Neurogenic potential of human mesenchymal stem cells revisited: analysis by immunostaining, time-lapse video and microarray

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Summary

The possibility of generating neural cells from human bone-marrow-derived mesenchymal stem cells (hMSCs) by simple in vitro treatments is appealing both conceptually and practically. However, whether phenotypic modulations observed after chemical manipulation of such stem cells truly represent a genuine trans-lineage differentiation remains to be established. We have re-evaluated the effects of a frequently reported biochemical approach, based on treatment with butylated hydroxyanisole and dimethylsulphoxide, to bring about such phenotypic conversion by monitoring the morphological changes induced by the treatment in real time, by analysing the expression of phenotype-specific protein markers and by assessing the modulation of transcriptome. Video time-lapse microscopy showed that conversion of mesenchymal stem cells to a neuron-like morphology could be reproduced in normal primary fibroblasts as well as mimicked by addition of drugs eliciting cytoskeletal collapse and disruption of focal adhesion contacts. Analysis of markers revealed that mesenchymal stem cells constitutively expressed multi-lineage traits, including several pertaining to the neural one. However, the applied 'neural induction' protocol neither significantly modulated the expression of such markers, nor induced de novo translation of other neural-specific proteins. Similarly, global expression profiling of over 21,000 genes demonstrated that gene transcription was poorly affected. Most strikingly, we found that the set of genes whose expression was altered by the inductive treatment did not match those sets of genes differentially expressed when comparing untreated mesenchymal stem cells and immature neural tissues. Conversely, by comparing these gene expression profiles with that obtained from comparisons between the same cells and an unrelated non-neural organ, such as liver, we found that the adopted neural induction protocol was no more effective in redirecting human mesenchymal stem cells toward a neural phenotype than toward an endodermal hepatic pathway.

Key words: Bone-marrow-derived stem cells, Neurogenesis, Transdifferentiation

Introduction

The notion that phenotypic fate is indissolubly bound to the embryonic germ layer derivation of the cell has been challenged by several observations on different types of pluripotent stem cells. A number of authors have shown that several adult tissues harbour rare populations of stem cells with mesenchymal characteristics. When grown in vitro under specific conditions, or after genetic modification, these cells populations show an extensive capacity for self-renewal and differentiate into cell types not necessarily restricted to the mesodermic lineage (Blau et al., 2001; Prockop, 2003; Rafii and Lyden, 2003; Verfaillie, 2002). Similar results have been obtained after syngenic or xenogenic transplantations of such cells in a number of animal species, including primates, and in most cases it was possible to demonstrate integration of the transplanted donor cells in different host tissues (Krause et al., 2001; Liechty et al., 2000). In parallel, a number of studies have reported cases of chimerism after bone marrow infusions in humans (Gussoni et al., 2002; Mezey et al., 2003; Quaini et al., 2002; Weimann et al., 2003b), suggesting that the behaviour of the cells observed in animal models could to some extent be reproduced in man.

Cells with neural characteristics appear to have been generated in vitro from adult stem cells of putative mesodermal origin and isolated from a variety of connective tissues, including bone marrow, umbilical cord blood, dermis and adipose tissue (Hermann et al., 2004; Jin et al., 2003; Kang et al., 2004; Padovan et al., 2003; Sanchez-Ramos et al., 2000; Sanchez-Ramos, 2002; Wislet-Gendebien et al., 2005; Wislet-Gendebien et al., 2003). However, attempts to assert transdifferentiation of adult bone-marrow-derived cells into neural lineages in vivo have produced discordant results. Some authors have reported the integration and differentiation of such cells in the brain (Munoz-Elias et al., 2004), whereas others have found that the few cells that were capable of engrafting into the nervous tissue fused with endogenous cells

Journal of Cell Science

and thereby acquired the phenotype of the partner host cell (Alvarez-Dolado et al., 2003; Weimann et al., 2003a; Weimann et al., 2003b). The beneficial effects observed following transplantation of bone marrow stromal cells into injured nervous tissue are now believed to occur independently of tissue integration and to be due to a potential local release of stimulating factors by the transplanted cells (Hofstetter et al., 2002; Zhao et al., 2002). Several recent reports propose that mesenchymal stem/progenitor cells may generate both endodermal derivatives, such as hepatocytes (Alison et al., 2000; Krause et al., 2001; Petersen et al., 1999; Schwartz et al., 2002; Wang et al., 2004) and ectodermal ones, such as various types of neural cells (Deng et al., 2001; Hung et al., 2002; Jiang et al., 2003; Jiang et al., 2002; Kim et al., 2002; Kohyama et al., 2001; Levy et al., 2003; Munoz-Elias et al., 2003; Reyes et al., 2001; Sanchez-Ramos et al., 2000; Tzeng et al., 2004).

Of these investigations, some have suggested that this capacity may be restricted to a rare subpopulation of bone-marrow-resident cells, termed multipotent adult progenitor cells (MAPCs), which can be selected on the basis of immunological criteria and expanded extensively in vitro (Jiang et al., 2003; Reyes et al., 2001). Others have reported that neural differentiation can be observed in the predominant population of MSCs isolated according to standard procedures if these cells are sequentially exposed to a series of inducing factors (Deng et al., 2001; Dezawa et al., 2004; Levy et al., 2003; Munoz-Elias et al., 2003; Qian and Saltzman, 2004; Rismanchi et al., 2003; Sanchez-Ramos et al., 2000; Suon et al., 2004; Tzeng et al., 2004; Wislet-Gendebien et al., 2005; Wislet-Gendebien et al., 2003; Woodbury et al., 2002; Woodbury et al., 2000). Significantly, some of these studies claim that neural cells may be obtained rapidly and with high yield from cultured human and rodent MSCs by applying simple chemical treatments (Munoz-Elias et al., 2003; Woodbury et al., 2002; Woodbury et al., 2000). This latter observation would have a high conceptual relevance as it suggests that MSCs might be somehow precommitted toward a neural fate. However in such studies the accomplishment of in vitro neurogenic differentiation was concluded exclusively on the basis of changes of cell morphology and de novo expression of few markers, such as NSE, NeuN and Tau. In addition, some authors have raised doubts about the interpretation of these results, arguing about the authenticity of the observed neuron-like morphology and the reliability of the expression of the various markers (Lu et al., 2004; Neuhuber et al., 2004; Tondreau et al., 2004).

This apparent controversy has not confirmed whether MSCs truly harbour the potential to be converted into neural cells with the proposed chemical methods and, hence, whether they are likely to be precommitted toward a neural phenotype. Using MSCs, we have therefore revisited the problem at the cellular and molecular level.

Materials and Methods

Cell isolation and culture

Human mesenchymal stem cells (hMSCs) were isolated from bone marrow aspirates taken from the iliac crest of healthy male volunteers aged 35 to 50 years after informed consent. Aspirates were diluted 1:1 with standard growth medium, consisting of DMEM supplemented with penicillin/streptomycin and 10% foetal bovine serum (FBS; Invitrogen) and centrifuged through a density gradient (Ficoll-Plaque 1.077 g/ml, GE Biosciences) for 30 minutes in a centrifugal field of 9000 m/second^2 to remove lymphocyte-erythrocyte populations. The mononucleated cell fraction was plated at a density of 3×10^6 cells/cm^2 in 60 cm^2 culture dishes and the non-adherent cells were removed by repeated replacement of medium. When cultures reached confluence, the cells were replated at a density of 5×10^3 cells/cm^2 in standard growth conditions. Primary fibroblasts from human prostate were kindly provided by Saverio Bettuzzi (Department of Experimental Medicine, University of Parma, Italy) and were cultured in the same medium as used for hMSCs.

