Differential Expression of Shh and BMP Signaling in the Potential Conversion of Human Adipose Tissue Stem Cells Into Neuron-Like Cells In Vitro

ALEJANDRA CARDOZO,* MARCELO IELPI,* DANIEL GÓMEZ,† AND PABLO ARGIBAY*

**Instituto de Ciencias Básicas y Medicina Experimental, Hospital Italiano de Buenos Aires, Buenos Aires, Argentina*

†Laboratorio de Oncología Molecular, Universidad Nacional de Quilmes, Buenos Aires, Argentina

The nervous system (NS) has a limited self-repair capability and adult neurogenesis is limited to certain regions of the brain. This generates a great interest in using stem cells to repair the NS. Previous reports have shown the differentiation of adipose tissue-derived mesenchymal stem cells (ASCs) in neuron-like cells when cultures are enriched with growth factors participating in embryonic and adult neurogenesis. Therefore, it could be thought that there exists a functional parallelism between neurogenesis and neuronal differentiation of ASCs. For this reason, the goal of this work was to study the differential gene expression of Shh and BMP genetic pathways involved in cell fate determination and proliferation. In this study we demonstrated that hASCs are endowed with active Hedgehog and BMP signaling pathways through the expression of genes of both cascades and that their expressions are downregulated after neuronal induction. This idea is in accordance with the facts that Shh and BMP signaling is involved in the maintenance of cells with stem cells properties and that proliferation decreases during the process of differentiation. Furthermore, Noggin expression was detected in induced hASCs whereas there was no expression in noninduced cells, which indicates that these cells are probably adopting a neuronal fate because noggin diverts neural stem cells from glial to neuronal fate. We also detected FM1-43 and synaptophysin staining, which is evidence of the presence of putative functional presynaptic terminals, a neuron-specific property. These results could partially contribute to the elucidation of the molecular mechanisms involved in neuronal differentiation of adult human nonneural tissues.

Key words: hASCs; Neuronal differentiation; Shh signaling; BMP signaling

INTRODUCTION

Neural tissue has long been regarded as incapable of regeneration. Hence, the identification of cell populations capable of neuronal differentiation has generated great interest (58,60). Stem cells from embryonic tissue as well as from adult brain can undergo expansion and neuronal differentiation in vitro and in vivo (5,19,20,49). However, the inaccessibility of these stem cells limits their clinical utility and has

led to investigate alternative cells with the ability to differentiate to neuronal lineage.

Adipose tissue is derived from the mesodermal germ layer and contains a supportive stroma that can be easily isolated (24,28). This stromal fraction consists of a heterogeneous mixture of cells, such as endothelial cells, smooth muscle cells, pericytes, fibroblasts, mast cells, and preadipocytes. In addition, this fraction contains a multipotent adipose tissue-derived mesenchymal stem cell (ASC) population (79). Hu-

Address correspondence to Pablo Francisco Argibay, Instituto de Ciencias Básicas y Medicina Experimental, Hospital Italiano de Buenos Aires, Potosí 4240—8° piso—C1199ACL, Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina. Tel: +54 11 4959 0200 # 8919; Fax: +54 11 4959 0200 # 5355; E-mail: pablo.argibay@hospitalitaliano.org.ar

man ASCs (hASCs) have the capacity to differentiate in vitro into mesodermal and nonmesodermal lineages, between them into neuron-like cells (2,65); and in vivo may contribute to improvements in neuronal functions (36). hASCs express genes across the three germ layers, thus supporting a differentiation potential towards nonmesodermal lineages (9,37).

Decisions regarding self-renewal versus commitment are based on microenvironmental cues, which predominantly use the Notch, Wnt, BMP, and Shh signaling pathways (10,21,43,56). Understanding each step of differentiation and characterizing differentiation phenotypes are the basis of stem cell engineering. Future stem cell research is likely to focus on improving the ability to guide the differentiation of stem cells and to control their survival and proliferation for clinical application.

