Gene 511 (2012) 427–436

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Short Communication

# Neurogenic differentiation of human adipose-derived stem cells: Relevance of different signaling molecules, transcription factors, and key marker genes

Alejandra Johana Cardozo [a], Daniel Eduardo Gómez [b,1], Pablo Francisco Argibay [a,*]

[a] Instituto de Ciencias Básicas y Medicina Experimental, Hospital Italiano de Buenos Aires, Potosí 4240, Ciudad Autónoma de Buenos Aires (1199), Argentina
[b] Laboratorio de Oncología Molecular, Universidad Nacional de Quilmes, R. S. Peña 352, Bernal (1876), Buenos Aires, Argentina

ARTICLE INFO

*Article history:*
Accepted 12 September 2012
Available online 18 September 2012

*Keywords:*
Adipose-derived stem cells
Neuronal differentiation
Proneural genes
Patterning molecules
Neurogenesis

ABSTRACT

Since numerous diseases affect the central nervous system and it has limited self-repair capability, a great interest in using stem cells as an alternative cell source is generated. Previous reports have shown the differentiation of adipose-derived stem cells in neuron-like cells and it has also been proved that the expression pattern of patterning, proneural, and neural factors, such as Pax6, Mash1, Ngn2, NeuroD1, Tbr2 and Tbr1, regulates and defines adult neurogenesis. Regarding this, we hypothesize that a functional parallelism between adult neurogenesis and neuronal differentiation of human adipose-derived stem cells exists. In this study we differentiate human adipose-derived stem cells into neuron-like cells and analyze the expression pattern of different patterning, proneural, neural and neurotransmitter genes, before and after neuronal differentiation. The neuron-like cells expressed neuronal markers, patterning and proneural factors characteristics of intermediate stages of neuronal differentiation. Thus we demonstrated that it is possible to differentiate adipose-derived stem cells in vitro into immature neuron-like cells and that this process is regulated in a similar way to adult neurogenesis. This may contribute to elucidate molecular mechanisms involved in neuronal differentiation of adult human non-neural cells, in aid of the development of potential therapeutic tools for diseases of the nervous system.



## 1. Introduction

Since numerous diseases affect the central nervous system, identifying regulators that control stem cell self-renewal and neuronal differentiation is essential for the development of stem cell-based cell replacement therapies. It is known that the neurodegenerative diseases and brain injuries lead to neuronal loss and that the neural tissue has long been regarded as restricted in regeneration capacity (Reynolds and Weiss, 1992; Richards et al., 1992). But stem cells, with ability to self-renew and differentiate into multiple lineages, are found in different tissues and adipose tissue is an abundant source of adipose-derived stem cells (ASCs) (Gronthos et al., 2001; Hauner et al., 1987; Zuk et al., 2001). Human ASCs have capacity to differentiate in vitro into neuron-like cells (Anghileri et al., 2008; Ashjian et al., 2003; Cardozo et al., 2010; Dhar et al., 2007; Jang et al., 2010; Safford et al., 2002), and in vivo may contribute to functional benefits in a wide range of neurological insults (Chi et al., 2010; Kang et al., 2003; Kim et al., 2007; Kulikov et al., 2008; Wei et al., 2009).

One of the most important issues in stem cell biology is to uncover molecular mechanisms underlying stem cell self-renewal and differentiation. The transition from an undifferentiated to a fully differentiated neural cell comprises a series of sequential steps: proliferation, commitment, specification, and terminal differentiation (Temple, 2001). Throughout neuronal differentiation process different transcription factor families are expressed, such as proneural genes, patterning factors, neuronal and neurotransmitter genes.

Proneural genes are a set of transcriptional regulators that code for basic Helix–Loop–Helix (bHLH) proteins, which play a central role in the differentiation of neural progenitors into neurons and influence the particular neuronal subtypes, produced in a region-specific manner. The expression of proneural bHLH proteins is both necessary and sufficient to promote the generation of differentiated neurons from undifferentiated progenitor cells (Bertrand et al., 2002; Brunet and

*Abbreviations:* ASCs, adipose-derived stem cells; bHLH, basic Helix–Loop–Helix; Mash, mammalian achaete-scute homolog 1; Math, mammalian atonal homolog 1; Ngn, neurogenin; NeuroD, neurogenic differentiation; Tbr, T-box brain; Pax6, paired box gene 6; BHA, butylated hydroxyanisole; RA, retinoic acid; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; CP, crossing point; DCX, doublecortin; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; NSE, neuron specific enolase; NF200, neurofilament 200 kDa; M1, cholinergic muscarinic receptor 1; GABA, gamma-aminobutyric acid; GABRA1, GABA receptor type A subunit α1; GABAbR1, GABA receptor type B subunit 1; GABAbR2, GABA receptor type B subunit 2; GABRD, GABA receptor type A subunit δ; NKCC1, $Na^+$, $K^+$, $2Cl^-$ co-transporter 1; KCC2, $K^+/Cl^-$ co-transporter 2; GluK5, glutamatergic receptors type kainate subtype 5; mGluR5, metabotropic glutamate receptor 5; NMDA, N-methyl-D-aspartic acid; NR1, NMDA 1 receptor.

* Corresponding author at: Instituto de Ciencias Básicas y Medicina Experimental, Hospital Italiano de Buenos Aires, Potosí 4240 – 8° piso – C1199ACL, Ciudad Autónoma de Buenos Aires, Argentina. Tel.: +54 11 4959 0200x8919; fax: +54 11 4959 0200x4155.
*E-mail addresses:* degomez@unq.edu.ar (D.E. Gómez), pablo.argibay@hospitalitaliano.org.ar (P.F. Argibay).
[1] Tel./fax: +54 11 4365 7257.

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http://dx.doi.org/10.1016/j.gene.2012.09.038

Ghysen, 1999; Nieto et al., 2001; Ross et al., 2003; Sun et al., 2001; Tomita et al., 2000). Two classes of proneural genes can be distinguished: the determination factors, such as mammalian achaete-scute homolog 1 (Mash1), mammalian atonal homolog 1 (Math1), and neurogenin (Ngn), expressed early in mitotic neural precursor cells; and the differentiation factors, including neurogenic differentiation 1 (NeuroD1), NeuroD2, T-box brain 1 (Tbr1), Tbr2 and Math2, expressed later in post-mitotic cells (Bertrand et al., 2002; Morrison, 2001). These proneural genes are downstream effectors of paired box gene 6 (Pax6), a transcription factor that promotes neurogenesis and it is involved in the establishment of the progenitors that produce different classes of neurons followed by oligodendrocytes and astrocytes (Hack et al., 2005; Heins et al., 2002; Sugimori et al., 2007).

