Continuous Monitoring of Cell Transfection Efficiency with Micropatterned Substrates

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ABSTRACT

The effect of cell-cell contact on gene transfection is mainly unknown. Usually, transfection is carried out in batch cell cultures without control over cellular interactions, and efficiency analysis relies on complex and expensive protocols commonly involving flow cytometry as the final analytical step. Novel platforms and cell patterning are being studied in order to control cellular interactions and improve quantification methods. In this work, we report the use of surface patterning of fibronectin for the generation of two types of mesenchymal stromal cells patterns: single cell patterns without cell-to-cell contact, and small cell-colony patterns. Both scenarios allowed the integration of the full transfection process and the continuous monitoring of thousands of individualized events by fluorescence microscopy. Our results showed that cell-to-cell contact clearly affected the transfection, as single cells presented a maximum transfection peak 6 hours earlier and had a 10 % higher transfection efficiency than cells with cell-to-cell contact.

Keywords: Surface Patterning; Cell Patterning; Gene Transfection; Cell-cell Contact; Continuous Monitoring; GFP; Mesenchymal Stromal Cell.
1. INTRODUCTION

The process of gene transfection refers to the manipulation of eukaryotic cell’s genome. Most commonly, gene transfection is based on the insertion of exogenous genetic material into a cell for its expression using the cell’s own gene expression process. In some cases, the exogenous gene is completely integrated into the genome of the cell. More recently, gene transfection has also been centered in the incorporation of editing tools for cell’s genome through the generation of sequence-specific strand breaks and the synthetically guided modification of a desired gene (Rose, 2003; Yang et al., 2014). It constitutes the base of gene therapy, which aims to cure and treat genetic-related diseases and disorders through the insertion of specific genes or the manipulation and editing of existing, pathological genes (Kaufmann et al., 2013). Cell cultures are commonly used to carry out the transfection of cells and test, quantify and optimize transfection protocols that could lead to the development of efficient gene therapies (Azuaje-Hualde et al., 2017a; Hamann et al., 2019).

One major parameter studied to address the potential of a transfection protocol is the gene transfection efficiency, defined as the proportion of cells that, after exposure to transfection reagents, incorporate the genetic material inside their genome and express the desirable product. The efficiency of a transfection is directly related to the delivery system used, the design of the nucleic acid sequence, the target cell type and the cell cycle (Kim et al., 2015; Yin et al., 2014). Usually, analysis of the gene transfection efficiency consists on the transfection of a gene that encodes the expression of a fluorescent protein reporter, such as green fluorescence protein (GFP), to cells seeded in conventional cell culture plates. Quantification of the efficiency is most commonly carried out by analysis of detached cells or non-adherent cells using flow cytometry, which allows the accurate quantification of the total number of cells as well as the quantification of the number of cells expressing the fluorescent protein. Microscopy imaging of attached cells is also widely used, where the images are compared to address the ratio of transfected cells over the total number of
cells, usually relying on complex imaging processes or secondary dies for quantification (Marjanović et al. 2014; Usaj et al. 2011; Peng et al. 2017).

While conventional analysis of the transfection efficiency is undeniably optimal in many cases, it also presents two major limitations that could affect the analysis. The first one is the lack of control over cell-cell interactions. Conventional cell culture lack control over the many cell-cell interactions and therefore, may not represent the best conditions for gene transfection. Moreover, it has been demonstrated that cell-substrate and cell-matrix interactions as well as cell architecture can affect cells behavior and in turn affect cell transfection (Azuaje-Hualde et al., 2017b; Mantz and Pannier, 2019). Over the past decade, several studies helped to better understand and control the interactions that may affect gene transfection. For example, Dhaliwal et al. demonstrated that the interaction with the extracellular matrix heavily influences the process of transfection, since different mechanisms of gene transfection could be observed in regular seeded cells compared to cells cultured in a three-dimensional hydrogel scaffold (Dhaliwal et al., 2013). Modaresi et al. demonstrated that the stiffness of the substrate influenced plasmid internalization due to the role of stress fibers associated with the endocytosis of the carriers (Modaresi et al., 2018). Shui et al. showed that surface patterning on superhydrophobic areas improved cell adhesion, separation of cell colonies and transfection efficiency in comparison to flat poly-D-lysine substrates (Shiu et al., 2010). The effect of the substrate hydrophobicity of patterned cells (Ueda et al., 2016) and the chemical interactions (Fujita et al., 2013) of high density cell-clusters on the transfection efficiency were also studied. Combined pattern of transfection reagents and extracellular matrix proteins into the surface, commonly known as reverse transfection, were employed. Recently, Yang et al. demonstrated that cell spreading affects and enhances gene transfection on patterned single cells (Yang et al., 2019).

