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## A two-step strategy that combines epigenetic modification and biomechanical cues to generate mammalian pluripotent cells

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Dear Editor,

please find attached the revised version of our manuscript #JoVE61655 entitled “*A simple, two-step strategy that combines epigenetic modification and biomechanical cues to generate mammalian pluripotent cells*”, by Pennarossa et al.

We addressed all the comments made by the Editor and modified the manuscript accordingly.

We prepared a highlighted version of the revised manuscript, in order to easily trace the changes made in the text.

We thank you for your attention and support  
Best regards

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**TITLE:**

A Two-Step Strategy that Combines Epigenetic Modification and Biomechanical Cues to Generate Mammalian Pluripotent Cells

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**KEYWORDS:**

5-aza-CR, 3D culture system, epigenetic erasing, fibroblast, mechanosensing-related cue, pluripotency, PTFE microbio reactor

**SUMMARY:**

We here present a method that combines the use of chemical epigenetic erasing with mechanosensing-related cues to efficiently generate mammalian pluripotent cells, without the need of gene transfection or retroviral vectors. This strategy is, therefore, promising for translational medicine and represents a notable advancement in stem cell organoid technology.

**ABSTRACT:**

Cell phenotype can be reversed or modified with different methods, with advantages and limitations that are specific for each technique. Here we describe a new strategy that combines the use of chemical epigenetic erasing with mechanosensing-related cues, to generate mammalian pluripotent cells. Two main steps are required. In the first step adult mature (terminally differentiated) cells are exposed to the epigenetic eraser 5-aza-cytidine to drive them into a pluripotent state. This part of the protocol was developed, based on the increasing understanding of the epigenetic mechanisms controlling cell fate and differentiation; and involves the use of the epigenetic modifier to erase cell differentiated state and then drive into a transient high plasticity window.

In the second step, erased cells are encapsulated in polytetrafluoroethylene (PTFE) micro-bioreactors, also known as Liquid Marbles, to promote 3D cell rearrangement to extend and stably maintain the acquired high plasticity. PTFE is a non-reactive hydrophobic synthetic compound and its use permits the creation of a cellular microenvironment, which cannot be achieved in traditional 2D culture systems. This system encourages and boosts the maintenance of pluripotency through bio-mechanosensing-related cues.

The technical procedures described here are simple strategies to allow for the induction and maintenance of a high plasticity state in adult somatic cells. The protocol allowed the derivation of high plasticity cells in all mammalian species tested. Since it does not involve the use of gene transfection and is free of viral vectors, it may represent a notable technological advance for translational medicine applications. Furthermore, the micro-bioreactor system provides a notable advancement in stem cell organoid technology by *in vitro* re-creating a specific micro-environment that allows for the long-term culture of high plasticity cells, namely as ESCs, iPSCs, epigenetically erased cells and MSCs.

## **INTRODUCTION:**

During the last decades, the widely accepted concept of unidirectional progression towards cell commitment and differentiation was completely revised. It has been demonstrated that cell specification can be reversed, and a terminally differentiated cell can be pushed towards a less committed and higher permissive state, using different methods.

Among the several methods proposed, one of the most promising method involves the use of chemical compounds to induce cells into a so called chemically induced pluripotency. The small molecules used in this approach are able to interact and modify the epigenetic signature of an adult mature cell, avoiding the need of any transgenic and/or viral vector<sup>1-10</sup>. Numerous studies have recently shown that it is possible to switch cells from one phenotype to another by providing specific biochemical and biological stimuli that induce the reactivation of hypermethylated genes<sup>11-15</sup>. These demethylating events allow for the conversion of terminally differentiated cells into a primitive progenitor, a multipotent or a high plasticity/pluripotent cell<sup>1-10</sup>.

In parallel, many studies have been recently focussing on the understanding of mechanosensing-related cues and, more specifically, on the possibility to use mechanical forces to directly influence cell plasticity and/or differentiation<sup>16-19</sup>. Indeed, it has been clearly demonstrated that the extracellular matrix (ECM) plays a key role in the control of cell fate. In particular, the biomechanical and biophysical signals produced by ECM directly regulate molecular mechanisms and signaling pathways, influencing cell behavior and functions<sup>20,21</sup>. These recent data have paved the way to the development of novel 3D culture systems that more closely mimic the *in vivo* cell microenvironment, replicating mechanical and physical stimuli driving cell behaviour.

We here describe a two-step protocol that combines the use of chemical epigenetic erasing with mechanosensing-related cues, to generate mammalian pluripotent cells. In the first step, cells are incubated with the demethylating molecule 5-aza-cytidine (5-aza-CR). This agent is able to induce a significant global DNA demethylation through a combined effect of the direct ten-eleven

translocation 2 (TET2)-mediated action<sup>8,10</sup> and the indirect inhibition of the DNA methyltransferases (DNMT)<sup>22,23</sup>. This step induces the removal of the epigenetic blocks with a subsequent re-activation of pluripotency-related gene expression and, therefore, the generation of high plasticity cells<sup>1-3,8,10</sup>, hereinafter referred as “epigenetically erased cells”. In the second step, cells are encapsulated in a 3D culture system. To this end, the non-reactive hydrophobic synthetic compound polytetrafluoroethylene (PTFE; with particle size of 1 µm) is used as micro-bioreactor, that permits the creation of a cellular microenvironment unachievable through the use of traditional 2D culture systems<sup>10</sup>. The PTFE powder particles adhere to the surface of the liquid drop in which cells are re-suspended and isolate the liquid core from the supporting surface, while allowing gas exchange between the interior liquid and the surrounding environment<sup>24</sup>. The “PTFE micro-bioreactor” thus obtained, also known as “Liquid Marble”, encourages cells to freely interact with each other, promoting 3D cell rearrangement<sup>25-27</sup>, and extends and stably maintains the acquired high plasticity state through bio-mechanosensing-related cues<sup>10</sup>.

## **PROTOCOL:**

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 isolation from healthy adult individuals was 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.

### **1. Skin fibroblast isolation**

NOTE: All the procedures described below can be applied to fibroblasts isolated from different mammalian species, including mouse, porcine, and human. Murine cells were isolated from 7-week-old male mice and porcine skin tissue were collected at local slaughterhouse. Human cells were isolated from adult patients, after written informed consent.

1.1. Prepare 0.1% porcine gelatin solution.

1.1.1. Weigh 0.1 g of porcine gelatin and dissolve it in 100 mL of water. Sterilize gelatin solution by autoclaving before use.

1.1.2. Coat 35 mm Petri dish with 0.1% porcine gelatin by adding 1.5 mL of the prepared solution. Incubate for 2 h at room temperature.

1.2. Cut mammalian (mouse, porcine, and human) skin biopsies of approximately 2-5 cm in length and place them in Dulbecco's Phosphate Buffered Saline (PBS) containing 2% antibiotic antimycotic solution. Store at + 4 °C until use.

NOTE: Biopsy collections must be carried out in agreement and after the Ethical Committee's approval, in accordance with the established guidelines.

1.3. Extensively wash the collected biopsies 3x in fresh sterile PBS containing 2% antibiotic antimycotic solution.

1.4. Collect biopsies from the last wash and place them into a sterile 100 mm Petri dish. Use a sterile scalpel to cut them into pieces of approximately 2 mm<sup>3</sup> size.

1.5. At the end of 2 h incubation, remove the excess of gelatin solution from the 35 mm Petri dish (described in step 1.1.2) and, using a sterile surgical tweezer, immediately place 5-6 skin fragments into each pre-coated culture dish.

1.6. Wet the fragments by adding 100 µL droplets of fibroblast isolation medium (**Table 1**) over each of them. Culture at 37 °C in 5% CO<sub>2</sub> incubator.

NOTE: To prevent the medium from evaporation, place the 35 mm Petri dish within a 100 mm or bigger Petri dish containing sterile water. Ensure to cap both Petri dishes.

1.7. After 24 h of culture, check the quantity of the medium in the 35 mm culture Petri dish. If needed, add 500 µL of fibroblast isolation medium to keep wet the fragments.

1.8. Carefully remove the medium and refresh it at least every 2 days of culture using a pipette.

1.9. When fibroblasts start to grow out of the skin fragments placed in the 35 mm Petri dish (described in step 1.5.) and begin to form a cell monolayer (usually 6 days). Remove skin pieces using a sterile surgical tweezer and culture in 2 mL of fibroblast isolation medium.

1.10. Continue to culture the cell monolayer at 37 °C in 5% CO<sub>2</sub> incubator until 80% confluence and refresh medium every other day.

## **2. Fibroblast primary cell line culture**

2.1. When fibroblasts reach 80% confluence, carefully remove fibroblast isolation medium and wash cells three times with 3 mL of PBS containing 1% antibiotic antimycotic solution.

