Reversion of neuronal differentiation induced in human adipose-derived stem cells

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SUMMARY

Adipose-derived mesenchymal stem cells are a great alternative to other types of stem cells obtained from other tissues, since they are found in large numbers and are easy to obtain by liposuction and do not have ethical connotations. These cells can differentiate into neuronal lineage using induction media, although the efficacy of these media is determined by their composition. In previous studies, we have demonstrated the differentiation of human adipose-derived mesenchymal stem cells to neuronal lineage by using three induction media, Neu1, Neu2 and Neu3, each one showing a series of neuronal markers in the treated cells. In the present study, a further step is taken, since once a neuronal differentiation is obtained with these three induction media, they are removed from the medium and both morphology and neuronal markers and the ability to maintain neurosphere formation are analyzed. The results obtained show that when these induction media are withdrawn in the three cases, both the neuronal morphological characteristics and the neuronal markers are lost at different times depending on the medium used. In the case of neurospheres, the ability to maintain this shape is also lost when they are not in contact with neuronal induction factors. Therefore, although the neuronal differentiation mechanism is very promising in this type of cells, it is necessary to carry out more studies to elucidate an induction medium that allows cells differentiated into neurons to maintain neuronal characteristics over time without the need to continue applying neuronal differentiation factors.

Key words: Human adipose mesenchymal stem cells – Neurological disorders – Neuronal differentiation

ABBREVIATIONS

cAMP	Adenosine 3',5'-cyclic monophosphate
CHAT	Choline O-acetyltransferase
DMEM	Dulbecco's Modified Eagle Medium
EGF	Epidermal growth factor
ENS	Enolase
FBS	Fetal bovine serum

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FGF	Fibroblast growth factor
FOXO-4	O4 forkhead box
GalC	Galactosylceramidase
GFAP	Glial fibrillary acidic protein
hASC	Human adipose-derived mesenchymal stem cells
HGF	Hepatocyte growth factor
IBMX	3-isobutyl-1-methylxanthine.
LIF	Leukemia inhibitory factor
MAP2	Microtubule-associated protein-2
MSCs	Mesenchymal stem cells
Neu1	Neuronal differentiation medium 1
Neu2	Neuronal differentiation medium 2
Neu3	Neuronal differentiation medium 3
NeuN	Neuronal nuclear protein
NFH	Neurofilament heavy chain
NFL	Neurofilament light chain
NFM	Neurofilament
NGF	Nerve growth factor
NPBM	Neuronal progenitor basal medium
NSE	Neuron specific enolase
PBS	Phosphate-buffered saline
SCN9A	Sodium voltage-gated channel alpha subunit-9
SNAP25	Synaptosome-associated protein-25
TAU	Tau protein
TH	Thyroxine hydroxylase
VEGF	Vascular endothelial growth factor

INTRODUCTION

The use of tissue-derived mesenchymal stem cells has great advantages for clinical use. Among them, we can find the absence of ethical implications, unlike other cell types such as embryonic stem cells (Hanley et al., 2010), their easy obtaining from different tissues such as adipose tissue (ideal tissue to obtain these cells by liposuction) (Minteer et al., 2013) and the availability of mesenchymal stem cells in fat compared with bone narrow (2% versus 0.001-0.004%, respectively) (Mahmoudifar and Doran, 2015). Human adipose-derived mesenchymal stem cells (hASCs) can give rise to different cell lines (cardiomyocytes, endothelial cells, osteoblast-like cells, etc.), which had been used in several clinical fields such as cardiovascular diseases, reconstructive plastic surgery or spine degenerative conditions, among others (Ma et al., 2017; Naderi et al., 2017; Caliogna et al., 2020). In addition, these cells exert a neuroprotective effect that may be relevant in neurological pathologies such as head trauma, ischemic stroke, and Alzheimer's disease (Sánchez-Castillo et al., 2022) and were able to improve controlled cortical impact injury in rats reducing neuroinflammation (Ruppert et al., 2020). Furthermore, hASCs can also derive in a neuronal lineage, as demonstrated by Safford et al. (2002), using valproic acid, hydrocortisone, insulin, butylated hydroxyanisole, and epidermal and fibroblast growth factor (EGF and FGF). However, this differentiation was unstable (between 3 and 4 days), and was not accompanied by a functional evaluation. Krampera et al. (2007) used a differentiation medium (which included valproic acid and butylated hydroxyanisole, among others) and Schwann cells, demonstrating a significant increase in the expression of typical markers of differentiated cells. B-mercaptoethanol has also been used to produce neuronal differentiation associated to factors such as EGF, retinoic acid or butylated hydroxyanisole (Cardozo et al., 2011).

