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# Influence of biological matrix and artificial electrospun scaffolds on proliferation, differentiation and trophic factor synthesis of rat embryonic stem cells

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ABSTRACT

Two-dimensional *vs* three-dimensional culture conditions, such as the presence of extracellular matrix components, could deeply influence the cell fate and properties. In this paper we investigated proliferation, differentiation, survival, apoptosis, growth and neurotrophic factor synthesis of rat embryonic stem cells (RESCs) cultured in 2D and 3D conditions generated using Cultrex® Basement Membrane Extract (BME) and in poly-(L-lactic acid) (PLLA) electrospun sub-micrometric fibres. It is demonstrated that, in the absence of other instructive stimuli, growth, differentiation and paracrine activity of RESCs are directly affected by the different microenvironment provided by the scaffold. In particular, RESCs grown on an electrospun PLLA scaffolds coated or not with BME have a higher proliferation rate, higher production of bioactive nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) compared to standard 2D conditions, lasting for at least 2 weeks. Due to the high mechanical flexibility of PLLA electrospun scaffolds, the PLLA/stem cell culture system offers an interesting potential for implantable neural repair devices.



## 1. Introduction

The influence of tridimensional (3D) microenvironment ("niche") in stem cells biology is well known and established (Brizzi et al., 2012; Zapata et al., 2012). The chemical composition of the extracellular matrix (ECM), free- and ECM-linked bioactive molecules, and mechanic and adhesive forces generated in this dynamic space are key players in stem cell proliferation, differentiation and migration (Blancas et al., 2011; Pineda et al., 2013). In spite of this, 3D *in vitro* systems considering ECM properties are still poorly used for stem cell biology studies, as compared to conventional 2D culture systems. This is even more surprising when *in vitro* studies are directed to understand the possible use of stem cells for regenerative medicine purposes.

Tissue engineering technologies and material science are offering a wide range of possibilities for tailoring mechanical and chemical properties of new scaffolds to host stem cells according to specific purposes, *i.e.* "*in vitro*" niche reproduction, stem cell properties regulation, transplant of scaffold/cell mixed devices, *etc.* (Dickinson et al., 2011; Edalat et al., 2012).

In this study we investigated properties of rat embryonic stem cells (RESCs) (Fernandez et al., 2011) cultured in different 2D and 3D systems, conjugated or not to ECM components, focusing on the synthesis of growth and trophic factors of interest for neural repair. To create 3D systems, we used Basement Membrane Extract (BME) as "natural" microenvironment and poly(L-lactic acid) (PLLA) electrospun sub-micrometric fibers as artificial scaffold possibly usable for *in vivo* application. Cultrex® BME is a soluble form of basement membrane purified from Engelbreth–Holm–Swarm (EHS) tumor, including laminin, collagen IV, entactin, and heparin sulfate proteoglycan. PLLA fibrous scaffolds were fabricated by electrospinning. The properties of RESCs grown on these 3D systems were compared to standard conditions (2D culturing on either glass or plastic) with regard to proliferation, differentiation, survival, apoptosis and paracrine properties useful for neural repair.

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## 2. Results

The following culture conditions were used and compared in the study: (i) es-PLLA fibrous scaffolds (3D); (ii) es-PLLA fibrous scaffolds coated with BME (3D); (iii) 3D-BME (3D); (iv) plastic/glass (2D); and (v) plastic/glass coated with BME (2D). For growth factor expression analysis, also embryonic bodies (EB) derived from RESCs have been included. RESC growth, early differentiation and paracrine properties were assessed in short (3 DIV) and long (15 DIV) culture conditions.

### *2.1. Proliferation, survival, stemness and differentiation of RESCs in different 2D and 3D culture conditions*

In the first part of the study the effect of culturing conditions on the main biological characteristics of RESCs, such as viability and proliferation, general morphology and the expression of pluripotency markers, were investigated.

The MTT viability assay was used to study cell viability and proliferation at different days in culture (up-to 5 DIV). Since the cell death, as investigated by nuclear morphology and caspase expression, is similar in the different conditions (except for 3D-BME), the MTT results can reasonably reflect cell proliferation. Results are reported in Fig. 2A. In a 5-day cell culture, BME coated PLLA and es-PLLA scaffolds promoted an increase in RESC growth compared to plastic (Fig. 2A). The comparative analysis of pyknotic nuclei in all culture conditions performed at 3, 6 and 12 DIV indicated a significant increase in nuclear condensation and fragmentation in 3D-BME at 6 DIV and a 10-fold increase at 12 DIV *vs* glass (similar to plastic, *data not shown*) (Fig. 2B). Panel 2C illustrates pyknotic (head of arrow) and fragmented (thin arrows) nuclei after Hoechst 33342 staining. According to this, a high percentage of active-caspase-3 positive cells was counted in long-term 3D culture (Fig. 2D). Panel 2E illustrates a caspase 3-pyknotic nucleus (arrow).

