

# World Journal of *Stem Cells*

*World J Stem Cells* 2018 March 26; 10(3): 23-33





**REVIEW**

- 23      Physiologically based microenvironment for *in vitro* neural differentiation of adipose-derived stem cells  
*Graziano ACE, Avola R, Perciavalle V, Nicoletti F, Cicala G, Coco M, Cardile V*

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*World Journal of Stem Cells*  
Volume 10 Number 3 March 26, 2018

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*World Journal of Stem Cells* is now indexed in PubMed, PubMed Central, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports/Science Edition, Biological Abstracts, and BIOSIS Previews.

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NAME OF JOURNAL  
*World Journal of Stem Cells*

ISSN  
ISSN 1948-0210 (online)

LAUNCH DATE  
December 31, 2009

FREQUENCY  
Monthly

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PUBLICATION DATE  
March 26, 2018

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## Physiologically based microenvironment for *in vitro* neural differentiation of adipose-derived stem cells

Adriana Carol Eleonora Graziano, Rosanna Avola, Vincenzo Perciavalle, Ferdinando Nicoletti, Gianluca Cicala, Marinella Coco, Venera Cardile

Adriana Carol Eleonora Graziano, Rosanna Avola, Vincenzo Perciavalle, Marinella Coco, Venera Cardile, Department of Biomedical and Biotechnological Sciences, Section of Physiology, University of Catania, Catania 95123, Italy

Ferdinando Nicoletti, Department of Biomedical and Biotechnological Sciences, Section of Pathology and Oncology, University of Catania, Catania 95123, Italy

Gianluca Cicala, Department of Civil Engineering and Architecture, University of Catania, Catania 95125, Italy

**ORCID number:** Adriana Carol Eleonora Graziano (0000-0003-1652-9577); Rosanna Avola (0000-0002-3094-2416); Vincenzo Perciavalle (0000-0003-0614-2969); Ferdinando Nicoletti (0000-0002-4570-8462); Gianluca Cicala (0000-0002-4680-152X); Marinella Coco (0000-0002-1797-2717); Venera Cardile (0000-0001-9902-3736).

**Author contributions:** All authors had full access to all the data in the study and take responsibility for the integrity of the data; Graziano ACE and Cardile V contributed to the study concept and design; Graziano ACE, Avola R and Coco M contributed to the acquisition of data; Graziano ACE, Avola R, Coco M and Cicala G contributed to the analysis and interpretation of data; Graziano ACE and Cardile V contributed to the drafting of the manuscript; Perciavalle V, Nicoletti F and Cardile V contributed to the critical revision of the manuscript for important intellectual content; all the authors have given final approval of the version to be published and agree to be accountable for all aspects of the work.

**Supported by** University of Catania, Italy, FIR, No. 21040104, UPB: 20722142002; 20130143007 (partially).

**Conflict-of-interest statement:** All authors declare no potential conflict of interest.

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**Manuscript source:** Invited manuscript

**Correspondence to:** Dr. Adriana Carol Eleonora Graziano, PhD, PharmD, Research Fellow, Department of Biomedical and Biotechnological Sciences, Section of Physiology, University of Catania, Via Santa Sofia 97, Catania 95123, Italy. [acegraz@unict.it](mailto:acegraz@unict.it)  
Telephone: +39-09-54781324

**Received:** February 19, 2018

**Peer-review started:** February 19, 2018

**First decision:** March 13, 2018

**Revised:** March 20, 2018

**Accepted:** March 21, 2018

**Article in press:** March 21, 2018

**Published online:** March 26, 2018

### Abstract

The limited capacity of nervous system to promote a spontaneous regeneration and the high rate of neurodegenerative diseases appearance are keys factors that stimulate researches both for defining the molecular mechanisms of pathophysiology and for evaluating putative strategies to induce neural tissue regeneration. In this latter aspect, the application of stem cells seems to be a promising approach, even if the control of their differentiation and the maintaining of a safe state of proliferation should be troubled. Here, we focus on adipose tissue-derived stem cells and we seek out the recent advances on the promotion of their neural differentiation, performing a critical integration of the basic biology and physiology of adipose tissue-derived stem cells with the functional modifications that the biophysical, biomechanical and biochemical microenvironment induces to cell phenotype. The pre-

clinical studies showed that the neural differentiation by cell stimulation with growth factors benefits from the integration with biomaterials and biophysical interaction like microgravity. All these elements have been reported as furnisher of microenvironments with desirable biological, physical and mechanical properties. A critical review of current knowledge is here proposed, underscoring that a real advance toward a stable, safe and controllable adipose stem cells clinical application will derive from a synergic multidisciplinary approach that involves material engineer, basic cell biology, cell and tissue physiology.