Osteogenic and adipogenic differentiation

Osteogenic and adipogenic differentiation of hMSCs were induced according to a published protocol (Pittenger et al., 1999). To achieve osteogenic differentiation, cells were seeded at a density of 3×10^3 cells/cm^2 and then cultured for 10 days in the presence of 100 nM dexamethasone, 50 μM ascorbic acid 2-phosphate and 10 mM β-glycerophosphate. Osteoblast formation was assessed by alkaline phosphatase (AP) staining using Sigma Diagnostic Kit 85, according to the manufacturer's instructions. For adipogenic differentiation, cells were grown until they reached confluence and were then induced by three cycles of induction/maintenance with 1 μM dexamethasone, 0.2 mM indomethacin, 10 μg/ml insulin and 0.5 mM 3-isobutyl-l-methylxanthine. Adipogenesis was assayed by staining of intracellular lipid droplets with Oil Red O (Sigma) as previously described (Sottile et al., 2002).

Neural induction and drug treatments

The protocol for neural induction of hMSCs (DMSO/BHA neural induction) was as reported (Woodbury et al., 2002; Woodbury et al., 2000). Briefly, cells at passage 4-7 were cultured in standard conditions on plastic or glass coverslips until they became subconfluent and then incubated for 24 hours in pre-induction medium consisting of DMEM supplemented with 20% FBS and 10 ng/ml FGF-2 (Calbiochem). Thereafter, the medium was replaced with an equal volume of the induction medium composed of DMEM supplemented with 2% DMSO, 100 μM butylated hydroxyanisole (BHA), 10 μM forskolin, 2 mM valproic acid, 10 mM KCl, 5 nM K252a (Sigma) and N2 supplement (Invitrogen). The cells were treated for 6 hours, 48 hours or 6 days before fixation or harvesting. In some cases, hMSCs at a similar density were incubated for 1 hour in serum-free DMEM with 10 μM cytochalasin D (Sigma) or 4 mM EDTA. Dose-dependent tests determined these concentrations were suitable to cause contraction and substrate-detachment of the cells.

The effects of DMSO/BHA neural induction protocol on cell morphology were evaluated in hMSCs derived from three different donors. For each donor, two independent experiments were performed in duplicate by treating cells for 6 and 48 hours (24 cultures in total). Two additional cultures for each donor were maintained in the induction medium for 6 days. In each culture the percentage of affected or unaffected cells was evaluated by scoring more than 500 cells from five randomly selected fields. Results are reported as the mean percentage±s.e.m. calculated across all the cultures for any given treatment period.

Immunocytochemistry

Immunocytochemical staining was performed in cells grown on glass coverslips and fixed with 4% paraformaldehyde as previously described (Malatesta et al., 2003; Malatesta et al., 2000). The following antibodies were used: mouse monoclonal antibodies against CD31, CD44, CD73, CD105, CD117 and CD147 (1:100, BD Biosciences), αSMA (1:400, Sigma), calponin (1:500, Sigma),

caldesmon (1:100, Sigma), fibronectin (1:50, Sigma), the myofibroblastic antigen 1B10 (1:500, Sigma), nestin (1:4, Dev. Hybridoma Bank), NeuN (1:50, Chemicon), 200 kDa neurofilament isoform (NF-200; 1:200, Sigma), synaptophysin (1:100, Sigma), Tau protein (1:100, Sigma), βIII-tubulin (1:100, Sigma), GFAP (1:200, Sigma), S100 (1:500, Sigma), CNPase (1:500, Sigma), O4 (1:40, Chemicon) and β-galactosidase (1:500, Promega); rabbit polyclonal antibodies against MyoD (1:100, Santa Cruz), parvalbumin (1:500, Swant), NSE (1:2, Sigma) and GFP (1:500, RDI).

Binding of primary antibodies was revealed with specific secondary antisera, conjugated with FITC, TRITC (Immucor) or biotin (Dako), and subsequently developed with the ABC kit from Vector laboratories, according to the procedure provided by the suppliers. Primary antibodies against CD34, CD45, CD10, CD38, CD90 and CD133 directly conjugated with phycoerythrin or FITC (BD Biosciences) were also used at 1:100 dilution.

Western blotting

For western blot analysis, confluent cultures of either untreated hMSCs or hMSCs treated with the DMSO/BHA neural induction medium were lysed with RIPA buffer (Sigma) supplemented with 2 mM EDTA and 'Complete Mini' protease inhibitor kit (Roche) at the concentration recommended by the supplier. Total protein content of the lysates was quantified using Bradford reagent (Sigma). Approximately 30 μg of each cell lysate were diluted in sample buffer (50 mM Tris-HCl pH 6.8, 7% glycerol, 0.5% SDS, 25 mM DTT, 0.005% Bromophenol Blue) and separated on 8% SDS-polyacrylamide gels. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane to perform the antibody detection. The primary antibodies were used at the following concentrations: anti-βIII-tubulin (1:1000), anti-NF-200 (1:800), anti-NeuN (1:500), anti-NSE (1:200), anti-αSMA (1:3000) and anti-fibronectin (1:200). Rabbit polyclonal anti-β-actin (1:3000, Abcam) was used for normalization. Primary antibodies were detected with specific antisera conjugated with HRP (Sigma) and developed with the ECL Plus chemiluminescent detection system (Amersham Biosciences). Quantification of protein expression was performed on digitized images using ImageJ (Wayne Rasband, NIH, USA). The intensity of β-actin bands was used to normalize the densitometric measurements of each band analysed for different samples.

Time-lapse video recordings

For time-lapse analysis hMSCs cultured in standard growth conditions were placed in a temperature- and CO_2-controlled chamber under an Axiovert phase-contrast microscope with a 10× magnification objective. Medium changes were made during the time-lapse recordings using a tubing system. The images were acquired at 2-minute intervals for 1-3 hours with a Nikon digital camera controlled by Nikon ACT-1 software.

DNA microarray analysis

Total RNA from hMSC cultures was extracted with the RNeasy Mini kit (Qiagen) and total RNA of human foetal brain and adult liver were purchased from Chemicon. Representative amplification of mRNA and fluorescent labelling were carried out using an Amino Allyl MessageAmp aRNA kit (Ambion) and NHS ester fluorescent dyes (Amersham Bioscience) following the protocol suggested by the supplier. Hybridization was performed on a human Operon oligo V2.0 microarray platform printed by MicroCRIBI service at CRIBI facility (University of Padua, Italy) containing 21,329 human gene probes (GEO Platform accession number GPL 2136). The arrays were previously blocked in hybridization buffer [5× SSC, 0.1% SDS, 0.2 mg/ml Torula Yeast RNA (Sigma), 5× Denhardt's solution, 25% formamide] for 2 hours. 120 pmol of each dye-labelled aRNA sample,