The spontaneous aggregation and culture attitude of the RESCs grown on different substrates was then analyzed using actin immunostaining. When seeded on plastic, glass or BME coated plastic/glass, cells were arranged in a monolayer of either round or spindle-shaped cells (Fig. 2G). A 3D-BME environment promoted the growth of spheric-like structures; after a few days culture, the spheres developed empty central cavities resembling embryoid bodies (Fig. 2F). On es-PLLA scaffolds, closely connected round-shaped cells grew forming a ring-like structure with a diameter ranging from 20 to 40 μm (Fig. 2H). The es-PLLA scaffold was characterized by a rather narrow fibrous mesh, so that the cells remained spread over the surface, without infiltrating deep into the fiber network (Fig. 1B, C).

In order to assess whether culturing conditions affects RESCs stemness, the expression of octamer-binding transcription factor 4 (Oct4), a marker associated with pluripotency (Li et al., 2010; Fernandez et al., 2011; Ou et al., 2011), was investigated at 15 DIV in the different culture conditions. Results are presented in Fig. 3. While 2D-culture did not change the Oct4 mRNA expression in long-term culture, 3D long-term grown culture in 3D-BME increased Oct4 expression level, thus supporting the stemness properties of these cells. Conversely, the long-term grown on either uncoated or BME-coated es-PLLA reduced the Oct4 expression level, thus suggesting a pro-differentiative action. Oct4 protein was expressed in all cells when visualized by immunocytochemistry (Fig. 3B–E).

The expression of nestin and alpha-fetoprotein (AFP), which are lineage-associated markers, was then analyzed and the results are illustrated in Fig. 4. Nestin showed a cytoplasmic or peri-nuclear distribution, while AFP displayed a dotted cytoplasmic localization in all investigated conditions. In glass and BME-glass (Fig. 4A, B) almost all cells were Nestin-positive, while in es-PLLA and 3D-BME (Fig. 4C, D) few cells were Nestin-negative. Eventhought AFP immunoreactivity was homogeneously distributed within cell population in all the investigated conditions, few differences in the staining intensity could be appreciated only in RESCs cultured on es-PLLA, especially in the internal side of the ring. When cultured in 3D conditions, either es-PLLA or 3D-BME, RESCs spontaneously aggregated to form follicle-like structures, thus suggesting the 3D microenvironment favors shaping multicellular aggregates.

### *2.2. Paracrine properties*

An important issue in regenerative medicine is the role of growth factors in neuroprotection and damage repair. In order to assess how the culture conditions influence the paracrine properties of RESCs, the production of growth (VEGF) and neurotrophic factors (NGF, BDNF) was investigated by semiquantitative real-time PCR at 3, 7 and 15 DIV (days *in vitro*). The growth factor expression profile is shown in Fig. 5, using 3 DIV plastic as the reference mRNA level. The statistical analysis was performed using two-ways ANOVA to analyze both time and culture condition effects; at each time point, one-way ANOVA and post-hoc test *vs* the respective control (plastic) culture condition were also performed and presented in Fig. 5.

A time-dependent, 10- to 30-fold increase in the VEGF mRNA levels occurred in all the culture conditions (Fig. 5A). Notably, manipulation of

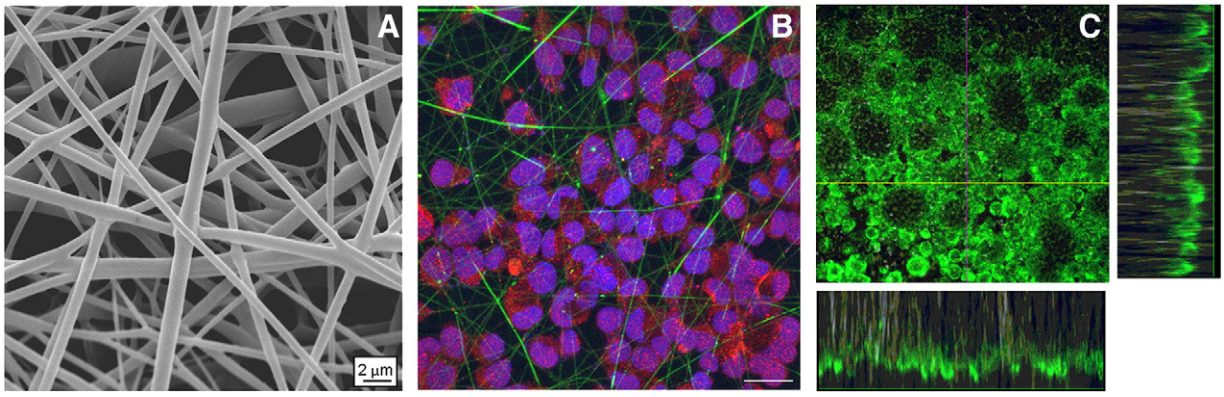


**Fig. 1.** es-PLLA scaffold and RESCs on scaffold. A: SEM micrograph of PLLA electrospun scaffold; Scale bar: 2μm. B: Representative picture of RESC cells grown on es-PLLA scaffold; B: cells were visualized by actin staining (red) and nuclear Hoechst33258 staining (blue); es-PLLA fibers were labeled with FITC (green); *Scale bar:* 20 μm. C: 3D visualization of RESCs by Oct4 immunostaining (green) in confocal microscopy; Purple and yellow lines represent *xy* visualization of *yz* and *xz* orthogonal planes, respectively. Side and bottom panels represent purple line *yz* projection and yellow line *xz* projection, respectively; cells grew as a monolayer on es-PLLA scaffold and are localized on the scaffold surface.