**Key words:** Adipose stem cells; Biomaterials; Neurodegeneration; Neural differentiation; Physiological microenvironment

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**Core tip:** Adipose-derived stem cells are easily accessible from liposuction, obtained in large quantity and cultured for several months with low levels of senescence. Moreover, they could be induced toward a neural phenotype *in vitro*. The preclinical studies show that microenvironment has a predominant role. Our objective is to consolidate the current literature to better delineate the functional response of adipose-derived stem cells to biochemical, biophysical or dimensional stimuli. Specifically, chemicals - like drugs and growth factors-biomaterials and microgravity are here discussed as both single and co-applied parameters for inducing a neural lineage.

Graziano ACE, Avola R, Perciavalle V, Nicoletti F, Cicala G, Coco M, Cardile V. Physiologically based microenvironment for *in vitro* neural differentiation of adipose-derived stem cells. *World J Stem Cells* 2018; 10(3): 23-33 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v10/i3/23.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v10.i3.23>

## INTRODUCTION

The majority of neurological diseases are characterized by primary or secondary neurodegeneration with the concomitance of different degree of inflammation<sup>[1,2]</sup>. Parkinson disease<sup>[3,4]</sup>, multiple sclerosis<sup>[5]</sup>, traumatic injury<sup>[6]</sup> or lysosomal storage disease with neurological symptoms like Krabbe disease<sup>[7]</sup> represent conditions in which the neural cells disappearance turn into decline of patient quality of life. Therapeutic approaches are mostly symptomatic and not restorative.

Due to the skills to immunomodulation, to support the brain parenchyma and to transdifferentiate, stem cells (SC) are under evaluation in preclinical tests for the promotion of neural regeneration.

Among the different sources of SC, the adipose stem cells (ASCs) are becoming more and more popular and

attract the researchers' interest because they are easily accessible from subcutaneous liposuction, obtained in large quantity<sup>[8]</sup>, cultured for several months *in vitro* with low levels of senescence<sup>[9,10]</sup> and applicable without ethical and political issues<sup>[11]</sup>. Moreover, ASCs have been shown to possess self-renewal property and multipotential differentiation toward adipocytes<sup>[12]</sup>, chondrocytes<sup>[13,14]</sup>, osteoblasts<sup>[15]</sup>, myocytes<sup>[16]</sup>, neurocytes<sup>[17]</sup>, and other cell types<sup>[18]</sup>, including neurons<sup>[19]</sup> and neural cells<sup>[20]</sup>. All these hallmarks give to ASCs potential application in regenerative medicine and clinical studies<sup>[21,22]</sup>. As regard the transdifferentiating potential of ASCs into neural cells, the transduction properties need to be further characterized. Longtime, the stimulation of ASCs by growth factors-enriched media has been the most applied procedure to induce a specific cell lineage<sup>[23]</sup>, but recently it has been enlightened that the conventional two-dimensional systems do not mimic the cellular connections and the space distribution that occur *in vivo*<sup>[24,25]</sup>, especially if compared with the structural complexity of nervous system. A reliable solution to this question resides in three-dimensional (3D) biomaterial scaffolds that show a great potential as engineered neural tissue for cell-based therapy<sup>[26,27]</sup>.

This review integrates the basic physiology of ASC with the functional modifications of cell phenotype furnished by enrichment of microenvironment with appropriate biophysical, biomechanical and biochemical stimuli. In particular, the effects of chemicals-like drugs and growth factors-biomaterial and microgravity are discussed as both single and co-applied parameters for inducing ASCs toward the neural lineage.

## NEURAL CELLS FROM ASCS, WHY IS IT DESIRABLE?

SCs are defined as unspecialized cells capable of self-renewing and of giving rise to a wide range of mature cell types<sup>[28]</sup>. During their proliferation, SCs do not follow the classical asymmetric cell division that generates a SC and a differentiated daughter at each division. Their "potential" resides in generating more SC and differentiated daughters<sup>[29]</sup>. Two types of SCs have been classified following their origin and their potential of differentiation: Embryonic stem cells (ESCs) and somatic SCs.

The ESCs derive from the early blastocyst and the inner cell mass of the embryo and are able to differentiate into cell types of the three germ layers<sup>[30]</sup>. Even if they represent the most powerful tool for cell therapy in animal models, their application is associated to ethical issue and to high degree of variation with regard to differentiation potential due to their genetic and epigenetic instability.

