Novel triblock copolymer nanofiber system as an alternative support for embryonic stem cells growth and pluripotency

Journal:	*Journal of Tissue Engineering and Regenerative Medicine*
Manuscript ID:	TERM-13-0131.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Perestrelo, Ana; Universidade do Algarve, Departamento de Ciências Biomédicas e Medicina Mouffouk, Fouzi; National Guard Health Affairs, King Abdullah International Medical Research Centre (KAIMRC) Rosa da Costa, Ana; Universidade do Algarve, Departamento de Química e Farmácia, Faculdade de Ciências e Tecnologia Belo, José; Universidade do Algarve, Departamento de Ciências Biomédicas e Medicina
Keywords:	embryonic stem cells, polymeric nanofibers, pluripotency, ES cell culture, growth support, MEFs substitute

Novel triblock copolymer nanofiber system as an alternative support for embryonic stem cells growth and pluripotency

Ana Rubina Perestrelo[1,2,3], Fouzi Mouffouk[4], Ana M. Rosa da Costa[5], José António Belo[1,2 *]

[1] Laboratory of Embryology and Genetic Manipulation, Regenerative Medicine Program, Departamento de Ciências Biomédicas e Medicina (DCBM), Universidade do Algarve, Portugal

[2] IBB/CBME - Institute for Biotechnology and Bioengineering, Centro de Biomedicina Estrutural e Molecular, Universidade of Algarve, Portugal.

[3] PhD Program in Biomedical Sciences, Universidade do Algarve, Portugal.

[4] National Guard Health Affairs (NGHA), King Abdullah International Medical Research Centre (KAIMRC), Jeddah, Saudi Arabia.

[5] Centro de Investigação em Química do Algarve (CIQA) & Departamento de Química e Farmácia, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Portugal.

* Corresponding author

Address correspondence to:

José António Belo

Laboratório de Embriologia e Manipulação Genética, Regenerative Medicine Program, Departamento de Ciências Biomédicas e Medicina (DCBM), IBB/CBME, Universidade do Algarve, Ed 8, Lab 3.29, 8005-139 Faro, Portugal

Tel: + 351 289 800 040; jbelo@ualg.pt

Running head: Novel copolymer nanofibers for ES cell culture

Keywords: embryonic stem cells, polymeric nanofibers, pluripotency; ES cell culture; growth support; Gelatin substitute.

Abstract

Conventionally, embryonic stem (ES) cells are cultured on gelatin or over a mitotically inactivated monolayer of mouse embryonic fibroblasts (MEFsi). Considering the lack of versatile, non-animal derived and inexpensive materials for that purpose, we aimed to find a biomaterial able to support ES cell growth in a pluripotent state that avoids the need of laborious and time consuming MEFsi culture in parallel with mouse ES (mES) cell culture. Undifferentiated mES cells were cultured in a new nanofiber material designed for ES cell culture, which is based on the self-assembly of a triblock copolymer, poly (ethyleneglycol-*b*-trimethylsilyl methacrylate-*b*-methacrylic acid), conjugated with the peptide Glycine-Arginine-Glycine-Aspartate-Serine, to evaluate its potential application in ES cell research.

Morphology, proliferation, viability, pluripotency and differentiation potential of mES cells was assessed. Compared to conventional stem cell culture methodologies, the nanofibers promoted a higher increase in mES cell number, enhanced pluripotency and were able to support differentiation after a long term culture.

This newly developed synthetic system allows elimination of animal-derived matrices and provides an economic method of ES cell culture made of a complex network of nanofibers in a scale similar to

the native extracellular matrices where the functional properties of the cells can be observed and manipulated.

1. Introduction

Stem cell research has grown from unexplored to becoming an important field in biomedical sciences today. ES cells have theoretically two unique abilities, an unlimited self-renewal capacity and multi-lineage differentiation potential (pluripotency; Evans and Kaufman 1981). Indeed, a deeper understanding of the basic biology of stem cells holds the key to unlock new hopes to various so far incurable human diseases (Silva *et al.*, 2012).

Even though the first mES cell lines were derived three decades ago (Evans and Kaufman 1981) and standard protocols for ES cell derivation and maintenance are widely used today, the technical difficulties of these protocols still pose a challenge for many investigators attempting to produce pluripotent mES cells with high quality levels. The gold standard supportive material for *in vitro* mES cell culture is MEFsi, which allows mES cells to continue proliferating without differentiating (E. Michalska, 2007). Besides the immunity problems and batch-to-batch variation, the use of MEFsi as a support

for ES cell culture is a laborious and time-consuming process. MEFs are primary cells and stop dividing after a couple of passages and hence they need to be isolated freshly from time to time and one week is required to reach the correct confluency to perform ES cell culture (E. Michalska, 2007). Concerning that, many efforts are being made to avoid the use of MEFs without losing ES cell pluripotency through replacing the supportive MEFsi by synthetic systems. The major challenge is to find a cheap, defined, user-friendly and feeder-free condition that mimics the embryonic stem cell niche properly, in order to obtain high quality undifferentiated mES cells. Many efforts have been made to closely mimic the real microenvironment of cells. So far, a wide range of approaches have been explored, including the use of the new 2i defined medium (Ying *et al.*, 2008), coating with proteins (Heng *et al.*, 2012) and peptides (Klim *et al.*, 2010), carbon nanotubes (Lizundia *et al.*, 2012), hydrogels (Geckil *et al.*, 2010), a diversity of natural and synthetic scaffolds from different sources (Li *et al.*, 2010) and nanofibers (nf; Nur-E-Kamal *et al.*, 2006). Nanofibers have exciting geometry properties that have drawn much attention recently, particularly in the field of ES cells and tissue engineering. Special properties of nanofibers, such as the ability of mimicking the arrangement of fibers and fibrils of the extracellular matrix (ECM)

makes them suitable for a wide range of biomedical applications that are improved when combined with ES cells (Kanani and Bahrami 2010).

Here, we report the synthesis of poly(ethyleneglycol-*b*-trimethylsilyl methacrylate-*b*-methacrylic acid)-Glycine-Arginine-Glycine-Aspartate-Serine (PEG-PTMSMA-PMAA-GRGDS) - based nanofibers and the capability of this new artificial nanofiber network to support mES cell culture.

2. Materials and Methods

2.1. Nanofiber synthesis and characterization

2.1.1. Materials and instruments

All reagents and solvents for synthesis were reagent grade and were used without further purification, unless stated otherwise. Methacrylic acid (MAA) (Fluka) was distilled at low pressure in a Büchi Glass Oven B-585 micro distiller before use. Azo*bis*isobutyronitrile (AIBN) (Fluka) was recrystallized from methanol and dried under

vacuum at room temperature. GRGDS (Sigma) was dissolved in miliQ water to a concentration of 5 mg/mL. Polymer isolation and identification was performed as described in reference (Mouffouk *et al.*, 2011).

