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Influence of fabrication parameters in cellular microarrays for stem cell studies

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Abstract Lately there has been an increasing interest in the development of tools that enable the high throughput analysis of combinations of surface-immobilized signaling factors and which examine their effect on stem cell biology and differentiation. These surface-immobilized factors function as artificial microenvironments that can be ordered in a microarray format. These microarrays could be useful for applications such as the study of stem cell biology to get a deeper understanding of their differentiation process. Here, the evaluation of several key process parameters affecting the cellular microarray fabrication is reported in terms of its effects on the mesenchymal stem cell culture time on these microarrays. Substrate and protein solution requirements, passivation strategies and cell culture conditions are investigated. The results described in this article serve as a basis for the future development of cellular microarrays aiming to provide a deeper understanding of the stem cell differentiation process.

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1 Introduction

In the last few years, the success of using DNA microarrays has lead to researchers attempting to transfer the technology to protein microarrays, therefore allowing high throughput studies of protein interactions [1] and, more recently, studies of cell–microarrayed protein [2, 3] and cell–microarrayed DNA [4] interactions. These studies aim to provide screening tools for the design of improved biomaterials for applications in medicine, among other applications. Different approaches to culturing cells arranged in discrete microarrays can be found in the literature [5]. Microfluidic devices have been used to place cells on the substrate [6, 7], microcontact printing techniques have been used to pattern cell adhesion proteins in a discrete configuration [8, 9] and protein microarrays have also been performed by using standard photolithography techniques [10] and self-assembled monolayers [11].

In particular, the development of cell culture techniques on artificial microenvironments ordered in a microarray format, referred to as cellular microarrays, has been proposed as a major breakthrough in the study of factors affecting stem cell biology and differentiation and has been recently reviewed by several authors [12, 13]. Through these microarrays, a large number of differentiation factors can be immobilized on a surface in multiple combinations, thus creating complex microenvironments which differ at the biomolecular level, for interaction with stem cells [2, 3, 14, 15]. Such

microenvironments can be produced by varying the composition of extracellular matrix (ECM) proteins [2, 3] and growth factors [14, 16, 17]. Additionally, cellular microarrays may be implemented on a substrate that allows topographical structuring, thus allowing more complex signaling possibilities based on both chemical and topographical cues. For example, poly(methylmethacrylate) (PMMA) is a suitable material for this purpose. It has the advantage of being easily structured [18] while allowing the use of standard activation procedures to generate amino-reactive groups on its surface [19]. Both of these properties, combined with excellent optical properties, make PMMA a good candidate as a substrate for cell microarray assays.

Despite the potential for using cellular microarrays as a powerful high-throughput technique for the study of stem cell differentiation, real applications are still scarce. This is because they are hindered by a lack of consensus about the optimum parameters for standard microarray preparation procedures; the results published so far being highly application-dependant. Parameters such as the process for linking proteins to the substrate [10, 17, 19], the passivation of the non-printed surface [20, 21], and the medium used for cell culture [14, 20] must be optimized for particular cell types and periods of cell culture.

A highly attractive cell model with which to apply the potential capabilities of cellular microarrays are the mesenchymal stem cells (MSCs). The ability of MSCs to differentiate into several cell fates (such as osteoblasts, adipocytes and chondrocytes) has been widely explored during the last decade [22]. As a result, a lot of information is now available on the ways of inducing their differentiation to several cell fates [23, 24]. The usual differentiation strategies involve culture in tissue culture plates with a medium enriched by a combination of soluble differentiation factors and animal serum. This leads to the induction of predominant phenotypes for the desired fate after a certain period of time [22, 25, 26]. Current state of the art in MSC differentiation to osteoblasts allows following the differentiation process along several stages of the pathway, thus providing differentiation markers which could be interrogated from 48 h (Osterix expression) [27, 28], 8–14 days (ALP) [22, 29] or 21 days (matrix mineralization) [29]. MSC differentiation processes have also been shown to be responsive to both surface physical properties [9, 30] and specific growth factors added to the culture medium [28, 31]. MSC biology and differentiation by surface-immobilized molecules has already been studied using microcontact printing to immobilize fibronectin (Fn) and create islands of single cells [9]. However, a non-microcontact printing technique would be an advantage when dealing with cellular microarrays, since it would allow tailoring the composition of each individual spot within the array, and hence the high throughput study of cell–microarrayed protein interactions in each particular experiment.