The somatic SCs are obtained from fetal (after gastrulation) or adult tissues and traditionally differentiate only toward cell types that belong to the tissue which



they originate from. Among the adult tissues, somatic SC have been isolated from bone marrow<sup>[31]</sup>, brain<sup>[32]</sup>, blood<sup>[33]</sup>, epidermis<sup>[34]</sup>, skeletal muscle<sup>[35]</sup> and fat<sup>[10]</sup>. In each tissue, somatic SCs guarantee the maintaining of tissue homeostasis, but their action in replacing damaged cells after intense insults is limited by a mostly quiescent status or a weak activity. This is the case of neural stem cells (NSCs), located in adult mammals within a cellular niche<sup>[36]</sup> in the sub-ependymal layer of the ventricular zone and in the dentate gyrus of the hippocampus<sup>[37]</sup>. However, even if these differentiate *in vitro* into neurons, astrocytes and oligodendrocytes<sup>[38]</sup>, they are not effective in containing neurodegenerative process.

The adult SCs offer the potential for autologous stem cell donation, reducing the risk of immune rejection and complications<sup>[39]</sup> and are additionally far from ethical and religious debates. We underscore that these advantages represent a solid basis for cultural renaissance and for scientific efforts to define the best source of adult SCs and to optimize methods for a safe, controlled and long-lasting differentiation.

According to our experience, the first description of a population of cells derived from human adipose tissue with a multilineage differentiation and high proliferation capacity *in vitro*<sup>[9]</sup> represents the milestone for scientific awakening and for overcoming specific tissue-linked limitations. Compared to bone marrow, adipose tissue is obtained with a not invasive, well-tolerated and safe procedure such as liposuction surgery. Moreover, the yield of obtained cells is relatively higher than other stem cell sources<sup>[40]</sup> and the digestion of lipoaspirate permits to isolate approximately from  $0.5 \times 10^4$  to  $2 \times 10^5$  stem cells per gram of adipose tissue<sup>[41]</sup>. Furthermore, ASCs can be cultured for several months *in vitro* with low levels of senescence<sup>[10]</sup>. The latter aspect is essential because it turns into a reduction of permanent post-mitotic states and the cells remain viable and proliferative over extensive periods during which the terminal differentiation could be stimulated. Thus, the critical point is the induction of a stable phenotype not restricted to mesodermal cells but including the ectodermal ones.

There is a diffuse disagreement about pluripotential properties of ASCs, but in our experience the phenotype of ASCs can be addressed toward mesodermal<sup>[12,14]</sup> and non-mesodermal lineages<sup>[20]</sup>. In these observations reside the scientific efforts in the evaluation of ASCs as tools for generation of neural cells to apply in cell therapy strategies and in cell models for various neurodegenerative disorders<sup>[42]</sup>.

Because non-neural differentiation potential falls outside of the scope of the present review, we focus on *in vitro* methods to induce neural differentiation. A systematic literature search was conducted using PubMed, WoS, and Scopus. Studies providing only results for *in vitro* neural phenotype induction from ASCs and preclinical examination were included. When preliminary tests on animal model of diseases have

been performed, the major relevant findings were discussed.

## **IN VITRO METHODS TO INDUCE NEURAL DIFFERENTIATION OF ASCs**

The experimental conditions of ASCs neural induction and differentiation contemplate at least three main categories or microenvironment factors: (1) The elaboration of chemically-defined or growth factors-enriched media; (2) the creation of a functionalized tree-dimensional structure by biomaterials; and (3) the application of appropriate biophysical forces.

## **GROWTH FACTORS AND CHEMICALS FOR ASCs NEURAL DIFFERENTIATION**

The most applied protocols for neural differentiation of ASCs are designed as "run-through" procedures, in which ASCs are sequentially propagated in different media enriched by growth factors or chemicals until they transdifferentiate into a desired phenotype. These approaches should be defined "physiological-inspired" or "chemical-based" as they try to mimic *in vitro* the complex environment of nervous system by adding growth factors or chemicals.

In the earlier reports, a two-steps method has been adopted and a phase of cellular preconditioning or induction was followed by application of differentiation stimuli. As preconditioning media, Safford *et al.*<sup>[43]</sup> tested the enrichment with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), whereas Zuk *et al.*<sup>[10]</sup> used DMEM supplemented by 20% of fetal bovine serum and  $\beta$ -mercaptoethanol. After this step, the neuronal differentiation was performed by medium composed of DMEM plus butylated hydroxyanisole, KCl, valproic acid, forskolin, hydrocortisone, and insulin or by a serum-free and  $\beta$ -mercaptoethanol-enriched medium, respectively. In both experimental conditions, ASCs developed to an early neuronal stage, as no expression of established oligodendrocyte and astrocyte markers or mature neuronal markers were observed. These two works are milestones for neuronal differentiation of ASCs, but they lacked in electrophysiological tests. Indeed, a delayed-rectifier type K<sup>+</sup> current (an early developmental ion channel) concomitantly with morphologic changes and increased expression of neural-specific markers suggested that ASCs differentiate toward early progenitors of neurons and/or glia after 2 wk in differentiating medium with isobutylmethylxanthine, indomethacin, and insulin<sup>[44]</sup>. The pre-induction was also performed by bFGF for seven days<sup>[19]</sup> or for twenty-four hours<sup>[45]</sup>, following the incubation with forskolin alone<sup>[19]</sup> or in combination with N2 supplement, butylated hydroxyanisole, KCl, valproic acid<sup>[45]</sup>. Despite the similar protocol, the relevant findings were different. Krampera *et al.*<sup>[45]</sup> reported a transient and reversible differentiation within 48-72 h