The functionality of this differentiation was also not studied, although some different markers were established for neuronal cells. Li et al. (2013) obtained neuronal differentiation markers after differentiation with β-mercaptoethanol, butylated hydroxyanisole and platelet-rich plasma. We have recently shown that three media of neuronal induction, using EGF and FGF combined with leukemia inhibitory factor (LIF), vascular endothelial growth factor (VEGF) or nerve growth factor (NGF), among others, induced a significant differentiation of hASCs to neural-type cells (Hernández et al., 2021). However, whatever the induction method, it is vital to verify the maintenance of the neuronal markers expression to verify the functionality of these differentiated cells. In this regard, NeuN, MAP2, NSE, NFM, NFH and NFL markers stand out (Jang et al., 2010; Salehi et al., 2016). For neuronal differentiation to last over time, the expression of these neuronal

markers must also be prolonged. Therefore, it is important to know their expression changes since a loss of these markers would imply a reversal of this differentiation.

The aim of this study is to determine the degree of reversion of the neuronal characteristics acquired by mesenchymal cells induced by different culture media and to determine if there are permanent changes in their phenotype. This would allow us to determine the degree of neuronal phenotype that is maintained in cells that could be used in neurological disorders.

MATERIALS AND METHODS

Obtention of hASCs from human adipose tissue

Adipose tissue samples were obtained by liposuction from three healthy patients (aged 30 to 55 years). hASCs lines were extracted and characterized as described in our study (Hernández et al., 2021). Informed consent and approval of the Ethics Committee (Nuestra Señora de la Salud Hospital, Granada, Spain, 111430/31; date 9/11/2011) was obtained. The assay was conducted in accordance with the declaration of Helsinki.

hASCs neuronal differentiation and dedifferentiation

hASCs differentiated to neuronal lineage and subsequently dedifferentiated. As described previously (Hernández et al., 2021), we used Neu1, Neu2 and Neu3 differentiation media. For Neu1 induction (Gong et al., 2018), the hASCs were maintained in DMEM, supplemented with 25 µg/ml fibroblast growth factor (FGF, Sigma-Aldrich), 0.1 mg/ml epidermal growth factor (EGF, Sigma-Aldrich) and 10 µg/ml leukemia inhibitory factor (LIF, Sigma). The medium was changed every three days for 21 days. For Neu2 induction, hASCs were maintained in HAM-F12 medium (Sigma-Aldrich), supplemented with 20 ng/ml hepatocyte growth factor (HGF, Sigma-Aldrich), 20 ng/ml vascular endothelial growth factor (VEGF, Sigma-Aldrich) and 10 ng/ml epidermal growth factor. The medium was changed every three days for 15 days. For Neu3 induction based in the procedure of Tondreau et al. (2008), hASCs were maintained in neuronal progenitor basal medium (NPBM, Lonza), supplemented with 10 mg/ml adenosine 3',5'-cyclic monophosphate (cAMP, Sigma-Aldrich), 25 ng/ml nerve growth factor (NGF, Sigma-Aldrich), 2.5 µg/ml insulin (Sigma-Aldrich) and 5 µM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich) changing the medium every three days for 10 days. All cells were preserved at 37 °C in 95% humidity and 5% CO_2 atmosphere. All media were supplemented with 1% penicillin/streptomycin solution, 1% ciprofloxacin (Fresenius Kabi). In addition, after neuronal differentiation, cells were induced to undergo dedifferentiation by removal of neuronal culture media Neu1, Neu2 and Neu3, and replacement with standard medium (10 days in the case of Neu1, 7 days in the case of Neu2, and 5 days in the case of Neu3).

hASCs neurospheres formation

hASCs neurospheres were induced following previously described procedure (Yang et al., 2015). Briefly, hASCs (90% confluent) were harvested with trypsin/EDTA and plated in 6 wells plate (2.5 x 10^5 cells/ml) in DMEM/F12 medium with 2% B27 serum-free supplement (*Gibco*) for 7 days. 96-well of matrigel or agarose were prepared. Agarose (*Sigma-Aldrich)* in distilled water (0.5%) was boiled (10 minutes). 100 µl of liquid agarose was added to each well of 96-wells plate and kept at 37ºC for 30 minutes. Matrigel (*BD-Biosciences*) in distilled water (50%) was added to the 96-wells plate (100 µl). Differentiation and dedifferentiation assays were carried out only with Neu 1 medium. Neurospheres (1 neurosphere/well) in 150 µl Neu1 medium were maintained for 4 days. DMEM/F12 medium was used as control.

Western blot analysis of neuronal marker modulation

Western blot analysis using proteins obtained from total differentiated and dedifferentiated cells was performed. Firstly, cells were collected, centrifuged, and total proteins were extracted with Laemmli buffer to finally determine protein concentrations (Bradford method). For electrophoresis, 35 µg protein of each sample were heated at 95ºC for 5 min and separated in 7.5% SDS-PAGE gel. Fractions were transferred

to a nitrocellulose membrane with a 45 µm pore size (Millipore), blocked in 5% milk in PBS supplemented with 0.1% Tween-20 (Bio-Rad) (1 hour) and incubated with respective primary antibodies overnight at 4ºC: NES, GFAP, TUB-III, SCN8A, SNAP-25, FOXO-4, CHAT, MAP2, TH, ENS, TAU, and GalC (Table 1). Antibody binding was revealed by incubation with 1:2000 dilution secondary antibody (Goat anti-mouse IgG-HRP, sc-2005). Signals were detected with an ECLTM Western blot detection reagent (GE Healthcare) and β-actin (A3854, Sigma-Aldrich) 1:10000 dilution served as an internal control. Each western was performed at least two times.