2.1.2. Polymer synthesis

PEG-PTMSMA-PMAA was obtained from the diblock copolymer poly(ethyleneglycol-*b*-trimethylsilylmethacrylate) (PEG-PTMSMA), which synthesis is described elsewhere (Mouffouk *et al.*, 2011).

To 1.83 g of PEG-PTMSMA dissolved in 5 mL of THF, 1.8 mL (106 eq) of methacrylic acid and 0.033 g of AIBN were added. After three freeze-pump-thaw cycles, the mixture was incubated in an oil bath at 60 ºC, under stirring, for 24 h. The flask was then cooled down to room temperature and 20 mL of THF added to dilute the mixture, which had become very viscous, and its content precipitated by pouring into diethyl ether. The polymer was separated from the supernatant by centrifugation at 15 344 × *g* for 10 min followed by decantation. This procedure was repeated once more, affording a white solid that was vacuum dried, at 40 ºC overnight, yielding 3.5 g (96%). ^{1}H NMR (CD_3OD) δ 0.06 (s, $(CH_3)_3Si$), 0.10 (s, $(CH_3)_3Si$), 1.09 (s, $CH_3C–C=O$), 1.19 (s, $CH_3C–C=O$), 1.86 (s, $CH_2C–C=O$), 1.99 (s,

$CH_2C–C=O$), 3.35 (s, $CH_3O–(CH_2CH_2O)_n$), 3.64 (s, $(CH_2CH_2O)_n$), 3.72 (s, $(CH_2CH_2O)_n$). DP_n (NMR) = 94. IR (KBr): 1706 (ν(C=O)), 1255 ($\delta(Si(CH_3)_3)$, 1181 and 846 ($\nu(Si(CH_3)_3)$ cm^{-1}. GPC analysis reveals a monomodal molecular weight distribution; t_e = 16.00 min.

2.1.3. Polymeric fiber formation and bioconjugation with the peptides

Twenty mg of the polymer were added to 1 mL of miliQ water containing Pen/Strep (100 U/ml) and sonicated for 30 min. The solution becomes opaque white as the polymer dissolves and self-assembles into nanostructures that were confirmed by Cryo-TEM. Then, 15 µL of GRGDS were added to the previous solution (corresponding to a 1:10 peptide/polymer molar ratio), and the resulting solution sonicated for an additional 5 min.

2.1.4. Cryo-Transmission Electron Microscopy (TEM)

The structure of the self-assembled nanofibers was observed on a Fei Titan Krios™ Cryo-Transmission Electron Microscope (TEM). Cryo-TEM is a technique used to visualize specimens that are suspended in an aqueous medium. Nanofibers were suspended in a fluid staining

medium (1% uranyl acetate) and applied to a standard pre-treated support film. Then, specimen grid is blotted with filter paper to remove excess fluid and rapidly plunged into liquid ethane that has been cooled to liquid nitrogen temperature (freezing rate on the order of 1.000.000 K/sec) to prevent the formation of ice crystals. Images were recorded under low electron dose conditions (10 – 25 $e^-/Å^2$).

2.2. Stem cell culture, proliferation and viability in an alternative support

2.2.1. Cell Culture

E14GPF8 mES cells, cells that constitutively express GFP protein, were propagated on 0.1% gelatin coated plates at 37ºC and 5 % CO_2 with a growth medium consisting of Glasgow Minimum Essential Medium (GMEM) without pyruvate and glutamine (Gibco) supplemented with 10% (v/v) of ES screened and defined FBS (HyClone), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 50 μM β-mercaptoethanol (Gibco), 100 U/ml Penicillin and 100 μg/ml Streptomycin (Gibco), 0.1mM non-essential aminoacids (Gibco) and 1000 U/ml of leukemia inhibitory factor (LIF) (ESGRO®, Millipore). mES cells were subcultured every 2 to 3 days through incubation of

0.05 % trypsin/EDTA solution for 5 min and the culture medium was replaced daily.

To assess the potential of polymeric nanofibers to support ES cells growing in an undifferentiated state, the polymeric nanofibers solutions were placed into 6-well culture plate to cover the entire growth area, which will allow the nanofibers to form a mesh and adsorb to TCPS surface. Upon a 12 h incubation at 4ºC, the wells were sterilized for 30 minutes in UV light. mES cells resuspended in maintenance medium were seeded directly onto nanofibers covered wells and half of the medium was changed every day.

2.2.2. Proliferation and viability assays

The competence of the nanofibers to support mES cells in an undifferentiated state was evaluated by assessing the cellular morphology, growth and viability. E14GFP8 undifferentiated mES cells (22 000 cells/well) were seeded on 100, 50 and 10 µg/ml of PEG-PTMSMA-PMAA-GRGDS nanofibers, on MEFsi and 0.1% of gelatin and in standard TCPS 6-well plate for 3, 5 and 15 days. Cells were counted by flow cytometry and the viability was determined using propidium iodide. For flow cytometry, the maintenance medium was removed, cells were washed and trypsinized. One portion of the cells solution

was collected to a polystyrene tube, incubated with propidium iodide (5 µg/ml), and analyzed with a FACSCalibur™ flow cytometer (BD Sciences). Viability ratio is the ratio between the number of viable cells and total number of cells. Cell proliferation was calculated as the ratio between viable cells and initial cell number.

2.2.3. Alkaline Phosphatase assay

The pluripotency of mES cells was also assessed by an Alkaline Phosphatase (AP) staining performed at day 3 and day 15 of culture, using an Alkaline Phosphatase staining kit (86R; Sigma) according to the producer instructions. In brief, the cells were fixed with the citrate:acetone:formaldehyde solution for 2 min, washed twice with water, stained with the AP staining solution for 30 min and counterstained with Hematoxylin for 2 min. The morphology of the colonies and AP positive cells were observed with an inverted light microscope (Leica DMIL) and photographed by the coupled digital camera (Leica DC 500).

2.2.4. Differentiation potential *in vitro*

Differentiation potential of mES cells cultured on nanofibers was compared to cultures in standard conditions over multiple passages. Determination of the differentiation potential was assayed by the quantification of the expression of gene markers from the three germ layers in embryoid bodies (EBs) collected at day 10 of differentiation. To do so, undifferentiated mES cells were seeded and passaged every 3 days at the same cell density for 10 passages. At day 30, correspondent to day 0 of differentiation, cells were resuspended in differentiation medium, which consists of growth medium with 15% (v/v) FBS and without LIF supplementation, and a three-dimensional environment was created to induce spontaneously differentiation of undifferentiated mES cells. For that, cells were dissociated in mES cell culture differentiation medium and drops of 20 µl of mES cell suspension (22 cells/µl) plated onto the base of a bacteriological Petri dish. Cells were placed in hanging droplets by inverting the base of a Petri dish. After 48 hour incubation, mES cell differentiation medium was added so that the cells were cultured in suspension for 4 days. The EBs were then plated on 0.1% gelatin coated 6-well plates at day 6 and cultured up to day 10 of differentiation.

