Effect of the nichoid substrate on mesenchymal stem cell structure and function

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***Abstract*— Stem cells fate and behavior are affected by the bidirectional communication of cells and their local microenvironment (stem cell niche), which include biochemical cues, as well as physical and mechanical factors. Stem cells are normally cultured in conventional two-dimensional systems with the consequent alteration of mechanical cues received by cells. Here we cultured rat mesenchymal stem cells on standard culture supports and on the "nichoid", an innovative three-dimensional substrate nano-engineered to recapitulate the physiological niche *in vitro*. Our results demonstrate that mesenchymal stem cells adhered and grew in the nichoid, occupying not only the surface but also the internal structure of the niche. After seeding on conventional supports, cells displayed large and spread nucleus and cytoplasm, while cells cultured on the nichoid were spatially organized in three dimensions, with smaller but spherical nuclei. The transcriptional co-activator YAP was localized in the nucleus in cells cultured on both rigid substrates. Preliminary results on gene expression revealed differences in the expression of genes related to stemness and to mesenchymal stem cells' function.**

***Keywords*—3D culture, artificial niche, nichoid.**

I. Introduction

Cell therapies represent the most appealing application of stem cells. Adult stem cells, such as mesenchymal stem cells (MSCs), possess great potential as an autologous therapy source since they are ethically sourced and simple to obtain from adult tissues as the bone marrow. Moreover, MSCs can secrete potent combinations of trophic and immunomodulatory factors that are able to modulate responses from surrounding cells.

The natural microenvironment that surrounds stem cells, called stem cell niche, provides a complex array of physical and biochemical signals. The integration of both local and systemic cues in the niche guides these cells to proliferation and fate specification [1]. Physical and mechanical factors, including substrate stiffness, surface nanotopography, microgeometry, and extracellular forces can all play a crucial role in triggering stem cell responses *in vitro* [2], [3].

The transcriptional co-activators YAP and TAZ are involved as downstream elements in cells perception of their physical microenvironment, in a process called mechanotransduction. It has been reported that forces originating on the cell surface are transmitted to the nucleus, resulting in chromatin stretching and changes in gene transcription [4]. Through these processes, mechanical signals can direct MSCs' fate and behavior even in the absence of chemical signals [5], [6].

Conventional cell-culture used to investigate stem cell behavior is based on two-dimensional (2D) monolayers. However, 2D cultures strongly alter cell biological functions, as they don't reproduce the complex three-dimensional (3D) environment of stem cell niche. In addition, 2D substrate confine cells to a planar environment, in which the mechanotransduction process is strongly altered.

The aim of the present work is to investigate whether the culture of MSCs on standard 2D glass supports versus on an innovative nano-engineered synthetic 3D substrate [7] affect cell structure and function.

II. Methods

The innovative culture substrate nichoid was fabricated via two-photon laser polymerization in a biocompatible photopolymer called SZ2080. A total of 168 blocks of niches were laser written directly onto circular glass coverslips of 150 μm thickness and 12 mm diameter (BioOptika, Italy).

MSCs were obtained from bone marrow of 6 weeks-old male Sprague Dawley rats (Charles River). Bone marrow was obtained by flushing the femurs and tibias with cold medium and filtered through a 100-μm sterile filter (BD Biosciences, Milan, Italy). Filtered bone marrow cells were plated in α-MEM (Invitrogen, Scotland) supplemented with 20% FBS (Invitrogen), 100U penicillin and 100 μg/mL streptomycin (Invitrogen) and allowed to adhere at 37∘C in a humidified atmosphere containing 5% CO2. After reaching 80%–90% confluence, cells were detached by 0.25% trypsin-EDTA and cryopreserved in liquid nitrogen. Isolated cells were characterized for their ability to differentiate into osteoblast, chondrocytes and adipocytes. All experiments were performed with cells at passage 2-4.

MSCs were seeded at concentration of 1-2*10^4 cells/cm^2 on 2D glass substrates and on the 3D nichoid substrates and maintained in culture up to 2 weeks. During culture, cell adherence and growth were verified using a phase-contrast microscope. Moreover, we recorded time-lapse videos, using CytoMate digital imaging system positioned into the incubator, in order to monitor cell movement and migration on the substrates. Images were captured every 2 minutes. Cell viability and proliferation were assessed after 3.5 hours incubation with the non-toxic, water-soluble resazurin dye (Sigma Aldrich srl, Italy) at day 1, 4, 7, 10 and 14.