Sonic hedgehog (Shh) is an intercellular signaling morphogen, which plays an important role in many developmental stages and stem cell regulation (31,50,70). Recent studies have involved Shh in the proliferation and cell fate specification of several stem cells (1,8,10,39,56,77). It transduces its signal to cells interacting with the 12-transmembrane protein, Patched (Ptc), that serves as a receptor for Shh (47). Smoothed (Smo), a 7-transmembrane protein, is a signal transducer that, in the absence of Shh, interacts with Ptc. This Smo–Ptc interaction represses Smo-signaling activity, therefore also acting as a repressor of Shh signaling. Binding of Shh to its receptor Ptc releases the repression exerted on Smo and transfers the signal activating transcription factors of the Gli family, thus activating a number of downstream targets of the Shh pathway. In mammals, Shh signaling involves two Ptc receptors (Ptc1 and Ptc2) and at least three Gli proteins (Gli1, Gli2, and Gli3) (23,30,64). Gli1 is one of Shh target genes and has been characterized as a reliable marker of Hedgehog signaling activity (29,63).

Noggin belongs to a class of polypeptides that bind to bone morphogenetic proteins (BMPs) and consequently prevents the activation of BMP receptors (71). Signaling from BMP family instructs adult NSCs to adopt a glial fate and Noggin diverts stem cells from glial to neuronal fate. It was reported that ectopic Noggin expression promoted neuronal differentiation (44).

The main downstream target genes of BMP signaling are the Id proteins. The Ids (inhibitors of DNA binding) are members of the helix–loop–helix (HLH) family, but they lack the basic DNA binding domain (6,57). Heterodimerization of Ids with other bHLH transcription factors results in active transport of such complexes into the nucleus and in simultaneous inhibition of their binding to DNA (12). Concomitantly,

Ids can act as dominant-negative regulators that interfere with the transcriptional activities of proneural proteins in neuronal progenitors (76).

Among the four members of Id family, Id1 seems to be specifically required for proliferation of neuroepithelial cells and for timing of neuronal differentiation in the embryonic stage (16,22,34,45,57). In mice, Id1 is expressed in proliferating neural precursors in the ventricular zone (VZ), but is not found in differentiated cells at the early stages of embryogenesis (14,18). Id2 is expressed by early newborn neurons in the developing nervous system and continues to be expressed in specific neurons throughout development and adulthood in the mouse brain (54,68), suggesting that Id2 may be involved in other cellular processes besides proliferation. Id3 expression is more widespread and apparent throughout embryogenesis; it is present in various regions of embryo, including areas of the developing brain. Finally, Id4 transcripts are mainly detected in neuronal tissues (61,68). Based on this evidence, it is clear that the Id gene family plays a significant role in neural progenitor cell proliferation and differentiation.

In general, in the mammalian nervous system, the expression of Id1 and Id3 is located in the less differentiated neuroblasts, whereas Id2 and Id4 are expressed in different sets of more mature presumptive neurons. Specifically, Id2 and Id4 are expressed in the presumptive interneurons and motor neurons, respectively, during spinal cord development. These results indicate that the expression of two subclasses of the Id family (i.e., Id1, Id3 and Id2, Id4) may have different physiological consequences (i.e., the specification of different differentiation states of neuronal cells during development).

Our working hypothesis was that neuronal differentiation of adult mesenchymal stem cells could recreate some of the molecular mechanisms involved in neural stem cells differentiation. Therefore, the objective of the present work was to evaluate the differential expression of some Shh and BMP signaling genes involved in proliferation and differentiation of human adipose tissue mesenchymal stem cells to neuron-like cells.