**Table 1 Functions of growth factors on neural induction of adipose stem cells**

Growth factors	Profile	Physiological activity	Effect on ASCs	Remarks
EGF	Small polypeptide of 53 amino acid residues and a molecular mass of approximately 6000 daltons <sup>[57]</sup>	Development of the oral cavity, lungs, gastrointestinal tract, epidermis, derma, eyelids and central nervous system <sup>[56]</sup>	Promotion of proliferation with delays of senescence and insurance of differentiation potency <sup>[55]</sup>	EGF and bFGF co-administration limits ASCs differentiation abilities by inducing ASCs into an ectodermal lineage rather than the mesodermal one <sup>[53]</sup>
bFGF	Non-glycosylated polypeptide of 18 kDa and 155 amino acid in length (heparin-binding growth factor family)	Stimulator of tissue repair and cellular viability released from an injured extracellular matrix <sup>[64]</sup>	Enhancement of proliferation, differentiation and hepatocyte growth factor expression ability <sup>[58]</sup> . Induction of the adipogenic <sup>[59]</sup> and chondrogenic <sup>[60]</sup> potential, with inhibition of osteogenic differentiation <sup>[61]</sup>	
PDGF	Dimeric glycoprotein	Potent mitogen for cell of mesodermal lineage and stimulator of tissue repair released from activated platelets on bleeding <sup>[65]</sup>	Supporting of cell proliferation <i>in vitro</i> : It increases ASCs yield. Promotion of neural differentiation in an antioxidant microenvironment <sup>[48]</sup>	Receptor- $\beta$ signalling is involved primarily in ASCs stimulation <sup>[62]</sup> . ASCs stimulation with autologous platelet-rich plasma reduces the cost of differentiation <sup>[48]</sup>

EGF: Epidermal growth factor; bEGF: Basic fibroblast growth factor; PDGF: Platelet-derived growth factor; ASCs: Adipose stem cells.

of culture with basal medium. Indeed, in the protocol of Jang *et al.*<sup>[19]</sup>, the acquired neuron-like functions were demonstrated by evaluation of voltage-dependent tetrodotoxin (TTX)-sensitive sodium currents, outward potassium currents, and prominent negative resting membrane potentials. These events underscore that the *in vitro* microenvironment is capable to infer with the multiple functional ion channel currents that are physiologically present in undifferentiated ASCs<sup>[46]</sup>.

Another approach showed morphological, immunocytochemical and electrophysiological evidences of stable neuronal differentiation of ASCs. It is based on the induction of floating sphere in serum-free medium in presence of bFGF, and EGF. The spheres were dissociated in single cells and cultured with brain derived neurotrophic factor (BDNF) and retinoic acid<sup>[47]</sup>. It was also investigated the possibility to transdifferentiate ASCs by using neural induction medium (high glucose DMEM,  $\beta$ -mercaptoethanol, and butylated hydroxyanisole) supplemented with and 10% of autologous platelet-rich plasma (PRP) isolated and prepared from venous blood of the same patient underwent liposuction<sup>[48]</sup>. Some reports showed an induction toward a neural-like phenotype by media previously conditioned thought incubation with neuroblastoma or olfactory ensheathing cells (OECs)<sup>[49]</sup> or with ASCs induced to secrete neurotrophic factors<sup>[50]</sup> in presence of estrogen<sup>[51]</sup>, also.

All the major protocols considered for differentiation of ASCs to neural cells have been reviewed by linking them to the neural markers that should be used in each procedure and the possible pathways that are involved in this process<sup>[52]</sup>. Here we focus on the physiological input trying to define a profile that links chemicals and growth factors to ASCs fate.