2.2. For cell detaching, add 600 µL of 0.25% trypsin-EDTA solution in the culture dish and incubate at 37 °C for 3-5 min.

2.3. Add 5.4 mL of fibroblast culture medium to neutralize trypsin when cells start to detach from the culture dish (**Table 1**).

2.4. Dislodge cells by repeated and gentle pipetting. Plate cells in new culture dishes (without gelatin), keeping the passage ratio between 1:2 and 1:4 (depending on growth rate).

NOTE: Centrifugation is not necessary.

2.5. Maintain cells in culture and change medium every 2 days, until they have reached 80% confluency and passage them.

NOTE: Propagate fibroblasts twice a week to maintain vigorous growth.

### **3. Fibroblast exposure to 5-aza-CR**

#### **3.1. Prepare fresh 1 mM 5-aza-CR stock solution.**

3.1.1. Weigh 2.44 mg of 5-aza-CR and dissolve it in 10 mL of DMEM high glucose. Resuspend the powder by vortexing. Sterilize the solution with 0.22  $\mu$ m filter.

NOTE: 5-aza-CR stock solution must be prepared immediately before use.

3.1.2. Prepare 5-aza-CR working solution by diluting 1  $\mu$ L of 5-aza-CR stock solution (3.1.1.) in 1 mL of fibroblast culture medium.

NOTE: The concentration of 5-aza-CR working solution is 1  $\mu$ M<sup>1-3, 8, 9</sup>.

3.2. Trypsinize cells as previously described (2.1.-2.3.) and dislodge cells by repeatedly and gently pipetting.

3.3. Collect the cell suspension and transfer it into a conical tube.

3.4. Count cells using a counting chamber under an optical microscope at room temperature. Calculate the volume of medium needed to re-suspend cells to obtain  $4 \times 10^4$  cells in 30  $\mu$ L of fibroblast culture medium supplemented with 1  $\mu$ M 5-aza-CR (see step 3.1.2.).

NOTE: The formula to be used depends on the specific type of chamber.

*Cells/ $\mu$ L*

*= Average number of cells per small grid  $\times$  chamber multiplication factor  $\times$  dilution.*

3.5. Centrifuge the cell suspension at 150  $\times g$  for 5 min at room temperature. Remove the supernatant and resuspend pellet with the fibroblast culture medium supplemented with 1  $\mu$ M 5-aza-CR (see step 3.1.2.). For the volume of the fibroblast culture medium to be used see step 3.4.

NOTE: As a negative control, resuspend cells at the same concentration in fibroblast culture medium without 5-aza-CR and proceed with cell encapsulation in PTFE powder (step 4.1.-4.13.).

### **4. Fibroblast encapsulation in PTFE micro-bioreactors**

221 4.1. Fill a 35 mm Petri dish with polytetrafluoroethylene (PTFE) powder to produce a bed (**Figure**  
222 **1A**).

223  
224 NOTE: Use 35 mm bacteriology Petri dishes to avoid liquid marble adhesion. In order to obtain a  
225 thin hydrophobic and porous shell, use a PTFE powder with an average particle size of 1  $\mu\text{m}$  and  
226 produced with a maximum grind of 2.0 NPRI. This allows for the creation of gas-permeable liquid  
227 marbles. Furthermore, the translucent coating facilitates the observation of cell aggregation  
228 processes in real-time. Larger particle size leads to high polydispersity that can cause elevated  
229 evaporation, deformity and loss of the spherical shape, and the premature dissolution of the  
230 micro-bioreactors.

231  
232 4.2. Dispense 30  $\mu\text{L}$  single droplet containing  $4 \times 10^4$  cells (see steps 3.4.- 3.5.) onto the powder  
233 bed (**Figure 1B**).

234  
235 4.3. Gently rotate the 35 mm Petri dish in a circular motion to ensure that PTFE powder entirely  
236 cover the surface of the liquid drop to form a liquid marble micro-bioreactor (**Figure 1C**).

237  
238 4.5. Pick up the liquid marble micro-bioreactor using a 1,000  $\mu\text{L}$  pipette tip, cut at the edge, to  
239 accommodate the diameter of the marble (**Figures 1D,E**). Plate the liquid marble micro-  
240 bioreactor onto a clean bacteriology Petri dish to stabilize it (**Figures 1F**).

241  
242 NOTE: To create a friction to grip the marble inside the tip, cut the pipette tips with a diameter  
243 approximately slightly less than the liquid marble diameter.

244  
245 4.6. Transfer the liquid marble micro-bioreactor from the Petri dish into a 96 well plate (one  
246 marble/well) (**Figure 1G**).

247  
248 4.7. Slowly add 100  $\mu\text{L}$  of media from the margin of the well. The micro-bioreactor starts to float  
249 on top of the media (**Figure 1H**).

250  
251 NOTE: The micro-bioreactor breaks in direct liquid contact, due to the disruption of PTFE  
252 hydrophobicity. As an alternative approach, the liquid marble micro-bioreactors can be  
253 individually placed in a 35 mm bacteriology culture dish. In this case, in order to prevent liquid  
254 marble evaporation, the 35 mm Petri dish containing the micro-bioreactor must be inserted in a  
255 100 mm Petri dish, previously aliquoted with sterile water

256  
257 4.8. Incubate liquid marble micro-bioreactor for 18 h at 37  $^{\circ}\text{C}$  in 5%  $\text{CO}_2$  incubator<sup>1-3,8,9</sup>.

258  
259 NOTE: The PTFE particle size of 1  $\mu\text{m}$  can ensure an optimal gas exchange between the interior  
260 liquid and the surrounding environment.

261  
262 4.9. After 5-aza-CR incubation for 18 h, collect the liquid marble micro-bioreactor using a 1,000  $\mu\text{L}$   
263 pipette tip cut at the edge (see step 4.5).



4.10. Place the micro-bioreactor in a new 35 mm bacteriology Petri dish (**Figures 1 D-F**).

4.11. Use a needle to puncture the liquid marble and break it.

4.12. Recover formed spheroids with a 200  $\mu$ L pipette tip, cut at the edge, under a stereomicroscope (**Figures 1 I,J**).

NOTE: Epigenetically erased cells encapsulated in PTFE form a 3D spherical structure (one aggregate in each liquid marble).

4.13. To assess the acquisition of pluripotent state in response to 5-aza-CR, check the onset of the pluripotency- related gene expression, OCT4, NANOG, REX1, and SOX2, by qualitative PCR (**Table 2**).

4.14. Proceed with the second step of the protocol as described below.

## **5. Culture in PTFE micro-bioreactors of epigenetically erased cells**

5.1. Prepare fresh ESC culture medium (**Table 1**).

5.2. Transfer organoids in a Petri dish containing ESC medium for washing 5-aza-CR residuals (see steps 5.1.-5.2.).

5.3. Prepare a new 35 mm bacteriology Petri dish containing PTFE powder bed (see also step 4.1.).

5.4. Dispense a single organoid in a droplet of 30  $\mu$ L ESC culture medium onto the powder bed using a 200  $\mu$ L pipette tip, cut at the edge (see steps 4.9.; 5.3.).

5.5. Gently rotate the 35 mm Petri dish in a circular motion to form a new liquid marble micro-bioreactor, pick up it using a 1,000  $\mu$ L pipette tip, cut at the edge, and place the newly formed micro-bioreactor into a well of 96-well plate (one marble/well) (see steps 4.3.-4.6.).

5.6. To float the micro-bioreactors, add 100  $\mu$ L of media from the margin of the well to slowly bathe the marble (see note 4.7.).

5.7. Culture liquid marble micro-bioreactors at 37 °C in 5% CO<sub>2</sub> incubator for as long as required. Change medium every other day, following the procedure described in 5.3.-5.7.

NOTE: In the present manuscript, results obtained with organoids culture for 28 days are provided. However, if needed longer culture period can be performed.

## **REPRESENTATIVE RESULTS:**

The present protocol describes all the steps to be performed to generate and stably maintain

mammalian pluripotent cells from adult somatic cells. This method has been successful with fibroblasts isolated from different mammalian species, namely mouse, porcine and human. The representative results here reported are obtained from all cell lines, irrespectively of the species of origin.