RESULTS AND DISCUSSION

Morphological changes in hASCs during neuronal differentiation and dedifferentiation

Morphological changes were observed with the three neuronal differentiation protocols – Neu1, Neu2, and Neu3 – (see Methods) (Fig. 1). Neu1 induced morphological changes from day 5 (Fig. 1b) to the final induction point, in which cells with neural-like morphology appeared (Fig. 1e), i.e., showing cytoplasmic retraction towards the nucleus and a spherical cell body with long extensions that interacted with neighboring cells (Fig. 1f). Neu2 induced fewer morphological changes than Neu1 (and later -day 10-), in the form of a tendency to form organized clusters with membrane contacts between surrounding cells (Fig. 1g-k). Finally, Neu3 caused minor morphological changes, including a spindled shape involving cytoplasmic retraction towards the nucleus. Furthermore, the induction of cytoplasmic extensions was observed sometimes forming long cell chains at the end of the differentiation period (Fig.1 lm). Marei et al. (2018) verified evident morphological changes from the sixth day of induction using retinoic acid. In this differentiation process, the cells acquired a morphological modification from the third day and a neuronal appearance from the sixth day, reaching a total neuronal appearance on the fourteenth day. Compared with our study, the Neu1 differentiation media showed significant morphological changes from day 5. These changes were less noticeable when Neu2 and Neu3 were used. Thus, Neu1 differentiation medium with EGF and FGF factors improves neuronal differentiation relative to other media, including those that used retinoic acid or heparin. This may be related to the relevant role of both EGF and FGF growth factors on hASCs differentiation to a neuronal lineage (Hu et al., 2013).

Table 1. mAb antibodies used in western blot (WB).

mAbs	WB Dilution	Reference
sodium voltage-gated channel alpha subunit-9 (SCN9A)	1:400	*Na+ CP type IXa Antibody H-17, sc-130096; Santa Cruz*
synaptosome associated protein-25 (SNAP-25)	1:400	*SNAP-25 Antibody C-18, sc7538; Santa Cruz*
tubulin-III (TUB-III)	1:1000	*Anti-tubulin Antibody beta III isoform, MAB1637; Millipore*
nestin (NES)	1:500	*Nestin Antibody, sc-23927; Santa Cruz*
choline O-acetyltransferase (CHAT)	1:400	Choactase antibody E-7, sc-55557; Santa Cruz
glial fibrillary acidic protein (GFAP)	1:500	*GFAP Antibody GA-5, sc-58766; Santa Cruz*
microtubule associated protein-2 (MAP2)	1:500	*MAP2 antibody A-4, sc74421; Santa Cruz*
O4 forkhead box (FOXO-4)	1:100	*Anti-FOXO-4, sab4501887; Sigma*
tyroxine hydroxylase (TH)	1:4000	*Anti-tyrosine hydroxylase antibody, T2928; Sigma*
enolase (ENS)	1:400	*Enolase 5G10, sc-51882; Santa Cruz*
Tau protein (TAU)	1:100	*Anti-Tau antibody TAU-5, ab3931; ABCAM*
Neurofilament (NFM)	1:800	*Anti-Neurofilament antibody, 05744; Millipore*
galactosylceramidase (GalC)	1:500	*Anti-GalC antibody, mab342; Millipore*
β-actin	1:10000	*β-actin antibody,, A3854, Sigma Aldrich*

