1 **TITLE:**
2 A Two-Step Strategy that Combines Epigenetic Modification and Biomechanical Cues to Generate
3 Mammalian Pluripotent Cells
4
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24 **KEYWORDS:**
25 5-aza-CR, 3D culture system, epigenetic erasing, fibroblast, mechanosensing-related cue,
26 pluripotency, PTFE microbioreactor.
27
28 **SUMMARY:**
29 We here present a method that combines the use of chemical epigenetic erasing with
30 mechanosensing-related cues to efficiently generate mammalian pluripotent cells, without the
31 need of gene transfection or retroviral vectors. This strategy is, therefore, promising for
32 translational medicine and represents a notable advancement in stem cell organoid technology.
33
34 **ABSTRACT:**
35 Cell phenotype can be reversed or modified with different methods, with advantages and
36 limitations that are specific for each technique. Here we describe a new strategy that combines
37 the use of chemical epigenetic erasing with mechanosensing-related cues, to generate
38 mammalian pluripotent cells. Two main steps are required. In the first step adult mature
39 (terminally differentiated) cells are exposed to the epigenetic eraser 5-aza-cytidine to drive them
40 into a pluripotent state. This part of the protocol was developed, based on the increasing
41 understanding of the epigenetic mechanisms controlling cell fate and differentiation; and
42 involves the use of the epigenetic modifier to erase cell differentiated state and then drive into
43 a transient high plasticity window.
44

In the second step, erased cells are encapsulated in PTFE micro-bioreactors, also known as Liquid Marbles, to promote 3D cell rearrangement to extend and stably maintain the acquired high plasticity. Polytetrafluoroethylene (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 though 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 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 toward 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[1–10]. 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 [11–15]. These demethylating events allow for the conversion of terminally differentiated cells into a primitive progenitor, a multipotent or a high plasticity/pluripotent cell[1–10].

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[16–19]. 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[20,21]. 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[8,10] and the indirect inhibition of the DNA methyltransferases (DNMT)[22,23]. 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[1–3,8,10], 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[10]. 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[24]. The "PTFE micro-bioreactor" thus obtained, also known as "Liquid Marble", encourages cells to freely interact with each other, promoting 3D cell rearrangement[25–27], and extends and stably maintains the acquired high plasticity state though bio-mechanosensing-related cues[10].

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

133 approval, in accordance with the established guidelines.
134
135 1.3. Extensively wash the collected biopsies 3x in fresh sterile PBS containing 2% antibiotic
136 antimycotic solution.
137
138 1.4. Collect biopsies from the last wash and place them into a sterile 100 mm Petri dish. Use a
139 sterile scalpel to cut them into pieces of approximately 2 mm^3 size.
140
141 1.5. At the end of 2 h incubation, remove the excess of gelatin solution from the 35 mm Petri dish
142 (described in step 1.1.2) and, using a sterile surgical tweezer, immediately place 5-6 skin
143 fragments into each pre-coated culture dish.
144
145 1.6. Wet the fragments by adding 100 µL droplets of fibroblast isolation medium (**Table 1**) over
146 each of them. Culture at 37 °C in 5% CO_2 incubator.
147
148 NOTE: To prevent medium evaporation place the 35 mm Petri dish within a 100 mm or bigger
149 Petri dish containing sterile water. Ensure to cap both Petri dishes.
150
151 1.7. After 24 h of culture, check the quantity of medium in the 35 mm culture Petri dish. If needed,
152 add 500 µL of fibroblast isolation medium to keep wet the fragments.
153
154 1.8. Carefully remove the medium and refresh it at least every 2 days of culture using a pipette.
155
156 1.9. When fibroblasts start to grow out of the skin fragments placed in the 35 mm Petri dish
157 (described in step 1.5.) and begin to form a cell monolayer (usually 6 days), remove skin pieces,
158 using a sterile surgical tweezer, and culture in 2 mL of fibroblast isolation medium.
159
160 1.10. Continue to culture the cell monolayer at 37 °C in 5% CO_2 incubator until 80% confluence
161 and refresh medium every other day.
162
163 **2. Fibroblast primary cell line culture**
164
165 2.1. When fibroblasts reach 80% confluence, carefully remove fibroblast isolation medium and
166 wash cells three times with 3 mL of PBS containing 1% antibiotic antimycotic solution.
167
168 2.2. For cell detaching, add 600 µL of 0.25% trypsin-EDTA solution in the culture dish and incubate
169 at 37 °C for 3-5 min.
170
171 2.3. Add 5.4 mL of fibroblast culture medium to neutralize trypsin when cells start to detach from
172 the culture dish (**Table 1**).
173
174 2.4. Dislodge cells by repeated and gentle pipetting. Plate cells in new culture dishes (without
175 gelatin), keeping the passage ratio between 1:2 and 1:4 (depending on growth rate).
176