Morphological analyses show that, after 18 h incubation with the demethylating agent 5-aza-CR, fibroblasts encapsulated in PTFE micro-bioreactors (3D Post 5-aza-CR) aggregate and formed 3D spherical structures displaying a uniform size geometry, in all the three species considered. (**Figure 2A-C**, 3D Post 5-aza-CR).  $86.31 \pm 4.13\%$  of encapsulated cells remarkably modified their phenotype, showing features typically related to a high plasticity phenotype<sup>8</sup>. In contrast, post 5-aza-CR cells cultured into 2D standard conditions, although replaced the typical fibroblast elongated shape with a round or oval one, were considerably smaller in size with larger and granulated nuclei, retain a monolayer distribution (**Figure 2**). The morphological changes were accompanied by the onset of pluripotency-related gene expression both in 3D and 2D Post 5-aza-CR cells. Transcription for POU class 5 homeobox 1 (OCT4), Nanog homeobox (NANOG), ZFP42 zinc finger protein (REX1), and sex determining region Y-box 2 (SOX2) was also observed, which is absent in untreated fibroblasts (T0), was detected (**Figure 3, Figure 4, Figure 5**). Furthermore, quantitative PCR analysis demonstrated a significant up-regulation of the above mentioned genes, as well as of the ten-eleven translocation-2 (TET2), epithelial cell adhesion molecule (EPCAM), and cadherin 1 (CDH1) genes in 3D Post 5-aza-CR cells (**Figure 3, Figure 4, Figure 5**, blue bars) compared to cells cultured in 2D standard plastic dishes (2D Post 5-aza-CR) (**Figure 3, Figure 4, Figure 5**, orange bars). In parallel, a significant downregulation of the fibroblast specific marker Thy-1 cell surface antigen (THY1) was clearly detectable in 3D and 2D Post 5-aza-CR cells (**Figure 3, Figure 4, Figure 5**).

The achievement of a high plasticity state was also confirmed by ELISA analysis of DNA global methylation, that demonstrates a significant decrease of methylation levels in both 3D and 2D Post 5-aza-CR cells (**Figure 6 A-C**). Moreover, in agreement with the gene expression results, DNA methylation levels were significantly lower in 3D Post 5-aza-CR cells (**Figure 6 A-C**, blue bars), compared to 2D Post 5-aza-CR ones (**Figure 6 A-C**, orange bars).

Even more interestingly, 3D Post 5-aza-CR cells retain the acquired 3D spherical structure (**Figure 2A**, 3D 28d) and maintain high expression levels of pluripotency-related genes (**Figures 3, 4, and 5**, blue bars) as well as low DNA methylation levels (**Figure 6 A-C**, blue bars), during all the subsequent culture period and, specifically, until 28 day, when culture was arrested. In contrast, although 2D Post 5-aza-CR cells transcribe for the same pluripotency genes after treatment with the demethylating agent, they turned down such expression by day 7 (**Figure 3, Figure 4, Figure 5**, nd). Similarly, the decrease in methylation levels was maintained for the first 72 hours; then methylation slowly increased, returning comparable to untreated fibroblasts (**Figure 6 A-C**, T0, white bars) by day 7 of culture (**Figure 6 A-C**, orange bars).

#### FIGURE AND TABLE LEGENDS:

**Figure 1: Cell encapsulation in PTFE micro-bioreactor and organoid recovery. (A)** A bacteriology

Petri dish was filled with PTFE to prepare a powder bed. (B) A single droplet of medium containing cells was dispensed on top of the PTFE bed. (C) The Petri dish was gently rotated with circular movements to coat the droplet and produce the micro-bioreactor. (D) A 1000  $\mu$ L pipette tip was cut at the end (red arrow) and (E) used to collect the micro-bioreactor. (F) The liquid marble was transferred to a clean Petri dish to stabilize it, (G) placed into a 96 well plate (one marble/well) and (H) floated onto the media. (I) To collect newly formed organoid, the micro-bioreactor was punctured with a needle and (J) the obtained cell aggregates were recovered under a stereomicroscope.

**Figure 2: Mammalian epigenetically erased cells encapsulated in PTFE micro-bioreactors form 3D spherical structures.** Murine (A), porcine (B) and human (C) cells encapsulated in PTFE and treated with 5-aza-CR form 3D spherical structures (3D Post 5-aza-CR), that were stably maintained during all the subsequent culture period (3D 28d; Scale bar, 100  $\mu$ m). In contrast, murine (A), porcine (B) and human (C) cells plated onto plastic dishes and treated with 5-aza-CR replaced the typical fibroblast elongated shape (T0) into a round epithelioid phenotype and retained a monolayer distribution (2D Post 5-aza-CR). By day 7 of culture, 2D cells reverted to their original elongated shape which was stably maintained for the subsequent culture period (2D 28 d; Scale bar, 100  $\mu$ m).

**Figure 3: Murine epigenetically erased cells encapsulated in PTFE micro-bioreactors show high level and long-term maintenance of pluripotency-related gene expression.** Transcription levels for *Oct4*, *Nanog*, *Rex1*, *Sox2*, *Tet2*, *Epcam*, *Cdh1* and *Thy1* genes in murine untreated fibroblasts (T0, white bars), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR), and at different time points of culture for PTFE encapsulated (blue bars) and standard plastic dish (orange bars) cultured cells. Gene expression values are reported with the highest expression set to 1 and all others relative to this. Different superscripts denote significant differences ( $P < 0.05$ ).

**Figure 4: Porcine epigenetically erased cells encapsulated in PTFE micro-bioreactors show high level and long-term maintenance of pluripotency-related gene expression.** Transcription levels for OCT4, NANOG, REX1, SOX2, TET2, EPCAM, CDH1 and THY1 genes in porcine untreated fibroblasts (T0, white bars), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR), and at different time points of culture for PTFE encapsulated (blue bars) and standard plastic dish (orange bars) cultured cells. Gene expression values are reported with the highest expression set to 1 and all others relative to this. Different superscripts denote significant differences ( $P < 0.05$ ).

**Figure 5: Human epigenetically erased cells encapsulated in PTFE micro-bioreactors show high level and long-term maintenance of pluripotency-related gene expression.** Transcription levels for OCT4, NANOG, REX1, SOX2, TET2, EPCAM, CDH1 and THY1 genes in human untreated fibroblasts (T0, white bars), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR), and at different time points of culture for PTFE encapsulated (blue bars) and standard plastic dish (orange bars) cultured cells. Gene expression values are reported with the highest expression set to 1 and all others relative to this. Different superscripts denote significant differences ( $P < 0.05$ ).

**Figure 6: The PTFE micro-bioreactor enhances 5-aza-CR demethylating effect and maintains**

**long-term DNA hypomethylation in mammalian epigenetically erased fibroblasts.** Global DNA methylation levels of murine (A), porcine (B) and human (C) cells encapsulated in PTFE micro-bioreactors (blue bars) or plated on standard plastic (orange bars) exposed to 5-aza-CR (Post 5-aza-CR) and cultured in ESC medium for 28 days. Untreated fibroblasts (T0; white bars). Results represent the mean  $\pm$  SD of three independent experiments with five independent biological replicates. Different superscripts denote significant differences ( $P < 0.05$ ).

**Table 1: Composition of fibroblast isolation medium, fibroblast culture medium and ESC culture medium.**

**Table 2: Primer information.**

## **DISCUSSION:**

During the last decades, several studies focused on the development of strategies to revert a terminally differentiated cell towards a less committed and higher permissive state. The protocol here described allow the generation and long-term maintenance of pluripotent cells starting from adult mature terminally differentiated cells. The method combines two independent steps that involve the induction of a high permissive state which is achieved through chemical epigenetic erasing and its subsequent maintenance ensured using a 3D culture system.

The formation of 3D spheroid structures observed in PTFE encapsulated cells (**Figure 2**) is consistent with other studies demonstrating PTFE ability to efficiently encourage cell aggregation, facilitating the establishment of olfactory ensheathing cell (OEC) spheroid structures<sup>25</sup> or the formation of 3D toroidal aggregates<sup>26</sup>. These morphological changes are paralleled by the onset of pluripotency-related gene expression (**Figure 3, Figure 4, Figure 5**) that shows significantly higher levels in 3D Post 5-aza-CR cells, when compared to 2D Post 5-aza-CR cells (**Figure 3, Figure 4, Figure 5**). Consistently, 3D Post 5-aza-CR cells display a higher DNA hypomethylation than 2D Post 5-aza-CR ones (**Figure 6**). Overall, these results indicate 5-aza-CR ability to induce a high plasticity state, regardless to the cell culture system used. However, the chemically induced pluripotency state achieved by the cells, is significantly promoted using a PTFE micro-bioreactor which boosts pluripotency gene transcription and increases 5-aza-CR demethylating effects. Even more interestingly, only 3D Post 5-aza-CR cells stably retain the acquired 3D spherical structure (**Figure 2**) and maintain high expression levels of pluripotency-related genes (**Figure 3, Figure 4, Figure 5**) as well as low DNA methylation levels (**Figure 6**), during all the subsequent period of culture. Altogether, the representative results here reported demonstrate that this two-step strategy is highly efficient and robust, and the use of a PTFE micro-bioreactor not only boosts high plasticity, but also allows its stable, long-term maintenance in the mammalian species considered. We recently demonstrated that these beneficial effects are related to the activation of the Hippo-signaling pathway and its mechanotransduction-related cues<sup>10</sup>, that have been previously shown to have a key role in the active regulation of cell pluripotency<sup>28-30</sup>.

