

1 **Analysis of the behavior of 2D monolayers and 3D spheroid human pancreatic**
2 **beta cells derived from induced pluripotent stem cells in a microfluidic**
3 **environment**

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6 Amal Essaouiba^{1,2}, Rachid Jellali¹, Marie Shinohara³, Benedikt Scheidecker³, Cécile
7 Legallais¹, Yasuyuki Sakai^{2,3}, Eric Leclerc^{1,2(*)}

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10 ¹*Université de technologie de Compiègne, CNRS, Biomechanics and Bioengineering,*
11 *Centre de recherche Royallieu CS 60319, 60203 Compiègne Cedex*

12 ²CNRS UMI 2820; Laboratory for Integrated Micro Mechatronic Systems, Institute of
13 Industrial Science, University of Tokyo; 4-6-1 Komaba; Meguro-ku; Tokyo, 153-
14 8505, Japan

15 ³Department of Chemical Engineering, Faculty of Engineering, University of Tokyo,
16 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

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*** Corresponding author**

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Eric Leclerc: eleclerc@iis.u-tokyo.ac.jp, eric.leclerc@utc.fr

Rachid Jellali: rachid.jellali@utc.fr

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40 **Abstract**

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42 The limited availability of primary human β -cells/islets and their quality (due to donor
43 diversity) restrict the development of *in vitro* models for diabetes research. Human
44 induced pluripotent stem cells (hiPSCs) may be a promising cell-source for diabetes
45 studies, anti-diabetic drug screening and personalized therapies. However, achieving
46 levels of maturity/functionality that are comparable to the *in vivo* situation and islets
47 rebuilt from iPSCs is still challenging. Here, we compare and discuss two strategies
48 for culturing human pancreatic β -cells derived from hiPSCs in microfluidic biochips.
49 First, we confirmed that the protocol in conventional Petri 2D monolayer led to insulin,
50 PDX1 and MAFA positive staining, to C-Peptide productive cells, and to tissue
51 responsive to high/low glucose and GLP1 stimulation. This protocol and its
52 subsequent modifications (including extracellular matrix coating, cell adhesion time,
53 cell inoculation density, flow rate) was not successful in the 2D biochip culture. We
54 proposed a second strategy using 3D spheroids created from honeycomb static
55 cultures. Spheroids in static experiments carried out over 14 days demonstrated that
56 they expressed high levels of β -cell markers (*INS* mRNA) and higher α -cell markers
57 (*GCG* mRNA and glucagon positive staining), when compared to Petri 2D cultures.
58 Furthermore, the 3D spheroids were specifically able to secrete insulin in response to
59 both high/low glucose stimulation and GLP1 exposure. The spheroids were
60 successfully inoculated into biochips and maintained for 10 days in perfusion. The 3D
61 biochip cultures increased mRNA levels of *GCG* and maintained high levels of β -cell
62 markers and responsiveness to both high/low glucose and GLP1 stimulation. Finally,
63 C-peptide and insulin secretion were higher in biochips when compared to static
64 spheroids. These results illustrate the promising potential for hiPSCs derived β -cells
65 and their spheroid-based pancreas-on-chip model for pancreatic disease/diabetes
66 modeling and anti-diabetic drug screening.

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68 **Keywords:** human induced pluripotent stem cells, β -pancreatic cells, microfluidic
69 culture, 3D spheroids.

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Introduction

In 2019, the International Diabetes Federation (IDF) reported that 466 million people worldwide (9.3 % of adults aged 20-79 years) have diabetes mellitus (DM) (IDF Diabetes Atlas, 2019). The predictions for DM are worrying, with 700 million people affected (10.9% of the population) by 2047 (IDF Diabetes Atlas, 2019). The annual healthcare cost of diabetes was estimated at approximately 760 billion USD in 2019 and is predicted to reach 800 billion USD by 2040 (IDF Diabetes Atlas, 2019; Rogal et al., 2019). There are two main types of DM: type 1 DM (T1DM, 5-10 % of diabetic patients) and type 2 DM (T2DM, more than 90% of cases, Essaouiba et al., 2020). T1DM results from autoimmune destruction of pancreatic islet beta cells leading to a lack of insulin secretion (Jellali et al., 2020; Rogal et al., 2019). The standard practice for T1DM treatment is daily scheduled or continuous insulin administration, based on monitoring of glucose levels (Galderisi et al., 2017). T2DM is caused by the insensitivity of target tissues to insulin and impaired insulin secretion (DeFronzo et al., 2015). T2DM can be managed by lifestyle adjustments and oral antidiabetic agents such as thiazolidinediones, metformin, sulphonylureas, meglitinides and GLP-1 receptor agonists (Kahraman et al., 2016).

For both types of DM, there is a real need to develop relevant models for cell therapy, investigation of the underlying mechanisms in diabetes and the etiology of beta cell dysfunction, and anti-diabetic drug screening. Several animal models (in particular rodents) with the characteristics of T1DM and T2DM have been used for DM studies (King and Bowe 2016). However, animal models have their limitations because of species differences, resulting in poor extrapolation from animal research to human (Cota-Coronado et al., 2019; Merlier et al., 2017). With the development of tissue-engineering 3D cultures, dynamic organ-on-chip cultures, and co-culture models, *in vitro* cell-based models have the potential to mimic the *in vivo* physiological microenvironments of organs and provide relevant models for diabetes modelling. The type of cells and their source are a key factor for the development of *in vitro* models (Rogal et al., 2019). Primary human β -cells or islets are considered a gold standard for *in vitro* models in DM research (Kaddis et al., 2009). However, the

108 limited availability, the high cost of islet isolation and inter-donor differences remain
109 major limitations to using primary islets/ β -cells (Balboa et al., 2019; Amirruddin et al.,
110 2020). Furthermore, primary islets rapidly lose their specific functions when cultured
111 *in vitro* (Rogal et al., 2019). β -cell lines are a potential alternative to primary β -cells as
112 they have an infinite life span, low cost and have reduced variability (Scharfmann et
113 al., 2019). Nevertheless, these cells have limited functionality, lack plasticity and
114 there are differences in the gene expression of β -cell markers when compared to
115 primary cells (Amirruddin et al., 2020; Bakhti et al., 2019).

116

117 In 2007, Takahashi et al., achieved a major breakthrough by reprogramming
118 patient somatic cells into human induced pluripotent stem cells (hiPSCs) (Takahashi
119 et al., 2007). The availability of these cells, along with their ability to both self-renew
120 indefinitely *in vitro*, and generate different cell types, provide great insight for
121 investigating the pathogenic mechanisms of diseases and for contributing to cell
122 therapies and drug development (Balboa et al., 2019; Amirruddin et al., 2020).
123 Furthermore, unlike human embryonic stem cells (hESCs), hiPSCs do not raise any
124 ethical problems and offer the possibility of developing patient-specific models
125 (Balboa et al., 2019). hiPSC differentiation into mature selected tissue is a strategy
126 based on translational embryology (Spence *et al.*, 2007). This sequential process
127 makes it possible to direct the hiPSCs from the endoderm stage to specific cell types,
128 such as β -cell pancreatic progenitors and as far as pancreatic β -like cells. In light of
129 this, several protocols following this philosophy have been proposed (Hosoya, 2012;
130 Zhu et al., 2016). While it was demonstrated that partially functional pancreatic β -cell
131 tissue could be achieved, attaining levels of maturity and functionality comparable to
132 those of the *in vivo* situation is still challenging. Nevertheless, it is reported that iPSC
133 derived pancreatic cells may be a source of cells for pancreatic disease models
134 (Kahraman et al., 2016; Hohwieler et al., 2019).