MATERIALS AND METHODS

Cell Isolation and Culture

After informed consent and approval of ethics committee of research protocols from Hospital Italiano de Buenos Aires, adipose tissue samples were obtained during abdominal and mammary plastic surgeries of 23 healthy donors between 26 and 56 years old. The adipose tissue was extensively washed with

Hank's balanced salt solution (HBSS, Sigma, Buenos Aires, Argentina) to remove blood and fibrous material and vessels were carefully dissected and discarded. The remaining tissue was finely minced and digested with 0.1% of Collagenase Type I (Gibco, California, USA) for 45 min with gentle agitation. Enzyme activity was neutralized with a twofold volume of standard medium containing Dulbecco's modified Eagle medium (DMEM, Gibco) with 20% of fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin (ATB, Gibco), and centrifuged for 12 min at 400 × g. The supernatant containing the lipid droplets was discarded. The stromal vascular fraction (SVF) settled at the bottom was resuspended in standard medium and seeded in culture dishes (Nunc International, Roskilde, Denmark).

SVF cultures were incubated at 37°C in a 5% CO₂ atmosphere. After 48 h, nonadherent cells were removed. When they reached 70–80% of confluence, adherent cells were trypsinized (0.25% at 37°C for 5 min, Sigma), harvested, and washed with standard medium to remove trypsin and were then expanded in larger dishes. A homogenous cell population of hASCs was obtained after 2 or 3 weeks of culture. Cells at early passages (3–5) in culture were used for the experiments.

Neuronal Differentiation

Neuronal differentiation of 12 samples was initiated at passage 3–5 using a modification of previous neuronal induction protocols (42,46,67,73,78). Briefly, the cells were plated in dishes until they were subconfluent. Preinduction was performed for 48 h after discarding the medium, washing the cells, and adding new DMEM containing 20% FBS and 1 mM β-mercaptoethanol (BME, Riedel, De Haën, Germany). Then, the preinduction medium was removed and the induction medium was added to the culture. The composition of induction medium was: DMEM with 100 µM butylated hydroxyanisole (BHA, Sigma), 10⁻⁶ M retinoic acid (RA, Sigma), 10 ng/ml epidermal growth factor (EGF, Invitrogen, Brazil), and 10 ng/ml basic fibroblast growth factor (bFGF, Invitrogen). Cells were incubated in this medium during 14 days. The medium was changed every 3 days. The cells were monitored continually after neuronal induction and were lysed for RNA extraction or fixed for immunostaining. One noninduced culture dish was also analyzed with every experiment as a control.

Staining With Endocytotic Marker FM1-43

To visualize synaptic vesicle accumulations, cells were loaded with 15 µM styryl dye {N-[3-(triethyla-

monio)propyl]-4-(4-dibutylaminostyryl)pyridinium dibromide (FM1-43, Molecular Probes, Invitrogen)} in depolarizing extracellular solution (70 mM K⁺) during and up to 90 s. After loading, the cells were washed in low Ca²⁺ solution for 5–10 min. Dishes were visualized under a fluorescence microscope (NIKON ECLIPSE E400).

Immunocytochemistry

Noninduced and induced hASCs were fixed with 4% paraformaldehyde at room temperature. After blocking to prevent nonspecific antibody binding, plates were incubated with primary antibodies at 4°C overnight. Following a PBS washing, the plates were incubated with avidin/biotin blocking kit with fluorescein isothiocyanate (FITC) or Texas Red avidins (1:50, 1:200, Vector Laboratories, Burlingame, CA, USA) at room temperature during 1 h in order to label the antibodies. In some samples, nuclei were counterstained with Hoechst 33258 (Sigma).

The primary antibodies used were anti-glial fibrillary acidic protein (1:200, GFAP, Chemicon International, Inc., Temecula, CA, USA), anti-neurofilament 200 (1:100, NF-200, Chemicon), anti-βIII tubulin (1:100, Chemicon), anti-nestin (1:200, Chemicon), anti-synaptophysin (1:25, Dako, Glostrup, Denmark), and anti-neuronal nuclei (1:200, NeuN, Chemicon). Monoclonal antibodies against CD90 (1:10, Thy-1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), CD34 (1:20, Abcam, Cambridge, UK), CD45 (1:20, Abcam), and Stro-1 (1:100, R&D System, Minneapolis, MN, USA) were used to characterize hASCs. All the conditions were maintained in negative controls, except that the primary antibodies were eliminated.