Cells cultured on different substrates were fixed in 0.5% glutaraldehyde for scanning electron microscopy (SEM) or 2% paraformadehyde (PFA) in 4% sucrose for immunofluorescence analysis. For SEM observation, cells

were postfixed with osmium tetroxide, dehydrated through a series of passages in increasing ethanol baths and dried in pure hexamethyldisilazane (HMDS, Fluka Chemie AG, Buchs, Switzerland). At the end, samples were mounted on stubs, coated with gold in a sputter coater (Agar Scientific, UK) and then examined on a Cross-Beam 1540EsB electron microscope (Carl Zeiss GmbH, Germany). For immunofluorescence analysis, samples fixed in PFA were permeabilized with Triton X-100 (Sigma Aldrich srl, Italy) and blocked with 3% bovine serum albumin. MSCs were then labeled with 2 µg/ml rabbit policlonal anti-YAP1 antibody (Abcam, Cambridge, UK) overnight at 4°C. Goat anti-Rabbit IgG (H+L) secondary antibody Alexa Fluor® 647 conjugate (Thermo Fisher Scientific Inc., Milan, Italy) was used at a concentration of 2 µg/ml in phosphate buffered saline for 1 hour at room temperature. Finally, cells were treated with FITC-labeled phalloidin for 45 minutes at room temperature and counterstained with DAPI (1 mg/ml) for 15 minutes at room temperature. Samples were then examined by laser confocal microscopy (LSM 510 Meta, Carl Zeiss).

Gene expression analysis was performed at the end of the observation period. Briefly, total RNA was extracted from MSCs using the RNeasy micro kit, as described by the manufacturer (QIAGEN S.r.l., Italy) and quantified by Nanodrop. The RT^2 first strand kit was used for cDNA synthesis according to manufacturer's guidelines (Qiagen). Next, the cDNA was mixed with the appropriate RT^2 SYBR green master mix and the resulting mixture aliquoted into the wells of the Rat Mesenchymal Stem Cell RT^2 profiler PCR array (Qiagen). qPCR was then performed on the real time cycler Viia7 (Applied Biosystems, UK). Normalization and fold changes were calculated using the ΔΔCt method.

III. Results and Discussion

Live images of cell culture revealed that the 3D nichoid was suitable for adherence and growth of rat MSCs. Moreover, proliferation curves of cells cultured under 2D and 3D conditions, documented by the Alamar Blue assay, were similar.

SEM analysis revealed differences in cell morphology depending on whether the cells were cultured on 2D or 3D support (Fig. 1 and 2).

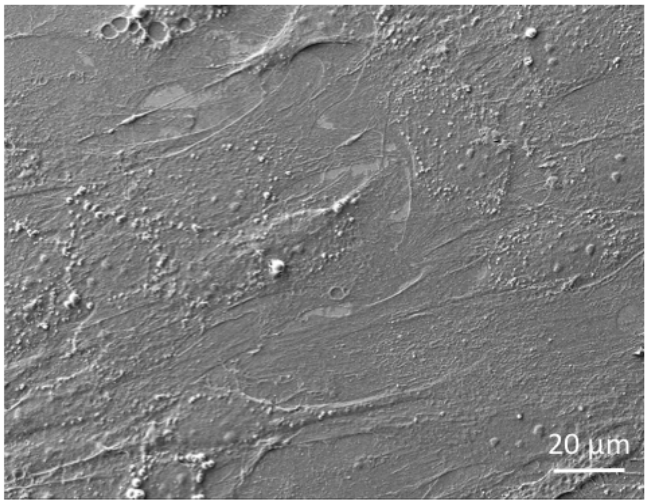

Fig. 1. SEM image of rat MSCs on standard glass substrate after 2 weeks of culture

SEM images showed that MSCs cultured on glass substrates form a confluent monolayer, with spread and flat cells covering all the available surface (Fig.1).

Differently from 2D culture, cells seeded on the 3D nichoid penetrate into the internal structure of the niche, with an elongated morphology and well distributed organization in all the three dimensions (Fig. 2). This conformation allowed the cells to establish connections both with the surrounding cells and with the niche's 3D structure.