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136 Organ-on-chip is one of the more promising techniques for investigating
137 complex human diseases that allow human physiological *in vitro* responses (Esch et
138 al., 2015). These microfluidic platforms improve the exchange and transport of
139 nutrients, oxygen, metabolic waste and hormones, and create “physiological-like”
140 situations such as cell-cell interaction, shear stress and chemical gradients (Merlier
141 et al., 2017; Rogal et al., 2019). Several previous studies have reported that perfused

142 microfluidic cultures enhance the long-term viability and functionality of pancreatic
143 islets and β -cell spheroids (Jun et al., 2019; Bakhti et al., 2019). Last but not least,
144 organ-on-chip technology makes possible the co-cultures of two or more organs in
145 separate micro-bioreactors, connected by soluble factors exchanged through the
146 microfluidic network (Merlier et al., 2107). This system can be used to study inter-
147 organ crosstalk such as interactions between pancreatic islets and hepatic cells
148 (Bauer et al., 2019; Essaouiba et al., submitted). The co-culture of two or more
149 organs is a powerful tool for modulating multi-organ diseases such as diabetes.
150 Although organ-on-chip technology has been used to reproduce *in vitro* pancreas-on-
151 chip models using pancreatic islets or β -cell spheroids (Jun et al., 2019; Lee et al.,
152 2018; Li et al., 2017; Mohammed et al., 2009; Bauer et al., 2017; Schulze et al.,
153 2017; Zbinden et al., 2020), only very few studies have already coupled iPSC derived
154 pancreatic-like cells with organ-on-chip technology (Rogal et al., 2019; Hirano et al.,
155 2017; Tao et al., 2019).

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157 Our group has developed organ-on-chip technology contributing to
158 investigations into human liver metabolism (Prot et al., 2011; Jellali et al., 2016), the
159 human liver regeneration process (Danoy et al., 2019), as well as crosstalk and
160 synergy between different organs such as the liver's interaction with the intestine and
161 kidneys (Bricks et al., 2014; Choucha-Snouber et al., 2013). Recently, we have
162 investigated the behavior of rat islets of Langerhans and their interaction with
163 hepatocytes in microfluidic biochips (Essaouiba et al., 2020; Essaouiba et al.,
164 submitted). In this paper, we propose extending those microfluidic developments to
165 pancreatic human β -cells derived from induced pluripotent stem cells. We
166 investigated and compared several protocols for biochip cultures, as well as 2D and
167 3D culture configurations.

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169 **2. Material and methods**

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171 **2.1. Cell source**

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173 The cells used in this work (Cellartis hiPSCs derived β -cells) were provided
174 by Takara Bio (Japan). Cellartis hiPSC beta cells were differentiated from ChiPSC12

175 lines and provided in stage 1 of maturation (Fig.1). The hiPSCs derived β -cells were
176 differentiated into insulin-producing cells using the hiPSCs beta cell media kit (cat. N°
177 Y10108, Takara Bio, Japan), according to the manufacturer's instructions.

178

179 **2.2. 2D Petri pancreatic β -cell culture protocol**

180

181 Culture dishes (24-well plates) were coated with a Cellartis beta cell coating
182 (cat. N° Y10103) and incubated at 37°C. After 1h, the coating solution was removed
183 and 500 μ L of maintenance culture medium (Cellartis beta cell basal medium Y10104,
184 supplemented with beta cell supplement Y10102) containing cells were added to
185 each well. The cells were inoculated at a density of 2×10^5 cells/cm² and the plates
186 incubated at 37°C in an atmosphere supplied with 5% CO₂. The maintenance culture
187 medium was used for 12 days and changed every day. The assay medium (Cellartis
188 beta cell medium 2 Y10105, supplemented with beta cell supplement Y10102) was
189 then used from day 12 to day 15 (Fig.1).

190

191 **2.3. 3D spheroid cultures using honeycomb technology**

192

193 To create the spheroids, we used the honeycomb technology previously
194 developed by Shinohara et al., 2014, 2017. Briefly, the honeycomb polygons were
195 made of PDMS and had the geometric characteristics of 126 μ m in width and 129 μ m
196 in depth (Fig.2A). The PDMS honeycomb sheet was seeded on to a bottomless 24-
197 well plate. Each well of the 24-well plate contained 6750 honeycombs. The plates
198 were sterilized with ethanol for one hour, coated with pluronic-PBS solution overnight
199 (Pluronic® F-127 Sigma) and rinsed three times with phosphate-buffered saline (PBS,
200 Gibco) and once with maintenance culture medium. After thawing, the β -cells were
201 dropped into the honeycomb in 500 μ L of maintenance medium and incubated at
202 37°C in an atmosphere supplied with 5% CO₂. Two densities were tested: 2×10^5 cells
203 per well (low-density, LD) and 6×10^5 cells per well (high-density, HD). The sequence
204 of the culture medium change was exactly the same as the 2D Petri monolayer
205 cultures. Nevertheless, after 24h, the medium was adjusted to 1 mL. We then
206 removed 600 μ L at each culture medium change, replacing them with 600 μ L of fresh

207 medium (thus resulting in there always being 400 μ L in the honeycombs to prevent
208 the spheroids suction).

209 **2.4. Dynamic cultures in biochips**

210

211 We tested two biochip culture strategies. The first was a “2D monolayer”,
212 where cells adhered to the surface culture inside the biochip. The second strategy
213 consisted of the dynamic culture of 3D β -cell spheroids.

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215 **2.4.1. Microfluidic biochip manufacture**

216

217 In the 2D culture, the biochip consisted of a cell culture chamber manufactured
218 with two polydimethylsiloxane (PDMS) layers. The microstructured bottom layer, with
219 series of microchambers and microchannels (depth of 100 μ m, Fig.2B), was used as
220 a support for cell attachment. The second layer, with a reservoir (depth of 100 μ m),
221 was placed on top of the first layer and included an inlet and outlet microfluidic
222 network for homogenous culture medium distribution (Fig.2B). The design and
223 dimensions of the biochip were described in our previous work (Baudoin et al., 2011;
224 Jellali et al., 2016). For the 3D β -cell spheroid cultures, construction of the biochip
225 included a first PDMS layer (bottom layer) for trapping islets, which contained a
226 series of crescent-shaped structures of 600 μ m in diameter (height of 300 μ m), and
227 spaced 300 μ m apart (Fig.2B). The second PDMS layer (top layer) was the same as
228 described above for 2D biochip.

229

230 The biochips were made of PDMS using the conventional replica molding
231 process. The mold masters for the bottom and top layer of the biochips were
232 manufactured using photolithography with SU-8 photosensitive resin. The PDMS
233 prepolymer (Sylgard 184, Dow Corning in a mixture of 10:1 base polymer: curing
234 agent) was poured on to the SU-8 master and cured for 2 h at 75°C. The surfaces of
235 the PDMS layers obtained were activated with reactive air plasma (1 min; Harrick
236 Scientific) and brought together immediately to form an irreversible seal.

237

238 **2.4.2. Biochip cultures**

239

240 Before the cell experiments, the biochips and perfusion circuits (silicone tubing
241 and bubble trap) were sterilized by autoclaving and dried in an oven. Then, the
242 biochips were assembled with the perfusion system and filled with culture media in
243 order to remove the air bubbles and moisturize the circuits. The bubble trap was
244 used as a reservoir interconnected to the biochips by the silicone/Teflon tubing with a
245 diameter of 0.65 mm. The assembled experimental setup (biochip, tubing, reservoir,
246 and peristaltic pump) is presented in Fig.S1 (supplementary file).

