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Dynamic adhesive environment alters the differentiation potential of young and ageing mesenchymal stem cells

Maria Valeska Bianchi [a], Firas Awaja [b], George Altankov [a,c,*]

[a] Institute for Bioengineering of Catalonia, Barcelona, Spain
[b] Department of Orthopaedic Surgery, Experimental Orthopaedics, Medical University Innsbruck, Innrain 36, Innsbruck, Austria
[c] Institució Catalana de Recerca i Estudis Avancats (ICREA), Barcelona, Spain

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ABSTRACT

Engineering dynamic stem cell niche-like environment offers opportunity to obtain better control of the fate of stem cells. We identified, for the first time, that periodic changes in the adhesive environment of human adipose derived mesenchymal stem cells (ADSCs) alters dramatically their asymmetric division but not their ability for symmetric renewal. Hereby, we used smart thermo-responsive polymer (PNIPAM) to create a dynamic adhesive environment for ADSCs by applying periodic temperature cycles to perturb adsorbed adhesive proteins to substratum interaction. Cumulative population doubling time (CPDT) curves showed insignificant decline in the symmetric cell growth studied for up to 13th passages accompanied with small changes in the overall cell morphology and moderately declined fibronectin (FN) matrix deposition probably as a functional consequence of ADSCs ageing. However, a substantial alteration in the differentiation potential of ADSCs from both early and late passages (3rd and 14th, respectively) was found when the cells were switched to osteogenic differentiation conditions. This behavior was evidenced by the significantly altered alkaline phosphatase activity and Ca deposition (Alizarin red) assayed at 3, 14 and 21 day in comparison to the control samples of regular TC polystyrene processed under same temperature settings.

1. Introduction

Stem cell-based therapies are hindered by insufficient understanding of the signals that govern cellular behavior. There is limited understanding of what variables influence stem cells fate and how it can be controlled [1]. Stem cells state is tightly regulated by spatiotemporally complex signals originating from the surrounding physical and chemical milieu, i.e. the stem cell niche [2]. Consequently, precise engineering of the niche environment is an important tool for controlled production of stem cells in vitro and guiding of their subsequent behavior in vivo - both critical issues for most cell based therapies [3].

Engineering particular niches is a strategy in which a biomaterial is constructed to mimic the natural cell-extracellular matrix (ECM) interactions through simple adsorption or selective bounding of important adhesive matrix proteins and signaling molecules [1,4]. A promising kind of materials that could change our understanding for the niche microenvironment is the stimuli-responsive polymers. These materials that undergo radical changes of their internal structure upon external stimuli (e.g. temperature, pH, light) could be used as "on-off"-switches between different functions providing dynamic environment for the adhering cells. For example, the thermo-responsive polymer poly (*N*-isopropyl acrylamide) (PNIPAM) has been widely used to indirectly control cell attachment and detachment by modifying the surface wettability via subtle changes of the culture temperature [5,6]. Decreasing the temperature from 37 °C to room temperature, i.e. below the LCST (Lower Critical Solution Temperature), which for PNIPAM is 32 °C, the swelling of the material increases and cells and matrix are released together from the surface without the need for digestive enzymes [6–9].Yang et al. [10] extended this strategy by fabricating a micro heater array underneath a PNIPAM layer to locally control cell adhesion. Recently, many authors report on using thermo-responsive systems to harvest whole cell sheets [10] exploring the idea for broad tissue engineering application [10] including the preparation of sophisticated constructs ready for implantation [11,12].

The stem cell niche is described as a dynamic system that is able to regulate multiple cellular processes [13]. Hereby, we explore, for the first time, the dynamic attributes of stem cell niche by using an innovative approach utilizing thermo-responsive polymers. Thermo-responsive polymers, such as PNIPAM, have strong potential to control the ECM - substrate interactions, but may also provide conditions that switch from attraction and retraction of cells. We anticipate that the

* Corresponding author at: Institute for Bioengineering of Catalonia, Feixa Llarga, s/n, Pavello Govern, 1° Planta, Room 1121, 08907 L'Hospitalet de Llobregat, Barcelona, Spain.
E-mail address: george.altankov@icrea.cat (G. Altankov).

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thermo-responsive effect can be explored in both directions (including for periodic alternative cycling) and thus implicated for mimicking the dynamic environment of an artificial niche. Both static and cyclic mechanical strain has been explored previously as a means to create alignment in cell sheets [14]. Endothelial cells responded to cyclic shear-stress with an increase in stress filaments that led to the regulation of autocrine and paracrine signaling for angiogenesis and vascular remodeling [15,16]. Cyclic stretch was also shown to affect the osteogenic differentiation of MSCs [17] as well as the bone homeostasis [18]. The use of PNIPAM for culturing stem cells has also been previously explored: Shi et al. [19] studied the proliferation and multi-differentiation potentials of human mesenchymal stem cells (MSCs) on thermo-responsive PNIPAM grafted PDMS surface showing that the proliferation is not significantly different from that on a surface coated with gelatin. They demonstrated however that PNIPAM-grafted surface was favorable for the osteogenic differentiation of MSCs, an effect confirmed in a recent investigation utilizing PNIPAM microgels [49] although without using the thermos-responsive property of the polymer for cell harvesting. In the biomaterials field, PNIPAM is regarded as potential cell culture substrata not only because of its biocompatibility, but also due to its special property of allowing controlled detachment of cells [49]. Zhang et al. [20] described a number of chemically defined thermo-responsive hydrogels as substrata for long-term culture of human embryonic stem cells and evaluated 2-(dimethylamino)ethyl acrylate to be strongly in favor. Multi-layered cell sheet comprised of human ADSCs detached by lowering the temperature was also described in the context of wound healing [21]. The dynamic aspects of stem cells culture on thermo-responsive substrata, however, have not been explored.