Cooperation among patterning, proneural and inhibitory HLH proteins establishes a molecular code that determines both the spatial and the temporal patterns of neurogenesis and gliogenesis, by establishing distinct profiles of proneural gene expression in different progenitor domains and by modulating the neurogenic or gliogenic activity of proneural proteins (Scardigli et al., 2001; Sugimori et al., 2007).

Although the molecular control of neuronal differentiation of hASCs is unknown, we suggest that it would exist a parallelism with the differentiation of adult generated neural cell-types, and that many of the regulatory control genes expressed during adult neurogenesis are also present in our neuronal differentiation protocol, providing an in vitro model of how generation of neuronal-like cells from adult stem cells might occur.

The aim of the present work is to study the expression of different patterning factors, proneural, neuronal, and neurotransmitter genes, before and after the neuronal differentiation of hASCs.

## 2. Materials and methods

### 2.1. Cell isolation and culture

After informed consent and approval of the ethics committee of research protocols from the 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 (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (Gibco), and centrifuged for 12 min at 400×*g*. The supernatant containing the lipid droplets was discarded. The stromal vascular fraction settled at the bottom was resuspended in standard medium and seeded in culture dishes (Nunc International, Roskilde, Denmark). Stromal vascular fraction cultures were incubated at 37 °C in a 5% CO2 atmosphere. After 48 h, no adherent 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 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.

### 2.2. Neuronal differentiation

Neuronal differentiation of 12 samples was initiated at passages 3–5 using a modification of previous neuronal induction protocols (Levy et al., 2003; Mareschi et al., 2006; Tao et al., 2005; Woodbury et al., 2000; Zuk et al., 2002). 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% fetal bovine serum and 1 mM β-mercaptoethanol (Riedel, De Haën, Germany). Then, the preinduction medium was removed and the induction medium was added to the culture. The composition of the 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 ribonucleic acid (RNA) or protein extraction or fixed for immunostaining. One non-induced culture dish was also analyzed in every experiment as control.

### 2.3. RNA isolation and reverse transcription

Total RNA from hASCs before and after 14 days of 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 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 deoxyribonucleic acid (DNA), no amplification was detected in a PCR using treated RNA as sample. One microgram of treated RNA was used as template in a 20 μl volume cDNA synthesis reaction using ImProm-II™ Reverse Transcriptase (Promega).

### 2.4. Quantitative real-time PCR

Quantification was performed using real-time polymerase chain reaction (real-time PCR) to determine the relative expression levels of proneural, neuronal and neurotransmitter genes involved in neural commitment and terminal differentiation. For this purpose between 6 and 12 samples of induced and non-induced hASCs were analyzed. Real-time PCR reactions were optimized to proceed without the formation of primer dimmers or ectopic bands, which would interfere with quantification. For quantitative real-time PCRs, SYBR Green (Invitrogen), Platinum Taq Polymerase (Invitrogen), and LightCycler 2.0 Instrument (Roche Applied Science) were used. After 45 PCR cycles, a melting curve of the PCR product was obtained. A smooth sigmoid was an indication that the only doubled-strand DNA present in the PCR product was the dimerized product, and no primer dimmers or ectopic bands were contaminating the reaction. To verify the identity of amplified cDNAs, the size of the PCR products was checked on agarose gel.

A calibration standard curve was created for each primer set by serial dilution of a cDNA pool of all samples analyzed. The number of cycles after which the fluorescence of a reaction rose above baseline was designated as crossing point (CP). As more cDNA was included in a reaction, the CP dropped, and a calibration curve of volume of cDNA against CP was plotted. The CP of each cDNA sample was then plotted onto this calibration curve, thus allowing relative cDNA quantification across PCR reactions.

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).

The expression of human β-actin gene was used to standardize gene expression levels. The experiments were done in quadruplicates. Control experiments without cDNA template revealed no non-specific 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.

### 2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, blocked to prevent nonspecific antibody binding and incubated with primary antibodies at 4 °C overnight. Following a PBS washing, the plates were incubated with avidin/biotin blocking kit with FITC or Texas Red avidins (1:50, 1:200, Vector Laboratories, Burlingame, USA). 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., California, 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, Glostrap, Denmark) and anti-neuronal nuclei (1:200, NeuN, Chemicon). All the conditions were maintained in negative controls, except that the primary antibodies were eliminated. Dishes were examined under the fluorescence microscope (NIKON ECLIPSE E400). ImageJ software (National Institute of Health) was used to pseudo-color images, adjust contrast, and add scale bars.

### 2.6. FM1-43 staining

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

### 2.7. Western blot analysis

Total protein was obtained from the cells using cold lysis buffer with protease inhibitor cocktail (Sigma). Protein concentration was determinate using Bio-Rad Protein Assay kit. Protein samples were separated by reducing SDS-PAGE on an 8-15% gel (Bio-Rad), and then transferred to a polyvinylidene difluoride (PVDF, Millipore, Billerica, USA) or nitrocellulose membrane (Bio-Rad). The membrane was incubated with primary antibodies and then horseradish conjugated secondary antibodies. Detection was performed using enhanced chemiluminiscence reagents as described by the supplier (Pierce, Rockford, USA). Primary antibodies that were used in this study were: anti-TAU (Dako), anti-synaptophysin (Dako), anti-βIII tubulin (Chemicon), anti-neuron specific enolase (NSE, Dako) and actin (Sigma). ImageJ software (National Institute of Health) was used to process and analyze the images.

### 2.8. Statistical analysis

All data are presented as mean ± 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 non-induced hASCs was done using the Student's *t*-test (to compare two groups). Differences were considered statistically significant when $p<0.05$. Statistical analysis was performed using the software Primer of Biostatistics Version 5.0 (McGraw-Hill, 2002).

