

1 A comparative study of the capacity of mesenchymal

2 stromal cell lines to form spheroids

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18 Abstract

19 Mesenchymal stem cells (MSCs)-derived spheroid models favor maintenance of stemness, *ex*
20 *vivo* expansion and transplantation efficacy. Spheroids may also be considered as useful
21 surrogate models of the hematopoietic niche. However, accessibility to primary cells, from bone
22 marrow (BM) or adipose tissues, may limit their experimental use and the lack of consistency
23 in methods to form spheroids may affect data interpretation. In this study, we aimed to create a
24 simple model by examining the ability of cell lines, from human (HS-27a and HS-5) and murine
25 (MS-5) BM origins, to form spheroids, compared to primary human MSCs (hMSCs). Our
26 protocol efficiently allowed the spheroid formation from all cell types within 24 hours. Whilst
27 hMSCs-derived spheroids began to shrink after twenty-four hours, the size of spheroids derived
28 from cell lines remained constant during three weeks. The difference was partially explained
29 by the balance between proliferation and cell death, which could be triggered by hypoxia and
30 induced oxidative stress. Our results demonstrate that, unlike hMSCs, MSC cell lines make
31 reproducible spheroids that are easily handled. Thus, this model could help in understanding
32 mechanisms involved in MSC functions and may provide a simple model by which to study
33 cell interactions in the BM niche.

34 Introduction

35 Over the last two decades, extensive studies have attempted to characterize
36 mesenchymal stem cell (MSC). Initially described in the bone marrow (BM), MSCs were later
37 found in almost all adult and fetal tissues [1]. Their classification rapidly suffered from a lack
38 of clear phenotypical definition. Therefore, in 2006, the International Society for Cellular
39 Therapy (ISCT) defined MSCs according to three minimal criteria: adherence to plastic,
40 specific cell surface markers and multipotent potential. Indeed, MSCs are classically described
41 as stem cells that are able to differentiate into osteoblasts, adipocytes and chondroblasts [2],
42 making them an attractive source of cells in regenerative medicine. Subsequent studies have
43 also established their ability to differentiate into cardiomyocytes [3], neurons [4], epithelial
44 cells [5] and hepatocytes [6]. The discovery of the multiple functions of MSC, such as those
45 involved in the anti-inflammatory response [7] and in injury repair [8,9] confirmed them as
46 promising cellular tools in regenerative medicine.

47 Furthermore, MSCs represent a key component of the BM microenvironment
48 supporting normal hematopoiesis through the regulation of stem cell renewal and differentiation
49 processes, but also fueling malignant cells and protecting them from therapeutic agents [10].
50 As such, primary MSCs have often been used as feeder layers in long-term co-culture of
51 hematopoietic cells *in vitro* in preclinical studies [11]. With the aim of standardization, the
52 murine MS-5 cell line became the gold-standard for both normal or malignant hematopoietic
53 cell culture [12]. This robust co-culture model has been widely used and has contributed to the
54 characterization of hematopoietic stem cells (HSC) [11]. This 2D system, while closer to BM
55 physiology than the culture of hematopoietic cells alone, still lacks the three-dimensional
56 complexity of the BM niche. Thus, although widely used, it is certainly not sufficiently

57 consistent at predicting *in vivo* responses [13]. Therefore, a 3D system might be a better
58 alternative to mimic the BM microenvironment.

59 Critically, the culture leads to rapid loss of MSC pluripotency and supportive functions.
60 Therefore, a wide range of techniques to form 3D MSC structures, from the simplest spheroids
61 to the more complex matrix-based structures, have been proposed [14]. Studies of spheroids,
62 also called mesenspheres, were mostly dedicated to the examination of MSC stemness and
63 differentiation abilities, such as osteogenesis, in order to improve their *in vitro* expansion and
64 transplantation efficacy in regenerative medicine [15,16]. Furthermore, this model has also
65 been tested as a surrogate niche for hematopoietic cells [17–23]. Spheroids take advantage of
66 the ability of MSCs to self-aggregate, which is improved by using various approaches such as
67 low adhesion plates, natural and artificial (centrifugation) gravity, cell matrix or more complex
68 scaffolds [13,14,24,25]. Classically, studies have used human primary MSCs, from BM, cord
69 blood and lipoaspirate, or rodent sources [15,26].

70 Although immortalized MSCs, or well characterized cell lines, could bypass the lack of
71 primary cells and avoid the variability involved with use of primary human MSCs (hMSCs)
72 samples, they are rarely employed to make spheroids [27,28]. Cell lines would also allow better
73 standardization of the spheroid formation protocol. In this study, we examined the spheroid-
74 forming capacity of two human cell lines (HS-27a and HS-5) and the murine gold-standard
75 MS-5, in comparison with hMSCs. We defined a simple and fast method using standard matrix
76 to form spheroids and characterized them in terms of physical features, cell proliferation and
77 death.

78

79 Materials and methods

80 Cell culture and reagents

81 Murine MS-5 bone marrow (BM) stromal cell line was kindly provided by Mori KJ
82 (Niigata University, Japan) [29]. Human HS-27a and HS-5 BM stromal cell lines were
83 purchased from the American Type Culture Collection (ATCC CRL-2496 and CRL-11882,
84 respectively). Primary human MSCs (hMSCs) were obtained by iliac crest aspiration from
85 informed consent patients undergoing orthopedic surgery (Cardiovascular Surgery Department,
86 Trousseau Hospital, Tours, France). HS-27a and HS-5 cell lines were cultured in RPMI 1640
87 (Life Technologies, Villebon-sur-Yvette, France) and hMSCs and MS-5 in MEM Alpha (Life
88 Technologies). All medium were supplemented with 10 % heat-inactivated fetal bovine serum
89 (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all from Life
90 Technologies). For hMSCs culture only, 0.004 % of recombinant human FGF basic (FGF-2,
91 R&D Systems, Abingdon, United Kingdom) was added. Cells were maintained in a saturated
92 humidified atmosphere at 37°C and 5 % CO₂. HS-27a, HS-5 and MS-5 cell lines were used for
93 experiments between passages 5 and 20, and hMSCs at passage 2.

94

95 Spheroids formation

96 For one spheroid, 30,000 cells were cultured in 100 µL of medium, supplemented by
97 0.25 % to 1 % of either MethocultTM SF H4236 or H4100 (StemCell, Grenoble, France), and
98 seeded in U-bottomed 96-well plate (Sarstedt, Marnay, France). The medium was the same as
99 that of the normal culture for each cell line but supplemented with heat inactivated FBS to reach
100 15 %. At days as detailed, microscopic analysis was performed using a Leica DMIL microscope

101 (Leica, Nanterre, France), coupled to a DXM1200F camera (Nikon, Champigny-sur-Marne,
102 France). To determine the number of cells in each spheroid over time, 12 spheroids per
103 experiment were pooled and dissociated with 2 mg/mL collagenase 1A (Sigma-Aldrich, Saint-
104 Quentin-Fallavier, France), 10 min at 37°C, with agitation every two minutes, and then counted
105 by the trypan blue exclusion assay.

106

107 **Time-lapse video**

108 Automatic acquisitions were performed on a Nikon Eclipse TI-S microscope, coupled
109 to a DS Qi2 camera (Nikon). The system includes a cage incubator (Okolab, Pozzuoli, NA,
110 Italy) controlling temperature and level of CO₂. Analyses were performed using both NIS
111 Element BR (Nikon) and Fiji/ImageJ softwares.

112

113 **Scanning electron microscopy**

114 Spheroids were fixed by incubation for 24 h in 4 % paraformaldehyde, 1 %
115 glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Samples were then washed in phosphate-
116 buffered saline (PBS) and post-fixed by incubation with 2 % osmium tetroxide for 1 h.
117 Spheroids were then fully dehydrated in a graded series of ethanol solutions, and dried in
118 hexamethyldisilazane (HMDS, Sigma-Aldrich). Finally, samples were coated with 40 Å
119 platinum, using a PECS 682 apparatus (Gatan, Evry, France), before observation under an Ultra
120 plus FEG-SEM scanning electron microscope (Zeiss, Marly-le-Roi, France).

121

122 **Transmission electron microscopy**

123 Spheroids were fixed by incubation for 24 h in 4 % paraformaldehyde, 1 %
124 glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Samples were then washed in phosphate-
125 buffered saline (PBS) and post-fixed by incubation with 2 % osmium tetroxide for 1 h.
126 Spheroids were then fully dehydrated in a graded series of ethanol solutions and propylene
127 oxide. Impregnation step was performed with a mixture of (1:1) propylene oxide/Epon resin,
128 and then left overnight in pure resin. Samples were then embedded in Epon resin, which was
129 allowed to polymerize for 48 h at 60°C. Ultra-thin sections (90 nm) were obtained with an EM
130 UC7 ultramicrotome (Leica). Sections were stained with 5 % uranyl acetate (Agar Scientific,
131 Stansted, United Kingdom), 5 % lead citrate (Sigma-Aldrich) and observations were made with
132 a transmission electron microscope (Jeol, JEM 1011, Croissy-sur-Seine, France).

133

134 **Immunohistochemistry**

135 At least five spheroids per conditions were pooled, fixed in formalin, embedded in
136 paraffin and cut in 3-4 µm sections on Superfrost Plus slides. Slides were deparaffinized,
137 rehydrated and heated in citrate buffer pH 6 for antigenic retrieval. After blocking for
138 endogenous peroxidase with 3 % hydrogen peroxide, the primary antibodies were incubated.
139 The panel of primary antibody included anti-HIF-1 α (Abcam ab51608, Paris, France) (dilution
140 1/200, incubation 1 h), VEGF-A (Abcam ab1316, dilution 1/200, incubation 1 h), HO-1
141 (Abcam ab52947, dilution 1/1 000, incubation 1 h), CA-IX (Novocastra clone TH22, Nanterre,
142 France) (dilution 1/100, incubation 20 min), and Ki-67 (DakoCytomation clone 39-9, Glostrup,
143 Denmark) (dilution 1/50, incubation 30 min). Immunohistochemistry was performed with
144 either the automated BenchMark XT slide stainer (Ventana Medical System Inc.) using

145 OptiView Detection Kit (Ventana Medical System Inc.) (for CA-IX and Ki-67), or manually
146 using the streptavidin-biotin-peroxidase method with diaminobenzidine as the chromogen (Kit
147 LSAB, DakoCytomation). Slides were finally counterstained with haematoxylin. Negative
148 controls were obtained after omission of the primary antibody or incubation with a non-specific
149 antibody.

150

151 **Quantitative real-time PCR**

152 Total RNAs were extracted using TRIzol reagent (15596-026, Life Technologies) and
153 reverse transcription was performed with the SuperScriptTM VILOTM cDNA Synthesis Kit
154 (11754-050, Invitrogen, Villebon-sur-Yvette, France), both according to the manufacturer's
155 procedures. qRT-PCR was performed on a LightCycler[®] 480 (Roche, Switzerland) with the
156 LightCycler[®] 480 Probes Master (04887301001, Roche). *GAPDH*, *ACTB*, *RPL13A* and *EF1A*
157 genes were used as endogenous genes for normalization. Primer sequences (S1 Table) were
158 designed with the ProbeFinder software (Roche), and all reactions were run in triplicate.

159

160 **Cell cycle analysis**

161 Spheroids were dissociated with 2 mg/mL collagenase 1A (Sigma-Aldrich), 10 min, at
162 37°C, with agitation every two minutes. Cells were fixed with 2 % paraformaldehyde/0.03 %
163 saponin for 15 min at room temperature (RT), and washed three times for 5 min with
164 10 % FBS/0.03 % saponin. Cells were then stained with 7-Aminoactinomycin D (7-AAD,
165 Sigma-Aldrich) and an AF488-conjugated anti-KI-67 antibody (BD Biosciences, Le Pont de
166 Claix, France) or the AF488-conjugated IgG₁ isotype control (BD Biosciences). Experiments

167 were performed on AccuriTM C6 flow cytometer (BD Biosciences) and data were analyzed with
168 the FlowJo V10.4.1 software (Tree Star Inc.).