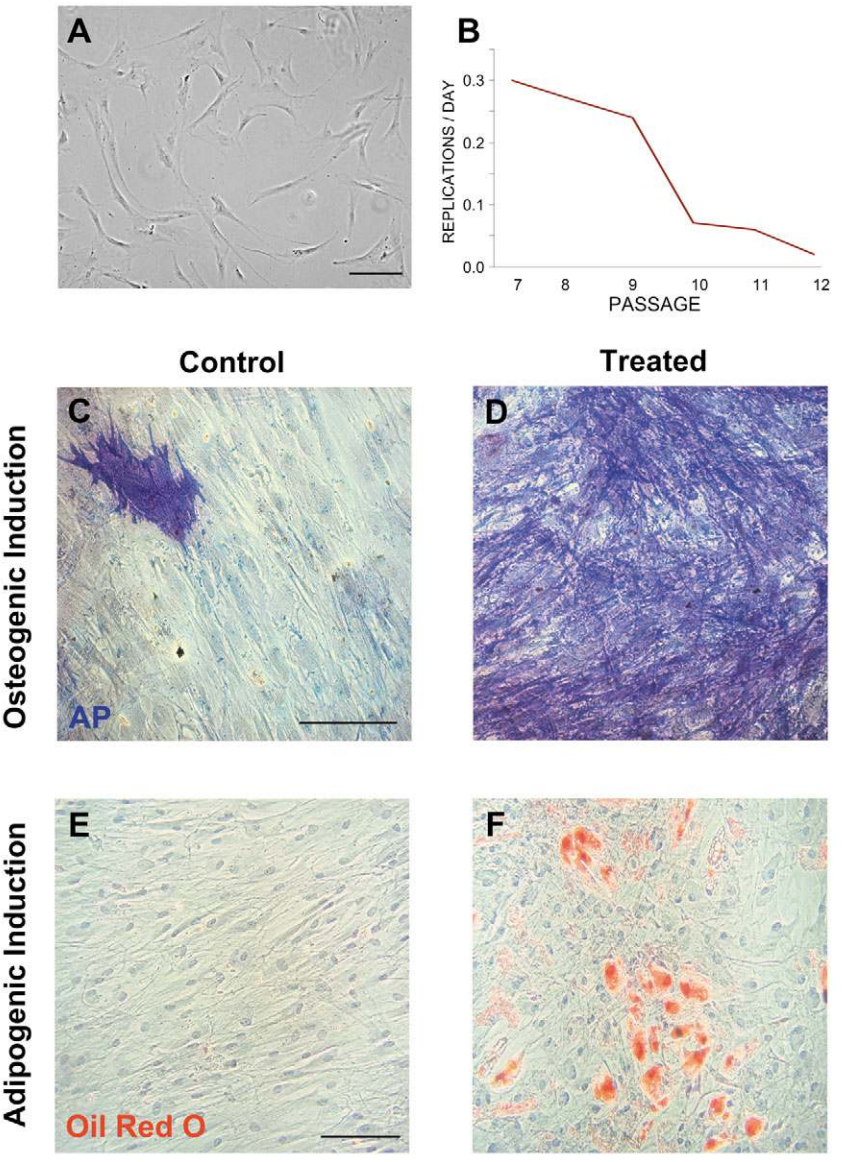

Fig. 1. In vitro characterization of hMSCs. (A) Undifferentiated hMSCs cultured in standard growth medium show a well-spread or spindle-shaped morphology. (B) hMSCs at early passages display an average doubling time of about 72 hours, but their proliferation rate gradually decreased during ex vivo cell expansion with a replication arrest between passages 10 and 12. (C-F) Analysis of osteogenic and adipogenic differentiation. AP activity of hMSCs cultured in standard conditions (C) and after 10 days of osteogenic induction treatment (D). (E) No lipid droplet formation was seen in hMSCs cultured in control medium. (F) Oil Red O staining after 21 days of adipogenic induction. Bar, 50 μm.

diluted in 100 μl hybridization buffer, were denatured for 5 minutes at 65∞C and were then applied to the glass slide. A 60-mm glass coverslip was used to uniformly spread the sample solution. Incubation was carried out for 24 hours at 48°C in a humid chamber and was followed by six washing steps with decreasing concentrations of SSC and SDS. Array-scanning was performed by using a ScanArray Lite (PerkinElmer Life Sciences). Background subtraction, normalization and statistical analysis were performed using the TIGR TM4 suite (Saeed et al., 2003) and SAM (Tusher et al., 2001). The non-linear LOWESS-based lockfit algorithm was used for normalization. Principal component analysis (PCA) was performed using SPSS13.

Results

Phenotypic characterization of hMSCs

HMSCs isolated and grown according to standard protocols displayed the characteristic spindle-shape or flat-polygonal morphology (Fig. 1A) and replicated exponentially for up to ~30 population doublings. Whereas their proliferation rate showed a tendency to progressively decrease with increasing number of passages (Fig. 1B), their phenotypic traits remained relatively stable. Immunodetection of a set of cell surface antigens, recognized to be characteristic of both human and rodent MSCs, showed that our cells were positive for CD44, CD73, CD90, CD105 and CD147, but failed to express the haematopoietic marker CD34, the endothelial marker CD31 and the lymphocyte/leukocyte markers CD10, CD38, CD45 and CD117 (Table 1). A relative stability was also found in the expression of several cell phenotype-specific markers associated with mesodermic lineages, such as αSMA and fibronectin (Table 1). HMSCs showed weak constitutive expression of the putative neuronal marker βIII-tubulin, which in the mouse is strongly associated with neuronal phenotypes, but which in human shows a broader distribution encompassing also non-neural tissues as reflected by the pattern of the transcriptional activity of the gene tubb3 (Pontius et al., 2003). Although expressed at detectable levels, markers typical of non-mesodermic lineages, such as βIII-tubulin, nestin, parvalbumin and S100, were found to be expressed at considerably lower levels than αSMA, fibronectin and other mesodermic markers (Table 1).

It was also confirmed that hMSCs maintained their ability to undergo osteogenic and adipogenic differentiation with increasing number of passages. Following osteogenic induction with the appropriate medium, hMSCs changed their morphology, expressed alkaline phosphatase within 7-10 days (Fig. 1D) and deposited a mineralized matrix (data not shown). Following the adipogenic induction, cells showed a reduced proliferation rate and within 10 days accumulated lipid vacuoles in their cytoplasm that were evidenced by Oil Red O staining (Fig. 1F). Taken together these results indicate that the population of hMSCs under examination was comparable to that described by other authors (Colter et al., 2001; Conget and Minguell, 1999; Deans and Moseley, 2000; Pittenger et al., 1999; Vogel et al., 2003; Woodbury et al., 2002).

'Neural induction'

When hMSCs were exposed to the DMSO/BHA neural induction medium, they rapidly underwent dramatic morphological changes. Within a few hours, the majority of the cells rounded up and showed thin radial processes vaguely reminiscent of neurites (Fig. 2B). Some of the cells partially lost contact with the substratum and others completely detached, whereas a limited fraction of hMSCs appeared to be unaffected by the treatment (15±5%, mean±s.e.m.) (Fig. 2B). These morphological changes were transitory and reverted spontaneously even if the cells were maintained in the same medium. After 48 hours only 20±5% (mean±s.e.m.) of cells still displayed a pseudo-neural appearance (Fig. 2C) and after 6 days of continuous treatment cultures were almost indistinguishable from untreated ones (data not shown).

