypic traits.

Although our immunocytochemical results indicated the presence of both mesenchymal and CNS markers in hMSCs, regardless of their differentiative state, because immunocytochemistry is at best a semiquantitative procedure, it was not clear whether the amounts of these proteins and/or their mRNAs were changing with differentiation or whether there was merely a reorganization of intrinsic substances. Therefore, we re-examined this issue using western blot analysis (Fig. 5) and real-time RT-PCR (Table 2). We found that the FN protein was present in much higher quantities in undifferentiated hMSCs (Fig. 5), while differentiated hMSCs (Fig. 5) conversely contained greater amounts of β-tub III, NF, NSE, and GFAP. Similarly, when mRNA levels were semiquantitated by real-time RT-PCR, we found that, consistent with changes in proteins, the mRNA levels for FN decreased while CNS mRNAs increased with the a neuron-like morphology in differentiated hMSCs.

We next tested whether the increased amounts of neuron-related mRNAs and proteins found in differentiated hMSC cultures reflected an increase in synthesis of these substances. To do so, cultures of hMSCs were incubated for 30 min with the mRNA inhibitor actinomycin D (100 ng/ml) or the protein synthesis inhibitor cycloheximide (100 ng/ml) prior to a 1-h incubation with the differentiation cocktail. Cultures were then imaged using phase-contrast optics, fixed, and stained immunocytochemically or sister cultures were harvested for Western analysis (Fig. 5) or real-time RT-PCR (Table 2). Importantly, cycloheximide (Fig. 5) and actinomycin D (Table 2) partially prevented cocktail-induced increases in proteins and mRNAs above undifferentiated levels. However cells continued to differentiate into neuronal-looking cells by phase microscopy (Fig. 6), despite the presence of cycloheximide and actinomycin D in the cultures, suggesting that the morphological and biochemical responses to the differentiation cocktail were independently regulated in hMSCs.

The fact that the morphological conversion of hMSCs into process-bearing cells did not depend on changes in proteins and mRNAs suggested that these effects might instead be due to a reorganization of existing cytoskeletal proteins. To test this possibility, cultures were co-incubated with the differentiation cocktail and increasing doses of colchicine, a drug known to disrupt micro-tubules (35). As seen in Fig. 7, hMSCs were prevented from assuming a neuron-like appearance with the addition of increasing concentrations of colchicine (up to 1 μg/ml which is toxic to cells). Thus, at 100 ng/ml of colchicine, the morphology of nearly all hMSCs remains mesenchyme-like despite the presence of differentiation cues in the cocktail, suggesting that these cues normally mediate their effects through a functioning microtubule system.

DISCUSSION

The results of the present study demonstrate that hMSCs derived from adult human bone marrow express low levels of neuronal (β-tub III, NF, NSE) and glia- (GFAP) specific proteins and mRNAs, even when maintained in the undifferentiated mesenchymal state. However, incubation with certain differentiation cocktails, particularly those containing forskolin, produce a rapid and reversible, though transient, transformation of nearly all hMSCs into neuronal-like cells bearing elaborate neuritic processes. Moreover, differentiated hMSCs express increased quantities of neuronal and glial proteins and mRNAs, and, conversely, decreased levels of the mesenchymal marker fibronectin. As might be expected, these biochemical changes could be partially inhibited by incubation of differentiated hMSCs with protein and mRNA synthesis inhibitors. Surprisingly, however, these inhibitors did not prohibit the acquisition of a neuron-like morphology in differentiation medium. Only disruption of the microtubular cytoskeleton with colchicine prevented the conversion of hMSCs into neuron-like cells.

Although previous studies had established that murine and rodent MSCs were capable of undergoing a transformation in appearance if incubated with certain reagent cocktails (17,18, 20), interestingly, those differentiation protocols yielded little or no change in human MSCs in our study. Indeed, only the inclusion of reagents that increased cAMP levels [or activated protein kinase A (PKA)] such as forskolin or IBMX, resulted in a significant morphological transdifferentiation of hMSCs (nearly 50%) into neuron-like cells (21,22). Rodent MSCs are similarly responsive to the presence of forskolin (26) or dibutyryl cAMP (27). In the present study, we further show that the addition of the protein kinase C (PKC) activator, TPA, which itself has no effect on the morphology of hMSCs, works in synergy with PKA activators, increasing from 50% to nearly 100% the number of hMSCs that adopt a neuronal appearance.