169

170 **Statistical analysis**

171 All statistical analyses were performed using R software. The Mann-Whitney test was
172 used to compare two conditions and Kruskall-Wallis for multiple comparisons, followed by a
173 Dunn's *post hoc* test. The threshold for significance was set up to a p-value of 0.05.

174

175 **Results**

176 **Establishment of hMSC-derived spheroids by cell aggregation**
177 **method**

178 Among different methods to form MSC-spheroids, we followed an approach based on
179 cell aggregation in methylcellulose-based medium [27] (Fig 1A). To establish a protocol that
180 is simple, reproducible and compatible with hematopoietic cell culture, two commercial
181 methylcelluloses commonly used for hematopoietic cell assays were tested. In general, a range
182 from 0.01 to 1 % of methylcellulose was used [27,30–33], so we tested three different
183 concentrations (0.25, 0.5 and 1 %). We also tested a hanging drop technique [31,33–35] and
184 the previously described U-bottomed 96-well plates methods [27,30,32,33,36]. Both techniques
185 worked well for primary hMSCs but the second was more appropriated for further analyses and
186 offered lesser dehydration (data not shown). The SF H4236 methylcellulose at a concentration
187 of 0.5 % was adopted because it generated one spheroid per well with lower condensation

188 aspect for primary hMSCs (Fig 1B). Under these culture conditions, hMSCs were able to form
189 spheroids rapidly, in as little as five hours of culture (S1 Video), which is consistent with
190 previous studies [27,32,37].

191

192 **Fig 1. Spheroids formation from hMSCs.** (A) Schematic representation of experimental plan.
193 (B) 30,000 hMSCs per well were seeded into U-bottomed 96-well in medium containing
194 0.25 %, 0.5 % or 1 % of methylcellulose (Methocult™ H4100 or SF H4236). Microscopy
195 analysis was performed after 24 h (scale bars = 500 μ m).

196

197 **Formation of spheroids from MSC cell lines**

198 The spheroid-forming capacity was followed for two human cell lines, HS-27a and HS-
199 5, and compared to that of hMSCs. These cell lines have been obtained by immortalization of
200 hMSCs with the papilloma virus E6/E7 genes [38,39]. HS-27a cells support hematopoietic stem
201 cell maintenance (self-renewal, formation of cobblestone areas), whereas HS-5 cells mainly
202 sustain proliferation and differentiation [38–40]. Unlike hMSCs, they retained the ability to
203 form spheres but required about 10 hours to make rounded spheroids (S2 and S3 Videos).
204 Although MSCs of various origins formed spheroids of equivalent sizes (about 300 μ m of
205 diameter) after 24 hours, hMSCs-derived spheroids rapidly condensed and reached half of their
206 initial perimeter after 14 days of culture (Fig 2A and B). In contrast to hMSCs, the perimeter
207 of spheroids resulting from both cell lines remained constant during three weeks. Knowing that
208 hMSCs and cell lines may differ in their growth properties, we used the murine MS-5 cell line
209 that has contact inhibition [29]. This cell line was able to quickly form spheroids similarly to
210 the other cell lines (S4 Video). It is noteworthy that MS-5 cells initially formed a flat multilayer

211 disk of cells prior to contracting into spheres. Similarly to the spheroids derived from human
212 cell lines, spheroids from MS-5 cells kept the same size over time (S1A and S1B Fig). This
213 suggests that shrinking might be an intrinsic property or extracellular matrix (ECM)
214 composition of primary cells rather than related to cell proliferation control. We thus examined
215 whether the difference in the size maintenance between various MSCs might be attributed to
216 the cell number per spheroid. In order to quantify the viable cells, spheroids were dissociated
217 at different timepoints after seeding. In accordance with the decrease in circumference, the
218 number of cells per spheroid for hMSCs dramatically dropped within seven days (Fig 2C),
219 which was in agreement with other studies [31,35]. Remarkably, although keeping the same
220 size, HS-27a-derived spheroids, as well as the MS-5 ones, had lost viable cells similarly to
221 hMSCs (Fig 2C and S1C Fig). In contrast, HS-5-derived spheroids had less obvious decrease
222 in cell number with time (Fig 2C). Overall, the size reduction does not seem to be strictly
223 attributable to reduced cell number in spheroids and could be possibly attributed to other factors
224 such as the ECM composition.

225

226 **Fig 2. Follow up of the spheroids derived from various MSCs.** (A) Microscopy analysis of
227 hMSCs-, HS-27a- and HS-5-derived spheroids over 21 days in culture (scale bars = 100 μ m).
228 (B) Perimeter was measured with an arbitrary unit; each experiment is the mean of at least
229 10 spheroids from $n = 3$ experiments. Data are mean \pm SD; * compared to day 1; * $p \leq 0.01$.
230 (C) Number of living cells per spheroids over 21 days in culture (hMCSs $n = 3$; HS-27a and
231 HS-5 $n = 4$; each experiment is the mean of 12 spheroids).

232

233 **Electron microscopy observation of the MSCs-derived spheroids**

234 Scanning electron microscopy (SEM) confirmed the shrinking of hMSC-derived
235 spheroids (Fig 3A and S1D Fig). SEM also revealed at higher magnification that spheroids from
236 hMSCs are highly cohesive, showing tight intercellular connections forming a flat surface,
237 whereas HS-27a, HS-5 and MS-5 spheroids exhibited more rounded cells at their surface
238 (Fig 3B and S1E Fig). This phenomenon intensified over the time and, in line with the
239 assumption that ECM composition is different, may explain the size reduction of hMSC-derived
240 spheroids compared to the cell lines. From day 7 for cell lines and day 14 for primaries, spheroid
241 structure began to change, suggesting a progressive cell death. Further analysis by transmission
242 electron microscopy (TEM) to investigate the ultrastructure of the cells within the spheroids
243 showed the appearance of a progressive cell injury, thus confirming induced cell death (Fig 3C
244 and S1F Fig).

245

246 **Fig 3. Electron microscopy observation of MSCs-derived spheroids.** (A, B) Scanning
247 electron microscopy (SEM) and (C) transmission electronic microscopy (TEM) analysis of
248 spheroids derived from hMSCs, HS-27a and HS-5 cells, over 14 days (scale bars = 100 μ m (A),
249 20 μ m (B and C)).

250

251 **Cell death and proliferation analyses of the MSCs-derived** 252 **spheroids**

253 To explain why spheroids had decreased cell number over time, we proposed an
254 imbalance between cell death and cell proliferation. Thus, apoptosis and cell cycle were

255 measured by flow cytometry using 7-AAD/Ki-67 staining (Fig 4A). First, increasing sub-G₀/G₁
256 cell population revealed a strong induction of cell death after 14 days in spheroids obtained
257 with hMSCs, while a more moderate cell death was observed after seven days for the two
258 human cell lines (Fig 4B). Although harvested at the same confluence, primary cells appeared
259 already much more quiescent than HS-27a or HS-5 cells at day 0 (Fig 4C). Then, a significant
260 proportion of cells remained proliferating in spheroids until day 3 for HS-27a and day 7 for HS-
261 5 cells. Remarkably, while closer to HS-27a cells in terms of perimeter and number of cells,
262 MS-5 cells had a massive increase in cell death and almost no proliferation (S1G and S1H Fig).
263 This suggests that, based on proliferation and cell death, the MS-5 cell line is more similar to
264 primary cells than others, probably due to their contact inhibition, which limits their
265 proliferation capacity. Ki-67 detection by immunochemistry, in hMSCs and human cell lines,
266 revealed homogeneous staining at day 1 indicating proliferation in the whole spheroid (Fig 4D)
267 in agreement with a previous study [41]. It also confirmed a lower proliferation rate of hMSCs
268 compared to cell lines and a rapid proliferation arrest with only few Ki-67-positive cells
269 remaining at the periphery of the spheroid at day 3. A progressive decrease in the proliferation
270 for the two human cell lines supported the results obtained by flow cytometry. Interestingly,
271 decreased proliferation appears in the entire spheroid and is not restricted to in-depth
272 localizations. These data showed that spheroids are characterized by imbalance between cell
273 death and proliferation, which may explain the highest loss of cells over time.

274

275 **Fig 4. Determination of proliferation and apoptosis of MSCs-derived spheroids. (A-C)** Cell
276 cycle analysis of spheroids over 21 days in culture. (A) Representative gating strategy from
277 hMSCs at day 0, (B) sub-G₁ apoptosis quantification (hMSCs n = 6; HS-27a and HS-5 n = 3)
278 and (C) cell cycle quantification (hMSCs n = 6; HS-27a and HS-5 n = 5; * for G₀; † for G₁;

279 # for S/G₂/M) (data are mean \pm SD; *#/# compared to day 0; */# p \leq 0.05; **#/## p \leq 0.01).

280 (D) Immunohistochemistry of Ki-67 at days 1, 3 and 7 for hMSCs-, HS-27a- and HS-5-derived

281 spheroids (scale bars = 100 μ m). Arrows indicate Ki-67-positive cells.

282

283 **Hypoxia and oxidative stress in MSCs-derived spheroids**

284 Like in tumor spheres [42,43], the appearance of an oxygen gradient and hypoxia in

285 MSCs-derived spheroids [44] has been demonstrated. Carbonic anhydrase IX (CA-IX), a

286 mediator of hypoxia-induced stress response, is commonly used as marker in tumors [45].

287 Increased CA-IX has been observed in MSCs-derived spheroids, particularly in HS-27a cells

288 (Fig 5A). The pro-survival adaptation to hypoxia occurs mainly through the stabilization of the

289 hypoxia-inducible factors (HIFs). HIFs are key regulators of multiple cell processes, including

290 cell cycle, metabolism, pH control and autophagy. Increasing expression of HIF-1 α protein

291 expression has been observed in spheroids over the time, as well as at the mRNA level mainly

292 in hMSCs (Fig 5B). Finally, we examined the expression of *VEGFA*, a standard HIF

293 transcriptionally regulated gene [46]. Its expression in hMSCs- and HS-27a-derived spheroids

294 was already elevated at day 1, but strongly increased at both protein and mRNA levels over

295 time (Fig 5C).

296

297 **Fig 5. Hypoxia detection of hMSCs- and HS-27a-derived spheroids over 7 days in culture.**

298 (A) Immunohistochemistry of CA-IX. (B) Immunohistochemistry and mRNA of HIF-

299 1 α . (X) Immunohistochemistry and mRNA expression of VEGF-A. (hMSCs n = 5; HS-27a

300 n = 3; * p \leq 0.05; ** p \leq 0.01; scale bars = 100 μ m).

301 In certain circumstances, very low level of oxygen (anoxia) or long exposure to hypoxia
302 may provoke DNA damage and oxidative stress that trigger apoptosis [42]. Besides hypoxia
303 appearance in spheroids, cell aggregation may also stress the cells by itself and increase reactive
304 oxygen species (ROS). Heme oxygenase 1 (HO-1) is induced by a variety of stressors, and is
305 therefore a marker of hypoxia and oxidative stress [47]. Indeed, oxidative stress triggers nuclear
306 relocation of NRF-2, a HO-1 transcription factor, which then leads to antioxidant response
307 through induced expression of antioxidants by HO-1. In the spheroids, we observed a high
308 expression of HO-1 at day 1, which increased over time (Fig 6A). Conversely, among the 24
309 antioxidant genes (Patent WO2016083742), we found a total of seven genes upregulated in
310 spheroids from the hMSCs and the HS-27a cell line (Fig 6B). Remarkably, of these genes, four
311 (*GPX1*, *PRDX2*, *SOD1* and *SOD2*) were commonly upregulated in both cell types irrespective
312 of their initial expression level.