In order to determine the timing of the morphological transformation, we monitored the process by time-lapse video microscopy. Within 25 minutes of addition of the DMSO/BHA induction medium, flattened hMSCs progressively shrank; their lamellipodia were retracted, their cell soma rounded up and became iridescent (Fig. 2D). Cytoplasmic retraction did not involve the entire cell perimeter and the few adhesion points maintained by the cells in contact with the substratum became the extremities of filopodium-like processes that other authors have judged to represent neurites (Fig. 2D). However, this pattern of morphological change is clearly distinct from neurite outgrowth, where a well-defined structure, the growth cone, leads an active extension of the cell process. Moreover, in agreement with observations reported by Woodbury and

Table 1. Immunophenotypic characterisation of untreated hMSCs

CD markers	Previous reports	Present report
CD10	– *	–
CD31	– *,†	–
CD34	– *,†,‡	–
CD38	– * / low §	–
CD44	low * / + †,‡	+
CD45	– *,†,‡	–
CD73 (SH3)	+ †	+
CD90	low * / + †,‡	+
CD105 (SH2)	+ †	+
CD117	– *,§	–
CD147	+ §	+
αSMA	+ ¶,**	+
Calponin	+ **	+
Caldesmon I	+ **	+
Myo D		+
Fibroblast Mab1B10		+
Fibronectin Mab IST-3	+ ‡	+
Calretinin		–
Parvalbumin		+
Nestin	– ‡ / + †	+ / –
NeuN	– ‡	–
Neurofilaments	– ‡	–
NSE	low ‡	–
Peripherin		–
Synaptophysin	– ‡	–
Tau	– ‡	–
β-III-TUB	+ †,††	+
GFAP	– ‡ / low†	–
S100	– ‡‡	+
CNPase		–
O4		–

Constitutive expression of CD and lineage-specific markers by hMSCs cultured in standard growth medium as determined by immunostaining: +, most cells positive; low, few cells positive; – , negative. Our data are compared to those of previous studies: *Reyes et al., 2001; †Tondreau et al., 2004; ‡Woodbury et al., 2000; §Colter et al., 2001; ¶Kinner et al., 2002; **Galmiche et al., 1993; ††Lodie et al., 2002; ‡‡Deng et al., 2001.

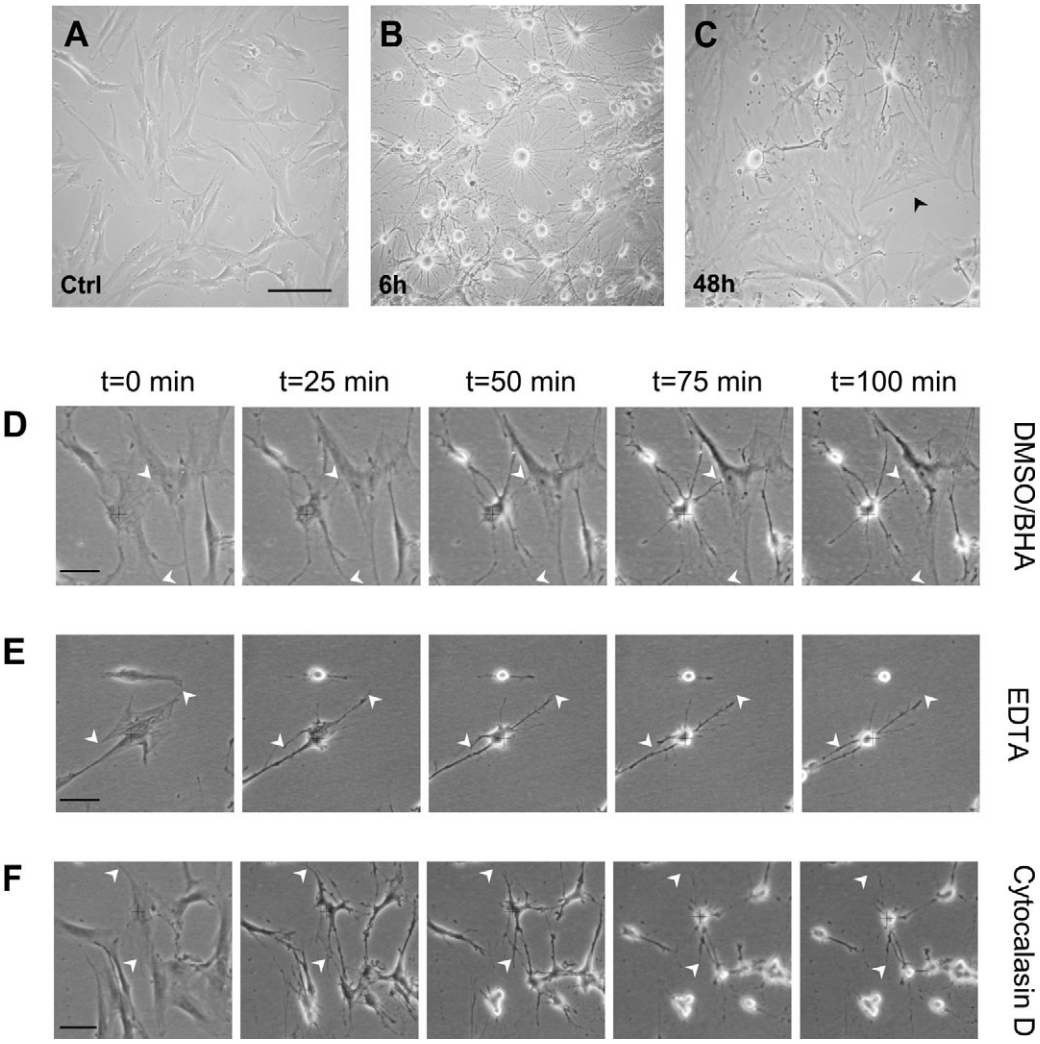

Fig. 2. Morphological changes of hMSCs following different treatments. (A) Cultures of untreated hMSCs display a characteristic fibroblastic morphology. (B) Within 6 hours of DMSO/BHA neural induction treatment about 80% of hMSCs undergo morphological changes and acquire a pseudo-neuronal shape with refractile soma and neurite-like processes. (C) A spontaneous reversion to fibroblastic shape (black arrowhead) is evident 48 hours later. (D-F) Representative photomicrographs extracted from time-lapse sequences, each corresponding to the indicated time points. (D) Time-lapse analysis of DMSO/BHA treatment inducing hMSCs to retract their cytoplasm in the perikaryal region leaving only a few cytoplasmic extensions whose tips still adhere to substrate (white arrowheads). No outgrowth of processes from the cell soma was ever observed. (E,F) The morphological rearrangements can be mimicked by incubating hMSCs with 4 mM EDTA (E) or 10 μM cytochalasin D (F). Bar, 50 μm (A-C); 20 μm (D-F).

collaborators (Woodbury et al., 2002), video time-lapse analysis showed that the timing of this transformation was extremely rapid. This feature must be deemed to be incompatible with a neuronal differentiation event involving neurite extension, where the expression of a vast set of genes needs to be accurately regulated.

Rather than being representative of a differentiation event, the morphological alterations exhibited by hMSCs following incubation with the DMSO/BHA neural induction medium seemed to be a consequence of cytoskeletal collapse and/or disruption of focal contacts. We therefore assayed by video time-lapse whether drug-induced interference of cell-substratum contacts or cytoskeletal rearrangement evoked similar effects. Treatment of hMSCs with both EDTA, causing loss of cell-substratum anchorage, and cytochalasin D, known to disrupt the cytoskeletal architecture, resulted in shape changes virtually indistinguishable from those caused by treatment with DMSO/BHA (Fig. 2E-F).

