TITLE: Use of a PTFE micro-bioreactor to promote 3D cell rearrangement and maintain high plasticity in epigenetically erased fibroblasts

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ABSTRACT

Phenotype definition is driven by epigenetic mechanisms as well as directly influenced by the cell microenvironment and by biophysical signals deriving from the extracellular matrix. The possibility to interact with the epigenetic signature of an adult mature cell, reversing its differentiated state and inducing a short transient high plasticity window, was previously demonstrated. In parallel, in vitro studies have shown that 3D culture systems, mimicking cell native tissue, exert significant effects on cell behavior and functions. Here we report the production of "PTFE micro-bioreactors" for long-term culture of epigenetically derived high plasticity cells. The system promotes 3D cell rearrangement, global DNA demethylation and elevated transcription of pluripotency markers, that is dependent on WW domain containing transcription regulator 1 (TAZ) nuclear accumulation and SMAD family member 2 (SMAD2) co-shuttling. Our findings demonstrate that the use of 3D culture strategies greatly improves the induction and maintenance of a high plasticity state.

Keywords: epigenetic erasing, high plasticity, micro-bioreactor, PTFE, SMAD2, TAZ

INTRODUCTION

Embryo development, gene regulation and cell specification are driven by complex epigenetic mechanisms, that lead to a gradual loss of potency [1] and a progressive restriction in cell options [2]. However, the differentiation process is reversible and it has been recently demonstrated that it is possible to interact with the epigenetic signature of an adult mature cell, switching its original phenotype into a different one [3-10]. This is achievable through biochemical and biological manipulations that are able to reactivate hypermethylated genes [11-15]. In previous studies, we used the epigenetic modifier 5-azacytidine (5-aza-CR) to interfere with DNA methylation and induce a transient high plasticity state, sufficient to allow a complete, directed differentiation of an adult mature cell into a different functional cell type [3-5, 8]. We also demonstrated that 5-aza-CR is able to decrease methylation levels through a direct ten-eleven translocation 2 (TET2)-mediated action [10], beside the well-known indirect DNA methyltransferase (DNMT) inhibition mechanism [16, 17]. The transient high plasticity state induces the transcription of pluripotency-related genes, that, however gradually decreased, even when epigenetically erased cells were maintained in embryonic stem cell (ESC) medium, and disappeared by day 6 of culture [10].

Parallel studies addressed their attention to tissue architecture and mechanical forces that are involved in the control of cell plasticity and/or differentiation. Indeed, it is known that changes in cell fate are directly influenced by cell microenvironment and biophysical signals conveyed by the extracellular matrix (ECM) [18, 19]. In vitro studies have shown that 3D culture systems, mimicking cell native tissue, exert significant effects on cell behavior, influencing cellular molecular mechanisms and related functions. Furthermore, a recent report highlighted the possibility and advantage to use a fiber bioreactor to increase efficiency of mesenchymal stem cells (MSC) expansion, allowing a GMP-compliant process and leading to cost effective manufacturing procedure [20].

In the present work, we describe a new method to promote 3D cell rearrangement and specifically extend and maintain the transient high plasticity window in epigenetically erased cells. To this end we use polytetrafluoroethylene (PTFE), a non-reactive hydrophobic synthetic compound, to produce an easy to generate and efficient micro-bioreactor, that permits the production of cellular microenvironment unachievable through the use of traditional 2D culture systems.

We assess whether biophysical effectors linked to 3D cell confinement may positively impact on cell plasticity, induced by epigenetic erasing. In particular, we examine if the use of the micro-bioreactor may influence global DNA methylation, boost pluripotency gene transcription and maintain long-term high plasticity. In order to better understand the mechanisms linking 3D cell rearrangement and the maintenance of high plasticity, we investigate the involvement of the Hippo signaling mechanotransduction pathway and demonstrate that 3D cell confinement induces the activation of WW domain containing transcription regulator 1 (TAZ, also known as WWTR1), which is one of the key molecule in the pathway. We then evaluate TAZ interaction with the crucial pluripotency regulator SMAD family member 2 (SMAD2) and demonstrate co-accumulation of the two molecules in the cell nuclear compartment. Furthermore, we show that TAZ knockdown causes loss of SMAD2 nuclear retention and loss of pluripotency marker transcription. Altogether, our findings indicate that the use of a micro-bioreactor and 3D cell confinement activates the Hippo signaling machinery, which, in turn, is required to maintain high plasticity.