**Table 1**
Primers used for real-time PCR experiment.

| Gene | GenBank | Forward | Reverse | Annealing T (°C) |
|---|---|---|---|---|
| *Proneural genes* | | | | |
| Pax6 | NM_000280.3 | TTACgAgACTggCTCCATCA | CCgCTTATACTgggCTATTT | 55 |
| Mash1 | NM_004316.3 | CCAgTTgTACTTCAgCACC | TgCCACTTTgAgTTTggAC | 55 |
| Ngn2 | NM_024019.2 | CCTggAAACCATCTCACTTCA | TACCCAAAgCCAAgAAATgC | 55 |
| NeuroDl | NM_002500.2 | CCACggATCAATCTTCTCAg | CATgATgTgAATggCTATCg | 55 |
| NeuroD2 | NM_006160.3 | CAgTAAggACTTTTAgAA | TgATTATTggTAgTAgTg | 45 |
| Mathl | NM_005172.1 | gAgAgAgCATCCCgTCTA | ggAATgTAgCAAATACTg | 53 |
| Math2 | NM_022728.2 | TgAATCCTTCTATgAAAgTA | gAAAATATCCCATTATAgTTA | 45 |
| Tbrl | NM_006593.2 | gAATCAgTCAgATACAgA | AAgACAggAgAgAgTTTA | 50 |
| Tbr2 | NM_005442.2 | CAAggTTCTgTATTTATTT | TTTAACTCATCTgATAgC | 48 |
| *Neuronal genes* | | | | |
| Nestin | NM_006617.1 | AgAgCgTAgAggCAgTAA | CACAgTggTgCTTgAgTT | 55 |
| GFAP | NM_002055.3 | gCCTCAAggACgAgATgg | TCgCCCTCTAgCAgCTTC | 57 |
| βIIItubulin | NM_006086.2 | gCCAAgTTCTggAAgTCA | gCCTCgTTgTAgTAgACgC | 56 |
| NSE | NM_001975.2 | TAACTTCCgTAATCCCAgTgT | AAgAggTCAggTAAgCCAA | 54 |
| TAU | NM_016835.3 | TACAgACCTgCggCTTCATAA | CCAgAAATAgTCCTgCTCAACA | 56 |
| Synaptophisin | NM_003179.2 | AggAggACAgggAgggAA | gggAgAgAgAggAgAgAgT | 64 |
| DCX | NM_000555.2 | gAAggCATTAgCgTTTCTCAT | gATACACAAgAggTAgAgCgg | 55 |
| MAP2 | NM_002374.3 | AACCCTTTgAgAACACgACA | TCTTTCCgTTCATCTgCCA | 54 |
| *Neurotransmitter genes* | | | | |
| GABRA1 | NM_000806.5 | TgATTCTCTCACAAgTCTCCT | gAgCACAgTTgTTACTCCAAA | 53 |
| GABRbR1 | NM_001470.2 | CCCTggTCATCAAgACATTC | gTTAAAggACAgACAgACAACA | 55 |
| GABRbR2 | NM_005458.7 | TgACACggAgTgCgACAA | TggATgTgACggATggAC | 55 |
| GABRD | NM_000815.4 | CCgACTACAggAAgAAgCA | AgAgggAgAAgAggACAAT | 55 |
| GAT1 | NM_003042.3 | gATCggTCTCTCTAACATCAC | TCAAAgAACACgAggAACAg | 53 |
| NKCC1 | NM_001046.2 | AgTAAAggAgTCgTgAAgTT | TTgACCCACAATCCATgACA | 53 |
| KCC2 | NM_020708.4 | CCTTCATCAACAgCACCgA | gTCCATCTCCTCCTCAAAC | 53 |
| M1 | NM_000738.2 | gCTACTTCTCCgTgACTCg | gCCCAgAgCACAAAggAAA | 56 |
| G1uK5 | NM_002088.3 | ggAATgACCTCAgCgTTT | TCCACAATACCgTCCAgAT | 52 |
| mGluR5 | NM_000842.3 | CCCTgTTTgTTACTgTAgTCTT | ggATAATgTAgCAgAgTTCCC | 56 |
| NR1 | NM_000832.6 | gTCCACCAgACTgAAgATT | CgTTgACTgTgAACTCCTC | 53 |
| *Housekeeping gene* | | | | |
| β-actin | NM_001101 | CCCTTgCCATCCTAAAAgCC | TgCTATCACCTCCCCTgTgT | 58 |

## 3. Results and discussion

### 3.1. Neuronal differentiation and neuronal genes

Human adipose-derived stem cells were obtained from healthy donors and then were subjected to neuronal differentiation. Following 2 weeks in neural induction medium, the cells changed from flat, spindle-shaped cells to neural-like cells with retraction of the cytoplasm towards the nucleus and several cytoplasmic extensions. Some of these cell processes make contact with other surrounding neural-like cells (Fig. 1).

Then we evaluated the relative expression of different genes typically expressed in neuronal cells using real-time PCR in hASCs before and after 14 days of neuronal differentiation. The analyzed genes were: nestin, βIII-tubulin, doublecortin (DCX), glial fibrillary acidic protein (GFAP), synaptophysin, TAU, microtubule-associated protein 2 (MAP2) and neuron specific enolase (NSE). We detected high expression of GFAP and nestin in non-induced cells and their expression was significantly decreased after neuronal differentiation ($P=0.009$; $P=0.006$, respectively). Immature and mature neuronal markers were detected in hASCs and there were a significant increase of βIII-tubulin ($P=0.057$), NSE ($P=0.046$), TAU ($P=0.001$), MAP2 ($P=0.007$) and synaptophisin ($P=0.036$) expression level after the neuronal differentiation protocol. We could not detected DCX expression before or after the induction protocol (Fig. 2).

We next confirmed neuronal differentiation by the expression of neuronal markers using immunocytochemistry and western blot analysis. Both methods revealed the expression of markers representative of different stages of neural lineage commitment, including the immature neural cell markers nestin and GFAP. Cells immunoreactive for βIII-tubulin, neurofilament 200 kDa (NF200), TAU, NSE and synaptophysin, markers of committed neural cells, were observed two weeks after neuronal differentiation. We could not detect the expression of mature neuron marker NeuN. The expression of βIII-tubulin, TAU, NSE and synaptophysin was increased significantly after neural induction compared with non-induced hASCs. We also detected the punctuated labeling of FM1-43 dye (recycling synaptic vesicles staining) in induced cells, which could probably indicate the presence of functional presynaptic terminals (Fig. 3).

This expression pattern is similar to that observed for neural stem cells, immature and mature neurons during the process of adult neurogenesis (Gotz and Barde, 2005; Kempermann et al., 2004; Merkle et al., 2004). Stem cells in adult brain exhibit characteristics of radial glia (Gotz and Barde, 2005; Kempermann et al., 2004; Merkle et al., 2004). They express the intermediate filament protein nestin and GFAP (Gotz and Barde, 2005). These stem cells undergo symmetric division giving rise to two identical stem cells. They can also divide asymmetrically and give rise to a new stem cell and one neuronal progenitor. The latter expresses nestin, PSA-NCAM and DCX (Kronenberg et al., 2003). Once the neuronal progenitors are committed, they become immature neurons expressing βIII-tubulin and calretinin markers. And finally, when these cells receive the correct neurogenic stimulation, they terminally differentiate and acquire the mature phenotype expressing NeuN, MAP2, NSE, TAU and synaptophisin (Abrous et al., 2005; Cameron et al., 1993; Mullen et al., 1992; Nacher et al., 2001).

### 3.2. Screen for patterning, proneural factors and neurotransmitters genes

To gain insight into the molecular mechanisms involved in proliferation and neuronal differentiation of hASCs and given their important roles in neuronal cell fate determination, we first analyzed the relative gene expression before and after 14 days of neuronal induction of proneural, patterning and neurotransmitters genes by real-time PCR.