Besides the lack of control over cell-cell interactions, the second main limitation found in conventional transfection efficiency studies is related to the methods of analysis. Flow cytometry, the most commonly used analytical methodol, has a relative high cost of materials and equipment,
while requires trained personnel. On top of that, the different steps required during the analytical process, like detachment of cells or incorporation of secondary dyes, may affect cell state in a way that can affect the analysis of gene transfection. Furthermore, any process that requires cell fixation or detachment functions as end-of-assay analysis, disabling the possibility for real-time and continuous monitoring of gene transfection (de Carvalho et al., 2018; Cho et al., 2010; Hahnenberger and Chan, 2001; Han et al., 2016; Marjanović et al., 2014). The current trend to facilitate and speed up data collection, as well as to incorporate non-invasive analytical measurements, is the design of platforms that provide high throughput real time screening capabilities. Microfabrication has enabled the development of miniaturized platforms with multiple interrogation sites that allow high throughput screening of transfection conditions. For example, microfluidics platforms such as droplet microfluidic systems enabled single cell transfection of hard-to-transfect cell lines with improved transfection efficiency (Li et al., 2018). Woodruff et al. developed a microfluidic device based on individual cell culture chambers that allowed the simultaneous monitoring of 280 independent transfections, achieving 99% transfection efficiency (Woodruff and Maerkl, 2016). Later, Guipponi et al. also developed an easy to use lab-on-a-chip device with cylindrical culture chambers that allowed the high-throughput analysis of transfection to compare vector efficiencies (Giupponi et al., 2018).

Overall, there is an increasing interest in developing new platforms for the analysis of gene transfection efficiency in order to improve the control over cell interactions, facilitate the method of analysis, or a combination of both. In particular, surface micropatterning is a methodology to produce localized deposition of adhesion proteins on a surface and to enable the formation of cell patterns with controlled cell-cell contact. These cell arrays have been proven to be suitable for gene transfection, having the potential to overcome the limitations of conventional cell cultures (McConnell et al., 2011). Thanks to the versatility of the technique, great control over cell interactions can be achieved, including cell-material and cell-cell interactions, while also enabling the easy generation of high number of data points in a small substrate area (Garcia-Hernando, Calatayud-Sanchez, Etxebarria-Elezgarai, De Pancorbo, et al., 2020; Gonzalez-Pujana et al., 2019a; Hamon
et al., 2016; Hu, Chen, Zhao, Wang, & Lam, 2018, (Kobel and Lutolf, 2010).. While previous studies have addressed how various types of cell interactions affect gene transfection, the degree in which cell-cell interaction and physical contact between cells affect the transfection processes is still mostly unknown. As cell-cell contact is a major factor in several transcriptional and translation pathways, it is expected to influence gene transfection. Cells patterns can be directly transfected and monitored, without the need of detaching and transporting the cells to a different setup, as the pattern itself simplifies quantification due to the highly controlled localization of the cells.

In this paper, we report on the use of surface micropatterns to create cell arrays and assess the effect of cell-cell contact on the efficiency of gene transfection, as well as to monitor the progress of the transfection process over time. Primary human Hair Follicles Mesenchymal Stromal Cells (hHF-MSCs) were chosen due to their potential use in regenerative medicine and their hard-to-transfect nature (Li et al., 2018).