247

248 The protocol for 2D cultures in biochips was similar to the protocol used in 2D
249 Petri cultures (section 2.2). In this protocol, the biochips were coated using the
250 extracellular matrix solution provided in the β -cell kit (Cellartis beta cell coating, cat.
251 N° Y10103) and incubated at 37°C. Then, several parameters were tested to
252 establish the best attachment protocol as shown in Table S1 (supplementary file). It
253 included modulation of the inoculation cell density, incubator oxygen concentration,
254 composition of the culture medium, and time of adhesion before perfusion. In 3D
255 biochip cultures, the β -cell spheroids were formed using the honeycomb technology
256 as described in the section above. After 4 days of culture in the honeycombs, the
257 spheroids formed were collected and seeded in biochips (Fig.1). In order to minimize
258 any spheroids damage, wide orifice pipette tips with low binding were used during the
259 entire handling process. After spheroids seeding, the biochips were incubated at
260 37°C in a 5% CO₂ supplied incubator for 1h to allow the crescent-shaped structures
261 to trap the spheroids. The biochips were then connected to the perfusion circuits and
262 peristaltic pump, and the perfusion started at 20 μ L/min. The entire setup was
263 continuously incubated at 37°C in a 5% CO₂ supplied incubator. A similar protocol
264 was used in 3D spheroid honeycomb static cultures for comparative purposes. For
265 that purpose, the spheroids formed (after 4 days of culture in the honeycombs) were
266 collected and seeded in a new honeycomb plate (using the same density as the
267 biochip). The culture medium change sequence was the same as for the 2D Petri
268 monolayer cultures and 3D static cultures.

269

270 **2.5. RTqPCR assays**

271

272 Total RNAs were extracted and purified from samples using a hybrid protocol
273 that combined Trizol™ Reagent (Life Technologies) and the RNeasy Mini Kit

274 (QIAGEN 74104) following the manufacturer's instructions. The concentrations and
275 qualities of the RNAs extracted were assessed using a BioSpec-nano (Shimadzu
276 Scientific Instruments). Reverse-transcription into cDNA was performed from 0.5 µg
277 of total RNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover
278 (TOYOBO). Real-time quantitative PCR was then performed with the
279 THUNDERBIRD SYBR qPCR Mix (TOYOBO) according to the manufacturer's
280 protocol and a StepOnePlus Real-Time PCR system (Applied Biosystems). Primer
281 sequences of genes are shown in Table S2 (supplementary file). β-Actin was used as
282 the reference gene.

283

284 **2.6. Immunostaining**

285

286 After transfer to an untreated TCPS 24-well plate, the spheroids were washed
287 with phosphate buffer saline solution (PBS) and fixed in paraformaldehyde 4% at 4°C
288 overnight. In order to perform the immunohistochemistry (IHC) staining in a 3D
289 structure, the spheroids were permeabilized with 1% Triton X100 in PBS for 3 hours
290 at 4°C and washed 3 times with PBS for 30 min. Then, the spheroids were blocked
291 with a gelatin buffer for 24 hours at 4°C. Primary antibodies (Table S3,
292 supplementary files) were incubated for 24 hours at 4°C in a BSA/PBS solution. After
293 washing with PBS, secondary antibodies (Table S3, supplementary files) were further
294 incubated overnight in a BSA/PBS solution at 4°C in the dark. Finally, the nuclei were
295 stained with DAPI (342-07431, Dojindo) at 1/1000 for 30 min at room temperature
296 (RT) in the dark. All the incubations and washing steps were carried out using a
297 shaker. Observations were made using an Olympus IX-81 confocal laser-scanning
298 microscope.

299 The IHC staining of the 2D monolayer β-cells followed a similar protocol in
300 which the period of permeabilization, first antibody incubation and second antibody
301 incubation were reduced to 15 min at RT, overnight at 4°C, and 2 hours at RT,
302 respectively. The primary and secondary antibodies used are listed in Table S3
303 (supplementary file).

304

305 **2.7. Insulin and C-peptide measurements**

306

307 The insulin and C-peptide released into the culture medium from the different
308 culture conditions were assessed using ELISA assays, following the manufacturer's
309 protocol. The following kits were used: insulin (human insulin ELISA kit, 10-1113-01,
310 Mercodia) and C-peptide (human C-peptide ELISA kit, 10-1136-01, Mercodia). The
311 results were obtained using an iMark microplate reader (Bio-Rad, Osaka, Japan) set
312 to a wavelength of 450nm.

313

314 **2.8. Insulin secretion by low / high glucose assays**

315

316 At the end of the cultures, we carried out a low / high glucose stimulation to
317 check insulin production. In the biochips, the culture medium was removed from the
318 bubble trap, and the perfusion circuits with the culture chamber containing the
319 spheroids were washed with a 0-glucose solution (DMEM, No Glucose, Wako) for 2
320 hours. Then, the washing 0-glucose solution was removed from the bubble trap and
321 1 mL of fresh 0-glucose was added and perfused for 2 additional hours. After this low
322 glucose perfusion, the spheroids were exposed to a high glucose culture medium for
323 2 hours (25 mM of glucose; DMEM, 25 mM high Glucose, Wako). This involved the
324 low glucose solution being removed from the bubble trap, replaced with 1mL of high
325 glucose solution and the perfusion launched for 2h. In the Petri dishes (2D and 3D
326 spheroids), this protocol led to 2h 0-glucose exposure (washing), followed by another
327 2h 0-glucose exposure and finally 2h of high glucose stimulation. At the end of the
328 assays, basal media were re-established for all conditions.

329

330 **2.9. Glucagon-like peptide-1 (GLP1) stimulations**

331

332 In order to test the response of the β -cells to drug stimulations, we exposed
333 the cultures to GLP1. To do so, we added 100 nM of GLP1 to the culture medium for
334 the last 24h of culture, on day 13, until day 14. This provided 24h of exposure to the
335 drug.

336

337 **2.10. Statistical analysis**

338

339 All experiments were repeated at least three times. The data are presented as
340 the mean \pm standard deviations (SD) of 9 biochips (3 biochips from 3 different
341 experiments, $n=3 \times 3$). The data were analyzed statistically using GraphPad prism 8
342 software (San Diego, USA). The Kruskal Wallis test was performed to determine any
343 significant differences between the samples (P values < 0.05 were identified as
344 statistically significant).

345

346 **3. Results**

347

348 **3.1 The 2D monolayer strategy derived β -cells in Petri dishes but failed in** 349 **biochips**

350

351 The protocol recommended by Cellartis (cells plated in Petri 2D) led to
352 successful cells adhesion and 16 days of cell culture (Fig.3 A-C). The β -cells profile,
353 at the protein level, was confirmed by the expression of PDX1, MAFA and insulin, as
354 demonstrated by the immunostaining in Fig.3 D-G. The RTqPCR analysis illustrated
355 successful β -cells differentiation in Petri dishes, as demonstrated by the upregulation
356 of the mRNA levels of *INS*, *PDX1*, *NGN3*, *NKX6.1* and *NKX2.2* at the end of the
357 differentiation, when compared to the first day of culture and to the iPSCs standard
358 (Fig.3H). Finally, the functionality of the cells was confirmed by the secretion of the
359 C-peptide (Fig.3I). Secretions reached 4.5 ± 0.5 pmol/ 10^5 of inoculated cells (8600
360 pmol/L/ 10^5 cells). The β -cells culture was also responsive to high / low glucose
361 stimulation, leading to a 2.6 ± 0.9 ($n=4$ assays) times more insulin secretion in high
362 glucose stimulation when compared to low glucose stimulation (data not shown).
363 Finally, glucagon production was not detected (either by ELISA, or by
364 immunostaining, data not shown). This set of results confirmed that the β -cells
365 differentiated in 2D Petri conditions.

366

367 The same strategy was investigated in the biochips by directly seeding the
368 hiPS β -cells, after thawing, inside the 2D biochips. To try to attach the cells to the
369 bottom surface of biochips, we investigated several conditions including (i) the
370 extracellular matrix coating; (ii) the presence of a rock inhibitor in the seeding
371 medium; (iii) adjusting oxygen concentrations during the adhesion phase; (iv) and the

372 density of the seeded cells. The complete set of parameters tested is summarized in
373 Table S1 (supplementary file). After 24h of adhesion, the cells were not able to attach
374 in most of the conditions tested. The typical morphology is presented in Fig.4A (24h
375 after seeding, ↓ density). When using the high cell density, few cells managed to
376 attach but they quickly formed aggregates, as shown in Fig.4A (24h after seeding, ↑
377 density). Then, once the perfusion was launched, the cells were detached after 5h of
378 culture (Fig.4A, 5h after perfusion, ↑ density). Finally, no optimized condition was
379 found to make successful 2D monolayer biochip cultures possible (n=3 cryotubes
380 used in 3 independent experiments, leading to 26 biochips).