313

314 **Fig 6. Oxidative stress detection of hMSCs- and HS-27a-derived spheroids over 7 days in**
315 **culture.** (A) Immunohistochemistry of HO-1 (scale bars = 100 μ m). (B) Expression of
316 antioxidant genes ($n = 3$; data are mean; * compared to 2D control (CTL); * $p \leq 0.05$;
317 ** $p \leq 0.01$).

318

319 Together, these data indicate concomitant appearance of hypoxia and oxidative stress in
320 established MSCs-derived spheroids, which could therefore explain initial cell cycle arrest and
321 further apoptosis in prolonged hypoxia [48].

322

323 **Stemness in MSCs-derived spheroids**

324 The 2D culture of MSCs critically leads to rapid loss of their pluripotency and
325 supportive functions. In contrast, MSCs-derived spheroids have the potential to maintain
326 stemness that could be demonstrated by the expression of three classical embryonic markers,
327 OCT-4, SOX-2 and NANOG [32]. Furthermore, it has been described that hypoxia
328 transcriptionally regulates these factors in a HIFs-dependent manner [49]. Therefore, in order
329 to validate whether HS-27a behave similarly to hMSCs, we examined the expression of the
330 genes coding for the three factors, over time. Results showed that hMSCs formation was
331 accompanied by upregulation of *OCT4* and *SOX2*, in agreement with previous studies, but
332 surprisingly showed no upregulation of *NANOG* (Fig 7A). HS-27a had similar expression level
333 of the three genes to hMSCs in 2D culture and had progressive increased expression of all three
334 markers (Fig 7B). These data confirmed that, like hMSCs, HS-27a had preserved a stemness
335 capacity that could also be (re)activated during spheroid formation.

336

337 **Fig 7. Stemness detection of hMSCs- and HS-27a-derived spheroids over 7 days in culture.**
338 (A and B) Gene expression of *OCT4*, *NANOG* and *SOX2* for (A) hMSCs- and (B) HS-27a-
339 derived spheroids (hMSCs n = 5; HS-27a n = 3; * p ≤ 0.05; ** p ≤ 0.01).

340

341 **Discussion**

342 In the last decade, studies have shown that MSC spheroids could be a promising model
343 for *in vitro* culture. Indeed, some have demonstrated their benefits in studying cardiac ischemia
344 [50], cerebral ischemia [51], hindlimb ischemia [52] or bone repair [53]. In addition, spheroids

345 may be a good model to study the interaction of normal [17–19,21,22] or malignant
346 hematopoietic cells [20,23] with their microenvironment. For instance, spheroids could be used
347 to study the mechanisms triggering chemoresistance in leukemias [20,23]. However, studies
348 might be limited by the availability of primary human MSCs and the reproducibility due to the
349 different sources, while 2D co-cultures have been for a long time established with cell lines,
350 mostly murine, such as MS-5 or M2-10B4 [11]. In this study, we chose the HS-27a and HS-5
351 cell lines for their human origin and their capacity to sustain hematopoiesis in co-culture
352 (Roecklein & Torok-Storb, 1995). Nonetheless, in contrast to the murine MS-5 cell line, they
353 do not retain contact inhibition that certainly, although of human origin, have limited their use
354 for long-term culture. We found that both human and murine cell lines, independently of their
355 contact inhibition capacity, were able to provide quick and reproducible spheroids using
356 standard methylcellulose, similarly to hMSCs, with the advantage of keeping the same size over
357 time. The delay to achieve a complete spheroid, 5 h *versus* 10 h for hMSCs and cell lines,
358 respectively, could certainly be attributed to sedimentation speed. In fact, cell lines are much
359 smaller than primaries that could hence sediment faster. On the other hand, this phenomenon
360 might also be attributed to spheroid condensation that could depend on ECM composition.
361 Indeed, hMSCs-derived spheroids appeared more cohesive by SEM.

362 ECM may also explain, at least partially, shrinking of hMSC-derived spheroids.
363 Shrinking has been previously reported for hMSCs [31,32,35,37,54–56] and has been attributed
364 to induced autophagy [32]. Therefore, we could hypothesize that transformed cell lines may
365 have lower autophagy, which is often induced in reduced or arrest cell growth [57]. Indeed,
366 HS-27a and HS-5 cell lines continue to proliferate until 7 days, unlike hMSCs, and could block
367 autophagy and compensate cell death. However, the number of viable HS-27a decreased over
368 time and no apoptosis has been detected for any of the MSCs before seven days, which could

369 not explain the loss of cells. In agreement, others studies have also demonstrated an induction
370 of apoptosis only after several days [35,54], but not at short term [58].

371 Studies have already reported oxygen gradients in tumor-spheres [42,43] as well as in
372 MSCs-derived spheroids [44]. The hypoxia response mainly happens through the stabilization
373 of hypoxia-inducible factors (HIFs), which are regulators of multiple biological processes, such
374 as angiogenesis or energetic metabolism. HIFs have an essential pro-survival role by promoting
375 genes, such as those involved in metabolism and autophagy [46]. However, acute and prolonged
376 hypoxia may also trigger cell death through blocking DNA replication and induced oxidative
377 stress [42]. Interestingly, cell lines showed increased hypoxia markers over time, and
378 concomitant decreased cell cycle prior induced apoptosis. This is consistent with induced
379 oxidative stress revealed by increased expression of HO-1 and antioxidant response.

380

381 **Conclusions**

382 Overall these data indicate that, like hMSCs, MSC cell lines make reproducible and
383 easily handled spheroids. Remarkably, the HS-27a cell line more closely resemble primary cells
384 than the HS-5 line. This is of a particular interest, since HS-27a has been shown to provide
385 better support to HSCs [38–40]. Thus, this model could help in understanding mechanisms
386 involved in MSC physiology and may be a simple model to study cell interactions in the
387 hematopoietic niche. The model could also be extended to research metastatic process as
388 previously described for breast cancer [28].

389

390 **Acknowledgements**

391

392 **Supporting information**

393 **S1 Fig. Spheroids formation of mouse MS-5 cell line.** (A) Microscopy analysis over 21 days
394 in culture (scale bars = 100 μ m). (B) Perimeter was measured with an arbitrary unit; each
395 experiment is the mean of at least 10 spheroids (n = 3; data are mean \pm SD). (C) Number of
396 living cells per spheroids over 21 days in culture (n = 3; each experiment is the mean of
397 12 spheroids). (D, E) Scanning electron microscopy (SEM) and (F) transmission electronic
398 microscopy (TEM) analysis over 14 days (scale bars = 100 μ m (D), 20 μ m (E and F). (G) Sub-
399 G₁ apoptosis quantification (n = 3) and (H) cell cycle quantification over 21 days in culture
400 (n = 3; data are mean \pm SD).

401

402 **S1 Video.** A representative time-lapse video of spheroid formation. 30 000 primary MSCs
403 seeded into U-bottomed 96-well, in medium containing 0.5 % of methylcellulose (MethocultTM
404 SF H4236) were followed via a Nikon Eclipse TI-S microscope for 24 hours.

405

406 **S2 Video.** A representative time-lapse video of spheroid formation. 30 000 HS-27a cells seeded
407 into U-bottomed 96-well, in medium containing 0.5 % of methylcellulose (MethocultTM SF
408 H4236) were followed via a Nikon Eclipse TI-S microscope for 24 hours.

409

410 **S3 Video.** A representative time-lapse video of spheroid formation. 30 000 HS-5 cells seeded
411 into U-bottomed 96-well, in medium containing 0.5 % of methylcellulose (MethocultTM SF
412 H4236) were followed via a Nikon Eclipse TI-S microscope for 24 hours.

413

414 **S4 Video.** A representative time-lapse video of spheroid formation. 30 000 MS-5 cells seeded
415 into U-bottomed 96-well, in medium containing 0.5 % of methylcellulose (MethocultTM SF
416 H4236) were followed via a Nikon Eclipse TI-S microscope for 24 hours.