2. MATERIALS AND METHODS

2.1. Materials

Primary human Hair Follicles Mesenchymal Stromal Cells (hHF-MSCs) were obtained from human follicles (passages 6 to 9). Bovine plasma fibronectin, Dulbecco’s modified eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (P/S), Gibco trypan blue solution (0.4 %), 4’,6-diamino-2-phenylin (DAPI) dye, phalloidin dye, lipofectamine stem transfection reagent and optiMEM I reduced serum medium were purchased from Fisher Scientific, Spain. Green Fluorescence Protein (GFP) encoding plasmid pCXLE-EGFP was purchased from Addgene, USA. Bovine serum albumin and propidium iodide were purchased from Sigma Aldrich, Spain. Polydimethylsiloxane silicone (PDMS) elastomer and curing agent were purchased from Ellsworth adhesives, Spain. Paraformaldehyde 4 % for fixation was purchased from Panreac Quimica, Spain. Brightfield and fluorescence images were taken with a modified Nikon Eclipse TE2000-S (USA) microscope with a LUMENCOR laser light source (USA) and Zylar sCMOS camera (Oxford
Instruments, UK). Flow cytometry analysis were carried out with a FACS Calibur system from Becton Dickinson. Microscopy images were processed by Fiji/ImageJ software. Flow cytometry data was analyzed by Summit Software v4.3 (Dako) and Gallios software v1.2 (Beckman Coulter). Rocker Vari-Mix steep angle rocker (Thermo Fisher) was used for cell patterning.

2.2. Patterning of single hHF-MSCs and small hHF-MSC-colonies

Patterning of hHF-MSCs was obtained by incubation of a cell suspension inside a cell culture-well containing a pattern of fibronectin dots created by microcontact printing. Microcontact printing of fibronectin was performed as described elsewhere (Gonzalez-Pujana et al., 2019; Langan and Chou, 2011a). Briefly, PDMS (ratio 10:1 of silicone and curing agent) was polymerized on top of silicon wafers containing holes of 20 µm or 100 µm diameter holes with 50 µm of separation between two holes, creating stamps with either 20 µm or 100 µm diameter pillars. PDMS stamps were wetted with 50 µL of a 50 µg·mL⁻¹ fibronectin solution in PBS for 30 min. Afterwards, the ink was removed and the PDMS stamps were rinsed with distilled water and dried with compressed air. Each PDMS stamp was put in contact with the bottom plate of the well in a 12-well microtiter plate for another 30 min, in order to transfer the protein from the PDMS stamp to the substrate and create small dots of fibronectin. Finally, PDMS stamps were removed and the wells were blocked with 1 mL of BSA solution 1% (w/v). The total area of the printed pattern in each well was 1 cm². 20 µm (D₂₀) patterns were comprised of 20000 fibronectin dots with a 20 µm diameter and a 50 µm separation between dots. 100 µm (D₁₀₀) patterns were comprised of 4500 fibronectin dots with a 100 µm diameter and a 50 µm separation between dots. D₂₀ patterns were used to create single cell patterns. D₁₀₀ patterns were used to create small cell-colony patterns.

To attach the cells to the printed fibronectin D₂₀ and D₁₀₀ patterns, hHF-MSCs were incubated with complete medium (CM) in cell culture flasks until reaching 80 % confluence. CM consisted of DMEM supplemented with 30 % FBS and 10 % P/S. hHF-MSCs were detached from the flask, centrifuged and resuspended in serum-free medium (0% FBS) for a concentration of 100000 cells mL⁻¹. Serum-free medium for incubation on patterning and maintenance consisted in DMEM with 10 % P/S. 1 mL
of the cell suspension was added to each printed well and were left for 2 h on constant oscillation in a rocker inside an incubator at 37 °C and 5 % CO₂ air atmosphere. Afterwards, the remaining suspension was removed and the wells were rinsed 3 times with PBS, Figure 1A.

For the quantification of the number of cells per dot (dot occupancy), cell area and cell aspect ratio, cells were fixated with formaldehyde and then dyed with phalloidin for 30 min and with DAPI for 5 min. Images were taken by brightfield and fluorescence microscopy and were analyzed using ImageJ.

In order to evaluate the stability of the hHF-MSCs patterns, different maintenance mediums, varying the FBS concentration, were tried on hHF-MSCs D₁₀₀ patterns. After adhesion of the cells to the fibronectin pattern, the culture medium was changed to maintenance medium, containing different concentrations of FBS (0 %, 5 %, 10 % and 20 %), and samples were left inside of the incubator for 24 h. Afterwards, images were taken by brightfield microscopy to check the conservation of the cell pattern.