2.3. Reverse Transcription and Real Time quantitative PCR

Total RNA was isolated from mES cell samples at different time points of cell culture using TRIzol Reagent (Invitrogen) according to manufacturer instructions. RNA quantity and RNA quality indicator (RQI) were determined using the NanoDrop 2000c spectrophotometer (Thermo Scientific) and Experion automated electrophoresis system (Bio-Rad), respectively. The RQI classification, revealed to be reproducible between all the samples and approximately 10, which corresponds to intact RNA. The Reverse Transcription was performed through First strand cDNA synthesis kit (Fermentas) in which the cDNA was synthesized from a volume equivalent to 1 µg of RNA sample.

Real Time quantitative PCR was performed by means of SsoFast EvaGreen Supermix (Bio-Rad) on a CFX96 Real-Time PCR Detection System (Bio-Rad). Table 1 contains a brief description of the primers and qRT-PCR programs used to evaluate the effect of nanofibers in the expression levels of markers of pluripotency, cell adhesion and differentiation markers.

Co-culture of mES cells in MEFSi was used as control and gene expression was normalized to *Gapdh* and *Tbp* expression. All samples, standards, positive and no template controls were run in triplicate and in at least two biological replicates of each qRT-PCR reaction. Data

were presented as the mean of four selected values (extreme values were excluded) and standard error of the mean.

2.4. Statistical analysis

Unless otherwise specified, results are expressed as mean ± standard deviation (SD). Data were statistically analyzed using One-way analysis of variance test (ANOVA) to test for significant differences between the experimental conditions and t-student for pairwise comparison, using Sigma Stat software. Differences were considered significant for $p<0.05$.

3. Results

3.1. Synthesis and characterization of nanofibers

The mechanism of PEG-PTMSMA-PMAA-GRGDS nanofiber formation involves three phases: the first phase represents the self-assembly of the amphiphilic copolymer into vesicles, followed by a second one in which polymeric vesicles get converted into nanofibers via a stacking process, and lastly, GRGDS is incorporated in the nanofibers surface by electrostatic interaction (Fig. 1 A.).

PEG-PTMSMA-PMAA was synthesized by Reversible Addition-Fragmentation chain Transfer (RAFT) polymerization (Chiefari *et al*, 1998) from the diblock copolymer α-(O-ethylxanthate)-ω-methylPEG-PTMSMA as macro-Chain Transfer Agent (macro-CTA) and methacrylic acid (MAA). A monomer to macro-CTA ratio of 106:1 and AIBN (1 mol% of the monomer), as radical initiator were used (Fig. 1 A.). The polymer was characterized by ^{1}H NMR spectroscopy triple-detection GPC, FTIR, and MALDI-TOF. A DP_n value of 94 was inferred from the NMR peak area at 3.64-3.72 ppm (CH_2 in PEG) and those at 1.09-1.19 ppm (CH_3 in TMSMA and MAA) and 0.06-0.10 ppm ($(CH_3)_3Si$ in TMSMA). GPC analysis reveals a monomodal molecular weight distribution. The presence of TMSMA units is patent on both the FTIR and NMR spectra as evidenced by the bands at 1255, 1181 and 846 cm^{-1} (δ and $\nu(Si(CH_3)_3$, respectively) and the singlets at 0.06 and 0.10 ppm ($(CH_3)_3Si$). On the MALDI-TOF spectrum, interpeak distances corresponding to the masses of the three repeating units (44 for EG, 86 for MAA, and 158 for TMSMA) were observed.

Self-assembly of the polymer into nanofibers was achieved by means of its solubilization in an aqueous medium through sonication. The synthetic nanofibers were observed by Cryo-TEM and presented 1-2 µm long and approximately 30 nm diameter (Fig. 1 B.).

3.2. Cell adhesion

Analysis of the expression of *β1-Integrin* revealed that culture of undifferentiated mES cells in 10 and 100 µg/ml of nanofibers for 13 days results in a significantly higher expression of *β1-Integrin* (1.55 and 1.32 fold, respectively) when compared to gelatin and MEFsi (1 and 1.10, respectively) (Fig. 2 A.). Furthermore, mES cells cultured in 100 µg/ml of nanofibers presented significantly higher expression levels of Collagen type I alpha 1 (*Col1a1*; 0.12 fold) than standard polystyrene culture plates and gelatin, 0.08 and 0.09 fold respectively (Fig. 2 B.). Furthermore, previous experiments already revealed the importance of GRGDS bioconjugation with PEG-PTMSMA-PMAA nanofibers in mES cells adhesion and growth (Fig. S1.). These data suggest that nanofibers bioconjugated with GRGDS are able to improve cell adhesion by inducing the expression of adhesion molecules that will allow the reorganization of the microenvironment and incorporation in a fiber-based matrix.

3.3. Cell Proliferation

As a first approach to evaluate the capacity of the nanofibers to sustain pluripotent ES cells cultures, we tested if the nanofibers were able to support mES cells adhesion and growth. Undifferentiated E14GFP8 ES cells were cultured for 3 and 5 days on different concentrations of PEG-PTMSMA-PMAA-GRGDS nanofibers and compared to cultures in MEFsi, gelatin and TCPS used as control conditions. The morphology, viability and the proliferation of the cells were assessed.

Morphological analysis of mES cells cultured for 5 days on nanofibers (Fig. 3 A: A'; B'; C') showed that colonies were tightly-packed, dome-shaped and presented clear and defined borders similar to the colonies obtained when using MEFsi as substrate (Fig. 3 A: D'). Conversely, mES cultured in gelatin and in TCPS plates lost the capacity of colony-formation, presented an irregular shape and acquired undefined borders, a typical characteristic of loss of pluripotency (Fig. 3 A: E; E'; F; F').

After 3 days in culture, the cell growth in 100 and 50 µg/ml of nanofibers was 16.04 and 15.27 fold respectively, which was significantly higher than the 10.62 fold observed in TCPS plates. In addition, no significant difference was observed in proliferation between 100, 50, 10 µg/ml of nanofibers, gelatin and MEFsi, at day 3

of culture (Fig. 3 B). On the other hand, mES cell proliferation in nanofibers for 5 days was higher than not only the cultures in TCPS but also than cultures in gelatin. Concurrently, 50 μg/ml of nanofibers promoted a 27-fold increase in cell number, which is significantly higher than that observed in gelatin (17.63-fold) or TCPS (14.53-fold) (Fig. 3 B). Therefore, the results obtained for the proliferation of mES cells in PEG-PTMSMA-PMAA-GRGDS nanofibers in a short-time culture were similar or, in some cases, even better than gelatin. This suggests that the nanofibers may be used to replace gelatin in supporting mES cell growth.

The viability ratio of mES cells was approximately 1 for all the tested conditions indicating that viability maintenance was independent from the culture conditions and the time of culture (Figure 3 C).