In the present work we detected the expression of the patterning and determination proneural factors before and after the neuronal differentiation protocol. Pax6, Mash1 and Ngn2 were expressed at low levels in hASCs but after neuronal differentiation there was a significant increase of Pax6 expression level ($P=0.039$) and a trend was observed for Mash1 ($P=0.17$) and Ngn2 ($P=0.21$) (Fig. 4). The expression of Math1 gene was not detected using real-time PCR. These results are in accordance with previous reports of mammalian neurogenesis, where it has been shown that the earliest steps of this process involve expression of patterning and proneural bHLH genes such as Pax6, Sox2, Mash1, Math1 and Ngn2 (Bertrand et al., 2002). Pax6 regulates neuronal differentiation and its overexpression in brain cells promotes neurogenesis, whereas loss of Pax6 results in reduced neurogenesis and precocious formation of oligodendrocytes and astrocyte precursors (Heins et al., 2002). The proneural determination factors Mash1 and Ngn2 are present transiently in neural progenitor cells and if it is ectopically expressed in neural progenitor cells, the cells exit the cell cycle and begin expressing neuronal differentiation markers (Bertrand et al., 2002; Farah et al., 2000; Lee, 1997; Nakada et al., 2004; Parras et al., 2002; Sommer et al., 1996).

Based on previous reports (Hodge et al., 2008; Roybon et al., 2009), we also examined the expression pattern of the differentiation factors NeuroD1, NeuroD2, Math2, Tbr1 and Tbr2, which are expressed at intermediate and late stages of neurogenesis. We found that before neuronal differentiation there was a low expression of NeuroD1 and Tbr2, which increased after the differentiation protocol ($P=0.03$; $P=0.18$, respectively). The non-induced cells did not express Tbr1 but it was detected after the induction protocol (Fig. 4). However, we could not detect the expression of Math2 and NeuroD2 neither before nor after differentiation, both of them expressed in later stages of neurogenesis, in mature neurons. Neuronal progenitor expresses Tbr2 and immature granule neurons express Tbr1 and NeuroD1. While Tbr1 expression ceases during granule maturation, NeuroD1 is weakly maintained when

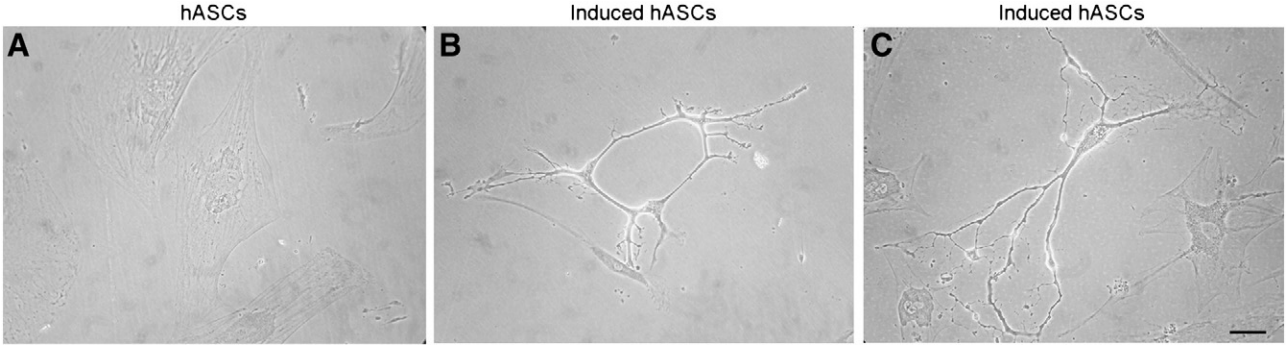


**Fig. 1.** Morphologic changes following neuronal differentiation of hASCs. (A) Human ASCs morphology grown under standard medium. (B–C) Induced hASCs adopt neuronal-like morphology with cytoplasm retraction towards the nucleus and cells increasingly displayed neuronal traits of pyramidal, perikaryal appearances. Scale bar: 100 μm.