MATERIALS AND METHODS

All reagents were purchased from Thermo Fisher Scientific unless otherwise indicated.

Ethics statement

Murine cells were isolated from 7-week-old male mice carrying Oct4–GFP (B6;129S4-Pou5f1^{tm2Jae}/J, stock #008214) obtained from Jackson Laboratory. All studies were reviewed and approved by the Ethical Committee of the University of Milan. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH).

Human cells were isolated from adult patients, after written informed consent and approved by the Ethical Committee of the Ospedale Maggiore Policlinico, Milano. All the methods in our study were carried out in accordance with the approved guidelines.

Isolation, culture and transfection of dermal fibroblasts

Murine dermal fibroblasts were isolated from skin biopsies of four Oct4-GFP transgenic mice (B6;129S4-Pou5f1^{tm2Jae}/J; Jackson Laboratory, stock #008214) [21].

Human dermal fibroblasts were established from skin biopsies of five patients.

Fragments of murine and human dermal tissue of approximately 2 mm^3 were transferred onto 0.1% gelatin (Sigma) pre-coated Petri dish (Sarstedt) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% Fetal Bovine Serum (FBS), 2 mM glutamine (Sigma) and antibiotics. After 6 days of culture, fibroblasts started to grow out of the tissue fragments and the latter were carefully removed. Cells were maintained in the medium described above, grown in 5% CO2 at 37°C, and passaged twice a week in a 1:3 ratio.

siRNA transfection was performed using Lipofectamine RNAi-MAX in antibiotics-free Opti-MEM® medium according to manufacturer's instructions. Validated Stealth RNAi siRNA used in the present study were specific for TAZ/WWTR1 (Human: HSS119545).

All experiments were independently repeated at least three times for each cell line derived from mouse (n=4) and human patients (n=5).

Treatment of dermal fibroblasts with 5-aza-CR

Murine and human fibroblasts were randomly allocated in two groups: A) standard plastic dish group, and B) polytetrafluoroethylene (PTFE) group. Cells belonging to the two groups were epigenetically erased with 1 μM 5-aza-CR (Sigma) for 18 hours. Concentration and time of exposure were selected according previous works [3, 22].

Group A- standard plastic dish

Cells were plated in 4-well multidish (Nunc) previously treated with 0.1% gelatin (Sigma) at concentration of 7.8 X 10^4 cell/cm^2. 24 hours after plating, cells were erased with 5-aza-CR as described above.

Group B- PTFE

The PTFE micro-bioreactor was created inside a Petri dish by preparing PTFE powder bed with particle size of 1 μm (Sigma 430935). Spatula was used to gently make a curved gully at the centre of the powder bed. A micropipette was used to dispense 4 X 10^4 cells resuspended in 30 μl of 1 μM 5-aza-CR, on the PTFE powder bed. The Petri dish was then gently shaken in a circular motion to ensure that the powder particles completely covered the surface of the liquid drop. PTFE drops were incubated in 35- mm Petri dishes at 37 °C in 5 % CO_2 in air. To increase humidity and avoid dehydration, the Petri dish was placed in a larger Petri dish containing sterile water.

Culture of epigenetically erased fibroblasts

At the end of the 18-hour exposure, 5-aza-CR treated cells belonging to the two experimental groups (A and B) were cultured in ESC culture medium [22, 23]. More in details, group B liquid marbles were broken by puncturing with a needle. Formed organoids were recovered

using a 200 µl pipette tip cut at the edge, washed in ESC medium and re-encapsulated in PTFE micro-bioreactors. Culture medium was refreshed daily until day 28, when culture was arrested.

Ultrastructural analysis

Cells encapsulated in PTFE were collected for transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

TEM analysis samples were fixed for 2 hours in 0.1 M cacodylate buffer pH 7.2, containing 2% glutaraldehyde. Specimens were then washed in the same buffer and post-fixed for 2 hours with 1% osmic acid in cacodylate buffer. After standard serial ethanol dehydration, specimens were embedded in an Epon-Araldite 812 mixture. Sections were obtained with a Reichert Ultracut S ultratome (Leica). Semi-thin sections were stained by conventional methods (crystal violet and basic fuchsin) and observed under a light microscope (Olympus). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 EX electron microscope (Jeol).