3.4. Maintenance of Pluripotency

The pluripotent status of stem cells was detected by Alkaline Phosphatase test performed in mES cells cultured in 100, 50 and 10 μg/ml of PEG-PTMSMA-PMAA-GRGDS nanofibers and in control conditions. Undifferentiated pluripotent stem cells presented elevated levels of alkaline phosphatase, therefore alkaline phosphatase

staining is used to distinguish between pluripotent and differentiated cells (Tsuji *et al*, 2008). mES cells were passaged every 3 days at the same density and alkaline phosphatase test was performed at the first passage (day 3) and at passage 5 (day 15). Alkaline phosphatase staining revealed that, contrarely to ES cells cultured in gelatin (Fig. 4 B: E; E') or TCPS (Fig.4 B: F; F'), alkaline phosphatase activity is present in all cells cultured in nanofibers independently of the time of culture (3 or 15 days) (Fig. 4 B: A; A'; B; B'; C, C'). Indeed, mES cells cultured on nanofibers (Fig. 4 B: A; A'; B; B'; C, C') resemble more the colonies that form when mES cells are cultured on a monolayer of fibroblasts where all cells are alkaline phosphatase positive and colonies are dome-shaped (Fig. 4 B: D; D'). These results suggest that nanofibers may be used as an alternative to conventional gelatin to support the growth of undifferentiated ES cell cultures.

Quantitative RT-PCR for stem cell markers was performed using total RNA isolated from mES cells at passage 4. After 13 days, mES cells cultured in 10 and 100 μg/ml of nanofibers presented *Oct4* expression levels of 1.37 and 1.24 fold, which were significantly higher than mES cells cultured in MEFsi (1 fold), gelatin (1.09 fold) or TCPS (0.99 fold) (Fig. 4 A.). Being *Oct4* one of the major regulators of ES cell stemness (Shi and Jin 2010), these results suggest that the

nanofibers not only support but also stimulate further mES cell "stemness". Furthermore, mES cells cultured in 10 μg/ml of nanofibers also presented *Nanog* expression level similar to that of mES cells cultured in MEFsi (Fig. 4 A.). Moreover, the expression levels of *Sox2* in mES cells cultured in 10 μg/ml of nanofibers (0.72 fold) were higher than mES cells cultured in gelatin (0.65 fold) or TCPS (0.59 fold) (Fig. 4 A.). Taken together, these data suggest that mES cells cultures in the nanofibers remain self-renewable.

3.5. Tri-lineage differentiation

Determination of long-term effects of nanofibers is important to screen nanomaterials for their potential benefits or pathogenic properties. Therefore, we tested whether the nanofibers could support mES differentiation potential over multiple passages.

mES cells were subcultured in nanofibers and in standard conditions for 10 passages, then induced to form EBs by hanging drops method, and differentiated for 10 days. At day 10 of differentiation, some fibroblast-like cells had migrated out to form a halo around the EBs, cultures become dense, confluent and multilayered (Fig. 5 A.). Different structures with diverse type of organization and arrangement had formed (e.g. beating foci) suggesting that cells cultured for a long

term in nanofibers retain their tri-lineage differentiation capacity (Fig. 5 A.). Indeed, these results were confirmed by qRT-PCR for markers of ectoderm (*βIII-Tubulin* and *Sox 1*), mesoderm (*Hand 1* and *αSma*) and endoderm (*Afp* and *Gata 4*) (Fig. 5 B). According to our qRT-PCR data, mES cells cultured in 50 and 10 μg/ml nanofibers for 30 days were able to give rise to the three germ layers more efficiently than the MEFsi and gelatin (Fig. 5 B.).

4. Discussion

The PEG-PTMSMA-PMAA-GRGDS nanofibers were designed taking advantage of the ideal and diverse properties of each block. PEG is a flexible, water-soluble well-established biodegradable polymer with many applications from industrial manufacturing to medicine (Duncan, 2003). On the other hand, a combinatorial library of biomaterials formed from different acrylate and methacrylate monomers has proved to be useful for identifying environments suitable for ES cell differentiation (Anderson *et al.*, 2004). Furthermore, methacrylates have been used in biomedical applications for many years and have been shown to promote adhesion and proliferation of endothelial cells when tethered to adhesive proteins (Fussell and Cooper 2004). Polymers containing methacrylic acid and

trimethylsilyl methacrylate have also been described to improve adhesion between the resist film and the wafer surface, as well as dry etch resistance in photolithographic applications (Mormann and Ferbitz 2002). RGD is a well-known cell attachment peptide (Bellis, 2011). Taking this into account, our nanofibers have many features that may promote stem cell adhesion and control of cell proliferation. In addition to these potential benefits, they represent a good substitute for the existing systems, due to the advantages of being cheap and easy to produce, presenting chemical and physical properties tailored via molecular synthesis.

We successfully synthetized PEG-PTMSMA-PMAA by RAFT polymerization and the polymer self-assembly into nanofibers also succeeded (Fig. 1). In the self-assembly process, it is expected that the neutral hydrophilic segment of the copolymer (PEG) gets majorly oriented to the core of the particles, and the negatively charged one (PMAA) to their surface, in order to minimize charge repulsion. Attachment of GRGDS to the fibers surface would occur by interaction between the positively charged terminal amino group of the former and the negatively charged carboxylate groups of the latter, leaving the guanidium group of the arginine residue available for cell adhesion (Fig. 1 A). After polymerization, nanofiber assembly and

bioconjugation with GRGDS, our purpose was to test the authentic effect of the nanofibers culture system not only in supporting high quality mouse embryonic stem cells growth but also in keeping cells undifferentiated and pluripotent.

Cell-biomaterial interaction mechanisms are poorly understood and many efforts have been made to clarify this matter. Nonetheless, several studies have demonstrated that cells do not interact with surfaces directly, but via deposition of adhesive proteins secreted by themselves and adsorbed on the adhesive surface, forming and remodeling their own ECM (Vladkova, 2010). Therefore, an increased expression of ECM and adhesion proteins, such as integrins, is evidence that surrounding microenvironment fosters cell adhesion and interaction with the support system. Our hypothesis was that the functionalization of the nanofibers with GRGDS may promote the expression of integrins, like e.g. *β1-Integrin*. Indeed, culture of ES cells in 100 µg/ml of nanofibers lead to an increase in *β1-Integrin* expression and *Col1a1* (Fig. 2) suggesting that integrins mediate the interaction between cells and the surrounding engineered bioadhesive motifs (GRGDS) of the nanofiber matrix. This indicates that the nanofibers play an important role in the regulation of synthesis, secretion and deposit of endogenous ECM proteins. This newly

developed synthetic system allows the establishment of more realistic and controlled microenvironment for mES cell culture, by providing a complex network of nanofibers, gaps and pores through which oxygen and nutrients can be delivered and metabolites can be filtered away in a scale similar to the native ECM, where the functional and biological properties of mES cells could be understand more precisely and manipulated.