There remained the possibility that the morphological changes observed in hMSCs following the putative neural induction could still reflect a specific trait of pluripotent cells and were, in fact, a display of an incipient in vitro neurogenesis process. We therefore incubated normal human prostate fibroblasts in the same neural induction medium and, in parallel, challenged them with the osteogenic induction protocol to verify that they diverged from hMSCs in their multipotency. These fibroblastic cells failed to differentiate into osteoblasts (Fig. 3D), but, under DMSO/BHA treatment, showed an analogous retraction of the cell margins and rapidly assumed a similar stellate pseudo-neural morphology (Fig. 3B), as displayed by hMSCs.

Immunodetection of cell lineage markers

Besides the morphological evidence, other authors have presented data suggesting that chemical manipulation of MSCs is able to induce de novo or enhanced expression of neural-specific markers (Munoz-Elias et al., 2003; Qian and Saltzman, 2004; Rismanchi et al., 2003; Woodbury et al., 2000). We therefore compared the expression pattern of characteristic neural antigens in hMSCs, i.e. NeuN, Tau, NF-200, NSE, GFAP, prior to and after treatment of the cells with the DMSO/BHA neural induction medium. In accordance with previous reports, pseudo-neurally shaped cells showed an apparent increase of immunoreactivity (Fig. 4A′-D′), whereas the rare hMSCs that preserved a flat morphology did not

change their immunocytochemical staining pattern (Fig. 4C′-D′, arrowheads). A similar apparent increase in immunoreactivity levels as that seen for neural markers was, however, also observed using antibodies directed against markers of non-neural lineages, such as αSMA and fibronectin (Fig. 4E′,F′), MyoD, caldesmon, calponin and the myofibroblast antigen 1B10 (data not shown). Analogously, an enhanced staining intensity was seen after labelling with antibodies directed against a bacterial protein, i.e. β-galactosidase (Fig. 4G′), and against the GFP reporter molecule (data not shown), both of which would be absent from normal non-manipulated human cells. Even in these cases, a markedly high background staining was observed in pseudo-neurally shaped hMSCs, despite the absence of the corresponding antigen. Moreover, this enhanced staining was reminiscent of that detected after labelling with antibodies to neural markers. These observations suggested that a true transdifferentiation had not occurred.

We then suspected that the apparent increase in immunoreactivity could simply be an artefact deriving from immunolabelling of shrunken cells having potentially altered membrane properties. To test this hypothesis, we immunolabelled hMSCs that had been partly detached from their substrate by treatment with EDTA, or in which their focal adhesions had been disrupted through treatment with cytochalasin D (Fig. 4H-K), but which had not been exposed to the DMSO/BHA neural induction medium. These cells too exhibited enhanced staining for the neural markers. In further support of the idea of a possible pitfall and misinterpretation of the previously reported immunocytochemical patterns was the aberrant localization of the neural markers NeuN and Tau. The former should have shown a preferentially nuclear distribution and the second a microtubule-associated localization along the pseudo-neurites. Both in our immunostaining experiments and in those reported by other investigators adopting DMSO/BHA neural induction protocol (Munoz-Elias et al., 2003; Woodbury et al., 2002; Woodbury et al., 2000), immunoreactivity for these two antigens was evenly distributed in the cytoplasm of the treated hMSCs indicating that the staining patterns did not reflect proper immunolocalization of these markers.

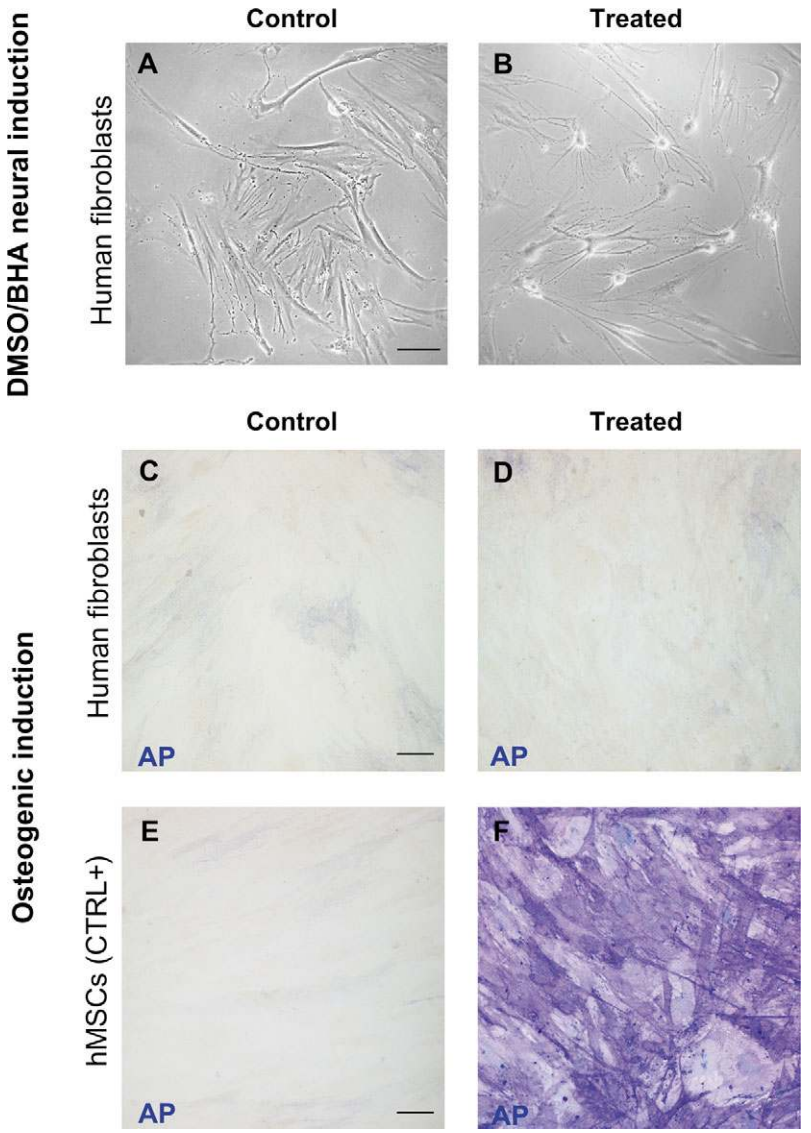

Fig. 3. Differentiation potential of human fibroblasts. (A) Untreated cells in subconfluent standard culture. (B) Human fibroblasts exposed for 6 hours to the DMSO/BHA neural induction protocol assumed a pseudo-neural morphology. AP staining of human fibroblasts (C,D) and hMSCs as positive control (E,F). Before osteogenic induction, both human fibroblasts and hMSCs showed low levels of AP activity (C,E). After 10 days treatment in appropriate differentiation media hMSCs increased their level of AP as consequence of osteogenic differentiation (F) whereas human fibroblasts did not undergo osteogenic differentiation and failed to upregulate AP (E). Bar, 20 μm.