It is important to distinguish between different approaches when studying the effect of printed protein microarrays on cells. While some authors create protein microarrays but culture cells in a monolayer over the whole substrate [10, 32], cellular microarrays are based on individual cell spots, whose composition can be easily adjusted to provide a high-throughput screening of mutually isolated cell microenvironments. Consequently, one desired property in cellular microarrays is isolation of the cell spots during the culture period. Previous studies on cellular microarrays report a large variation in the properties affecting the fabrication process [2, 3, 14, 17]. In this work, cellular microarrays were produced by means of protein deposition through a commercial microarray plotter machine. A range of parameters was studied, including the substrate, the cell culture media, the protein density on the microarray, the cell seeding density and the cell seeding time. These parameters were optimized to achieve MSC culture time periods long enough to allow following, in a future, some differentiation stages (up to 8 days) in Fn microarrays.

2 Materials and methods

2.1 Cell isolation and culture

Rat MSCs were obtained as primary cultures from the bone marrows of healthy 10–12 weeks old rats by means of standard procedures [33, 34]. Only early passaged cells (passages 3–6) were used in all the experiments to avoid changes in their stem cell phenotype. After cell seeding in the microarray, cell cultures were maintained in control medium (Advanced DMEM (GIBCO), 1% penicillin/streptomycin, 1% L-Glutamine and 10% FBS) or in a completely defined medium (Advanced DMEM, 1% penicillin/streptomycin, 1% L-Glutamine and 1% ITS (composed by insulin, transferrin, sodium selenite, BSA and linoleic acid, ITS + 1 Liquid media supplement, Sigma)). Culture medium was changed every 2 or 3 days.

2.2 Microarray fabrication

Cellular microarrays were fabricated by Fn deposition, on spots in a microarray format, followed by passivation of the non-printed surface and cell seeding (Fig. 1a). For the production of MSC cellular microarrays, several parameters affecting the array configuration and cell survival were assayed and optimized (see below).

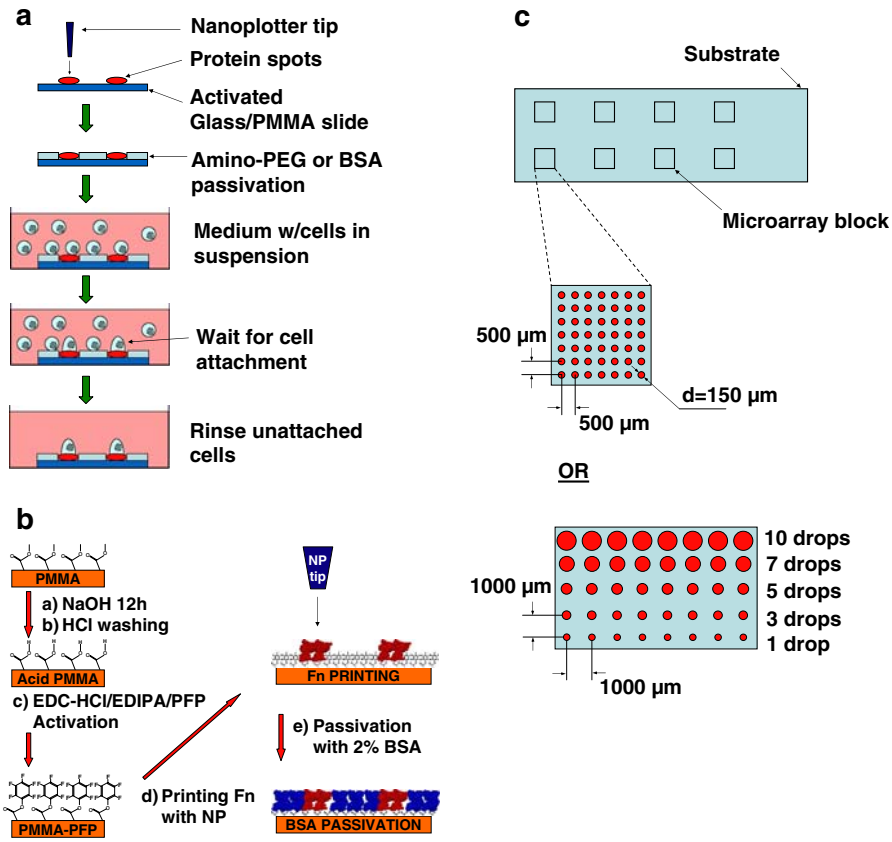

Fig. 1 Cellular microarray preparation. **a** Cellular microarray fabrication steps. **b** PMMA activation and printing. **c** Printed protein microarray layouts