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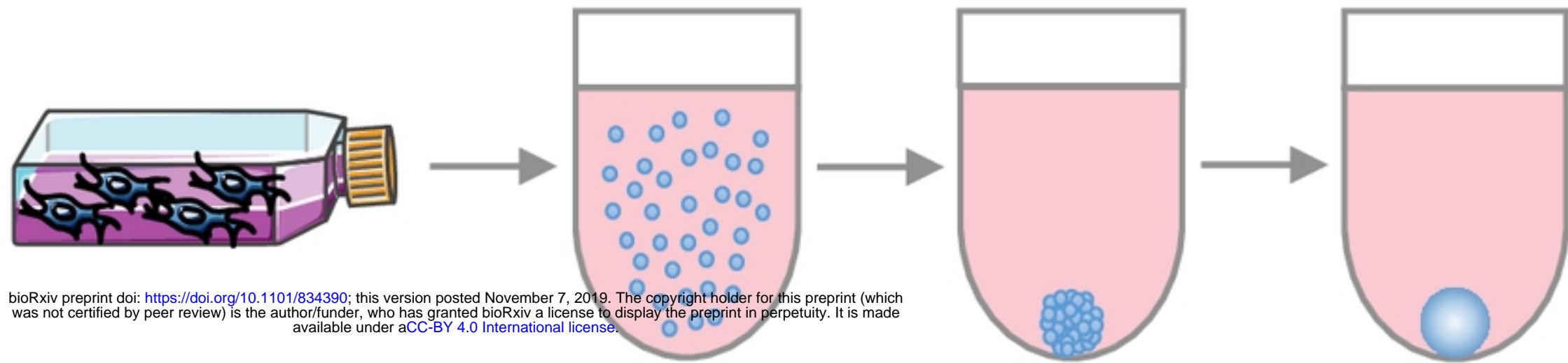
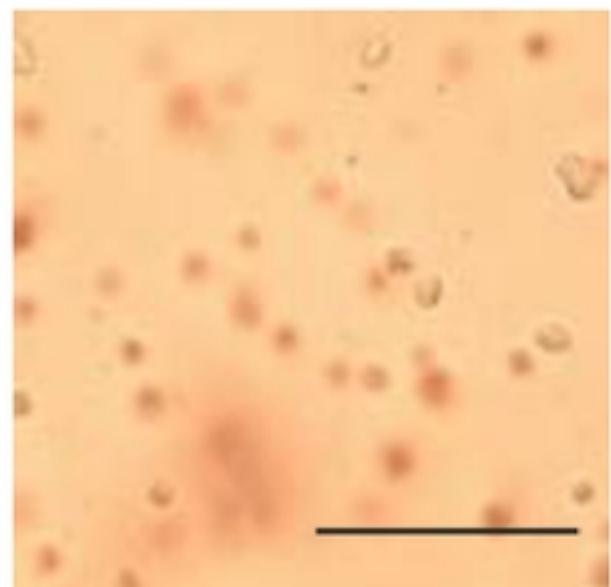
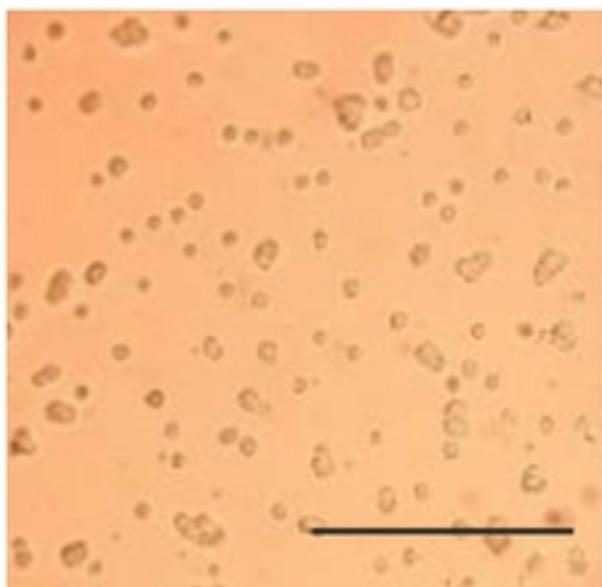
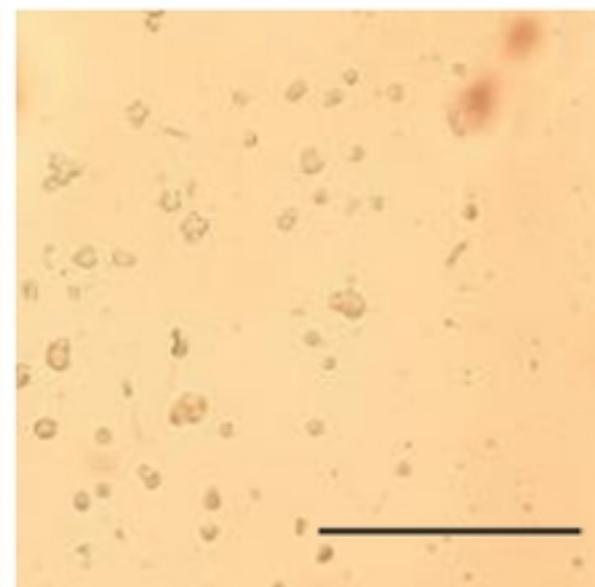
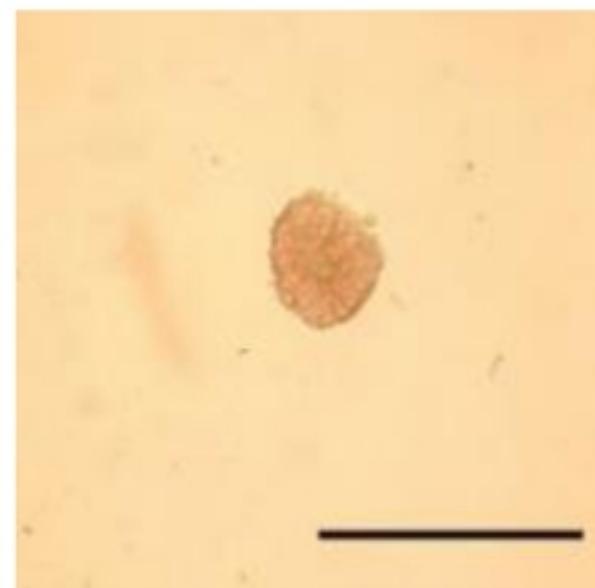
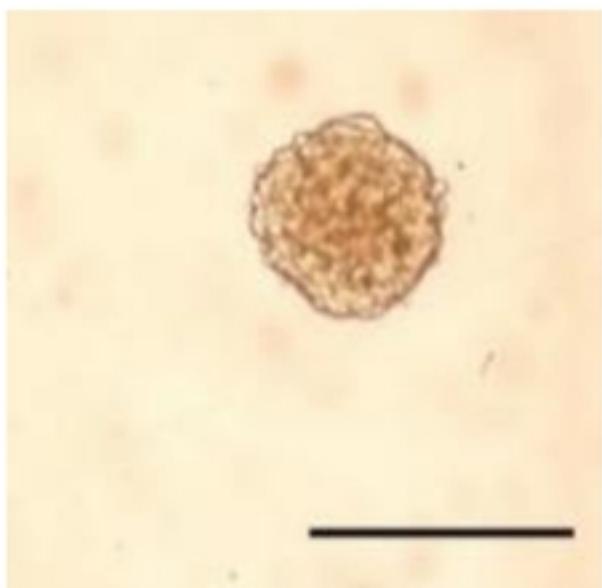
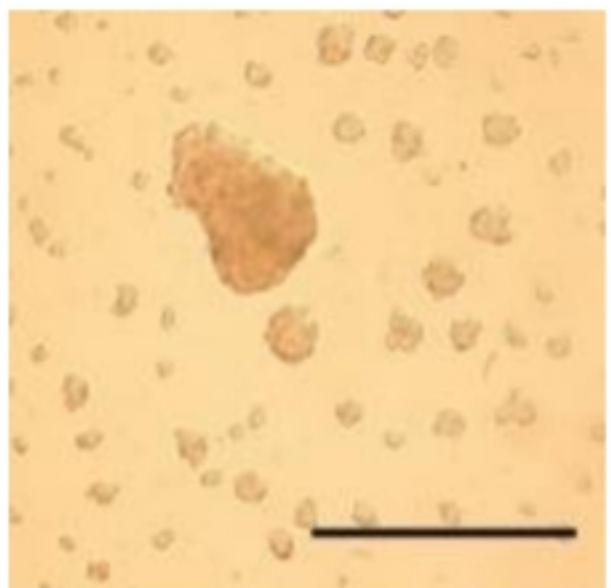
