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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23
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

45　In the second step, erased cells are encapsulated in PTFE micro-bioreactors, also known as Liquid
46　Marbles, to promote 3D cell rearrangement to extend and stably maintain the acquired high
47　plasticity. Polytetrafluoroethylene (PTFE) is a non-reactive hydrophobic synthetic compound and
48　its use permits the creation of a cellular microenvironment, which cannot be achieved in
49　traditional 2D culture systems. This system encourages and boosts the maintenance of
50　pluripotency though bio-mechanosensing-related cues.
51
52　The technical procedures described here are simple strategies to allow for the induction and
53　maintenance of a high plasticity state in adult somatic cells. The protocol allowed the derivation
54　of high plasticity cells in all mammalian species tested. Since it does not involve the use of gene
55　transfection and is free of viral vectors, it may represent a notable technological advance for
56　translational medicine applications. Furthermore, the micro-bioreactor system provides a
57　notable advancement in stem cell organoid technology by in vitro re-creating a specific micro-
58　environment that allows for long-term culture of high plasticity cells, namely as ESCs, iPSCs,
59　epigenetically erased cells and MSCs.
60
61　**INTRODUCTION:**
62　During the last decades, the widely accepted concept of unidirectional progression toward cell
63　commitment and differentiation was completely revised. It has been demonstrated that cell
64　specification can be reversed, and a terminally differentiated cell can be pushed towards a less
65　committed and higher permissive state, using different methods.
66
67　Among the several methods proposed, one of the most promising method involves the use of
68　chemical compounds to induce cells into a so called chemically induced pluripotency. The small
69　molecules used in this approach are able to interact and modify the epigenetic signature of an
70　adult mature cell, avoiding the need of any transgenic and/or viral vector[1–10]. Numerous studies
71　have recently shown that it is possible to switch cells from one phenotype to another by providing
72　specific biochemical and biological stimuli that induce the reactivation of hypermethylated genes
73　[11–15]. These demethylating events allow for the conversion of terminally differentiated cells into
74　a primitive progenitor, a multipotent or a high plasticity/pluripotent cell[1–10].
75
76　In parallel, many studies have been recently focussing on the understanding of mechanosensing-
77　related cues and, more specifically, on the possibility to use mechanical forces to directly
78　influence cell plasticity and/or differentiation[16–19]. Indeed, it has been clearly demonstrated that
79　the extracellular matrix (ECM) plays a key role in the control of cell fate. In particular, the
80　biomechanical and biophysical signals produced by ECM directly regulate molecular mechanisms
81　and signaling pathways, influencing cell behavior and functions[20,21]. These recent data have
82　paved the way to the development of novel 3D culture systems that more closely mimic the in
83　vivo cell microenvironment, replicating mechanical and physical stimuli driving cell behaviour.
84
85　We here describe a two-step protocol that combines the use of chemical epigenetic erasing with
86　mechanosensing-related cues, to generate mammalian pluripotent cells. In the first step, cells
87　are incubated with the demethylating molecule 5-aza-cytidine (5-aza-CR). This agent is able to
88　induce a significant global DNA demethylation through a combined effect of the direct ten-eleven

89 translocation 2 (TET2)-mediated action[8,10] and the indirect inhibition of the DNA
90 methyltransferases (DNMT)[22,23]. This step induces the removal of the epigenetic blocks with a
91 subsequent re-activation of pluripotency-related gene expression and, therefore, the generation
92 of high plasticity cells[1–3,8,10], hereinafter referred as "epigenetically erased cells". In the second
93 step, cells are encapsulated in a 3D culture system. To this end, the non-reactive hydrophobic
94 synthetic compound polytetrafluoroethylene (PTFE; with particle size of 1 μm) is used as micro-
95 bioreactor, that permits the creation of a cellular microenvironment unachievable through the
96 use of traditional 2D culture systems[10]. The PTFE powder particles adhere to the surface of the
97 liquid drop in which cells are re-suspended and isolate the liquid core from the supporting
98 surface, while allowing gas exchange between the interior liquid and the surrounding
99 environment[24]. The "PTFE micro-bioreactor" thus obtained, also known as "Liquid Marble",
100 encourages cells to freely interact with each other, promoting 3D cell rearrangement[25–27], and
101 extends and stably maintains the acquired high plasticity state though bio-mechanosensing-
102 related cues[10].
103 .
104
105 **PROTOCOL:**
106 All studies were reviewed and approved by the Ethical Committee of the University of Milan. All
107 animal experiments were performed in accordance with the Guide for the Care and Use of
108 Laboratory Animals, published by the US National Institutes of Health (NIH). Human cells isolation
109 from healthy adult individuals was approved by the Ethical Committee of the Ospedale Maggiore
110 Policlinico, Milano. All the methods in our study were carried out in accordance with the
111 approved guidelines.
112
113 **1. Skin fibroblast isolation**
114
115 NOTE: All the procedures described below can be applied to fibroblasts isolated from different
116 mammalian species, including mouse, porcine, and human. Murine cells were isolated from 7-
117 week-old male mice and porcine skin tissue were collected at local slaughterhouse. Human cells
118 were isolated from adult patients, after written informed consent
119
120 1.1. Prepare 0.1% porcine gelatin solution:
121
122 1.1.1. Weigh 0.1 g of porcine gelatin and dissolve it in 100 mL of water. Sterilize gelatin solution
123 by autoclaving before use.
124
125 1.1.2. Coat 35 mm Petri dish with 0.1% porcine gelatin by adding 1.5 mL of the prepared solution.
126 Incubate for 2 h at room temperature.
127
128 1.2. Cut mammalian (mouse, porcine, and human) skin biopsies of approximately 2-5 cm in length
129 and place them in Dulbecco's Phosphate Buffered Saline (PBS) containing 2% antibiotic
130 antimycotic solution. Store at + 4 °C until use.
131
132 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**).