2.2.1 *Substrates*

Two kind of substrates with different linking chemistries have been tested: commercial aldehyde derivatized (AD) glass slides (SuperAldehyde, Array It, USA) and in-house activated PMMA slides (Goodfellow, UK). For the glass slides, the proteins were linked to the substrate via covalent binding of the protein amino groups to the substrate aldehyde groups [1]. For the PMMA slides, the polymer was treated as described by Hyun et al. [19], and the treatment is presented in brief in Fig. 1b.

2.2.2 *Protein deposition in a microarray format*

Protein solutions of Fn at different concentrations (40, 100 and 200 µg/ml) were prepared in PBS, with and without glycerol. Volumes of 50–70 µl of these solutions were placed in wells of a 384 well culture plate. A robotic non-contact piezoelectric plotter (Nano-Plotter, GeSiM GmbH, Germany) was used to dispense the protein solutions onto the activated substrates in a square microarray format (8 blocks of 7 × 7 or 5 × 8 spots, Fig. 1c). The addition of glycerol (2% or 20%) to the Fn solution was used to delay spot dry out and different spot sizes were produced by overprinting single and multiple drops (1–10 consecutive drops, 0.4 nl in volume each). The distance between spots was set to 1 mm to avoid spot overlapping due to the increasing spot diameter. The printed slides were transferred to a light-tight sealed box and kept at 4°C for 2 (PMMA slides) or 24 h (AD glass slides) to ensure a proper protein–surface interaction. The protein density deposited (in µg/cm^2) is used in this article to refer to the spots Fn composition. This value may differ from effective protein immobilized on the substrate due to protein wash off. Here, it has been calculated from the Fn concentration printed, the drop volume, the number of drops printed per spot and the spot area measured empirically.

2.2.3 *Surface passivation*

In order to block the non-printed surface area two passivation strategies were tested. The printed slides were placed in Petri dishes and filled with 2% BSA in PBS or, alternatively, with an amino-PEG6000 (*O,O′*-Bis(2-aminoethyl)polyethylene glycol M.W. = 6000, from Sigma) solution in PBS (38 mg/ml), and incubated for 90 min to block the non-printed surface. Afterwards, the blocking solution was removed and the slides were allowed to dry for 120 min.

2.2.4 *Cell culture on the protein microarrays*

A flexiPERM (Greiner Bio-One GmbH, Germany), previously immersed for 20 min in 70% ethanol and exposed to

UV light for 15 min, was placed on top of the passivated slides and aligned with the blocks of microarrays to create 8 individual wells per slide. This allowed testing several parameters in the same slide. Each flexiPERM well was seeded with cells at densities ranging from 5,500 cells/cm^2 to 110,000 cells/cm^2 and cultured over different seeding times. Afterwards, the flexiPERM was removed, the slide was placed into a Falcon tube filled with pre warmed medium and centrifuged at 1000 rpm for 5 min to remove unbound cells between spots. Cellular microarrays were further cultured for periods of time ranging from 7 to 15 days, and medium, either 10% FBS or 1% ITS, was replaced every 2 or 3 days.

2.3 Cellular microarray characterization

Microarray layout and cell morphology were examined by optical microscopy during cell culture. At days 0 and 8 the cellular microarrays were characterized. For immunostaining of Fn and nuclei, cells were fixed (3% paraformaldehyde), permeabilized for 10 min in Triton 100× (0.05% solution in PBS-Glycine) and the slides were blocked with BSA (1% in PBS-Glycine) for 20 min. Afterwards, the slide was incubated with primary antibodies (anti-fibronectin, Sigma), followed by incubation with secondary antibodies (goat anti-rabbit Alexa Fluor 568, Molecular Probes) and Hoechst. Dried samples were mounted in Mowiol plus antifade and imaged using a fluorescence microscope.