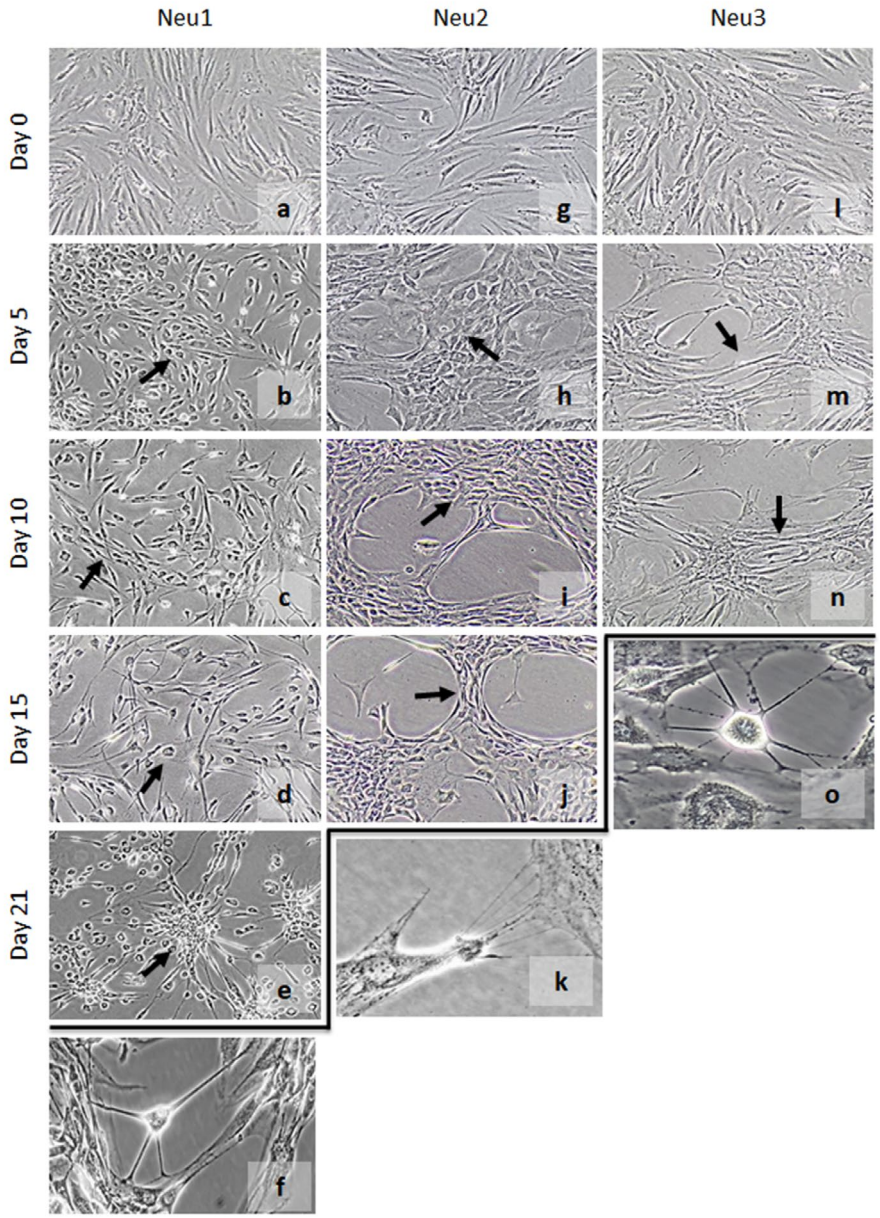

Fig. 1.- hASCs neuronal differentiation. hASCs were exposed to Neu1 (**a-e**) (21 days), Neu2 (**g-j**) (15 days) and Neu3 (**k-n**) media (10 days) (magnification 4x). Images **f, k** and **o** (magnification 20x) show intercellular cytoplasmic bridges in the last day of differentiation. Arrows point to cell morphology changes during differentiation.

Interestingly, dedifferentiation analyses showed that all cells induced with Neu1, Neu2 and Neu3 returned to their initial fibroblastic appearance, which was more pronounced in the case of Neu1. Abundant intercellular cytoplasmic bridges are also observed towards the end of dedifferentiation, more abundant in the case of Neu2 (Fig. 2). Interestingly, dedifferentiation analyses showed that all cells induced with Neu1, Neu2 and Neu3 returned to their initial fibroblastic appearance, which was more pronounced in the case of Neu1. Abundant intercellular cytoplasmic bridges are also observed towards the end of dedifferentiation, more abundant in the case of Neu2 (Fig. 2). Following Yu et al. (2011), the dedifferentiation may be related to selection that FGF and EGF in exert on these cells. In fact, Anghileri et al. (2008) demonstrated a significant loss of functionality in hASCs differentiated to neuronal lineage after seven days in culture. In our study, after remove Neu2 (HGF and VEGF included), a significant dedifferentiation was observed at the same time to Anghileri's study. However, after remove Neu1 (FGF and EGF included) the dedifferentiation was observed at 10 days. Finally, Neu3 (NGF included) was the least effective observing a cell dedifferentiation after removing media at 5 days.