For the study of the viability of the patterned hHF-MSCs on serum free medium, substrates containing D₂₀ and D₁₀₀ cell patterns were kept for 72 h in maintenance medium 0 % FBS with daily medium changes. Every 24 h, brightfield microscopy images were taken. On the last day, cells were dyed with trypan blue to verify the survival of the remaining cells. Cell viability was calculated following equation 1. Images were analyzed using ImageJ.

\[
\text{Patterned cells viability} = \frac{\text{Patterned cells (tx)}}{\text{Patterned cells (t0)}} \times 100
\]  
(eq. 1)

2.3. Transfection of hHF-MSCs

Transfection process was adapted from the commercial lipofectamine protocol. Both, the lipofectamine Stem Reagent and the pCXLE-EGFP were diluted in the serum free Opti-MEM medium at different concentrations, to generate four different transfection mixtures, the Transfection mix-1 containing, 0.80 % (v/v) lipofectamine and 0.40 % (v/v) GFP plasmid in Opti-MEM medium;
the transfection mix-2 containing 0.80 % (v/v) lipofectamine and 0.80 % (v/v) GFP plasmid in Opti-MEM; the transfection mix-3 containing 1.60 % (v/v) lipofectamine and 0.40 % (v/v) GFP plasmid in Opti-MEM; and transfection mix-4 containing 1.60 % (v/v) lipofectamine and 0.80 % (v/v) GFP plasmid in Opti-MEM. Transfection mixtures were incubated at room temperature for 10 min to form the DNA-lipid complexes.

To study the transfection efficiency of mix-1, 2, 3 and 4 in hHF-MSCs cultured in conventional cell culture well plates, hHF-MSCs were cultured in 24-well plates at 20000 cells per well and incubated to reach a confluence of about 70 % or 90 %. Before transfection, the culture medium was replaced by one of the previously listed lipofectamine/GFP plasmid mixtures. 4 h after transfection, the medium containing the mixtures was replaced by DMEM culturing medium and cells were cultured for another 24 h. GFP expression was quantified using flow cytometry. Cytotoxicity and cell viability was evaluated with propidium iodide using flow cytometry. Two tailed t-student statistical analysis was carried out using Excel software.

The same transfection methodology was followed for patterned hHF-MSCs in all assays. To test the transfection of patterned cells, cell-D100 patterns were incubated after adhesion with 600 µL of either transfection mix-3 or transfection mix-4 for 4 h. Afterwards, patterned cells were put in maintenance medium 0 % FBS. Brightfield and fluorescence images were taken after 24 h, Figure 1B.

For the analysis of the effect of cell-cell contact on the gene transfection efficiency, cell-D20 and cell-D100 patterns were transfected with mix-4 as previously indicated. Brightfield and fluorescence images were taken at 0, 12, 18, 24, 30 and 42 h after transfection. For the quantification of the absolute transfection efficiency on patterned single cells, D20 cell patterns were transfected with mix-4. A grid was marked on the bottom of the 12-well microtiter plates dividing the cell pattern in 16 areas to facilitate continuous monitoring over the same area and the same cells over time. Brightfield and fluorescence images were taken at 0, 12, 18, 24, 30 and 42 h after transfection.
2.4. Quantification of transfection efficiency on patterned hHF-MSCs by microscopy images analysis

For the quantification of transfection efficiency on patterned cells, two microscopy images of the patterns were taken after transfection: a brightfield image, to count the total number of patterned cells at a specific time, and a fluorescence microscopy image to count the number of cells expressing GFP, Figure 1C. Total number of cells and transfected cells were counted using Image J. Images of non-transfected cells patterned and cultured under similar conditions were taken as negative controls. Fluorescence images were taken using an inverted Nikon microscope, equipped with a LUMENCOR laser light source with 4 emission filters (446, 523, 561 and 677 nm) and Zylar sCMOS camera. DAPI images were taken at an excitation wavelength of 395 nm. GFP images were taken at an excitation wavelength of 480 nm. Phalloidin images were taken at an excitation wavelength of 640 nm. Background signal, mainly coming from cells autofluorescence, was subtracted from all images before the transfection evaluation. Two tailed t-student statistical analysis was carried out in Excel.