The first approach to evaluate the capacity of the nanofibers to sustain ES cells culture was to test if the nanofibers were able to support mES cells growth for 3 and 5 days. According to cell adhesion and proliferation results, the cell proliferation in 100 µg/ml nanofibers for a short term culture revealed to be higher or similar than in gelatin and in TCPS (Fig. 3 B). Besides the similarity of proliferation results between gelatin and in nanofibers, mES cells cultured in gelatin revealed to be less pluripotent than in nanofibers which could be explained by a selective process in which nanofibers potentiate the growth of pluripotent cells. Indeed, according to our morphological analyses of the ES cells cultures at different time points of culture, the ability of nanofibers to support growth of undifferentiated mES cells seemed to be better than gelatin as, in the latter, mES cells were rearranged in irregular colonies resembling a spontaneous

differentiation process (Fig. 3 A.). Nevertheless, despite the translational effects that cell morphology has on different cell functions, a study pointed out that the association of the undifferentiated state of the ES cells with their pluripotency might not necessarily be related to a specific cellular morphology (Tsuji *et al.*, 2008). The regulation of pluripotency in mES cells is provided by a complex network of transcription factors, cell-ECM interactions, cell-cell contacts and niche-support cells. Due to the complexity of pluripotency maintenance mechanism, we performed an extensive assay using nanofibers and standard culture conditions to more accurately verify the effect of the nanofibers on mES cells pluripotency. The maintenance of pluripotency was verified through alkaline phosphatase activity test, analysis of the quantitative expression of pluripotency marker genes (*Nanog*, *Oct4* and *Sox2*) and lastly the differentiation potential of mES cells after long term culture in nanofibers. Results of alkaline phosphatase staining showed that independently of the time of culture, the alkaline phosphatase activity was higher in mES cells cultured in the nanofibers than in gelatin in which there seem to be much more differentiated cells (Fig. 4 B.). In addition, mES cells long-term cultured in 10 μg/ml of nanofibers exhibited *Nanog* and *Oct4* expression levels similar or higher than mES

cells cultured in MEFsi, emphasizing that nanofibers not only support but also promote mES cells to retain their stemness (Fig. 4 A.).

The differentiation potential of mES cells was tested after 30 days of culture in nanofibers, through hanging drop method. EBs formation stimulates disordered and heterogeneous patterns of differentiated ES cells into three germ lineages. Consequently, in some cases there are a preponderance of a specific germ layer derived cells whereas only a small fraction of cells differentiate into the other lineages (Kim *et al.*, 2010). Therefore, to clarify the differentiation status of EBs, the global gene expression profile of mES cells population differentiated in nanofibers was quantitatively analyzed by qRT-PCR, as in (Koike *et al.*, 2007; Sakai *et al.*, 2011).

The *in vitro* differentiation assay showed that mES cells cultured in 50 and 10 µg/ml of nanofibers for 10 passages were able to preserve their tri-lineage differentiation capacity, which validates the authenticity of mES cells cultured in nanofibers (Fig. 5 B.). Interestingly, the higher levels of pluripotency markers and differentiation were observed in ES cell cultures in 10 and 100 µg/ml of nanofibers as well as the higher levels of *β1-Integrin* and *Col1α1* expression suggesting that nanotopography may play a role in regulation cell attachment, spreading, proliferation and, most

importantly, in regulation of self-renewal of undifferentiated mES. This phenomenon of influence of nanometric scale surface topography and roughness of biomaterials in cell fate is also observed in other studies (Park *et al*, 2007). In summary, this newly developed synthetic system brings an alternative substrate for mES cell culture with the advantage of being inexpensive, easy to produce, and researchers can really control their chemical and physical properties via molecular synthesis. The major advantage of this system is that mES cells cultured on PEG-PTMSMA-PMAA-GRGDS nanofibers maintain their pluripotent state and present increased expression of *Col1a1* and *β1-Integrin*, which may help establishing a microenvironment that supports ES cell attachment, proliferation, and pluripotency.

5. Conclusion

We developed a new artificial support that can be used to grow pluripotent stem cells by converting an amphiphilic biocompatible peptide-copolymer into a nanofiber mesh, through a molecular self-assembly process. The developed nanofibers structure was shown to support mES cells proliferation in an undifferentiated state for short- and long-term culture, indicating that this system is an alternative

substrate and possible candidate to substitute gelatin for mES cell culture.

The nanofibers promoted self-renewal of mES cells without the requirement of a matrix coating with high levels of proteins and polymers, which not only provides a route for easier and economical stem cell culturing, but also promotes production of higher quality undifferentiated pluripotent stem cells with better control of cell proliferation and differentiation in a chemically defined matrix.

Acknowledgements

E14GPF8 mES cells were gently provided by Dr. Tristan Rodriguez.

We thank all the members of Embryology and Genetic Manipulation lab, staff of Cytometry and Gene Expression facilities by technical support.

A. R. Perestrelo is a F.C.T. doctoral fellow (SFRH/BD/65624/2009). This work has been supported by research grants from F.C.T. and from IBB/CBME, LA to J. A. Belo in the frame of Project refª PEst-OE/EQB/LA0023/2011. Also Part of this work has been supported by The National Plan for Sciences & Technology (NPST), King Saud University Saudi Arabia, grant number 11-BIO1578-02.

Conflict of interest

The authors have declared that there is no conflict of interest.

References

Anderson DG, Levenberg S & Langer R (2004) Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. *Nature biotechnology* **22:** 863–6

Bellis SL (2011) Advantages of RGD peptides for directing cell association with biomaterials. *Biomaterials* **32:** 4205–10

Chiefari J, Chong YKB, Ercole F, Krstina J, Jeffery J, Le TPT, Mayadunne RTA, Meijs GF, Moad CL, Moad G, Rizzardo E, Thang SH & South C (1998) Living Free-Radical Polymerization by Reversible Addition-Fragmentation Chain Transfer□: The RAFT Process. *Macromolecules* **31:** 5559–5562

Duncan R (2003) The dawning era of polymer therapeutics. *Nature reviews. Drug discovery* **2:** 347–60

E. Michalska A (2007) Isolation and propagation of mouse embryonic fibroblasts and preparation of mouse embryonic feeder layer cells. *Current protocols in stem cell biology* **Chapter 1:** Unit1C.3

Evans MG & Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos.