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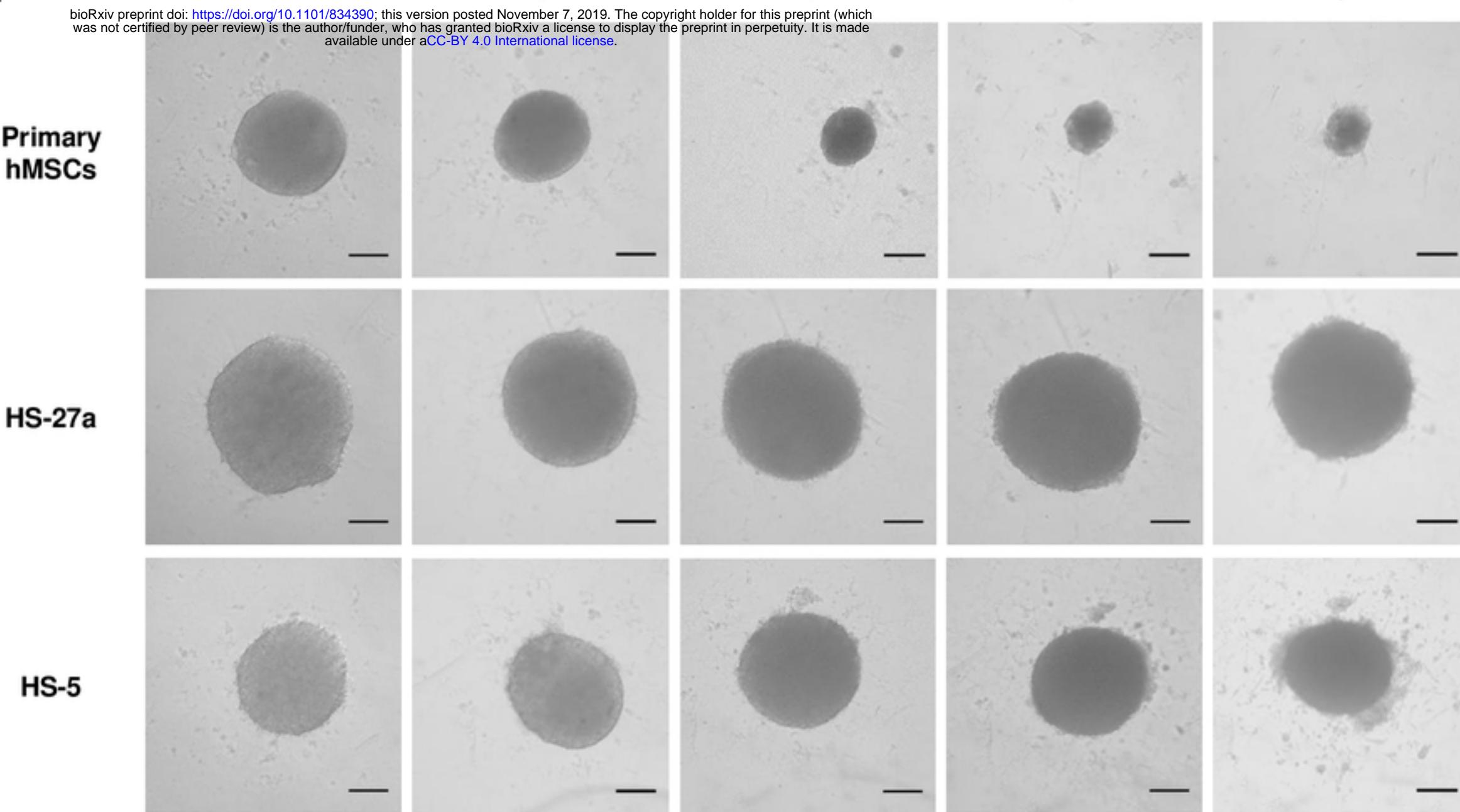
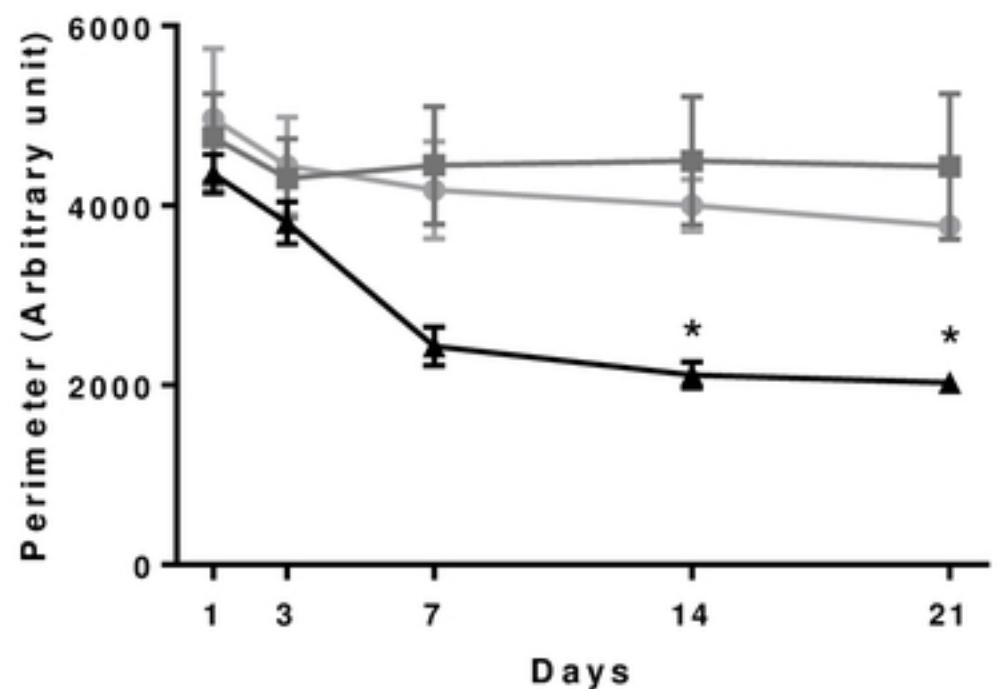
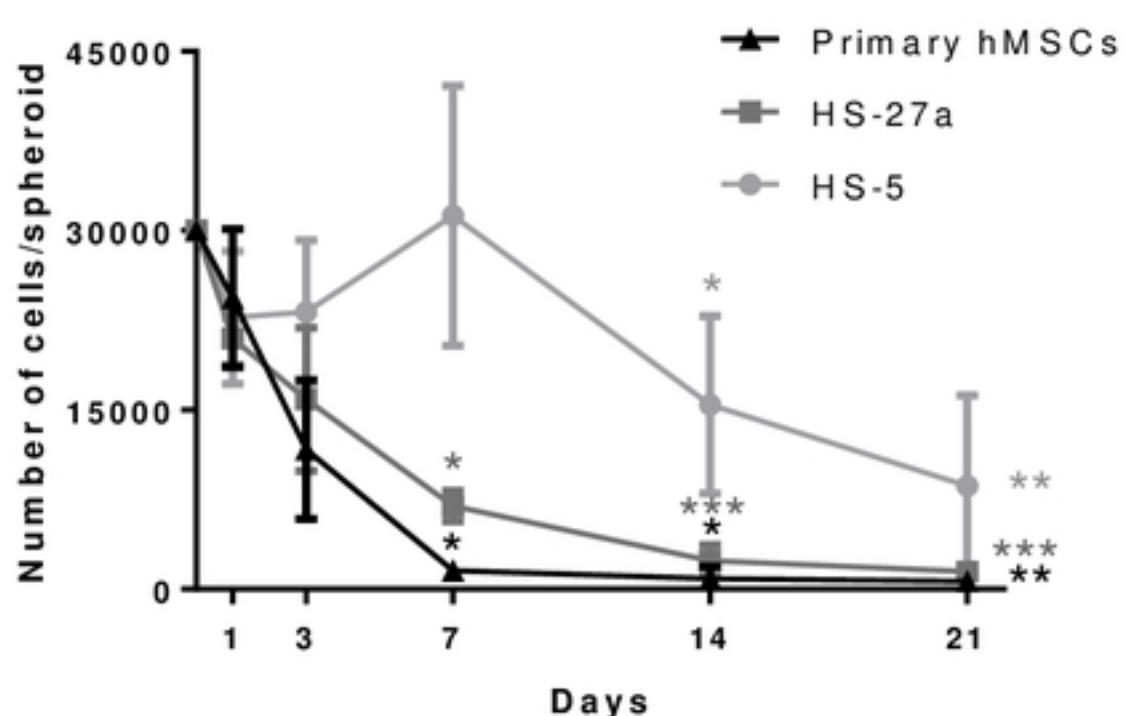
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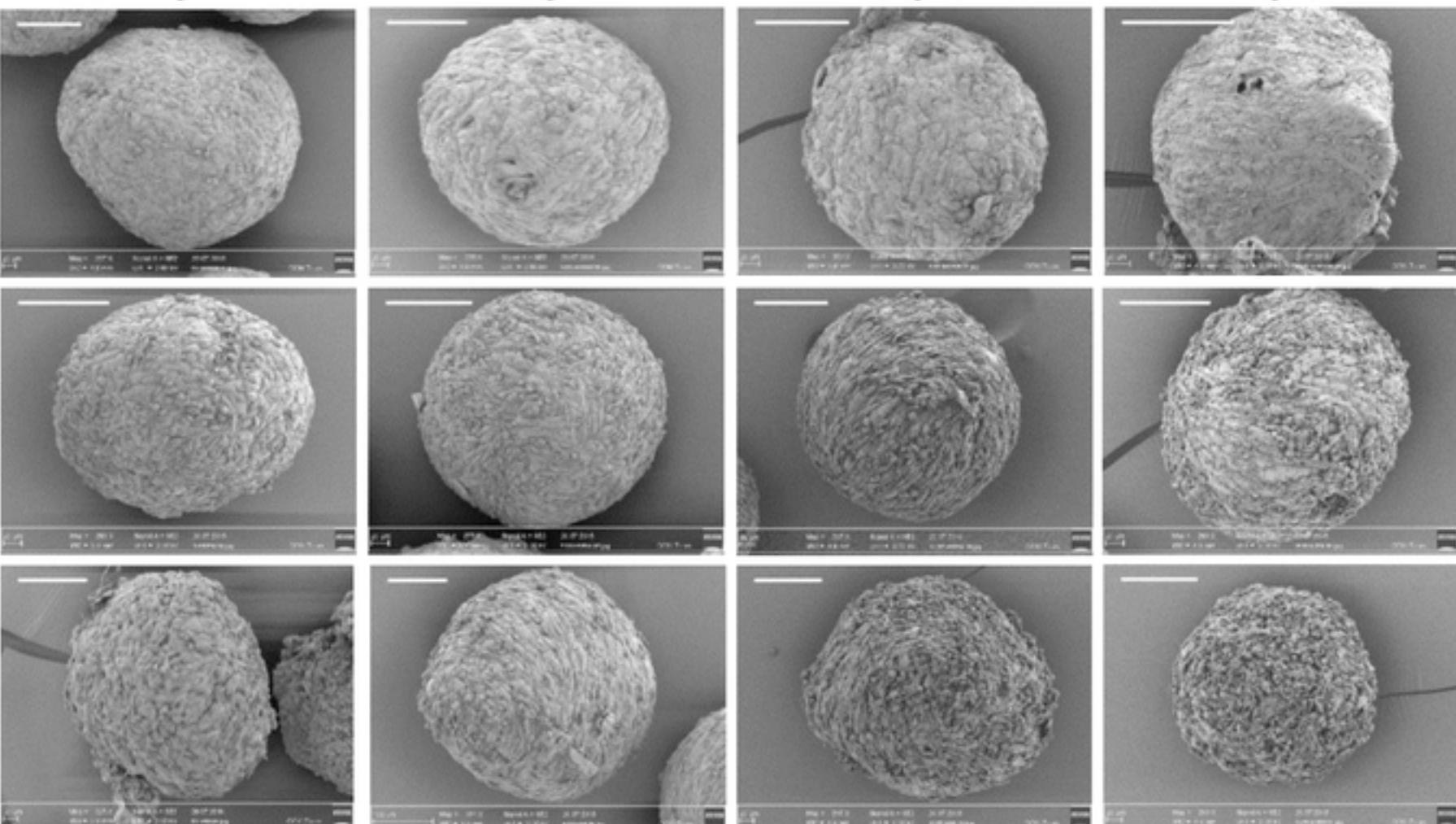
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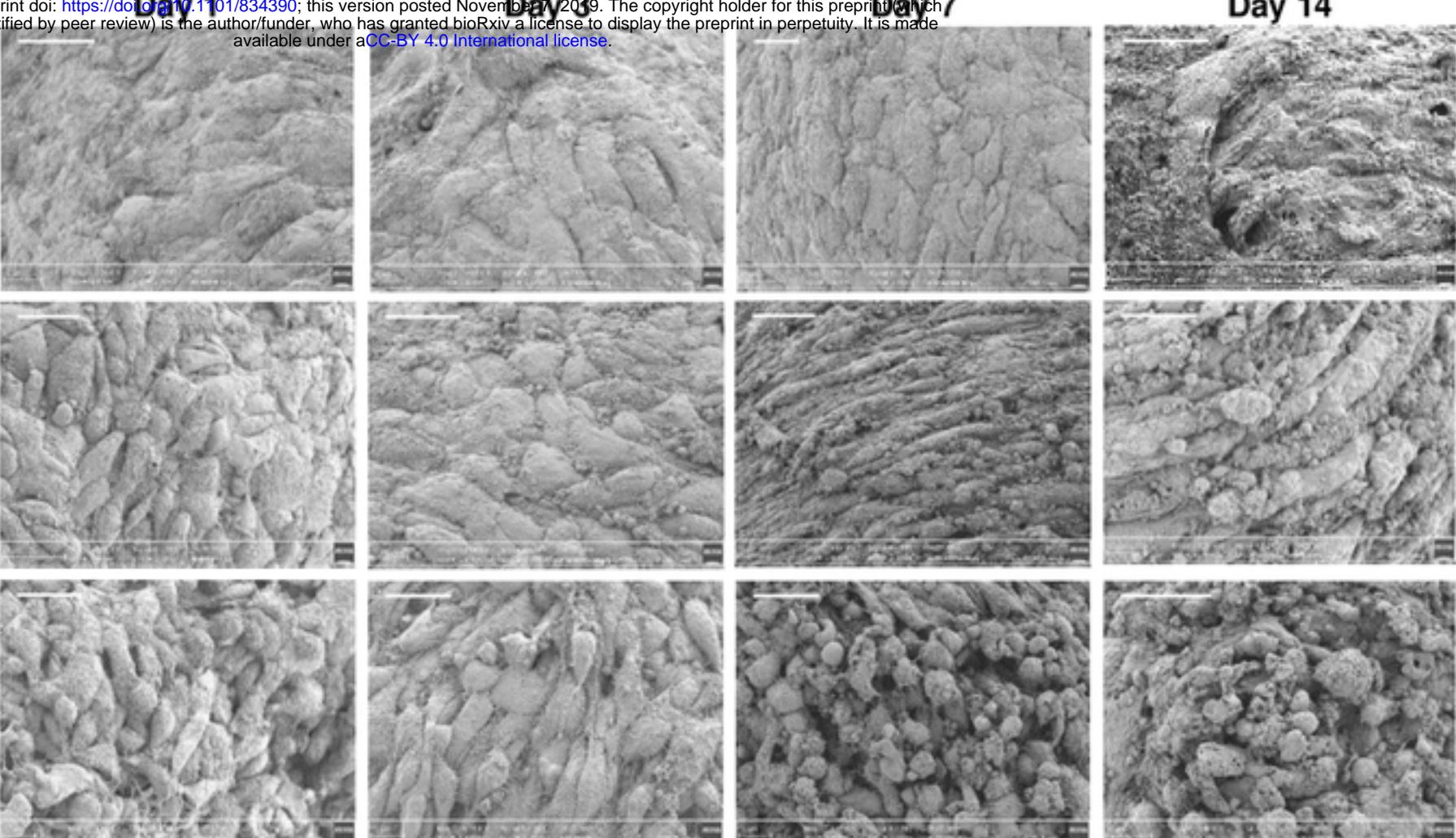
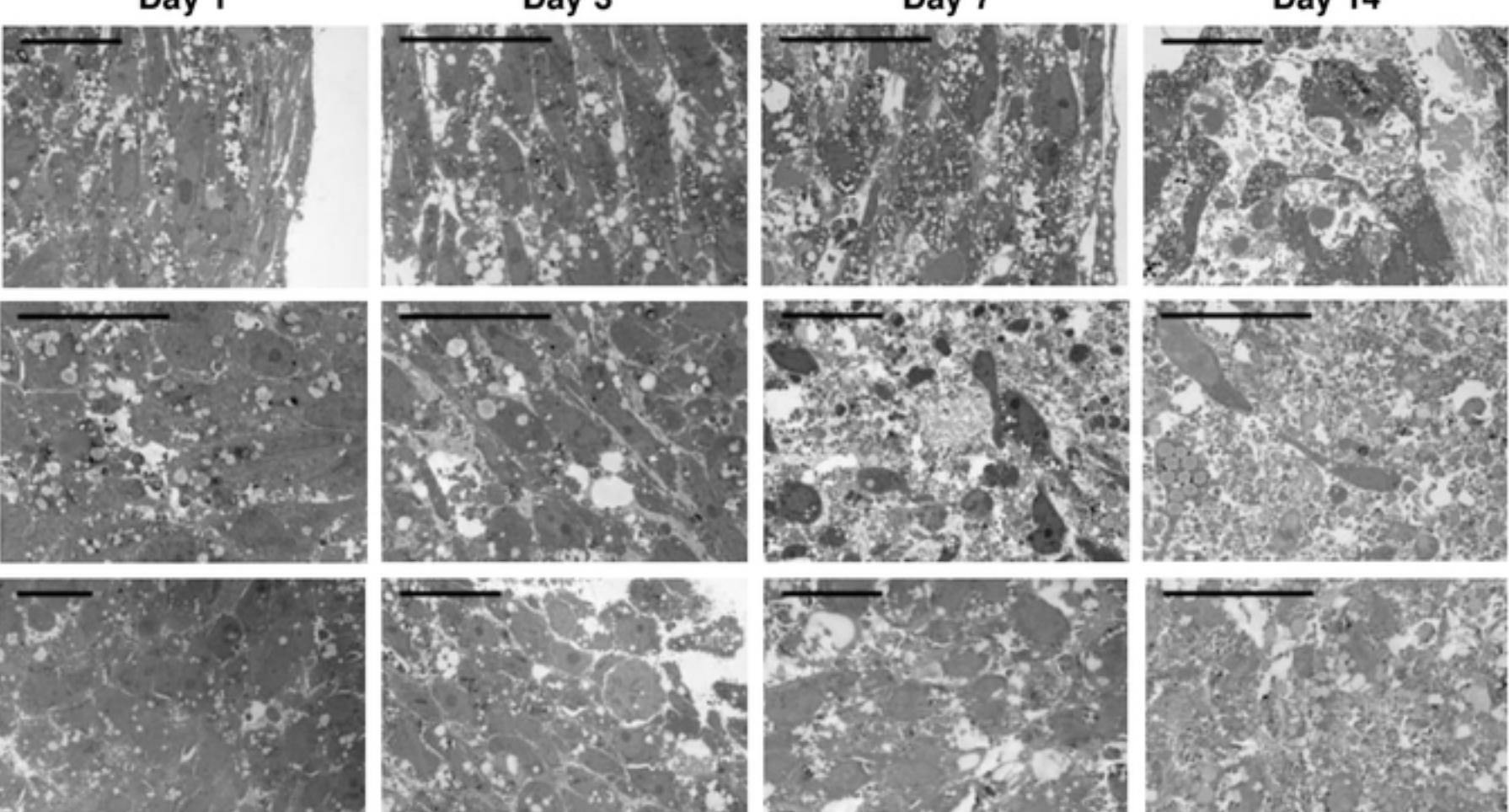
581