For SEM, cells were fixed and dehydrated as described above, then treated with hexamethildisilazane and mounted on polylysinated slides, air dried and subsequently covered with a 9 nm gold film by flash evaporation of carbon in an Emitech K 250 sputter coater (Emitech). Specimens were examined with a SEM-FEG Philips XL-30 microscope (Philips).

Karyotyping

Cells were cultured in medium containing Colcemid (0.1 µg/ml) for 3 h, incubated for 30 min in hypotonic solution, fixed, and stained using Giemsa (Kario MAX Giemsa). Metaphases were examined under a Leica HC microscope equipped with a digital camera Leica DC250. Images were analyzed using Leica CW4000 Karyo software.

Global methylation analysis

Genomic DNA was extracted using PureLink® Genomic DNA Kits following the manufacturer's instructions. DNA was converted to single-stranded DNA by incubation at 95°C for 5 min, followed by rapid chilling on ice. Samples were then digested to nucleosides by incubating the denatured DNA with nuclease P1 for 2 h at 37°C in 20 mM sodium acetate (pH 5.2). Alkaline phosphatase was added and incubated for 1 h at 37°C in 100 mM Tris (pH 7.5). After centrifugation, the supernatant was used for ELISA assay using Global DNA Methylation ELISA Kit (5'-methyl-2'-deoxycytidine Quantitation; CELL BIOLABS) according to the manufacturer's protocol.

Gene expression analysis

RNA was extracted using the TaqManGene Expression Cells to Ct kit (Applied Biosystems), and DNase I was added in lysis solution at 1:100 concentration, as indicated by the manufacturer's instructions. Quantitative PCR was performed on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories) using predesigned gene-specific primers and probe sets from TaqManGene Expression Assays, listed in Table 1. *GAPDH* and *ACTB* were used as internal reference genes. CFX Manager software (Bio-Rad Laboratories) was used for target gene quantification.

Western blot analysis

Cells were lysed and constitutive proteins were extracted using a ReadyPrep Protein Extraction Kit (Bio-Rad). Nuclear extracts from the cells were isolated using the NXtract CelLytic NuCLEAR Extraction Kit (Sigma). Protein concentration was assessed by Coomassie Blue-G Dye-binding method. 100 μg of proteins were resuspended in sample buffer (1:1) consisting of 4% (wt/vol) SDS, 10% 2-mercaptoethanol, 20% (wt/vol) glycerol, 0.004% bromophenol blue, and 0.125 M Tris–HCl at pH 6.8. Equal amounts of total protein were loaded and

electrophoresed on a SDS-polyacrylamide gels. Proteins were then transferred onto 0.45 μm pore size nitrocellulose membranes (Hybond-C Extra, GE Healthcare Life Sciences) and probed with primary antibodies listed in Table 2. Protein bands were visualized by the WesternBreeze chemiluminescent kit. Densitometric analysis was performed with Quantity One 1-D analysis software (Bio-Rad).

Statistical analysis

Statistical analysis was performed using Student t-test (SPSS 19.1; IBM). Data were presented as mean ± standard deviation (SD). Differences of $p \leq 0.05$ were considered significant.

RESULTS

The PTFE micro-bioreactor promotes 3D cell rearrangement and maintain high plasticity in epigenetically erased murine fibroblasts: preliminary studies

Preliminary experiments were carried out using fibroblast primary cell lines, isolated from skin biopsies of four Oct4-GFP transgenic mice (B6;129S4-Pou5f1tm2Jae/J; Jackson Laboratory, stock #008214) [21]. After encapsulation in PTFE and 5-aza-CR exposure, cells formed 3 dimensional (3D) spherical structures (Figure 1A) and became GFP positive (Figure 1B), indicating the onset of *Oct4* gene expression. High plasticity state achievement was also confirmed by gene expression analysis. Indeed, the morphological changes were accompanied by the expression of pluripotency-related genes, namely POU class 5 homeobox 1 (*Oct4*), Nanog homeobox (*Nanog*), Zfp42 zinc finger protein (*Rex1*), and sex determining region Y-box 2 (*Sox2*), which were undetectable in untreated fibroblasts (T0, Figure 1C). Interestingly, GFP positivity as well as pluripotency gene transcription were high for the entire length of the experiments.

