UNIVERSITY OF CATANIA

INTERNATIONAL PhD PROGRAM IN NEUROSCIENCE

XXXII Cycle

GIULIANA MANNINO

CULTURE STRATEGIES FOR NEUROGENIC INDUCTION OF ADIPOSE DERIVED STEM CELLS. INFLUENCE OF MELATONIN

PhD Thesis

Coordinator:

Prof. Salvatore Salomone

Supervisors:

Prof.ssa Debora Lo Furno

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SUMMARY

LIST OF ABBREVIATION

MSCs	Mesenchymal Stem Cells
ASCs	Adipose-derived mesenchymal Stem Cells
NSCs	Neural Stem Cells
OECs	Olfactory Ensheathing Cells
SCs	Schwann Cells
CM	Conditioned Medium
GFs	Growth Factors
bFGF	Fibroblast Growth Factor
EGF	Epidermal Growth Factor
BDNF	Brain-Derived Neurotrophic Factor
NGF	Nerve Growth Factor
GDNF	Glial cell Derived Neurotrophic Factor
CNTF	Ciliary Neurotrophic Factor
PGP 9.5	Protein Gene Product 9.5
MAP2	Microtubule-Associated Protein 2
MTr	Melatonin Receptor
Cx	Connexins
CNS	Central Nervous System
PNS)	Peripheral Nervous System
MEM-H	Minimum Essential Medium-H
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Foetal Bovine Serum
PFA	Paraformaldehyde
OPBA	Organismo Preposto al Benessere Animale
MFI	Mean Fluorescence Intensity

ABSTRACT

The ability of Mesenchymal Stem Cells (MSCs) to differentiate in several cell lines has been extensively investigated in the last decades in order to develop therapeutic strategies in the field of cell-based regenerative medicine. MSCs from bone marrow have been the first to be identified and studied. Successively, many other sources have been successfully tested. Adipose-derived MSCs (ASCs) have been lately more investigated since they feature several advantages. They can be easily obtained with minimally invasive procedures, such us liposuction; they are characterized by a higher cell yield; they show higher proliferation rate and differentiation ability; they can be readily available for autologous applications.

In this PhD thesis, the ability of ASCs to differentiate toward a neural phenotype has been investigated. A neural differentiation of ASCs is of particular interest for future treatments of traumatic or degenerative diseases affecting the nervous system, in which repair mechanisms are not efficient. In fact, only a limited amount of neural stem cells reside in the brain and their restricted localization cannot ensure a satisfactory recovery in case of injured or dead neural cells.

To induce a neural differentiation of ASCs, instead of supplementing the culture medium with potentially toxic chemical molecules, the use of

conditioned media derived from glial cell was preferred. In this way, the culture medium contains several cytokines/growth factors and more closely mimic the physiological composition of a neural microenvironment *in vivo*. In particular, conditioned media from Olfactory Ensheathing Cells or Schwann Cells were used. In additional experiments, the effects of melatonin were also investigated, since it has been reported that this hormone can improve cell growth and differentiation of neural stem cells.

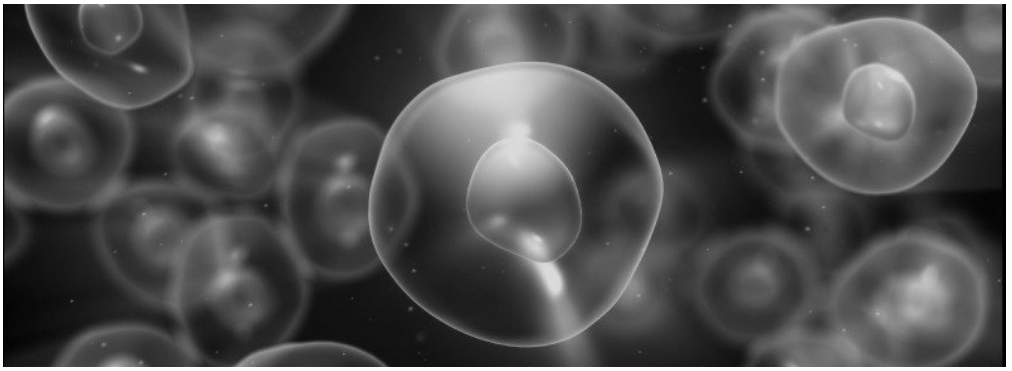
Results were obtained by immunofluorescence protocols and flow cytometry analysis. The modification of typical neural markers and connexins were observed. Overall, it has been found that the protocol adopted has provided successful outcomes. In fact, a variety of neuronal and glial markers were overexpressed after a few days of treatment (3-6 days). At the same time, a typical neural pattern of connexin expression could be induced. These modifications were earlier induced when melatonin was added to the culture medium. The interaction with Melatonin receptor 1 was likely responsible for these effects.

In conclusion, results obtained in the present investigations contribute to elucidate effects of molecules, normally present in the physiological environment, on ASC differentiation toward a neural-like phenotype. It is reasonable to expect that more studies will provide further information useful

for developing stem cell therapeutic application in case of nervous system damage, to restore disease-disrupted brain circuitry.

CHAPTER 1

General Introduction & Aims



Adipose-derived Stem Cells and their differentiation ability

Stem cells are unspecialized cells that indefinitely proliferate and may differentiate towards different cell lineages. When a cell division occurs, the two daughter cells may remain as stem cells or become specialized cells. In adult life, stem cells reside in virtually all tissues, where they replace dead or worn out cells. Stem cells from some tissues show a particular capability to differentiate not only into cells specific of the resident tissue, but also into other cell types. In particular, Mesenchymal Stem Cells (MSCs) are adult stem cells with self-renewal ability that can differentiate into cells of the mesodermal germinal line, such as adipocytes, osteoblasts, chondrocytes, muscular cells and others. The first MSCs were discovered as bone-marrow stromal cells, but several other tissues contain MSCs, for example dermis, periosteum, dental pulp, adipose tissue. All MSCs share some features, such as adherence during culture conditions, the ability to differentiate into adipocytes, osteoblasts, and chondroblasts, the expression of some surface markers (CD73, CD 90 and CD105) and the lack of others (CD45, CD34, CD14, CD11b, CD79a or CD19, and HLA-DR).

Among MSC sources, adipose tissue has recently received great interest due to its high yield of Adipose-derived Stem Cells (ASCs) and because it is easily

obtained with minimally invasive procedures, such us liposuction. ASCs, first isolated in 1976, were identified and characterized by Zuk et al. in 2001. They show the ability to differentiate into mesodermal lineage cells, but numerous studies also report their capability to differentiate into cells belonging to endodermal and ectodermal lineages.

A neural differentiation of ASCs (Figure 1) is of particular interest in the field of regenerative medicine, especially for the treatment of traumatic or degenerative diseases affecting the nervous system, in which repair mechanisms are not efficient. In fact, only a limited amount of neural stem cells is present in the brain and their restricted localization cannot ensure a satisfactory recovery in cases of injured or dead neural cells (Lo Furno et al., 2016; 2018).

For the first time, a neural differentiation of ASCs was reported by Safford et al. by using a culture medium supplemented with a cocktail containing valproic acid, butylated hydroxyanisole, insulin, and hydrocortisone. In their experiments, they showed a modification of the ASC phenotype toward a neuronal lineage after 1-3h. Subsequently, other protocols were adopted to differentiate the ASCs towards neural phenotypes. For example, Zuk et al. (2002) used a pre-induction medium for 24h and then a neurogenic medium. In another study, two protocols were tested. The first included the use of a specific

induction medium; in the second one cells were co-cultured with human Schwann cells (Krampera et al., 2007). Moreover, also some neurotrophic factors, such as basal Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF), and Brain-Derived Neurotrophic Factor (BDNF), in combination with retinoic acid, were used to induce a neural differentiation of ASCs (Anghileri et al., 2008).

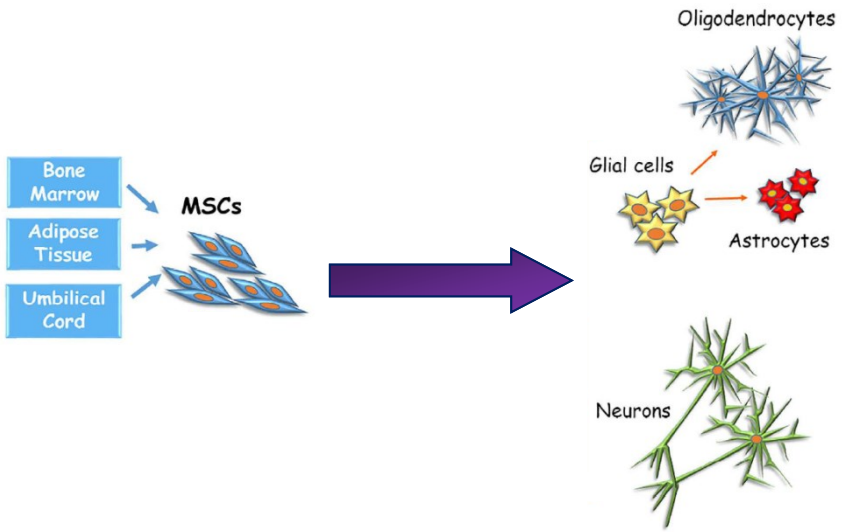


FIGURE 1 - Sources and neural differentiation ability of Mesenchymal Stem Cells. *Modified from Lo Furno et al., 2018.*

Conditioned Media from Glial Cells

More recently, the effects of media conditioned by cultured neural cells were tested. Encouraging result were observed using conditioned media from rat Olfactory Ensheathing Cells (OECs) or human B104 neuroblastoma cells (Lo Furno et al., 2013). These studies show that ASCs acquire a neuronal-like morphology, expressing typical neuronal markers such as nestin, Protein Gene Product 9.5 (PGP 9.5), and Microtubule-Associated Protein 2 (MAP2). Although both conditioned media were able to induce positive outcomes, the one from OECs was more effective. It was concluded that various cytokines and/or neurotrophic factors contained in the culture medium might be responsible for these effects.

Indeed, other glial cells, such as Schwann cells (SCs), are also able to release these products. In fact, OECs and SCs share many features and functional properties (Kocsis et al., 2009; Gao et al., 2018).OECs, located both inside and outside the central nervous system, favor neurogenesis in the mammalian olfactory system (Ramón-Cueto & Avila, 1998) and support new sensory neurons and axonal regeneration and remyelination (Boyd et al., 2005; Mackay-Sim, 2005; Sasaki et al., 2011). SCs, located in the peripheral nervous system, also support neuron functions and form myelin around axons.

Both cell types produce BDNF, FGF, Nerve Growth Factor (NGF), Glial cell Derived Neurotrophic Factor (GDNF), and Ciliary Neurotrophic Factor (CNTF). In particular, BDNF and GDNF promote the survival and the differentiation of neural stem cells and neurons (Li et al., 2017; Ma et al., 2016). CNTF and bFGF enhance the proliferation of Muller glia-derived progenitor cells (Todd et al., 2016).

Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is secreted by the pineal gland. Its levels in blood increase during the night in mammals (Reiter, 1991). It has effects on circadian rhythms, sleep-wake cycle, tumor growth inhibition, immune functions (Cardinali et al., 2012; Hardeland et al., 2012; Mauriz et al., 2012; Rodriguez-Garcia et al., 2013; Jarzynka et al.,2009) and provides antioxidant protection and redox homeostasis in tissues (Acu-Na-Castroviejo et al., 2001; Tan et al., 2002; Salido et al., 2013). Melatonin receptors, MT1 and MT2, are ubiquitously distributed and are G-protein-coupled (Dubocovich, 2015; Slominski et al., 2012).

It was recently shown that melatonin can influence the cell growth and differentiation of Neural Stem Cells (NSCs) (Moriya et al., 2007; Kong et al., 2008; Sotthibundhu et al., 2010). In fact, melatonin treatment can improve NSC viability in a dose-dependent manner and the expression of typical neuronal markers such as MAP2, especially at 7 days differentiation. In particular, it was also reported that melatonin regulates NSC function through a specific membrane receptor (MT). The use of luzindole, an MT antagonist, decreased the expression of MT1 as well as the stimulatory effects of melatonin on NSC proliferation.

Aims of the PhD work

The aim of the present PhD thesis is to identify different strategies for *in vitro* neural differentiation of ASCs. Instead of supplementing the culture medium with potentially toxic chemical molecules, the use of glial cell conditioned media is preferred, since it more closely mimic the physiological composition of a neural microenvironment *in vivo*.

Specific aims are described in the published papers included in the present thesis:

AIM I

To investigate the effects of either SC-CM or OEC-CM on ASC differentiation towards a neural phenotype by the expression of typical neural markers, such as MAP2, PGP9.5, Synapsin I, and Glial Fibrillary Acidic Protein (GFAP).

AIM II

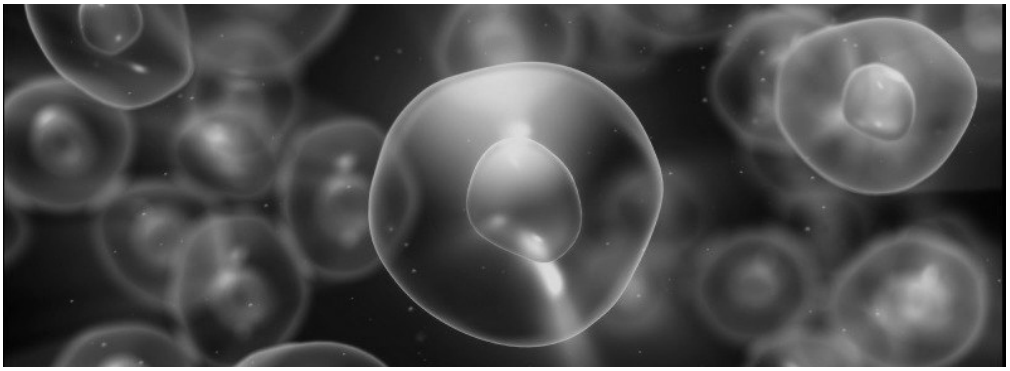
To evaluate the effects of glial conditioned media on the expression of Connexins (Cx) that are typically present in nervous elements, such as Cx32, Cx36, Cx43, and Cx47.

AIM III

To test melatonin effects on conditioned media-induced neural differentiation of ASCs. To elucidate the involvement of melatonin receptors.

CHAPTER 2

Neural Differentiation of human Adipose-Derived Mesenchymal Stem Cells Induced by Glial Cell Conditioned Media



Lo Furno et al., J Cell Physiol (2018)doi: 10.1002/jcp.26632.

Neural Differentiation of Human Adipose-Derived Mesenchymal Stem Cells Induced by Glial Cell Conditioned Media

Debora Lo Furno[1], **Giuliana Mannino[1]**, Rosario Giuffrida[1*], Elisa Gili[2], Carlo Vancheri[2], Maria Stella Tarico[3], Rosario Emanuele Perrotta[3], Rosalia Pellitteri[4]

[1]Dept Biomedical and Biotechnological Sciences, Section of Physiology, University of Catania, Italy

[2]Dept Clinical and Experimental Medicine, University of Catania, Catania, Italy

[3]Dept of General Surgery and Medical-Surgical Specialties, University of Catania, Italy

[4]Inst Neurological Sciences, National Research Council, Section of Catania, Catania, Italy

[1]Debora Lo Furno and Giuliana Mannino equally contributed to this paper

* Author for correspondence: Rosario Giuffrida (giuffros@unict.it)

Tel. +39 095 4781310

Department of Biomedical and Biotechnological Sciences, Section of Physiology.