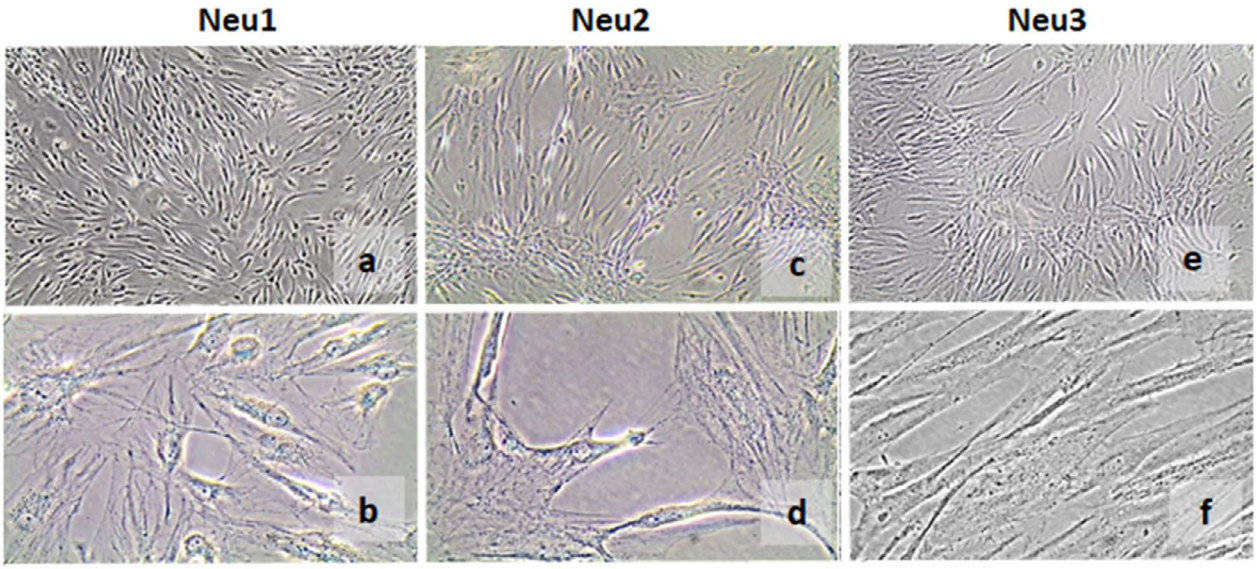

Fig. 2.- hASCs neuronal dedifferentiation. After hASCs differentiation medium was removed and cells growth until 29 days (Neu 1), 21 days (Neu2) and 15 days (Neu3). Images show dedifferentiation of hASCs cells. Magnification 4x (a,c,e) and 20x (b,d,f).

Neuronal marker modulation in differentiation and dedifferentiation of hASCs

Western blot analysis indicated a significant modulation in the expression of neuronal markers in the induced-hASCs as compared to non-induced cells. Western blot analysis showed the modulation of mature neuron markers (TAU and TH), glial markers (GFAP), and functional markers (SNAP25) cells treated with Neu1 (Fig. 3). Neu2 and Neu3 also induced an increased expression of TH and SNAP25 levels but significantly lower than those induced by Neu1; they did not induce TAU modulation and caused a significant decrease in the expression of ENS, suggesting lower neuronal differentiation compared to Neu1 (Fig. 3). For example, it has been recently observed how human-derived adipose stem cells (hASC) transdifferentiate into neuronal-like cells and increase the expression of the neuronal marker protein MAP-2 after neuronal induction. Interestingly, MAP-2 increase

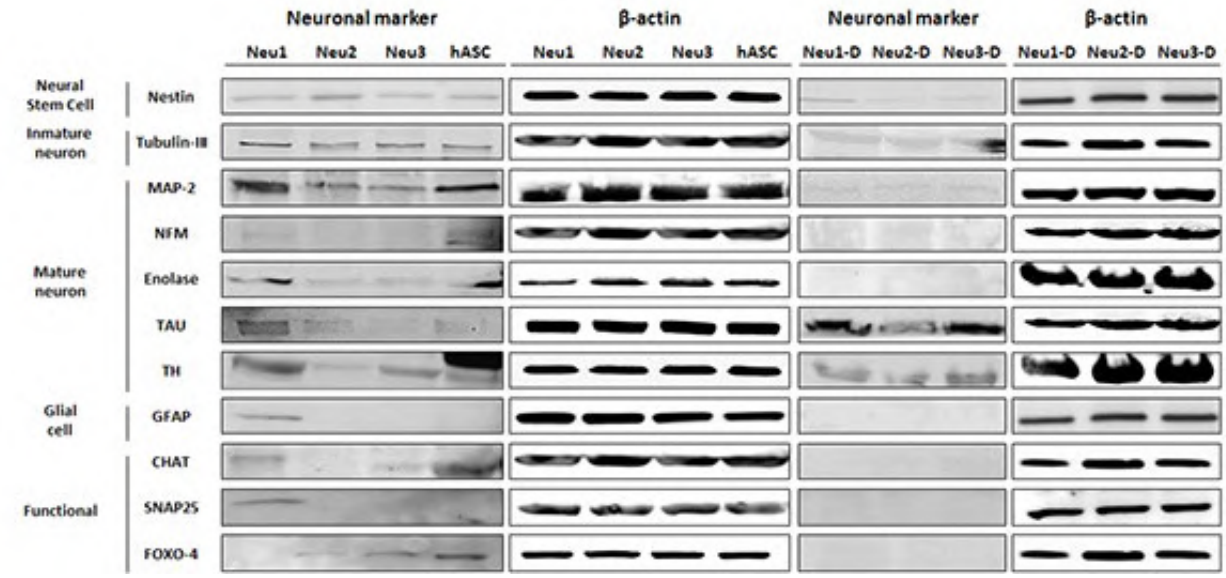

Fig. 3.- Neuronal markers determined by Western blot in hASCs control, hASCs induced with the media Neu1 (21 days), Neu2 (15 days) and Neu3 (10 days) as well as dedifferentiated cells after the differentiation process, Neu1-D (31 days), Neu2-D (21 days) and Neu3-D (15 days). β-actin served as an internal control.