Fussell GW & Cooper SL (2004) Endothelial cell adhesion on RGD-containing methacrylate terpolymers. *Journal of biomedical materials research. Part A* **70:** 265–73

Geckil H, Xu F, Zhang X, Moon S & Demirci U (2010) Engineering hydrogels as extracellular matrix mimics. *Nanomedicine (London, England)* **5:** 469–84

Heng BC, Li J, Chen AK-L, Reuveny S, Cool SM, Birch WR & Oh SK-W (2012) Translating human embryonic stem cells from 2-dimensional to 3-dimensional cultures in a defined medium on laminin- and vitronectin-coated surfaces. *Stem cells and development* **21:** 1701–15

Kanani AG & Bahrami SH (2010) Review on Electrospun Nanofibers Scaffold and Biomedical Applications. *Trends in Biomaterials & Artificial Organs* **24:** 93–115

Kim PTW, Hoffman BG, Plesner A, Helgason CD, Verchere CB, Chung SW, Warnock GL, Mui ALF & Ong CJ (2010) Differentiation of mouse embryonic stem cells into endoderm without embryoid body formation. *PloS one* **5:** e14146

Klim JR, Li L, Wrighton PJ, Piekarczyk MS & Kiessling LL (2010) A defined glycosaminoglycan-binding substratum for human pluripotent stem cells. *Nature Methods* **7:** 989–94

Koike M, Sakaki S, Amano Y & Kurosawa H (2007) Characterization of embryoid bodies of mouse embryonic stem cells formed under various culture conditions and estimation of differentiation status of such bodies. *Journal of bioscience and bioengineering* **104:** 294–9

Li Z, Leung M, Hopper R, Ellenbogen R & Zhang M (2010) Feeder-free self-renewal of human embryonic stem cells in 3D porous natural polymer scaffolds. *Biomaterials* **31:** 404–12

Lizundia E, Sarasua JR, D'Angelo F, Orlacchio A, Martino S, Kenny JM & Armentano I (2012) Biocompatible poly(L-lactide)/MWCNT nanocomposites: morphological characterization, electrical properties, and stem cell interaction. *Macromolecular bioscience* **12:** 870–81

Mormann W & Ferbitz J (2002) Copolymers from Methacrylic Acid and Trimethylsilyl Methacrylate - Synthesis , Reactivity Ratios and Thermal Properties. *Macromolecular Chemistry and Physics* **203:** 2616–2623

Mouffouk F, Da Costa AMR, Martins J, Zourob M, Abu-Salah KM & Alrokayan SA (2011) Development of a highly sensitive bacteria detection assay using fluorescent pH-responsive polymeric micelles. *Biosensors & bioelectronics* **26:** 3517–23

Nur-E-Kamal A, Ahmed I, Kamal J, Schindler M & Meiners S (2006) Three-dimensional nanofibrillar surfaces promote self-renewal in mouse embryonic stem cells. *Stem cells (Dayton, Ohio)* **24:** 426–33

Park J, Bauer S, Von der Mark K & Schmuki P (2007) Nanosize and vitality: TiO2 nanotube diameter directs cell fate. *Nano letters* **7:** 1686–91

Sakai Y, Yoshiura Y & Nakazawa K (2011) Embryoid body culture of mouse embryonic stem cells using microwell and micropatterned chips. *Journal of bioscience and bioengineering* **111:** 85–91

Shi G & Jin Y (2010) Role of Oct4 in maintaining and regaining stem cell pluripotency. *Stem cell research & therapy* **1:** 39

Silva FDS, Almeida PN, Rettore JVP, Maranduba CP, Souza CM De, Souza GT De, Zanette RDSS, Miyagi SPH, Santos MDO, Marques MM & Maranduba CMDC (2012) Toward Personalized Cell Therapies by Using Stem Cells: Seven Relevant Topics for Safety and Success in Stem Cell Therapy. *Journal of Biomedicine and Biotechnology* **2012:** 1–12

Tsuji Y, Yoshimura N & Aoki H (2008) Maintenance of undifferentiated mouse embryonic stem cells in suspension by the serum- □ and feeder-□ free defined culture condition. *Developmental Dynamics* **237:** 2129–2138

Vladkova TG (2010) Surface Engineered Polymeric Biomaterials with Improved Biocontact Properties. *International Journal of Polymer Science* **2010:** 1–22

Ying Q, Wray J, Nichols J, Batlle-morera L, Doble B, Woodgett J, Cohen P & Smith A (2008) The ground state of embryonic stem cell self-renewal. *Nature* **453:** 519–524

Table 1

Gene name	*Forward sequence (5′–3′)*	*Reverse sequence (5′–3′)*	*Annealing temperature (°C)*	*Amplicon size (bp)*
Gapdh	GGGAAGCCCATCACCATCTTC	AGAGGGGCCATCCACAGTCT	59	356
Tbp	ACAGGAGCCAAGAGTGAAGAAC	GGAGAACAATTCTGGGTTTGA	56	244
Nanog	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG	61	353
Oct 4	AGTATGAGGCTACAGGGACA	CAAAGCTCCAGGTTCTCTTG	58	251
Sox 2	CGAGATAAACATGGCAATCAAATG	AACGTTTGCCTTAAACAAGACCAC	56	236
Col1α1	GCAGACGGGAGTTTCTCCTC	TCAAGCATACCTCGGGTTTC	61	247
β1-Integrin	AATGTGTTCAGTGCAGAGCC	TTGGGATGATGTCGGGA	53	261
βIII-Tubulin	CCTGGAACCATGGACAGTGTT	CAGCACCACTCTGACCAAAGA	55	85
Sox 1	CCAAGAGACTGCGCGCGCTG	GGGTGCGCCGGGTGTGCGTG	60	362
Hand 1	CCAGTTACATCGCCTACTTG	CCTGGTCTCACTGGTTTAGT	56	240
αSma	ATCGTCCACCGCAAATGC	AAGGAACTGGAGGCGCTG	56	89
Afp	ATGTATGCCCCAGCCATTCTGTCC	GAGATAAGCCTTCAGGTTTGACGC	60	442
Gata 4	GAAAACGGAAGCCCAAGAACC	TGCTGTGCCCATAGTGAGATGAC	60	163

Figure Legends

Figure 1: Synthesis and characterization of nanofibers. **A.** Synthetic approach to PEG-PTMSMA-PMAA and nanofibers formation: self-assembly of the amphiphilic copolymer into vesicles, conversion into nanofibers via stacking, and GRGDS incorporation. **B.** Cryo-Transmission electron microscopy images of polymeric nanofibers by negatively staining with 1% uranyl acetate. These nanofibers are predisposed to form a mesh.

Figure 2: Cell adhesion. Expression of endogenous extracellular matrix proteins and adhesion proteins in mES cells grown for 4 subcultures on PEG-PTMSMA-PMAA-GRGDS nanofibers and in standard conditions. **A**. Adhesion marker. Levels of *β1-Integrin* gene expression. (*) indicates significantly different expression levels between nanofibers, MEFsi and gelatin ($p<0.05$). **B.** Endogenous extracellular matrix protein marker. Levels of *Col1a1* gene expression. (#) indicates significantly different expression levels between nanofibers and gelatin and tissue culture polystyrene plates (TCPS) ($p<0.05$). The relative expression was normalized to *Gapdh* and to MEFsi control.

Figure 3: Cell proliferation. A. Representative images of E14GFP8 embryonic stem cells during 3 and 5 (‘) days in culture. The wells were covered by: A. 100 μg/ml of nf, B. 50 μg/ml of nf, C. 10 μg/ml of nf. D. MEFsi E. 0.1% Gelatin, F. TCPS. Magnification is 100x.