Via S. Sofia, 97 - 95123 Catania, Italy

Running head: Glia-derived factors on ASC neural differentiation

Conflict of interest disclosure. The authors declare no conflict of interest.

Abstract

Adipose-derived mesenchymal stem cells (ASCs) may transdifferentiate into cells belonging to mesodermal, endodermal and ectodermal lineages. The aim of this study was to verify whether a neural differentiation of ASCs could be induced by a conditioned medium (CM) obtained from cultures of Olfactory Ensheathing Cells (OECs) or Schwann Cells (SCs). ASCs were isolated from the stromal vascular fraction of adipose tissue and expanded for 2-3 passages. They were then cultured in OEC-CM or SC-CM for 24 h or 7 days. At each stage, the cells were tested by immunocytochemistry and flow cytometer analysis to evaluate the expression of typical neural markers such as Nestin, PGP 9.5, MAP2, Synapsin I and GFAP. Results show that both conditioned media induced similar positive effects, since all tested markers were overexpressed, especially at day 7. Overall, an evident trend toward neuronal or glial differentiation was not clearly detectable in many cases. Nevertheless, a higher tendency toward a neuronal phenotype was recognized for OEC-CM (considering MAP2 increases). On the other hand, SC-CM would be responsible for a more marked glial induction (considering GFAP increases). These findings confirm that environmental features can induce ASCs toward a neural differentiation, either as neuronal or glial elements. Rather than supplementing the culture medium by adding chemical agents, a “more

physiological" condition was obtained here by means of soluble factors (cytokines/growth factors) likely released by glial cells. This culture strategy may provide valuable information in the development of cell-based therapeutic approaches for pathologies affecting the central/peripheral nervous system.

Keywords

conditioned media, human adipose mesenchymal stem cells, neural differentiation, olfactory ensheathing cells, Schwann cells.

Introduction

The stromal vascular fraction of adipose tissue has been extensively investigated in the last decades since it represents a rich source of multipotent Mesenchymal Stem Cells (MSCs). Adipose-derived MSCs (ASCs) are able to differentiate not only into cells of mesodermal lineage such as adipocytes (Dicker et al., 2005; Lo Furno et al., 2016), chondrocytes (Musumeci et al., 2011) and osteocytes (Calabrese et al., 2016), but also into cells of other lineages. For example, ASC differentiation toward endocrine pancreatic cells or neural elements has been also reported (Schäffler and Büchler, 2007). This multilineage differentiation may represent a valuable tool for potential therapeutic applications in the field of regenerative medicine (Lo Furno et al., 2016).

Neural differentiation of ASCs has been reported in numerous studies. Safford et al. (2002) were the first to induce ASCs into neural phenotypes, indicated by the expression of Nestin and Neuronal Nuclei protein (NeuN). Moreover, under particular environmental conditions, they can express other neuronal differentiation markers, such as type III β-tubulin (Romanov et al., 2005).In our previous study, ASC differentiation into neural phenotypes was obtained by using conditioned medium (CM) from Olfactory Ensheathing Cells (OECs) or from B104 neuroblastoma cells (Lo Furno et al., 2013).

OECs are particular glial cells responsible for the continuous neurogenesis in the mammalian olfactory system (Ramón-Cueto and Avila, 1998). They are located both outside and inside the central nervous system (CNS), support new sensory neurons throughout adult life and play an important role in axonal regeneration and remyelination (Mackay-Sim, 2005; Boyd et al., 2005; Sasaki et al., 2011). Since Schwann Cells (SCs) share many features with OECs (Barton et al., 2017), the aim of the present *in vitro* study was to investigate whether, and to which extent, SC-CM is also able to induce a neural differentiation of ASCs. At the same time, a comparison with OEC-CM cultured cells seemed useful to highlight similar or different influences. SCs are also able to synthesize various growth factors (GFs) and extracellular matrix molecules (Frostick et al., 1998), which promote neuronal survival and functional characteristics (Pellitteri et al., 2001).

In the present study, the differentiation toward a neural-like phenotype was tested by evaluating the expression of specific markers such as Nestin, Protein Gene Product (PGP) 9.5, Microtubule-Associated Protein 2 (MAP2), Synapsin I and Glial Fibrillary Acidic Protein (GFAP). Nestin is an intermediate filament, which is considered a marker of neural stem cells (Wong et al., 2014). It increases during the early stages of development in the central and peripheral nervous system (PNS) and, upon differentiation, is replaced by cell type-specific neurofilaments or glial fibrillary acidic protein (GFAP). PGP

9.5, also known as ubiquitin carboxyl-terminal hydrolase L1, is mainly expressed in sympathetic and sensory ganglion neurons (Genç et al., 2015). It is considered a reliable marker to evaluate the peripheral axonal projections, because of its strong expression in neuronal axons and cell bodies. MAP2 is an early marker of neuron differentiation, likely involved in microtubule assembly during axonal formation (Lauzon et al., 2017). Its increased expression is associated with neuronal differentiation. Synapsin I, mainly distributed on synaptic vesicle membranes, is the most abundant phosphoprotein present in the synapses of the CNS (Zhang et al., 2017). It links synaptic vesicles and actin filaments and can be considered a specific marker of axon terminals. GFAP is a cytoskeletal element of astrocytes and plays a functional role in astrocyte plasticity, neurotransmitter absorption and neuronal interactions (Wang et al., 2017).

The expression of these markers was investigated by immunocytochemical procedures and flow cytometry analysis on ASCs after 1 and 7 days of culture with OEC-CM or SC-CM.

Material and Methods

OEC cultures and preparation of OEC-CM

As previously described (Pellitteri et al., 2009; Lo Furno et al., 2013), OECs were isolated from olfactory bulbs of 2-day old rat pups. Briefly, the bulbs were removed and dissected (+4 C) in Leibowitz L-15 cold medium (Sigma-Aldrich, Milan, Italy). Subsequently, they were digested in Minimum Essential Medium-H (MEM-H, Sigma-Aldrich) containing collagenase (Invitrogen, Milan, Italy) and trypsin (Sigma-Aldrich). Enzymatic activity was stopped by adding Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS, Sigma-Aldrich). Cells were finally resuspended, plated in flasks and fed with fresh complete DMEM supplemented with 10% FBS. The antimitotic agent cytosine arabinoside (10^{-5}M), was added to reduce the number of dividing fibroblasts. In the last passage, OECs were plated on 25 cm^2 flasks and cultured in DMEM/FBS supplemented with bovine pituitary extract. Cells were incubated at 37 °C in fresh complete medium, which was replaced twice a week. OECs were characterized by immunocytochemistry using S-100 as a marker. OEC-CM was collected 24–48 h after reaching confluence. It was filtered to remove debris and detached cells, aliquoted and stored at -20 °C until further use.

SC cultures and preparation of SC-CM

To harvest SCs, rat sciatic nerves were exposed, removed, and kept in DMEM with the addition of glutamax (Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Pellitteri et al., 2001). Sciatic nerves were then treated with 0.1% collagenase and 2.5% trypsin in DMEM, mechanically dissociated by trituration and filtered through a 150 µm nylon mesh. Cells were then resuspended in fresh complete medium and plated on 25 cm2 flasks. The antimitotic agent cytosine arabinoside (10−5 M) was added 24 h after the initial plating to reduce the number of dividing fibroblasts, whose contamination after this stage was minimal (about 1.5%). After 24-48 h from confluence, SC-CM was collected, filtered to remove debris and detached cells, aliquoted and stored at -20° C until further use.

Cultures of human ASCs

Adipose tissue was harvested from healthy young donors undergoing liposuction procedures at the Cannizzaro Hospital, Catania (Italy). Lipoaspirate was obtained after donors signed an informed consent to use the lipoaspirate for experimental procedures, following the standards of the local ethics committee and in accordance with Declaration of Helsinki (2000). The donors were not smokers and occasionally took non-steroidal anti-inflammatory drugs.

The raw lipoaspirate (50–100 ml) was washed with sterile phosphate-buffered saline (PBS; Invitrogen) to remove red blood cells and debris, and incubated for 3 h at 37 °C with an equal volume of serum-free low-glucose DMEM (DMEM-1g; Sigma-Aldrich) containing 0.075% of type I collagenase (Invitrogen). After inactivation of collagenase activity by adding an equal volume of DMEM-1g containing 10% of heat-inactivated FBS, the digested lipoaspirate was centrifuged at 1200 rpm for 10 min. The pellets were then resuspended in PBS and filtered through a 100-μm nylon cell strainer (Falcon BD Biosciences, Milan, Italy). After a further centrifugation (1200 rpm for 10 min), cells were resuspended and plated in T75 culture flasks (Falcon BD Biosciences) with DMEM-1g containing 10% FBS, 1% penicillin/streptomycin, 1% MSC growth supplement (MSCGS; ScienCell Research Laboratories, Milan, Italy). After 24 h of incubation at 37°C with 5% CO_2, non-adherent cells were removed by replacing the growth medium. After reaching confluence (about 80% of total flask surface), all cultures were trypsinized and, after resuspension, cells were expanded for 2-3 passages and plated for the following procedures.

Some cell samples were used to verify their MSC nature, according to procedures previously described (Lo Furno et al. 2013, 2017). In particular, by immunocytochemistry and flow cytometry, their immunopositivity for typical

MSC markers (CD 44, CD 90 and CD 105) was confirmed. At the same time, their immunonegativity for typical hematopoietic stem cell markers (CD 14, CD 34 and CD 45) was verified.

For the specific purpose of the present investigation, two sets of cultures were used. Each set consisted of three culture samples: one served as control, being kept in basal growth medium; in the two other culture samples, the basal growth medium was replaced with OEC-CM or SC-CM. One set of cultures was stopped at 24 h, whereas the other was maintained for 7 days. At each time point, fluorescence immunocytochemistry and flow cytometry procedures were carried out for signal detection.

Immunofluorescence

Immunocytochemical staining was carried out following the same procedures previously described (Lo Furno et al., 2013). Briefly, cells were washed with PBS, fixed with 4% paraformaldehyde (PFA) and incubated for 30 min with a 5% solution of normal goat serum (Sigma-Aldrich) in PBS containing 0.1% Triton (Sigma-Aldrich). They were then exposed overnight at 4°C to primary antibodies: anti-Nestin (1:100; Abcam, Cambridge, UK), anti-PGP 9.5 (1:100; Novus Biologicals, Milan, Italy), anti-MAP2 (1:100; Abcam), anti-GFAP (1:100; Abcam) and anti-Synapsin I (1:100; Abcam). The following day, cells

were washed with PBS and incubated for 60 min at room temperature with secondary antibodies conjugated to different fluorochromes: FITC-conjugated goat anti-rabbit (Abcam) and/or Cy3-conjugated goat anti-mouse (Abcam). The specificity of immunostaining was verified by omitting the primary antibody. Finally, DAPI was used to stain the cell nuclei (10 min).

Flow Cytometry

For flow cytometry, cultures of each sample were trypsinized, fixed with 2% PFA in PBS for 20 min at 4 °C and permeabilized with 1% Triton for 5 min at 4 °C. After a 30 min treatment with 1% BSA to block nonspecific sites, cells were incubated for 60 min at room temperature with the same primary antibodies mentioned above, at the same dilutions. Cells were then incubated for 60 min at room temperature in the dark with goat anti-mouse or goat anti-rabbit secondary antibodies conjugated with fluorescein (FITC; 1:200; Abcam). Samples were analyzed using a Coulter Epics Elite ESP flow cytometer (Coulter, Miami, FL, USA). A maximum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at 488 nm and fluorescence was monitored at 525 nm. Mean Fluorescence Intensity (MFI) values were calculated and recorded automatically.

Statistical analysis

Percentages of positive cells are expressed as mean ± standard deviation of at least three values. Statistical analysis [Student's t-test for paired and unpaired data; variance analysis (ANOVA)] was performed using the statistical software package SYSTAT, version 11 (Systat Inc., Evanston, IL, USA) Tukey's 'Honest Significant Difference' method was used as a post hoc test. A difference was considered significant at $P<0.05$.

Results

Cell morphology

Observed at the microscope at 24 h, ASCs cultured in the basal growth medium for stem cells showed a typical fibroblast-like shape.

Cells cultured in OEC-CM or in SC-CM appeared more elongated, featuring a more complex morphology.

After 7 days, much denser cell populations were present in control cultures; these cells showed similar shape as those at 24 h. Instead, exposition to conditioned media induced smaller increases in the number of cells, characterized by a higher cytoplasmic complexity.

Supporting results were obtained by flow cytometry (Fig. 1). In control cultures, cells were characterized by a simple shape, either at 24 h (Fig. 1A) or after 7 days (Fig. 1B). Marked differences were observed using each CM. Cells cultured with OEC-CM or SC-CM were characterized by a more complex morphology and less dense cell population when compared to controls, especially at 7 days. Data close to the origin of diagrams, related to the smallest sizes and lowest complexity, are likely indicative of debris and were not considered. In all likelihood, the decreased cell density is indicative of their differentiation progress, when cells lose their self-renewal ability to acquire a better defined fate.

Expression of neural markers

Potential differentiation of ASCs into neurons or glial cells was examined at 24 h and 7 days of culture by immunofluorescence and flow cytometry analysis of typical neural marker expression. Nestin, Protein Gene Product 9.5 (PGP 9.5), Microtubule-Associated Protein 2 (MAP2), Synapsin I and Glial Fibrillary Acidic Protein (GFAP) were tested. Overall, observations at the fluorescence microscope were supported by data obtained by flow cytometry. Table 1 summarizes percentages of positive cells and mean fluorescence

intensity (MFI) for the different markers, observed at each stage in controls and in cultures treated with OEC-CM or SC-CM.

Nestin

Conspicuously detectable in control cultures, Nestin immunoreactivity was significantly more expressed in ASCs cultured in either OEC-CM or SC-CM (Fig 2A). At 24 h, the percentage of positive cells was about 40% in control cultures, whereas it was significantly higher by about 130% using each CM. At 7 days, nestin positive cells in controls were higher than those at 24 h (70%) but, also at this stage, an increase of 40% was observed for cells cultured in conditioned media (Tab. 1). Similarly, MFI values were higher after using each CM (Fig. 2B). Such an increase was over 200% at 24 h (+257% and +219% for OEC-CM and SC-CM, respectively) and even greater at 7 days (+388% and +513% for OEC-CM and SC-CM, respectively).

PGP 9.5

High levels of PGP 9.5 were expressed in all samples (Fig. 3A), although with some differences. As indicated by flow cytometry data (Table 1), percentages of positive cells at 24 h were comparably high in every culture condition (above 70%). At 7 days, higher percentages of positivity were observed for each CM. Positive cells were also characterized by considerably higher (over 200%) MFI values (Fig. 3B).

MAP2

Both microscope observation and flow cytometry data showed that MAP2 immunopositivity, very low in controls, was significantly enhanced in CM-treated cultures (Fig. 3A, Table 1). In particular, a more pronounced increase was detected for OEC-CM vs SC-CM cultures, either at 24 h (+340% vs +260%) or at 7 days (+360% vs +250%). Comparable low MFI values were detected in all cultures at 24 h, whereas a slight increase could be observed for each CM after 7 days (Fig. 4B).

Synapsin I

Immunopositivity for Synapsin I was almost undetectable, except for CM-treated cultures after 7 days (Fig. 5A, Table 1). At this stage, flow cytometry

data indicated a considerable increase (about +400% for either OEC-CM or SC-CM). Low MFI values were found in all cell cultures. Only modest increases were observed for CM-treated ASCs at 7 days (Fig. 5B).