2.4 Statistics

All measurements of cell survival were performed on duplicate samples of two separate experiments ($n = 4$) and the data presented consist of representative results. Cell counting and spot size measurements were completed with the aid of Photoshop software. Parametric one-way ANOVA tests were performed on the statistical analysis of variables plotted. All graphical data is reported as mean ± standard deviation. Significance levels were set at $P < 0.05$ and $P < 0.01$.

3 Results

3.1 Protein deposition in microarray format

Fn was found to adhere strongly to the activated PMMA and AD glass slides, as the protein spots remained clearly identifiable after several cycles of incubation with 2% BSA and rinsing with medium. Within the assayed range, an increase in Fn concentration within the protein solution was translated to a higher density of immobilized Fn (shown as an increase in the fluorescence signal in Fig. 2a).

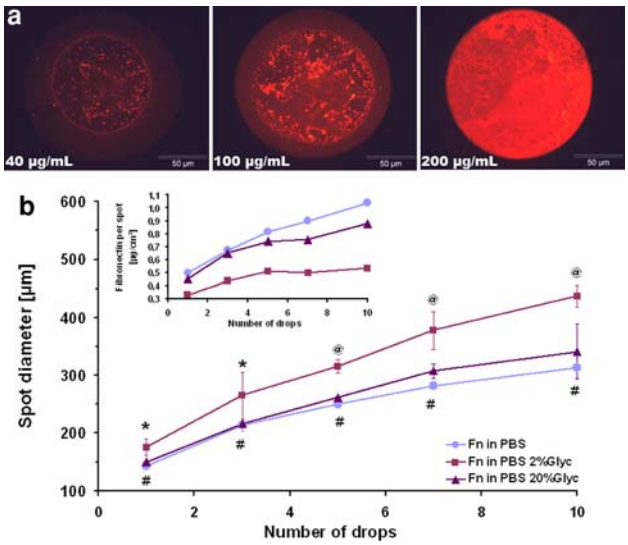

Fig. 2 Effect of printed Fn concentration. **a** Immuno fluorescent staining images of Fn spots (*red*) printed at 40, 100 and 200 µg/ml in PBS. **b** Relationship between the number of printed drops and the spot diameter for 200 µg/ml of Fn. Significant differences: * $P < 0.01$ and @ $P < 0.05$ (Fn in PBS with 2% glycerol), # $P < 0.05$ (Fn in PBS with 20% glycerol and Fn in PBS, One-way ANOVA test). *Inset*: Relationship between the number of printed drops and the amount of Fn deposited per spot. Lines are included as a guide only [refer colour figure in online]

Protein spot size in the microarray can be tailored by increasing the number of drops of protein solution deposited at a single position. Figure 2b shows the relationship found between drop number (for 1–10 consecutive drops) and the resulting spot diameter. The Fn density per spot as a function of the number of deposited drops is also presented in Fig. 2b (Inset). As the number of drops increases, the density of Fn deposited in the spot also increases, in the range 0.3–1 µg/cm^2.

The inclusion of glycerol in the protein solution resulted in an additional enlargement in the diameter of the printed spots (Fig. 2b). Interestingly, this increase was statistically different only for low (2%) glycerol concentration (26% larger spot diameter, measured value for 5 drops, $P < 0.05$). When a higher glycerol concentration was used (20%), no differences in the spot size were observed when compared to the glycerol-free protein spots.

3.2 Surface passivation

The efficiency of 2% BSA and Amino-PEG 6000 solutions as passivation agents was tested for both AD glass and PMMA substrates. It was found that BSA efficiently blocked cell adhesion outside the printed area in both substrates. The use of amino PEG 6000 did not prevent cell colonization outside the spots for cellular microarrays on AD glass slides, while showing passivation efficiency similar to that of 2% BSA when using activated PMMA substrates.