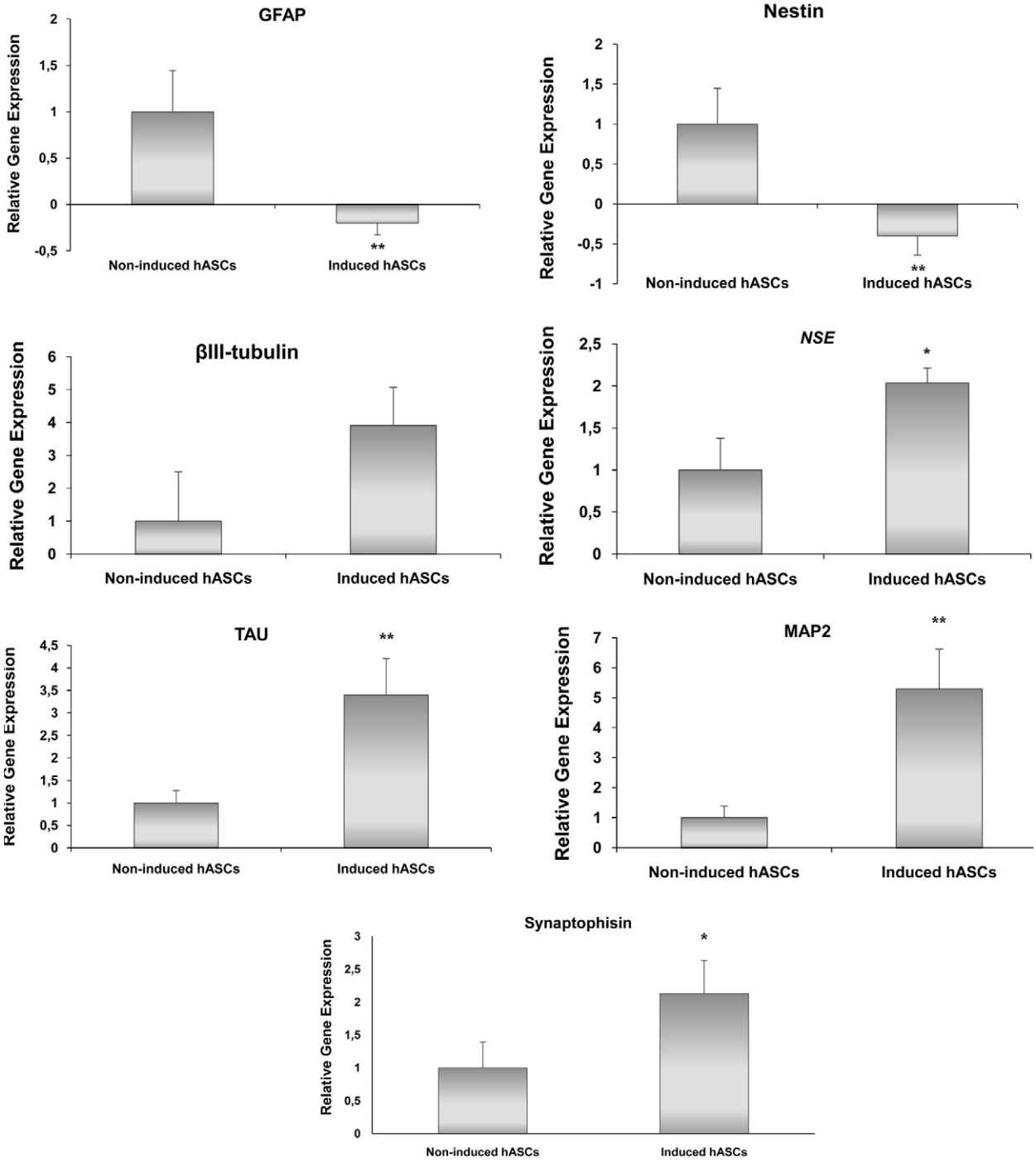


**Fig. 2.** hASCs express the neural stem cell markers GFAP and nestin genes at high levels, then they are downregulated after neuronal differentiation. After the induction protocol there is an increased expression of neuronal markers characteristic of immature and mature neurons such as βIII-tubulin, MAP2, NSE, synaptophisin and TAU. To carry out the real-time PCR analysis the hASCs were cultured in neuronal induction medium during 14 days. Relative gene expression of each gene (mean ± error deviation), normalized to the expression of the housekeeping gene β-actin, for induced and non-induced hASCs is shown. *$p<0.05$, **$p<0.01$ significantly different from control.

NeuN expression starts. Finally, NeuroD2 is highly expressed in mature neurons (Brandt et al., 2003; Hevner et al., 2006; Roybon et al., 2009).

Taken together our results and previous reports, we could confirm that adipose-derived stem cells after neuronal differentiation are committed to neural lineage and support the idea that we probably obtain an intermediate stage of differentiation between neuronal progenitor and immature neurons.

Several extracellular molecules, such as neurotransmitters, have been implicated in the extrinsic regulation of cell proliferation and neuronal differentiation in the central nervous system (CNS). For this reason, we decided to study specific neurotransmitter receptors that are expressed at different stages of adult neurogenesis such as the cholinergic muscarinic receptor 1 (M1), the gamma-aminobutyric acid (GABA) receptors type A subunit α1 (GABRA1), type B subunit 1 (GABAbR1), type B subunit 2 (GABAbR2), type A subunit δ (GABRD), $Na^+$, $K^+$, $2Cl^-$ co-transporter 1 (NKCC1) and $K^+/Cl^-$ co-transporter 2 (KCC2), and the glutamatergic receptors type kainate subtype 5 (GluK5), metabotropic glutamate receptor 5 (mGluR5) and N-methyl-D-aspartic acid (NMDA) 1 receptor (NR1) in hASCs, before and after neuronal differentiation.

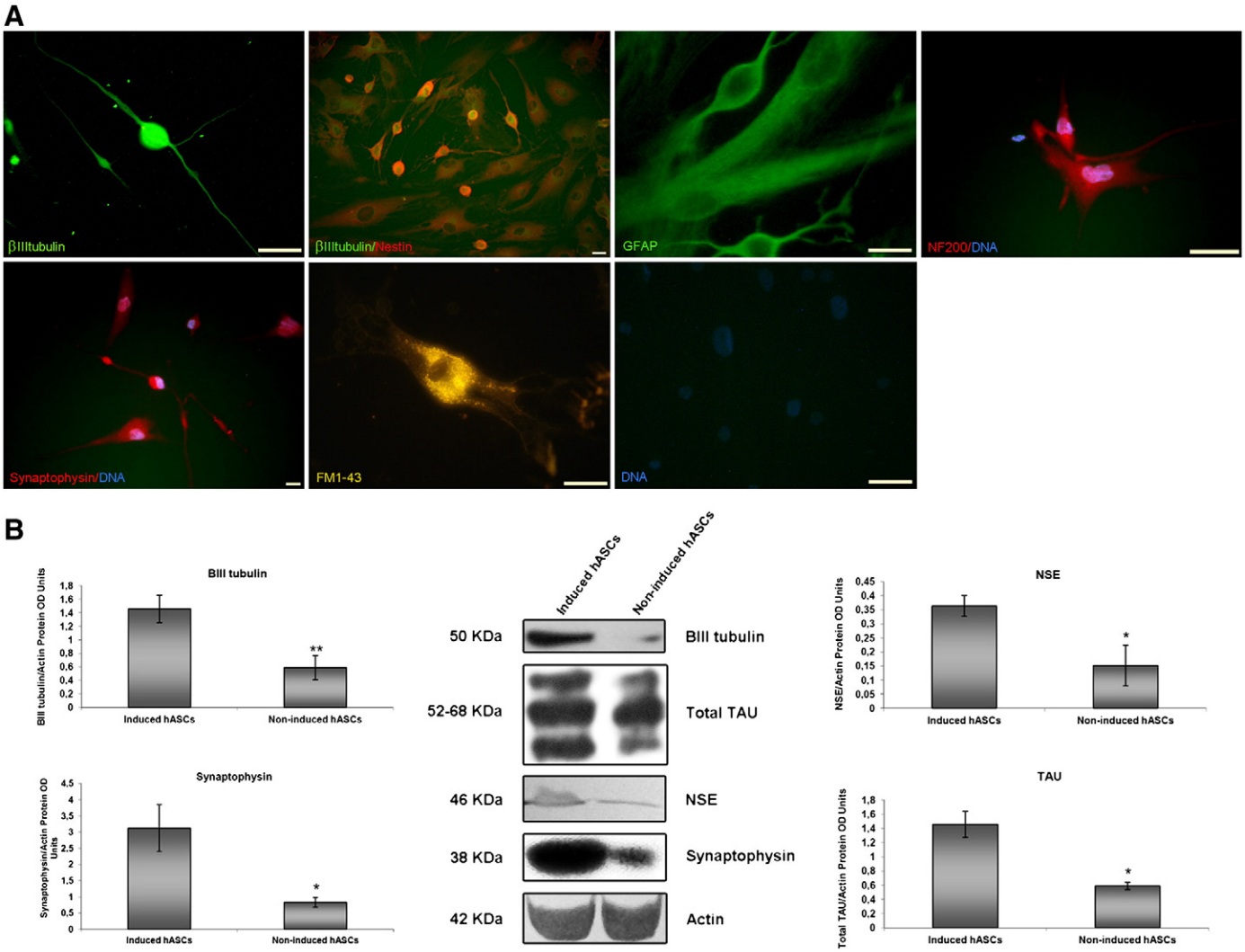


**Fig. 3.** Induced-hASCs express neural lineage-related proteins. (A) Immunocytochemistry of induced-hASCs using antibodies specific for early neural markers, nestin and GFAP, and for immature and mature markers, βIII-tubulin, NF200 and Synaptophysin. Synaptic vesicle staining with FM1-43 dye was positive in induced cells. Bar = 100 μm. (B) Western blot analysis of induced and non-induced hASCs for βIII-tubulin, TAU, NSE and synaptophysin proteins. The expression of all proteins analyzed was increased after the neuronal differentiation protocol. Protein OD Units for each protein (mean ± error deviation), normalized to the expression of actin, for induced and non-induced hASCs is shown. *$p<0.05$, **$p<0.01$ significantly different from control.

