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Nano-scale control of cellular environment to drive embryonic stem cells selfrenewal and fate

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ABSTRACT

Embryonic stem cells (ESC) are pluripotent cells capable to give rise to any embryonic cell lineage. In culture, these cells form colonies creating their own niche. Depending upon the molecular and physico-chemical environment, the pluripotent cells oscillate between two metastable states of pluripotency either reminiscent of the inner cell mass of the embryo or the epiblast, a stage of development which give rise to the three embryonic layers, ectoderm, endoderm and mesoderm. Herein, we used PLL/HA nanofilms cross-linked to various degrees to modulate the nanoenvironment of ESCs. Adhesion of ESC on nanofilms increased from native films to highly cross-linked films. The adhesion process was associated with cell proliferation. Expression of genes markers of the ICM decreased with adhesion of cells to cross-linked films. In parallel, genes more reminiscent of the epiblast, were turned on. ESC differentiation within embryoid bodies further revealed that cell pluripotency was better retained when cells did not adhere on native films. We further report that both the stiffness and the chemistry of nanofilms play a key role in modulating the niche of ESC and in turn govern their selfrenewal and fate.

1. Introduction

Embryonic stem cells (ESC) are derived from the inner cell mass (ICM) of the blastocyst at an early stage of embryonic development following the segregation of the embryo into the ICM and the trophectoderm [1]. In contrast to the cells located in the inner cell mass, mouse ESC lines remain pluripotent *in vitro* and self-renew in the presence of the cytokine LIF. For many years ESCs were thought to be a homogeneous cell population. More recently, this concept has been challenged first by Cui et al. [2] looking at the spatial distribution of adhesion molecules within ESCs colonies and more recently by the derivation of epiblast stem cells [3] and by the existence of cell subpopulations in culture of mouse ESCs [4]. Indeed, while derived at a slightly later stage of development than the ICM of the blastocyst, epiblast stem cells can still remain pluripotent *in vitro* in the presence of activin and FGF2 signalling. Second, heterogeneous expression of stella in ESCs, a marker of germ cells also present in the preimplantation embryo but repressed in the epiblast led first to the suggestion that ESCs might be closer to germ cells than to the ICM [5]. Finally, expression of stella in ESCs was associated to their reminiscent origin in the ICM [6]. In fact, ESCs are commuting between metastable states from the ICM to the epiblast stage. These reversible states are associated with distinct potential of differentiation [6,7] [4]. This is thus a highly dynamic self-renewing cell population which responds to environmental clues to maintain their pluripotency or to differentiate. The latter clues are several and include growth factors in the culture medium surrounding ESCs colonies or secreted within the colonies, signals arising from adhesion to the substrate (i.e. extracellular matrices, ECM), and/or stiffness of the substrate [8]. Little attention has been paid so far to the impact of the substrate on cell pluripotency as most ESCs lines are grown on feeder cells or in feeder free conditions on gelatin-coated dishes. More specifically, the influence of culturing ESC in gelatin-coated plates on the metastable state of ESCs has until now been largely ignored.

Furthermore, the formation of ESCs colonies thanks to cell–cell tight contacts together with the surrounding physiochemical environment, provide them with a niche, a locally restricted environment, required to maintain their pluripotency and to support their proliferation.

Several means exist to modulate the cellular environment. One of the major perturbation of stem cell niche is the capability or not

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for the cells to adhere to the substratum and to sense the environment in regard to both chemistry and stiffness. Indeed, if adherent or in suspension, the cells will presumably not respond similarly and will be more or less capable to sense and interpret the signals from the close environment. A pioneering work by Discher and coworkers suggested that multipotent stem cells might be driven in their differentiation by their tight or loose attachment depending upon the stiffness of the substrate [9], in this case a polyacrylamide gel.

