Exploring Embryonic Stem Cell fate using Cellular Microarrays

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***Abstract* —** This work addresses the development of artificial cellular niches for studying the mechanisms that affect stem cell pluripotency, and aims at contributing to a better understanding of the cellular and molecular events that regulate stem cell functions.

Embryonic stem (ES) cells were used as model systems to establish *in vitro* artificial microenvironments that better mimic the extracellular matrix. Microscale-based systems were designed for 3D culture of these cell types, and high-throughput screening platforms were developed to unveil the effects of complex interactions on cell fate. Particularly, We have developed a novel 3D cellular microarray platform to enable the rapid and efficient tracking of stem cell fate and quantification of specific stem cell markers. Our results revealed that this platform is suitable for studying the expansion of mouse ES cells as they retain their pluripotent and undifferentiated state. In addition to the influence of mechanical and matrix-related responses, the effects of microenvironmental conditions (*e.g.* small molecules and growth factors) were also analyzed due to their capacity of modulating intracellular pathways.

This novel platform is a powerful new tool for investigating cellular mechanisms involved in stem cell expansion and differentiation and provides the basis for rapid identification of signals and conditions that can be used to direct cellular responses.

Index Terms — Embryonic stem cells; Cellular microarrays; High-throughput Screening; Small Molecules.

I. CONTEXT

Stem cells are characterized by their unlimited self-renewal capacity and potential to generate fully differentiated, mature cells [1]. Due to these unique characteristics, stem cells are at the forefront of potential regenerative medicine therapies and may also provide an unlimited source of cells for pharmaceutical applications [2]. However, several major challenges hinder the development of new stem cell-based technologies. These challenges include the identification of new signals (*e.g.*, small molecules, hormones, proteins, etc.) and conditions that regulate and influence cell function, and the application of this information toward the design of reproducible stem cell bioprocesses and therapies. Therefore, the ability to interrogate signals that influence stem cell fate in a high-throughput manner will greatly impact our understanding of the mechanisms that regulate cellular responses [3].

In this context, microscale technologies are emerging as powerful tools for tissue engineering and high-throughput screening, and as cell-based probes in chemical biology [4]. With the advent of robotic spotting technology, it is now possible to distribute nanoliter volumes of different samples in a spatially addressable footprint. As a result, cellular microarrays can enable high-throughput, high-content (*e.g.*, protein-specific) parallel screening of a large number of small molecules. Such cell-based microarrays are particularly promising for studying the growth and differentiation of stem cells, and for investigating the influence of small molecules and cell growth conditions on cell physiology and function [5].

Herein we present a high-throughput, 3D cell-based microarray platform designed to allow the expansion and differentiation of embryonic stem (ES) cells, while being compatible with high-throughput screening applications. As a model system, we have studied the expansion and neural commitment of mouse ES cells on arrays of microscale 3D alginate spots. As a result, the identification of novel 3D microenvironments that regulate stem cell fate may be achieved using this platform.

II. GOALS

The main goal of this project is the development of microscale technologies to analyze the effect of soluble molecules and other signals during *in vitro* expansion and differentiation of ES cells. Small molecules and growth factors are being analyzed due to the reported capacity of those molecules to modulate intracellular pathways. We expect to contribute for a better understanding of the mechanisms that regulate cell fate providing a new opportunity for their use in transplantation therapies.

The originality of this work resides in the use of microscale technologies to analyze the effects of soluble factors and physical cues in the maintenance, expansion and differentiation of pluripotent stem cells. These cells provide new perspectives

for a multitude of potential therapies and treatments for a wide range of diseases and traumatic injuries.

III. Team and Institutions

This work is within the frame of the scientific activities currently being developed at the Department of Bioengineering (DBENG, IST) and Institute for Biotechnology and Bioengineering (IBB). In particular, it is being partially conducted at the Stem Cell Bioengineering Laboratory, which has been focusing for several years in the development of highly controlled culture systems for the *ex-vivo* expansion of stem cells. The research activities are also being conducted at the Center for Biotechnology and Interdisciplinary Studies (CBIS), Rensselaer Polytechnic Institute (RPI), Troy, NY, USA.

This project is being coordinated by Prof. Joaquim Sampaio Cabral, the IBB director and current head of the DBENG at IST, in collaboration with Prof. Jonathan Dordick, the director of the CBIS at RPI.

IV. Implementation

We have developed a microarray platform to enable the rapid and efficient tracking of stem cell fate and quantification of specific stem cell markers.

This platform consists in a miniaturized 3D cell culture arrayed on a functionalized glass slide for spatially addressable high-throughput screening. A non-contact microarrayer is used to spot cells on poly(styrene-co-maleic anhydride) (PS-MA) spin-coated glass slides to yield an array that consists of cells encapsulated in alginate gel spots with volumes as low as 20 nL. The utility of this platform was already demonstrated in high-throughput toxicity screening of drug candidates and their metabolites. To extend the use of this platform to other applications, we have developed a method based on an immunofluorescence technique scaled down to function on a cellular microarray. This on-chip, in-cell "Western-type" analysis allows quantification of specific cell marker protein levels on a microarray [4].