Dishes were examined under the fluorescence microscope. For each marker, five random nonoverlapping fields (±60 hASCs per field) per dish were photographed. Data are reported as a percentage of total cells expressing positive marker labeling among total labeling cells. All assays were repeated in three to six independent experiments.

Quantitative Real-Time PCR

Quantification was performed using real-time PCR to compare the levels of expression of Shh and BMP signaling genes involved in proliferation and neuronal differentiation. For this purpose between 6 and 12 samples of induced and noninduced hASCs were analyzed.

Total RNA from hASCs before and 14 days after neuronal induction was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's recommendations. The purity and integrity of the extracted RNA were evaluated by optical density

measurements (260:280 nm ratios) and by visual observation of samples electrophoresed on agarose (Biodynamics, Buenos Aires, Argentina) gels. Two micrograms of each total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) to eliminate possible contamination of genomic DNA. One microgram of treated RNA was used as template in a 20- μ l volume cDNA synthesis reaction.

Primer sequences were designed using LightCycler Probe Design Software 2.0 (Roche Applied Science, Mannheim, Germany) using gene sequences obtained from the GeneBank database (Table 1).

Quantitative real-time PCR was performed using SYBR Green (Invitrogen), Platinum Taq Polymerase (Invitrogen), and LightCycler 2.0 Instrument (Roche Applied Science). The expression of human β -actin was used to standardize gene expression levels. Each sample was run four times. Control experiments without template cDNA revealed no nonspecific amplification. When PCR results were negative, cDNAs from human cell lines or tissues were run as positive controls in order to eliminate the possibility of false-negative results.

To verify the identity of amplified DNAs, the size of the PCR products were checked on agarose gel.

Statistical Analysis

All data are presented as mean $\pm$ error deviation. The values obtained from the Real Time PCR were analyzed with Relative Standard Curve method and the error deviations were obtained according to the Applied Biosystems User Bulletin No. 2 (P/N 4303859). Statistical comparison of the results obtained with induced and noninduced hASCs was carried out according to the Student's *t*-test (to compare two treatment groups). Differences were considered statistically significant when $p < 0.05$. Statistical analysis was

performed using the program Primer of Byostatistics Version 5.0 (McGraw-Hill, 2002).

RESULTS

hASCs Characterization

When plated, the SVF exhibited a heterogeneous cell population with different morphologies that included spindle-shaped, large, flat, and small round cells. The spindle-shaped cells gradually became predominant with continued cell growth and division in culture (Fig. 1A–C). Immunocytochemistry analysis of hASCs at passages 3–5 demonstrated that the cells were negative for CD34 and CD45, which are cell surface markers associated with hematopoietic cells. In contrast, $49.3 \pm 0.2\%$ of hASCs expressed CD90 and $40.8 \pm 1.2\%$ of the cells expressed Stro-1, which are two typical markers of mesenchymal stem cells. There were also cells that coexpressed both markers (Fig. 1D–I). When cultured in lineage-specific differentiation culture medium, hASCs could undergo osteogenic, adipogenic, and chondrogenic differentiation (data not shown).

Thus, we concluded that morphology, plasticity and expression of the characteristic membrane markers were indicative of a mesenchymal stem cell population.