381

382 **3.2 3D spheroid strategy in static honeycombs**

383

384 As the biochip cultures failed with the monolayer of β -cells, we cultured the
385 cells into spheroids to create aggregates and allow us to seed them in the biochips
386 with crescent-shaped microstructures. The 3D spheroids were created using
387 honeycomb microwells. Two cell densities, 0.6×10^6 and 0.2×10^6 cells per well, were
388 tested (Fig. 4 B). The aggregates were formed after 7 hours of culture but still
389 presented a rough circumference (Fig.4B, 7h after seeding). They started to present
390 a round shape after 3 to 4 days of culture. The highest density led to spheroids of
391 $90 \pm 15 \mu\text{m}$ in diameter (Fig.4B, 14 days). The lowest density led to smaller spheroids,
392 of $50 \pm 25 \mu\text{m}$ in diameter, but with greater dispersion (Fig.4B, 14 days). Based on the
393 number of honeycombs (6750), we estimated about 30 and 90 cells/spheroid in low-
394 and high-density, respectively.

395

396 The immunostainings are presented in Fig.5 for both types of spheroid. They
397 confirmed that the spheroids were positive for β -cell markers: insulin, MAFA and
398 PDX1. When compared to 2D cultures, the spheroids appeared to be positive for
399 glucagon (in both high- and low-density).

400

401 The β -cells spheroids led to positive C-peptide secretion, as shown in Fig. 6 A.
402 When normalized by the number of seeded cells, we found that the secretion of C-
403 peptide was similar in both high- and low-density experiments (Fig.6A). Peak
404 concentrations of around $5 \text{ pmol}/10^5$ inoculated cells were achieved after 13 days of

405 culture. Furthermore, at the end of the experiment (day 14), we detected higher
406 secretion of insulin in the low-density spheroids cultures (210 ± 65 pmol/ 10^5 cells)
407 when compared to the high-density spheroids cultures (98 ± 20 pmol/ 10^5 cells), as
408 shown in Fig.6B. Both culture modes were responsive to the high/low glucose
409 stimulations (Fig.6C). Namely, the high glucose stimulation led to 4.5 ± 1.3 times more
410 insulin production when compared to the low glucose condition in high-density
411 spheroids (GSIS index). In the low-density spheroid cultures, the GSIS index was
412 11.5 ± 5 . Finally, both types of spheroid were also responsive to GLP1 drug
413 stimulation, leading to double the insulin secretion (Fig.6D). The ratios of insulin
414 secretion (GLP1 treated/control) were 1.96 and 1.6 in high- and low-density,
415 respectively.

416

417 **3.3. Critical transfer of 3D β -cells spheroids into microfluidic biochips**

418

419 After 4 days of culture in the honeycomb, once the spheroids had presented a
420 round shape, they were collected and inoculated into the 3D biochips. The low-
421 density spheroids were very fragile, and we were not able to collect the spheroids
422 without damaging them (loss during pipetting, loss during centrifugation, spheroids
423 destroyed during handling, $n=6$ honeycomb microwell dishes were tested for transfer).
424 As a result, we only transferred the high-density spheroids into the biochips.

425

426 Although it was possible to inject the high-density spheroids, we still noticed
427 significant loss: we counted only 150 ± 50 spheroids entering the biochips. As we used
428 one honeycomb Petri to fill 6 biochips, this led to about 82% - 91% of spheroids lost
429 ($600/6750 - 1200/6750$, this will be discussed below). The perfusion was started for
430 10 additional days, leading to 14 days of culture (4 days in static conditions to create
431 the spheroids and 10 days of dynamic culture). At the end of the perfusion, we
432 confirmed the presence of the spheroids (number similar to the inoculation density,
433 about 160 ± 66 spheroids), illustrating successful perfusion culture as shown by their
434 morphologies, which are presented in Fig.4C.

435

436 **3.4. High functionality of the 3D pancreatic spheroids in microfluidic biochips**

437

438 Analyzing the mRNA levels revealed major modifications to the profile of the
439 cells when we compared the 2D Petri, 3D Petri honeycomb (3D-HD) and the 3D
440 biochip cultures (Fig.7). The spheroid culture, in 3D Petri, appeared to increase the
441 gene expression of β -cell markers such as *PDX1*, *NKX2.2*, *NKX6.1* and *INS* (Fold
442 change, FC of 3.3, 2.6, 3 and 3.8, respectively when compared to Petri 2D). In
443 parallel, alpha or delta cell markers such as *GCG* (FC 2.6), *SST* (FC 6.6) and
444 glucose transporter *GLUT2* (FC 19) were higher in static 3D spheroid conditions than
445 in 2D Petri conditions. Finally, *GCK*, *UCN3* and *NGN3* were downregulated in static
446 3D spheroids (in comparison with Petri 2D).

447

448 Once cultivated in the biochip, we found an increase in mRNA levels of alpha
449 cells markers (*GCG*, FC 6.8), delta cell markers (*SST*, FC 3.5) and glucose
450 metabolism markers (*GLUT2*, FC 12.7), when compared to 2D cultures (Fig.7).
451 Furthermore, we found clear upregulation of *GCG* when compared to the 3D Petri
452 cultures. Although β -cells markers such as *NKX6.1* and *NKX2.2* were 2.5-2.7 times
453 higher in the biochips when compared to 2D (and similar to 3D Petri levels), the
454 levels of *PDX1* and *INS* mRNA were similar in the 2D cultures and biochips. Finally,
455 *MAFA* (FC 0.42), *GCK* (FC 0.11), *UCN3* (FC 0.12) and *NGN3* (FC 0.08) were lower
456 in the biochips, when compared to 2D cultures.

457

458 Immunostaining confirmed that the spheroids cultures in biochips expressed
459 typical β -cells markers, as illustrated by the detection of insulin, *MAFA* and *PDX1*
460 positive cells (Fig.5A). However, we also found cells positive for glucagon,
461 demonstrating the presence of alpha-like cells as well. As mentioned above, in the
462 2D Petri cultures, we never detected glucagon positive cells.

463

464 The kinetics of C-peptide secretion in biochips presented in Fig.8A
465 demonstrates the functionality of the spheroids. To be able to compare the dataset in
466 biochips and honeycombs (3D-HD), we normalized by number of spheroids at the
467 end of the experiments. When normalized by the number of spheroids, C-peptide
468 secretion in the biochips was measured at around 0.02-0.05 pmol/islet between day
469 9 and the end of the perfusion (day 14, Fig.8A). Furthermore, we detected higher
470 quantities of C-peptide in biochip cultures when compared to 3D honeycombs (about

471 10-30 times higher). We also observed higher secretions of insulin in the biochip
472 spheroids cultures when compared to the static spheroids cultures (3D-HD), as
473 shown in Fig.8B. Insulin secretion was about 2.55 times higher in the biochips at the
474 end of the experiment (day 14).

475

476 The functional assays performed using high/low glucose stimulation (glucose-
477 stimulated insulin secretion, GSIS) and GLP1 stimulation demonstrated that the
478 biochip spheroids were able to adapt their insulin response, as shown in Fig.8C and
479 8D, respectively. However, in terms of the induction ratio itself, no difference between
480 the biochip and the static honeycomb cultures was observed. The GSIS index
481 (glucose-stimulated insulin secretion: insulin measured in high-glucose divided by
482 insulin in low-glucose) values were of 3.2 ± 1.1 and 4.5 ± 1.2 in the biochip and static
483 honeycomb, respectively (Fig.8C). Concerning the GLP1 effect, the levels of insulin
484 were 1.5 (static spheroids) and 2 (biochip spheroids) times higher after GLP1
485 stimulation, when compared to the control (Fig.8D).