Western blot analysis of cell lineage markers

Biochemical techniques based on protein extracts are not influenced by cell morphology and we therefore examined the relative levels of marker expression in treated and untreated hMSCs by western blotting using human brain as a positive control and a human kidney cell line HEK293 as a negative control. Neither NeuN nor NF-200 was detectable in hMSCs before the treatment or after 6 and 48 hours of neural induction (Fig. 5A). Analogously, the levels of markers that are constitutively expressed in untreated cells, such as NSE (Fig. 5A) and Tau (data not shown), were not significantly increased following treatment with the neural induction medium (Fig. 5A). A slight upregulation after DMSO/BHA treatment was seen for βIII-tubulin. However, this could hardly be considered as an indication of a neural differentiation programme as the expression of this tubulin isoform is not strictly neurospecific in humans (Pontius et al., 2003; Tondreau et al., 2004). A modulation of the expression of fibronectin and the smooth muscle marker αSMA was also observed (Fig. 5B). Rather than being a trait in favour of a differentiation process, the

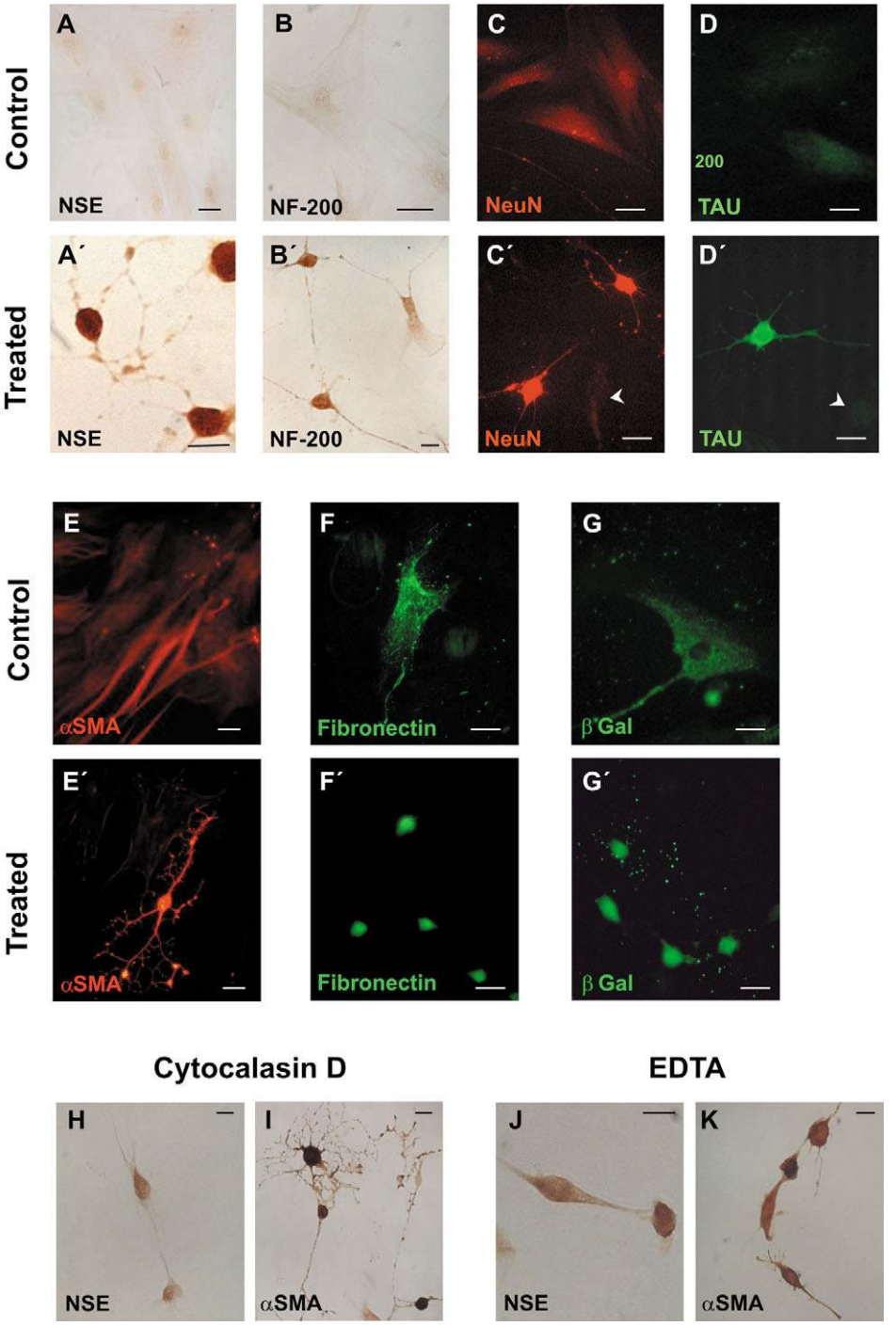

Fig. 4. Immunostaining of hMSCs. (A-G) Untreated hMSCs. (A′-D′) hMSCs treated for 6 hours with DMSO/BHA. The apparent immunoreactivity of neuronal markers (A′-D′) increases in parallel to the immunoreactivity of non-neuronal markers (E′,F′) and for the unrelated bacterial protein β-galactosidase (G′) compared to that in untreated cells (A-G). Adherent cells with fibroblastic morphology do not show any increase in immunoreactivity (arrowheads). Similarly increased immunoreactivity is also shown by hMSCs treated with cytochalasin D (H-I) and EDTA (J-K). Bar, 10 μm.

down-regulation of αSMA was probably due to collapse of the actin microfilaments.

Microarray gene profiling

The most severe limitation of previous studies is that very few markers were examined in the induced MSCs. In order to overcome these limitations, we performed a DNA microarray-based global expression profiling of treated and untreated hMSCs using a 21,329 oligonucleotide platform. RNA extracted from untreated hMSCs from different donors and RNA isolated from hMSCs treated for 6 or 48 hours with DMSO/BHA neural induction medium were used as reference and test samples, respectively. Control experiments were performed with RNA derived from human foetal brain, in order to provide information on the set of genes that would be differentially expressed in human neural tissue compared to untreated hMSCs. Data obtained from microarray experiments are expressed as the logarithm (base 2) of the ratio between the expression level in the probe compared to that of the reference sample (fold change). Eight independent experiments (GEO accession number: GSE2776), each one with an internal replicate, were used to derive 16 datasets that were first evaluated for their consistency by a Principal Component Analysis (PCA) statistical approach. The contribution of each dataset to the two principal components was determined (Fig. 6A). The datasets are tightly clustered into three groups, corresponding to the three different probes that were used. The cluster containing the datasets relative to the comparison between foetal brain and untreated hMSCs is well separated from the others and contributes almost entirely to the first principal component. Such data sets, therefore, constitute a coherent group that is the main source of variance between all the data sets. As expected, the other two clusters are more similar to each other, contributing only to the second principal component. These results show that the set of genes modulated by the DMSO/BHA neural induction treatment is different from the set of genes differentially expressed between untreated hMSCs and neural tissue. Moreover, the number of genes that show a significant modulation after the 6 or 48 hours treatment is extremely small compared to the number of genes differentially expressed between untreated hMSCs and neural tissue (Fig. 6B). In