In the case of the absolute transfection efficiency quantification, images were taken for each sample in the exact same position of the cell pattern on all times studied.

2.5. Proliferation of patterned and transfected hHF-MSCs

In order to check the capability of patterned and transfected hHF-MSCs to form a new culture after transfection, a proliferation assay was carried out. 48 h after transfection with mix-4. Cells patterns were kept in a complete medium (30 % FBS), in the incubator for another 48 h. Brightfield images were taken to check cell spreading and proliferation.

3. RESULTS AND DISCUSSION

hHF-MSC primary cells, which have enormous clinical potential for regenerative therapies, were used in this study. In general, these cells are difficult to transfect thus the development of new methods to improve its transfectability could have a big clinical impact (de Carvalho et al. 2018).
Our hypothesis was that cell-cell contact might affect the efficiency of transfection in adherent cells. The quantification of the transfection efficiency of a green fluorescence protein encoding a DNA plasmid in hHF-MSCs was used as a model to study the feasibility of the method. The presence of fluorescence inside of a cell indicates that the transfection of the GFP plasmid was successfully carried out. This could be easily monitored by flow cytometry, or in the case of the patterned cells, by fluorescence microscopy. Lipofectamine, a cationic liposome previously reported for MSCs (Madeira et al., 2010), was chosen as the carrier for the incorporation of the plasmid inside of the cell.

The formation of protein patterns using microcontact printing allows the generation of cell arrays with different interactions between cells. This procedure facilitates the comparison of the transfection efficiency, in arrays of single cells and of small cell-colonies, the later resembling conventional cultures. Fibronectin was chosen as the adhesion protein as established in previous works (Garcia-Hernando et al., 2020; Gonzalez-Pujana et al., 2019).

3.1. Cells seeding on custom protein pattern to control cell-cell contact.

First, we evaluated the formation of cell patterns with controlled cell-cell contact. Two types of fibronectin patterns were produced on the bottom plates of 12-well microtiter plates. D20 patterns with single cells and D100 patterns with small cell-colonies. The images showed that cells adhered specifically to the fibronectin adhesion dots. In both types of patterns, 99 % of the dots were filled by cells. In D20 substrates, more than 85 % of the dots contained only one cell per dot (the calculated mean occupancy value was 1.1 ± 0.1 cells per dot), while in the D100 substrates more than 50 % of the dots contained 4 or 5 cells (the calculated mean occupancy value was 4.6 ± 0.8 cells per dot) with a range of 2 to 8 cells per dot, Figure SI-1. The cells adhered to the fibronectin dots did not contact to each other; since the distance between dots was 50 µm. The size of the fibronectin dots affected the spreading of the cells. Single cells in the D20 substrates had a more rounded shape and filled a smaller area compared to the cells on D100 substrates (see Figure SI-1).
Cells in culture media containing serum migrated out of the pattern in less than 24 h because it contained growth, migration and proliferation factors that directly affected the attachment of cells to the protein patterns (see SI-2A). For this reason, next experiments were performed in serum-free culture conditions in order to keep the cells in the protein pattern. Although the lack of serum may not be optimal for mesenchymal stromal cells maintenance, it synchronizes the cell culture, and equalizes the cell phase of all cells (Langan and Chou 2011). This aspect may be important because cell transfection can be affected by the cell cycle (Brunner et al. 2000).

The stability and viability of hHF-MSCs on both D_{20} and D_{100} cell patterns was evaluated during 72 h. Viable cells were defined as alive cells that remain adhered to the substrate. Trypan blue stained less than 5 % of the cells, indicating than most of the cell in the pattern were alive. Our results showed that the 60 % and 53 %, after 48 h, and the 44 % and 41 %, after 72 h, of the patterned cells in the D_{20} and D_{100}, respectively, remained alive on the pattern, Figure SI 2-B.