has recently been pointed out as a critical fact in hASCs transdifferentiate into neuronal-type cells (González-Casacuberta et al., 2022). Thus, an expression decrease after dedifferentiation may represent a serious limitation for its applicability. To determine the reversibility of the differentiation process and corroborate the modulation of biomarkers and gene expression, an analysis of hASCs after dedifferentiation was conducted. Western blot showed a loss of expression in functional markers and in mature neuron markers (inclunding MAP2 and ENS). On the other hand, dedifferentiation after Neu2 and Neu3 exposure provoked a reduction in the expression of MAP2 and FOXO4 (Fig. 3). The expression of proteins such as TAU, GFAP and Map2 have been reported by several authors after neuronal differentiation in hASCs (Cardozo et al., 2011; Jang et al., 2010; Mostafavi et al., 2014), which indicates the acquired neuronal compromise of these cells after differentiation. NAP25 is considered a key factor to promote neuronal differentiation in rats (Bailey and Lahiri, 2006). In our study, the expression of three of these four fundamental proteins is increased by the Neu1 induction medium, suggesting that it is the best medium of the three for neuronal differentiation. In the other media studied, the modulation of these proteins is lower

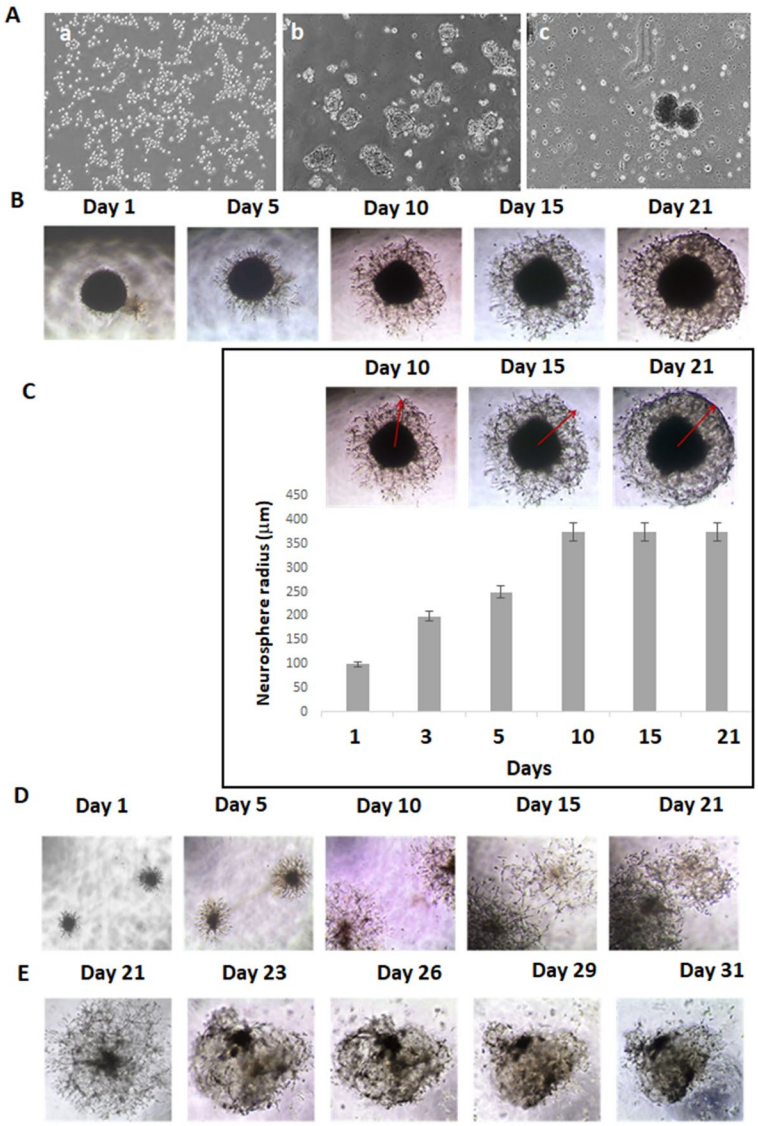

Fig. 4.- Neuronal differentiation/dedifferentiation of hASC neurospheres. **A.** Representative optical microscope image of neurosphere formation in DMEM/F12 medium supplemented with B27 during days 1 (a), 4 (b) and 7 (c). **B.** Growth of neurospheres (agarose 0.5%) after exposure to Neu 1 media (21 days). **C.** Representative images of neurospheres on days 10, 15 and 21 with determination of the extension growth (red line) (Image J). Graphic representation of growth during the 21 days. **D.** Approach and aggregation of hASC neurospheres. **E.** Morphological changes during dedifferentiation of neurospheres after removing Neu1 medium (31 days) (4x magnification).

or even show a reduction in Map2, although there are authors such as Razavi et al. (2015) that did not find significant differences in terms of Map2 expression after neuronal induction in hASCs.