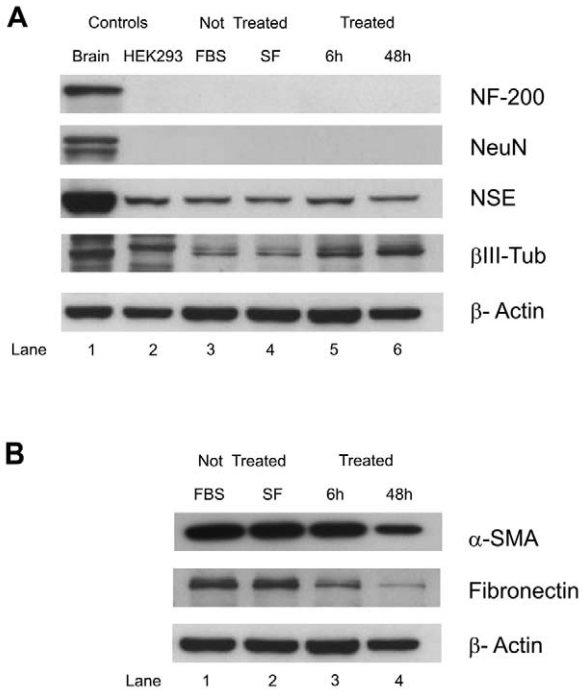

Fig. 5. Western blot analysis of neural and non-neural markers in hMSCs. (A) Neural marker expression in hMSCs cultured in standard growth medium with 10% FBS (Lane 3), in serum-free medium (Lane 4) and with DMSO/BHA for 6 hours (Lane 5) or 48 hours (Lane 6). Protein extracts from human foetal brain (Lane 1) and HEK293 (Lane 2) served as positive and negative controls, respectively. (B) Non-neural marker expression in hMSCs cultured in standard growth medium with 10% FBS (Lane 1), in serum-free medium (Lane 2) and with DMSO/BHA for 6 hours (Lane 3) or 48 hours (Lane 4). β-actin was used as a loading control (lower panels in A and B).

particular, the numbers of genes that showed a fold change in absolute value equal or greater to 1.5 were 55 after the 6-hour treatment and 104 after the 48-hour treatment. In comparison, 854 genes showed a significant differential expression between untreated hMSCs and neural tissue. These results show that the change in the transcriptome of hMSCs due to the DMSO/BHA neural induction treatment is dramatically different to the change that would be expected in the case of conversion between hMSCs and neural tissue.

The absolute number of genes differentially expressed after the treatment, however, may also depend on the number of cells that are influenced by the treatment. In order to rule out the

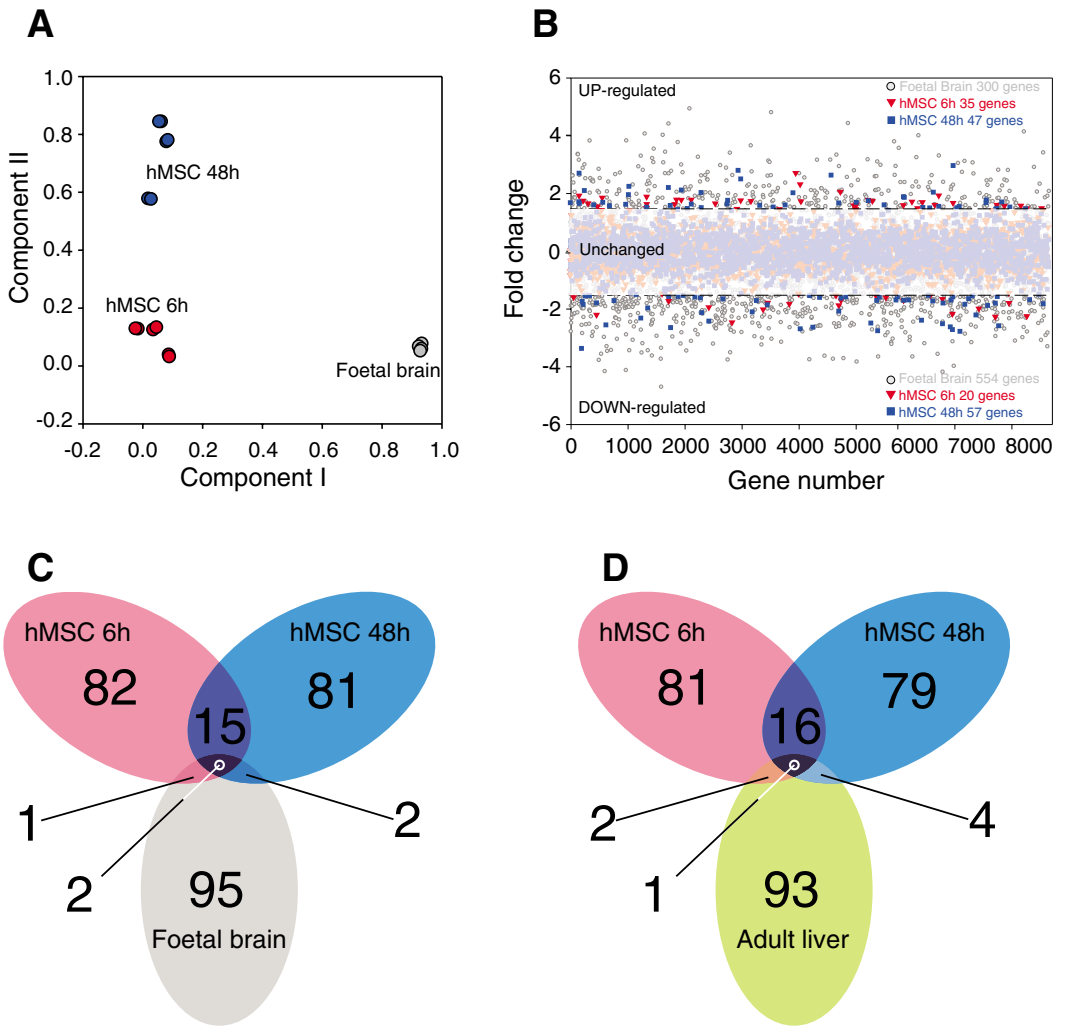

Fig. 6. Gene profiling by DNA microarray. (A) Loading plot projected on the first two principal components relative to the PCA of data from the three sets of arrays comparing the transcriptome of untreated hMSCs with 6 and 48 hour-treated hMSCs and foetal brain (positive control). (B) Plot of the average fold changes relative to all genes with detectable expression in all three sets of array experiments. The dimmed field comprising the range between –1.5 and 1.5-fold change contains the genes in which expression is not significantly modulated between the samples and the reference (i.e. uninduced hMSCs). (C,D) Intersections between the sets of the 100 genes most significantly modulated by DMSO/BHA treatment at the specified times and the set of genes differentially expressed between untreated hMSCs and foetal brain (positive control, C) or adult liver (negative control, D).

possibility that the DMSO/BHA neural induction medium was actually promoting the differentiation of a small subpopulation in the culture, we examined whether at least the set of genes that were most significantly modulated by the treatment matched the set of genes most differentially expressed between hMSCs and neural tissue. The sets containing the 100 genes that were most significantly modulated by the 6- and 48-hour treatments had extremely small intersections (respectively three and four genes) with the set of the 100 most significant genes differentially expressed between hMSCs and neural tissue (Fig. 6C). Noticeably, similar intersections could be found when comparing the set of genes modulated by the treatments and the set of genes differentially expressed between non-induced hMSCs and adult liver (three and five genes respectively) (Fig. 6D). Moreover, the genes belonging to the intersections (Table 2) do not show any obvious relation to neural differentiation pathways. Taken together, these data highlight that the modest changes in gene expression levels promoted by the treatment of hMSCs with the DMSO/BHA medium do not resemble at any level a differentiation cascade conducive to neurogenesis.