177 NOTE: Centrifugation is not necessary.
178
179 2.5. Maintain cells in culture and change medium every 2 days, until they have reached 80%
180 confluency and passage them.
181
182 NOTE: Propagate fibroblasts twice a week to maintain vigorous growth.
183
184 **3. Fibroblast exposure to 5-aza-CR**
185
186 3.1. Prepare fresh 1mM 5-aza-CR stock solution:
187
188 3.1.1. Weigh 2.44 mg of 5-aza-CR and dissolve it in 10 mL of DMEM high glucose. Resuspend the
189 powder by vortexing. Sterilize the solution with 0.22 µm filter.
190
191 NOTE: 5-aza-CR stock solution must be prepared immediately before use.
192
193 3.1.2. Prepare 5-aza-CR working solution by diluting 1 µL of 5-aza-CR stock solution (3.1.1.) in 1
194 mL of fibroblast culture medium.
195
196 NOTE: The concentration of 5-aza-CR working solution is 1 µM [1–3, 8, 9].
197
198 3.2. Trypsinize cells as previously described (2.1.-2.3.) and dislodge cells by repeatedly and gently
199 pipetting.
200
201 3.3. Collect the cell suspension and transfer it into a conical tube.
202
203 3.4. Count cells using a counting chamber under an optical microscope at room temperature.
204 Calculate the volume of medium needed to re-suspend cells to obtain 4 x 10^4 cells in 30 µL of
205 fibroblast culture medium supplemented with 1 µM 5-aza-CR (see step 3.1.2.).
206
207 NOTE: The formula to be used depends on the specific type of chamber.
208
209 Cells/µL = Average number of cells per small grid x chamber multiplication factor x dilution.
210
211 3.5. Centrifuge the cell suspension at 150 *x g* for 5 min at room temperature. Remove the
212 supernatant and resuspend pellet with the fibroblast culture medium supplemented with 1 µM
213 5-aza-CR (see step 3.1.2.). For the volume of the fibroblast culture medium to be used see step
214 3.4.
215
216 NOTE: As a negative control, resuspend cells ad the same concentration in fibroblast culture
217 medium without 5-aza-CR and proceed with cell encapsulation in PTFE powder (step 4.1.-4.13.).
218
219 **4. Fibroblast encapsulation in PTFE micro-bioreactors**
220

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 µm and
226 produced with a maximum grind of 2.0 NPIRI. 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 µL single droplet containing 4×10^4 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 PFTE powder entirely
236 cover the surface of the liquid drop to form a liquid marble micro-bioreactor (**Figure 1 C**).
237
238 4.5. Pick up the liquid marble micro-bioreactor using a 1,000 µL 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 µL 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 to be inserted in
255 a 100 mm petri dish, previously aliquoted with sterile water
256
257 4.8. Incubate liquid marble micro-bioreactor for 18 h at 37 °C in 5% CO_2 incubator[1–3, 8, 9].
258
259 NOTE: The PTFE particle size of 1 µm 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 µL
263 pipette tip cut at the edge (see step 4.5).
264