A**One week before**MSC expansion in
2D**Day 0**Plate 30 000
cells**Few hours later**Cell
aggregation**Day 1-21**Spheroid
analysis**B****H4100****0.25 %****0.5 %****1 %****SF H4236****Fig1**

A**B****C****Fig2**

A**Primary hMSCs****HS-27a****HS-5**

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B**Primary hMSCs****HS-27a****HS-5****C****Primary hMSCs****HS-27a****HS-5****Fig3**

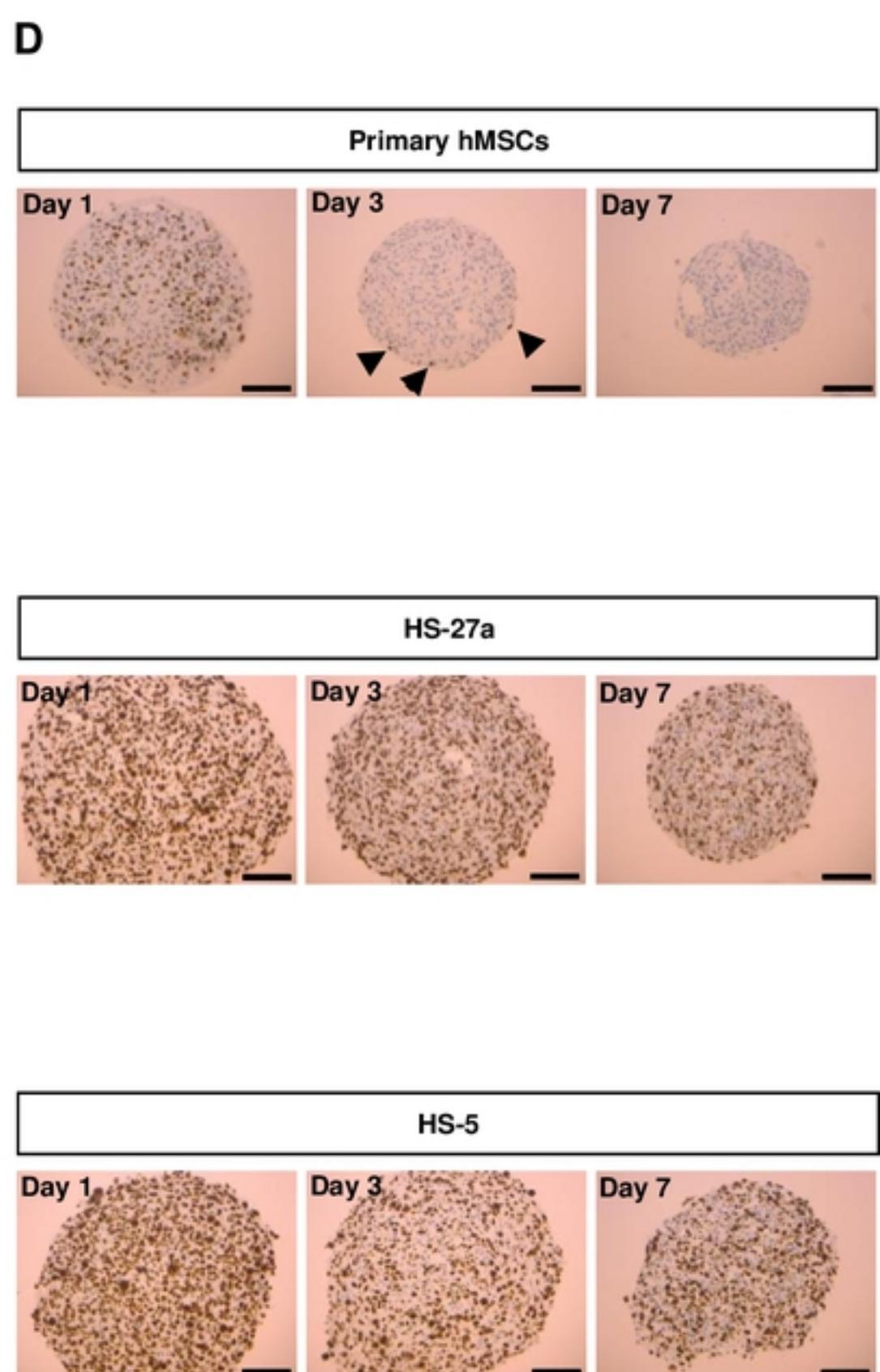