To challenge and modulate the adhesive properties of the cells, we designed experiments to tune the properties of thin multilayered films by covalently cross-linking them. These films are assembled by alternate adsorption of polycation and polyanions onto a surface [10]. There are already several studies investigating cell interactions with polyelectrolyte nanofilms [11,12] but very few investigated cell differentiation. The behaviour of progenitor cells and of adult stem cells but not of the most plastic embryonic stem cells on films has only recently been explored [13–15]. One of the most characterized multilayer films in terms of physico-chemical, mechanical and cell adhesive properties is that of poly(L-lysine) and hyaluronan (HA) [16,17]. Using carbodimide chemistry, one of us recently prepared a wide range of films of increased stiffness by simply varying the cross-linker concentration [18]. Cross-linking was found to have only minor effect on film roughness, wettability and serum protein adsorption in the range where important differences in cell adhesion were observed [18,19]. In this study, we used PLL/HA films cross-linked to various degrees to change the nanoenvironment and to investigate whether ESCs adhesion and formation of the niche can be regulated by the films.

2. Materials and methods

2.1. PLL/HA nanofilms

Poly(L-lysine) (MW : 70 kDa, P2636) and HA (MW 360 kDa) were purchased from Sigma. Polyelectrolyte multilayer nanofilms were built by alternately depositing *12* layer pairs of PLL and HA (noted as $(PLL/HA)_{12}$ film) on glass coverslips or plastic dishes as previously described [18] Briefly, PLL at 0.5 mg/mL and HA at 1 mg/mL were dissolved in 0. 15 M NaCl containing 20 mM of Hepes at pH 7.4. Adsorption was achieved for 8 min followed by rinsing for 2 min. After build-up, films were cross-linked following a previously published protocol using the water soluble carbodiimide, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) and N-Hydrosulfosuccinimide (sulfo-NHS) (both purchased from Sigma, St Quentin-Fallavier, France). EDC was varied from 10 to 100 mg/mL final concentration while sulfo-NHS was kept at 11 mg/mL. The films were incubated overnight at 4 °C with the cross-linking solution (in 0.15 M NaCl, pH 5.5). They were finally rinsed three times with a 0.15 M NaCl solution at pH 8.0.

2.2. Culture and differentiation of embryonic stem cells

ES cells (CGR8) were propagated in BHK21 medium supplemented with pyruvate, non-essential amino acids, mercaptoethanol, 7.5% fetal calf serum and LIF conditioned medium obtained from pre-confluent LIF-D cells stably transfected with a plasmid encoding LIF. The cells were trypsinized and replated every two days. Differentiation was carried out using the hanging drop method. Briefly, embryoid bodies (EBs) were formed in hanging drops of differentiation medium (BHK21 medium supplemented with pyruvate, non-essential amino acids, mercaptoethanol and 20% fetal calf serum without LIF) for two days (D0-2). Then, the EBs were incubated for three days in suspension (D2-5) and for at least seven days on gelatin-coated (D6-12) [20].

The Rex GFP clone was generated following electroporation of the REXGFP plasmid (a gift from Dr Benvenisty, Jerusalem, Israel). Cell clones were selected using G418 (300 μg/ml) for 10 days and clones were individually picked up for genotyping and then expansion.

2.3. Immunostaining and cell imaging

Embryoid bodies were fixed in 3% paraformaldehyde for 15 min, permeabilized for 10 min with 0.5% Triton X-100, and immunostained as described previously [21]. The antibodies used were sarcomere-specific α-actinin (Sigma France), Nkx2.5 (R&D MAB2444 1:100), Nestin (Abcam ab5968 1:1000), FoxA2 (Abcam ab40874 1:200).

Prior sectioning, fixed embryoid bodies were prepared by sequentially soaking them into PBS/5% Sucrose (Sigma France) for 30 min, PBS/30% Sucrose overnight, and finally in a 1:1 mixture of PBS/30% Sucrose and Tissue-Tek Optimal Cutting Temperature (O.C.T.) compound (Sakura, Japan) for at least 4 h. EBs were then transferred on a cryomold in pure OCT and cut into 10 μm sections. Finally sections were mounted onto SuperFrost Plus slides (Menzel-Gläser, Germany) for conventional Eosin/Haematoxylin staining (Sigma, France).