486

487 **Discussion**

488

489 In this work we investigated the behaviors of pancreatic β -cells derived from
490 human induced pluripotent stem cells. The 2D cultures in Petri dishes confirmed the
491 functionality of the derived tissue as a pancreatic-like β -cells. This was illustrated by
492 C-peptide production, the positive staining for insulin, negative staining for glucagon,
493 and insulin secretion in response to low/high glucose stimulation. Other 2D protocols
494 for iPSCs derived pancreatic β -cells, including different growth factor sequences, iPSC
495 cell lines and sources, have also reported successful insulin and C-peptide functional
496 tissues (Yabe et al., 2017; Southard et al., 2018; Pelligrini et al., 2018). Those works
497 attained C-peptide production of up to $5000 \text{ pmol/L}/2 \times 10^6$ cells after 23 days of
498 culture (Yabe et al., 2017), and ranging from 700 to $1500 \text{ pmol/L}/6.4 \times 10^4$ of plated
499 cells after 28 days of culture (Southard et al., 2018). Our differentiation took place
500 over 16 days after stage 1 of the Cellartis protocol (Fig 1), corresponding to 37 days
501 overall of differentiation from undifferentiated iPSCs. As we reached a peak of 8600
502 $\text{pmol/L}/10^5$ of plated cells ($4.5 \text{ pmol}/10^5$ of cells) after 35 days, our results appeared
503 consistent with the data in the literature.

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Although the 2D Petri cultures were encouraging, we failed to create 2D cultures of β -cells in our microfluidic devices (our primary goal). This strategy was first investigated because the Cellartis ChiPSC12 kit is recommended for use in monolayers. We could not identify the key parameters leading to this failure. First the cell adhesion, and then the cell monolayer could not be created in the biochips even though we tested several conditions, including the initial cell density, extracellular matrix coating and oxygen adhesion conditions (see Table S1, supplementary file). Several hypotheses can be formulated: (i) the first is related to choosing the extracellular matrix and its protocol of coating on PDMS. PDMS is a hydrophobic material that needs an appropriate coating of ECM to make cell adhesion possible. The Cellartis recommended ECM, when coated on PDMS, may require higher concentrations of ECM compounds and longer incubation times on the surface compared to the recommended protocol for polystyrene Petri cultures; it may also require other components, such as Matrigel (suitable for iPSC hepatocytes on PDMS for instance, Danoy et al., 2019) (ii) in addition, during the adhesion phase of cells in biochips, and the first hours of perfusion, our previous experiments (with cell lines) demonstrated that there was significant glucose consumption by the cells (Prot et al., 2011). As the biochip volume was 30 μ L, leading to a cell/volume ratio of 6 600 000 cells/mL in the biochip (200 000 cells/cm² in 30 μ L) and 400 000 cells/mL in Petri dishes (200 000 cells/cm² in 0.5 mL), we can hypothesize that there was a local shortage of a critical nutrient at the density inoculation tested. In this context, several reviews to help obtain successful microfluidic cultures have been proposed in the literature, exploring other 2D strategies (Yu et al., 2007, Young and Beebe 2010); (iii) we also previously reported some ROS production during the adhesion stage of cell culture in a microfluidic environment and during the first hours of perfusion (Leclerc et al., 2015). To avoid potential apoptosis, we tested the effects of a ROCK inhibitor, as it is used in several iPSC protocols during the plating stage after thawing (Emre et al., 2010; Watanabe et al., 2007), but it did not lead to improved adhesion. As a result, more extensive investigations are needed to solve the issues with the 2D biochip cultures.

536 As an alternative to the 2D biochip culture strategy, we proposed a 3D
537 spheroid protocol. In honeycomb static cultures, our spheroid protocol contributed to
538 generating β -cells-based spheroids secreting C-peptide and insulin. We found that
539 low cell density spheroids generated smaller spheroids (50 μm) compared to high cell
540 density ones (100 μm), although they produced similar levels of C-peptide. The
541 effects of cell density and spheroid diameter on functionality were documented with
542 β -cells line (Shinohara et al., 2014; Bernard et al., 2012). Microwells of 100 to 300
543 μm in diameter led to insulin levels close to 75ng/1000 cells in Min-6 (Bernard et al.,
544 2012). In our honeycomb geometry, previous works with Min-6 spheroids ranging
545 from 60 to 150 μm in diameter produced levels of insulin close to 60 ng/ng-DNA
546 (Shinohara et al., 2014). Furthermore, *in vitro* secretion of insulin from derived iPSCs
547 β -cells spheroids is reported as ranging from 1.6 to 2 $\mu\text{UI}/10^3$ cells (Millman and
548 Pagliuca 2017; Pagliuca et al., 2014, Millman et al., 2016). Based on the data in Figs.
549 6 and 8, our study contributed to generating β -cell-based spheroids secreting insulin
550 around 1.8 $\mu\text{UI}/10^3$ cells in the high density spheroids used in 3D Petri (with a
551 conversion of 0.144 $\mu\text{UI}/\text{mL} = 1 \text{ pmol}/\text{L}$). Finally, insulin secretion stimulated by high
552 glucose in primary human islets led to 4-fold induction (glucose 5.6 mM), 16-fold
553 (16.7 mM) after one hour of exposure (Mc Donald et al., 2011) and about 10-fold at
554 11 mM, 20 min of stimulation (Pelligrini et al., 2018). These results appear to be in
555 the range of our data in which the mean value of the induced insulin secretion ratio
556 was close to 4 and 11 in the 3D high- and low-density spheroids, respectively.

557

558 We then successfully applied our 3D spheroid cultures to the microfluidic
559 biochips. There was still a significant loss of spheroids, and a third strategy
560 consisting of generating the islets inside the biochips, to avoid having to transfer
561 them, needs to be investigated. Nevertheless, thanks to the biochips, we were able
562 to improve spheroid functionality when compared to 3D Petri controls in terms of
563 insulin and C-peptide secretion. The enhancements of basal pancreatic islets or
564 pseudo-islets functions such as insulin secretion and glucose-induced insulin
565 secretion under microfluidic flow have been observed consistently in the literature
566 (Jun et al., 2019; Tao et al., 2019; Li et al., 2013; Sankar et al., 2011). We suspect
567 that changing the continuous culture medium played a part in continuously
568 stimulating the spheroids with high glucose stimulation, and thus insulin secretion.

569 The spheroids in the biochip cultures were also responsive to both low/high glucose
570 stimulation and GLP1 exposure.

571

572 Focusing on the 3D spheroids experiments, we found that the 3D spheroids
573 had greater heterogeneity (in the 3D Petri and 3D biochip conditions), when
574 compared to the Cellartis optimized 2D protocol. The mRNA levels and
575 immunostaining analysis revealed partial loss of the β -cell specifications in the 3D
576 spheroids, and the potential orientation toward pancreatic α -cells and δ -cells sub-
577 lineages. This was illustrated by the positive staining of the glucagon, upregulation of
578 *SST* and *GCG*, and downregulation of *NGN3* in the spheroids in 3D conditions. It is
579 reported in the literature that *PDX1*, *MAFA*, *NGN3* and *NKX6.1* play a pivotal role in
580 β -cells differentiation, as well as in various processes within α -cell differentiation
581 (Schaffer et al., 2010; Matsuoka et al., 2017; Zhu et al., 2016; Brissova et al., 2018).
582 Furthermore, the *SST* gene (upregulated in the 3D Petri and 3D biochip cultures
583 when compared to 2D Petri) is a key player in δ -cell specification (Hauge Evans et al.,
584 2009).