265 4.10. Place the micro-bioreactor in a new 35 mm bacteriology Petri dish (**Figures 1 D-F**).
266
267 4.11. Use a needle to puncture the liquid marble and break it.
268
269 4.12. Recover formed spheroids with a 200 µL pipette tip, cut at the edge, under a
270 stereomicroscope (**Figures 1 I,J**).
271
272 NOTE: Epigenetically erased cells encapsulated in PTFE form a 3D spherical structure (one
273 aggregate in each liquid marble).
274
275 4.13. To assess the acquisition of pluripotent state in response to 5-aza-CR, check the onset of
276 the pluripotency- related gene expression, OCT4, NANOG, REX1, and SOX2, by qualitative PCR
277 (**Table 2**).
278
279 4.14. Proceed with the second step of the protocol as described below.
280
281 **5. Culture in PTFE micro-bioreactors of epigenetically erased cells**
282
283 5.1. Prepare fresh ESC culture medium (**Table 1**).
284
285 5.2. Transfer organoids in a Petri dish containing ESC medium for washing 5-aza-CR residuals (see
286 steps 5.1.-5.2.).
287
288 5.3. Prepare a new 35 mm bacteriology Petri dish containing a polytetrafluoroethylene (PTFE)
289 powder bed (see also step 4.1.).
290
291 5.4. Dispense a single organoid in a droplet of 30 µL ESC culture medium onto the powder bed
292 using a 200 µL pipette tip, cut at the edge (see steps 4.9.; 5.3.).
293
294 5.5. Gently rotate the 35 mm Petri dish in a circular motion to form a new liquid marble micro-
295 bioreactor, pick up it using a 1,000 µL pipette tip, cut at the edge, and place the newly formed
296 micro-bioreactor into a well of 96-well plate (one marble/well) (see steps 4.3.-4.6.).
297
298 5.6. To float the micro-bioreactors, add 100 µL of media from the margin of the well to slowly
299 bathe the marble (see note 4.7.).
300
301 5.7. Culture liquid marble micro-bioreactors at 37 °C in 5% CO2 incubator for as long as required.
302 Change medium every other day, following the procedure described in 5.3.-5.7.
303
304 NOTE: In the present manuscript, results obtained with organoids culture for 28 days are
305 provided. However, if needed longer culture period can be performed.
306
307 **REPRESENTATIVE RESULTS:**
308 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 form 3D spherical structures displaying a uniform size geometry, in all the three species considered. (**Figure 2A-C,** 3D Post 5-aza-CR). 86.31 ± 4.13% of encapsulated cells remarkably modified their phenotype, showing features typically related to a high plasticity phenotype[8]. In contrast, Post 5-aza-CR cells cultured into 2D standard conditions, although replacing the typical fibroblast elongated shape with a round or oval one, considerably smaller in size and with larger and granulated nuclei, retain a monolayer distribution (**Figure 2**). The morphological changes are 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) is also observed, which is absent in untreated fibroblasts (T0), is detected (**Figures 3, 4, and 5**). Furthermore, quantitative PCR analysis demonstrates 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 (**Figures 3, 4, and 5**, blue bars) compared to cells cultured in 2D standard plastic dishes (2D Post 5-aza-CR) (**Figures 3, 4, and 5,** orange bars). In parallel, a significant downregulation of the fibroblast specific marker Thy-1 cell surface antigen (THY1) is clearly detectable in 3D and 2D Post 5-aza-CR cells (**Figures 3, 4, and 5**).

The achievement of a high plasticity state is 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 are 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 turn down such expression by day 7 (**Figures 3, 4, and 5,** nd). Similarly, the decrease in methylation levels is maintained for the first 72 hours; then methylation slowly increases, 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 is filled with PTFE to prepare a powder bed. (**B**) A single droplet of medium containing