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.

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**).

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**).

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**).

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.

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

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**).

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

257 4.8. Incubate liquid marble micro-bioreactor for 18 h at 37 °C in 5% CO_2 incubator[1–3, 8, 9].

259 NOTE: The PTFE particle size of 1 µm can ensure an optimal gas exchange between the interior
260 liquid and the surrounding environment.

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).

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

　　　　　　　　　　　　　　　　　　　　revised November 2018

309   mammalian pluripotent cells from adult somatic cells. This method has been successful with
310   fibroblasts isolated from different mammalian species, namely mouse, porcine and human. The
311   representative results here reported are obtained from all cell lines, irrespectively of the species
312   of origin.
313
314   Morphological analyses show that, after 18 h incubation with the demethylating agent 5-aza-CR,
315   fibroblasts encapsulated in PTFE micro-bioreactors (3D Post 5-aza-CR) aggregate and form 3D
316   spherical structures displaying a uniform size geometry, in all the three species considered.
317   (**Figure 2A-C,** 3D Post 5-aza-CR). 86.31 ± 4.13% of encapsulated cells remarkably modified their
318   phenotype, showing features typically related to a high plasticity phenotype[8]. In contrast, Post 5-
319   aza-CR cells cultured into 2D standard conditions, although replacing the typical fibroblast
320   elongated shape with a round or oval one, considerably smaller in size and with larger and
321   granulated nuclei, retain a monolayer distribution (**Figure 2**). The morphological changes are
322   accompanied by the onset of pluripotency-related gene expression both in 3D and 2D Post 5-aza-
323   CR cells. Transcription for POU class 5 homeobox 1 (OCT4), Nanog homeobox (NANOG), ZFP42
324   zinc finger protein (REX1), and sex determining region Y-box 2 (SOX2) is also observed, which is
325   absent in untreated fibroblasts (T0), is detected (**Figures 3, 4, and 5**). Furthermore, quantitative
326   PCR analysis demonstrates a significant up-regulation of the above mentioned genes, as well as
327   of the ten-eleven translocation-2 (TET2), epithelial cell adhesion molecule (EPCAM), and cadherin
328   1 (CDH1) genes in 3D Post 5-aza-CR cells (**Figures 3, 4, and 5**, blue bars) compared to cells cultured
329   in 2D standard plastic dishes (2D Post 5-aza-CR) (**Figures 3, 4, and 5,** orange bars). In parallel, a
330   significant downregulation of the fibroblast specific marker Thy-1 cell surface antigen (THY1) is
331   clearly detectable in 3D and 2D Post 5-aza-CR cells (**Figures 3, 4, and 5**).
332
333   The achievement of a high plasticity state is also confirmed by ELISA analysis of DNA global
334   methylation, that demonstrates a significant decrease of methylation levels in both 3D and 2D
335   Post 5-aza-CR cells (**Figure 6 A-C**). Moreover, in agreement with the gene expression results, DNA
336   methylation levels are significantly lower in 3D Post 5-aza-CR cells (**Figure 6 A-C,** blue bars),
337   compared to 2D Post 5-aza-CR ones (**Figure 6 A-C,** orange bars).
338
339   Even more interestingly, 3D Post 5-aza-CR cells retain the acquired 3D spherical structure (**Figure
340   2A,** 3D 28d) and maintain high expression levels of pluripotency-related genes (**Figures 3, 4, and
341   5,** blue bars) as well as low DNA methylation levels (**Figure 6 A-C,** blue bars), during all the
342   subsequent culture period and, specifically, until 28 day, when culture was arrested. In contrast,
343   although 2D Post 5-aza-CR cells transcribe for the same pluripotency genes after treatment with
344   the demethylating agent, they turn down such expression by day 7 (**Figures 3, 4, and 5,** nd).
345   Similarly, the decrease in methylation levels is maintained for the first 72 hours; then methylation
346   slowly increases, returning comparable to untreated fibroblasts (**Figure 6 A-C,** T0, white bars) by
347   day 7 of culture (**Figure 6 A-C,** orange bars).
348
349
350   **FIGURE AND TABLE LEGENDS:**
351   **Figure 1. Cell encapsulation in PTFE micro-bioreactor and organoid recovery. (A**) A bacteriology
352   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).

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

406 **Table 2: Primer information.**

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.

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].

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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