The PTFE micro-bioreactor promotes 3D cell rearrangement in epigenetically erased human fibroblasts

Human dermal fibroblasts obtained from five patients displayed a dramatic change in their morphology, when exposed to 5-aza-CR, regardless of the culture system used. In particular, fibroblasts plated on standard plastic dishes (Group A) as well as those encapsulated in PTFE (Group B) became rounded, with large and granulated nuclei (Figure 2A). However, while Group A cells retained a monolayer distribution for the entire length of the experiments, cells encapsulated in PTFE (Group B) formed 3D spherical structures (Figure 2B), that were stably maintained for the entire length of the experiments.

The PTFE micro-bioreactor induces ultrastructural modifications in epigenetically erased human fibroblasts

The fine structure of cells encapsulated in PTFE was analyzed by SEM and TEM starting from T0, after 5-aza-CR exposure and up to 28 days. 5-aza-CR treatment induced changes in morphology. Cells lost their characteristic spindle shape (Figure 2D, E) and became ovoidal (Figure 2F, G), showing a cytoplasm filled with autophagic vacuoles (Figure 2G).

Starting from 24 hours after 5-aza-CR treatment and for the entire length of the experiments, cells within 3D structures maintained a roundish shape with a high nucleus to cytoplasm ratio, few organelles and large intercellular spaces (Figure 2H, I). Their cytoplasm contained free ribosomes, polyribosomes, elongated tubular mitochondria, well-developed rough endoplasmic reticulum (RER), Golgi complexes, few reticulum cisternae and lipid droplets. Furthermore, cells showed nuclei with euchromatin and large reticulated nucleoli that are typical of ESC [24]. Occasionally, scattered cells with cytoplasm occupied by large autophagic vacuoles were visible (Figure 2I).

The PTFE micro-bioreactor enhances the demethylating effect of 5-aza-CR in epigenetically erased human fibroblasts

5-aza-CR caused a significant decrease in global DNA methylation in fibroblasts belonging to the two experimental Groups (Figure 3A). However, Group B cells exhibited significantly lower DNA methylation levels compared to those of Group A, at all time points analyzed, and remained significantly hypomethylated for the entire length of the experiments. In contrast, cells plated on plastic dishes (Group A), although displayed a decreased methylation for 72 hours, slowly increased and returned comparable to untreated fibroblasts (T0) by day 7 of culture.

Interestingly, cells maintained a normal karyotype throughout the entire length of the experiments (Figure 2C), indicating that methylation changes did not cause chromosome copy number variations or cytotoxic effects.

The PTFE micro-bioreactor boosts pluripotency gene transcription and maintains long-term high plasticity in epigenetically erased human fibroblasts

Morphological changes and methylation decrease were accompanied by the onset of the expression of pluripotency-related genes, namely POU class 5 homeobox 1 (*OCT4*), Nanog homeobox (*NANOG*), ZFP42 zinc finger protein (*REX1*), and sex determining region Y-box 2 (*SOX2*), which were undetectable in untreated fibroblasts (T0; Figure 3B). Group B cells showed significantly higher expression levels of these genes, when compared to Group A. Furthermore, encapsulation in PTFE (Group B) maintained high expression levels for the entire length of the experiments. In contrast, Group A cells transcribed for pluripotency-related markers until 72 hours and turned down their expression by day 7 of culture.

5-aza-CR treatment also induced the up-regulation of *TET2*, epithelial cell adhesion molecule (*EPCAM*), and cadherin 1 (*CDH1*) genes in both groups (Figure 3B). More in detail, Group B cells showed significantly higher expression levels of these genes, compared to those of Group A. Furthermore, *TET2*, *EPCAM*, and *CDH1*, expression profile paralleled that described above for pluripotency- related genes.

In agreement with these observations, we detected a significant downregulation of a fibroblast specific gene (Thy-1 cell surface antigen, *THY1*) in Group B cells for the entire length of the experiments. Group A cells showed, in contrast, decreased levels for the first 72 hours of culture, and returned to values comparable to those of untreated fibroblasts by day 7 (Figure 3B).