Morphologic Changes After Neuronal Induction

During preinduction hASCs experienced few changes; the fibroblastic morphology was maintained but the cells became more elongated. After the neuronal induction, the morphology of hASCs began to change within a few hours. The cells changed from flat, elongated, spindle-shaped to round cell bodies with several branching extensions and retractile char-

TABLE 1
PRIMERS USED FOR REAL-TIME PCR EXPERIMENTS

Gene	Genebank	Forward	Reverse	Annealing Temp.
Gli1	NM_005269	GGAAGGAGTTCGTGTGCC	CACTTGTGTGGCTTCTCGC	60°C
Gli2	NM_030381	CAACTGCCACTGGGAAGAC	GTGGATGTGCTCGTTGTTG	59°C
Gli3	NM_000168	AGCAGGACCTCAGCAACAC	TTGGCTTCTCTGCCTTGAC	59°C
Ptc	NM_000264	TGGAGCAGATTTCCAAG	TTTGAATGTAAACAACCCAGT	58°C
Smo	NM_005631	GTCCTCACTGTGGCAATCC	CGCACGGTATCGGTAGTTC	59°C
Noggin	NM_005450	TGTAAATATAGAGAACAAATGGAATGACT	ACGGGATATTATAAAGAATAAATAGCAGAT	57°C
Id1	NM_181353.1	CTGCTCTACGACATGAACG	CTCACCTTGCGGTTCTG	57°C
Id2	NM_002166.4	AACATGAACGACTGCTACT	AGGATTTCCATCTTGCTCAC	58°C
Id3	NM_002167.3	CGACATGAACCACTGCTAC	GGATTTCCACCTGGCTAAG	58°C
Id4	NM_001546.2	TGCTACCAAAGGACAAACTC	TTCTCCCACTGTTGCCTA	58°C
β -Actin	NM_001101	CCCTTGCCATCCTAAAGC	TGCTATCACCTCCCCTGTGT	57°C

acteristics similar to those observed in cultured neuronal cells. After 12 h of neuronal induction, a mean of 13% of hASCs displayed retraction of the cytoplasm towards the nucleus, forming compact cell bodies with cytoplasmic extensions. The cell bodies became increasingly spherical with multiple cell processes (Fig. 2). Neuronal induction was carried out for 14 days. During this time the number of cells exhibiting the neuronal phenotype increased to a mean level of $59.7 \pm 2.5\%$.

Synaptic Vesicle Staining During Neuronal Differentiation

To test whether neuron-like cells develop synaptic activity, cells differentiated during 14 days were loaded with 15 μM FM1-43, which stains recycling synaptic vesicles. Saturating staining was performed using a depolarizing (70 mM K^+) extracellular solution to stimulate synaptic vesicle cycling in the presence of FM1-43. We observed a punctuate labeling

in induced cells, which could probably indicate the presence of functional presynaptic terminals. No labeling was detected in noninduced cells (Fig. 3).

Immunocytochemistry After Neuronal Induction

To characterize neuronal and glial differentiation, hASCs were stained with a panel of markers against neuronal and glial cells before and after neuronal induction. Before exposure to neuronal induction media, hASCs expressed high levels of nestin, a progenitor and glial marker. The nestin expression by mesenchymal stem cells has been reported in concordance with the acquisition of the ability to respond to extrinsic signals and cues driving their neuronal differentiation (72). Control hASCs did not express any other neuronal or glial marker examined.

Following exposure to induction media, hASCs exhibited immunocytochemical changes consistent with neuronal lineage cells. At the end of the induction treatment, hASCs stained brightly for nestin (100%),

Figure 1. hASCs morphology and characterization. Microscopic photographs of hASCs at passage 0 (A), 3 (B), and 5 (C) where the fibroblast-like and spindle-shaped cell morphology is observed. CD90 expression in hASCs at passage 5 with a cytoplasmic localization (D, E) and Stro-1 expression at the same stage with a perinuclear localization (G, H). There is coexpression of CD90 (Texas Red labeled, red) and STRO1 (FITC labeled, green) in hASCs at passage 5 (F, I). Scale bar: 100 μm .