3.3 Cell culture on protein microarrays

3.3.1 Effects of cell seeding time

The cellular microarrays were analyzed with respect to different cell seeding times, for a 110,000 cells/cm^2 seeding density (Fig. 3). The number of cells adhered to the spots increased with increased seeding time, resulting in an important increase in the number of cells per spot for each seeding time presented (4.5 cells/spot for 10 min, 9.6 cells/spot for 30 min and 16 cells/spot for 60 min, data from Fig. 3). However, more cells could also be found on the passivated areas with longer seeding times. Therefore, although a seeding time of 60 min forms a nice array, the layout is lost at day 1 due to cell migration from the spots. Shorter seeding times are preferred, since they allow cells to spread while still keeping the microarray layout.

3.3.2 Effects of cell seeding density

MSCs were seeded at densities of 5,500, 11,000 and 110,000 cells/cm^2 for 15 min and cultured in 1% ITS medium. The evolution of the number of cells attached to each spot is shown in Fig. 4.

For a 5,500 cells/cm^2 seeding density, only a few cells remained attached and uniformly distributed on the substrate at day 0. The array layout was not distinguishable and therefore cells located on the spots could not be determined. However, at day 1, cells migrated to the printed spots (Fig. 4).

For a cell seeding density of 11,000 cells/cm^2, the number of cells attached per spot did not show a statistically significant difference until day 6 ($P < 0.05$). This means that the number of cells in the spots remained stable for cell culture periods of 4–6 days, allowing us to conclude that an excellent cell survival occurs for this cell seeding density.

Finally, when seeding MSCs at a density of 110,000 cells/cm^2, after day 1, a 60% decrease in the mean value ($P < 0.05$) of the cells attached per spot was observed (Fig. 4, Inset). After 6 days of cell culture in the microarray, no cells could be seen on the spots. This cell seeding density therefore impedes cell culture beyond 4 days and cell survival is reduced when compared to the lower cell seeding densities.

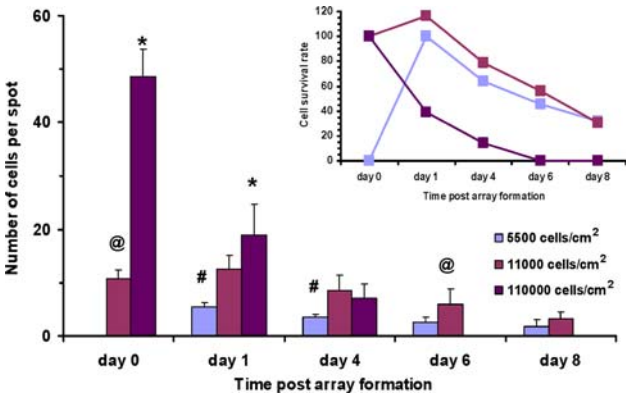

Fig. 4 Effect of cell seeding densities. Temporal plot of the number of cells per spot for increasing cell seeding densities, 8 days follow up ($n = 4$). Results are for cellular microarrays formed using AD glass slides and a spot size of five drops of Fn 200 μg/ml in PBS 2% glycerol. Bars marked with *, @, and # denote a statistical difference of $P < 0.05$ (One-way ANOVA test). *Inset*: Cell survival temporal plot expressed as a percentage of the initial number of cells per spot at day 0 (for 11,000 and 110,000 cells/cm^2) or at day 1 (for 5,500 cells/cm^2)

3.3.3 Effects of cell culture medium

After formation of the cellular microarrays, two culture media were assayed for consolidating the array layout over time. Medium containing 10% FBS had undesired effects from day 1, since cells migrated from the printed Fn spots and invaded the passivated area. Using a protein-free medium composed of 1% ITS, cells remained on the protein printed spots for the cell culture periods assayed (up to 8 days). This is a suitable alternative medium, which provides a very basic buffer for cell sustenance and culture detoxification.

3.3.4 Effect of spot size and spot composition

Results show that increasing the Fn concentration in the spotted printed solutions lead to a higher number of cells