However, this dramatic conversion in cells was short-lived, with cells either spontaneously reverting back into mesenchymal cells 48 h after differentiation or undergoing cell death. Although co-culture with glia or media conditioned by glia postponed by 1–2 days the reversion of hMSCs back to a mesenchymal phenotype, we could find no way to permanently maintain their neuronal appearance. Indeed, even in rodent MSC studies, the longest reported period of sustained cell transformation into neuron-like cells is 1 week (17,20), during which there is a down-regulation of neuronal markers such as nestin (17). Taken together, these data suggest that hMSCs “are disinclined” to forsake their usual fate permanently (as mesenchymal derivatives) for a more atypical fate (as neuroectodermal derivatives).

When we examined the biochemical make-up of hMSCs, we found as others working with rodent MSCs had seen previously (26,33), that even undifferentiated FN-containing MSCs expressed low levels of many of the markers usually associated with neurons (NF, NSE, β-Tub III) and glia (GFAP), suggesting that they may in fact be multipotent cells. Supporting this supposition is the finding that MSCs can differentiate in vivo into cells of all three germ layers,

including neurons and glia (2-9,11). When hMSCs were further differentiated with the protein kinase cocktail, cells not only acquired a more neuronal appearance, but there was a concomitant shift in their protein and corresponding mRNA profiles, with a decrease in FN and an increase in NF, NSE, β-Tub III, and GFAP. Thus, under varying differentiation conditions in vitro and in vivo, cell-specific markers are either up- or down-regulated at both the transcriptional and translational levels in MSCs.

Importantly, however, when we examined the mechanism by which this transformation occurred, we found that morphological changes were indeed dissociated from biochemical events in hMSCs. Thus, while protein and mRNA synthesis inhibitors (cycloheximide or actinomycin D, respectively) prevented the up-regulation of CNS proteins and mRNAs in differentiated hMSCs in predictable fashion, they did not hinder the conversion of cells to a phase-bright, process-bearing morphology. Only colchicine, which causes severe disturbances to the microtubular cytoskeleton (35), prevented in a dose-dependent manner the morphological conversion of cells following their incubation with the differentiation cocktail. In fact, several reagents present in the cocktail are known to have profound effects on the cytoskeleton. Forskolin, via PKA, increases CREB phosphorylation of micro-tubule-associated protein 2 (MAP2). Because MAP2 is an anchoring protein in dendrites, it serves to stabilize microtubules (36-38). Likewise, TPA, by disrupting actin stress fibers, can cause significant changes in fibroblast contractility (39,40). Thus, it is possible that the transfiguration of hMSCs into process-bearing cells that resemble neurons merely reflects a mechanical reorganization in cytoskeletal elements by components present in the differentiation cocktail. In such a case, the synthesis of new mRNAs and proteins would not be required, thus explaining the lack of effect by cycloheximide or actinomycin D in this regard. Although the differentiation cocktail used here may have the capacity both to affect the cytoskeleton mechanically and enhance the differentiation of neuronal proteins in hMSCs, the possibility exists that, under other circumstances, MSCs may be persuaded to ‘look’ like neurons but not differentiate biochemically along neuronal lines. Therefore, caution must be exercised in interpreting purely morphological changes in these cells as has been done frequently in the past.

Finally, despite the fact that a differentiation cocktail was used in these studies that is known to induce novel expression of TH in neural stem cells (24,25,30,31,41,42) and ES cells (unpublished observations), we found no evidence of TH differentiation in hMSCs. Possibly because gestation is relatively long in humans, there was not ample incubation time for the differentiation of DA traits in hMSCs in culture. Indeed, we have previously found that human carcinomal stem cells (43,44) and human neural progenitor cells (25) require several days of treatment with the differentiation cocktail for the induction of TH expression. Perhaps if it were possible to maintain hMSCs for longer periods in the differentiation cocktail without phenotypic reversion or death, the induction of TH expression may have been possible. Thus far, only one experimental paradigm has resulted in the appearance of TH in a substantial number (30%) of marrow stem cells in culture (8,32). In these studies, TH was expressed by cells that were sequentially incubated with various growth factors over an extended period (21–28 days). Of greater significance than the time of incubation or the cocktail ingredients, however, is the fact that, in these studies, the cells were derived from a rare cell type present in mouse bone marrow, called a MAPC (28). MAPCs behave both in vitro (8,32) and following injection into a blastocyst (28), like embryonic stem (ES) cells. Consequently, in vivo they give rise to derivatives of all three germ layers, and in vitro they can be differentiated into TH-expressing neurons according to published protocols first described by McKay and co-workers to induce expression of TH in ES cells (45,46). In contrast, the hMSCs used here did not express these neuronal traits after incubation with these agents (Table 1; DM + 100 ng/ml bFGF 7d, 10 ng/ml FGF8 + 100 ng/ml SHH 7d, 10 ng/ml BDNF 7d), suggesting that our cultures did not contain MAPCs. Because, in vivo, only an occasional MAPC has survived transplantation