Acetylcholine exerts its effects on the CNS throughout muscarinic and nicotinic receptors. Key roles in cell–cell communication have been suggested for muscarinic receptors during migration and differentiation of neural precursor cells in vitro and in vivo. Here we analyzed and detected M1 expression in hASCs and its increase after neuronal differentiation ($P = 0.024$), typical of progenitor and immature neurons. The M1 receptor subtype is expressed in nestin-positive progenitor cells and Tuj1-positive recently differentiated neurons. This data supports the idea that early expression of M1 subtypes participates in neural differentiation prior to synaptogenesis (Williams et al., 2004).

Glutamate is the most prominent neurotransmitter in the mammalian CNS and exerts its actions via ionotropic (NMDA, AMPA and kainate) and metabotropic mGluRs receptors. In this study we observed low expression of ionotropic NR1 and GluK5 and metabotropic mGluR5 receptors in hASCs and an increased expression after the neuronal induction protocol (NR1, $P = 0.049$; GluK5, $P = 0.026$; mGluR5, $P = 0.064$). The expression of ionotropic glutamate receptors was detected in immature neurons (Ambrogini et al., 2004; Overstreet Wadiche et al., 2005; Tozuka et al., 2005). The NR1 and NR2 subunits are expressed at early stages during adult neurogenesis (Moult, 2009; Nacher et al., 2007). The GluK5 is expressed in migrating neuroblast in the SVZ and RMS (Platel et al., 2007). The mGluR5 metabotropic glutamate receptors are expressed in neural progenitor cells, including neuroblasts, and endogenous activation controls the proliferation and/or survival of these cells both in vivo and in vitro (Di Giorgi-Gerevini et al., 2005; Platel et al., 2008a, 2008b). Our results and the previous data mention above confirm that hASCs bias their fate towards neuronal specification.

GABA is the major inhibitory neurotransmitter in the adult brain and acts primarily by binding to $GABA_A$ or $GABA_B$ receptors, but in early development GABA actions can be excitatory and may function as a trophic signal (Barbin et al., 1993; Behar et al., 1996; Ben-Ari et al., 1988; LoTurco et al., 1995). Changes in GABAergic function during development arise in part from alterations in the chloride ion reversal potential (Owens et al., 1996; Zhang et al., 1991) mediated by changes in expression of chloride transport proteins (Rivera et al., 1999). The $GABA_A$ receptor gates a $Cl^-$ channel, and in the mature brain, GABA activation causes $Cl^-$ influx and membrane hyperpolarization due to the low intracellular $Cl^-$ concentration established by the $K^+$–$Cl^-$ co-transporter KCC2 (Li et al., 2002; Rivera et al., 1999). However, during development, GABA exerts a different effect by depolarizing cortical progenitors (radial glia) and immature neurons. This is due to the $Cl^-$ gradient established by the $Na^+$–$K^+$–$2Cl^-$