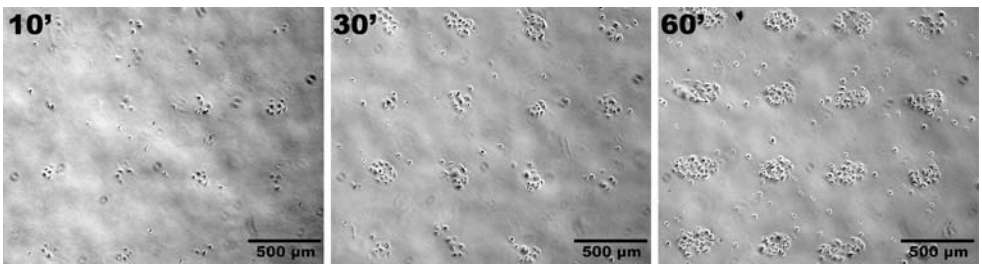

Fig. 3 Effect of cell seeding times. Phase contrast microscopy images of the cellular microarray for different cell seeding times (10, 30 and 60 min) and 110,000 cells/cm^2 seeding density. Microarray formed using PMMA substrates, Fn spots with a concentration of 40 μg/ml in PBS 2% Glycerol and 2% BSA passivation. After passivation the arrays were allowed to dry for 120 min

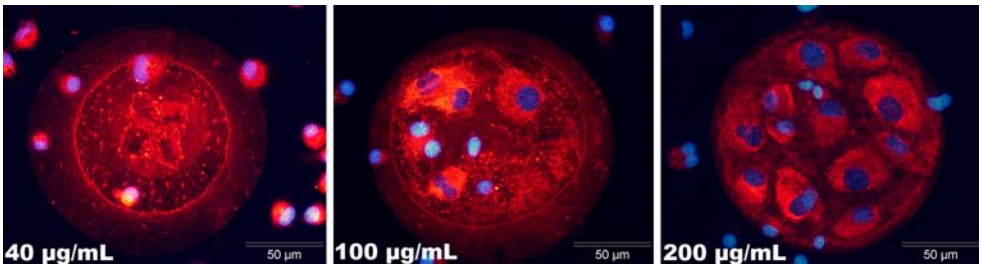

Fig. 5 Effect of spot composition. Fluorescence microscopy images of Fn spots (red immunostaining) printed at 40, 100 and 200 µg/ml in PBS. Cell nuclei are stained in blue. Microarray formed using AD glass substrates, 110,000 cells/cm^2 seeding density, 30 min cell seeding time and 2% BSA passivation [refer colour figure in online]

attached to the spots (5, 8 and 12 cells/spot for spots printed using Fn at concentrations 40, 100 and 200 µg/ml, respectively, Fig. 5). The Fn concentration yielding the best results in this study was 200 µg/ml. Results for cell survival related to the spot size are shown in Fig. 6. For 3 and 5 drops, cells attached on the spots at day 0 (Fig. 6b) and formed well defined cell spots at day 6 (Fig. 6c). Figure 6d shows that for 3 and 5 drops, at day 8, more than 40% of cells survived. Larger spot sizes resulted in a higher number of cells attached per spot at day 0, but at day 6 only few cells remained. Figure 6d shows that only 20% of cells survived at day 8. For a spot size of 1 drop, cells barely attached to the spots. The addition of glycerol to the protein spots resulted in a larger number of cells attached (Fig. 6e). However, the highest concentration (20%) led to a decrease in cell survival rate at day 8.

4 Discussion

One of the most interesting applications of cellular microarrays comes through the high throughput study of stem cell differentiation process at different stages by examining the response of cells to surface-immobilized factors. For this application, each spot in the cellular microarray functions as an artificial microenvironment that will have a particular effect in stem cell commitment. As previously demonstrated, stem cell differentiation is governed both by surface topography [35] and biochemical cues [14]. Therefore, in order to approach stem cell microenvironments found in nature, cellular microarray fabrication on substrates that allow 3D topography modifications and biochemical signaling is an asset. PMMA can be molded to incorporate micro and nanostructures on its surface [18] and can be chemically activated [19] to effectively support protein microarray formation. Here, we show that PMMA can be used for cellular microarray fabrication and it has been successfully tested with MSCs for cell culture periods up to 8 days.