Neuronal dedifferentiation in hASCs neurospheres

The differentiation/dedifferentiation assays in neurospheres were carried out only with Neu1 given the previous results. As shown in Fig. 4A, the neurospheres were formed within a few days (5 days) of the hASCs culture in DMEM / F12 medium supplemented with B27. Neurospheres maintained in the Neu1 medium grew by developing long cell extensions in the manner dendrites (Fig. 4B). These extensions grew fundamentally in the first 5 days of induction, stabilizing later (Fig. 4C). The neurospheres aggregated through long processes to finally form a single neurosphere (Fig. 4D). Analysis of the dedifferentiation process after Neu1 removal and the addition of the standard medium (10 days) showed that the neurospheres collapsed, acquiring an irregular morphology and losing the cell extensions formed during neuronal differentiation (Fig. 4E). Neurospheres derived from hASCs are associated to a greater extent with their possible clinical use in neurodegenerative diseases such as Alzheimer's. These neurospheres are characterized by high levels of Nestin (Peng et al., 2019; Yang et al., 2015). EGF and FGF together with B27 seems essential not only to the maintenance of differentiation but also the own neurospheres (Peng et al., 2019). In fact, as soon as these factors are removed from the environment, these neurospheres begin to lose neuronal differentiation.

CONCLUSION

In conclusion, previous studies demonstrated that three different media achieved effective neuronal differentiation of hASCs with remarkable results in terms of morphology changes and expression of typically neuronal markers. However, removal of the induction media induced a significant neuronal dedifferentiation. These results suggest that other strategies including a new composition of differentiation media will be necessary to avoid phenotype reversal in order to provide better therapeutic utility of the hASCs in some neurogical disorders.

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REFERENCES

ANGHILERI E, MARCONI S, PIGNATELLI A, CIFELLI P, GALIÉ M, SBARBAT A, KRAMPERA M, BELLUZZI O, BONETTI B (2008) Neuronal differentiation potential of human adipose-derived mesenchymal stem cells. *Stem Cells Dev,* 17(5): 909-916.

BAILEY JA, LAHIRI DK (2006) Neuronal differentiation is accompanied by increased levels of SNAP-25 protein in fetal rat primary cortical neurons: Implications in neuronal plasticity and Alzheimer's disease. *Ann N Y Acad Sci*, 1086: 54-65.

CALIOGNA L, BINA V, BOTTA L, BENAZZO FM, MEDETTI M, MAESTRETTI G, MOSCONI M, COFANO F, TARTARA F, GASTALDI G (2020) Osteogenic potential of human adipose derived stem cells (hASCs) seeded on titanium trabecular spinal cages. *Sci Rep,* 10(1): 18284.

CARDOZO AJ, GÓMEZ DE, ARGIBAY PF (2011) Transcriptional characterization of Wnt and Notch signaling pathways in neuronal differentiation of human adipose tissue-derived stem cells. *J Mol Neurosci,* 44(3): 186-194.

GONZÁLEZ-CASACUBERTA I, VILAS D, PONT-SUNYER C, TOBÍAS E, CANTÓ-SANTOS J, VALLS-ROCA L, GARCÍA-GARCÍA FJ, GARRABOU G, GRAU-JUNYENT JM, MARTÍ MJ, CARDELLACH F, MORÉN C (2022) Neuronal induction and bioenergetics characterization of human forearm adipose stem cells from Parkinson's disease patients and healthy controls. *PLoS One*, 17(3): e0265256.

GONG L, CAO L, SHEN Z, SHAO L, GAO S, ZHANG C, LU J, LI W (2018) Materials for neural differentiation, trans-differentiation, and modeling of neurological disease. *Adv Mat,* 30(17): e1705684.

HANLEY J, RASTEGARLARI G, NATHWANI AC (2010) An introduction to induced pluripotent stem cells. *Br J Haematol*, 151(1): 16-24.

HERNÁNDEZ R, JIMÉNEZ-LUNA C, ORTIZ R, SETIÉN F, LÓPEZ M, PERAZZOLI G, ESTELLER M, BERDASCO M, PRADOS J, MELGUIZO C (2021) Impact of the epigenetically regulated hoxa-5 gene in neural differentiation from human adipose-derived stem cells. *Biology*, 10(8): 802.

HU F, WANG X, LIANG G, LV L, ZHU Y, SUN B, XIAO Z (2013) Effects of epidermal growth factor and basic fibroblast growth factor on the proliferation and osteogenic and neural differentiation of adipose-derived stem cells. *Cell Reprogram*, 15(3): 224-232.

JANG S, CHO HH, CHO YB, PARK JS, JEONG HS (2010) Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin. *BMC Cell Biol*, 11(1): 25.