into the brain and none of these cells have expressed neuroectodermal markers (28), the potential utility of MAPCs remains questionable regardless of their capacity to express TH in culture.

Future studies on hMSCs will be directed at the further characterization of these cells and the mechanisms by which they express neuronal traits as well as their physiological function. Only when we find new ways to direct appropriately and, maybe more importantly, maintain the specification and neuronal function of hMSCs can these adult stem cells fulfill their potential use as autologous graft tissue for the treatment of neurodegenerative disease.

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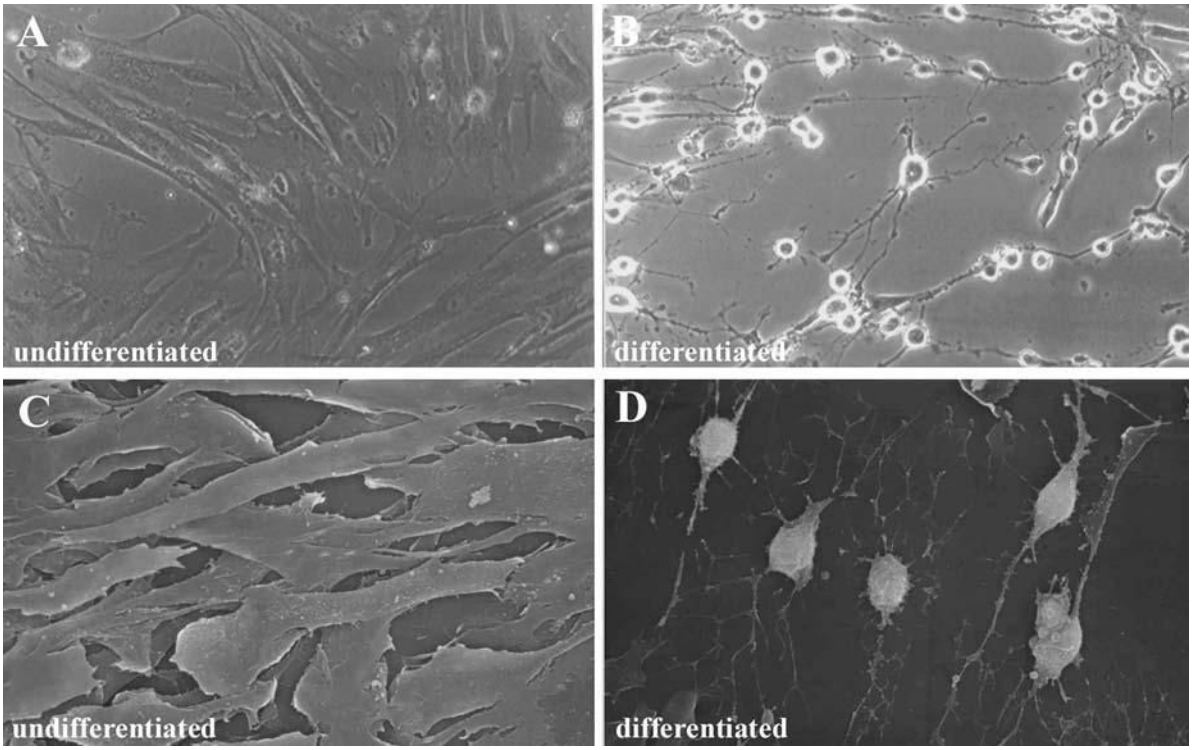

FIG 1.

Photomicrograph of human MSCs grown as undifferentiated cells in serum-free media (**A,C**) or following treatment with a differentiation cocktail containing 200 nM TPA 250 μM IBMX + 50 μM forskolin for 1 h (**B,D**). Cells are detected with phase-contrast optics (**A,B**) or by scanning electron microscopy (**C,D**).