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586 In parallel, modification to the differentiation pattern was concomitant with
587 high levels of *GLUT2* and *GCG*, and low levels of *GCK* in 3D cultures. We confirmed
588 the high level of glucose in the culture medium, even in 3D Petri and 3D biochip
589 cultures (in the culture medium from step 2, we measured 9.5 ± 0.5 mM, data not
590 shown). As a result, we can hypothesize that the secretion of glucagon in the
591 spheroids (detected by positive immunostaining) is due to local shortage of glucose
592 inside the spheroid, and thus to a modulation of glucose transport inside the spheroid
593 (*nb*: it has been reported that *GLUT2* is weakly expressed in β -cells and over-
594 expressed in α -cells, leading to the way the glucose is transported being modulated,
595 but not the fact of the transport itself, Heimberg et al., 1995). *GCK* is a glucose
596 sensor that regulates insulin release in β -cells, and glucose homeostasis in α and δ -
597 cells (Matschinsky et al., 2019). In addition, *GCK* governs an α -cell metabolic
598 pathway by suppressing glucose-related secretion of glucagon at/or above
599 normoglycemic levels (Basco et al., 2018). Downregulation of *GCK* in our 3D cultures
600 appeared consistent with glucagon secretion due to glucose shortage in the center of
601 the spheroids. As a result, in agreement with pancreas organogenesis (Puri et al.,

602 2015), our data suggest that there is major cell plasticity in the differentiation process
603 of the present iPSCs in response to the 3D spheroid culture conditions. Additional
604 investigations are now required to understand these phenomena. More particularly, it
605 would be interesting to see whether complex physiological islet differentiation into
606 multicellular pancreatic lineages including α , β and δ cells can occur in these 3D
607 spheroid microfluidic cultures.

608

609 **Conclusion**

610

611 In summary, we investigated the behaviors of β -cells derived from hiPSCs in various
612 culture conditions. 2D monolayer cultures generated typical β -cells profiles, as
613 shown by C-peptide production and undetected glucagon secretion. When cultivated
614 in 2D biochips in a monolayer, we did not find any stable protocol making their
615 microfluidic cultures possible. When the cells were cultivated in 3D spheroids, the
616 cells presented higher heterogeneity, as seen in the appearance of α , β and δ -cell
617 markers at the mRNA level, and glucagon positive immunostaining, in addition to the
618 secretion of C-peptide. The 3D spheroids were then successfully cultivated in a 3D
619 biochip under microfluidic conditions. The microfluidic culture established contributed
620 to increasing pancreatic maturation by improving C-peptide and insulin secretion
621 levels. The high level GLUT2 and low level GCK in 3D static spheroids and 3D
622 biochips, when compared to 2D Petri, suggested modulation of glucose metabolism
623 and transport as a potential regulator of pancreatic specification during differentiation
624 into 3D spheroids and 3D biochips. We believe that our results are encouraging for
625 the development of functional pancreas-on-chip in vitro models using the advantages
626 of organ-on-chip technology and hiPS cells, a promising source of cells.

627

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629

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638 **Conflict of interest**

639

640 The authors declare no conflict of interest.

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642 **References**

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Figures Captions

Fig.1. (A) Schematic of differentiation process from hiPSC to beta cells; (B) experimental procedures used for stage 2 of β -cells maturation.

Fig.2. Design and structure of (A) honeycomb and (B) biochips used for hiPSC derived β -cells cultures.

Fig.3. hiPSC derived β -cells cultures in static Petri (monolayer). (A-C) morphologies after 5 h, 12 and 16 days, respectively; (D-G) immunostainings of β -cells at the end of the experiment: DAPI, MAFA, PDX1 and insulin, respectively; (H) ratio of mRNA levels (iPSC derived β -cells/iPSC) of selected genes after 24h and 16 days of culture, $*P < 0.05$ mRNA level significantly different when compared to iPSCs; (I) daily C-peptide secretion.

Fig.4. Morphology of hiPSC derived β -cells cultivated in honeycomb wells and microfluidic biochips. (A) 2D (monolayer) dynamic culture in biochip; (B) 3D (spheroids) static culture in honeycomb wells seeded at low- and high-density of cells; (C) 3D (spheroids) dynamic culture in biochip after 14 days of culture (4 days in static honeycomb and 10 days in biochip).

Fig.5. Immunostainings (end of the experiments) of hiPSC derived β -cells spheroids cultivated in honeycomb wells and biochips (A) DAPI, insulin, glucagon and merge; (B) DAPI, PAFA, PDX1 and merge.

Fig.6. hiPSC derived β -cells spheroids cultivated at high- and low-density in static honeycomb wells. (A) daily c-peptide secretion between day 9 and day 14; (B) daily insulin secretion at day 14 ($*P < 0.05$); (C) ratio of insulin secretion (high/low, GSIS index) after high/low glucose stimulations ($*P < 0.05$, GSIS:

glucose-stimulated insulin secretion); (D) ratio (GLP1/control) of insulin secretion after GLP1 treatment.

Fig.7. Ratio of mRNA levels of selected genes at the end of culture. black bars: β -cells spheroids cultivated at high-density in static honeycomb wells versus β -cells cultures in static Petri (2D monolayer) and gray bars: β -cells spheroids cultivated in dynamic biochip versus β -cells cultures in static Petri (2D monolayer). $*P < 0.05$, mRNA level significantly different when compared to static Petri (2D monolayer).

Fig.8. hiPSC derived β -cells spheroids cultivated in honeycomb wells (high-density) and biochips. (A) daily c-peptide secretion between day 9 and day 14 ($*P < 0.05$); (B) daily insulin secretion at day 14 ($*P < 0.05$); (C) ratio of insulin secretion (high/low, GSIS index) after high/low glucose stimulations (GSIS: glucose-stimulated insulin secretion); (D) ratio (GLP1/control) of insulin secretion after GLP1 treatment.