According to the studies performed up to now, EGF and bFGF seem able to induce a useful pre-conditioning microenvironment for ASCs induction toward ectodermal lineage<sup>[53]</sup>. The co-administration of EGF and bFGF is essential because, as shown in Table 1, when tested alone on ASCs, EGF acts to promote ASCs proliferation by robust phosphorylation of SHC and ERK1/2<sup>[54]</sup>, to induce migration, to delay senescence, and to maintain differentiation potency by EGF-induced activation of STAT signal pathway<sup>[55]</sup>. Indeed, bFGF alone enhances the proliferation, and the hepatocyte growth factor expression ability of ASCs<sup>[58]</sup>, promoting the adipogenic<sup>[59]</sup> and chondrogenic<sup>[60]</sup> differentiation with the contemporary inhibition of the osteogenic one<sup>[61]</sup>. This biochemical event happens because ASCs express EGF and bFGF receptors<sup>[54]</sup>. Furthermore, they express PDGF receptors  $\alpha$  and  $\beta$ . PDGFR- $\alpha$  is highly expressed, but its ligand only slightly increases the proliferation of ASCs. Therefore, it is reasonable to assume that PDGF- $\beta$  and PDGF receptor- $\beta$  signalling is involved primarily in the stimulation of ASCs<sup>[62]</sup>. PDGF is released from activated platelets on bleeding, thus the stimulation with autologous platelet-rich plasma (PRP) represents an effective method to mediate stimulatory effect on cell proliferation, to increase the yield of ASCs and to reduce the cost of ASCs differentiation. In the same manner, the incubation with conditioned media appears a good technique for introducing an enriched cocktail of growth factors with positive remarks at both financial and practical point of view. Specifically, the secretome of neuroblastoma B104 cells has been reported to contain PDGF-AA, bFGF and IGF-1<sup>[63]</sup>, whereas brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-4/5 (NT-4/5), neuregulin, secreted

**Table 2** Effects of chemicals-enriched microenvironment on adipose stem cells fate

Action	Chemicals/drugs	Profile	Recognized function	Effects on ASCs
Antioxidant	β-mercaptoethanol	Water-soluble thiol used as a reducing agent for disulfide bonds to protect sulfhydryl groups from oxidation	In peripheral intestinal nervous system increases the number of synapses and the vesicle population in the nerve terminals <sup>[69]</sup> Improve meiotic maturation <i>in vitro</i> cultured oocytes <sup>[70]</sup>	Key elements for the neural induction medium: Reduction of oxidative stress and reactive oxygen species production could support neural population
	Butylated hydroxyanisole	Mixture of two isomeric organic compounds	Inhibition of 17 β-estradiol(E2)-mediated oxidative stress and of oxidative DNA damage	
	N-acetyl-L-cysteine	Synthetic derivative of endogenous amino acid, L-cysteine, precursor of the antioxidant enzyme Glutathione	Stimulator of glutathione synthase Activator of NMDA1 receptor	When co-administrated, reduction of ASCs-doubling time and increase of cell number compared with b-FGF alone supplementation <sup>[66]</sup>
	Ascorbic acid-2-phosphate (Vitamin C)	Water-soluble essential vitamin	Reducing agent and coenzyme in several metabolic pathways	
Interference on DNA	Valproic acid	Branch-chained fatty acid, acting as a histone deacetylase inhibitor	Wide range of neuroprotection <sup>[71,72]</sup> Inhibitor of glycogen synthase kinase-3 <sup>[73]</sup> Inducer of chromatin remodeling <sup>[74]</sup>	Promoter of neuron-like cells <sup>[75]</sup> <i>In vivo</i> , it improves homing of ASCs <i>via</i> overexpression of CXCR4 and CXCR6 <sup>[76]</sup>
	5-azacytidine	Analog of cytidine nucleoside, acting as demethylating agent <sup>[77,78]</sup>	Inducer of cell plasticity and active molecule for cellular differentiation into multiple phenotype <sup>[79]</sup> Inhibitor of COX1/2	Stimulated-cells ameliorate neurological deficits when injected in rats after cerebral ischemia <sup>[80]</sup>
Anti-inflammation	Indomethacin	Synthetic nonsteroidal indole derivative		Component of neural induction medium applied for two weeks <sup>[44]</sup>
Immuno modulation	Hydrocortisone	Glucocorticoid hormone	Suppressor of cell-mediate immunity	Form multi-nucleated myotubes, yielding protein markers for myocytes <sup>[9]</sup>
Energetic balance	N2 supplement	Chemically defined formulation containing insulin, transferrin, progesterone, putrescine and selenite	<i>In vitro</i> survival and expression of post-mitotic neurons in primary cultures from both the peripheral nervous system and the central nervous system	General promotion of neural cell survival
	B27 supplement	Mixture of vitamins (biotin, Tocopherol, Vitamin A), proteins (BSA, catalase, insulin transferrin, superoxide dismutase), Corticosterone, Galactose, Ethanolamine, Glutathione, Carnitine, Linoleic acid, linolenic acid, progesterone, putrescine, selenite, T3	Growth and maintenance of neurons. Differentiating Glial Precursor Cells into Astrocytes and Oligodendrocytes. Differentiating Neural Stem Cells into Neurons and Glial Cells	