KRAMPERA M, MARCONI S, PASINI A, GALIÈ M, RIGOTTI G, MOSNA F, TINELLI M, LOVATO L, ANGHILERI E, ANDREINI A, PIZZOLO G, SBARBATI A, BONETTI B (2007) Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. *Bone*, 40(2): 382-390.

LI H, HAN Z, LIU D, ZHAO P, LIANG S, XU K (2013) Autologous platelet-rich plasma promotes neurogenic differentiation of human adipose-derived stem cells in vitro. *Int J Neurosci*, 123(3): 184-190.

MA T, SUN J, ZHAO Z, LEI W, CHEN Y, WANG X, YANG J, SHEN Z (2017) A brief review: Adipose-derived stem cells and their therapeutic potential in cardiovascular diseases. *Stem Cell Res Ther*, 8(1): 124.

MAHMOUDIFAR N, DORAN PM (2015) Mesenchymal stem cells derived from human adipose tissue. In: Doran PM (ed.). *Cartilage tissue engineering: methods and protocols*. Springer, New York, pp 53-64.

MAREI HES, EL-GAMAL A, ALTHANI A, AFIFI N, ABD-ELMAKSOUD A, FARAG A, CENCIARELLI C, THOMAS C, ANWARUL H (2018) Cholinergic and dopaminergic neuronal differentiation of human adipose tissue derived mesenchymal stem cells. *J Cell Physiol*, *2*33(2): 936-945.

MINTEER D, MARRA KG, RUBIN JP (2013) Adipose-derived mesenchymal stem cells: biology and potential applications. In: Weyand B, Dominici M, Hass R, Jacobs R, Kasper C (eds.). *Mesenchymal Stem Cells-Basics and Clinical Application I*. Springer, Berlin, Heidelberg, pp 59-71.

MOSTAFAVI FS, RAZAVI S, MARDANI M, ESFANDIARI E, ESFAHANI HZ, KAZEMI M (2014) Comparative study of microtubule-associated protein-2 and glial fibrillary acidic proteins during neural induction of human bone marrow mesenchymal stem cells and adipose-derived stem cells. *Int J Preven Med*, 5(5): 584-595.

NADERI N, COMBELLACK EJ, GRIFFIN M, SEDAGHATI T, JAVED M, FINDLAY MW, WALLACE CG, MOSAHEBI A, BUTLER PE, SEIFALIAN AM, WHITAKER IS (2017) The regenerative role of adipose-derived stem cells (ADSC) in plastic and reconstructive surgery. *Int Wound J*, 14(1): 112-124.

PENG C, LU L, LI Y, HU J (2019) Neurospheres Induced from human adipose-derived stem cells as a new source of neural progenitor cells. *Cell Transplant*, *28*(1 Suppl): 66S-75S.

RAZAVI S, KHOSRAVIZADEH Z, BAHRAMIAN H, KAZEMI M (2015) Changes of neural markers expression during late neurogenic differentiation of human adipose-derived stem cells. *Adv Biomed Res*, 4: 209.

RUPPERT KA, PRABHAKARA KS, TOLEDANO-FURMAN NE, UDTHA S, ARCENEAUX AQ, PARK H, DAO A, COX CS, OLSON SD (2020) Human adipose-derived mesenchymal stem cells for acute and sub-acute TBI. *PLoS One*, 15(5): e0233263.

SAFFORD KM, HICOK KC, SAFFORD SD, HALVORSEN YD, WILKISON WO, GIMBLE JM, RICE HE (2002) Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochem Biophys Res Commun*, 294(2): 371-379.

SALEHI H, AMIRPOUR N, NIAPOUR A, RAZAVI S (2016) An overview of neural differentiation potential of human adipose derived stem cells. *Stem Cell Rev Rep*, 12(1): 26-41.

SÁNCHEZ-CASTILLO AI, SEPÚLVEDA MR, MARÍN-TEVA JL, CUADROS MA, MARTÍN-OLIVA D, GONZÁLEZ-REY E, DELGADO M, NEUBRAND VE (2022) Switching roles: beneficial effects of adipose tissue-derived mesenchymal stem cells on microglia and their implication in neurodegenerative diseases. *Biomolecules,* 12(2): 219.

TONDREAU T, DEJENEFFE M, MEULEMAN N, STAMATOPOULOS B, DELFORGE A, MARTIAT P, BRON D, LAGNEAUX L (2008) Gene expression pattern of functional neuronal cells derived from human bone marrow mesenchymal stromal cells. *BMC Genom*, 9: 166.

YANG E, LIU N, TANG Y, HU Y, ZHANG P, PAN C, DONG S, ZHANG Y, TANG Z (2015) Generation of neurospheres from human adipose-derived stem cells. *Biomed Res Int*, 2015: 743714.

YU JM, BUNNELL BA, KANG SK (2011) Neural differentiation of human adipose tissue-derived stem cells. In: Gimble JM, Bunnell BA (eds.). *Adipose-Derived Stem Cells: Methods and Protocols*. Humana Press, Totowa, NJ, pp 219-231.