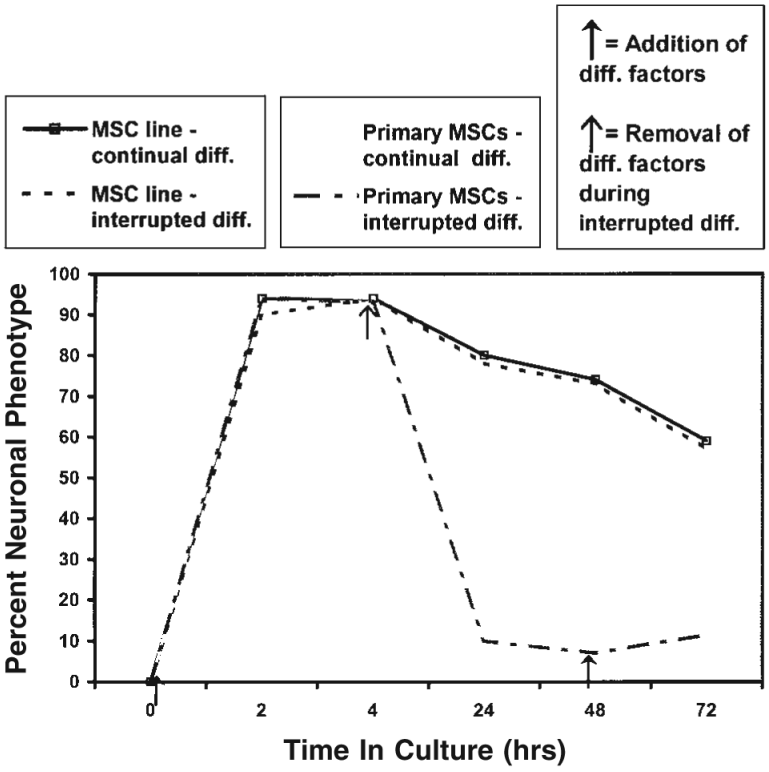

FIG 2.

Time course of differentiation of primary MSCs or hMPC 32F cell line after continual or interrupted treatment with the differentiation cocktail (200 nM TPA + 250 μM IBMX 50 + μM forskolin) for 72 h. Thick pointed arrows indicate the time point at which the differentiation cocktail was added during both the continual and interrupted differentiation protocols. The thin-pointed arrow indicates when the differentiation medium was removed and replaced with the control medium during the interrupted differentiation protocol.