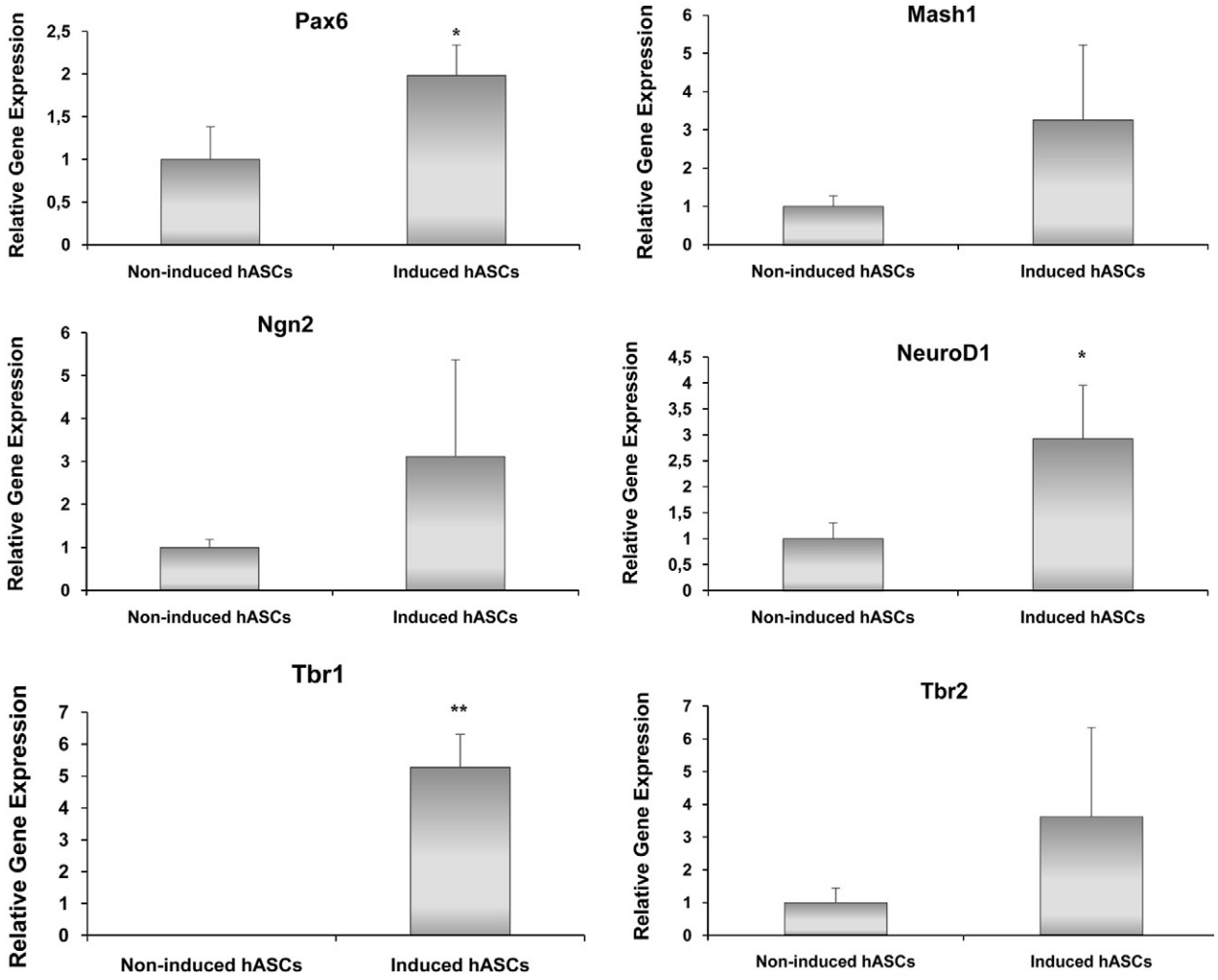


**Fig. 4.** hASCs express proneural determination and differentiation genes at low levels but after neuronal differentiation they are upregulated, demonstrating that they have acquired a neuronal fate. hASCs were cultured in neuronal induction medium during 14 days. Real-time PCR analysis shows increased mRNA levels of Pax6, Mash1, NeuroD1, Ngn2, Tbr1, and Tbr2 in induced relative to non-induced hASCs. Relative gene expression of each gene (mean ± error deviation), normalized to the expression of the housekeeping gene β-actin, for induced and non-induced hASCs is shown. *$p<0.05$, **$p<0.01$ significantly different from control.

co-transporter NKCC1, expressed from mid-embryonic stages until the first week of postnatal life in rodents. NKCC1 imports $Cl^-$ into immature cells, thereby causing $Cl^-$ efflux and membrane depolarization upon $GABA_A$ receptor activation (Li et al., 2002; Plotkin et al., 1997). Early in neuronal development there is a delayed expression of the co-transporter KCC2 and its expression is increased during neuronal maturation, which correlates with the shift from GABAergic excitation to inhibition (Kriegstein and Owens, 2001). We detected basal expression of both co transporter NKCC1 and KCC2 in hASCs and it was upregulated after neuronal differentiation ($P=0.036$; $P=0.049$, respectively), this is in accordance with previous works in adult brain where NKCC1 has been associated with neuronal proliferation in juvenile and some mature neurons (Plotkin et al., 1997; Wang et al., 2002). However, the mere expression of this co-transporter in our neuron-like cells does not necessarily imply that these are functionally active.

$GABA_BR$ may be functionally expressed by neural progenitor cells to preferentially promote the commitment toward a neuronal lineage after the activation of cellular proliferation toward self-replication in the developing mouse brain (Fukui et al., 2008). Our results are according with this previous report, since we detected GABAbR1 and GABAbR2 in hASCs and after neuronal induction there was a decrease of GABAbR2 ($P=0.022$) and an increase of GABAbR1 expression ($P=0.039$), which was previously associated with proliferation activity in neural progenitor cells before commitment and subsequent differentiation towards a neuronal lineage, respectively (Fukui et al., 2008) (Fig. 5). It is known that $GABA_B$ receptors influence neuronal activity by modulating numerous intracellular signaling pathways.

### 3.3. Molecular code model

Taken together, after the neuronal differentiation protocol, hASCs acquire a specific expression pattern of early and intermediate neural genes, such as patterning factors, proneural, neuronal, and neurotransmitter genes. In the presence of growth factors/cytokines known to induce neuronal differentiation, hASCs differentiate in vitro into neuron-like cells expressing MAP2, TAU, NSE, βIII-tubulin and synaptophisin.

A possible explanation of our findings is that the neuronal differentiation of hASCs results in an intermediate stage of neural differentiation, a transition between neural progenitors and immature neurons, with the establishment of fate determination and the expression of characteristic markers of these stages.

In our previous work we have demonstrated that Hes1 expression, a target gene of Notch signaling, was downregulated after neuronal differentiation (Cardozo et al., 2011). And it is known that Hes1