Figure 2. Morphologic changes following neuronal induction of hASCs. Human ASCs grown under standard medium (A). hASCs incubated during 48 h with preinduction medium (B, C). (D–F) During 14 days of neuronal differentiation, morphological changes were observed such as cytoplasm retraction towards the nucleus and cells increasingly displayed neuronal traits of pyramidal, perikaryal appearances. Some cells appeared to make contact with others. Scale bar: 100 μ m.

GFAP ($24.6 \pm 2.5\%$), NF-200 ($32.9 \pm 5.1\%$), synaptophysin ($42.1 \pm 8.4\%$), as well as β III-tubulin ($29.4 \pm 7.2\%$). Double staining of induced hASCs with nestin and β III-tubulin revealed cells that coexpressed both markers (Fig. 4). In contrast, the expression of the mature neuronal marker NeuN was not observed in induced hASCs. Because nestin, β III-tubulin, NF-200, synaptophysin, and GFAP are neuronal-glial markers, our data suggest that hASCs probably differentiate into some neuron-like cells in vitro.

Changes in hASCs Gene Expression

To gain insight into the molecular mechanisms involved in proliferation and neuronal differentiation, we evaluated the expression before and 14 days after of neuronal induction of Shh and BMP signaling genes known to have important roles during normal nervous system development. Using quantitative real-

time PCR, induced and noninduced hASCs gene expression profiles of the following factors were compared: sonic hedgehog transcription factors (Gli1, Gli2, and Gli3) as well as its receptors (Ptc and Smo), BMP target genes (Id1, Id2, Id3, and Id4), and Noggin (BMP signaling inhibitor).

The five genes of the sonic hedgehog signaling pathway were found to be expressed in hASCs, suggesting that these cells would be able to respond to sonic hedgehog signaling from the environment. After neuronal differentiation, Gli1, Gli2, Gli3, and Smo expression decreased significantly ($p < 0.01$), whereas only Ptc expression did not show significant changes (Fig. 5). These results are consistent with the fact that Shh pathway is involved in the maintenance of a pool of cells with stem cell properties in other tissues and that their expression is downregulated after differentiation (40,73,79).

One of the signaling pathways involved in commit-

Figure 3. FM1-43 staining. Fluorescence images of hASCs differentiated into neuron-like cells loaded with FM1-43. Note the punctuate appearance of dye loading (A, B). Noninduced hASCs did not load with FM1-43 (C). Scale bar: 100 μ m.

Figure 4. Immunocytochemical analysis of neuronally induced and noninduced hASCs. β III-tubulin expression in induced cells (A, B) and noninduced hASCs (C). GFAP expression in induced cells (D, E) and noninduced cells (F). Induced hASCs were costained with β III-tubulin (FITC labeled, green) (G) and nestin (Texas Red labeled, red) (H). Merged image confirming coexpression of β III-tubulin and nestin in the same cells (I). Synaptophysin expression in induced (J, K) and noninduced hASCs (L). Scale bar: 100 μ m.

ment and differentiation is the BMP pathway. In our experiment, we found Noggin expression in hASCs after neuronal differentiation, an antagonist of BMP, which was reported to inhibit BMP signaling promoting neuronal differentiation (44). Noggin expression was not detected in noninduced hASCs. Furthermore, we analyzed Id expression before and after neuronal differentiation and Id1, Id2, Id3, and Id4 expression was found in noninduced hASCs. However, upon exposure of cultures to neuromorphogens, when the cells stopped proliferating and started to differentiate morphologically, a decrease in the Id1 expression was detected. These results are similar to previous

reports where there was a deep reduction in Id1 protein in vitro when cells differentiated under treatment with serum and/or morphogens (55), a proneural gene expansion and premature neuronal differentiation when Id1 expression was inhibited by siRNA (4), and a significant inhibition of neuronal differentiation when Id1 was overexpressed (35). On the other hand, an increase in Id4 expression after neuronal differentiation was detected, which is in accordance with the fact that this gene is expressed in more mature phenotypes of neural cells (62,68). There were no significant changes in Id2 and Id3 expression after treatment (Fig. 6).