Figure 1

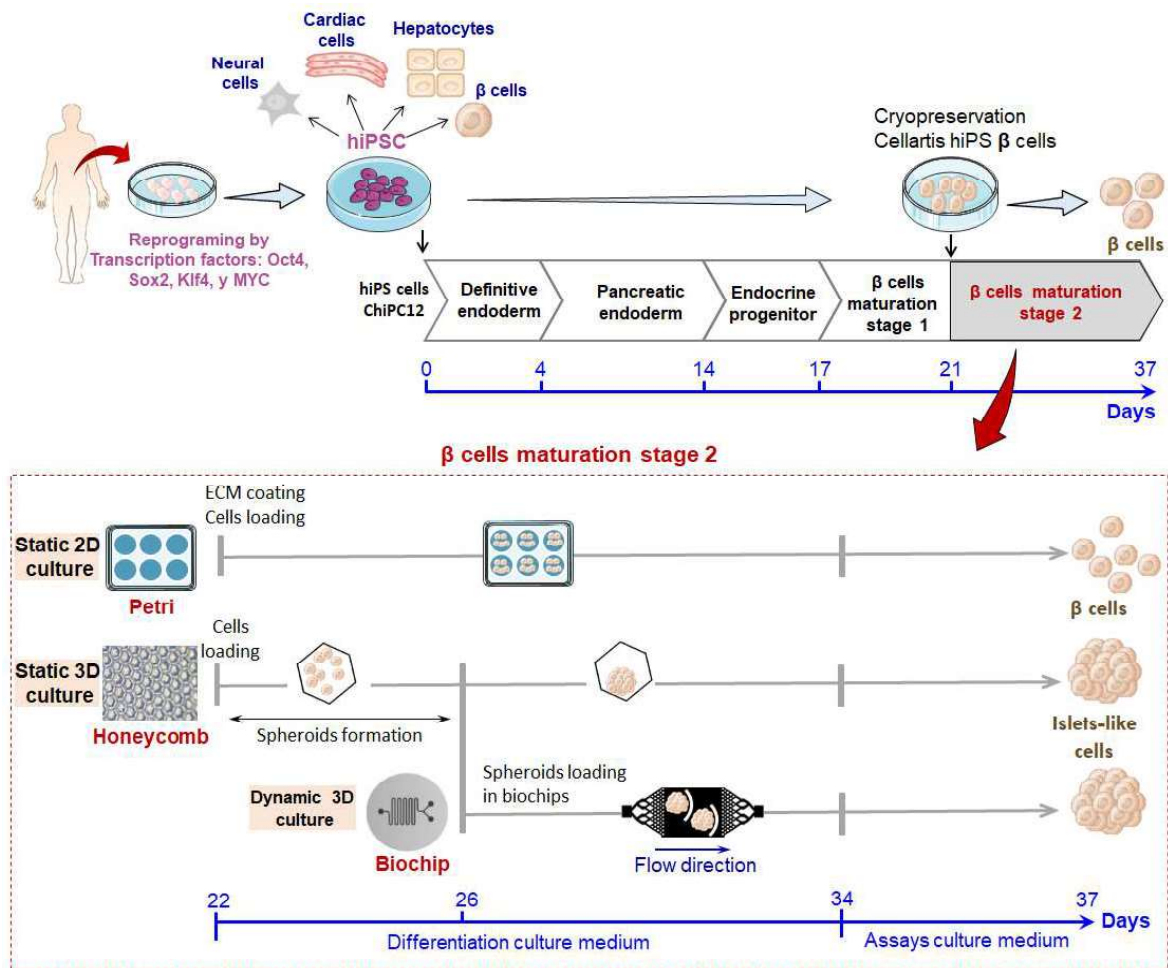


Fig.1

Figure 2

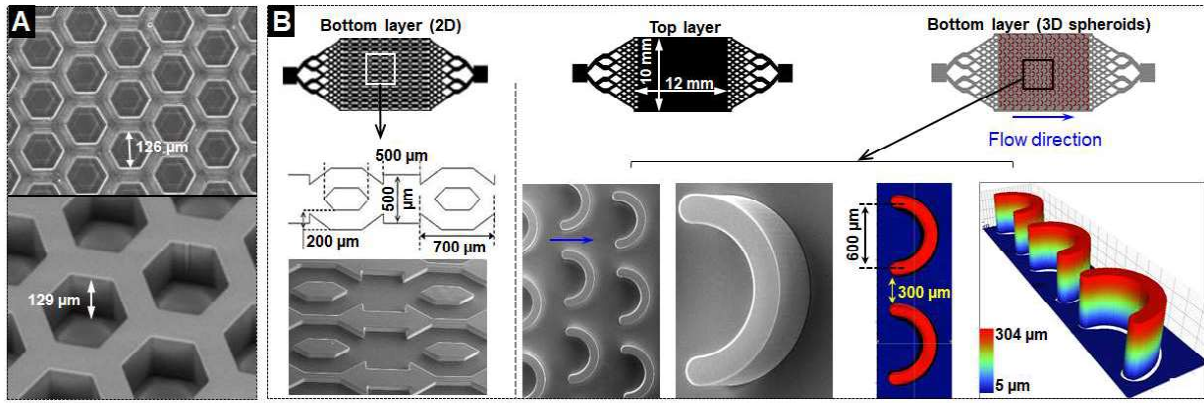


Fig.2

Figure 3

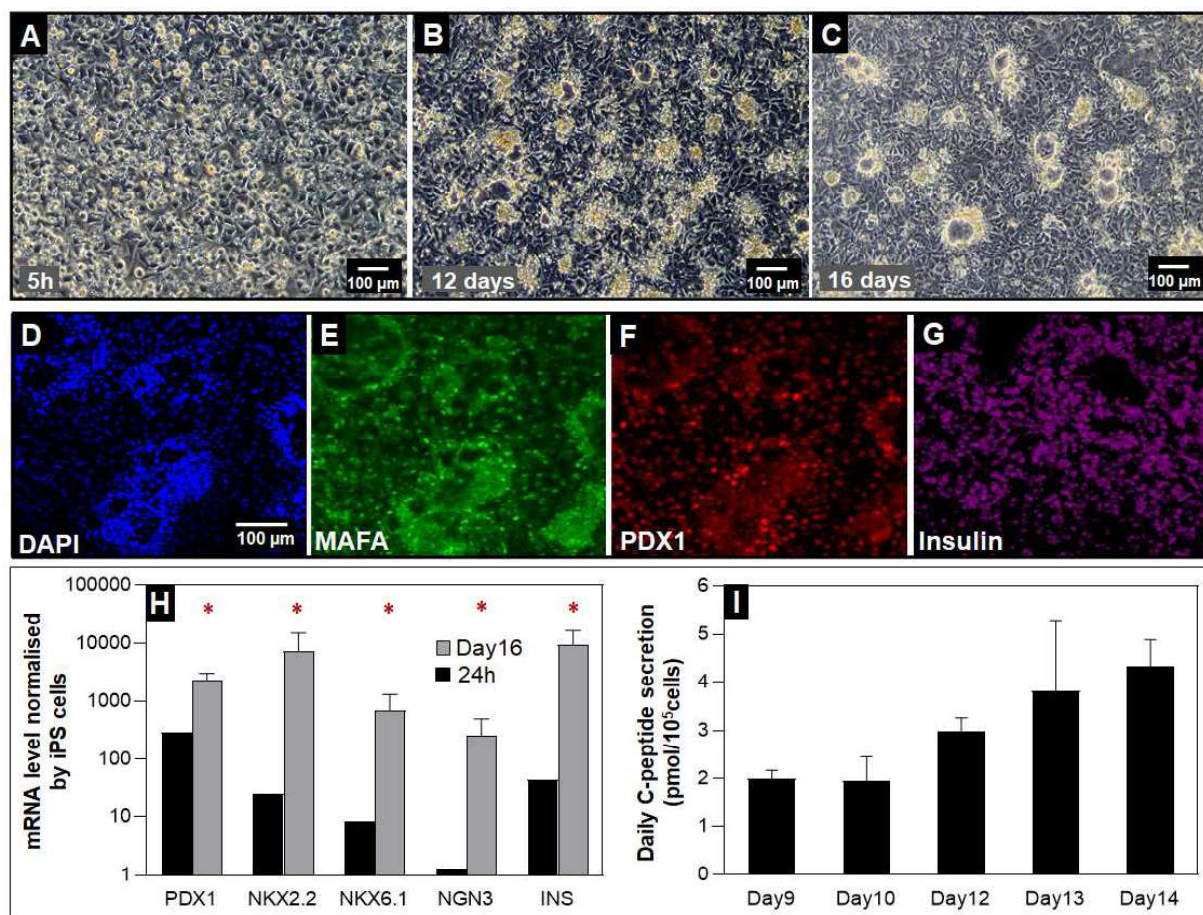


Fig.3