GFAP

Immunopositivity of GFAP, quite low in controls, was markedly enhanced in CM-treated cells, especially for SC-CM (Fig. 6A). Quantitative data by flow cytometry (Table 1) indicate at 24 h higher percentages of positive cells after using each CM (+80 and +160% for OEC-CM and SC-CM, respectively). Significantly higher percentages were found at 7 days (+227% and +291% for OEC-CM and SC-CM, respectively). MFI values, very low in control cultures, were slightly higher for CM-treated cells, particularly for SC-CM at 7 days (Fig. 6B).

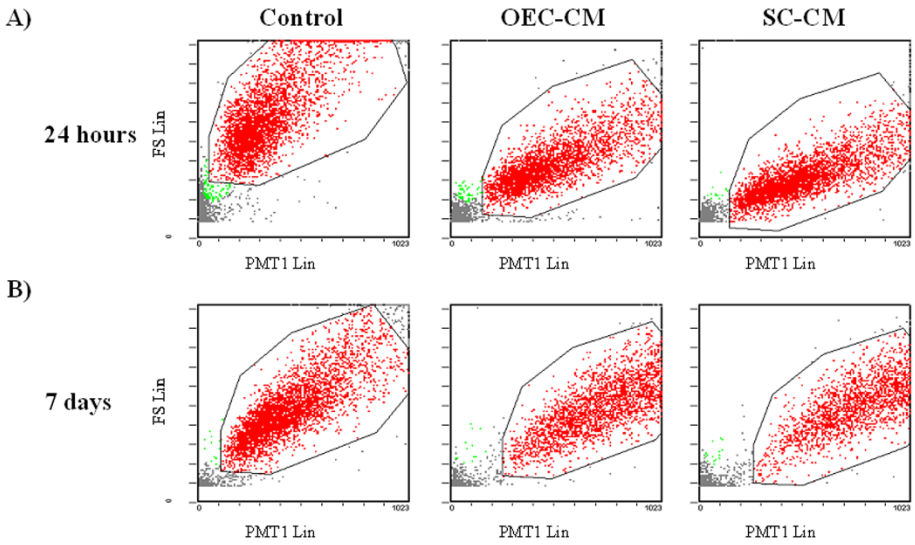


FIGURE 1 Cell morphology analysis by flow cytometry at 24 hr (a) and 7 days (b) of ASCs. An increased cell complexity is observed after CM treatment, especially at 7 days.

TABLE 1 Flow cytometry data showing effects of OEC-CM or SC-CM on marker expression in ASCs after 24 hr and 7 days of culture

	24 hr			7 days		
	Control	OEC-CM	SC-CM	Control	OEC-CM	SC-CM
Nestin						
% Positive cells	42 ± 6	98 ± 2**	96 ± 3**	70 ± 5	98 ± 2**	98 ± 2**
MFI	2.1 ± 0.4	7.5 ± 0.6	6.7 ± 0.5	4.3 ± 0.3	21.0 ± 1.2	26.4 ± 1.4
PGP9.5						
% Positive cells	70 ± 5	72 ± 6	80 ± 5	80 ± 4	95 ± 3*	97 ± 3*
MFI	2.0 ± 0.3	2.0 ± 0.2	3.0 ± 0.3	6.0 ± 0.2	19.0 ± 0.9	22.0 ± 1.2
MAP2						
% Positive cells	5 ± 2	22 ± 7*	18 ± 4*	10 ± 3	46 ± 6**	35 ± 4**
MFI	2.0 ± 0.2	2.5 ± 0.3	2.3 ± 0.2	2.2 ± 0.4	3.5 ± 0.4	3.0 ± 0.5
Synapsin I						
% Positive cells	5 ± 2	5 ± 3	5 ± 2	6 ± 3	30 ± 5**	31 ± 4**
MFI	1.0 ± 0.2	1.1 ± 0.3	1.1 ± 0.2	1.1 ± 0.1	2.1 ± 0.4	2.0 ± 0.4
GFAP						
% Positive cells	10 ± 3	18 ± 4	26 ± 5*	22 ± 4	72 ± 7**	86 ± 6**
MFI	1.8 ± 0.3	2.0 ± 0.2	2.0 ± 0.3	1.3 ± 0.2	2.8 ± 0.3	3.0 ± 0.4

MFI; mean fluorescence intensity.
All data are expressed as means and standard deviations (Means ± SDs) of at least three values.
*$p < 0.05$ compared to control.
**$p < 0.001$ compared to control.

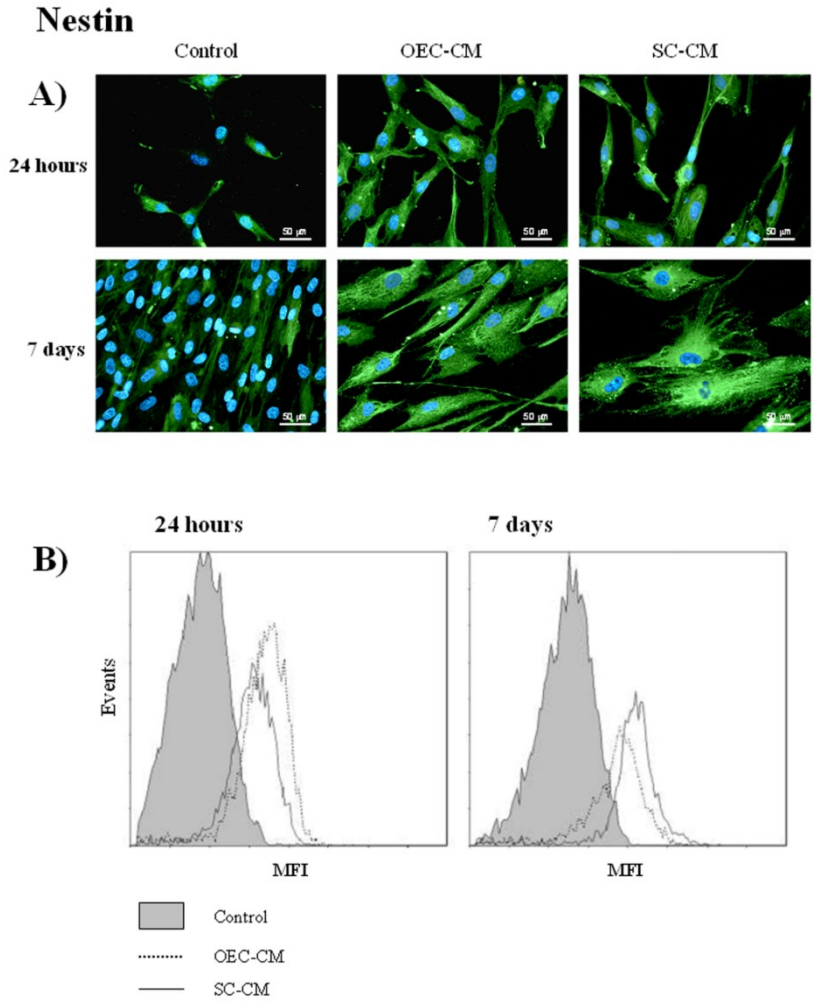


FIGURE 2 Nestin expression in ASCs at 24 hr and 7 days of culture. (a) photomicrographs of Nestin immunoreactivity of ASCs in control cultures (left), in OEC-CM cultures (middle), and in SC-CM cultures (right). Upper and lower rows show results after 24 hr and 7 days of culture, respectively. Magnification: 40×; Scale bar: 50 µm. B: Nestin expression by flow cytometry analysis of ASC cultures in control medium, OEC-CM or SC-CM at 24 hr (left) and 7 days (right). Nestin levels are considerably higher in both CM-treated cultures, especially for MFI values.

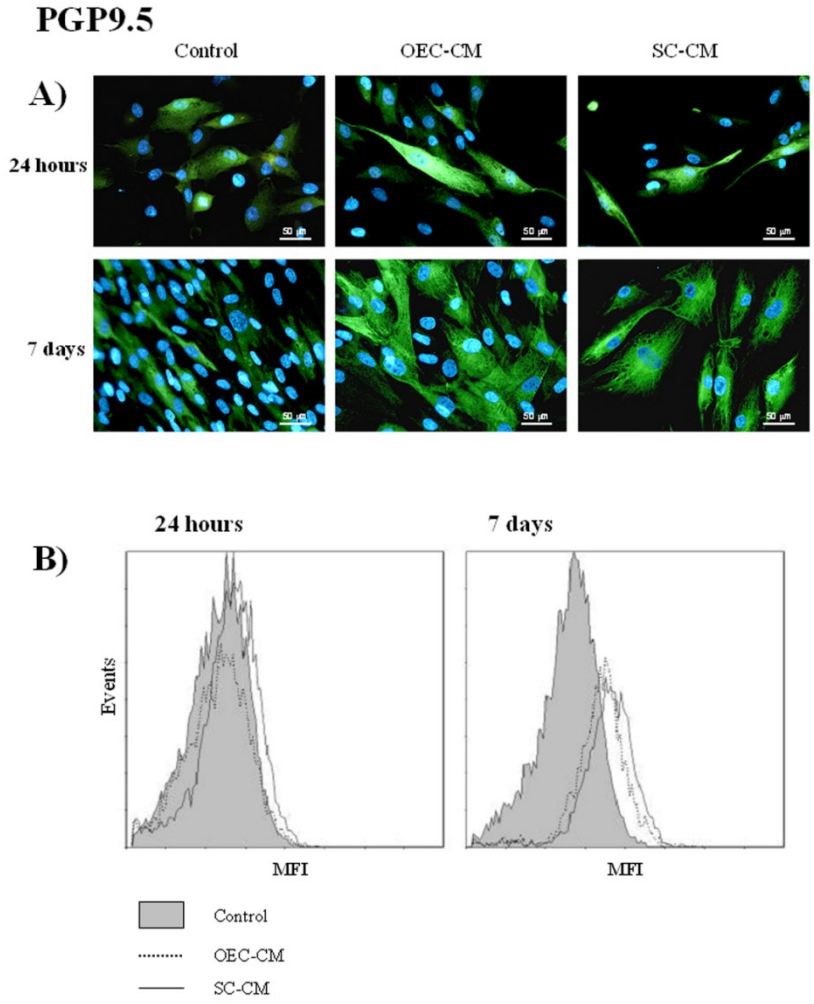


FIGURE 3 PGP9.5 expression in ASCs at 24 hr and 7 days of culture. (a) photomicrographs of PGP9.5 immunoreactivity of ASCs in control cultures (left), in OEC-CM cultures (middle), and in SC-CM cultures (right). Upper and lower rows show results after 24 hr and 7 days of culture, respectively. Magnification: 40×; Scale bar: 50 μm. (b) PGP9.5 expression by flow cytometry analysis of ASC cultures in control medium, OEC-CM or SC-CM at 24 hr (left) and 7 days (right). At 24 hr, PGP9.5 levels are comparable in all samples. Percentages of positive cells and MFI values are considerably higher at 7 days in CM-cultured ASCs

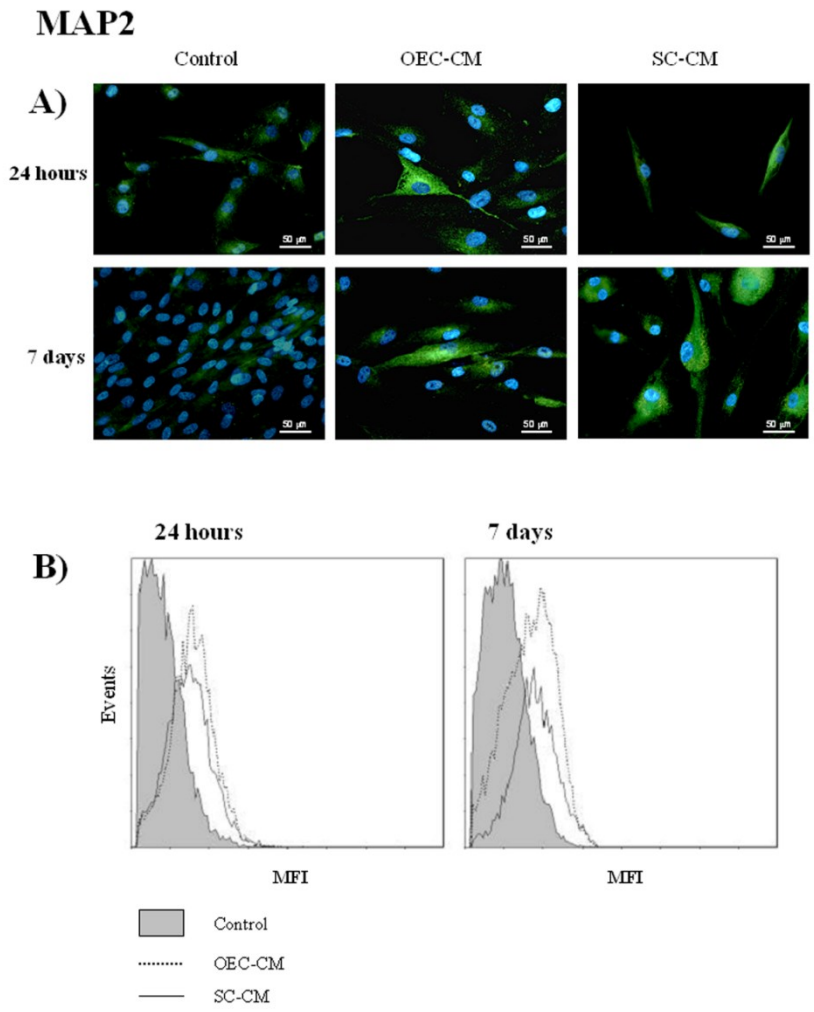


FIGURE 4 MAP2 expression in ASCs at 24 hr and 7 days of culture. (a) photomicrographs of MAP2 immunoreactivity of ASCs in control cultures (left), in OEC-CM cultures (middle), and in SC-CM cultures (right). Upper and lower rows show results after 24 hr and 7 days of culture, respectively. Magnification: 40×; Scale bar: 50 µm. (b) MAP2 expression by flow cytometry analysis of ASC cultures in control medium, OEC-CM or SC-CM at 24 hr (left) and 7 days (right). MAP2 levels are considerably low in control ASC cultures. Percentage of positive cells are considerably higher in CM cultured ASCs, especially at 7 days