The two most critical steps for a successful procedure are the rigorous maintenance of cells at 37 °C, at all times, including their handling under the sterile laminar flow and the microscope and

the use of a correct cell number/liquid volume rate during micro-bioreactor production, that may vary according to the specific cell type used. In our experience, it is also highly recommended to prepare reagents freshly, prior to their use in culture (this is absolutely crucial for 5-aza-CR stock solution). Furthermore, medium refreshing must be carried out under a stereomicroscope since the 3D spherical organoids may be lost during medium changes.

The main strengths of this method are no transgenic and/or viral vector requirement; the robustness and reproducibility in different mammalian species; low costs; and high flexibility to different cell types. On the other hand, a possible limitation could be represented by the restricted number of data obtained, due to the small volumes of the micro-bioreactors. In addition, the use of high cell density may cause low oxygen transfer rates and limited growth in suspension. To overcome these problems, further work on scale-up and/or scale-down strategies remains necessary.

It is important to highlight that the key aspects common to all 3D spheroid-based applications are the reproducibility, the production efficiency, the organoid size uniformity and the influence on cellular physiology. These features are strictly correlated to the mechanical forces generated within the culture system and vary according to the different methods used. For instance, multicellular organoids can be cultured using non-adherent dishes in stationary systems. This approach is primarily based on diffusion-limited conditions and, usually, results in the formation of loose-aggregated clusters. The hanging-drop technique shows the same limitation. Indeed, the deposition of cell suspension drops onto the underside of the lid of a tissue culture dish leads to the creation of microgravity environment that concentrates the cells at the free liquid-air interface, inducing the generation of low-aggregated multicellular spheres. A possible alternative is represented by the spinner flask technique. However, this method is highly expensive since it requires elevated quantities of culture medium. Furthermore, the formed organoids need to be transferred to stationary culture system when used for characterization or further in vitro tests. All these issues can be overcome by using liquid marble micro-bioreactors. Indeed, they provide a non-adherent liquid surface that combines the advantages of both stationary and spinning methods, inducing a rapid cell aggregation and the generation of compact spheroids. In parallel, the concave bottom, the spherical shape, and the internal liquid flow of each marble allow cells to settle onto the bottom of the micro-bioreactor, resulting in the formation of organoids uniform in size and shape. Another significant advantage in the use of the liquid marbles is represented by the optimal gas exchanges that, thanks to their spherical shape, can occur through the entire surface.

In conclusion, the protocol here described allows for an efficient and simple generation of mammalian pluripotent cells. Since this strategy is viral vector free and does not involve the use of any gene transfection, it is highly promising for translational medicine applications and may be considered a step forward in patient-specific cell therapy. Furthermore, the use of 3D micro-bioreactor culture systems may represent a notable breakthrough in stem cell organoid technology and may constitute an advantageous micro-environment for long-term culture of different cell types, such as ESCs, iPSCs, and MSCs. An additional advantage is represented by the small volume that allows to study the effect of paracrine/autocrine signalling of the rich

environment established within the micro-bioreactor.

#### ACKNOWLEDGMENTS:

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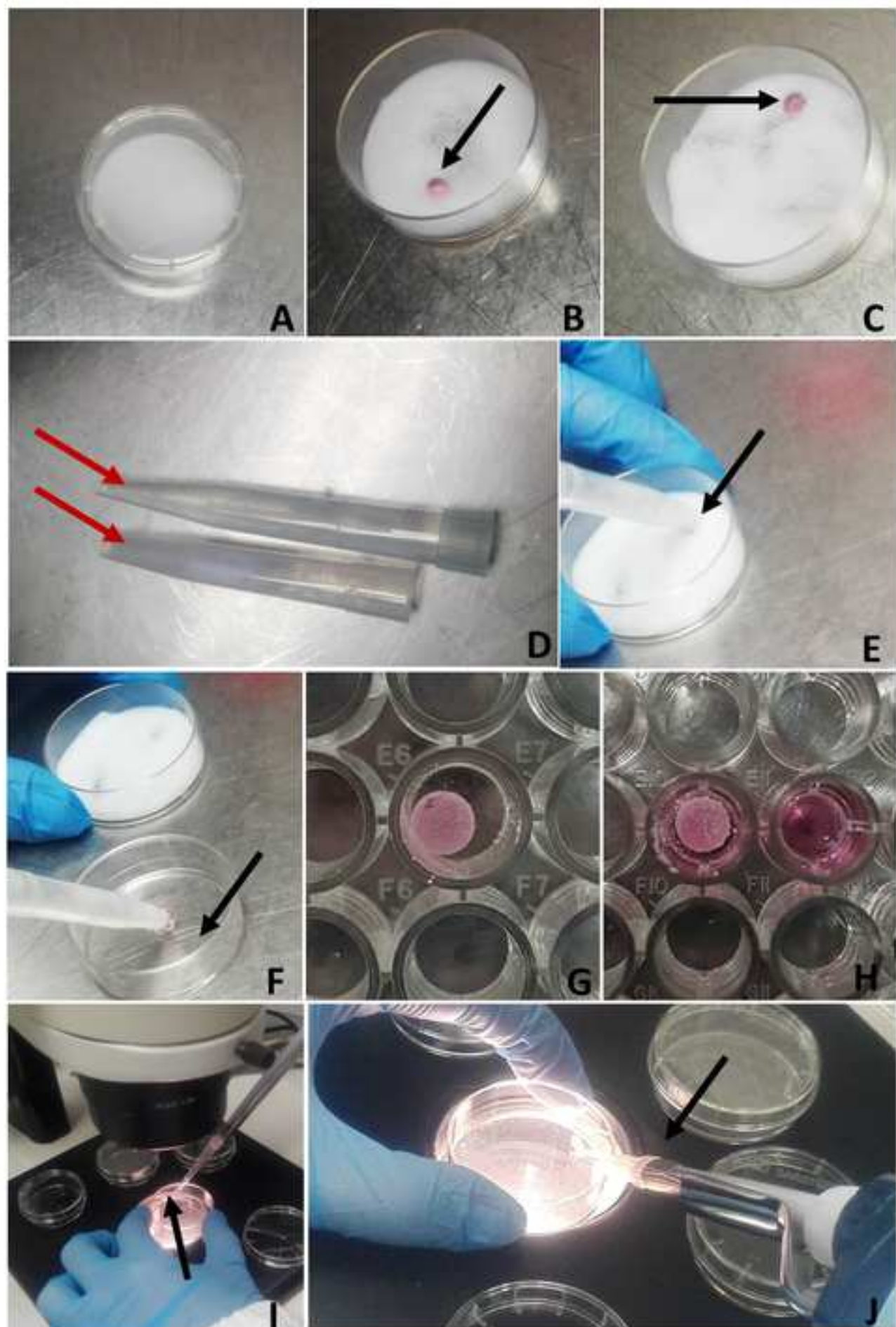
#### DISCLOSURES:

The authors have nothing to disclose.