ASCs: Adipose stem cells.

protein acidic rich in cysteine (SPARC) and matrix metalloproteinase-2 (MMP-2) have been reported as typical elements of OECs secretoma. Among these growth factors, nerve growth factor-β (NGF-β), BDNF, and neurotrophin-3 (NT-3) were applied on EGF plus bFGF-preconditioned ASCs to induce a neural-like phenotype<sup>[20]</sup>.

Thus, the growth factors that physiologically act in tissue rapid turnover<sup>[56,64,65]</sup> seem to stimulate proliferation and to improve responsiveness of ASCs toward ectodermal-derived stimuli. From this basic speculation, the major question must be opened is addressed to define what happens in humans with neurodegenerative syndromes, or in which way the loss

or the increase of a biochemical stimulus expression may interfere into ASCs phenotype by enhancing or limiting clinical application. Up to now, no scientific result can drive to answer.

Among the chemical reagents, the major applied ones were antioxidants or compounds active on DNA (Table 2).

An antioxidant microenvironment, obtained by N-acetyl-L-cysteine and ascorbic acid-2-phosphate, has been reported to reduce ASCs-doubling time and to increase cell number<sup>[66]</sup>. The β-mercaptoethanol sustained the induction of neural phenotype after pre-induction and differentiation<sup>[10]</sup>, whereas the butylated hydroxyanisole promoted the neural stem cell survival.



**Table 3 Biomaterials for neural phenotype of adipose stem cells**

Biomaterials	Profile	Effect on ASCs	Test on animal	Limitation for clinical
Chitosan films	Naturally derived polysaccharide from chitin <sup>[81,82]</sup>	Spontaneous cell organization in a 3D architecture	Yes, higher cellular retention ratio of ASC spheroids after intramuscular injection in nude mouse <sup>[81]</sup>	Not declared
Chitosan and gelatin	Elastic-dominant, porous scaffold	Conditioning toward a neuron-like phenotype	Yes, better repair in a mouse model of traumatic brain injury <sup>[83]</sup>	Not declared
Chitosan and silk	Complex structural framework	Efficient as delivery vehicle for ASCs	Yes, proposed as nerve grafts in the regeneration of injured rat sciatic nerve <sup>[84]</sup>	Not declared
Collagen gel	Engineered neural tissue	Cells must be aligned to collagen fibres	Yes, supported robust neural regeneration of injured rat sciatic nerve <sup>[85]</sup>	Not declared
Albumin	Serum-derived porous scaffold	Promotion toward neurons	Yes, filler effect on the spinal cord cavity in animal models of spinal cord injury <sup>[86]</sup>	Not declared
Matrigel	Commercially available hydrogel	Good cell encapsulation and delivery <sup>[87]</sup>	Yes, mouse models of spinal cord injury	Not applicable for its isolation from the basement membrane of a mouse sarcoma
Alginate	Hydrogel	Neurospheres encapsulation and neural promotion <sup>[88,89]</sup>		Good biocompatible profile
Nanosized graphene oxide-laminin hybrid patterns	Engineered tissue	Efficient neuron-like cells differentiation <sup>[90]</sup>		

ASCs: Adipose stem cells; 3D: Three-dimensional.

All these beneficial effects of antioxidants toward a neural phenotype should be related to the essential role that a pro-oxidant microenvironment exerts on induction of adipogenic phenotype<sup>[67]</sup>. It has been recently demonstrated that oxidative stress and reactive oxygen species (ROS) overproduction could drive the activation of molecular pathways that are able to convert myoblasts into brown adipocytes<sup>[68]</sup>. Nevertheless, the B27 reagent, that is routinely applied in laboratory's procedures for the growth and maintenance of neurons or for differentiating of SCs into neurons and glial cells, contains tocopherol, Vitamin A, catalase, superoxide dismutase, glutathione, that, among the other effects, are largely described for their propriety of oxidative stress-limiting agents.