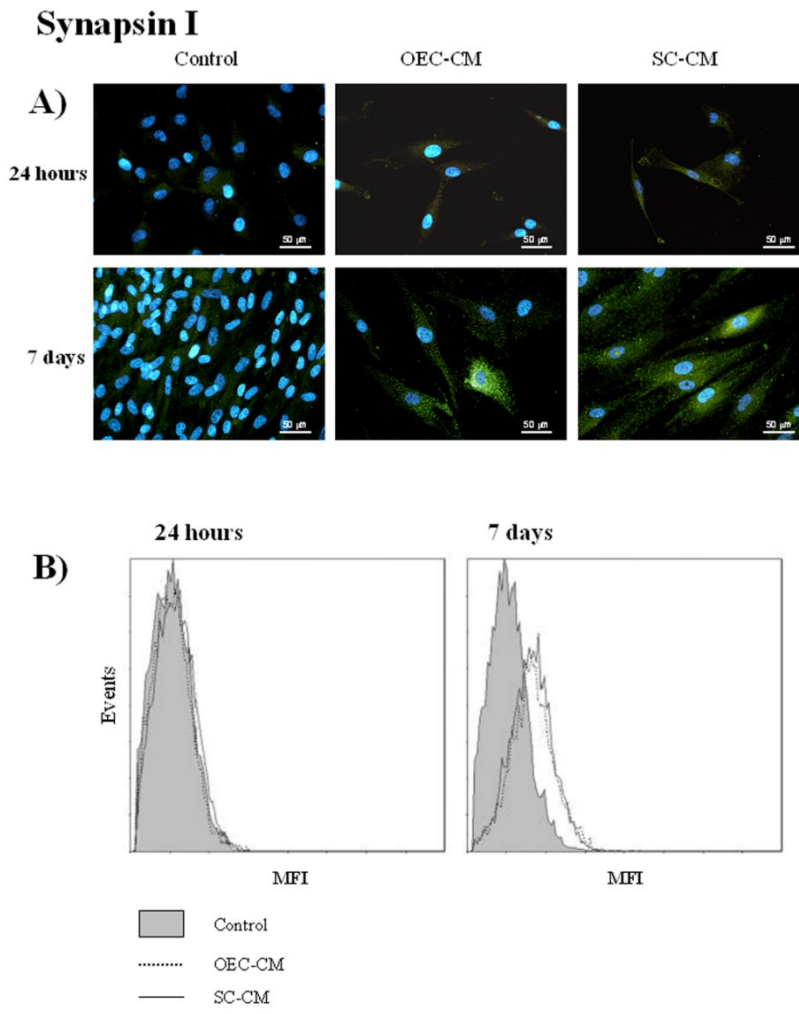


FIGURE 5 Synapsin I expression in ASCs at 24 hr and 7 days of culture. (a) photomicrographs of Synapsin I immunoreactivity of ASCs in control cultures (left), in OEC-CM cultures (middle), and in SC-CM cultures (right). Upper and lower rows show results after 24 hr and 7 days of culture, respectively. Magnification: 40×; Scale bar: 50 µm. (b) Synapsin I expression by flow cytometry analysis of ASC cultures in control medium, OEC-CM or SC-CM at 24 hr (left) and 7 days (right). Synapsin I levels are almost undetectable in all samples of ASC cultures at 24 hr. At 7 days, positive cells can be detected only in CM-cultured ASCs

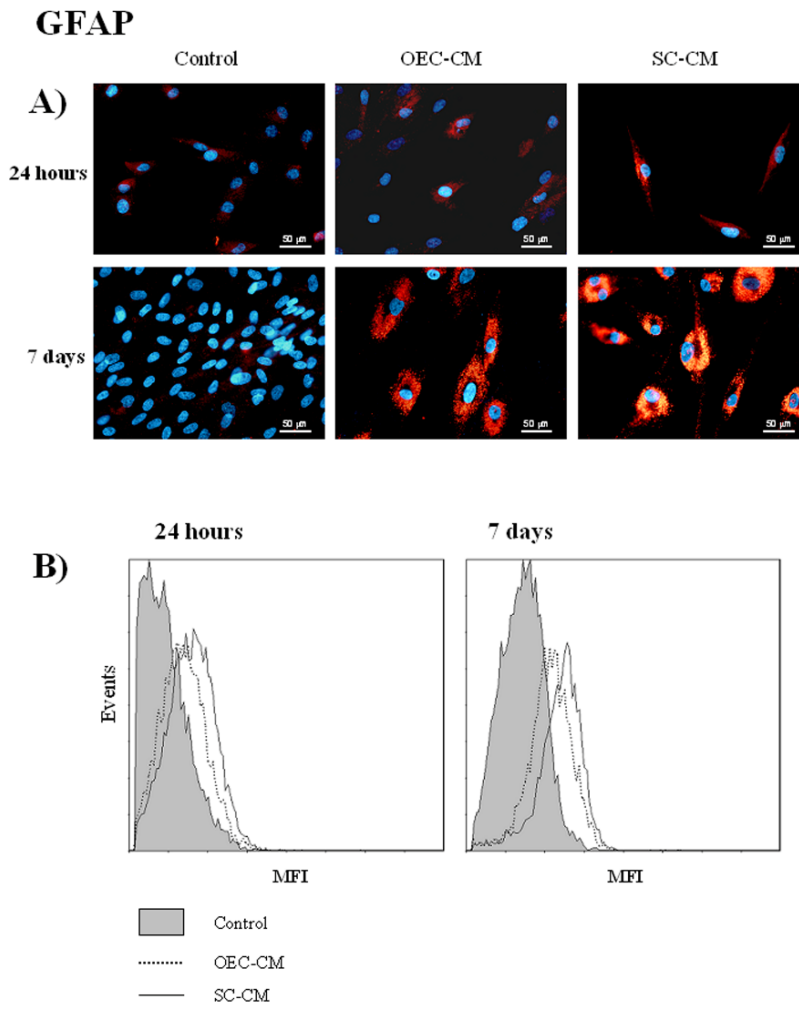


FIGURE 6 GFAP expression in ASCs at 24 hr and 7 days of culture. (a) photomicrographs of GFAP immunoreactivity of ASCs in control cultures (left), in OEC-CM cultures (middle), and in SC-CM cultures (right). Upper and lower rows show results after 24 hr and 7 days of culture, respectively. Magnification: 40×; Scale bar: 50 µm. (b) GFAP expression by flow cytometry analysis of ASC cultures in control medium, OEC-CM or SC-CM at 24 hr (left) and 7 days (right). GFAP levels are weakly detectable in ASC control cultures at both stages. Higher percentages of positive cells can be detected in CM cultured ASCs, particularly when using SC-CM.

Discussion

The present results indicate that ASCs can differentiate toward neural lineages by environmental features of the growth medium. In pioneer investigations, differentiation of ASCs into neuron- or glial-like cells have been reported by adding various induction molecules (valproic acid, butylated hydroxyanisole, insulin, hydrocortisone) to the culture medium (Safford et al., 2002). However, these strategies do not closely mimic the physiological composition of a neural microenvironment *in vivo*, especially when induction protocols include the use of potentially toxic molecules such as βmercaptoethanol or dimethyl sulfoxide (Zuk et al., 2002). This issue has been addressed by different strategies. In a work by Han et al. (2014), neural differentiation of ASCs was reported when cells were cultured in CM containing soluble factors derived from portions of whole nervous tissue (cerebellum, hippocampus, cerebral cortex). In a more focused manner, CM has been collected from cultures of specific neural cell types. In our previous study, a successful neural-like differentiation was achieved by using CM from OECs or neuroblastoma B104 cells, showing that more pronounced effects could be obtained by using OEC-CM (Lo Furno et al., 2013). In the present study, these effects have been compared with those obtained by using a CM from SC cultures. Overall, results show that, with some

differences, the use of SC-CM was equally effective. In fact, the expression of typical neural markers is considerably higher using each CM. These increases were noticeable for markers already conspicuously present in control cultures (Nestin, PGP 9.5), and were even more pronounced for the expression of markers which were only weakly detectable in undifferentiated ASCs (MAP2, Synapsin I and GFAP).

In many cases, an evident trend toward neuronal or glial differentiation was not clearly detectable. In fact, comparable increases were often observed for neuronal and glial markers, using each CM. This finding is not surprising, since both cell types share many features. They express similar markers, such as GFAP, p75, NGF, Vimentin, adhesion molecules, and S-100 protein (Pellitteri et al., 2010). Both support neuronal survival, differentiation and development, and are involved in axon myelination (Barton et al., 2107; Gu et al., 2017). Moreover, both secrete various growth factors (Kocsis et al., 2009) such as Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), Glial cell Derived Neurotrophic Factor (GDNF), Ciliary Neurotrophic Factor (CNTF), and Fibroblast Growth factor (FGF). These neurotrophins are generally recognized as potent stimulators of neural differentiation. For example, the addition of GDNF or FGF to the culture medium induced a neural differentiation of MSCs (Shokohi et al., 2018; Gonmanee et al., 2018). Notwithstanding, some differential effects can be

highlighted. A higher tendency toward a neuronal phenotype can be recognized for OEC-CM when taking into account MAP2 expression. Instead, SC-CM would be responsible for a stronger glial induction, considering GFAP increases.

Over the last decades, neural differentiation of ASCs has raised relevant interest in the field of regenerative medicine aimed at developing therapeutic strategies for disorders related to both CNS and PNS (Lo Furno et al., 2018; Yeh et al., 2015). ASCs promote axonal regeneration, myelination, and functional recovery (Tomita et al., 2012). The use of ASCs would be particularly suitable when peripheral nerve injuries are extensive and sufficient amounts of SCs are not available for transplantation (Guo et al., 2017). Moreover, it should be considered that SC harvesting is painful for patients and can cause complications at the donor site. Administration of ASCs has been successfully tested in the treatment of chronic degenerative diseases, since beneficial effects have been described in several preclinical investigations. In particular, better outcomes have been reported when transplanted MSCs were previously induced toward a neural differentiation (Yang et al., 2013).

Indeed, other sources of MSCs have been explored for a potential neural differentiation, especially those derived from bone marrow (Li and Ikehara, 2013). However, ASCs can be obtained by less invasive procedures and show a greater proliferation rate and differentiation ability (Zhang et al., 2013). In

conclusion, when safe culture protocols are developed and optimal functional neural differentiation of these cells is achieved, ASCs might provide a valuable tool in cell-based therapeutic strategies for neurological disorders.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ORCID

Rosario Giuffrida http://orcid.org/0000-0001-7494-3675

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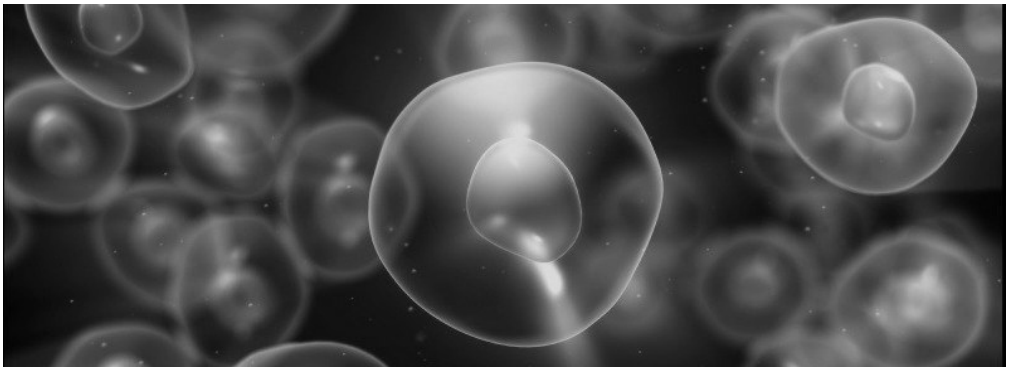
CHAPTER 3

Conditioned Media From Glial Cells Promote a Neural-Like Connexin Expression in Human Adipose-Derived Mesenchymal Stem Cells.



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Conditioned Media from Glial Cells Promote a Neural-Like Connexin Expression in Human Adipose-Derived Mesenchymal Stem Cells.

Debora Lo Furno[1*†], Giuliana Mannino[1†], Rosalia Pellitteri[2], Agata Zappalà[1], Rosalba Parenti[1], Elisa Gili[3], Carlo Vancheri[3], Rosario Giuffrida[1]

[1]Department of Biomedical and Biotechnological Sciences, Section of Physiology, University of Catania, Catania, Italy

[2]Institute of Neurological Sciences, National Research Council, Section of Catania, Catania, Italy

[3]Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy

[*]Correspondence: Debora Lo Furno lofurno@unict.it

[†]**These authors have contributed equally to this work**

Abstract

The expression of neuronal and glial connexins (Cxs) has been evaluated in adipose-derived mesenchymal stem cells (ASCs) whose neural differentiation was promoted by a conditioned medium (CM) obtained from cultures of olfactory ensheathing cells (OECs) or Schwann cells (SCs).

By immunocytochemistry and flow cytometer analysis it was found that Cx43 was already considerably expressed in naïve ASCs and further increased after 24 h and 7 days from CM exposition. Cx32 and Cx36 were significantly improved in conditioned cultures compared to control ASCs, whereas a decreased expression was noticed in the absence of CM treatments. Cx47 was virtually absent in any conditions.

Altogether, high basal levels and induced increases of Cx43 expression suggest a potential attitude of ASCs toward an astrocyte differentiation, whereas the lack of Cx47 would indicate a poor propensity of ASCs to become oligodendrocytes. CM-evoked Cx32 and Cx36 increases showed that a neuronal- or a SC-like differentiation can be promoted by using this strategy.

Results further confirm that environmental cues can favor an ASC neural differentiation, either as neuronal or glial elements. Of note, the use of glial products present in CM rather than the addition of chemical agents to achieve

such differentiation would resemble “more physiological” conditions of differentiation.

As a conclusion, the overexpression of typical neural Cxs would indicate the potential capability of neural-like ASCs to interact with neighboring neural cells and microenvironment.

Keywords: human adipose mesenchymal stem cells, neural-like differentiation, connexin expression, glial conditioned media, olfactory ensheathing cells, Schwann cells

Introduction

Mesenchymal stem cells (MSCs) are adult stem cells featuring self-renewal and differentiation abilities. They can typically give rise to mesodermal derivatives such as chondrocytes (Musumeci et al., 2011), osteocytes (Calabrese et al., 2015) or adipocytes (Lo Furno et al., 2013a) but, under appropriate conditions, they can also transdifferentiate into epithelial or neural cells (Zuk et al., 2002).

A neural differentiation of MSCs has been obtained by means of a variety of protocols. In 2002, Safford et al. were able to induce adipose-derived MSCs

(ASCs) into a neural phenotype by using a culture medium supplemented with valproic acid, butylated hydroxyanisole, insulin, and hydrocortisone. Several other supplements have been experimented thereafter, on MSCs of different sources (Krampera et. al., 2007). To date, a growing body of literature indicates the possibility of a neural differentiation of ASCs. This differentiation is supported by the induction of typical neural marker, evaluated both at protein expression and mRNA levels (Goudarzi et al., 2018), as well as for their functional characteristics (Ching et al., 2018; Guo et al., 2016; Jang et al., 2010). Moreover, some transcription factors such as Pax6, NeuroD1, Tbr2 and Tbr1, associated to different stages of neurogenesis, were found overexpressed in ASCs after neural induction (Cardozo et al., 2012).

In our previous studies (Lo Furno et al., 2013, 2018b), a successful differentiation of ASCs toward neural-like elements was achieved by using a conditioned medium (CM) from cultures of Schwann cells (SCs) or Olfactory Ensheathing Cells (OECs). This differentiation was documented by the increased expression of typical neural markers (Nestin, PGP9.5, MAP2, Synapsin I, GFAP).

In the present work, during a similar CM-promoted neural-like differentiation, the presence and/or the modification of the immunostaining of some connexins (Cxs) was investigated. In fact, Cxs are fundamental components of gap junctions which, though widely present in most tissues, play a crucial role in

nervous system physiology. They allow direct communication between neurons and/or glial cells. Other than electrical signals, they mediate intercellular propagation of ions, second messengers and other small metabolites (Evans and Martin, 2002).

In adjacent cells, gap junctions characteristically consist of two opposed hemichannels (connexons), each made up of six Cxs (Söhl et.al, 2005). Gap junctions consist of homotypic or heterotypic channels. In homotypic channels, both connexons are made by the same Cx subtype; in heterotypic channels, each connexon contains a different Cx subtype (Magnotti et al., 2011). Of note, they can connect cytoplasmic membranes of the same cell, forming autologous or reflexive gap junctions (Nualart-Marti et al., 2013). In addition, the presence of unpaired connexons (hemichannels) provides a communication device between the intra- and extra-cellular environments (Hartfield et al., 2011).

Neurons and glial cells of the mammalian brain are characterized by various Cx isoforms (Rozental et al., 2000; Rash et al., 2001; Vicario et al., 2017). Although with different cellular specificity, Cx32, Cx36, Cx43 and Cx47 are widely distributed.