The PTFE micro-bioreactor activates the Hippo signaling pathway to maintain high plasticity in epigenetically erased human fibroblasts

Fibroblasts exposed to 5-aza-CR (Post 5-aza-CR) showed TAZ nuclear accumulation both in cells plated onto plastic plates (Group A; Figure 4 A) and in those encapsulated in PTFE (Group B; Figure 4B). TAZ nuclear confinement was however lost by Group A cells, with the molecule relocating to the cytoplasm, by day 7 of culture (Figure 4A). In contrast, 3D cell confinement of Group B cells encouraged TAZ nuclear retention, that was stably maintained for the entire length of the experiments (Figure 4B).

Interestingly, TAZ localization was mirrored by a parallel compartmentalization of SMAD2. More in detail, cells displaying nuclear localized TAZ showed SMAD2 nuclear accumulation, while cells with cytoplasmic TAZ exhibited SMAD2 cytoplasmic distribution (Figure 4A, B).

In addition, siRNA knockdown of TAZ caused inhibition of SMAD2 nuclear accumulation (Figure 4C) as well as the loss of pluripotency marker transcription (Figure 4D), suggesting the

direct involvement of TAZ in SMAD2 shuttling and high plasticity maintenance, boosted by the PTFE 3D microenvironment.

DISCUSSION

The results obtained in the present study demonstrate that the use of the PTFE micro-bioreactor is able to induce significant morphological changes, 3D cell rearrangements and to enhance the acquisition and maintenance of high plasticity in 5-aza-CR exposed cells. These observations extend previous evidences indicating that 3D microenvironment may have a profound influence on cell phenotype and plasticity [25-28].

After treatment with the epigenetic eraser, both cells plated onto plastic plates (Group A) and those encapsulated in PTFE (Group B) displayed significant changes, compared to untreated fibroblasts (T0). In particular, the typical fibroblast elongated morphology, was replaced by a round or oval shape. Cells belonging to the two Groups became considerably smaller in size, with larger and granulated nuclei, acquiring the typical morphological features described for pluripotent cells [29, 30]. However, while Group A cells retained a monolayer distribution for the entire length of the experiments, the use of the micro-bioreactor allowed cells to self-assemble and form multicellular spheroids, displaying a uniform size geometry. This is consistent with previous studies indicating that the use of PTFE is able to efficiently encourage cell aggregation, facilitating the formation of embryoid bodies (EBs) from murine ESC [31] or the establishment of olfactory ensheathing cell (OEC) spheroid structures [32].

Ultrastructural analysis demonstrated that cells in the 3D spherical structures showed significant intercellular spaces, high nucleus to cytoplasm ratio, nuclei containing euchromatin and large reticulated nucleoli. Cytoplasm was characterized by the presence of free ribosomes, polyribosomes, elongated tubular mitochondria, well-developed rough endoplasmic reticulum (RER), Golgi complexes, few reticulum cisternae and lipid droplets. These features resemble

those described for human ESC [24] and induced pluripotent cells (iPS) [30], that have been shown to display large intercellular spaces, accessible euchromatin and morphology typical of undifferentiated cells, reminding of inner cell mass (ICM) cells of blastocysts [24, 29, 30, 33-36]. These observations suggest that the use of the PTFE micro-bioreactor encourages not only cell aggregation, but also boosts the formation and stable maintenance of morphological properties previously described in pluripotent cells.

Incubation with the epigenetic eraser significantly modified global DNA methylation. Indeed, cells belonging to both the two experimental Groups showed a significant decrease in methylation levels, compared to the starting cell population (T0). However, while Group A cell methylation returned comparable to untreated fibroblasts (T0) by day 7 of culture, PTFE encapsulated cells (Group B) remained significantly hypomethylated for the entire length of the experiments. Methylation changes have been previously described during fibroblast reprogramming [36-38], where the hypomethylated state is due to the effect of a direct and active demethylation mechanism controlled by TET proteins [38]. Indeed, it has been demonstrated that TET enzymes play an essential role in ESC pluripotency maintenance [39, 40] and are upregulated during mesenchymal to epithelial transition (MET) as well as during somatic cell reprogramming, affecting the establishment of the open chromatin and contributing to the re-activation of endogenous pluripotency genes [41]. The fundamental functions exerted by TET factors in iPS generation were also confirm by experiments demonstrating inability of *TET*-deficient fibroblasts to complete reprogramming processes [42]. In line with these observations, our results show that epigenetic erasing lead to an increased expression of the ten-eleven translocation family member *TET2*. This was accompanied by the onset of the pluripotency-related genes, *OCT4*, *NANOG*, *REX1*, and *SOX2*, as well as the up-regulation of *EPCAM*, and *CDH1* genes, confirming and expanding previous studies carried out in our laboratory [3-5, 10]. These changes in gene expression were detected in both