Figure 5. Relative gene expression of Sonic Hedgehog signaling pathway in hASCs treated with induction medium compared to noninduced hASCs. Hedgehog signaling is downregulated during neuronal differentiation. * $p < 0.05$; ** $p < 0.01$.

DISCUSSION

Cellular therapies are promising approaches not only in the treatment of several neurological diseases such as Parkinson's disease (33) and Huntington's disease (15), but also for spinal cord injury (27). One main problem concerns the origin and nature of the cells to be used for such procedures. The ideal cell should exhibit several key properties, including: (1) a high level of proliferation in vitro, allowing the production of a large number of cells from a minimal amount of donor material, (2) a good control of this proliferative activity in vivo, and (32) a phenotypic plasticity allowing the differentiation into appropriate neuronal or glial phenotype.

Several recent reports suggest that adipose tissue stem cells could be a nonembryonic or nonfetal source of stem cells suitable for cell replacement strategies in the treatment of CNS disorders (36,59).

In our study, hASCs were isolated from human adipose tissue using an adhesion procedure and were

selected by their capacity and rapid proliferation. We demonstrated that these stromal cells are able to express mesenchymal stem cell markers, CD90 and Stro-1, as well as nestin, a marker of the first step in the progression to neural lineage. We also observed that these cells have the ability to differentiate into multiple lineages, such as osteogenic, adipogenic, chondrogenic, and neurogenic, in accordance with previous studies (2,3,17,25,26,38).

Following the induction of neuronal differentiation, hASCs revealed biologic and morphologic characteristics of neural lineages and an increased expression of neuron specific proteins, β III-tubulin, NF-200, and synaptophysin, and of neural progenitor markers, GFAP and nestin. The critical epigenetic molecules in our chemically defined medium, which might contribute to this differentiation, are probably bFGF and RA, both of which are routinely used in the expansion of mammalian neural crest stem cells (41,52,66). Basic FGF produces neurogenic, proliferative, and patterning effects on adult CNS stem cells (11,13).

RA is a commonly used neural induction reagent in vitro that enhances expression of markers of neural crest (48,51).

Furthermore, FM1-43 staining in hASCs was observed after neuronal differentiation, which could probably be some evidence of the presence of functional presynaptic terminals because noninduced hASCs lacked this staining. This dye allows the visualization of the synaptic vesicle in living cells in real time. We used the fluorescent dye FM1-43 because it is employed to detect depolarization-induced synaptic vesicle recycling, which is a neuron-specific property (7). This dye enters the differentiated neurons in the presence of a high extracellular concentration of KCl when the synaptic vesicles are recycled back into the neurons after depolarization.

An understanding of the molecular regulation of such "mesenchymal-neural" transition may be very important when considering the use of stem cells in the treatment of CNS disorders. To study some of the pathways involved in this transition, we analyzed the gene expression of different factors like

Ptc, Smo, Gli1, Gli2, Gli3, Noggin, Id1, Id2, Id3, and Id4.

Shh, an important morphogen, is involved in a variety of cellular processes during development and disease, such as cell fate determination, proliferation, differentiation, and survival. It has widely been studied in the context of stem cells, with the expectation that specific modulation of the signal may provide an in vitro tool for stem cell expansion and manipulation of lineage specific differentiation in the future. The main finding of the present work is that Shh signaling represents an endogenous mechanism that regulates proliferation of cells with stem cells properties in the adipose tissue, the hASCs. We demonstrated that human ASCs are endowed with an active hedgehog signaling. Shh receptors, Ptc and Smo, and the downstream transcription factors (Gli1, Gli2, and Gli3) were expressed in hASCs during proliferation. However, their expression is downregulated after neuronal induction, in accordance with the fact that proliferation decreases during the process of differentiation. The decrease of Shh signaling could be associated