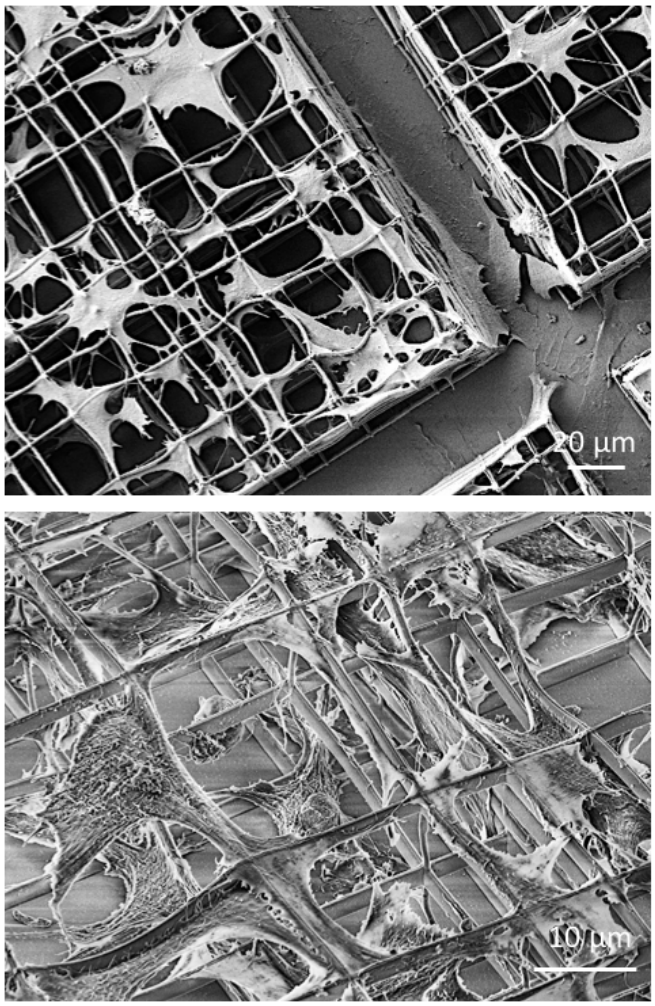

Fig. 2. SEM images of rat MSCs on the nichoid substrate after 2 weeks of culture

Time-lapse videos of the culture highlighted differences in the behavior of MSCs cultured on glass and on the nichoid. When cells were seeded on a standard glass substrate, they reached confluence in 3-4 days. Then, as documented by the image sequence in Fig. 3, MSCs stay still in the same position and only slightly change their morphology.

On the contrary, MSCs cultured in the nichoid assume a peculiar spatial organization and display a continuous movement. This movement was obtained by dynamic interaction with the surrounding cells and the structural elements of the synthetic niche (Fig. 4). In average, cells change completely the spatial organization and location in about 20 minutes.

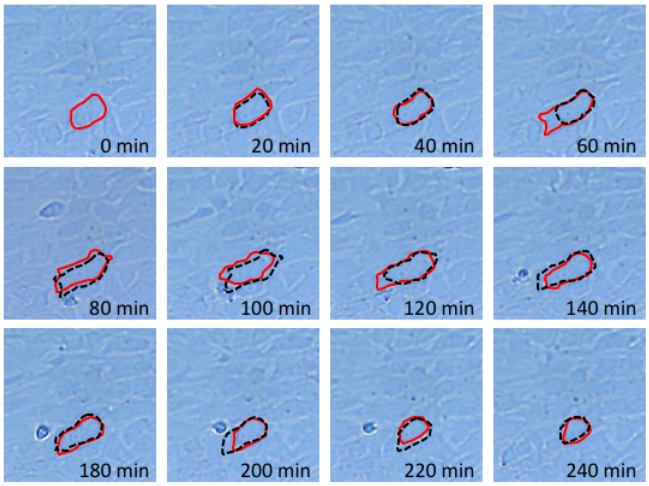

Fig. 3. Image sequence exported from time-lapse video of MSCs cultured in conventional glass support. The boundaries of the selected cell in every frame are designed with a red line, while the previous morphology and position are indicated by dashed black line.