Cx32 is expressed in the central nervous system (CNS) as well as in the peripheral nervous system (PNS). It is particularly found in oligodendrocytes and SCs, the two glial cells responsible for axon myelination (Mambetisaeva et al., 1999; Nualart-Marti et al., 2013).

Cx47 is characteristically expressed by oligodendrocytes (Parenti et al., 2010). It may exert compensatory effects when Cx32 is lacking. In fact, the loss of either Cx32 or Cx47 alone does not lead to evident alterations, whereas the absence of both Cxs significantly impairs axon myelination. Symptoms are characterized by gross tremors and tonic seizures, and death usually occurs after about 50 postnatal days (Minichella et al., 2003; Odermatt 2003).

Cx43 is mainly present in astrocytes (Dermietrel et al., 1991; Iacobas et al., 2005), where it mediates ionic and metabolic exchanges within single or between neighboring cells. Through heterotypic Cx43/Cx47 gap junctions, astrocytes may be coupled with oligodentrocytes (Xu et al., 2017). Cx43 is also present in OECs (Barnett et al., 2001; Rela et al., 2010) and neural progenitors at early embryonic development stages (Duval et al. 2001).

Cx36 is typically expressed in neurons (Condorelli et al., 1998;Parenti et al., 2000; Rash et al., 2000; Parenti et al., 2002). High levels of Cx36 have been detected in several regions of the CNS such as the inferior olive, cerebellum, hippocampus, hypothalamus and mammillary bodies (Condorelli et al., 2000). Although not exclusively, Cx36 is mainly found in GABAergic interneurons. This Cx is likely associated with the neuronal development, because its increased expression in several brain regions largely corresponds to interneuronal coupling in early postnatal weeks (Gulisano et al., 2000; Hartfield et al., 2011).

Herein, CM from OEC or SC cultures was used in the present investigation to promote a neural differentiation of ASCs. Both SCs and OECs are glial cells normally present in the nervous system, providing axon myelination. SCs are exclusively located in the PNS whereas OECs can also be found inside the CNS, where they support the continuous neurogenesis in the mammalian olfactory system (Ramón-Cueto and Avila, 1998). Both glial cells play an important role in axonal regeneration and remyelination (Sasaki et al., 2011; Gao et al., 2018). Their functional support is also provided by producing various growth factors and extracellular matrix molecules (Frostick et al., 1998; Pellitteri et al., 2001).

Connexin expression and their modifications in ASCs exposed to these conditioned media were evaluated by immunocytochemistry and flow cytometry analysis. The neural commitment of CM cultured ASCs was tested by the expression of Nestin, PGP9.5 and GFAP.

Data were gathered after 24 h and 7 days of culture and compared to control ASCs. Results suggest that, considering the pattern of Cx profile modification, a neural fate was promoted in ASCs under these conditions.

Material and Methods

Preparation of rat OEC-CM

Experimental procedures were carried out according to the Italian Guidelines for Animal Care (D. Lgs 26/2014), and the European Communities Council Directives (2010/63/EU), and were approved by the ethics committee of the University of Catania (Organismo Preposto al Benessere Animale, OPBA; Authorization n. 174/2017-PR). All efforts were made to minimize animal suffering and to reduce the number of animals used.

As previously described, OECs were isolated from olfactory bulbs of 2-day-old rat pups (Pellitteri et al., 2009). Briefly, the bulbs were removed and cold dissected (+4 °C) in Leibowitz L-15 medium (Sigma-Aldrich, Milan, Italy). Subsequently, they were digested by collagenase (Invitrogen, Milan, Italy) and trypsin (Sigma-Aldrich) in Minimum Essential Medium-H (MEM-H, Sigma-Aldrich). Enzymatic activity was stopped by adding Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS, Sigma-Aldrich; DMEM/FBS). The antimitotic agent cytosine arabinoside (10−5 M) was added to reduce the number of dividing fibroblasts. In order to increase OEC proliferation, bovine pituitary extract was added. Finally, cells were incubated at 37 C in fresh DMEM/FBS, which was replaced

twice a week. OEC-CM was collected 24-48 h after reaching confluence. It was filtered to remove debris and detached cells, aliquoted and stored at -20 °C until further use.

Preparation of rat SC-CM

To harvest SCs, sciatic nerves of 2-day-old rat pups were removed and treated with collagenase and trypsin in DMEM (Pellitteri et al., 2006). They were then mechanically dissociated by trituration and filtered through a 150 μm nylon mesh. After centrifugation, cells were resuspended and plated in 25 cm2 flasks containing fresh DMEM/FBS. The antimitotic agent cytosine arabinoside (10−5 M) was added to reduce the number of dividing fibroblasts.

After 24-48 h from confluence, SC-CM was collected, filtered to remove debris and detached cells, aliquoted and stored at -20 °C until further use.

Human ASC cultures

Adipose tissue was harvested from healthy young donors undergoing liposuction procedures at the Cannizzaro Hospital, Catania (Italy). Written informed consent was given by all donors to use the lipoaspirate for experimental procedures, which were carried out in accordance with the

Declaration of Helsinki (2000). The protocol was approved by the local ethics committee (Comitato etico Catania1; Authorization n. 155/2018/PO).

First, the lipoaspirate was digested by collagenase (Invitrogen) in DMEM before the following steps, as already described (Lo Furno et al., 2013b). Briefly, after centrifugation at 1200 rpm for 10 min, the pellet was resuspended in PBS and filtered through a 100-µm nylon cell strainer (Falcon BD Biosciences, Milan, Italy). Cells were then plated in T75 culture flasks (Falcon BD Biosciences) with DMEM/FBS and 1% MSC growth supplement (MSCGS; ScienCell Research Laboratories, Milan, Italy). After 24 h of incubation at 37 °C with 5% CO2, the growth medium was replaced to remove non-adherent cells. When about 80% of confluence was reached, cells were trypsinized and plated for the subsequent procedures.

As in previous works, the MSC nature of cells was tested by immunocytochemistry and flow cytometry. In particular, it was verified that cells were immunopositive for typical MSC markers (CD 44, CD 73, CD 90 and CD 105) and immunonegative for typical hematopoietic stem cell markers (CD 14, CD 34 and CD 45). Moreover, their multipotential differentiation toward chondrocytes, osteocytes and adipocytes was previously verified (Calabrese et al., 2015).

For the specific purpose of this investigation, some cultures were incubated with OEC-CM or SC-CM. Some others served as controls, maintained in DMEM/FBS. One sample of each culture type was stopped after 24 h, whereas other samples were kept for 7 days. At each time point, fluorescence immunocytochemistry and flow cytometry procedures were carried out for evaluating Cx expression. Additional samples were used to test the expression of neural markers (Nestin, PGP9.5 and GFAP). To evaluate cell morphology and growth rate, some cell cultures were stained with hematoxylin.

ASC immunocytochemistry

Immunostaining procedures were carried out following a protocol previously described (Lo Furno et al., 2013b). Briefly, cells were fixed with 4% paraformaldehyde and incubated for 30 min with a solution of PBS containing normal goat serum (5%; Sigma-Aldrich) and Triton (0.1%; Sigma-Aldrich). They were then exposed overnight at 4 °C to primary antibodies: anti-Cx32 (1:100; made in mouse; Novex), anti-Cx36 (1:100; made in mouse; Novex), anti-Cx43 (1:100; made in rabbit; Sigma-Aldrich), anti-Cx47 (1:100; made in mouse; Novex), anti-Nestin (1:100; made in rabbit; Abcam, Cambridge, UK), anti-PGP9.5 (1:100; made in rabbit; Novus Biologicals, Milan, Italy), anti-GFAP (1:100; made in mouse; Abcam). The following day, cells were

incubated for 1 h at room temperature with secondary antibodies conjugated to different fluorochromes: FITC-conjugated goat anti-rabbit (1:500; Abcam) or Cy3-conjugated goat anti-mouse (1:500; Abcam).

In some experiments, a double immunostaining procedure was carried out to verify the immunopositivity for Cx and neural markers in the same cells. In particular, some ASC cultures were tested for Nestin and Cx36; some others for PGP9.5 and Cx32. Immunostaining was revealed by FITC-conjugated and Cy3-conjugated secondary antibodies. As a final step, DAPI staining (10 min) was carried out to visualize cell nuclei. In some samples, the primary antibody was omitted to verify the specificity of immunostaining.

ASC flow cytometry

After trypsinization, cells were fixed (2% paraformaldehyde in PBS for 20 min at 4 °C) and permeabilized (1% Triton in PBS for 5 min at 4 °C). Cells were then incubated at room temperature for 30 min with 1% BSA to block nonspecific sites, and for 1 h with the primary antibodies. The same antibodies mentioned above were used, at the same dilutions. Successively, cells were exposed for 1 h to goat anti-mouse or goat anti-rabbit FITC-conjugated secondary antibodies (1:200; Abcam). A Coulter Epics Elite ESP flow cytometer (Coulter, Miami, FL, USA) was used. For each sample, a maximum

of 10,000 forward and side scatter gated events were collected. Excitation wavelength was 488 nm and fluorescence monitoring was at 525 nm wavelength. Mean Fluorescence Intensity (MFI) values were calculated and recorded automatically.

Statistical analysis

Quantitative data of percentages of positive cells were gathered from three experiments. They are expressed as mean ± standard deviations. Statistical analysis [Student's t-test for paired and unpaired data; variance analysis (ANOVA)] was performed using the statistical software package SYSTAT, version 11 (Systat Inc., Evanston, IL, USA). Tukey's 'Honest Significant Difference' method was used as a post hoc test. A difference was considered significant at $P<0.05$.

Results

The stem cell profile of ASCs was verified by immunocytochemistry and flow cytometry. In accordance with previous studies (Calabrese et al., 2015), cells were immunopositive for typical MSC markers (CD44, CD 73, CD90, and CD105) and immunonegative for typical hematopoietic stem cell markers

(CD14, CD34, and CD45). Moreover, as reported recently (Lo Furno et al., 2018), an ASC neural-like phenotype by using OEC-CM or SC-CM was here confirmed by the increased immunopositivity for Nestin, PGP9.5, and GFAP (Figure1).

Cell growth and morphology

After 24 h of growth in the basal MSC medium, ASCs exhibited a typical fibroblast-like morphology (Figure 2). Those cultured in OEC-CM or in SC-CM were bigger and showed a more complex cytoplasmic shape. At 7 days, ASCs in control cultures were much more numerous, showing a similar shape to that observed at 1 day. Instead, cells cultured in either OEC-CM or SC-CM were more scattered, featuring bigger and much more complex cell bodies; long and thin cytoplasmic branches were often detectable, more evident in OEC-CM treated cells.

Connexin expression

Immunofluorescence and Flow cytometry was used to evaluate the pattern of cellular Cx expression at 24 h and 7 days of culture. In particular, three conditions were investigated: a) control ASCs, kept in the basal medium, b) ASCs cultured in OEC-CM or c) SC-CM. Overall, observations at the

fluorescence microscope were consistent with flow cytometry outcomes. Quantitative data were gathered from three independent experiments. They are summarized in Table 1, where mean values and standard deviation of positive cells and mean fluorescence intensity (MFI) are reported for each condition at each stage of signal detections. Percentages of positive cells in the different conditions are also reported in the histograms of Figure 3, where significant differences between CM treated cultures and controls are highlighted.

Immunopositivity for Cx32 (Figure 4) was present in control ASC cultures both at 24 h and 7 days, although in a low percentage of cells (8% and 10%, respectively). In CM cultures, only slight increases could be observed at 24 h, whereas significantly higher percentages were found after 7 days, especially for SC-CM vs OEC-CM treatment (69% and 33%, respectively). MFI values in CM cultures were correspondently higher than those in control ASCs. Again, more pronounced increases were found in SC-CM samples at 7 days.

Cx36 immunopositivity (Figure 5) was modestly present in control ASC cultures at 24 h (8%), and it was almost undetectable at 7 days (less than 1%). In OEC-CM cultures, only slight increases were found at 24 h (14%), whereas significantly higher percentages were observed after 7 days (30%). More marked effects were detected in SC-CM samples. In these cases, the incidence of ASC immunopositive cells, both at 24 h and 7 days, was almost 50%. In addition, SC-CM was able to induce effects faster (already visible at 24 h),

whereas OEC-CM effects were more evident after a longer period (7 days). MFI values were correspondently higher in SC-CM cultures.

A considerably high incidence of Cx43 immunopositive cells (Fig.6) was present in control ASC cultures, both at 24 h and 7 days (about 70%). The treatment with OEC-CM or SC-CM evoked further increases of this Cx expression (above 90%). Flow cytometry data confirmed that MFI values, already considerable in control cultures, were even higher in CM samples.

Almost undetectable levels of Cx47 immunoreactivity (Fig.7) were present in control ASC cultures, both at 24 h and 7 days (less than 1%). Exposition to OEC-CM or SC-CM induced only very slight, if any, effects. The greatest increase, although of modest entity (up to 6%), was observed only after 7 days of SC-CM treatment. It can be assumed that this Cx, virtually absent in control ASCs, was not modified in all the conditions tested.

Double labelling experiments allowed us to verify that Cxs and neural markers may indeed be expressed by the same cells. Some examples are illustrated in Fig.8, where Nestin and PGP9.5 immunostaining was combined with Cx36 and Cx32 immunoreactivity, respectively.

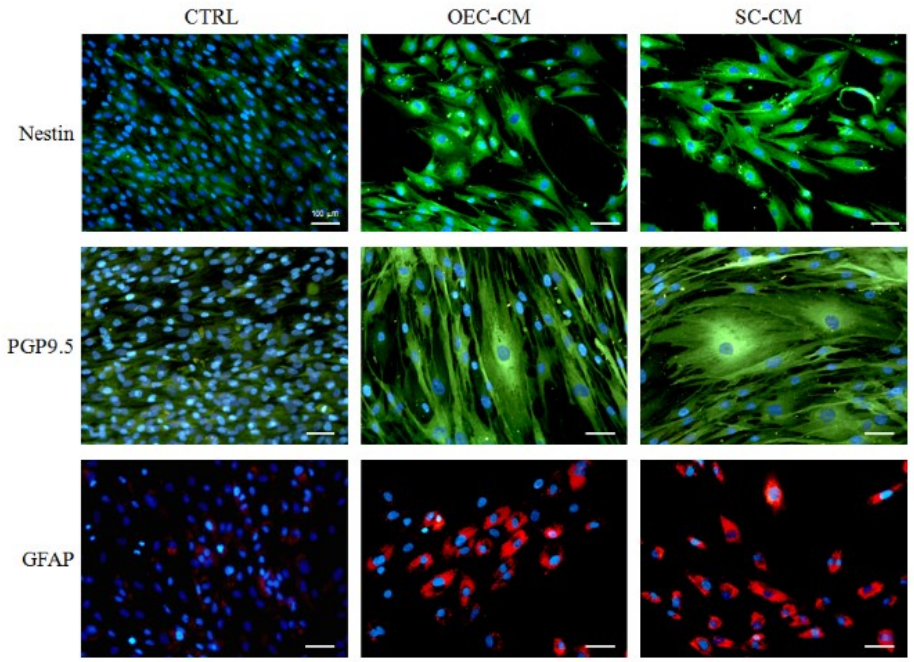

FIGURE 1 | Photomicrographs of ASC cultures after 7 days of growth in basal medium (CTRL), in OEC-CM or in SC-CM. Compared to controls, immunostaining for Nestin (upper row), PGP9.5 (middle row) and GFAP (lower row) is substantially increased in CM treated cultures. ASC, adipose-derived stem cell; OEC-CM, conditioned medium from olfactory ensheathing cells; SC-CM, conditioned medium from Schwann cells. Magnification: 20x; Scale bars: 100 μm.