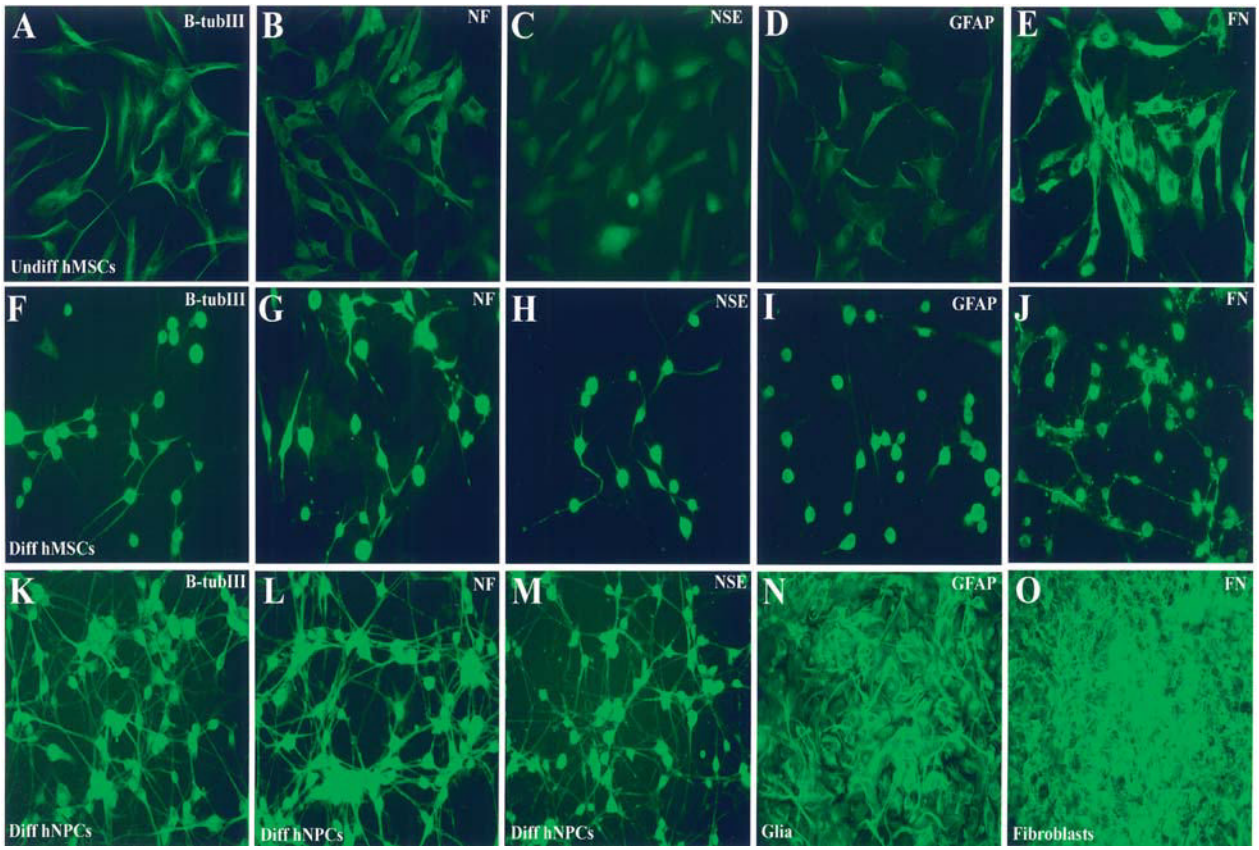

FIG 3.

Immunocytochemical localization of β-Tub III (**A,F,K**), NF (**B,G,L**), NSE (**C,H,M**), GFAP (**D,I,N**), and FN (**E,J,O**) in undifferentiated (**A–E**) or differentiated (**F–J**) MSCs. As positive controls for staining, human neuronal progenitor cells were stained for neuronal markers (**K–M**), newborn mouse astrocytes were stained for GFAP (**N**) and skin fibroblasts derived from newborn mouse was stained for FN (**O**). Differentiation was achieved by a 1- to 4-h incubation in media containing 200 nM TPA + 250 μM IBMX + 50 μM forskolin before fixation and staining.

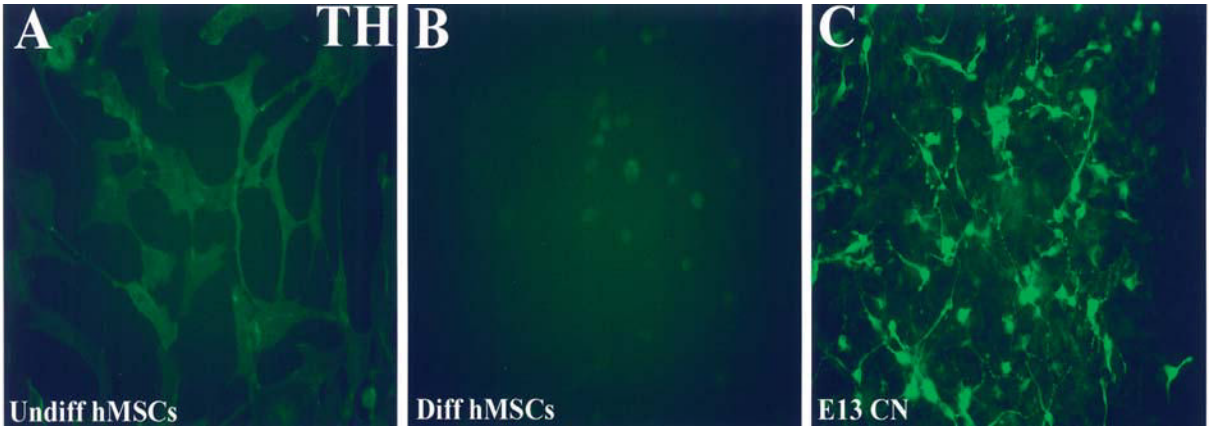

FIG 4.

Immunocytochemical localization of tyrosine hydroxylase (TH) in undifferentiated MSCs (**A**) and differentiated MSCs (**B**). Note the absence of specific staining compared to the positive control of TH-induced E13 caudate neurons (**C**). These cells have been previously shown to express TH when treated overnight with the differentiation cocktail (24,30,31).