Images were acquired on a Leica DRME-7 confocal microscope. Image channels assembly and overlays were performed with ImageJ. For field emission scanning electron microscopy (FESEM), the cells were fixed, prepared and observed as previously described [22].

2.4. Cell cytometry

Cells were trypsinized to obtain single cells suspensions from adherent cultures and were then transferred to cold PBS with 5% fetal calf serum for analysis with a FACScalibur (BD Biosciences). Gates were determined according to the autofluorescence levels of a preparation of wild type CGR8 cells with 7-amino-actinomycin D to exclude dead cells from the analysis.

2.5. Cell adhesion and proliferation assays

To quantify cell adhesion, cells were plated on films at a density of 25,000 cells/cm^2 and allowed to adhere for 24 h. The adherent and non-adherent fractions were collected separately and trypsinized to obtain single cells suspension. The number of cells contained in each fraction was then determined with a Flomax cytometer (Partec, France).

To determine the proliferation rate, 2000 cells/cm^2 were seeded on films and counted at 24 h intervals for 4 days. Each experiment was performed in triplicate and experimental points were fitted with a simple exponential function.

$$N(t) = N_0 e^{t/\tau} \quad (1)$$

$N(t)$ is the number of cells at a time t, N_0 is the initial number of cells and τ is the indicative proliferation rate.

2.6. RNA reverse transcription and real time quantitative PCR

Total RNA was prepared from ESC or EBs after cell lysis using a kit (Zymo research, Proteingene, France). After reverse transcription of 1 μg total RNA, PCR was carried out using a set of gene specific primers as previously described [21]. cDNA (equivalent to 10 ng) was used for real time quantitative PCR, performed with a lightcycler1.5 or LC480 and the SYBR Green fast start kit (Roche, Germany). The sequences of primers used for real time PCR are listed in Table 1. The 12 μL reaction mix contained 1 μL of Master SYBR Green I mix, including Taq DNA polymerase, buffer, deoxynucleoside trisphosphate mix, SYBR Green I dye, 3 mM $MgCl_2$ and 0.5 μM of each primer. 2 μL of 30-fold diluted cDNA was added to the mixture. Primer efficiency was established by a standard curve using sequential dilutions of gene specific PCR fragments. Data were normalised from RT-QPCR housekeeping gene ATP50 as an index of cDNA content after reverse transcription. Amplification included initial denaturation at 95 °C for 8 min, and 40 cycles of denaturation at 95 °C for 3 s (8s LC480°), annealing at 60–65 °C for 8–10 s (15s LC480), and extension at 72 °C for 7–10 s (15s LC480°). The temperature transition rate was 20 °C/s. Fluorescence was measured at the end of each extension step. After amplification, a melting curve was acquired by heating the product at 2.5 (LC480° or 20 °C/s to 95 °C), then cooling it at 20 °C/s to 70 °C. The reaction was maintained at 70 °C for 20 s followed by slow heating at 0.3 °C/s to 95 °C. Melting curves were used to determine the specificity of PCR products, and they were further confirmed by gel electrophoresis.

2.7. Statistics

Data were statistically analysed using a Student t-test and the Sigma Plot software. Differences were considered significant if $p \leq 0.05$.

2.8. Measurement of release of PLL and HA from films

The cell propagation medium was incubated onto $(PLL/HA)_{12}$ films built into 48 wells plates with or without cells and was collected after 24 h or 48 h. HA contained in this conditioned medium was then quantified by ELISA using the HA detection kit (Corgenix, France).

For measuring the release of PLL, (PLL/HA) films containing a mixture of PLL and PLL labelled with fluorescein-isothyocianate (FITC) (PLL^{FITC}) at 50:50 in mass were built on 14 mm glass slides as previously described. After their cross-linking and final rinsing, each slide was introduced in a 24-well plate, supplemented with 1 mL of Hepes-NaCl buffer at pH 7.4 and placed in an incubator at 37 °C for different time periods. At regular time intervals until 5 days, 200 μL of the buffer were taken and their fluorescence measured using a fluorescence microplate reader (TECAN Infinite 1000) using excitation and emission wavelengths of $494 \pm 2.5/518 \pm 2.5$ nm. To