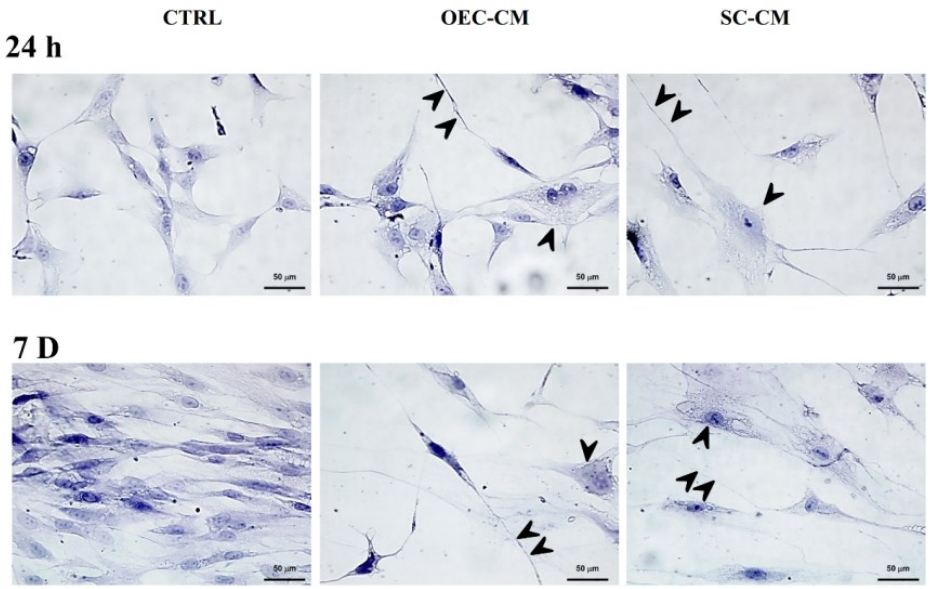

FIGURE 2 | Photomicrographs of hematoxylin stained adipose-derived stem cells (ASCs) cultured in basal medium (CTRL, left), in OEC-CM (middle) or in SC-CM (right). The three conditions are shown after 24 h (upper row) and 7 days (lower row) of growth. Typically, control ASC cultures show fibroblast-like cells, much more numerous after 7 days of growth. Cells cultured in either OEC-CM or SC-CM were less numerous, especially at 7 days. They are characterized by bigger cell bodies (arrowheads). In OEC-CM cultures, elongated cytoplasmic branches were frequently observed (double arrowheads). OEC-CM, conditioned medium from olfactory ensheathing cells; SC-CM, conditioned medium from Schwann cells. Magnification: 40x; Scale bars: 50 μm.

TABLE 1 | Flow cytometry data showing effects of OEC-CM or SC-CM on Cx expression in ASCs after 24 h and 7 days of culture.

		24 h			7 days		
		Controls	**OEC-CM**	**SC-CM**	**Controls**	**OEC-CM**	**SC-CM**
Cx32	% positive cells	8.12 ± 0.5	11.29 ± 2.1	15.46 ± 3.5	10.54 ± 2.3	33.10 ± 2.0	69.46 ± 2.2
	MFI	1.2 ± 0.4	1.4 ± 0.3	1.6 ± 0.5	1.4 ± 0.3	2.3 ± 0.2	2.8 ± 0.4
Cx36	% positive cells	8.26 ± 4.1	14.36 ± 3.3	47.2 ± 3.5	0.92 ± 0.2	30.02 ± 3.1	49.47 ± 3.3
	MFI	1.8 ± 0.3	1.9 ± 0.2	2.9 ± 0.3	1.5 ± 0.2	1.9 ± 0.4	2.3 ± 0.2
Cx43	% positive cells	66.10 ± 2.5	83.90 ± 3.2	91.29 ± 2.3	75.46 ± 3.2	95.31 ± 3.1	96.14 ± 2.3
	MFI	2.6 ± 0.5	3.3 ± 0.3	4.6 ± 0.4	2.6 ± 0.6	5.0 ± 0.4	5.4 ± 0.2
Cx47	% positive cells	0.77 ± 0.2	1.39 ± 0.3	1.31 ± 0.2	0.59 ± 0.1	1.33 ± 0.4	6.31 ± 0.3
	MFI	1.1 ± 0.2	1.2 ± 0.3	1.3 ± 0.2	1.2 ± 0.1	1.7 ± 0.4	1.6 ± 0.4

All data are expressed as means and standard deviations (Means ± SDs) of three values. Adipose-derived stem cells (ASCs) cultured in basal medium (controls), in conditioned medium from olfactory ensheathing cells (OEC-CM) or conditioned medium from Schwann cells (SC-CM). Cx, connexin; MFI, mean fluorescence intensity.

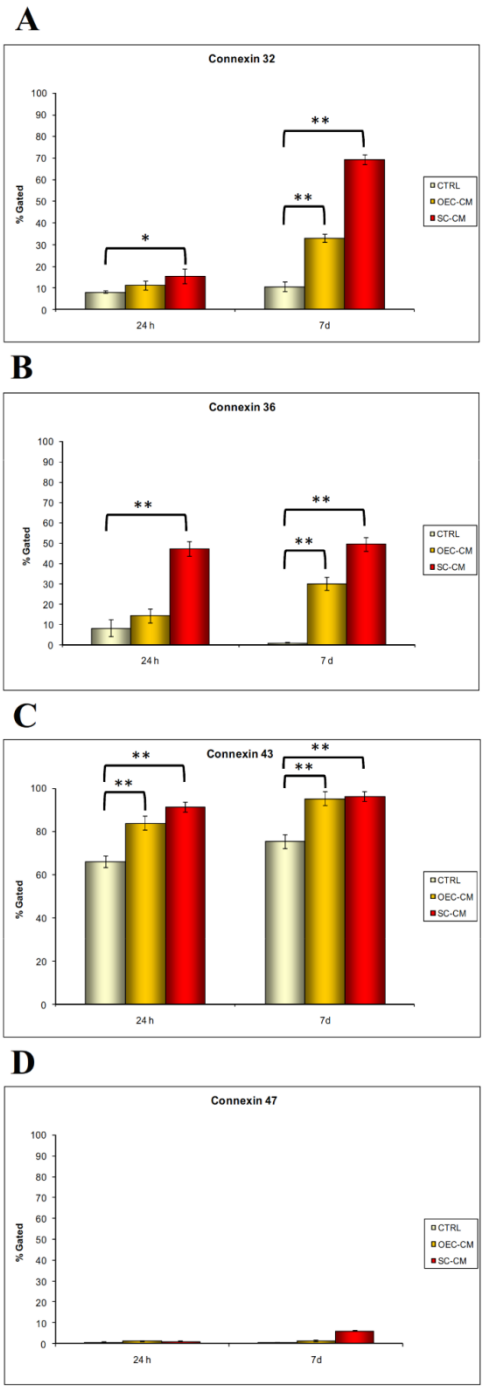

FIGURE 3 | Percentages of immunopositive cells for connexin 32 (A), 36 (B), 43 (C), and 47 (D) after 24 h and 7 days of culture. Data were gathered from three independent experiments where adipose-derived stem cells were cultured in basal medium (controls), in conditioned medium from olfactory ensheathing cells (OEC-CM) or in conditioned medium from Schwann cells (SC-CM). Significant differences between CM treated cultures and controls are indicated: *P < 0.05, **P < 0.001.

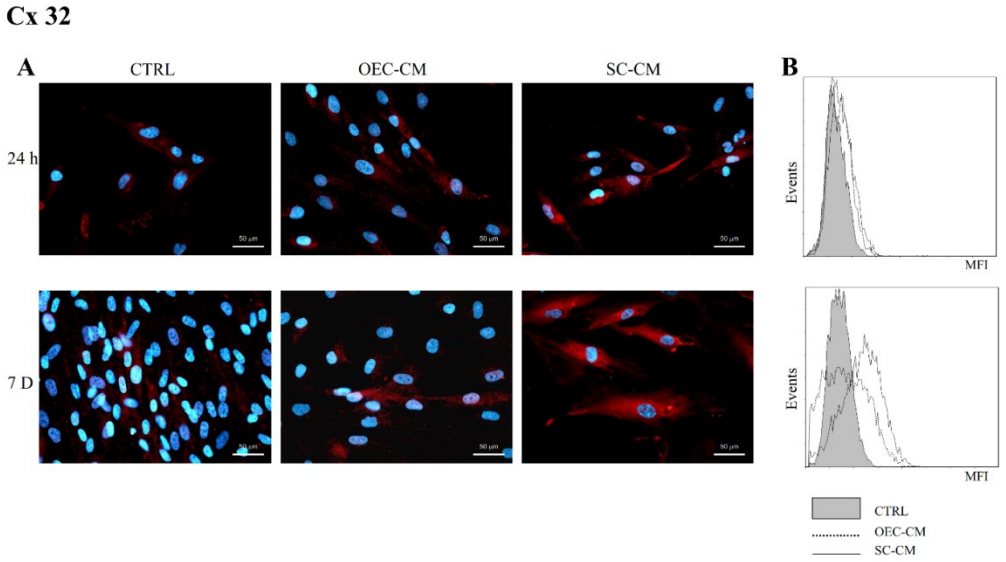

FIGURE 4 | Photomicrographs of Cx32 immunostaining (A) of ASC cultures in basal medium (CTRL), in OEC-CM or in SC-CM. Flow cytometry data are shown in (B). Data were collected after 24 h (upper row) and after 7 days (lower row). More evident increases are noticeable for SC-CM treated ASCs after 7 days. ASC, adipose-derived stem cell; OEC-CM, conditioned medium from olfactory ensheathing cells; SC-CM, conditioned medium from Schwann cells. Magnification: 40x; Scale bars: 50 μm.

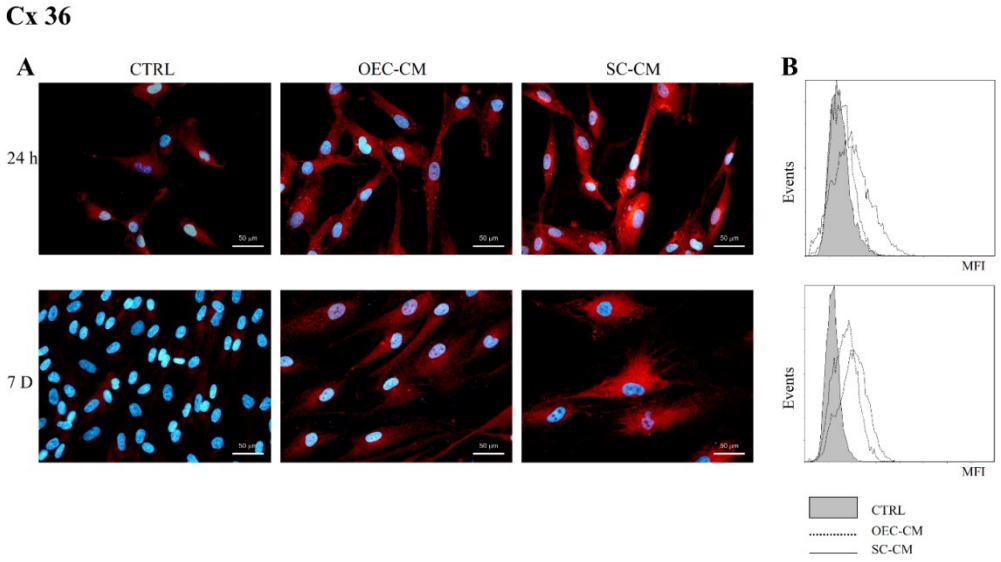

FIGURE 5 | Photomicrographs of Cx36 immunostaining (A) of ASC cultures in basal medium (CTRL), in OEC-CM or in SC-CM. Flow cytometry data are shown in (B). Data were collected after 24 h (upper row) and after 7 days (lower row). More evident increases are visible for SC-CM treated ASCs, already after 24 h. ASC, adipose-derived stem cell; OEC-CM, conditioned medium from olfactory ensheathing cells; SC-CM, conditioned medium from Schwann cells. Magnification: 40x; Scale bars: 50 μm.

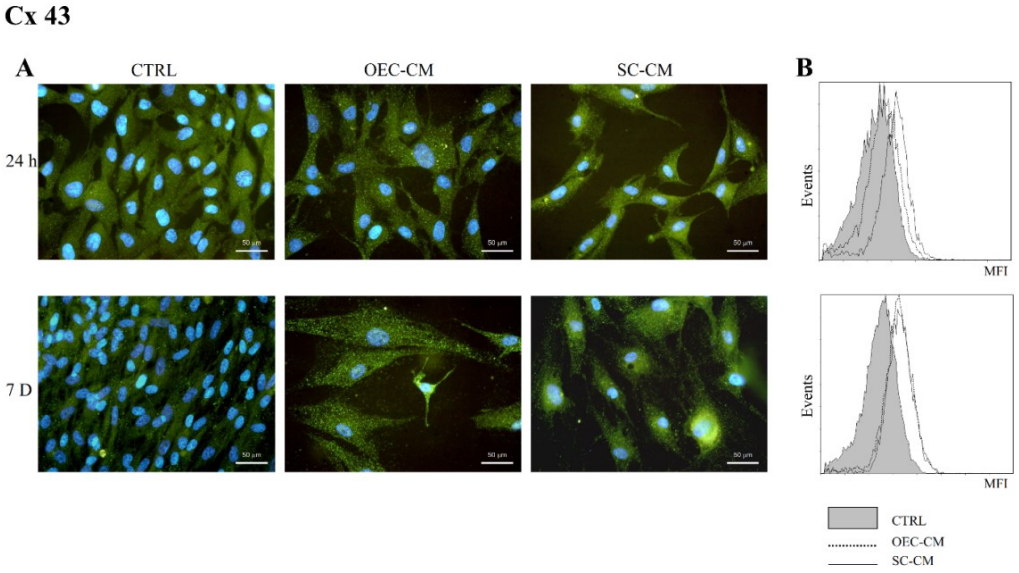

FIGURE 6 | Photomicrographs of Cx43 immunostaining (A) of ASC cultures in basal medium (CTRL), in OEC-CM or in SC-CM. Flow cytometry data are shown in (B). Data were collected after 24 h (upper row) and after 7 days (lower row). Immunoreactivity was evident already in control ASCs and further increased following OEC-CM or SC-CM treatment. ASC, adipose-derived stem cell; OEC-CM, conditioned medium from olfactory ensheathing cells; SC-CM, conditioned medium from Schwann cells. Magnification: 40x; Scale bars: 50 µm.