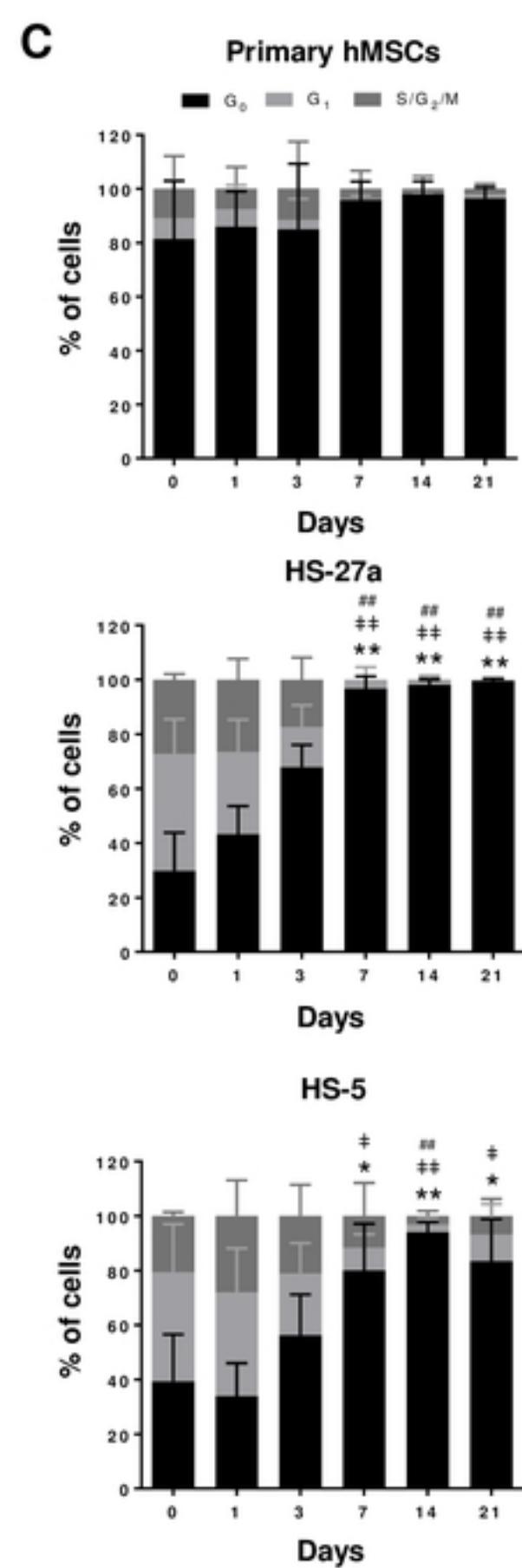
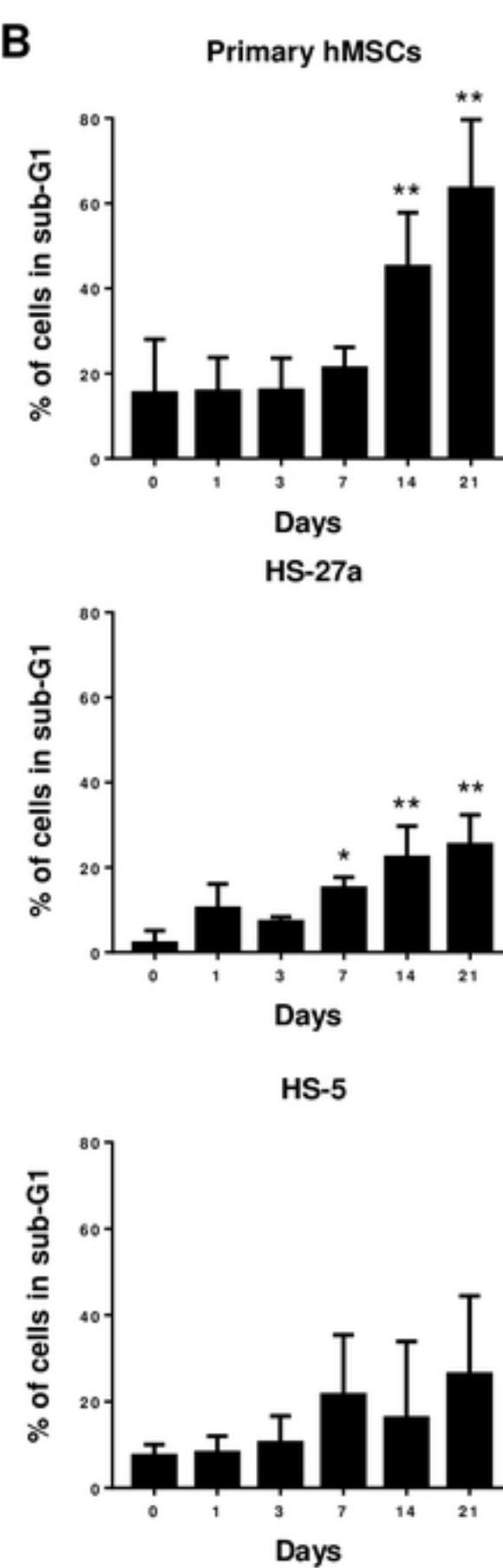
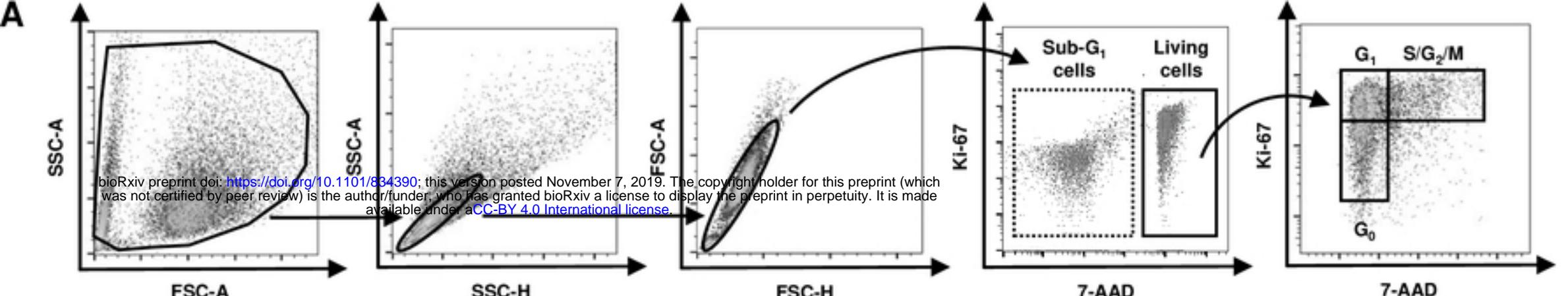
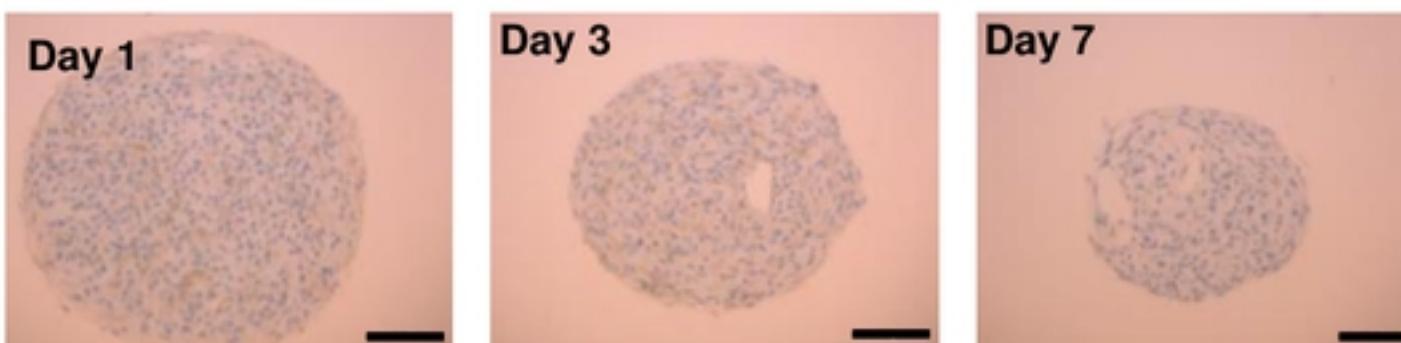
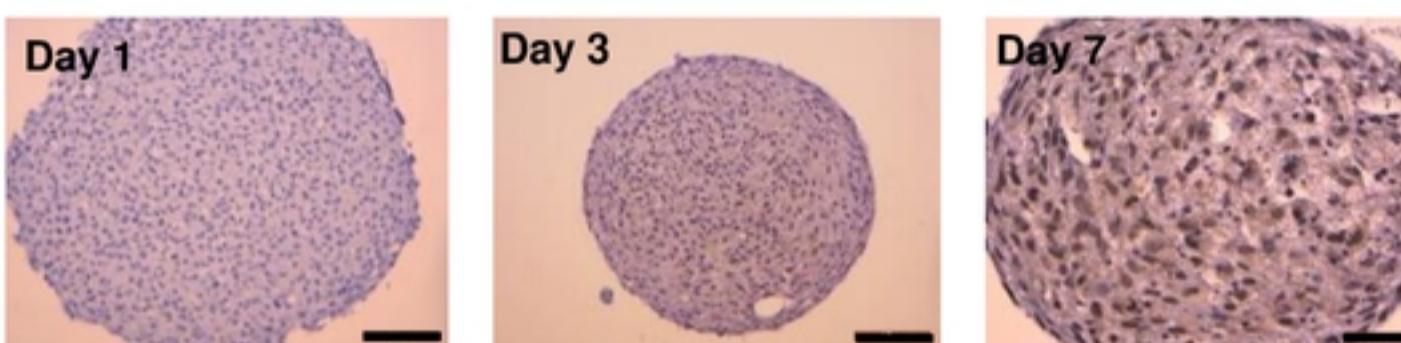
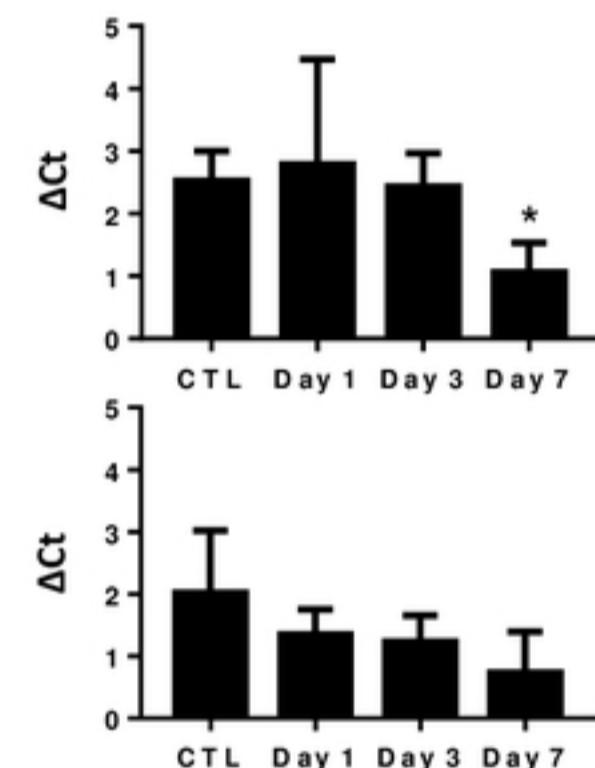
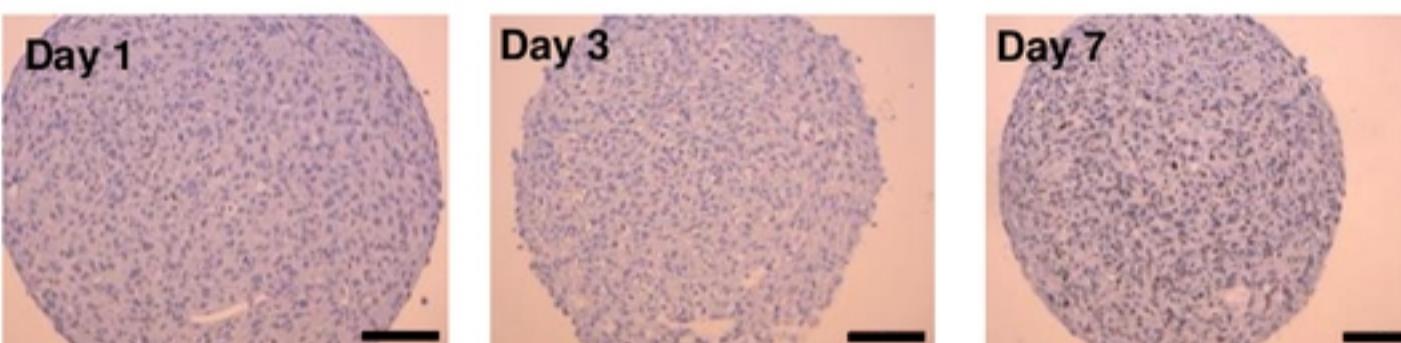
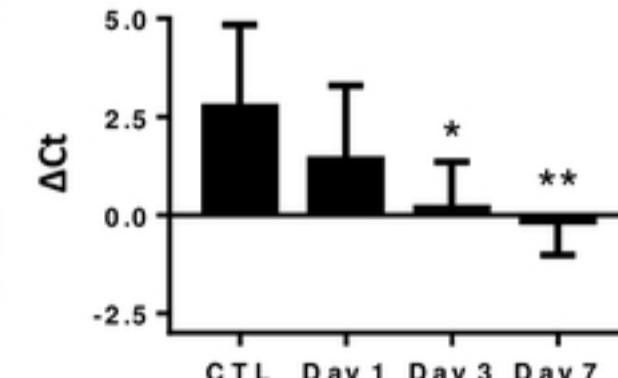
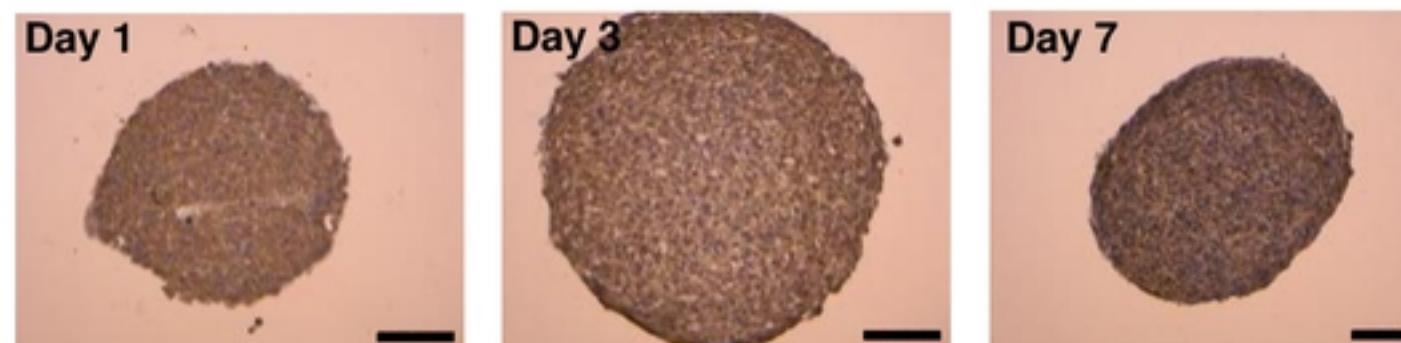
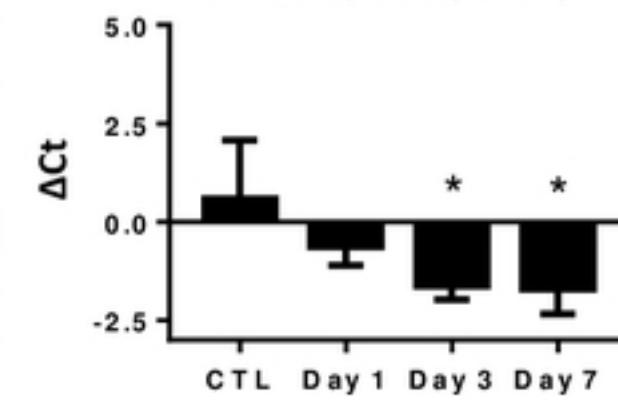
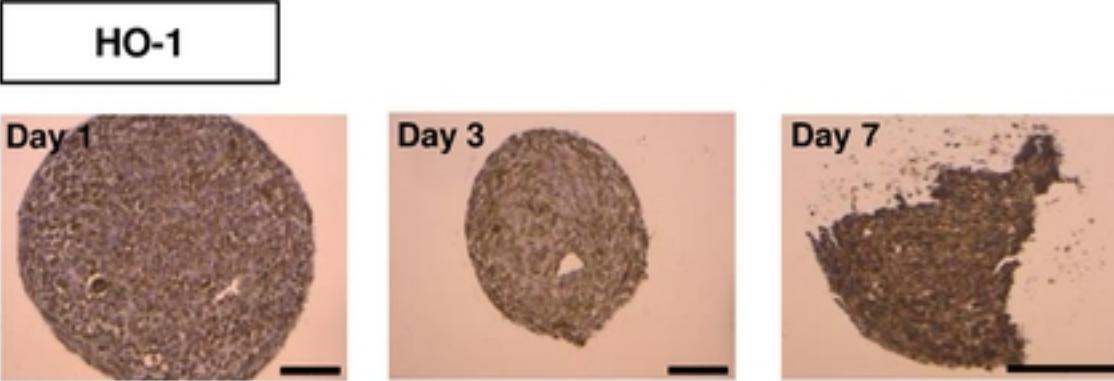