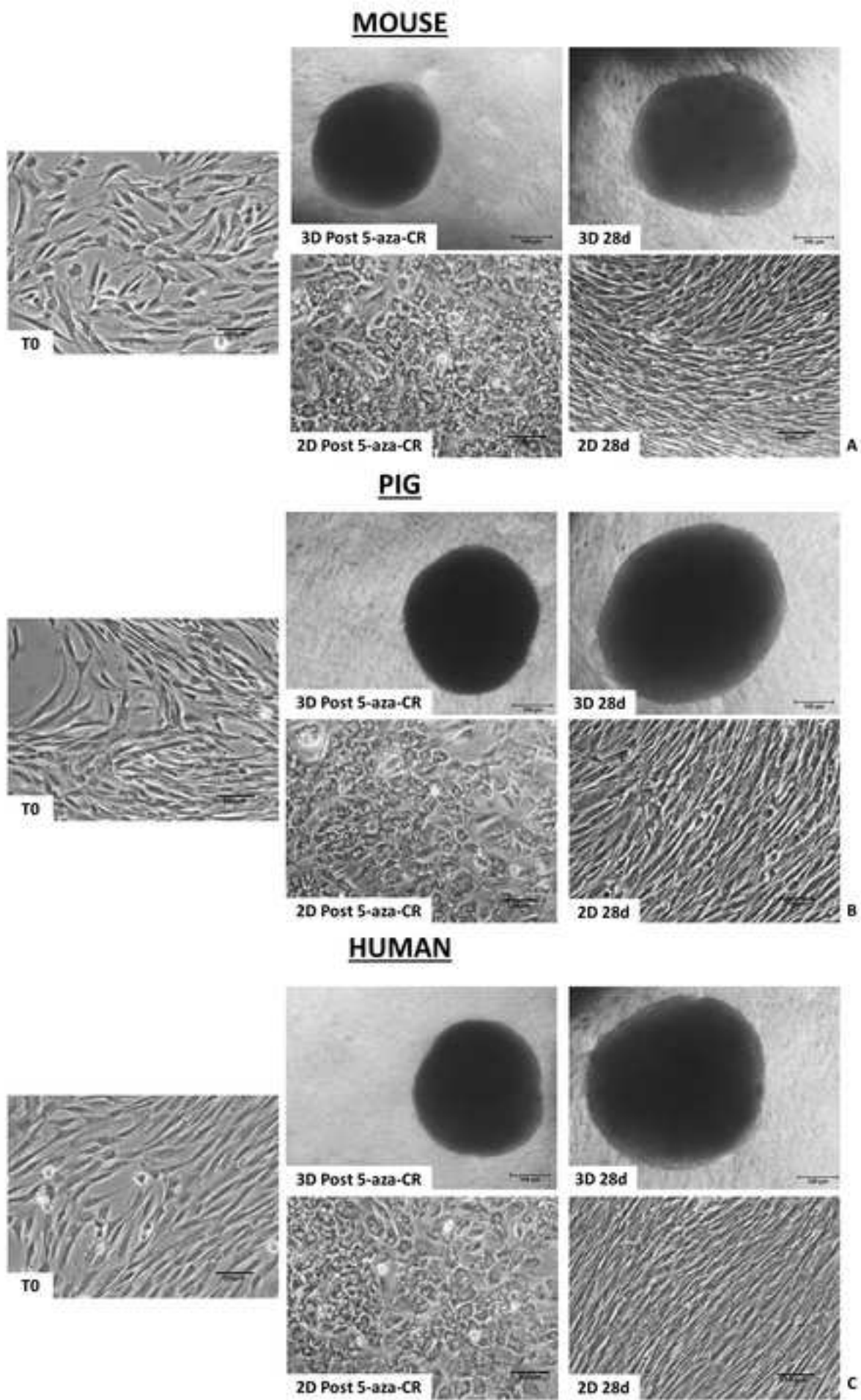
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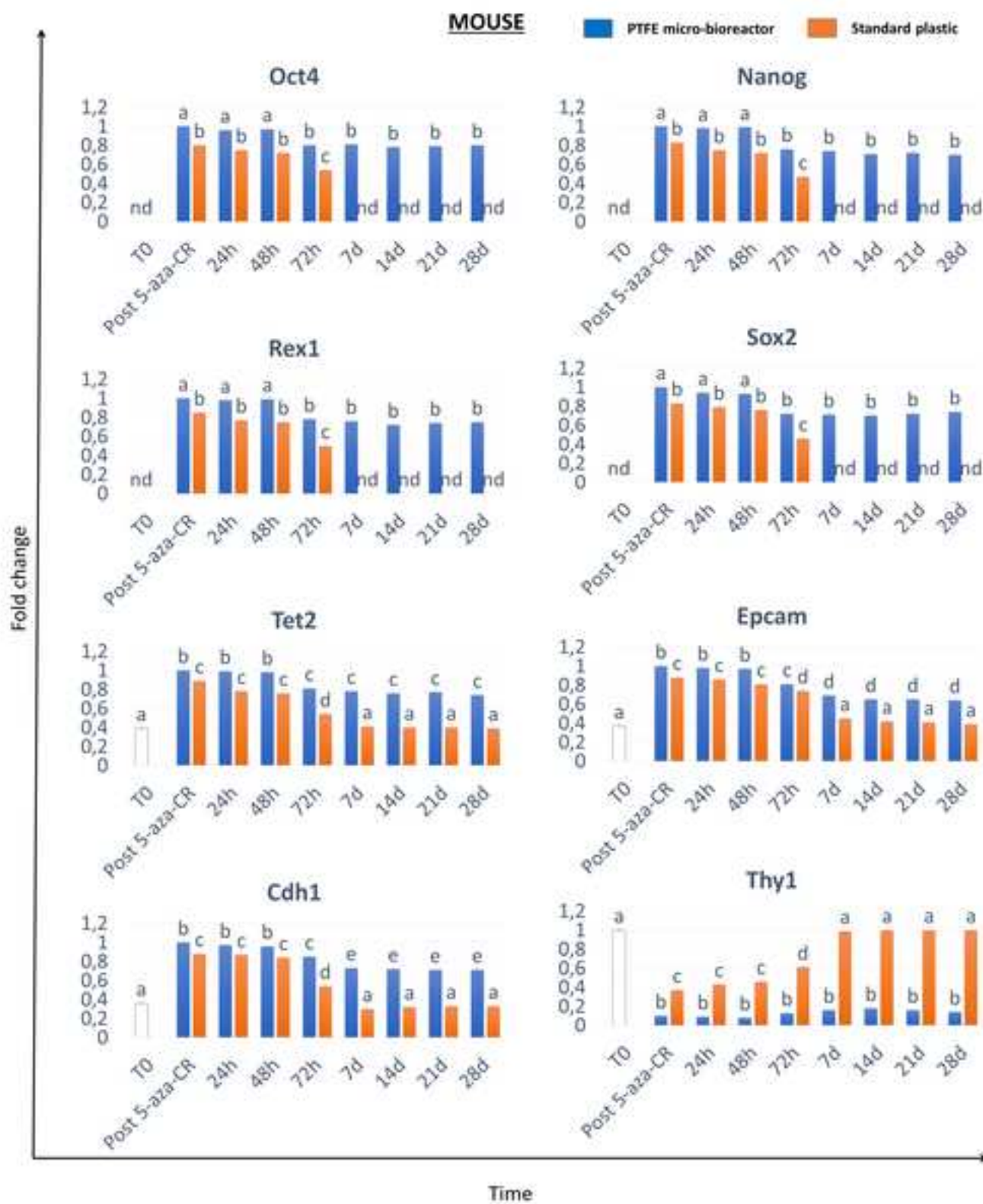
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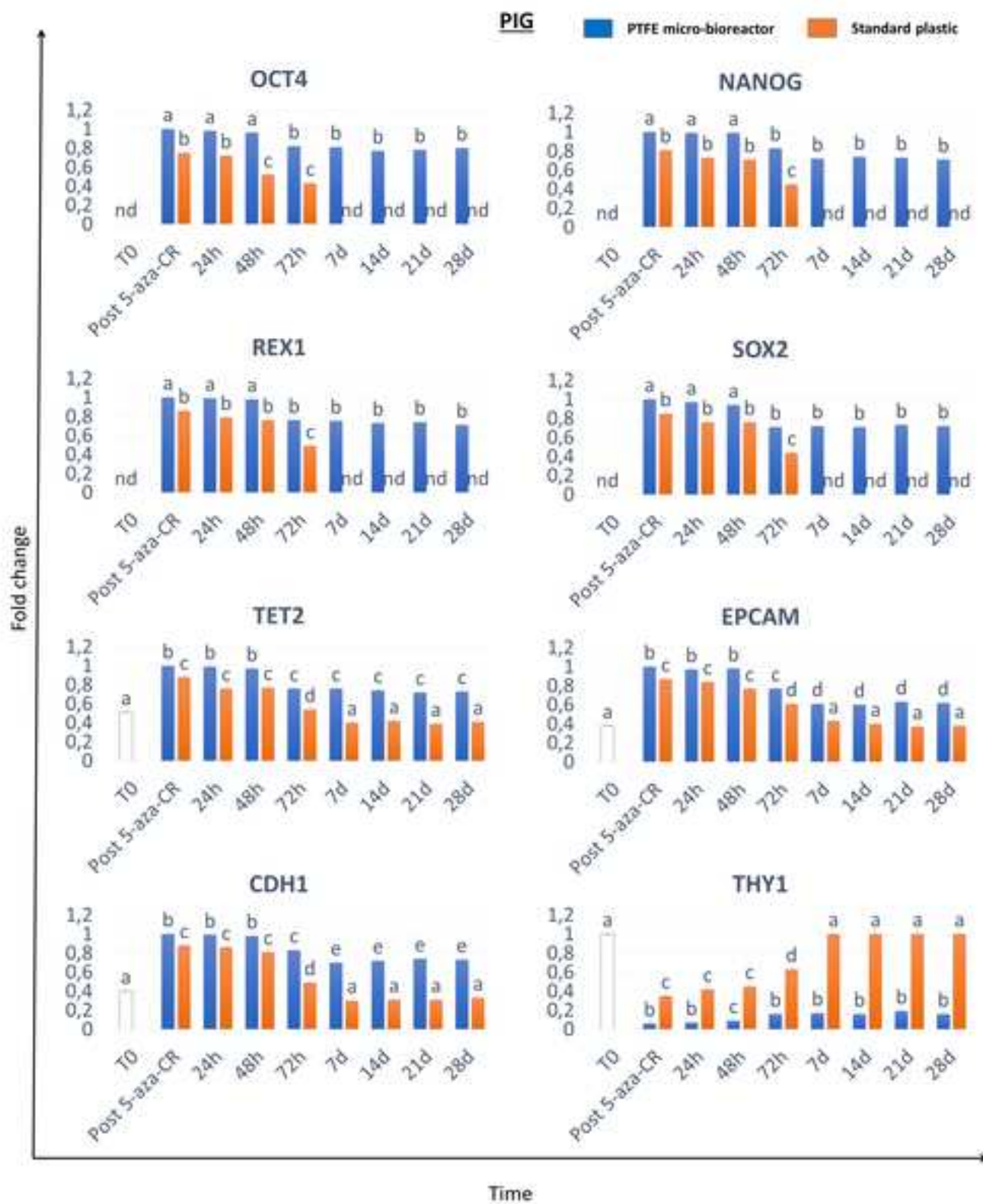
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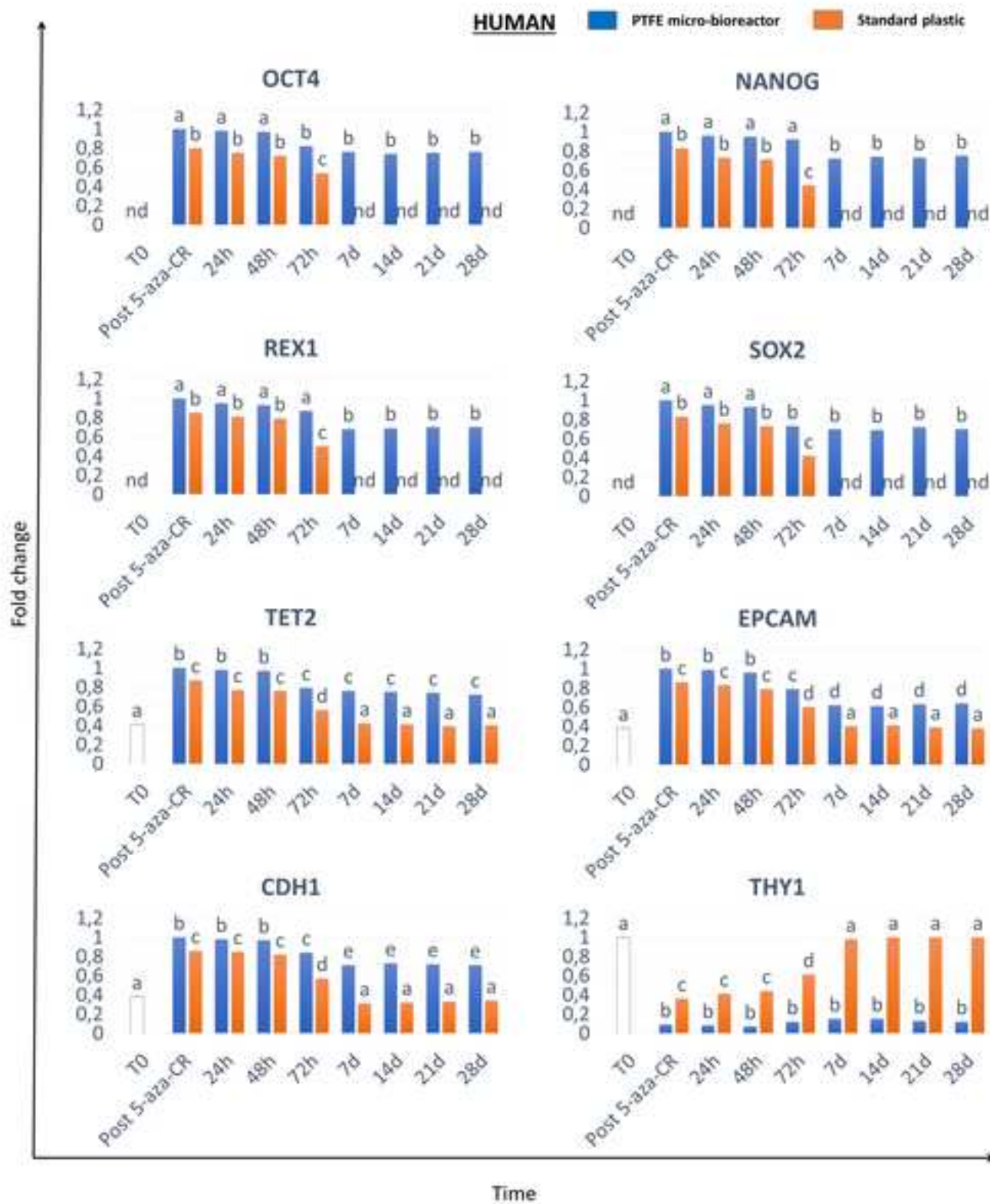