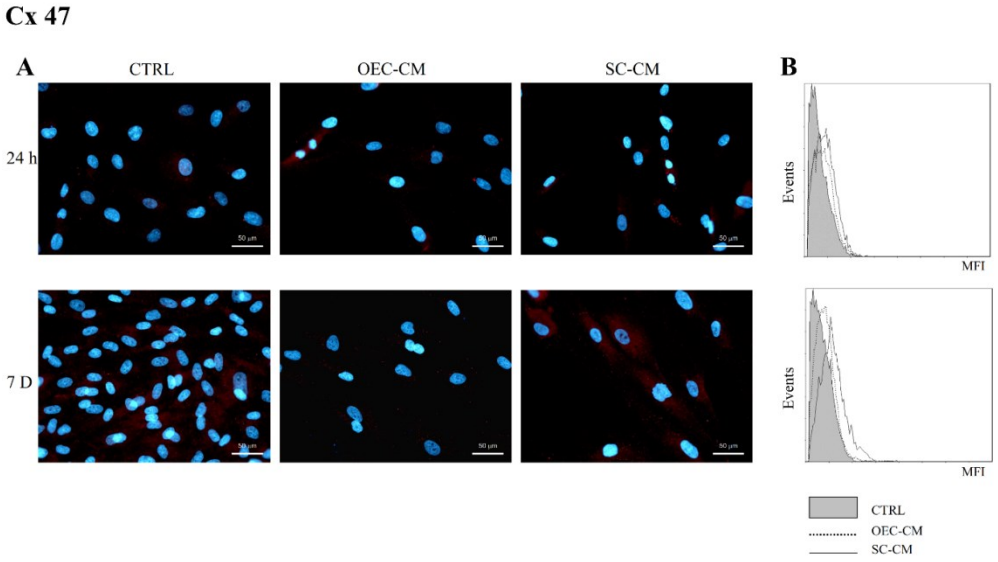

FIGURE 7 | Photomicrographs of Cx47 immunostaining (A) of ASC cultures in basal medium (CTRL), in OEC-CM or in SC-CM. Flow cytometry data are shown in(B). Data were collected after 24 h (upper row) and after 7 days (lower row). Immunoreactivity was virtually absent in all conditions. ASC, adipose-derived stem cell; OEC-CM,: conditioned medium from olfactory ensheathing cells; SC-CM, conditioned medium from Schwann cells. Magnification: 40x; Scale bars: 50 μm.

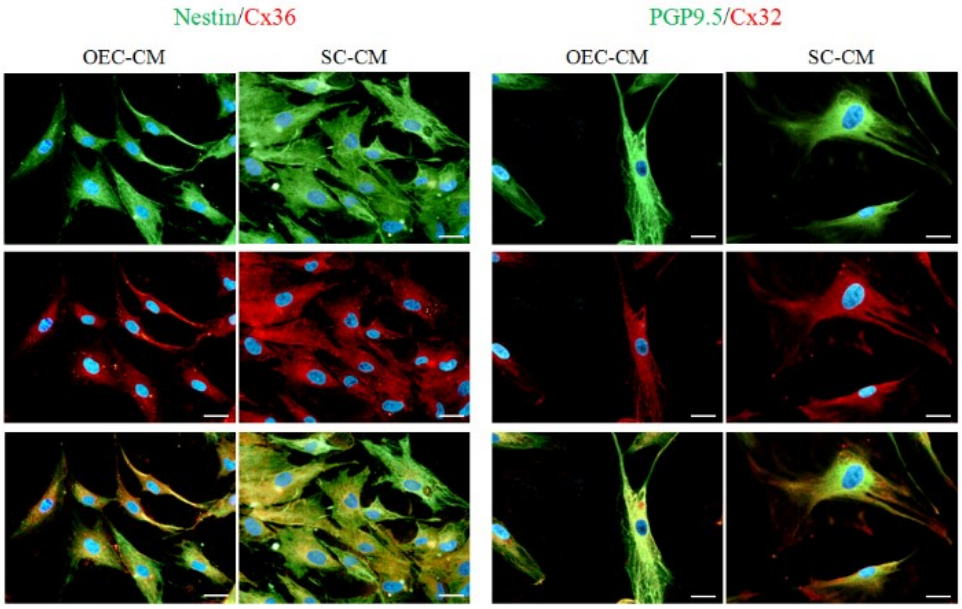

FIGURE 8 | Photomicrographs of double labeled ASC cultures exposed to OEC-CM or to SC-CM for 7 days. In the two columns on the left, Nestin immunostaining (green, upper row) was combined with Cx36 (red, middle row). In the two columns on the right, PGP9.5 immunostaining (green, upper row) was combined withCx32 (red, middle row). Note that the same cells are immunopositive both for neural markers and Cxs (merge in lower row). ASC, adipose-derived stem cell; OEC-CM, conditioned medium from olfactory ensheathing cells; SC-CM, conditioned medium from Schwann cells. Magnification: 40x; Scale bars: 50 μm.

Discussion

Results obtained in the present investigation support previous studies in which a neural-like differentiation of ASCs was promoted by conditioned media from OECs or SCs (Lo Furno et al., 2018).In fact, various Cxs, typical of neurons or glial cells, were found overexpressed by using a similar protocol.

In the absence of CM treatment, basal levels of Cx43 were considerably high; Cx47 was virtually absent; Cx32 and Cx36 were modestly found at 24 h and decreased at 7 days of culture. This decreased Cx expression is probably due to a reduced differentiation ability if ASCs are kept in the basal medium. The treatment with glial-derived conditioned media induced different degrees of modifications in the expression of the various Cxs, except for Cx47, whose immunostaining remained undetectable. In synthesis, high basal levels of Cx43 were further improved and significant increases were observed for Cx32 and Cx36.

Indeed, many of the Cxs tested can be found in tissues other than the nervous system. However, it is likely that only neurons or glial cells may exhibit this Cx combination. On the other hand, this interpretation is corroborated by the increased expression of neural markers such as Nestin, PGP9.5, and GFAP, and double labelling immunostaining. These findings are in keeping with a

previous work where, by using the same protocol, an increased expression of various neural markers was observed, either when conspicuously present (Nestin and PGP9.5) or weakly expressed (GFAP, MAP2 and Synapsin I) in ASC control cultures (Lo Furno et al., 2018).

The increased Synapsin expression already suggested the possibility to establish synaptic connections with other neural cells. The overexpression of various Cxs here observed further supports this interactive potential. Present in virtually every neural cell type, Cxs are essential for the constitution of gap junctions, and play an important role in brain homeostasis. They are crucial for cell-to-cell communication, as well as for interactions between cells and the surrounding microenvironment.

Cx32 is particularly important in myelinating glial cells, where it mainly establishes reflexive gap junctions (Nualart-Marti et al., 2013). In doing so, these junctions provide faster radial cytoplasmic communication between the several layers of myelin down to the axon membrane, thus ensuring nutritional support and functional integrity. Cx43 is the main Cx subtype in astrocytes. By homotypic/heterotypic coupling between astrocytes and/or oligodendrocytes, it contributes to the formation of the so-called “panglial syncytium” (Rash et al., 1997; Magnotti et al., 2011). This glial network helps to stabilize membrane potential by absorption and removal of extracellular potassium ions released during action potential firing. Cx36 in neuronal gap junctions is essential for

the formation of electrical synapses which are key elements for the electrotonic coupling between neurons and the synchronization of membrane potential (Condorelli et al., 2000; Nagy et al., 2018).

Altogether, our results indicate that high basal levels and increases of Cx43 expression suggest a strong predisposition of ASCs toward an astrocyte differentiation, as also suggested by high levels of GFAP previously reported (Lo Furno et al., 2018). On the other hand, the lack of Cx47 would indicate a lower propensity to differentiate into oligodendrocytes, at least by using the protocol adopted here. This kind of differentiation, however, cannot be ruled out, if using different strategies. Finally, observed increases of Cx32 and Cx36 would suggest a potential ASC differentiation toward neurons or SC.

Except for Cx32, whose increased expression was more pronounced in SC-CM cultures, comparable effects were observed for the other Cxs using each CM. This is probably due to the similarity of the two glial cell types, which play a similar functional role and are characterized by a similar immunophenotype. Similarly to astrocytes, SCs and OECs express GFAP (Bianchini et al., 1992; Pellitteri et al., 2010). Both SCs and OECs are characterized by their immunopositivity for S-100 and P75NTR (Chung et al., 2004; Pellitteri et al., 2009). Since they largely produce a similar variety of cytokines/growth factors, it can be assumed that these factors are responsible for the observed modifications. In this regard, it should be underlined that the same culture

medium was used both for control ASCs and for each of the two glial cells. Therefore, any CM-induced effect would not be related to different medium supplements, but only to glial products present in the CM.

Although the identification of these molecules was not the purpose of the present work, it is well established that both OECs and SCs are able to produce several growth factors (Kocsis et al., 2009; Gao et al., 2018) such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and fibroblast growth factor (FGF). These factors are known to promote a neural differentiation of various cell types. Positive effects have been described for BDNF on survival and differentiation of neurons and neural stem cells, likely through upregulation of Tropomyosin receptor kinase (Trk) B, phosphoinositide 3-kinase (PI3K), AKT and β-catenin (Li et al., 2017). CNTF and FGF2 were able to enhance the proliferation of Muller glia-derived progenitor cells (MGPCs), likely through the activation of Jak/Stat signaling pathway (Todd et al., 2016). A neural-like differentiation of BM-MSC has been promoted by GDNF (Ma et al., 2016). A stimulatory effect on Cx expression was also described (Cushing et al., 2005). For example, an overexpression of Cx43 was induced on PC12 cells by NGF through its receptor Trk A. Several pathways may be responsible for these effects, such as extracellular signal-regulated kinases (ERK-1/2) and protein kinase C (PKC). An increased

expression of Cx43 was also observed after treatment of C6 glioma cells with CNTF (Ozog et al., 2002).

Another hypothesis to explain CM-induced effects may take into account the presence in each CM of extracellular vesicles (EVs) and exosomes released by glial cells. EVs and exosomes have been recently recognized as intercellular messengers which can transfer mRNAs, microRNAs, proteins and lipids to recipient cells (Bátiz et al., 2016). If this is the case, ASC expression of Cx might result from the direct transfer of mRNA or proteins by EVs and exosomes to ASCs. Interestingly, this transfer likely involves exosomal Cx43, able to modulate the interaction and transfer of information between exosomes and acceptor cells (Soares et al., 2015). For example, this hypothesis would explain why SC-CM better stimulates Cx32 expression. In fact, Barnet et al. (2001) demonstrated that Cx32 protein was present in SCs but not in OECs, whereas Cx43 mRNA levels were similarly detected for both types of glial cells. According to other hypotheses, soluble factors bound to EVs/exosomes may also be responsible for intercellular communication mechanisms. In fact, it has been shown that multiple functional responses may be induced by signaling pathways which involve EV-associated cytokines (Cossetti et al., 2014). Probably, multiple mechanisms are involved in eliciting the final outcome. Further investigations are needed to elucidate whether different effects are exerted by different CM components.

Since high percentages of immunopositive cells were found in some instances both for typical glial and neuronal Cxs, it is reasonable to assume that two or more Cxs are simultaneously expressed in the same cells observed here. This event can however be explained if one takes into account that they might still be at early stages of neural differentiation. In fact, it has been reported that in neural progenitor cells both neuronal and glial markers still coexist (Wei et al., 2002; Kempermann et al., 2004; Vinci et al., 2016). Presumably, as the differentiation progresses, the expression of some Cxs would be favoured while some others would be downregulated, likely on the basis of microenvironmental cues. For example, during embryonic development, Cx36 expression becomes restricted to neuronal cells, while Cx43 expression becomes restricted to astrocytes (Sadowska et al., 2009; Hartfield et al. 2011).

Conclusion

In conclusion, this work and previous investigations confirm that a neural-like differentiation of ASCs can be achieved without adding to the culture medium chemical agents which could exert toxic effects. This seems of particular interest for potential therapeutic applications in the field of regenerative medicine, because newly differentiated neurons or glial cells would represent

a valuable tool for healing PNS injuries (Xie et al., 2017; Ching et al. 2018) or for the treatment of chronic neurodegenerative disorders affecting the CNS. Indeed, many encouraging results have been obtained worldwide not only in vitro but also in preclinical experiments and in clinical trials. In particular, better outcomes have been reported in vivo after administration of previously neural-induced elements rather than naïve stem cells. However, most studies attribute stem cell-induced progress to the paracrine production of cytokine/growth factors rather than to a real differentiation in functional nerve cells. In this context, every effort should be made to define differentiation protocols able to provide more permanently differentiated neurons or glial cells, whose target after administration would be to mend a disrupted brain circuitry or a loss of myelination. The use of conditioned media may help to develop some of these strategies because they mimic, in a more physiological way, the nervous tissue microenvironment in which neurons and glial cells normally work.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

DL and GM equally contributed to this work. DL, GM, and RG wrote the paper, designed and performed the experiments. RPE prepared the conditioned media and discussed experimental results. AZ and RPA contributed with their connexin expertise and provided essential reagents. EG and CV performed flow cytometry analysis. All authors read and approved the final version of the manuscript.

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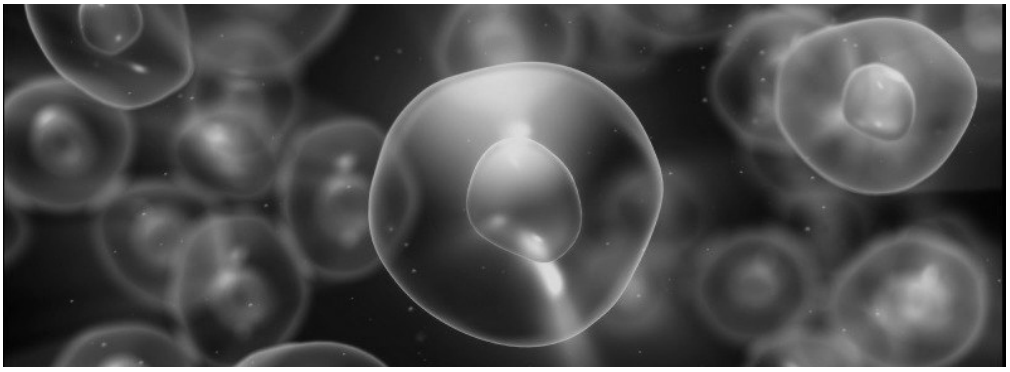
CHAPTER 4

Melatonin effects on Neural-like Phenotype and Melatonin Receptor Expression in human Adipose-derived Mesenchymal Stem Cells



Melatonin effects on Neural-like Phenotype and Melatonin Receptor Expression in human Adipose-derived Mesenchymal Stem Cells

G. Mannino[1], D. Lo Furno[1], R. Pellitteri[2], S. Salomone[1], R. Giuffrida[1]

[1]Dept. Biomedical and Biotechnological Sciences, University of Catania, Italy

[2]Institute of Neurological Sciences, National Research Council, Catania, Italy

Abstract

OBJECTIVE: Several studies show that a neural-like differentiation can be induced in Adipose-derived Stem Cells (ASCs) by various strategies. In a previous study, we showed that expression of neural markers was increased when ASCs were cultured in a conditioned medium (CM) from glial cells, such as Olfactory Ensheathing Cells (OEC-CM) or Schwann Cells. As Melatonin (Mel) plays an important role in survival and differentiation of Neural Stem Cells, in the present investigation the effect of this hormone was tested on ASC neural differentiation. The combination of Mel and OEC-CM treatment was also evaluated.

MATERIALS AND METHODS: ASCs were isolated from human lipoaspirate. After their stem cell characterization, four groups of cells were cultured: the first served as control, being ASCs cultured in the basal medium; in the second group, Mel was added (1μM) to the basal medium; the third group was cultured in OEC-CM; in the last group (OEC-CM-Mel), Mel (1μM) was added to the conditioned medium. Each group of cells was processed for immunofluorescence at day 1, 3 and 7 of growth. Antibodies for Neuron Specific Enolase (NSE), Microtubule Associate Protein 2 (MAP2), and for Melatonin Receptor 1 and 2 (MTr1 and MTr2) were tested.

RESULTS: A neural-like differentiation was confirmed after OEC-CM treatment. In fact, NSE expression, virtually absent in control cultures, was considerably increased in these conditions. In Mel-treated cultures, NSE increases were also observed, although to a lesser extent. As it was expectable, more evident immunostaining was induced in OEC-CM-Mel group. A similar trend was also found for MAP2 expression. Marked increases were particularly evident for MTr1 immunostaining, whereas MTr2 expression was only weakly increased by any of these treatments. The concentration of Mel at 1μM was chosen since it was the lowest to induce effects, without compromising cell growth.