Figure 6. Relative gene expression of Noggin and Id genes in hASCs treated with induction medium compared to noninduced hASCs. Id1 is downregulated during neuronal differentiation, whereas Id4 and Noggin are upregulated after morphogen treatment. * $p < 0.05$; ** $p < 0.01$.

with decreases in Gli1 expression. Indeed, Gli1 is the primary activator of hedgehog signaling and a decrease of its expression *in vivo* and *in vitro* leads to a decreased signaling (63). These findings show that Shh is a niche factor regulating the number of stem cells and they contribute to understand the mechanisms that modulate proliferation of stem cells. The controlled modulation of Shh signaling *in vitro* and *in vivo* is likely to lead to the development of protocols to increase the number of cells with stem cell properties in an effort to ameliorate the effects of degenerative diseases.

Both human and murine Ids are known to be the main targets of cell differentiation signaling (53,74,75). They have emerged as key regulatory intermediates for coordinating differentiation-linked gene expression with cell cycle control in response to extracellular signaling. There exists a paradox between the similar functional properties of different Id proteins in promoting cell growth and arresting differentiation and their apparent disparate mechanisms of action. During embryogenesis, an abundance of Id1, Id2, and Id3 transcripts was observed in VZ of mouse and rat brain. The expression of different Id genes fits well with the chronology of neuronal development: Id1 and Id3 expression is found in mitotically active and less differentiated neuroblasts. Their expression diminishes at later stages when mitotically active neuroblasts are no longer present. On the other hand, Id2 and Id4 are expressed first in presumptive neuroblasts and later in specific presumptive postmitotic neurons: Id4 mRNAs first appear in the motor neurons and later in the region where the sensory neurons reside. Id2 expression is first detected where the putative subset of motor neurons or/and interneurons are located. Thus, both Id2 and Id4 mark specific groups of neurons from very early stages, whereas Id1 and Id3 expression is present in most mitotically active neuroblasts (68). Our data, which confirm that the main function of Id1 is strictly connected with regulation of cellular differentiation, do not differ from data obtained for stem cells of neuroectodermal tissues and human cord blood induced to neural differentiation (35,75). Previous studies showed that Id1 protein could act as a dominant-negative regulator to interfere the transcriptional activities of proneural proteins and inhibit premature differentiation of neuronal progenitors (76). In this study, we found Id1 expression in hASCs, which probably maintain their self-renewal capacity and prevent neuronal differentiation. How-

ever, after neuronal induction, the Id1 expression decreased significantly, in accordance with previous reports (4,55). Furthermore, we demonstrated that there was a significant increase in Id4 expression after neuronal differentiation, which was associated with more mature phenotypes of neural cells (69).

Thus, distinct Id gene family members may have different functional roles that may be dependent on their expression levels and on the coexpression of certain Id-associated bHLH transcription factors. For this reason, it would be necessary to study some bHLH factors to associate this Id expression pattern with neuronal differentiation or cell proliferation.

The endogenous BMP antagonist Noggin is a potential molecule that controls neural commitment. It appears to support neurogenesis by binding to endogenous BMP and consequently preventing activation of BMP receptors, which would otherwise induce gliogenesis (44). The results of this study showed that only hASCs expressed Noggin after neuronal differentiation according to the acquisition of a neuronal commitment.

In conclusion, nestin expression by hASCs should be regarded as a first step in the progression to neural lineage. After neuronal induction, we probably reached the first step of neuronal differentiation, owing to the presence of neuronal markers and the increase in transcription factors expression typical of neural lineage cells. The differentiation of hASCs into excitable neuronal-like cells might require further steps of neuronal maturation process. It is, however, encouraging to notice that our procedure can induce the formation of possible synaptic vesicles, which is a specific-neuron property in functional presynaptic terminals. A better knowledge of the molecular mechanisms responsible for the regulation of the neuronal differentiation of hASCs is still needed before considering ASCs as an appropriate cellular material to be used for cell replacement therapies in CNS disorders.

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