353 cells is dispensed on top of the PTFE bed. (**C**) The petri dish is gently rotated with circular
354 movements to coat the droplet and produce the micro-bioreactor. (**D**) A 1000 µl pipette tip is
355 cut at the end (red arrow) and (**E**) used to collect the micro-bioreactor. (**F**) The liquid marble is
356 transferred to a clean petri dish to stabilize it, (**G**) placed into a 96-well plate (one marble/well)
357 and (**H**) floated onto the media. (**I**) To collect newly formed organoid, the micro-bioreactor is
358 punctured with a needle and (**J**) the obtained cell aggregates are recovered under a
359 stereomicroscope.
360
361 **Figure 2. Mammalian epigenetically erased cells encapsulated in PTFE micro-bioreactors form**
362 **3D spherical structures .** Murine (**A**), porcine (**B**) and human (**C**) cells encapsulated in PTFE and
363 treated with 5-aza-CR form 3D spherical structures (3D Post 5-aza-CR), that are stably maintained
364 during all the subsequent culture period (3D 28d; Scale bar, 100 µm). In contrast, murine (**A**),
365 porcine (**B**) and human (**C**) cells plated onto plastic dishes and treated with 5-aza-CR replace the
366 typical fibroblast elongated shape (T0) into a round epithelioid phenotype and retain a
367 monolayer distribution (2D Post 5-aza-CR). By day 7 of culture, 2D cells revert to their original
368 elongated shape which is stably maintained for the subsequent culture period (2D 28 d; Scale
369 bar, 100 µm).
370
371 **Figure 3. Murine epigenetically erased cells encapsulated in PTFE micro-bioreactors show high**
372 **level and long-term maintenance of pluripotency-related gene expression.** Transcription levels
373 for *Oct4*, *Nanog*, *Rex1*, *Sox2*, *Tet2*, *Epcam*, *Cdh1* and *Thy1* genes in murine untreated fibroblasts
374 (T0, white bars), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR), and at different time points of
375 culture for PTFE encapsulated (blue bars) and standard plastic dish (orange bars) cultured cells.
376 Gene expression values are reported with the highest expression set to 1 and all others relative
377 to this. Different superscripts denote significant differences ($P < 0.05$).
378
379 **Figure 4. Porcine epigenetically erased cells encapsulated in PTFE micro-bioreactors show high**
380 **level and long-term maintenance of pluripotency-related gene expression.** Transcription levels
381 for OCT4, NANOG, REX1, SOX2, TET2, EPCAM, CDH1 and THY1 genes in porcine untreated
382 fibroblasts (T0, white bars), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR), and at different time
383 points of culture for PTFE encapsulated (blue bars) and standard plastic dish (orange bars)
384 cultured cells. Gene expression values are reported with the highest expression set to 1 and all
385 others relative to this. Different superscripts denote significant differences ($P < 0.05$).
386
387 **Figure 5. Human epigenetically erased cells encapsulated in PTFE micro-bioreactors show high**
388 **level and long-term maintenance of pluripotency-related gene expression.** Transcription levels
389 for OCT4, NANOG, REX1, SOX2, TET2, EPCAM, CDH1 and THY1 genes in human untreated
390 fibroblasts (T0, white bars), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR), and at different time
391 points of culture for PTFE encapsulated (blue bars) and standard plastic dish (orange bars)
392 cultured cells. Gene expression values are reported with the highest expression set to 1 and all
393 others relative to this. Different superscripts denote significant differences ($P < 0.05$).
394
395 **Figure 6. The PTFE micro-bioreactor enhances 5-aza-CR demethylating effect and maintains**
396 **long-term DNA hypomethylation in mammalian epigenetically erased fibroblasts.** Global DNA

397 methylation levels of murine (**A**), porcine (**B**) and human (**C**) cells encapsulated in PTFE micro-
398 bioreactors (blue bars) or plated on standard plastic (orange bars) exposed to 5-aza-CR (Post 5-
399 aza-CR) and cultured in ESC medium for 28 days. Untreated fibroblasts (T0; white bars). Results
400 represent the mean ± SD of three independent experiments with five independent biological
401 replicates. Different superscripts denote significant differences ($P < 0.05$).
402
403 **Table 1: Composition of fibroblast isolation medium, fibroblast culture medium and ESC culture**
404 **medium.**
405
406 **Table 2: Primer information.**
407
408 **DISCUSSION:**
409 During the last decades, several studies focused on the development of strategies to revert a
410 terminally differentiated cell towards a less committed and higher permissive state. The protocol
411 here described allow the generation and long-term maintenance of pluripotent cells starting
412 from adult mature terminally differentiated cells. The method combines two independent steps
413 that involve the induction of a high permissive state which is achieved through chemical
414 epigenetic erasing and its subsequent maintenance ensured using a 3D culture system.
415
416 The formation of 3D spheroid structures observed in PTFE encapsulated cells (**Figure 2**) is
417 consistent with other studies demonstrating PTFE ability to efficiently encourage cell
418 aggregation, facilitating the establishment of olfactory ensheathing cell (OEC) spheroid structures
419 [25] or the formation of 3D toroidal aggregates [26]. These morphological changes are paralleled by
420 the onset of pluripotency-related gene expression (**Figures 3, 4, and 5**) that shows significantly
421 higher levels in 3D Post 5-aza-CR cells, when compared to 2D Post 5-aza-CR cells (**Figures 3, 4,**
422 **and 5**). Consistently, 3D Post 5-aza-CR cells display a higher DNA hypomethylation than 2D Post
423 5-aza-CR ones (**Figure 6**). Overall, these results indicate 5-aza-CR ability to induce a high plasticity
424 state, regardless to the cell culture system used. However, the chemically induced pluripotency
425 state achieved by the cells, is significantly promoted using a PTFE micro-bioreactor which boosts
426 pluripotency gene transcription and increases 5-aza-CR demethylating effects. Even more
427 interestingly, only 3D Post 5-aza-CR cells stably retain the acquired 3D spherical structure (**Figure**
428 **2**) and maintain high expression levels of pluripotency-related genes (**Figures 3, 4, and 5**) as well
429 as low DNA methylation levels (**Figure 6**), during all the subsequent period of culture. Altogether,
430 the representative results here reported demonstrate that this two-step strategy is highly
431 efficient and robust, and the use of a PTFE micro-bioreactor not only boosts high plasticity, but
432 also allows its stable, long-term maintenance in the mammalian species considered. We recently
433 demonstrated that these beneficial effects are related to the activation of the Hippo-signaling
434 pathway and its mechanotransduction-related cues[10], that have been previously shown to have
435 a key role in the active regulation of cell pluripotency [28–30].
436
437 The two most critical steps for a successful procedure are the rigorous maintenance of cells at 37
438 °C, at all times, including their handling under the sterile laminar flow and the microscope and
439 the use of a correct cell number/liquid volume rate during micro-bioreactor production, that may
440 vary according to the specific cell type used. In our experience, it is also highly recommended to