CONCLUSION: The present results show that Mel can favor a neural differentiation of ASCs, particularly in association with other induction strategies. Interestingly, it was found that MTr1 expression, already present in native ASCs, was considerably increased after Mel treatment. Since the concentration of Mel used in the present investigation did not affect cell viability and proliferation, it may be considered a suitable tool to be further developed. Future investigations will elucidate the molecular mechanisms underlying the observed effects.

Possible role of Melatonin receptors

Since native binding site densities of MTr (especially for the MTr2) in animal tissues are low or undetectable, their pharmacology and function is not fully understood. Moreover, it has to be considered that simultaneous activation of MTr1 and MTr2 could lead to synergistic or opposing responses that amplify or diminish each other. Although the minimal functional unit seems to be monomeric, it is possible that MTr1 and MTr2 form homo- and hetero-oligomers. However the formation of MTr1/MTr2 heterodimers is considerably lower than the formation of MTr homodimers. Monomeric forms of the MTr1 or MTr2 are differentially desensitized by exposure to melatonin

depending on its concentration, time of exposure, and receptor state (quiescent versus constitutive).

Recent data suggests that activation of the MTr1 and/or MTr2 may play a role on melatonin neuroprotective effects, often attributed to its free radical-scavenging properties. Melatonin receptor function may involve the induction of antioxidant genes, such as superoxide dismutase and catalase, through receptor-mediated transcriptional signaling. Thus, melatonin receptors may be useful tools for novel therapeutic strategies capable of counteracting the oxidative stress components of neuroinflammatory events.

It has been shown that melatonin can inhibit mitochondrial cell death pathways in a mutant striatal cell model of Huntington's disease, as well as primary cerebrocortical and primary striatal neuronal cultures. Since these effects are reversed by luzindole, they are likely mediated through the MTr1. On the other hand, activation of MTr2 has been associated to the melatonin-induced protection against neuronal damage caused by ischemic strokes. In addition, MTr2-dependent mechanisms would promote neurogenesis and cell proliferation (Liu et al., 2016).

Melatonin effects on Neural-like phenotipe and Melatonin Receptor Expression in human Adipose-derived Mesenchymal Stem Cells

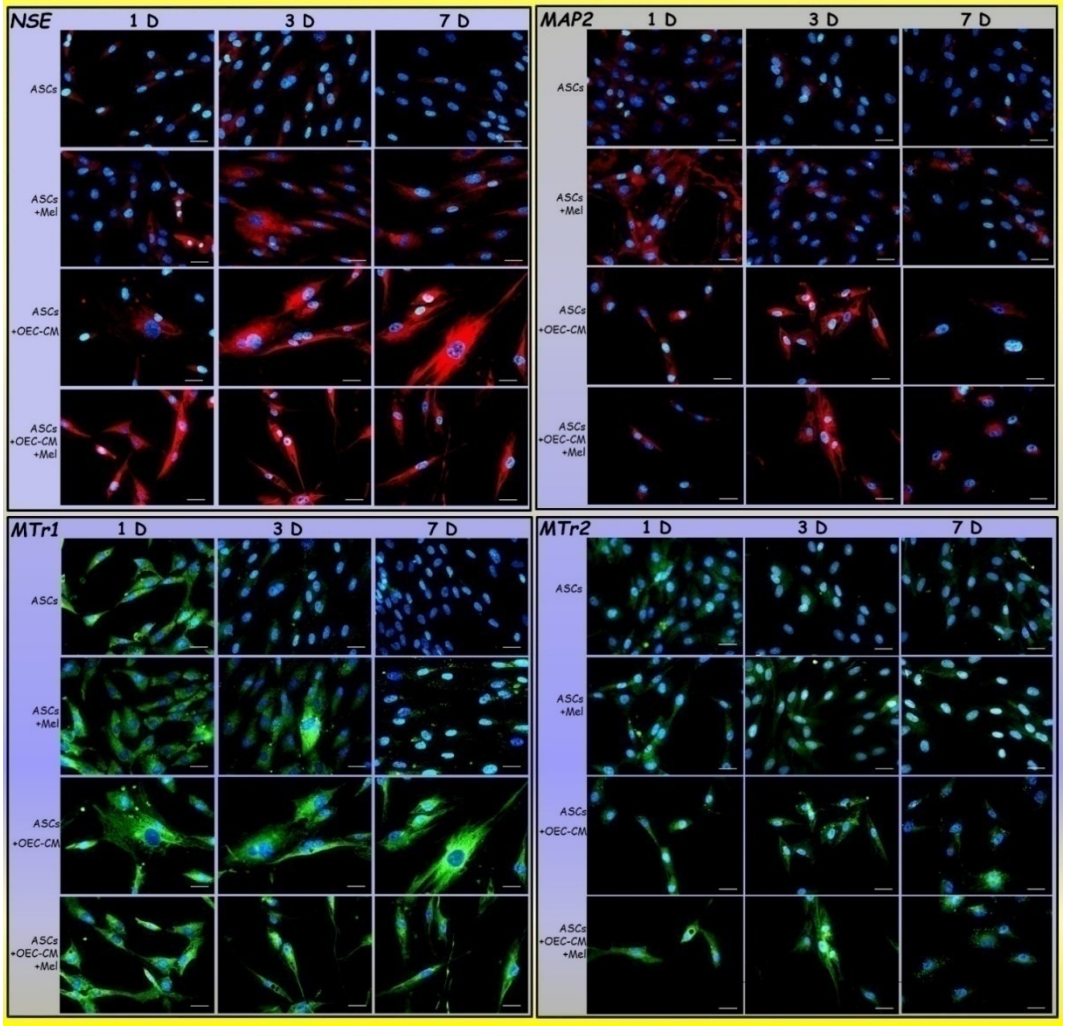
G. Mannino[1], D. Lo Furno[1], R. Pellitteri[2], R. Giuffrida[1]

[1]Dept. Biomedical and Biotechnological Sciences, University of Catania, Italy
[2]Inst. Neurological Sciences, National Research Council, Section of Catania, Italy

Previous studies showed an increased expression of neural markers on ASCs when cultured in a Conditioned Medium (CM) from Olfactory Ensheathing Cells (OEC-CM) or Schwann Cells. In the present investigation effects of Melatonin (Mel) were tested. This hormone seems to play an important role in survival and commitment of Neural Stem Cells. The combination of Mel and OEC-CM treatment was also investigated.

MATERIALS AND METHODS: human ASCs were isolated from lipoaspirates. Four groups were compared: ASCs cultured in the basal medium (ASCs); ASCs in which Mel 1 µM was added (ASC+Mel); ASCs cultured in OEC-CM (ASCs+OEC-CM); ASCs cultured in OEC-CM supplemented with Mel 1 µM (ASCs+OEC-CM+Mel). Immunofluorescence at day 1, 3 and 7 of growth was carried out to test the expression of Neuron Specific Enolase (NSE), Microtubule Associate Protein 2 (MAP2), Melatonin Receptor 1 (MTr1) and Melatonin Receptor 2 (MTr2).

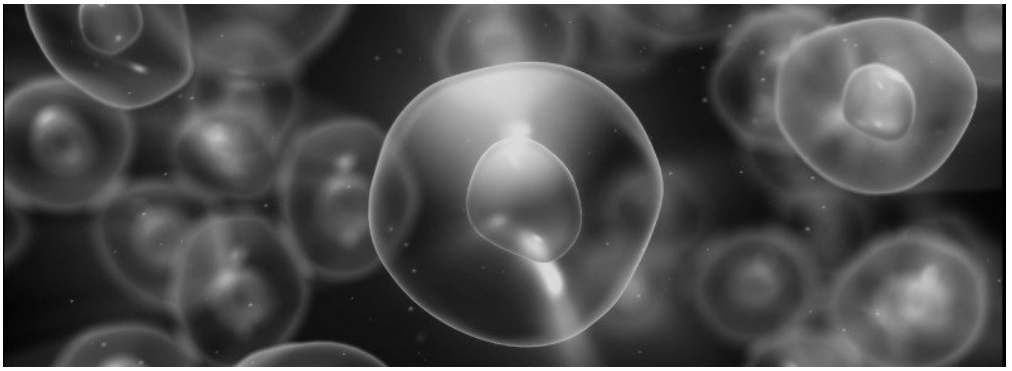
Results: A neural-like differentiation was confirmed after OEC-CM treatment by increased expression of NSE and MAP2. Weaker effects were observed by addition of Mel alone. Instead, more evident immunostaining was induced in OEC-CM+Mel group. Marked increases were found for MTr1 immunostaining, whereas MTr2 expression was only weakly increased by any of these treatments.



Conclusions: The present results show that Mel can favor a neural differentiation of ASCs, especially when associated with other induction strategies. Mel effects are likely exerted through MTr1, whose expression was strongly increased. Overall, Mel-based approaches may deserve to be further investigated and developed on neural differentiation of ASCs.

CHAPTER 5

Concluding remarks



General discussion and conclusion

Scientific investigations in humans and in animal models, by *in vivo* or *in vitro* methodologies, have shown the enormous potential of ASCs in cell-based regenerative medicine. Some clinical trials have already been performed and many others are currently in progress (clinicaltrials.gov). Many of them have been mainly designed to assess the treatment safety. However, available data show that the use of stem cells may induce appreciable amelioration in patients affected by neurodegenerative diseases, when only poor results can be achieved by pharmacological approaches or other therapeutic strategies.

Beneficial effects primarily rely on the particular tendency of stem cells to home onto injured areas, in particular to hypoxic, apoptotic, or inflamed areas. Notably, their ability to pass the BBB has permitted different administration routes (Kerkis et al., 2015; Lo Furno et al., 2018). In fact, stem cell engrafting at the injured nervous tissue has been reported not only by intracerebral injection, but also after intracerebroventricular, intravascular, or even intranasal administration.

Positive results have been obtained using different sources of MSCs, such as bone marrow or umbilical cord blood (Momin et al., 2010). MSCs from bone marrow were the first to be identified and have been the most studied.

However, their harvesting is characterized by limited cell yield and painful invasive procedures. Those from umbilical cord can be harvested with no discomfort for the newborn or the mother, from the already detached umbilical cord. However, they can mostly be used for allogeneic transplantation and the cell yield might be limited. For these reasons, the use of ASCs seems more suitable for translational medicine. First, their availability is virtually unlimited from subcutaneous deposits of adipose tissue (Yeh et al., 2015). Moreover, ASCs have often shown higher proliferation rates and differentiation ability for mesodermal and neural lineages (Calabrese et al., 2015; Zhang et al., 2012). Finally, thanks to their low immunogenicity and putative immunosuppressive effects, ASCs might be used for allogeneic transplantation. Therefore, they represent a valuable tool since, when appropriately cryopreserved and stored, as occurs for hematopoietic stem cells, they would be immediately ready for use, avoiding the necessary steps of fat harvesting and processing to obtain autologous ASCs.

Investigations described in this PhD thesis were designed to explore which physiological signals may improve the ability of ASCs to differentiate towards neural-like cells. The use of glial conditioned media may represent a valid strategy since it contains cytokine/growth factors released from other neural cells, normally present in nervous tissue. Furthermore, the influence of

melatonin has been tested, since it has been reported that this hormone favor the differentiation of neural stem cells.

Results obtained have shown that this protocol is able to induce a neural-like differentiation of ASCs. This was demonstrated by the paper reported in chapter 2, where a significant overexpression of typical neural markers was observed. Findings from the second work, reported in chapter 3, corroborated these observations, since a typical neural pattern of connexin expression was induced by using the same method. Although a clear difference between the two conditioned media was not observed, a higher tendency toward a glial differentiation was recognizable when using SC-CM, whereas a neuronal phenotype was more appreciable following OEC-CM treatment. However, the simultaneous expression of both neuronal and glial markers is indicative of an early phase of differentiation, at the stage of neural progenitor cells. Although the glial-derived molecules inducing these effects remain to be identified, some growth factors are likely responsible. For example, both glial cells produce BDNF, GDNF, CNTF, and FGF.

Preliminary data reported in chapter 4 show that the addition of melatonin slightly improve the expression of neural markers such as Neuronal Specific Enolase (NSE) and MAP2. The combination of melatonin and conditioned media seemed to induce earlier effects. This work also shows that melatonin effects would be likely exerted through MT1 receptors.

Overall, investigations reported in the present thesis contribute to elucidate effects of molecules, normally present in the physiological environment, on ASC differentiation toward a neural-like phenotype. It is reasonable to expect that more studies will provide further information useful for developing stem cell therapeutic applications in cases of nervous system damage, to restore disease-disrupted brain circuitry.

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List of Publications and Scientific Contributions

Articles

1. **Mannino G.**, Lo Furno D. Are ASCs Really Able to Differentiate into Nerve Cells? Annals Stem Cell Regenerat Med. 2019; 2(1): 1011.
2. Lo Furno D, **Mannino G**, Pellitteri R, Zappalà A, Parenti R, Gili E, Vancheri C, Giuffrida R. Conditioned Media From Glial Cells Promote a Neural-Like Connexin Expression in Human Adipose-Derived Mesenchymal Stem Cells. Front Physiol. 2018 Nov 29; 9:1742. doi: 10.3389/fphys.2018.01742. eCollection 2018.
3. Lo Furno D , **Mannino G**, Giuffrida R, Gili E, Vancheri C, Tarico MS, Perrotta RE, Pellitteri R. Neural differentiation of human adipose-derived mesenchymal stem cells induced by glial cell conditioned media. Journal of Cellular Physioogy 2018 May 8. doi: 10.1002/jcp.26632.
4. Lo Furno D, **Mannino G**, Giuffrida R. Functional role of mesenchymal stem cells in the treatment of chronic neurodegenerative diseases. Journal of Cellular Physioogy 2018 Oct; 233(10):7091-7100. doi: 10.1002/jcp.26632. Epub 2018 May 8.
5. Lo Furno D, Tamburino S, **Mannino G**, Gili E, Lombardo G, Tarico MS, Vancheri C, Giuffrida R, Perrotta R. Nanofat 2.0: experimental evidence for

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6. Lo Furno D, **Mannino G,** Cardile V, Parenti R, Giuffrida R. Potential Therapeutic Applications of Adipose Derived Mesenchymal Stem Cells. Stem Cells and Development. 12 agosto 2016 DOI: 10.1089/scd.2016.0135
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Conference Proceedings

Poster

Mannino G, Lo Furno D, Pellitteri R, Giuffrida R, Salomone S. Melatonin effects on Neural-like phenotype and Melatonin Receptor Expression in human Adipose-derived Mesenchymal Stem Cells. 2019 annual meeting GISM, congress of Gruppo Italiano Staminali Mesenchimali.

Lo Furno D , **Mannino G**, Giurdanella G, Gennuso F, Bucolo C, Salomone S, Giuffrida R. Functional interactions between Human Retinal Endothelial Cells and pericyte-like differentiated human Adipose-derived Mesenchymal Stem Cells. 2019 annual meeting GISM, congress of Gruppo Italiano Staminali Mesenchimali.

Mannino G, Lo Furno D, Giuffrida R. Melatonin-induced neural-like differentiation of human adipose mesenchymal stem cells. 69th SIF national congress, Florence Italy.

Lo Furno D, **Mannino G**, Giurdanella G, Bucolo C, Salomone S, Giuffrida R. Effects of various culture strategies on differentiation of human adipose-derived mesenchymal stem cells toward a pericyte-like phenotype 68th SIF national congress, Pavia Italy.

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