### 3.2. Optimization of GFP plasmid/lipofectamine transfection on hHF-MSCs

For the optimization of the transfection with lipofectamine and the GFP plasmid, we evaluated the transfection of hHF-MSCs cultured on conventional well plates using flow cytometry for the analysis as explained in experimental section 2.3. Cultures of hHF-MSCs with two confluences (70 % and 90 %) were transfected with the transfection mixtures (mix-1, 2, 3 and 4), containing different concentrations of GFP plasmid and lipofectamine. GFP expression was evaluated and analyzed by flow cytometry 24 h after cell transfection (Cheung et al., 2018; Madeira et al., 2010).

The highest transfection efficiency was observed for the 70% confluence cultures transfected with mix-3 and 4, indicating that cell-cell interactions affects the transfection efficiency. The samples treated with mix-3 and 4 resulted in similar transfection efficiency 19 ± 1 and 20 ± 1 %, respectively, which falls within the expected range for mesenchymal stromal cells (de Carvalho et al., 2018; Cheung et al., 2018). On the other hand, an increase on cell’s mortality was observed when the plasmid and lipofectamine concentrations were high, as in the case of mix-4, with a 17 ± 1 % of death, Figure 2 A.
The same transfection protocol was carried out on cell-D\textsubscript{100} patterns. Brightfield and fluorescence images were taken 24 h after transfection, in order to count the total number of attached cells and the GFP expressing cells, respectively. The efficiency of transfection using mix-3 and mix-4 on D\textsubscript{100} was 22 ± 4 % and 22 ± 2 % respectively, Figure 2B. These results were similar to those obtained for the 70 % confluence conventional culture, confirming that the transfection process could be done on patterned cells. Mix-4 was chosen as the optimum concentration for further experiments, as it presented a high transfection and low deviation between samples.

3.3. Transfection efficiency quantification on patterned single hHF-MSCs and small hHF-MSCs-colonies

In order to evaluate the effect of cell-to-cell contact in the transfection process of hHF-MSCs, cells from D\textsubscript{20} and D\textsubscript{100} patterns were treated with the transfection mix-4 and monitored over time, taking brightfield and fluorescence images at 0, 12, 24, 30 and 42 h, after transfection. Transfection efficiency was calculated using equation 1. Cells within the small cell-colonies in D\textsubscript{100} patterns presented clear and intimate cell-cell contact with other cells in the same colony after patterning and during transfection, showcasing an initial spreading similar to those found in conventional cultures (Figure 3A). Meanwhile, single cells in the D\textsubscript{20} patterns were completely isolated from others and presented a rounded architecture without spreading (Figure 3B). The transfection efficiency was calculated for each time, observing a Gaussian distribution of the transfection efficiency. Transfection efficiency was calculated using equation 2.

\[
\text{Transfection Efficiency} = \frac{\text{GFP expressing cells (tx)}}{\text{Total number of cells (tx)}} \times 100
\]  
(eq. 2)

The maximum transfection efficiency was found to be 28 ± 4 % at 18 h from D\textsubscript{20} and 22 ± 2 % at 24 h for D\textsubscript{100}, Figure 3B and 3C. These measurements indicate a faster expression of GFP when cells are isolated and suggest that cell-cell contact may participate in the regulation of the uptake, the transcription or the transduction of the plasmid, affecting the transfection process. This would be in
agreement with current publications showing that reduction of cell spreading may induce cellular stress (Koopman et al., 2006; Quan et al., 2015) and therefore affect DNA uptake, transcription and transduction processes (Hong et al., 2013; Natkańska et al., 2018), especially in regards of the secondary metabolism. Recently, Chang et al. reported that cell cytoskeleton is a fundamental factor on gene transfection, where nucleus reshaping and shorter actin microfilaments induces an increased transport of the plasmid inside the cells with smaller spreading (Chang et al., 2020). Furthermore, it has been reported that cell-cell contact affects different transcription and transduction pathways and therefore influence nuclear and genetic regulation and cell growth and survival (CHO, 2004; Jamora et al., 2003; Langan and Chou, 2011b). Regarding the viability of the patterned cells, both scenarios showcased low cell detachment, as 82 % and 72 % of cells remained at the D20 and D100 patterns respectively after 42 h.