441 prepare reagents freshly, prior to their use in culture (this is absolutely crucial for 5-aza-CR stock
442 solution). Furthermore, medium refreshing must be carried out under a stereomicroscope since
443 the 3D spherical organoids may be lost during medium changes.
444
445 The main strengths of this method are no transgenic and/or viral vector requirement; the
446 robustness and reproducibility in different mammalian species; low costs; and high flexibility to
447 different cell types. On the other hand, a possible limitation could be represented by the
448 restricted number of data obtained, due to the small volumes of the micro-bioreactors. In
449 addition, the use of high cell density may cause low oxygen transfer rates and limited growth in
450 suspension. To overcome these problems, further work on scale-up and/or scale-down strategies
451 still remains necessary.
452
453 It is important to highlight that the key aspects common to all 3D spheroid-based applications
454 are the reproducibility, the production efficiency, the organoid size uniformity and the influence
455 on cellular physiology. These features are strictly correlated to the mechanical forces generated
456 within the culture system and vary according to the different methods used. For instance,
457 multicellular organoids can be cultured using non-adherent dishes in stationary systems. This
458 approach is primarily based on diffusion-limited conditions and, usually, results in the formation
459 of loose-aggregated clusters. The hanging-drop technique shows the same limitation. Indeed, the
460 deposition of cell suspension drops onto the underside of the lid of a tissue culture dish leads to
461 the creation of microgravity environment that concentrates the cells at the free liquid-air
462 interface, inducing the generation of low-aggregated multicellular spheres. A possible alternative
463 is represented by the spinner flask technique. However, this method is highly expensive since it
464 requires elevated quantities of culture medium. Furthermore, the formed organoids need to be
465 transferred to stationary culture system when used for characterization or further in vitro tests.
466 All these issues can be overcome through the use of the liquid marble micro-bioreactors. Indeed,
467 they provide a non-adherent liquid surface that combines the advantages of both stationary and
468 spinning methods, inducing a rapid cell aggregation and the generation of compact spheroids. In
469 parallel, the concave bottom, the spherical shape, and the internal liquid flow of each marble
470 allow cells to settle onto the bottom of the micro-bioreactor, resulting in the formation of
471 organoids uniform in size and shape. Another significant advantage in the use of the liquid
472 marbles is represented by the optimal gas exchanges that, thanks to their spherical shape, can
473 occur through the entire surface.
474
475 In conclusion, the protocol here described allows for an efficient and simple generation of
476 mammalian pluripotent cells. Since this strategy is viral vector free and does not involve the use
477 of any gene transfection, it is highly promising for translational medicine applications and may
478 be considered a step forward in patient-specific cell therapy. Furthermore, the use of 3D micro-
479 bioreactor culture systems may represent a notable breakthrough in stem cell organoid
480 technology and may constitute an advantageous micro-environment for long-term culture of
481 different cell types, such as ESCs, iPSCs, and MSCs. An additional advantage is represented by the
482 small volume that allows to study the effect of paracrine/autocrine signalling of the rich
483 environment established within the micro-bioreactor.
484

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