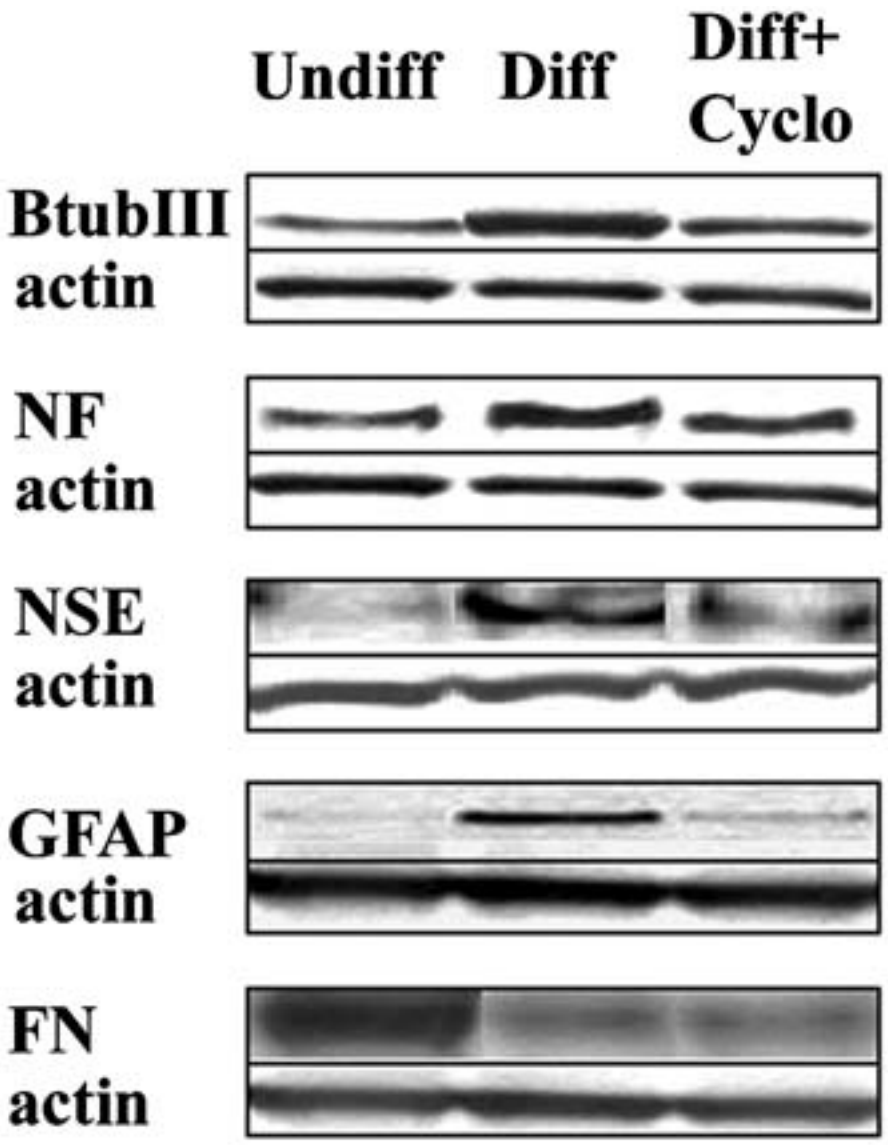

FIG 5.

Immunoblot analysis of undifferentiated (Undiff) and differentiated (Diff) MSCs, were incubated with the mRNA inhibitor actinomycin D (100 ng/ml ActD) or the protein synthesis inhibitor cycloheximide (100 ng/ml Cyclo) for 30 min prior to the differentiation step. β-Actin served as an internal standard to control for differences in gel loading.