Overall the implementation of this project was successful, and the strategy and procedures used were also very useful to reveal potential mechanisms of ES cell response to specific microenvironmental perturbation [5].

V. Results

Cellular Microarray Fabrication and Characterization

In an effort to mimic the cellular niche, we used a 3D cell-on-a-chip technique involving alginate-based matrixes on a microscope slide (Figure 1). The glass surface of a slide was treated and functionalized using a reactive copolymer of polystyrene and maleic anhydride (PS–MA; Fig. 1A). 3D spots were formed by the deposition of an alginate solution containing cells on top of a PLL/$BaCl_2$ bottom layer. Under these conditions, the positively charged PLL serves as a substrate to bind Ba^{2+} ions and assist the attachment of the negatively charged alginate. The Ba^{2+} ions cause instantaneous gelation of alginate chains to give rise to 3D cell-containing matrix spots (60 nL) with a diameter of <800 µm and a center-to-center distance between adjacent spots of <1,200 µm (Fig. 1B). Confocal microscopy images (of 30 nL spots) also

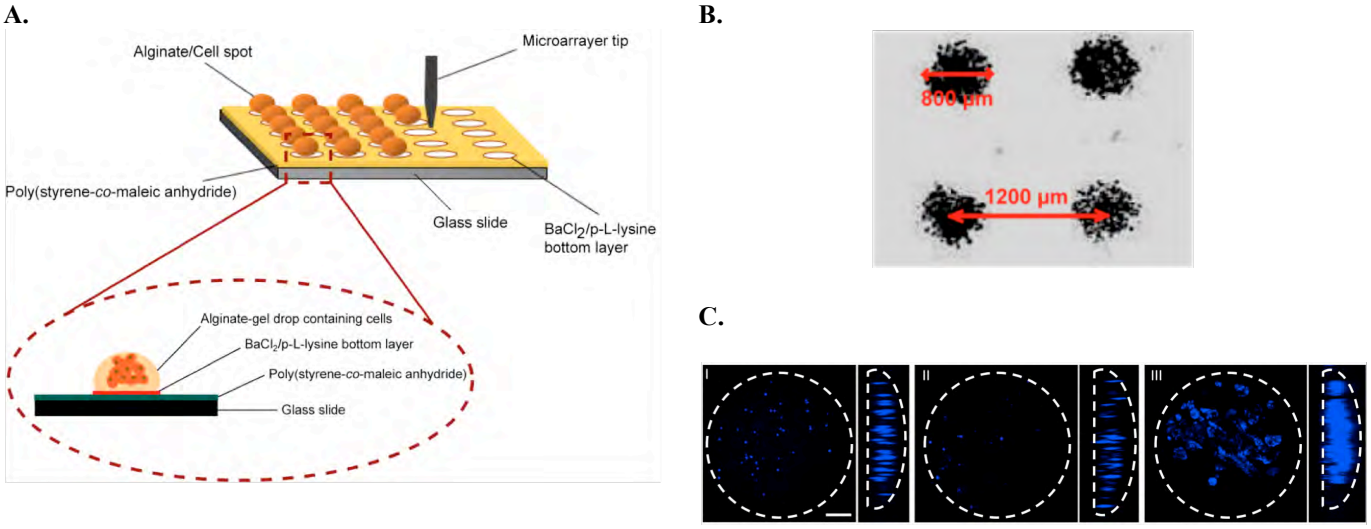


Figure 1. 3D cellular microarray platform for mouse ES cell culture. **A.** Cell encapsulation on a functionalized glass slide. The cells are spotted on PS–MA modified glass slides using a microarray spotter. **B.** Light microscope image of a portion of the cellular microarray (60 nL spots) depicting the spot diameter and center-to-center distance between adjacent spots. **C.** Confocal microscopy images showing the three-dimensional distribution of mouse ES cells on day 0 (I), day 2 (II), and day 5 (III) of incubation. Shown in each panel is the top view and side view of the spot, from a z-stack obtained with 4 µm sections. Scale bar is 100 µm. Each spot is 30 nL resulting in a diameter of 560 µm and a height of 150 µm.

indicate that encapsulated cells are evenly distributed inside the alginate spots and are in fact organized in a 3D environment (Fig. 1C). Cell viability was also confirmed after 5 days of culture, indicating that the hydrogel environment did not seriously affect cell viability, a result in agreement with previous studies of ES cell encapsulation in alginate hydrogels.

This spotting technique is therefore reproducible and requires low amounts of reagents for fabrication of the biochip, producing a functional microarray platform for high-throughput screening compatible with conventional microarray scanning for quantitative studies.