Different parameters can characterize the biological response of stem cells to dynamic environment, however, two parameters reflect their unique functionality, the capability for self-renewal (symmetric growth) and the competence to differentiate into multiple lineages (asymmetric cell division) [22,23]. Symmetric self-renewal is a mechanism by which each stem cell creates two daughter stem cells leading to an expansion of stem cell pool that is physiologically required for repair [24]. In vivo, this mechanism generally works in the stem cells niches, but might be translated also in vitro when propagating MSCs for tissue engineering applications [25]. The number of in vitro passaged cells however is limited due to ageing [26–27] manifested with the diminished cell growth, shortening of telomerase length and changes in the overall cell morphology [27].

In this work, we created dynamic cell adhesive conditions utilizing the smart thermo-responsive PNIPAM surface to reversibly affect cell-to-substratum interaction mediated by the adsorbed adhesive proteins. We further introduced this mechanically unstable environment to cultured ADSCs by applying periodic changes in temperature in order to investigate its particular effects on the symmetric and asymmetric division of stem cells.

2. Material and methods

2.1. Cells

Human ADSCs were obtained from Lonza Biowhittaker (Verviers, Belgium). After thawing the cells (Passage 1) were seeded in 25 cm^2 flasks (Thermo Scientific, 136196) and maintained in sterile DMEM/F12 (1:1) supplemented with 1% GlutaMAX™ (Gibco, 31331-028), 1% Antibiotic-Antimycotic solution (Gibco, 15240-062) and 10% Fetal Bovine Serum (FBS) (Gibco, 10270-106), and further incubated at 37 °C, 5% CO_2 and 98% humidity. The medium was replaced every 2 days until the cells reach confluency.

2.2. Cell sub-culturing

ADSCs were allowed to grow for one passage before the study. To start the experiment, the cells were harvested using standard enzymatic digestion with Trypsin/EDTA for 5 min and seeded at density of 2×10^4 cells/cm^2 on PNIPAM coated 60 mm Petri dishes or 24 well plates provided by Thermo Fisher Nunc UpCell™ (Cat No 174903 and 17489, respectively). As controls, were used same sized vessels from regular TC polystyrene (Sigma-Aldrich, P5481-500EA).

2.3. Thermo-cycling

After 24 h of cells seeding, both PNIPAM coated and control TC polystyrene dishes were exposed to thermo-cycling being placed on a custom made stainless steel thermo-plate with build in channels for circulating water. It was fitted to ultratermostate KH-4 (Biometra, 043-300) providing both heating and cooling regimes. A preliminary study (see Results and discussion section) indicated as optimal the temperature cycle of 20 min decrease (from 37 °C to 24 °C) and 20 min increase (from 24 °C to 37 °C) which was accepted as standard procedure to be applied twice a day.

2.4. Cells passaging

Three days after seeding the cells were detached from the thermo-responsive substrate via reducing the temperature to 20 °C for 60 min, followed by a gentle pipetting aimed to harvest single cells. Control group were detached by standard enzymatic procedure using Trypsin/EDTA. After harvesting the cells were counted and part of them seeded again at same density for the next passage using new vessels. The rest of the grown cells were used for determining the overall morphology and fibronectin (FN) matrix secretion (see below).

2.5. Cell proliferation and cell doubling assay

Cell proliferation was assayed by direct counting of their number at 3rd day of passaging using Neubauer chamber under phase contrast. To assure accuracy of the measurement counting were performed in triplicates. The calculation of the cumulative population doubling time (CPDT) was performed according to [28] as follows:

$$CPDT = CT/CPDN$$

$$CPDN = Ln(N_f/N_i)/\ ln2$$

where CPDN is the Cumulative Population Doubling Number, N_i and N_f are the initial and final cell numbers respectively, ln is the natural logarithm and CT is the culture time.

2.6. Overall cell morphology and visualization of focal adhesion complexes

To follow the effect of temperature on cell detachment, 4×10^4 cells were seeded on PNIPAM coated or control Petri dishes (as indicated in the text) for 5 h. The samples were further exposed to a standard temperature cycle, decreasing the temperature from 37 °C to 24 °C during 20 min and heating back to 37 °C. At the end of the descendent and ascendant cycles, the samples were fixed with 4% paraformaldehyde (Pareac, 141451-1210) and processed for immunofluorescence as explained below. Some of the samples were cooled for 2 h, as indicated in Results and discussion section and studied and photographed at inverted phase contrast at low magnification (10×).

To follow the focal adhesions formation and actin cytoskeleton reorganization, fixed samples were permeabilized with 0.5% Triton-X 100 (Sigma-Aldrich) and saturated with 1% BSA for 20 min to avoid unspecific binding before stained with a monoclonal anti-vinculin antibody (Sigma-Aldrich, V9264) followed by goat anti-mouse IgG AlexaFluor 448-conjugated secondary antibody (Life Technologies, A11001) both incubated for 30 min at 37 °C. The secondary antibody mixture contained FITC-Phalloidin 555 (Invitrogen, F432) and Hoechst (Invitrogen, 34580) for simultaneous staining of actin cytoskeleton