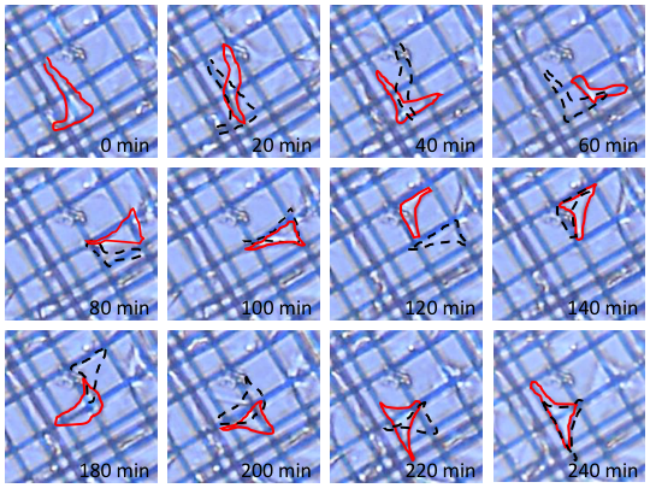

Fig. 4. Image sequence exported from time-lapse video of MSCs cultured in the innovative nichoid support. The boundaries of the selected cell in every frame are designed with a red line, while the previous morphology and position are indicated by dashed black line

Immunofluorescence staining for the actin cytoskeleton revealed that MSCs expanded on glass displayed large and flattened cytoplasm and nuclei (Fig. 5A). Conversely, when cells are cultured in the nichoid substrate, they exhibited long F-actin filaments organized in three dimensions and smaller but spherical nuclei (Fig. 5B).

Since the Hippo pathway effectors YAP and TAZ have been implicated in transducing mechanical signals and directing stem cell fate, we analyzed YAP expression and localization in MSCs cultured in 2D and 3D conditions. Immunofluorescence images showed that YAP is predominantly localized in the nucleus, independently from the used substrate (Fig. 5C-D). These results are in line with previous findings in literature that correlate YAP nuclear localization with stiff matrices [6]. However, quantification of nuclear YAP localization revealed a significant decrease in nuclear YAP percentage in MSCs cultured on the nichoid (Fig. 5E), suggesting that mechanical cues perceived by cells in the two culture conditions may differently affect YAP distribution.

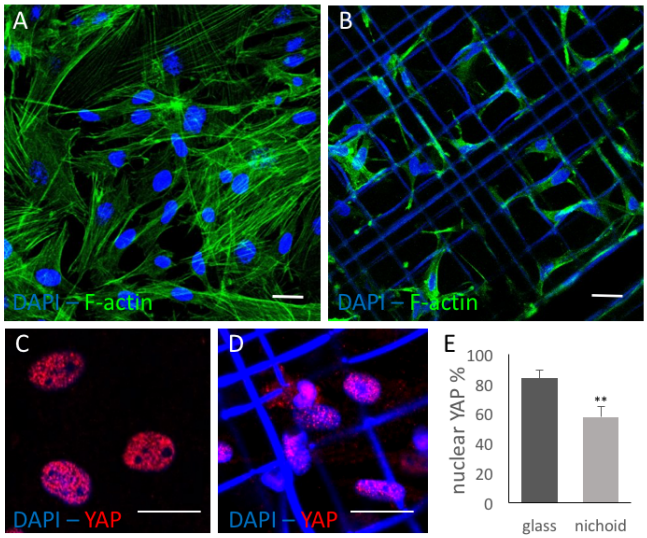

Fig. 5. Immunofluorescence staining for F-actin (A and B) and YAP (C and D) of rat MSCs cultured on standard glass substrate or on the nichoid. Scale bars 20 µm. (E) Quantification of the nuclear area positive for YAP staining, **p<0.01.

Preliminary data obtained by gene array assay revealed that the expression of several genes is significantly different in cells expanded in conventional supports or in the innovative nichoid substrate. The result of this analysis showed that 18 of the tested genes were significantly up- or down-regulated in MSCs cultured on the synthetic niche. These genes are related to MSCs' stemness, function and paracrine action. Future real-time PCR experiments will be performed in order to confirm differentially expressed genes.

IV. Conclusion

Our findings indicate that the nichoid is an innovative synthetic niche suitable for adherence and growth of MSCs. By better reproducing the 3D environment in which stem cells reside *in vivo*, the 3D substrate has pronounced effect on cell structure, nuclear dimensions and cytoskeletal organization, as compared to conventional 2D culture. Moreover, 3D substrate up-regulates the expression of stemness markers, of cytokines and growth factors. All together, these effects may enhance the potential beneficial action of MSCs in *in vitro* or *in vivo* studies.

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