3.4. Absolute transfection efficiency quantification on patterned single hHF-MSCs

The patterning of single cells also gives the opportunity to monitor particular cells over a lapse of time, allowing the constant observation of all individual cells during the full course of the assay, in contrast with conventional techniques, which usually measure different cell populations at specific time points.

Cell transfection on single cell patterns was monitored over the course of 42 h and the absolute transfection efficiency was obtained. Absolute transfection efficiency was calculated following the equation 3, where “New GFP Expressing Cells” means fluorescent cells not appreciable at previous time.

\[
\text{Absolute Transfection Efficiency} = \frac{\sum \text{New GFP expressing cells} (t_0, t_1, t_2 ... t_x)}{\text{Total number of cells} (t_0)} \times 100
\]

(eq. 3)

Figure 4 shows a section of a single cell pattern, at different times, after transfection. GFP expression was observed up to 30 h after transfection. The majority of the cells expressing GFP were appreciable within the first 18 h, obtaining the highest number of cells expressing GFP (25 ±
2 % in transfection efficiency). From 18 h onwards, the expression of GFP decreased rapidly. The absolute transfection efficiency was calculated to be 28 ± 2 %.

This results showcase that not all successfully transfected cells are analyzed when using a single time of analysis, the way conventional flow cytometer analysis are carried out. Our methodology not only allows the continuous monitoring of each cell or cell colony, but also allows a more accurate analysis of transfection efficiency.

3.5. Proliferation of the hHF-MSCs from the transfected patterns

In order to study the ability of patterned and transfected hHF-MSCs to produce a full cell culture, the maintenance cell medium of the transfected hHF-MSCs $D_{20}$ and $D_{100}$ patterns (0 % FBS) was changed to a complete DMEM medium 42 h after transfection. After another 48 h, cells replication, migration and expansion outside their pattern were observed, Figure 5. In general, higher proliferation was observed for cells patterned on $D_{20}$ substrates than on $D_{100}$ substrates.

This indicates that patterned cells can be fully cultivated after transfection, a desirable outcome that allows the growth and expansion of a culture of transfected cells. This can be achieved by the simply change in the serum concentration of the medium.

4. CONCLUSIONS

In this work, we demonstrated that cell patterns permit a quick and easy analysis of the transfection efficiency, opening the possibility to evaluate the effect of cell-cell contact during the cell transfection process. In terms of transfection efficiency, the results obtained using patterned substrates were comparable to those obtained in regular cell culture by flow cytometry. The transfection process was monitored for single cell $D_{20}$ and small cell-colony $D_{100}$ patterns up to 42 h. The time to reach a maximum transfection efficiency was 6 h earlier for single cells than for small cell-colonies. Additionally, the transfection efficiency on $D_{20}$ patterns was 10 % higher than on $D_{100}$ and conventional cultures. This confirms our hypothesis that cell-cell contact has an important effect during the transfection process.
Besides enabling the comparison of the transfection efficiency in isolated or non-isolated cells, the use of patterned substrates for transfection studies presented a series of additional advantages. The use of cell arrays allows the evaluation of the status of the cells and obtaining continuous transfection data for several days. In contrast to conventional techniques, it allows the quantification of the absolute transfection efficiency, counting transfected cells on by one, at a desired time. Cell patterning allows the generation of a large number of independent events, be single cells or small cell-colonies, in a tiny substrate. The high density of the array provides to the platform with a high analytical capacity, allowing statistical studies or even the possibility of analyzing cell subpopulations. All steps, cell adhesion, transfection and analysis can be done on the same substrate without manipulation, enabling real time monitoring. The cell arrays containing more than 4000 and 20000 independent elements (D_{20} single cells or D_{100} small cell-colonies, respectively) were generated in just 2 h, being ready for transfection and analysis. This significantly reduces the number of cells needed to perform the assay. In addition, the assay time is drastically reduced compared to methods based on conventional cultures and flow cytometry, where cells must be cultured for at least 24 h and detached from the culture dish to be analyzed. Furthermore, our results show that the cells transfected in the patterns proliferate, indicating that this methodology could be used for the selection of viable transfected cells, as in the case of stable transfection with antibiotic selection (Moore et al., 2010).