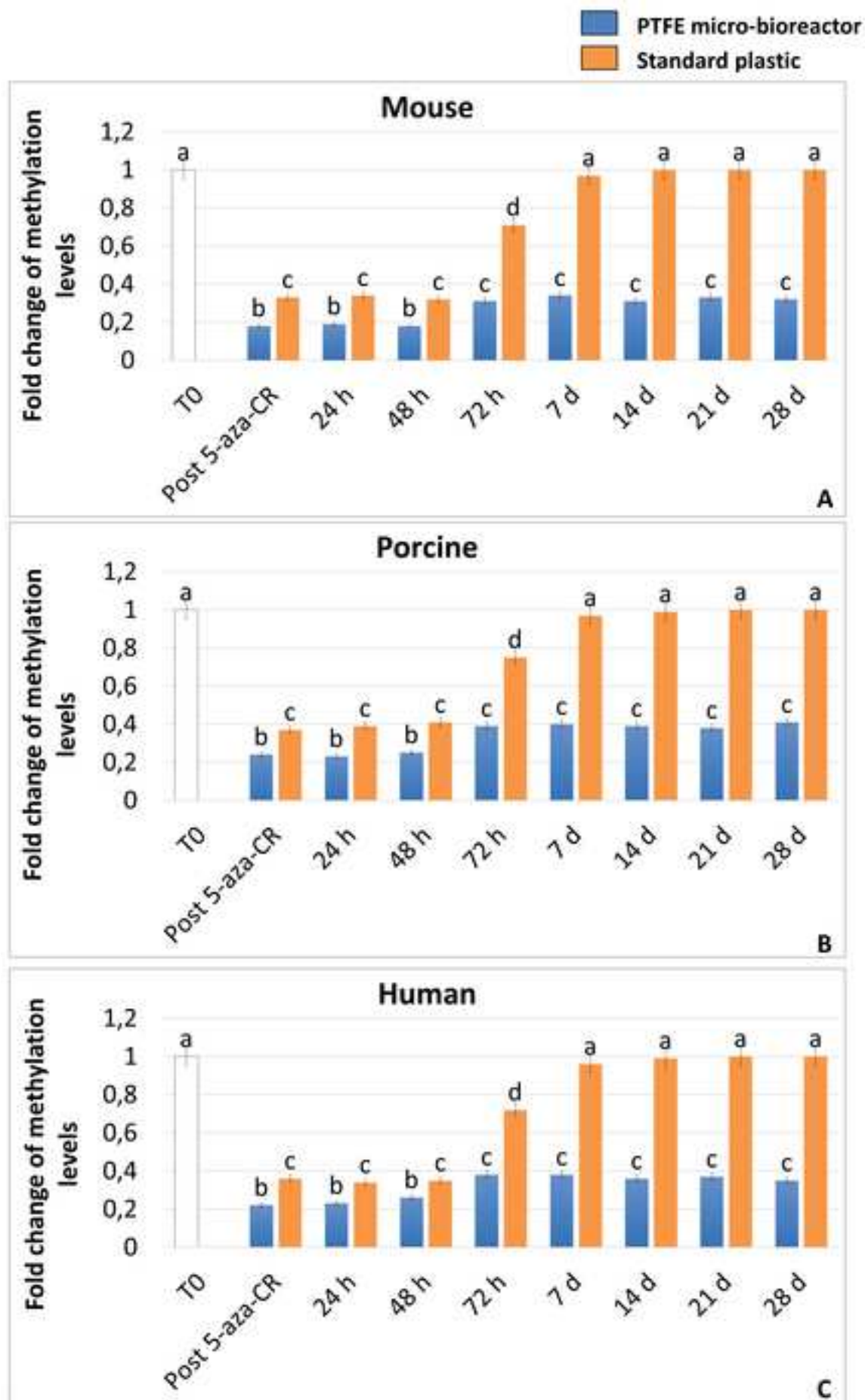












**Fibroblast Isolation Medium (100 mL)**

- DMEM, high glucose, pyruvate
- Fetal Bovine Serum, qualified, heat inactivated
- L-Glutamine solution
- Antibiotic Antimycotic Solution (100×)