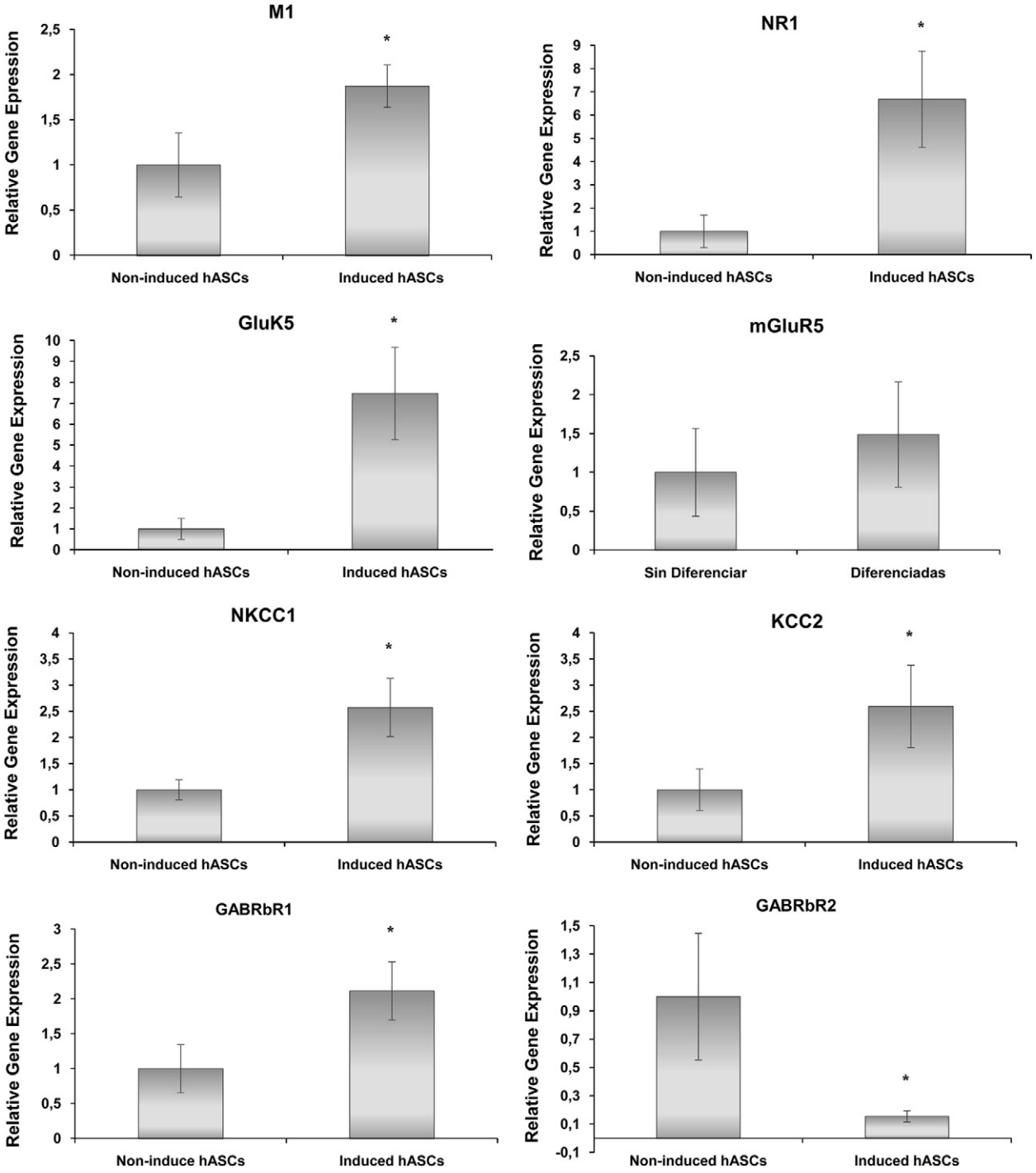


**Fig. 5.** hASCs express cholinergic, GABAergic and glutamatergic neurotransmitter receptor genes at basal levels and after neuronal differentiation most of them are upregulated significantly, demonstrating that these cells have acquired a neuronal fate but the mere expression of these receptors does not necessarily imply that they are functionally active. hASCs were cultured in neuronal induction medium during 14 days. Real-time PCR analysis shows increased mRNA levels of GABAbR1, NKCC1, KCC2, NR1 and M1 in induced relative to non-induced hASCs. Relative gene expression of each gene (mean ± error deviation), normalized to the expression of the housekeeping gene β-actin, for induced and non-induced hASCs is shown. $^{*}p<0.05$, $^{**}p<0.01$ significantly different from control.

negatively regulates neural differentiation by antagonizing bHLH activators (Ishibashi et al., 1994). In the present work we demonstrated that there is an upregulation of different proneural genes, including the bHLH transcription factor Mash1 and Ngn2. Taken together, our results indicate that a balance between positive and negative regulators, such as Mash1/Ngn2 and Hes1, may be critical for the neuronal differentiation of hASCs.

We propose a molecular code model to the neuronal differentiation of hASCs, based on the molecular similarities between the gene expression pattern detected in neuronal differentiation of hASCs and adult neurogenesis. Our model suggests that cells co-expressing patterning and inhibitory HLH factors are maintained as undifferentiated progenitors (hASCs) and these cells sequentially generate neural intermediate progenitors and immature neurons with the concomitant expression

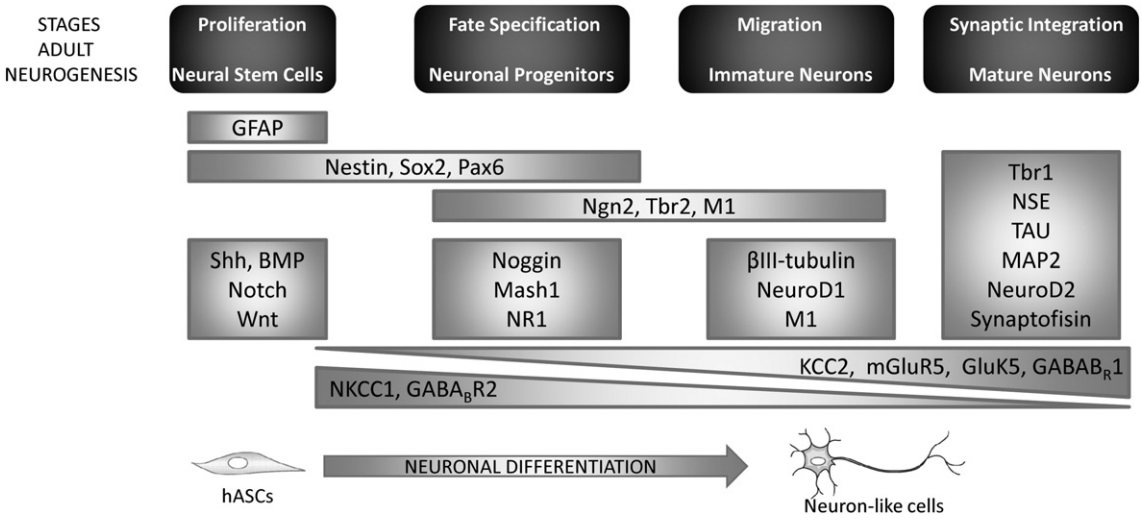


**Fig. 6.** Molecular code model of neuronal differentiation of hASCs based in the gene expression pattern similarities between this neuronal differentiation and adult neurogenesis.

of proneural factors and early neuronal genes. Finally, these intermediate progenitors differentiate to mature neurons with expression of late neuronal genes (Fig. 6).

According to our molecular code model of hASCs neuronal differentiation, GFAP, Sox2, Pax6 and nestin expressing hASCs differentiate into cells that are actively transitioning from neural progenitor cells expressing Mash1, Ngn2, Noggin and Tbr2, to differentiated immature neurons expressing βIII-tubulin, NeuroD1, MAP2, synaptophisin, Tbr1, NSE, and TAU. They also express different neurotransmitter receptors. Our model is according to a current model of adult neurogenesis (Hevner et al., 2006; Olson et al., 2001), supporting our hypothesis of certain parallelism between these two different processes.

## 4. Conclusions

It is known that hASCs exhibit properties like multipotency, expansion in vitro, and hypoimmunogenecity, making them ideal candidates for tissue engineering. But their complete potential would only be used when the molecular pathways regulating their self-renewal and differentiation will be completely deciphered. Directing the cells into a specific lineage, attaining complete terminal differentiation, and maintaining the differentiated state are essential requisites for tissue engineering. Unraveling the regulatory cascades that regulate adipose-derived stem cell maintenance, self-renewal and differentiation will facilitate the development of new and targeted therapies using adipose-derived stem cells for neurological disorders.

One long-term goal of our research is to develop strategies for replacing neurons lost from disease or injury. For this reason, studies in animal models of neurodegenerative diseases are needed to assess the function and safety of our neuron-like cells from hASCs in vivo. Future experiments are also needed to define further functional properties of these neuron-like cells and longer periods of differentiation to achieve more mature neurons. Our method provides the means to study autologous approaches in neurotransplantation using adult human adipose-derived stem cells, an accessible tissue in every individual.

## Acknowledgments

The authors have no conflict of interest. This study was funded by the Fundación para el desarrollo de las Ciencias Básicas (FUCIBA) and the Instituto de Ciencias Básicas y Medicina Experimental (ICBME), Hospital Italiano de Buenos Aires, Argentina. Financial support by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) through a scholarship to Alejandra Johana Cardozo is gratefully acknowledged. D.E. Gomez is a member of CONICET.

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