The drugs active on DNA usually applied for neural induction of ASCs are histone deacetylase (HDAC) inhibitors or methylation inhibitors, like the valproic acid and the 5-azacytidine, respectively. The valproic acid is commonly used for the treatment of seizures and bipolar disorder. Valproic acid demonstrated a wide range of neuroprotective properties in cellular and animal models of neurodegenerative diseases<sup>[71,72]</sup>, probably for the activity as both toward the inhibition of glycogen synthase kinase-3 (GSK-3)<sup>[73]</sup> and the enhancement of CXCR4 expression<sup>[74]</sup>. In ASCs, *in vitro* treatment with valproic acid resulted in a promotion of neuron-like differentiation<sup>[75]</sup> and *in vivo* an enhanced homing of ASCs was reported *via* overexpression of CXCR4 and CXCR6<sup>[76]</sup>. Indeed, the demethylating

agent 5-azacytidine is commonly employed to treat blood disorders such as myelodysplasia and leukemia<sup>[77]</sup>. It has been historically described as an inducer of cell plasticity and as an active molecule for cellular differentiation into multiple phenotypes<sup>[78,79]</sup>. The enrichment of ASCs microenvironment with 5-azacytidine has been effective to improve neural differentiation and to ameliorate neurological deficits after cerebral ischemia in rats<sup>[80]</sup>.

Thus, the media formulation for ASCs neural differentiation is very far from a "magic recipe", but its definition, amelioration and reproducibility should necessary start not only from ASCs physiology but also from the analysis of their reactivity toward environmental stimuli. We think this aspect is essential especially with a clinical application in mind.

## BIOMATERIALS FOR ASCS NEURAL DIFFERENTIATION

Chemicals and growth factors act as signal transducers to induce ASCs toward a neural-like phenotype, but the control of their differentiation toward a specific and stable lineage requires not only a controlled biochemical microenvironment, but also a milieu in which cell-cell and cell-environment interactions should be evaluated in a three-dimensional architecture. Biomaterials offer the possibility to deliver stem cell regulatory signals in a precise and near physiological manner without the exclusion of 3D space as parameter.

The effectiveness of biomaterials in driving ASCs differentiation has been already reported for their differentiation into epithelial cells. It was emblematic and noteworthy the geometric dependence of ASCs phenotype in fibrin culture<sup>[81]</sup>. In this well-conducted study, the experimental plan demonstrated clearly that the growth factor-enriched medium increased ASCs growth and chemotaxis, but the differentiation into epithelial cells was effective only in a 3D structure of fibrin. In this condition, the authors identified the formation of a bilayer of two segregate cell phenotypes: The superficial one with ASCs-derived epithelial cells and the deeper one with mesenchymal cells. This evidence strongly suggests that biomaterials may allow the control of proliferation and differentiation not only of ASCs, but also of their neural derivatives, because it is possible the hypothesis of biomaterials that should act by reducing ASCs propagation after differentiation and by maintaining a niche of ASCs with high level of stemness to be reprogrammed according to tissue necessity.

Table 3 summarizes the principal biomaterials for neural differentiation of ASCs investigated in preclinical studies. The spontaneous formation of three-dimensional spheroids has been reported by using chitosan, a naturally derived polysaccharide from chitin<sup>[82,83]</sup>. ASCs spheroids were formed on chitosan films because pure chitosan cannot support adequate cell adhesion for its biophysical parameter. This propriety enhanced spontaneous cell organization in a 3D architecture that permitted the close association of cells and a transmission of signal cues easier and faster than in monolayer cultures. Considering that chitosan lacks biological activity, the upregulation of pluripotency marker genes<sup>[82]</sup>, the transdifferentiation efficiency into neural-like cells<sup>[83]</sup> or neuron<sup>[82]</sup> *in vitro*, and the higher cellular retention ratio of ASC spheroids after intramuscular injection in nude mouse<sup>[82]</sup> could be justified by the 3D organization of cells.

Moreover, chitosan combinations with gelatin or silk were tested in animal models of neurodegeneration. In the case of elastic-dominant, porous scaffolds from photocurable, chemically modified chitosan and gelatin, ASCs were conditioned toward neuron-like cells capable to better repair a traumatic brain injury mouse model<sup>[84]</sup>. Indeed, the chitosan/silk fibroin scaffold has been proposed as nerve grafts for its efficiency as delivery vehicle for ASCs and as structural framework in the regeneration of injured rat sciatic nerve<sup>[85]</sup>. In the same animal model, an engineered neural tissue (EngNT) composed by collagen gel and aligned rat ASCs supported robust neural regeneration across the gap and into the distal stump<sup>[86]</sup>. Similar efficiency was proved in animal models of spinal cord injury after implantation of ASCs seeded on serum-derived albumin scaffold, a porous biomaterial that completely filled the spinal cord cavity and permitted the passage of descending and ascending neurons<sup>[87]</sup>. Similar results have been obtained by using Matrigel<sup>[88]</sup>, a commercially

available hydrogel that, because of its isolation from the basement membrane of a mouse sarcoma, is unlikely to be approved for clinical use. To select the hydrogels, the patient safety should be always considered in order to perform translational researches. For example, the alginate hydrogel that has been used to encapsulate neurospheres obtained from neural differentiation of ASCs<sup>[89,90]</sup> had a good biocompatible profile.