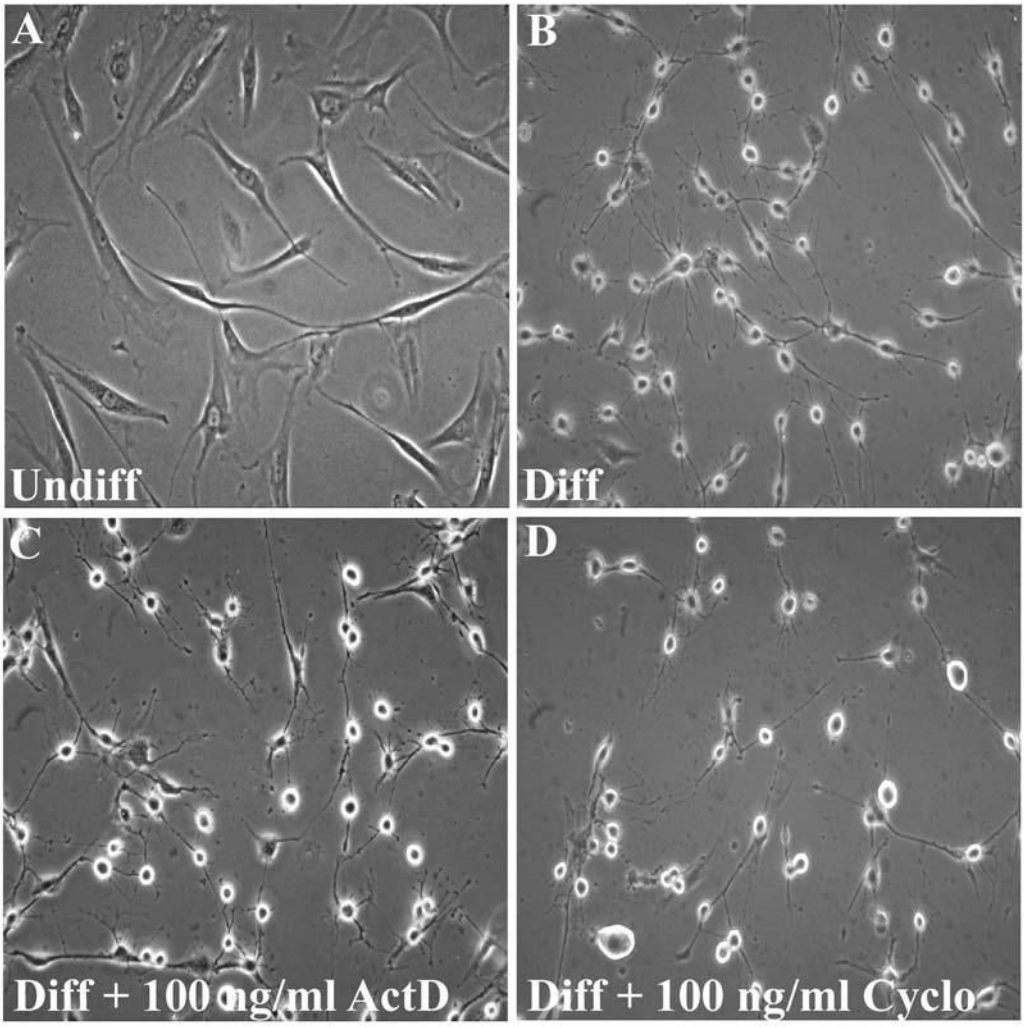

FIG 6.

Phase-contrast photomicrograph of hMSCs grown as undifferentiated cells (**A**) or following treatment with the differentiation cocktail (**B–D**) without pretreatment (**A,B**) or with a 30-min preincubation with 100 ng/ml actinomycin D (**C**) or cycloheximide (**D**). Note that neither protein nor mRNA synthesis inhibitors prevented the conversion of hMSCs to a neuronal morphology.

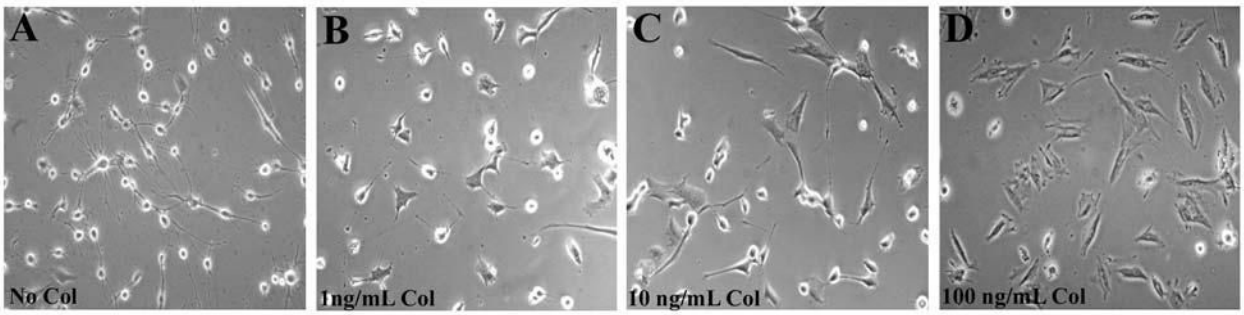

FIG 7.