Assessing ES Cell Fate in the Cellular Microarray

In order to quantify the levels of specific markers of pluripotency *in situ*, we used an in-cell, on-chip immunofluorescent methodology to enable rapid quantification of the levels of cell marker proteins on the microarray platform [4]. In the present work, this procedure was used to stain mouse ES cells encapsulated in the alginate spots. We evaluated the levels of the pluripotency markers Oct-4 and Nanog *in situ*, and the in-cell, on-chip immunofluorescence methodology was found to be both sensitive and accurate for measuring levels of these proteins in response to leukemia inhibitory factor (LIF) (Fig. 2A). Hence, mouse ES cells retained their undifferentiated state after expansion in the 3D alginate spots when LIF was exogenously supplemented to the culture medium. However, when signals for alternative fates are eliminated, ES cells commit efficiently to a neural fate as a consequence of autocrine FGF signaling. Such a cell fate could also be evaluated using our microarray platform.

The use of a Sox1-GFP knock-in mouse ES cell line allowed us to quantify the levels of Sox1 expression that resulted of the neural commitment of cells. The alginate-matrix provided a favorable microenvironment for this process to take place, and after 6 days of neural commitment cells showed high levels of Sox1 expression (Fig. 2B).

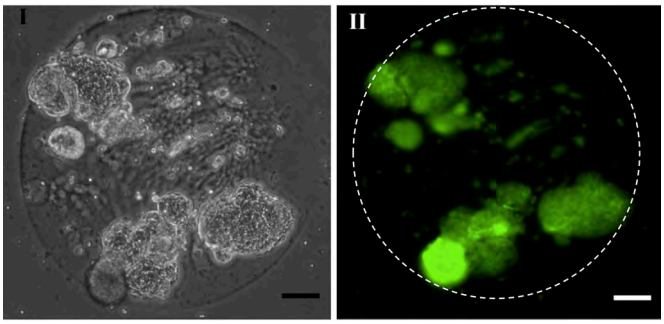
The high-throughput capacity of our microarray platform was also tested by contacting the 3D cell-spots with a mixture of retinoic acid and FGF-4. We used this approach to demonstrate the potential of this platform for screening small molecules and their effects in stem cell models. The small molecules were allowed to diffuse into the cell spots and induce a biological response. This resulted in irreversible differentiation of cells and consequent decrease in the expression of pluripotency markers even after incubation in LIF-containing medium for additional 3 days.

This procedure illustrates that the 3D cellular microarray can be tailored to function as a high-throughput screening platform to evaluate simultaneously the effects of different molecules, either alone or in combination, on stem cell fate.

VI. Conclusions

There exists an acute need to develop highly effective platforms for the rapid and quantitative study of stem cell fate. In this work we have addressed this need by fully integrating 3D cell culture with sensitive immunofluorescence detection in a microarray platform, enabling the assessment of potential stem cell fates in high-throughput. The 3D microarray spots provide individual cell cultures (without cross-talk between adjacent spots) and maintain the cells in a more *in vivo*-like microenvironment. As a result, it should be possible to identify parameters that regulate stem cell fate faster and more efficiently. This work takes us one step closer to stem cell-based high-throughput screening assays relevant to pharmacological and toxicological applications.

A.



B.

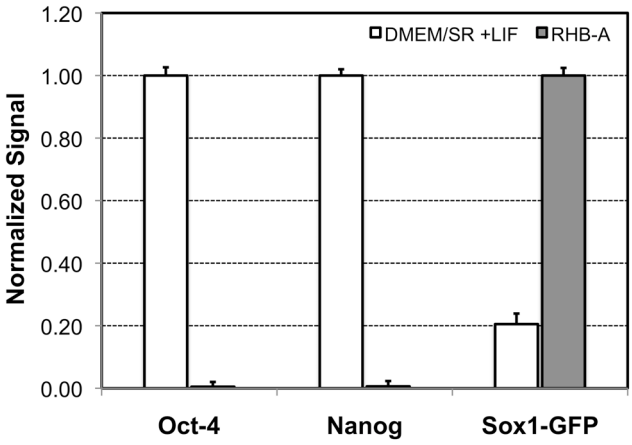


Figure 2. Assessing ES cell fate in the cellular microarray. **A.** Following expansion for 5 days in the alginate spots in serum-free medium containing LIF, mouse ES cells stain positively for Oct-4. Cells were inoculated at 100 cells/spot on 60 nL spots in DMEM/SR medium supplemented with LIF (I: bright field; II: fluorescence; scale bar: 100 μm). **B.** Quantification of Oct-4, Nanog, and Sox1-GFP for cells expanded in serum-free medium in the presence of LIF (DMEM/SR+LIF) and cells cultured for 6 days in neural commitment serum-free medium (RHB-A). Oct-4 and Nanog signals were normalized for β-actin and are relative to cells expanded in DMEM/SR+LIF. Sox1-GFP signals were normalized for β-actin and are relative to cells cultured in RHB-A medium.

VII. Planned Developments

Future developments will involve the use of rational and systematic approaches to study the influence of different stimuli in stem cell fate. In fact, rational approaches for stem cell culture and controlled differentiation in specific lineages are lacking, namely systematic analyses of the complex interactions that are involved in stem cell self-renewal and controlled differentiation. Such technological need represents the motivation for the continuation of this project and its overall goal is to establish robust and reproducible processes for the analysis of the expansion and differentiation of pluripotent stem cells.

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