A key point of concern when analyzing cell response to surface immobilized factors is to avoid undesired signaling from animal serum. It is well known that FBS contains ECM proteins, growth factors and hormones in unknown and variable quantities [36]. Therefore, its omission is highly desirable when dealing with cellular microarrays. It has been reported that BSA can activate cell adhesion proteins such as Fn by the modulation of its conformation [37], thus increasing its biological activity. When using FBS, our cells attached to the printed spots but then migrated onto the BSA passivated area due to the presence of adhesive proteins (Fn, vitronectin, collagen, etc.) at low concentrations on the BSA layer. A suitable serum replacement, ITS, is commonly used for chondrocyte differentiation because FBS contains factors that block cell differentiation towards this fate [38, 39]. In this work, ITS provides two advantages when culturing cells in a microarray format. Firstly, ITS is a completely defined serum substitute that prevents the adverse effects of factors affecting MSC differentiation. Secondly, ITS limits cell migration from the printed spots and the viability of attached cells on the passivated area is impeded due to the absence of adhesive proteins.

The density of the attached cells per spot (Fig. 4) is a critical factor when defining the application of the cellular microarray. For applications that require cell culture during short time periods (less than 24 h), a high initial cell density is preferred. This has the advantage of providing interactions between the cell and the printed factors throughout the whole spot from the beginning of the experiment. However, it can be detrimental for cell culture periods of several days, as cells proliferate and cover the area of the protein spots too quickly. This causes the stem cells to grow outside the spot circumference, or to form 3D structures and eventually detach [2], depending on the characteristics of the cellular microarray.

The density of cells per spot can be tailored by modulating the cell seeding time, the cell seeding density and the Fn density printed in the spot. Optimum values for these parameters are highly dependent on the passivation agent used and the cell type cultured in the microarray. Cell seeding times reported in the literature are extremely wide, ranging from a few minutes [14] to several hours [2]. Figure 3 shows an optimal seeding time ranging from 10 to

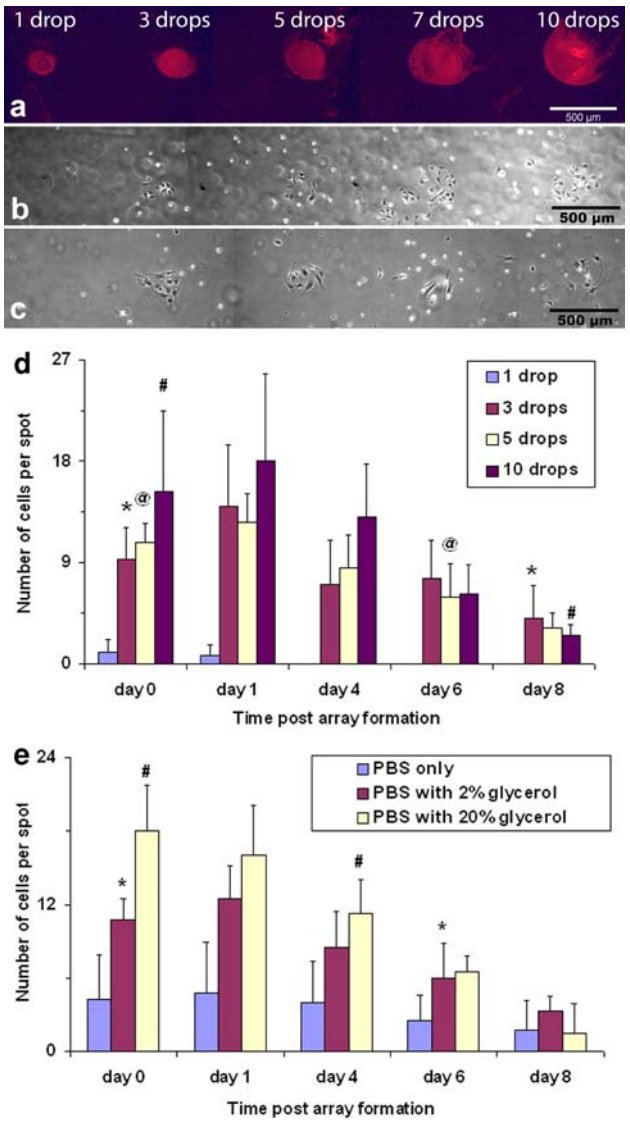

Fig. 6 Effect of spot size. Cellular microarray formed using AD glass substrates and 11,000 cells/cm^2 seeding density. Fluorescence (**a**) and phase contrast microscopy images of the microarray for increasing spot sizes of 200 µg/ml Fn in PBS 2% glycerol. (**b**, **c**) Images show the cell adhesion to each of the spots at day 0 (**b**) or at day 6 (**c**). **d**, **e** Temporal plot of cell survival for 8 days follow-up ($n = 4$). **d** Results for different spot sizes (Fn 200 µg/ml in PBS 2% glycerol). **e** Results for different glycerol percentages included in the spot (Fn 200 µg/ml, 5 drops spot size). Bars marked with *, and # denote a statistical difference of $P < 0.05$ (One-way ANOVA test)

30 min. Larger seeding times resulted in cells exceeding the spot circumference and loss of the microarray configuration.