Cell proliferation and viability tests performed during 3, 5 (**B and C**) of mES culture. Cell growth (fold) is presented as the ratio between viable cells and initial cell number. Viability ratio is the ratio between the number of viable cells and total number of cells. (*) indicates significantly different cell number in nanofibers compared with negative control value (TCPS) ($p<0.05$). (#) indicates significantly different cell number in nanofibers compared with gelatin. Data presented as mean + SD for n=6.

Figure 4: Maintenance of Pluripotency. A. Expression of the pluripotency markers *Nanog*, *Oct4* and *Sox2* in cells cultured on PEG-PTMSMA-PMAA-GRGDS nanofibers and in standard conditions, for 4 subcultures. (*) indicate significantly different expression levels in nanofibers compared with gelatin, MEFsi and polystyrene ($p<0.05$). (#) indicate significantly differentexpression levels compared with TCPS ($p<0.05$). **B.** Representative images of mES cells stained for Alkaline Phosphatase after 3 and 15 days (‘) of culture. The wells were

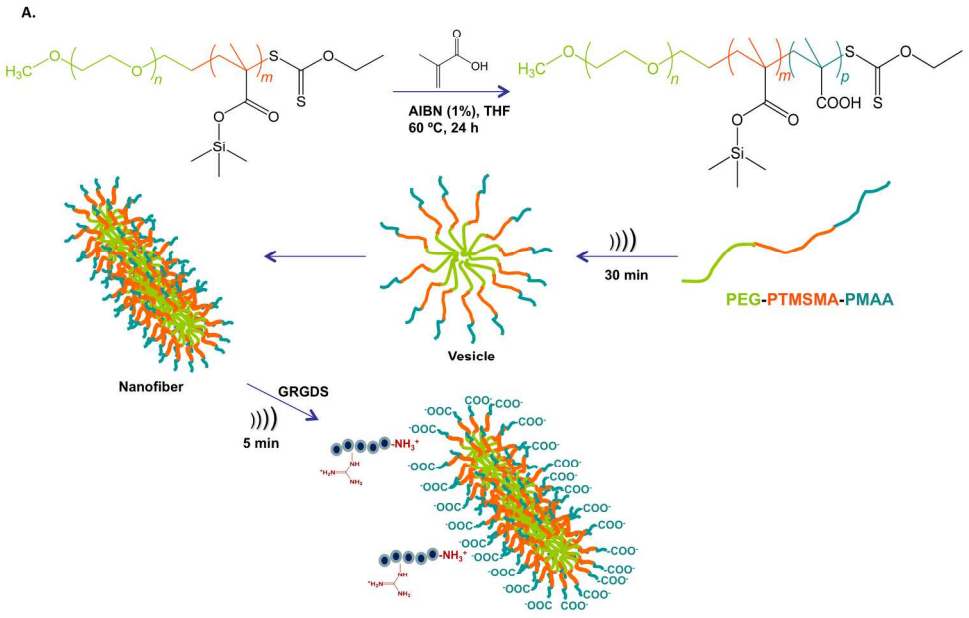
covered by: A. MEFsi B. 0.1% Gelatin, C. TCPS. D. 100 µg/ml of nf, E. 50 µg/ml of nf, F. 10 µg/ml of nf. Magnification is 100x. The red arrows indicate the differentiated cells and the green arrow indicates the pluripotent colonies with a regular shape.

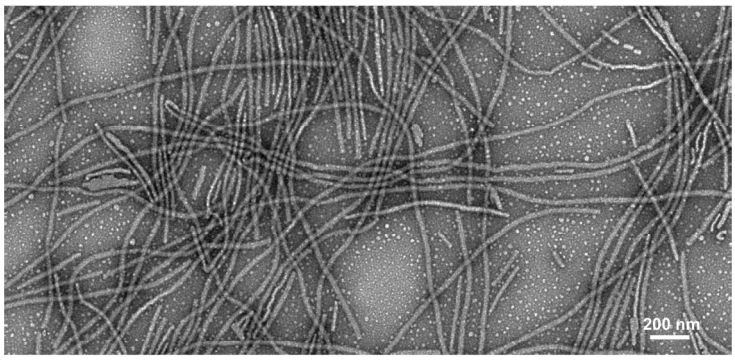
Figure 5: Tri-lineage Differentiation. Differentiation potential of mES cells cultured on nanofibers for 30 days. **A.** Morphology of EBs at day 10 of differentiation. **B.** Levels of gene expression of germ layer markers in EBs collected at day 10 of differentiation. *βIII-Tubulin* and *Sox 1* for ectoderm, *Hand 1* and *αSma* for mesoderm and *Afp* and *Gata 4* for endoderm. The relative gene expression was normalized to *Gapdh* and *Tbp* and to MEFsi control. Data are from two biological and two technical replicates, performed in triplicate (n=8).

Table 1: Primer sequences, product size and program used for qRT-PCR gene expression analysis. *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), *Tbp* (TATAA-box binding protein), *Col1α1* (collagen type I alpha 1), *αSma* (alpha smooth muscle Actin), Afp (alpha-fetoprotein).

Figure S1: Cell proliferation. Cell proliferation test performed at day 3 of mES culture in untreated polystyrene (PS), MEFsi, 0.1% of gelatin, 5 μM of GRGDS, PEG-PTMSMA-PMAA nanofibers and PEG-PTMSMA-PMAA nanofibers bioconjugated with 5 μM of GRGDS. (***) indicates statistically differences among the group ($p<0.001$).Data presented as mean + SD for n=6.