Discussion

The possibility of identifying a source of stem cells capable of generating neural cells is of primary interest for therapeutic approaches aimed at repairing injured nervous tissue and a number of investigations have suggested that bone-marrow-derived MSCs possess this ability when exposed to synthetic culture media. However, with the accumulation of reports emphasizing the plasticity of hMSCs, the level of criticism towards these observations has also grown, suggesting that the data used to support the transdifferentiation process are scanty and prone to misinterpretation (Jin et al., 2003; Lu et al., 2004; Neuhuber et al., 2004). The nature of the neural transdifferentiation phenomenon described by some investigators for cultured MSCs is peculiar as, according to the authors, it takes place in a surprisingly short time span producing neuron-like cells after a few hours (Woodbury et al., 2002; Woodbury et al., 2000). Such a short timeframe seems hardly to be compatible with a differentiation process that would involve cascades of transcriptional and translational events. Strikingly, Lu and colleagues (Lu et al., 2004) additionally suggest that murine MSCs may assume pseudo-neural shapes independently of protein synthesis when exposed to such neurogenesis-inducing conditions.

Our time-lapse recordings of the behaviour of hMSCs following treatment with the DMSO/BHA neural induction medium, are in agreement with recent data obtained on murine MSCs (Neuhuber et al., 2004) and show that the morphological changes are due to cell shrinkage rather than to a process of neurite extension typical of neuronal differentiation. These observations, therefore, suggest that the change in cell morphology could be an artefact rather than a differentiation trait. However, some doubts still remain as chemical treatment appears able to induce the expression of genes that are commonly considered as markers of neuronal differentiation. The most critical point in these studies is the reliability of these

Table 2. Members of the intersections between the sets of 100 genes most significantly modulated in each sample

Accession number	Name	hMSCs after 6 hours of induction	hMSCs after 48 hours of induction	Foetal brain	Adult liver	Function
NM_033138	Caldesmon 1	down	down	down	down	A calmodulin- and actin-binding protein essential in the regulation of smooth muscle and non-muscle contraction.
BE551792	EST, highly similar to A48118 major epidermal calcium-binding protein profilaggrin	down		down		Unknown
AK002171	011A15_Homo sapiens mRNA full-length insert cDNA clone EUROIMAGE 994846	down			down	Unknown
NM_020215	004O03_Hypothetical protein DKFZp761F2014	down			down	Unknown
BC015134	013E17_Homo sapiens, clone IMAGE:3934391, mRNA		down		down	Unknown
NM_018440	Phosphoprotein associated with glycosphingolipid-enriched microdomains	up	up	up		A type III transmembrane adaptor protein that binds to the tyrosine kinase csk protein. It is involved in the regulation of T-cell activation.
AK001971	Hypothetical protein FLJ11109		up	up		Unknown
BC004224	Hypothetical protein (clone MGC:4762 IMAGE:3537945), complete cds		up	up		Unknown
AB067500	022C12_KIAA1913 protein		up		up	Unknown
NM_001353	048L05_Aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1); 20-alpha (3-alpha)-hydroxyster		up		up	A member of the aldo/keto reductase superfamily. It catalyses the conversion of aldehydes and ketones to their corresponding alcohols.
NM_024539	005H16_Hypothetical protein FLJ23516		up		up	A type I transmembrane protein that localizes to the endocytic pathway. It has been shown to possess E3 ubiquitin ligase activity.

markers and in particular their capability to give information about the differentiation fate of cells found in a heterodox context. In most cases, cell type-specific markers have been deemed to be reliable in the identification of particular cell types in a given context, but they can be misleading in other contexts. For instance, in the CNS, the calcium binding protein calbindin is used as marker for the horizontal cells, a population of interneurons in the retina (Matsuda and Cepko, 2004), but it is also the standard marker for Purkinje projection neurons in the cerebellum (McAlhany et al., 1997). A cell expressing calbindin cannot be classified as a Purkinje cell (or horizontal cell) if its origin is not known or, worse, if it is different from both retina and cerebellum. In addition, some markers have a different specificity in different species. The well-established neuronal marker βIII-tubulin is extremely useful for identification of neurons in murine nervous system cultures, whereas in humans its expression is less restricted and can be found in different tissues including lung, pancreas and bone marrow (Pontius et al., 2003). Therefore, an increase of βIII-tubulin immunoreactivity, or expression levels, in a population of bone-marrow-derived cells hardly supports the notion of their transdifferentiation.

Moreover, many of the immunostaining patterns presented by other investigators do not show the expected localization of the corresponding antigens. For instance, NeuN immunoreactivity appears to be spread outside the perinuclear region, being present also in cell processes (Levy et al., 2003; Sanchez-Ramos et al., 2000; Sanchez-Ramos, 2002). Conversely, Tau, a typical axonal marker, appears to be more widely expressed in the cytoplasm of the cells and is not distinctly associated with cell protrusions (Munoz-Elias et al., 2003; Woodbury et al., 2002). Taken together with our observations highlighting that immunoreactivity for all tested antigens apparently increased in both the pseudo-neurally shaped cells and in the cells partially detached from the substratum by EDTA or cytochalasin D treatment, these considerations undermine the evidence in favour of a neural transdifferentiation event.

As, in the absence of functional data, the analysis of a few markers is not sufficient to allocate a cell, taken out of its normal physiological context, to a particular lineage, a more appropriate approach is to compare its entire transcriptome to that of cells belonging to the lineage of interest. We therefore compared the changes in gene expression profile of hMSCs treated with the protocol proposed by Woodbury and colleagues with the set of genes that are differentially expressed between hMSCs and brain tissue. This comparison showed that although the set of genes differentially expressed between hMSCs and brain was rather large, the set of genes modulated by the chemical treatment was about tenfold smaller and the two sets did not show significant correlation. Moreover, we found that the few genes in common among these sets were not overtly referable to a neural differentiation pathway. Strikingly, we found an even higher number of common elements in the set of genes modulated by the DMSO/BHA treatment and in the set of genes differentially expressed between untreated hMSCs and adult liver cells (Fig. 6D); in other words, the adopted neural induction protocol was not driving the cells toward the neural lineage more than toward the hepatic one.

The presence of common elements between the set of genes modulated by the induction and the genes differentially expressed between non-induced hMSCs and foetal brain (or adult liver) is probably due to the peculiar level of expression of such genes in the reference sample, i.e. untreated hMSCs. This may for example be the case for the caldesmon 1 gene *CALD1*, which encodes a calmodulin and actin binding protein, that appears to be expressed at high levels by bone marrow (Pontius et al., 2003) and untreated hMSCs, but is expressed at lower levels by both the nervous system and liver. The expression of the caldesmon 1 gene is probably reduced by the treatment with DMSO/BHA as a consequence of cytoskeleton collapse. These data provide a stronger basis for rejecting the idea that the adopted chemical treatment is able to induce a transdifferentiation process of hMSCs in vitro. We found that hMSCs undergo shape changes, assuming a pseudo-neural morphology when they are exposed to different kinds of chemical stresses that induce their detachment from the substratum and/or cause the collapse of the actin cytoskeleton. Taken together with the observation that a high percentage of apoptotic cells are present in the cultures exposed to the DMSO/BHA neural induction protocol (Rismanchi et al., 2003), our results support the notion that the morphological changes observed in hMSCs are caused by a cytotoxic effect of BHA and/or DMSO. Although this study cannot rule out the possibility that specific culture conditions, i.e. co-culturing with neural primary cells, as described recently (Wislet-Gendebien et al., 2005), may induce hMSCs to form neuronal elements in vitro, it unequivocally shows that the treatment with DMSO/BHA does not. Moreover, it raises doubts on other claims about transdifferentiation of hMSCs toward a neural fate based exclusively upon cell shape changes and analyses of a few precocious lineage markers.

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