Cell seeding density plays an important role in cellular microarray formation. However, the effect of this parameter was not so evident in previous studies [2, 3, 14]. These reports used high cell densities (>40,000 cells/cm^2) to produce almost confluent cell spots. In contrast, we show that lower cell seeding densities (11,000 cells/cm^2) produced microarrays which allowed cell culture periods up to 8 days using MSCs (Fig. 4). Higher cell seeding densities promoted the formation of confluent cell spots in the array, but the viability of these cells was reduced when cultured for several days. Probably, the attached cells compete for spreading room, and, as this is not supported by the culture medium and substrate passivation, many of them die, releasing toxic chemicals (e.g., proteases) that have a detrimental effect on their neighbor cells.

In this work, different Fn densities have been shown to affect the cell density per spot (Figs. 4, 6). The Fn density depends on the Fn concentration in the initial solution, the glycerol concentration and the number of drops printed on the microarray spot. Protein printing in PBS buffer [10] and in PBS buffer supplemented with 2% [14] or 20% [2] glycerol was chosen (Fig. 6) based on previous literature reports. Low Fn densities (0.08 µg/cm^2, produced when printing 1 drop of 40 µg/ml in PBS 2% glycerol) with glycerol, required a drying step before cell seeding to improve the interaction between the printed Fn and the surface. On the other hand, higher Fn densities (0.33 µg/cm^2, produced when printing 1 drop of 200 µg/ml in PBS 2% glycerol) allowed cellular microarray formation without the drying step. When compared to 2% glycerol, a 20% glycerol/Fn solution resulted in a smaller spot size. The printed spots do not dry at this glycerol concentration, thus some of the Fn printed could remain within the glycerol solution and be removed during passivation and washing of the slide. To optimize cell viability, a spot size of 3 or 5 drops (yielding spots of 265 and 315 µm diameter with 0.43 and 0.51 µg/cm^2 Fn density, respectively) was revealed to be the optimum dimension. This allowed a compromise to be struck which produced relatively small quantities of cells per spot but with a high rate of cell survival for periods up to 6 days. The Fn concentrations reported here are slightly lower than those described by the Fn provider (1–5 µg/cm^2) for use as cell culture substratum. Successful MSC attachment to these spots can be related to the highly adhesive characteristic of the cells used [33].

Finally, the washing step had to be customized in such a way that it removed many of the cells attached to the passivated area, while leaving arrayed cells attached to the protein spots. This prevents cells adhered to the passivated area corrupting the microarray layout. Centrifugation as a method to enhance cell seeding has recently been reported for cellular microarrays [40]. Here, an adapted centrifugation strategy was used to remove non-adhered cells, allowing us to standardize the washing step. This did not damage cells (tested using Calcein AM [data not shown]) and allowed us to culture MSCs for periods up to 8 days.

Summing up, cell survival up to 8 days for five different spot sizes (1, 3, 5, 7 and 10 drops), three Fn concentrations

(40, 100, 200 μg/ml), three buffer compositions (PBS and PBS with 2% or 20% glycerol) and three different cell seeding densities have been reported here, and were analyzed in up to 120 parameter combinations (40 spots for each cell seeding density) within a microscope slide in each experiment. The cellular microarray was successfully fabricated using PMMA substrates. Optimization experiments defined a cell density of $\sim$10 cells per spot and a Fn density of 0.43–0.51 μg/cm^2 (265–315 μm spot size) as the optimum values for a cell culture period of 8 days. Further efforts should be made to optimize the culture medium to allow a higher cell survival rate after 8 days in culture. The results described in this article are intended to serve as a base for future development of cellular microarrays aiming to provide a deeper understanding of the MSC differentiation process by introducing adequate growth factors in the pre-mixed protein solutions to be printed.

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