**Fibroblast Culture Medium (100 mL)**

- DMEM, high glucose, pyruvate
- Fetal Bovine Serum, qualified, heat inactivated
- L-Glutamine solution
- Antibiotic Antimycotic Solution (100×)

**ESC culture medium (10 mL)**

- Ham's F-10 Nutrient Mix
- DMEM, low glucose, pyruvate
- KnockOut Serum Replacement
- Fetal Bovine Serum, qualified, heat inactivated
- Antibiotic Antimycotic Solution (100×)
- L-Glutamine solution
- MEM Non-Essential Amino Acids Solution (100X)
- 2-Mercaptoethanol (10 mM)
- Nucleoside mix (0.3 M Guanosine, 0.3 M Adenosine, 0.3 M Cytidine, 0.3 M Uridine and 0.1 M Timidine)
- ESGRO Recombinant Mouse LIF Protein (1000 units/mL)
- Recombinant Human FGF basic (bFGF) (5 µg/mL)

77 mL  
20 mL  
1 mL  
2 mL

88 mL  
10 mL  
1 mL  
1 mL

3,99 mL  
3,99 mL  
1 mL  
500 µL  
100 µL  
100 µL  
100 µL  
100 µL (0.1 mM)  
100 µL (3 mM Guanosine, 3 mM Adenosine, 3 mM Cytidine, 3 mM Uridine and 1 mM Timidine)  
10 µL (1 unit/mL)  
10 µL (5 ng/mL)

Target	PCR Primer Sets	Species
<i>Cdh1</i>	Forward: GAGGAACCCACAGCCTCATA	Mouse
	Reverse: GTTGACCGTCCCTTCACAGT	Mouse
<i>Epcam</i>	Forward: TGGCAACAAGTTGCTCTCTG	Mouse
	Reverse: CTTGTGCGTTCTTCGGACTC	Mouse
<i>Nanog</i>	Forward: AGGCTGATTGTTGGTGTC	Mouse
	Reverse: CCAGGAAGACCCACACTCAT	Mouse
<i>Oct4</i>	Forward: ACACCTGGCTTCAGACTTCG	Mouse
	Reverse: AGTTGCTTTCCACTCGTGCT	Mouse
<i>Rex1</i>	Forward: CAGGTTCTGGAAGCGAGTTC	Mouse
	Reverse: GACAAGCATGTGCTTCCTCA	Mouse
<i>Sox2</i>	Forward: AGAACCCCAAGATGCACAAC	Mouse
	Reverse: CTCGGGAAGCGTGTACTTA	Mouse
<i>Tet2</i>	Forward: GCACAGGGAGCAAGAGATTC	Mouse
	Reverse: ATGTTGACATTGCCAGTGGA	Mouse
<i>Thy1</i>	Forward: AACTCTTGGCACCATGAACC	Mouse
	Reverse: GCACGTGCTTCCTCTTCTCT	Mouse
CDH1	Forward: GAATGACAATGGCCCCATAC	Porcine
	Reverse: AGGTGGTCACCTGGTCTTTG	Porcine
EPCAM	Forward: GCTTGGTCAGTGCCAGTGTA	Porcine
	Reverse: CTTCTGACCCCAGCAGTGTT	Porcine
NANOG	Forward: ATCCAGCTTGTCCTCCAAAG	Porcine
	Reverse: ATTCATTGCTGGTTCTGG	Porcine
OCT4	Forward: GTTCAGCCAAACGACCATCT	Porcine
	Reverse: CTCAGGTTGCCTCTCACTC	Porcine
REX1	Forward: CTTCAAGGAGAGCGCAAAAC	Porcine
	Reverse: TGTCCCAATCAAAAATGCT	Porcine
SOX2	Forward: GCCCTGCAGTACAACTCCAT	Porcine
	Reverse: GCTGATCATGTCCCGTAGGT	Porcine
TET2	Forward: CAGAAAACAATGCAGCCAGA	Porcine
	Reverse: GAATGGCTCGGTCTCTGAAG	Porcine
THY1	Forward: GGCATCGCTCTCTTGCTAAC	Porcine
	Reverse: GCTGGAGAAGTTGGTTCGAG	Porcine
CDH1	Forward: TGCCAGAAAATGAAAAAGG	Human
	Reverse: GTGTATGTGGCAATGCGTTC	Human
EPCAM	Forward: GCTGGTGTGTGAACACTGCT	Human
	Reverse: ACGCGTTGTGATCTCCTTCT	Human
NANOG	Forward: AGAAAAACAACCTGGCCGAAG	Human
	Reverse: TGCTCCAGGACTGGATGTTC	Human
OCT4	Forward: AATTGCCAAGCTCCTGAAG	Human
	Reverse: GTTGCCTCTCACTCGTTCT	Human
REX1	Forward: ACGTTTCGTGTGTCCCTTTC	Human
	Reverse: TATAACCGCTTTTGGGGTTG	Human
SOX2	Forward: ACACCAATCCCATCCACACT	Human
	Reverse: GCAAACCTTCCTGCAAAGCTC	Human
TET2	Forward: CCCACTTACCTGCGTTTCAT	Human
	Reverse: ACTGTGACCTTTCCCACTG	Human
THY1	Forward: AGATCCCAGAACCATGAACC	Human
	Reverse: GCACGTGCTTCTTTGTCTCA	Human



<b>Target</b>	<b>TaqMan Assays Catalog Number</b>	<b>Species</b>
<i>Actb</i>	Mm02619580_g1	Mouse
<i>Cdh1</i>	Mm01247357_m1	Mouse
<i>Epcam</i>	Mm00493214_m1	Mouse
<i>Gapdh</i>	Mm99999915_g1	Mouse
<i>Nanog</i>	Mm02019550_s1	Mouse
<i>Oct4</i>	Mm03053917_g1	Mouse
<i>Rex1</i>	Mm03053975_g1	Mouse
<i>Sox2</i>	Mm03053810_s1	Mouse
<i>Tet2</i>	Mm00524395_m1	Mouse
<i>Thy1</i>	Mm00493681_m1	Mouse
ACTB	Ss03376563_uH	Porcine
CDH1	Ss06942341_m1	Porcine
EPCAM	Ss03384752_u1	Porcine
GAPDH	Ss03375629_u1	Porcine
NANOG	Ss04245375_s1	Porcine
OCT4	Ss03389800_m1	Porcine
REX1	Ss03373622_g1	Porcine
SOX2	Ss03388002_u1	Porcine
TET2	Ss06880359_m1	Porcine
THY1	Ss03376963_u1	Porcine
ACTB	Hs01060665_g1	Human
CDH1	Hs01023895_m1	Human
EPCAM	Hs00901885_m1	Human
GAPDH	Hs02786624_g1	Human
NANOG	Hs02387400_g1	Human
OCT4	Hs00999632_g1	Human
REX1	Hs00399279_m1	Human
SOX2	Hs01053049_s1	Human
TET2	Hs00325999_m1	Human
THY1	Hs00174816_m1	Human