Fig4

A**CA-IX****Primary hMSCs****Day 1****Day 3****Day 7****HS-27a**

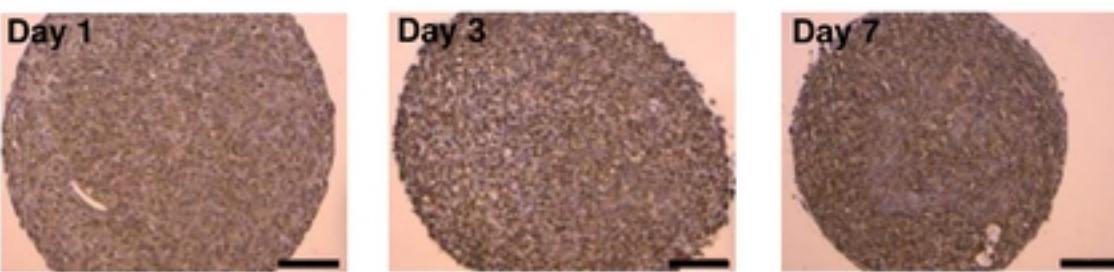
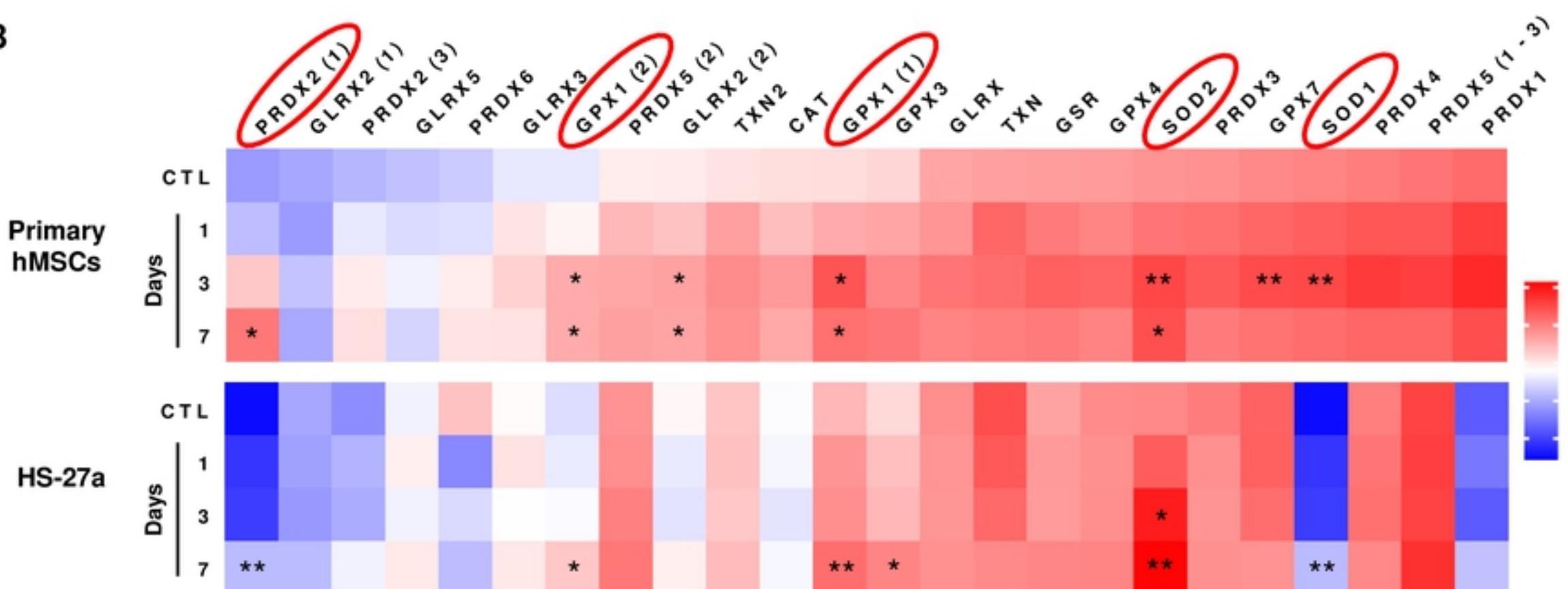
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**HIF-1 α** **Primary hMSCs****Day 1****Day 3****Day 7****HS-27a****C****VEGF-A****Primary hMSCs****Day 1****Day 3****Day 7****HS-27a****Day 1****Day 3****Day 7****Fig5**

A

Primary hMSCs

HS-27a

**B****Fig6**

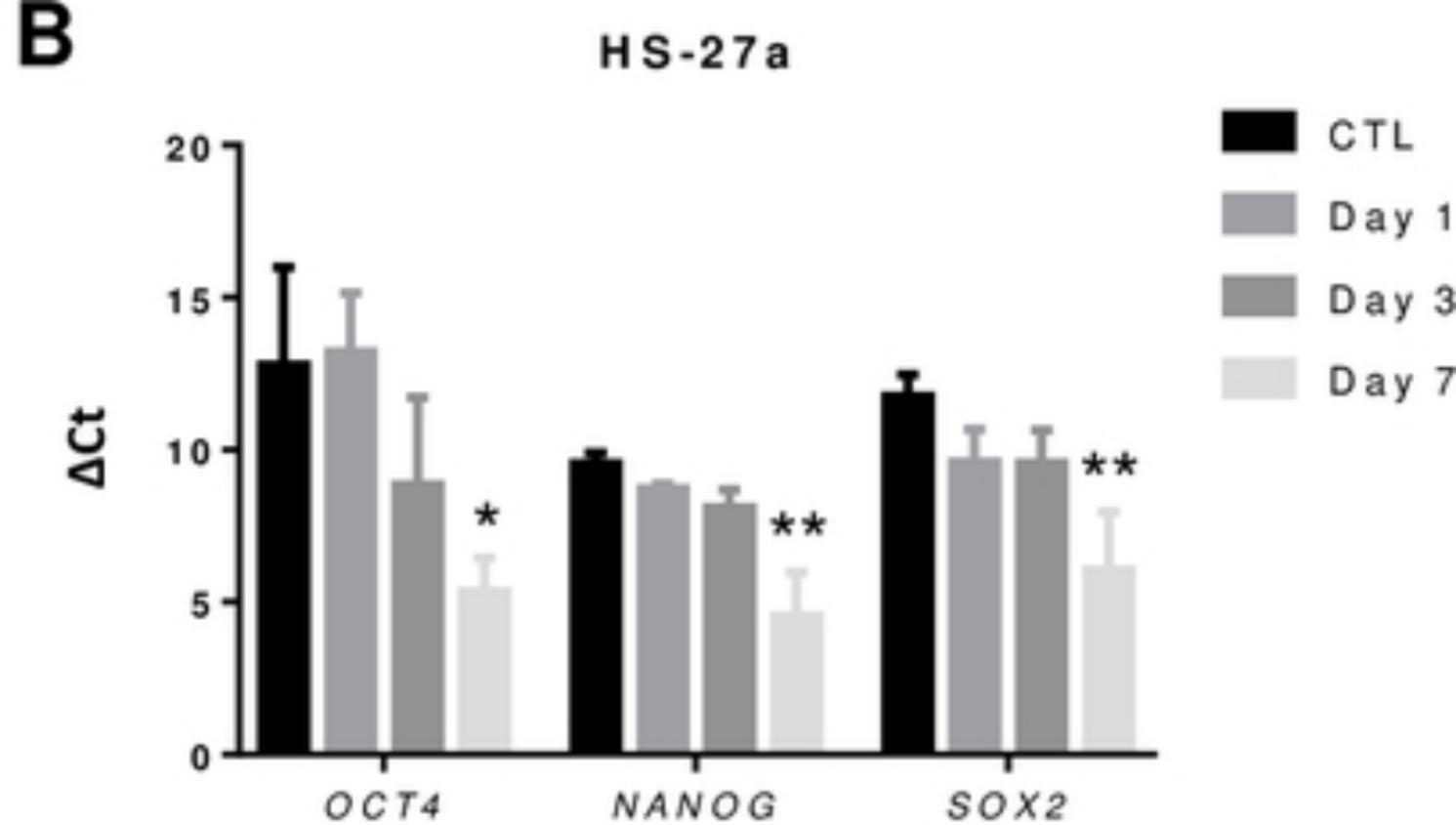
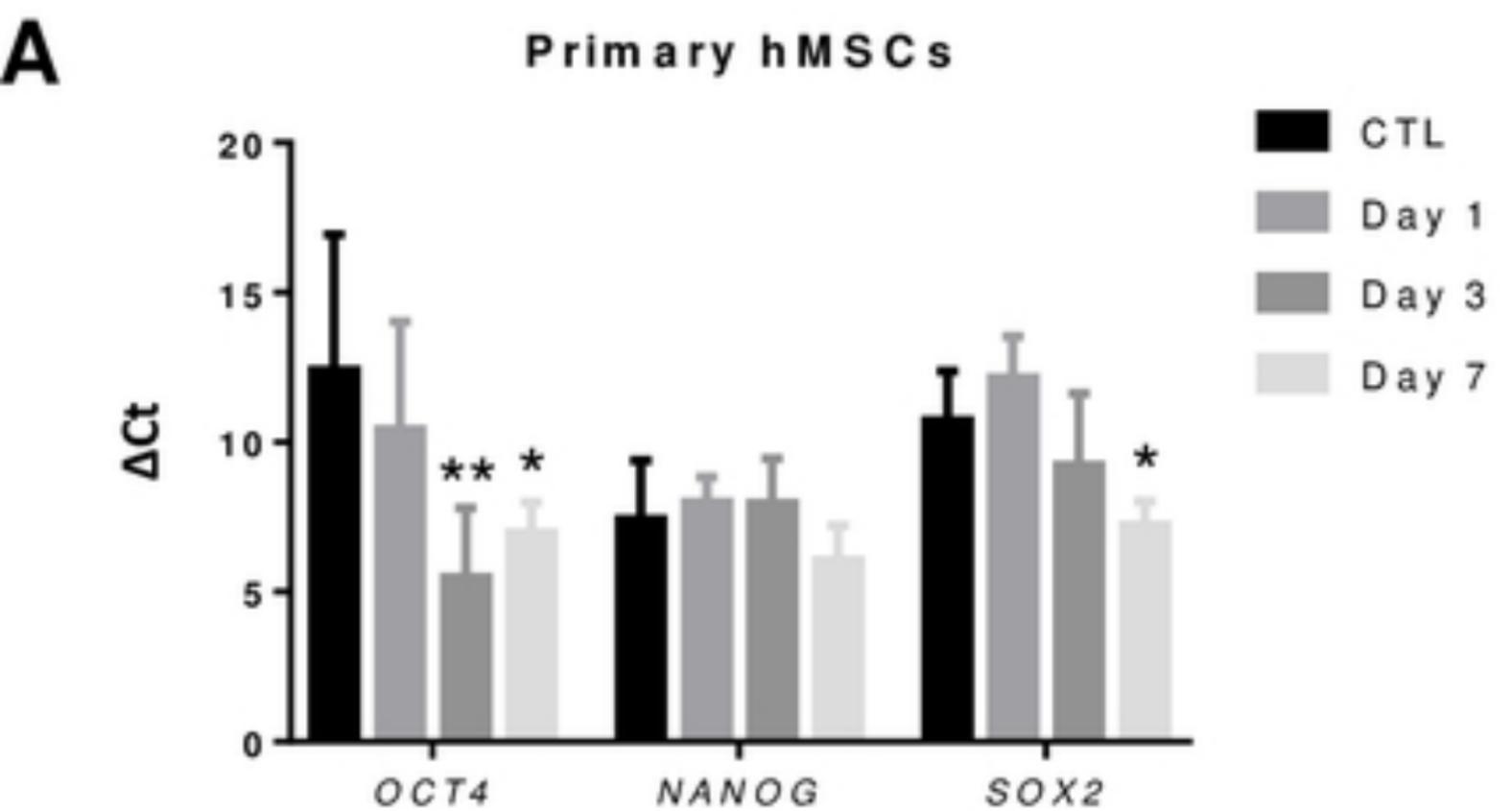


Fig7