experimental Groups. However, as described in methylation study, Group A cells returned to values comparable to those of untreated fibroblasts by day 7 of culture, while those encapsulated in PTFE (Group B) displayed high expression levels for the entire length of the experiments. These results are also well in line with the morphological observations and suggest that the acquisition of a high plasticity phenotype is paralleled by the concomitant decrease of fibroblast specific marker, *THY1*, the onset of pluripotency-related genes (*OCT4*, *NANOG*, *REX1*, and *SOX2*), and the upregulation of key MET markers (*EPCAM*, *CDH1*). Interestingly, these changes were promoted and stably maintained by the use of the PTFE micro-bioreactor, suggesting that 3D cell confinement boosts pluripotency gene transcription and maintains long-term cell plasticity.

A common denominator of all the experiments here described is the use the PTFE micro-bioreactor to generate a 3D cell confinement that favors the induction and maintenance of cell plasticity. In order to better understand the mechanisms associated, we investigated the possible involvement of mechanotransduction-related signaling pathways. Our results demonstrated that the morphological and molecular changes described in the previous paragraphs, were accompanied by the activation of the Hippo-signaling pathway with distinctive modifications in TAZ localization. In particular, after 5-aza-CR treatment, TAZ protein displayed a nuclear accumulation both in Group A and B. This localization was however lost by Group A cells, with TAZ molecule relocating to the cytoplasm, by day 7 of culture. In contrast, 3D cell confinement of Group B cells encouraged TAZ nuclear retention, that was stably maintained for the entire length of the experiments. This is consistent with a recent study showing a clear TAZ nuclear localization in large spheroids and organoid-like structures [43]. In our experiments, TAZ localization was mirrored by a parallel compartmentalization of SMAD2. In particular, we observed that cells displaying nuclear localized TAZ showed concomitant SMAD2 nuclear accumulation, while cells with cytoplasmic TAZ, also exhibited SMAD2

cytoplasmic distribution. This evidence is in line with previous reports that indicate a direct interaction between the transcriptional cofactor TAZ and SMAD proteins, where TAZ defines a hierarchical system, regulating SMAD complexes shuttling and coupling to the transcriptional machinery [44, 45]. Interestingly, Panciera et al. showed that transient expression of exogenous TAZ in terminally differentiated cells can induced conversion to a progenitor cell state [46] and that the loss of TAZ expression induces failure of SMAD nuclear accumulation, disappearance of *OCT4* and subsequent differentiation[44]. Consistent with this findings, our results showed that siRNA knockdown of TAZ, with the concomitant failure of SMAD nuclear accumulation, caused loss of pluripotency marker transcription in PTFE encapsulated cells. Base on this, we speculate that the activation of the Hippo-signaling pathway induced by the PTFE micro-bioreactor and the related TAZ-dependent SMAD shuttling may represent the base of the maintenance of high plasticity in encapsulated cells.

In conclusion, our findings demonstrated that the use of the PTFE micro-bioreactor boosts the induction and maintenance of high plasticity state in epigenetically erased cells. This model system could provide a novel in vitro culture technique that induces distinctive 3D cell rearrangement and specific cell-to-cell interactions. We are convinced that the PTFE micro-bioreactor may represent a notable advance in stem cell organoid technology and may constitute an advantageous micro-environment for long-term culture of different cell types, from epigenetically erased cells, to ESC and iPSC, as well as MSC.