<b>Name of Material/ Equipment</b>	<b>Company</b>
2-Mercaptoethanol	Sigma-Aldrich
5-Azacytidine	Sigma-Aldrich
Adenosine	Sigma-Aldrich
Antibiotic Antimycotic Solution (100x)	Sigma-Aldrich
CFX96 Real-Time PCR	Bio-Rad Laboratories
Cytidine	Sigma-Aldrich
DMEM, high glucose, pyruvate	Thermo Fisher Scientific
DMEM, low glucose, pyruvate	Thermo Fisher Scientific
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich
Dynabeads mRNA DIRECT Micro Purification Kit	Thermo Fisher Scientific
ESGRO Recombinant Mouse LIF Protein	Sigma-Aldrich
Fetal Bovine Serum, qualified, heat inactivated	Thermo Fisher Scientific
FGF-Basic (AA10-155) Recombinant Human Protein	Thermo Fisher Scientific
Gelatin from porcine skin	Sigma-Aldrich
GeneAmp PCR System 2700	Applied Biosystems
Global DNA Methylation ELISA Kit	CELL BIOLABS
GoTaq G2 Flexi DNA Polymerase	Promega
Guanosine	Sigma-Aldrich
Ham's F-10 Nutrient Mix	Thermo Fisher Scientific
KnockOut Serum Replacement	Thermo Fisher Scientific
KOVA glasstic slide 10 with grids	Hycor Biomedical
Leica MZ APO Stereo Microscope	Leica
L-Glutamine solution	Sigma-Aldrich
MEM Non-Essential Amino Acids Solution (100X)	Thermo Fisher Scientific
Millex-GS 0.22 µm pore filters	Millipore
M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant	Promega
Multiskan FC Microplate Photometer	Thermo Fisher Scientific
Nikon Eclipse TE300 Inverted Phase Contrast Microscope	Nikon
Perkin Elmer Thermal Cycler 480	Perkin Elmer
Poly(tetrafluoroethylene) 1 µm particle size	Sigma-Aldrich
PureLink Genomic DNA Mini Kit	Thermo Fisher Scientific
TaqMan Gene Expression Cells-to-CT Kit	Thermo Fisher Scientific
Thymidine	Sigma-Aldrich
Tissue Culture Dish 100X20 mm, Standard	Sarstedt
Tissue Culture Dish 35X10 mm, Standard	Sarstedt
Tissue Culture Dish 35X10 mm, Suspension	Sarstedt
Tissue Culture Plate 96 Well,Standard,F	Sarstedt
Trypsin-EDTA solution	Sigma-Aldrich
Tube 15ml, 120x17mm, PS	Sarstedt
Uridine	Sigma-Aldrich

<b>Catalog Number</b>	<b>Comments/Description</b>
M7522	Component of ESC medium
A2385	5-aza-CR, for fibroblast epigenetic erasing
A4036	Component of nucleoside mix for ESC medium
A5955	Component of fibroblast and ESC media
NA	Thermal cycler for quantitative PCR
C4654	Component of nucleoside mix for ESC medium
41966052	For fibroblast isolation and culture medium
31885023	For ESC medium
D5652	PBS; for biopsy and cell wash and for solution preparation
61021	mRNA estraction
ESG1106	Component of ESC medium
10500064	Component of fibroblast and ESC media
PHG0024	Component of ESC medium
G1890	For dish coating
NA	Thermal cycler for qualitative PCR
STA-380	Methylation study
M7801	Qualitative PCR
G6264	Component of nucleoside mix for ESC medium
31550031	For ESC medium
10828028	Component of ESC medium
87144	For cell counting
NA	For organoid observation
G7513	Component of fibroblast and ESC media
11140035	Component of ESC medium
SLGS033SB	For solution sterilization
M3681	mRNA reverse transcription
51119000	For ELISA plate reading
NA	For cell observation
NA	Thermal cycler for reverse transcription
430935	For generating micro-bioreactor
K182001	Genomic DNA estraction
AM1728	Quantitative PCR
T1895	Component of nucleoside mix for ESC medium
833902	For fibroblast isolation
833900	For Fibroblast isolation
833900500	Bacteriology petri dish for liquid marble culture
833924005	For liquid marble culture
T3924	For fibroblast dissociation
62553041	For cell suspension centrifugation
U3003	Component of nucleoside mix for ESC medium

**Editorial comments:**

**A1.** Please tone down the language.

*The sentence was modified to tone down the language:*

*“it may represent a notable technological advance for translational medicine applications.” (please see lines 55-56).*

**A2.** Please reword as it matches with previously published literature.

*The sentence was reworded:*

*“by in vitro re-creating a specific micro-environment that allows for long-term culture of high plasticity cells, namely as ESCs, iPSCs, epigenetically erased cells and MSCs.” (please see lines 57-59).*

**A3.** Please reword as it matches with previously published literature.

*The sentence was reworded:*

*“Indeed, it has been clearly demonstrated that the extracellular matrix (ECM) plays a key role in the control of cell fate. In particular, the biomechanical and biophysical signals produced by ECM directly regulate molecular mechanisms and signaling pathways influencing cell behavior and functions.” (please see lines 78-81).*

**A4.** Reworded to avoid previous match. Please check.

*The sentence was reworded:*

*“cells are incubated with the demethylating molecule 5-aza-cytidine (5-aza-CR). This agent is able to induce a significant global DNA demethylation through a combined effect of the direct ten-eleven translocation 2 (TET2)-mediated action and the indirect inhibition of the DNA methyltransferases (DNMT)”. (please see lines 86-90).*

**A5.** These details can be moved to the discussion.

*The details were moved from the introduction section to the discussion.*

**A6.** What kind of patients.

*Details were added.*

**A7.** Which fragment?

*Details were added.*

**A8.** Concentration?

*The concentration of 5-aza-CR stock solution was added.*

**A9.** Please reword as it matches with previously published literature.

*The sentence was reworded:*

*“To obtain a thin hydrophobic and porous shell, use a PTFE powder with an average particle size of 1  $\mu$ m and produced with a maximum grind of 2.0 NPRI. This allows for the creation of gas-permeable liquid marbles. Furthermore, the translucent coating facilitates the observation of cell aggregation processes in real-time.” (please see lines 232-236).*

**A10.** To completely cover the marble?

*The sentence was modified to allow a better understanding:*

*“Slowly add 100  $\mu$ L of media from the margin of the well. The micro-bioreactor starts to float on top of the media” (please see lines 256-257).*

**A11.** This needs clarity. Please reword.

*In order to clarify, the sentence was modified:*

*“In this case, in order to prevent liquid marble evaporation, the 35 mm Petri dish containing the micro-bioreactor must to be inserted in a 100 mm petri dish, previously aliquoted with sterile water” (please see lines 261-263).*

**A12.** Is this taken from the figure published previously? If not please reword.

*The figure is original and not previously published. The sentence was reworded:*

*“(A) A bacteriology petri dish is filled with PTFE to prepare a powder bed. (B) A single droplet of medium containing cells is dispensed on top of the PTFE bed. (C) The petri dish is gently rotated with circular movements to coat the droplet and produce the micro-bioreactor.” (please see lines 355-358).*

**A13.** Reworded to avoid match.

*The figure title was reworded. Please see line 365.*

**A14.** Figure 3, 4, 5: Are these taken from previously published literature? If yes, please include the reprint permission and cite the figures accordingly. If not please reword the legend for all these three figures.

*The figure is original and not previously published. The legend of figure 3 was reworded:*

*“Murine epigenetically erased cells encapsulated in PTFE micro-bioreactors show high level and long-term maintenance of pluripotency-related gene expression. Transcription levels for Oct4, Nanog, Rex1, Sox2, Tet2, Epcam, Cdh1 and Thy1 genes in murine untreated fibroblasts (T0, white bars), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR), and at different time points of culture for PTFE encapsulated (blue bars) and standard plastic dish (orange bars) cultured cells. Gene expression values are reported with the highest expression set to 1 and all others relative to this. Different superscripts denote significant differences ( $P < 0.05$ ).” (please see lines 375-381).*

**A15.** Please see my comment in figure 3.

*The figure is original and not previously published. The legend of figure 4 was reworded:*

*“Porcine epigenetically erased cells encapsulated in PTFE micro-bioreactors show high level and long-term maintenance of pluripotency-related gene expression. Transcription levels for OCT4, NANOG, REX1, SOX2, TET2, EPCAM, CDH1 and THY1 genes in porcine untreated fibroblasts (T0, white bars), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR), and at different time points of culture for PTFE encapsulated (blue bars) and standard plastic dish (orange bars) cultured cells. Gene expression values are reported with the highest expression set to 1 and all others relative to this. Different superscripts denote significant differences ( $P < 0.05$ ).” (please see lines 383-389).*

**A16.** Please see my comment marked in figure 3.

*The figure is original and not previously published. The legend of figure 5 was reworded:*

*“Human epigenetically erased cells encapsulated in PTFE micro-bioreactors show high level and long-term maintenance of pluripotency-related gene expression. Transcription levels for OCT4, NANOG, REX1, SOX2, TET2, EPCAM, CDH1 and THY1 genes in human untreated fibroblasts (T0, white bars), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR), and at different time points of culture for PTFE encapsulated (blue bars) and standard plastic dish (orange bars) cultured cells. Gene expression values are reported with the highest expression set to 1 and all others relative to this. Different superscripts denote significant differences ( $P < 0.05$ ).” (please see lines 391-397).*

**A17.** Please expand on the limitation of the protocol.

*The limitation of the protocol was expanded:*

*“On the other hand, a possible limitation could be represented by the restricted number of data obtained, due to the small volumes of the micro-bioreactors. In addition, the use of high cell density may cause low oxygen transfer rates and limited growth in suspension. To overcome these problems, further work on scale-up and/or scale-down strategies still remains necessary.” (Please see lines 447-451).*

**A18.** Some of these details can be moved to the result and removed from here.

*Some details were removed from the discussion section and moved to the result.*