Phase-contrast photomicrograph of hMSCs grown with the differentiation cocktail in the absence (**A**) or presence of 1 ng/ml (**B**), 10 ng/ml (**C**), or 100 ng/ml (**D**) of colchicine, a drug which disrupts microtubules. Note that the acquisition of a neuronal appearance by hMSCs is reduced with increasing concentrations of colchicine.

Table 1

Ability of Various Differentiation Protocols to Induce a Neuronal Phenotype in hMSCs in Culture

Treatment	*Degree of conversion*
Defined media (DM)	−
DMEM + 20% FCS (DMEM+)	−
DMEM + 1mM BME	+
DM + 0.5 μM RA	+
DMEM + 200 μM BHA	Cells died
DMEM + 2%DMSO	+
DMEM + 2% DMSO + 25 mM KCl + 10 μM Forskolin + 5 μg/ml insulin + 1 μm hydrocortisone	++
DM + glial CM 5d	−/+
Co-culture with glial beds 5d	+
DM + 100 ng/ml bFGF 7d, 10 ng/ml FGF8 + 100 ng/ml SHH 7d, 10 ng/ml BDNF 7 d	−/+
DM + 100 ng/ml bFGF 7d, 10 ng/ml FGF8 + 100 ng/ml SHH 7d, 10 ng/ml BDNF 7 d + Glia 5d	+
DM + 10 ng/ml FGF1	−
DM + 200 nM TPA	−
DM + 250 μM IBMX + 50 μM forskolin	++
DM + 200 nM TPA + 250 μM IBMX + 50 μM forskolin	++++
DM + 10 ng/ml FGF1 + 200 nM TPA + 250 μM IBMX + 50 μM forskolin	++++
DM + 10 ng/ml FGF1 + 200 nM TPA + 250 μM IBMX + 50 μM forskolin 1d + glial CM 3d	++++
DM + 10 ng/ml FGF1 + 200 nM TPA + 250 μM IBMX + 50 μM forskolin 1d + glial CM 5d	Cells died

Abbreviations: BME, β-mercaptoethanol; RA, retinoic acid; BHA, butylated hydroxyanisole; DMSO, dimethylsulfoxide; FGF, fibroblast growth factor; BDNF, brain-derived neurotrophic factor; SHH, sonic hedgehog; TPA, 4β-12-*O*-tetradecanoylphorbol 13-acetate; IBMX, 3-isobutyl-1-methylxanthine.

Glia derived from postnatal day-1 mice were grown to confluency, and CM was collected for incubation with MSCs or MSCs were seeded onto the glial beds. CM was replaced ever 2–3 days. Values: −/+, ∼10%; +, ∼25% conversion; ++, ∼50%; +++, ∼75%; ++++, ∼100%; −, 0%

Table 2

RT-PCR Analysis of Expression of Neuronal and Mesenchymal Markers in Undifferentiated and Differentiated hMSCs

mRNA	ΔC_T *undifferentiated*	ΔC_T *differentiated*	*Fold change from differentiated* $2^{-\Delta\Delta CT}$	ΔC_T *Actinomycin D*	*Fold change from actinomycin D* $2^{-\Delta\Delta CT}$
β-Tubulin III (100 pM)	2.627	2.331	1.228	2.343	−1.071
Neurofilament (100 pM)	6.367	5.403	1.951	6.970	−2.963
NSE (50 pM)	9.038	8.773	1.202	7.290	2.795
GFAP (100 pM)	8.082	5.473	6.101	6.772	−2.461
Fibronectin (100 pM)	−9.122	−6.999	−4.356	−8.470	2.772

C_T, The threshold cycle (C_T) is the cycle at which a statistically significant increase in ΔR_n is first detected. Threshold is the average standard deviation of R_n for the early cycles, multiplied by an adjustable factor.

ΔC_T, The ΔC_T value is determined by subtracting the average β-actin C_T value from the average C_T of an amplified target gene.

$\Delta\Delta C_T$, $\Delta\Delta C_T$ value is calculated by subtracting the ΔC_T of undifferentiated cell sample (as a calibrator) from the ΔC_T value of differentiated cell sample.

$2^{-\Delta\Delta CT}$, Therefore, the relative amplified amount of target gene expression, normalized to an endogenous reference (β-actin) and relative to the calibrator—same target gene expression in undifferentiated cells is given by $2^{-\Delta\Delta CT}$.