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FIGURE LEGENDS

Figure 1. The PTFE micro-bioreactor promotes 3D cell rearrangement and maintains high plasticity in epigenetically erased Oct4-GFP murine fibroblasts. (A) Cells encapsulated in PTFE and exposed to 5-aza-CR for 18 hours formed 3D spherical structures, that were stably maintained for the entire length of the experiments (Scale bar, 100 μm). **(B)** Fibroblasts became GFP positive, indicating the onset of *Oct4* gene expression (Scale bar, 100 μm). **(C)** Gene expression profiling of pluripotency-related genes (*Oct4*, *Nanog*, *Rex1*, and *Sox2*) in untreated fibroblasts (T0), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR) and at different time points of culture. Gene expression levels are reported with highest expression set to 1 and all other times relative to this. Different superscripts denote significant differences ($P<0.05$).

Figure 2. The PTFE micro-bioreactor promotes 3D cell rearrangement and induces ultrastructural modifications in epigenetically erased human fibroblasts. (A) After 5-aza-CR incubation, fibroblasts plated on plastic dishes (Group A) changed their typical elongated shape into a round epithelioid aspect and retained a monolayer distribution. Cell size was smaller, and nuclei became larger and granular. (Scale bar, 100 μm). **(B)** Cells encapsulated in PTFE (Group B) and treated with the demethylating agent formed 3D spherical structures, that

were stably maintained for the entire length of the experiments (Scale bar, 200 μm). **(C)** Cells maintained a normal karyotype. **(D-E)** Untreated fibroblasts displayed a spindle shaped morphology. **(F-G)** Cells encapsulated in PTFE and subjected to 5-aza-CR treatment became ovoidal with autophagic phenomena. **(H-I)** They showed a roundish shape, high nucleus to cytoplasm ratio, nuclei with euchromatin and large reticulated nucleoli, few organelles, and large intercellular spaces for the entire length of the experiments.

Figure 3. The PTFE micro-bioreactor enhances the demethylating effect of 5-aza-CR, boosts pluripotency gene transcription and maintains long-term high plasticity in epigenetically erased human fibroblasts. (A) Global DNA methylation levels of cells plated on standard plastic dishes (Group A) or encapsulated in PTFE (Group B), exposed to 5-aza-CR (Post 5-aza-CR) and cultured in ESC medium. Bars represent the mean ± SD of three independent experiments with five independent biological replicates. Different superscripts denote significant differences ($P<0.05$). **(B)** Gene expression changes in epigenetically erased fibroblasts plated on standard plastic dishes (Group A) or encapsulated in PTFE (Group B). Expression pattern of pluripotency-related genes (*OCT4*, *NANOG*, *REX1*, and *SOX2*), ten-eleven translocation family member *TET2*, MET markers (*EPCAM*, *CDH1*) and fibroblast specific marker (*THY1*). Gene expression levels are shown for untreated fibroblasts (T0), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR), and at different time points of culture. Values are reported with highest expression set to 1 and all other times relative to this. Different superscripts denote significant differences ($P<0.05$).

Figure 4. Activation of the Hippo signaling pathway in PTFE encapsulated cells. (A) Western blots for TAZ and SMAD2 proteins in epigenetically erased fibroblasts, plated on standard plastic dishes (Group A). The densitometry results (arbitrary units) of the Western

blots are shown as a bar graph. Bars represent the mean ± SD of three independent experiments with five independent biological replicates. Different superscripts denote significant differences ($P<0.05$). Representative Western blots for each protein are also shown. **(B)** Western blots for TAZ and SMAD2 proteins in epigenetically erased fibroblasts, encapsulated in PTFE (Group B). The densitometry results (arbitrary units) of the Western blots are shown as a bar graph. Bars represent the mean ± SD of three independent experiments with five independent biological replicates. Different superscripts denote significant differences ($P<0.05$). Representative Western blots for each protein are also shown. **(C)** Western blots for TAZ and SMAD2 proteins in PTFE encapsulated cells at day 28 of culture, after TAZ siRNA transfection (28d + siTAZ). The densitometry results (arbitrary units) of the Western blots are shown as a bar graph. Bars represent the mean ± SD of three independent experiments with five independent biological replicates. Different superscripts denote significant differences ($P<0.05$). Representative Western blots for each protein are also shown. **(D)** Pluripotency-related gene (*OCT4*, *NANOG*, *REX1*, and *SOX2*) expression levels in Group B cells at day 28 of culture (28d) and after TAZ siRNA transfection (28d + siTAZ). Values are reported with highest expression set to 1 and all other times relative to this. Different superscripts denote significant differences ($P<0.05$).