More recently, nanosized graphene oxide (NGO)-laminin hybrid patterns were reported to be useful for ASCs transdifferentiation into neuron-like cells that were up to 30% higher than the control group. In the same work, it was proved that cells grown on NGO grid patterns were more differentiated than the other ones grown on PLL-coated Au or on NGO-coated Au<sup>[91]</sup>.

Taken together, all these results strongly suggest that biomaterials provide a benefit for neural differentiation of ASCs: They should mimic the shape of interconnected neuronal network and the nanoscale topographical features of the extracellular matrix, as already reported for the nanoengineered polystyrene surface containing nanopore array-patterned substrate<sup>[92]</sup>. Moreover, biomaterials have been applied to realize an electrical cell culture system for selective induction of neurons. This *in vitro* technique is realized by cellular seeding on the conductive polypyrrole/chitosan membrane with a thickness of 0.4 mm. The membrane can be connected to an electric stimulator by two thin platinum electrodes. In this way, a defined electric intensity can be applied to cell culture (V/cm). It has been employed on Schwann cells<sup>[93]</sup>, on OECs<sup>[94]</sup> and on mouse bone marrow stromal cells<sup>[95]</sup> to induce and sustain their phenotype regulation. Yang *et al.*<sup>[96]</sup> applied it on ASCs; they tested the possibility to promote neuronal differentiation by using both electrical stimulation and *Nurr-1* gene transduction alone and in combination. The results clearly evidenced that both electrical extracellular microenvironments and intracellular pattern profile were capable of promoting neuronal differentiation in ASCs, but the best result was achieved by a synergistically combination of electrical forces and genetic modification.

## BIOPHYSICAL FORCES TO DRIVE NEURAL CELLS FROM ASCs

Biophysical forces, particularly the electro-mechanical coupling and the deformation forces are important physiological regulators of nervous systems. Actually, microgravity, as a mechanical factor, is more and more under investigation, especially for its implication in health of spaceflights and astronauts in orbit. As summarized by Mariggiò and Fanò-Illic<sup>[97]</sup>, the microgravity effects are not fully characterized and contrasting events have been reported: In some cases, cell differentiation and tissue assembly were not affected by microgravity, indeed in other cases alteration of cell morphology and function has been reported. For this reason, a three-dimensional glia-neuron co-culture cell model has been proposed as useful tool for the investigation of

microgravity as a new environment to successfully manipulate cell functions and phenotype. Generally, in monolayer tissue, an improvement of stem cell differentiation into neurons was reported for PC12 neuron-like cell<sup>[98]</sup> and for ESCs<sup>[99]</sup>.

As about ASCs, the microgravity effect is very little known. Up to now, only a single and recent study tried to define a mechanistic link between microgravity and neural induction of ASCs<sup>[100]</sup>. In this experimental setup, it was found that microgravity stimulation with a clinostat instrument increased ASCs differentiation toward neural-like cells in presence of the classical chemically defined and growth factor enriched medium. Even if the differentiation was proved by evaluation of neural cell lineage markers, no data about the specific effect of microgravity have been produced. Thus, it remains unclear if microgravity alone can modify the cell phenotype in absence of growth factors and biochemical stimuli, also.

## CONCLUSION

Since ASCs can be readily isolated, expanded and transplanted, their application in cell-based therapies is more and more under investigation. The differentiation of ASCs was initially considered restricted to mesodermal cells, but recent advances display ASCs ability to transdifferentiate, acquiring cell phenotype different from mesenchymal, including the ectodermal one. In the past decade, most of researches focused of the promotion of ASCs into neural-like cells for evaluating their potential application in neurodegenerative disorders. Different strategies were adopted. Among them, the cultures in chemically defined or growth factors-enriched media were applied to stimulate *in vitro* the physiological process of neural induction. This dynamic event involves many biological processes and signaling events that can be potentiated by the elaboration of an appropriate biophysical and biochemical microenvironment and should be evaluated in a 3D architecture. In this context, biomaterials provide a sophisticated microenvironment. Even if terminally differentiated, functional neurons have yet to be achieved, the reported data from animal tests have shown that some biomaterials have a great potential as nerve grafts. A synergic work between cell and tissue physiologists and biomaterial production experts seems to be useful for the future development of ASCs-based clinical therapeutics to be employed in neurodegenerative disorders.

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