Further investigation will allow to integrate these substrates into microfluidic platforms to leverage the use of flow for controlled delivery and removal of reagents and waste products in an automated manner. Novel studies could be directed to different applications such as, to discern the efficacy of different transfection vectors in different cell-cell contact scenarios, to study cell subpopulations, to understand which processes may cause the transfection efficiency variations, to evaluate toxicity for each transfection vector.
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CONFLICT OF INTEREST
Authors declare that there are no conflict of interest.

AUTHOR CONTRIBUTION
Enrique Azuaje-Hualde designed and carried out the transfection experiments on patterns, analyzed all data and wrote the manuscript. Melania Rosique designed and optimized the transfection protocol and carried out flow cytometry experiments. Enrique Azuaje-Hualde and Alba Calatayud-Sanchez carried out stability and viability experiments on patterned cells. Lourdes Basabe-Desmonts, Marian Martinez de Pancorbo and Fernando Benito-Lopez conceived the idea, supervised the project and revised the manuscript.
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Figure Captions

**Figure 1. Graphical scheme of cell patterning, transfection and continuous evaluation of cell transfection efficiency with micropatterned substrates.** A) Surface patterning of fibronectin features through microcontact printing and specific cell adhesion with controlled cell-cell contact to the fibronectin pattern. B) Direct transfection of patterned cells with GFP plasmid and liposome vector. Continuous observation of GFP expression is done through fluorescence microscopy. C) Transfection efficiency quantification through image analysis of total patterned cells and GFP expressing cells.

**Figure 2. Transfection in hHF-MSCs.** A) Top; graphics of the transfection efficiency (bars) and cell viability (lines) of conventional hHF-MSCs cultures with 70 % (left) or 90 % (right) confluence, transfected using four different mixtures (mix-1, 2, 3 and 4) of lipofectamine and GFP plasmid. The analysis was carried out through flow cytometry. Error bars represent mean ± SD (n = 3). Bottom; brightfield and fluorescence images of transfected cultured cells at 70% (left) and 90% (right) confluence with mix 4. B) Graph representing the transfection efficiency of D$_{100}$ patterns transfected using mix-3 and 4 (left) and brightfield and fluorescence images of a D$_{100}$ pattern (right). Error bars mean ± SD (n = 3, with ~4000 cells analyzed per sample).

**Figure 3. Transfection efficiency quantification on patterned single hHF-MSCs and small hHF-MSCs-colony patterns.** A) Microscopy images of D$_{20}$ (left) and D$_{100}$ (right) patterns. Top: Brightfield images; middle: fluorescence images showing DAPI staining of cell nucleus; bottom: fluorescence images of phalloidin dyed cell cytoskeletons. B) Brightfield and fluorescence microscopy images of single cell (left) and small cell-colony (right) after 18 and 24 h, respectively after transfection of GFP. C) Plots of transfection efficiency (bar chart) and patterned cell viability (dotted line) versus time of D$_{20}$ (left) and D$_{100}$ (right). Error bars mean ± SD (n = 3 patterns, with
~700 cells and ~4000 cells monitored for D$_{20}$ and D$_{100}$ respectively per sample and per time). Statistical significance; paired two-tailed t-test (* means significant statistical difference of $p < 0.05$ compared to D$_{100}$, 24 h).

**Figure 4. Absolute transfection efficiency quantification on single hHF-MSCs D$_{20}$ patterns. A)** Graphical representation of the absolute transfection efficiency over time for 42 h. Error bars correspond to, mean ± SD ($n = 3$ patterns with ~700 cells monitored per sample). B) Brightfield (left) and fluorescence (center) images of GFP expressing cells. Right: same fluorescence microscopy image than in center with colored circles in white (12 h), yellow (18h) or red (24h) on the transfected cells to calculate the absolute transfection efficiency following equation 2.

**Figure 5. Cell proliferation after patterning and transfection.** Brightfield microscopy images of substrates containing patterned and transfected D$_{20}$ (top) and D$_{100}$ (bottom) hHF-MSCs, 48 h after change from serum-free medium to complete medium, showing expansion and proliferation.