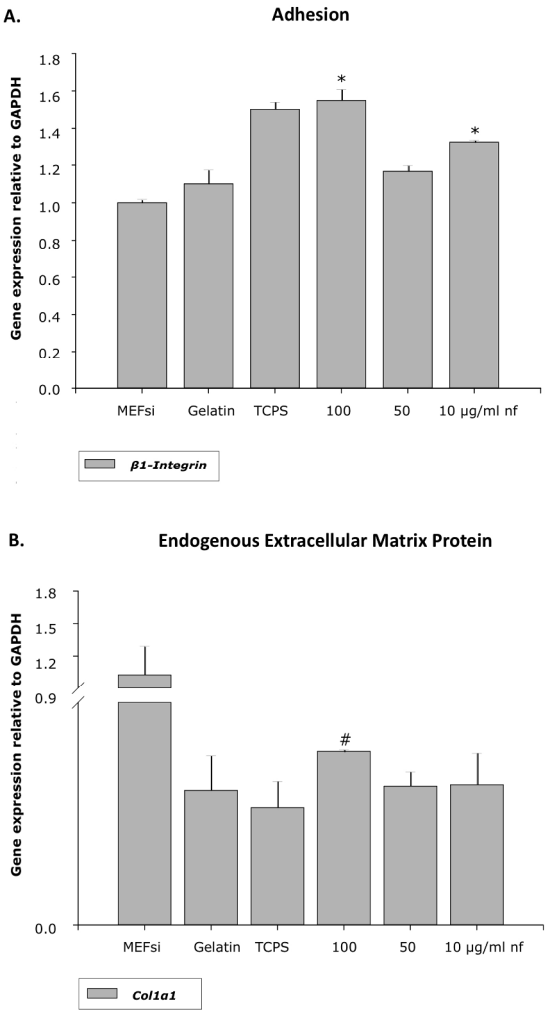
Figure 1





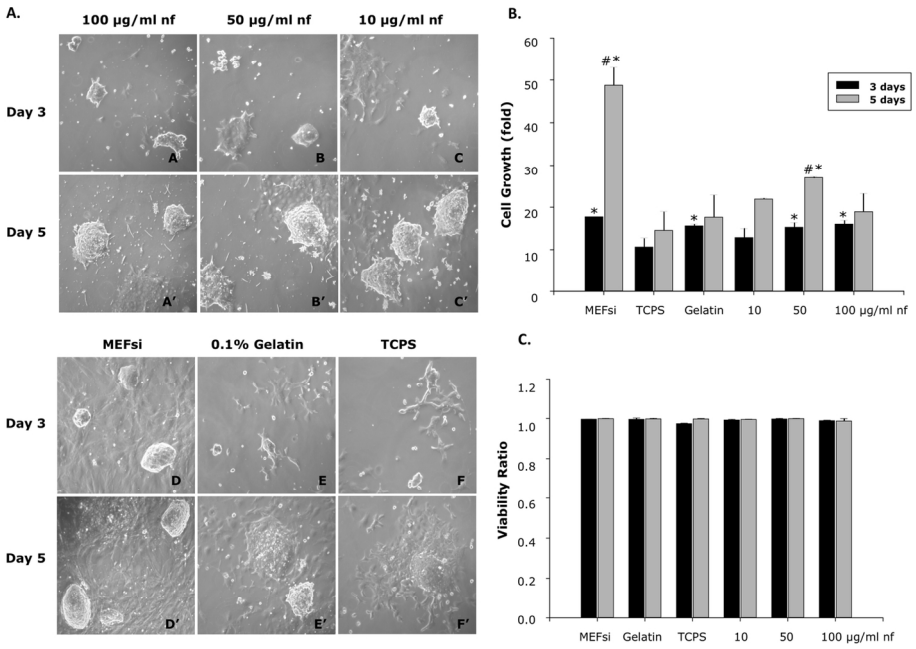
112x132mm (600 x 600 DPI)

Figure 2



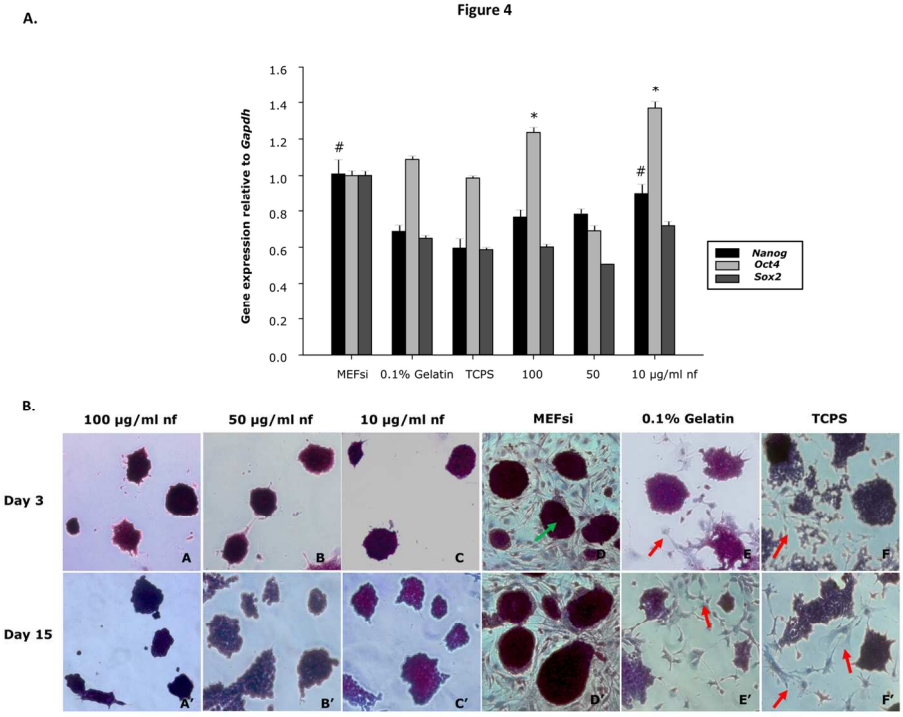
Perestrelo.Figure 2.tif
95x126mm (600 x 600 DPI)

Figure 3



Perestrelo.Figure 3.tif
71x53mm (600 x 600 DPI)

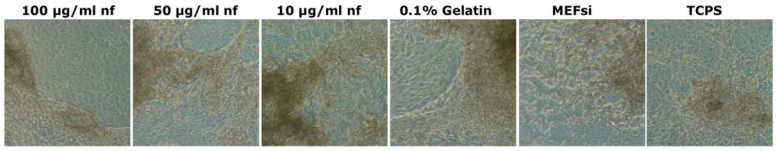
Figure 4



Perestrelo.Figure 4.tif
71x53mm (600 x 600 DPI)

Figure 5

A.



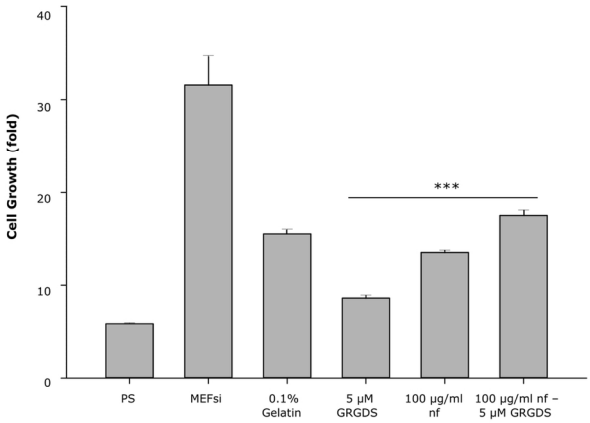
B.

EBs	ΔΔ Cq	100	50	10 µg/ml nf	MEFsi	0.1% Gelatin	TCPS
Ectoderm	βIII-tubulin	1,13	1,47	1,54	1,39	1,33	1,88
	Sox 1	0,22	0,40	0,65	1,02	0,58	0,61
Mesoderm	Hand 1	0,53	0,65	1,05	0,98	1,53	1,79
	αSMA	0,97	1,32	0,50	1,01	0,59	0,73
Endoderm	AFP	0,46	0,89	0,42	1,01	0,63	1,76
	Gata 4	0,06	0,44	0,47	1,14	0,47	0,34

ΔΔ Cq:
≥ MEFsi
≥ 0.1% Gelatin

71x53mm (600 x 600 DPI)

Figure S1



71x53mm (600 x 600 DPI)