Figure 4

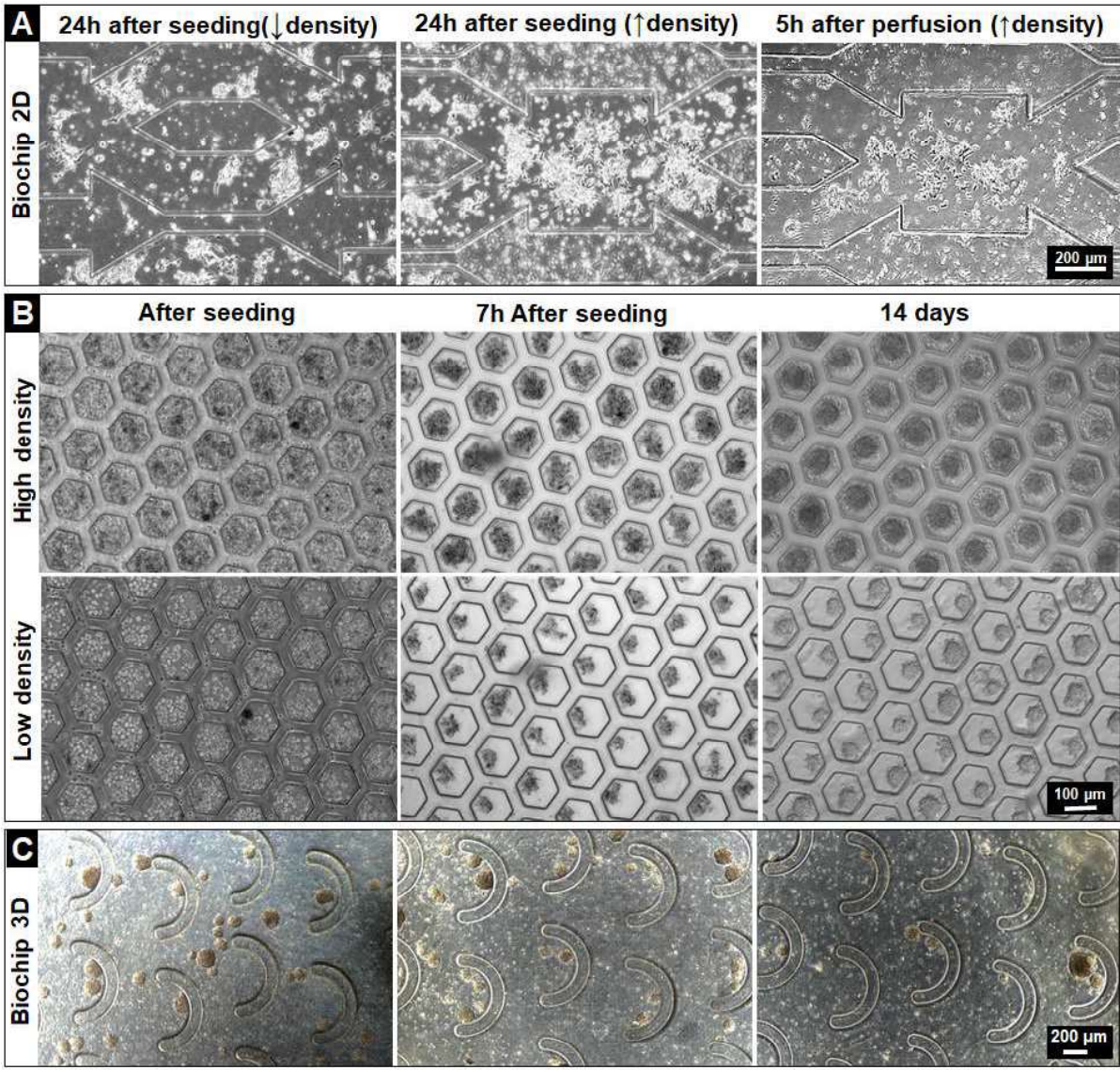


Fig.4

Figure 5

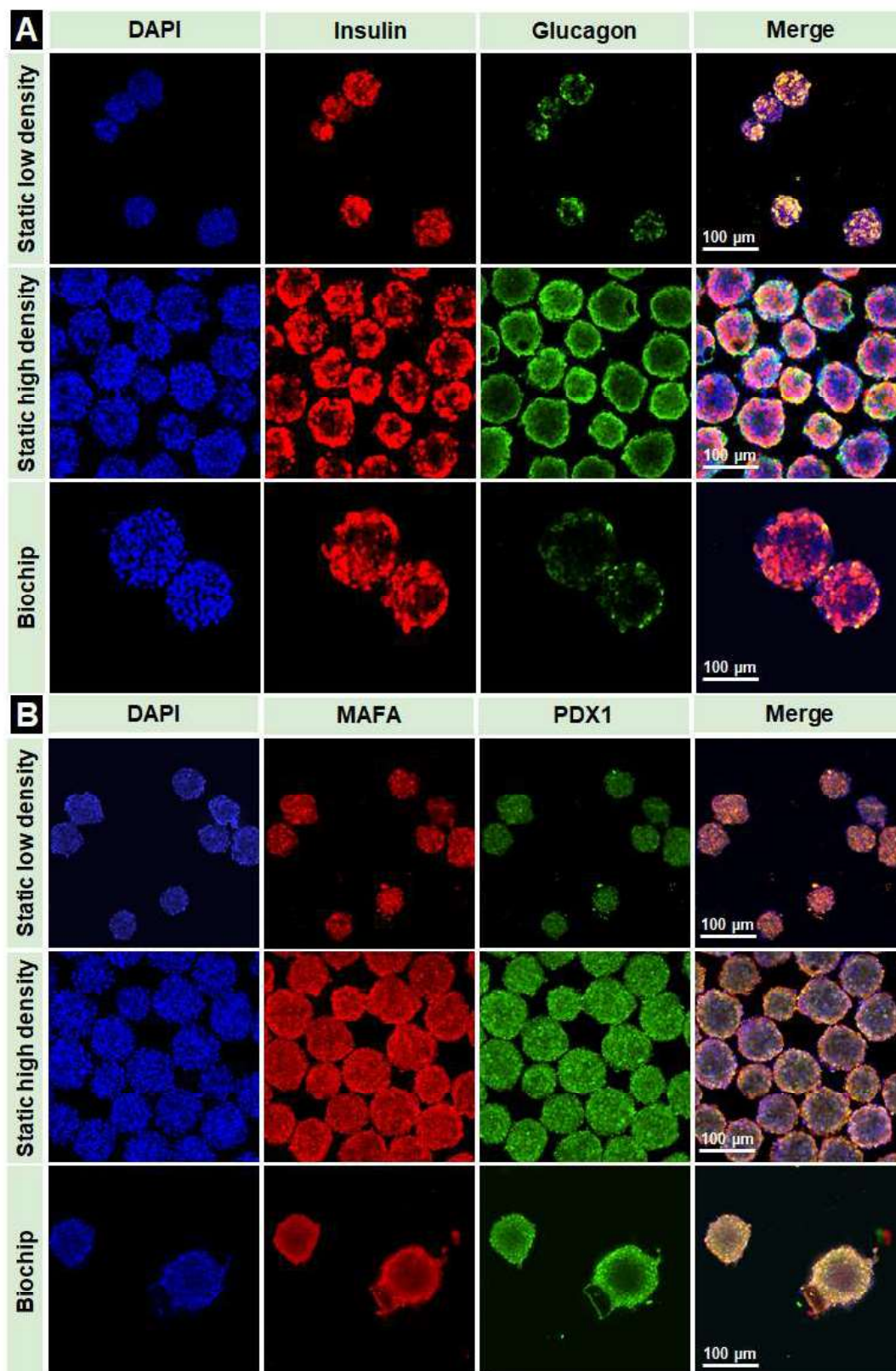


Fig.5

Figure 6

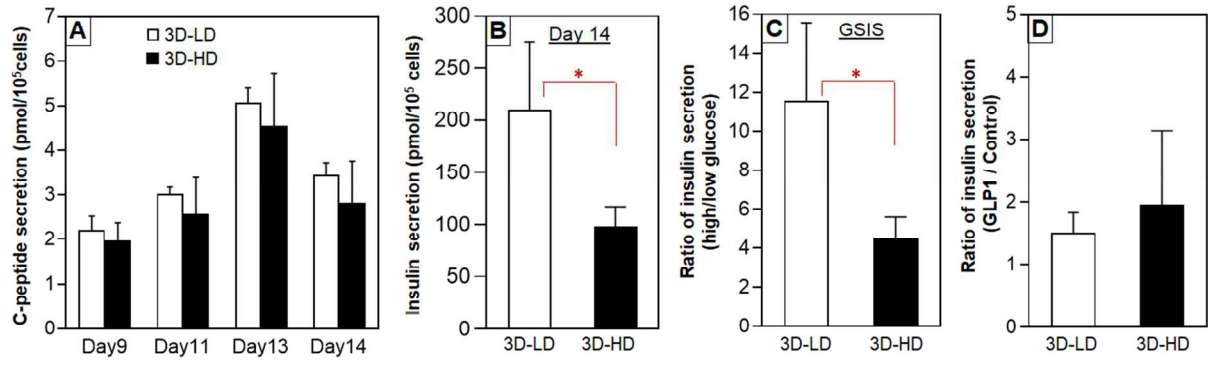


Fig.6

Figure 7

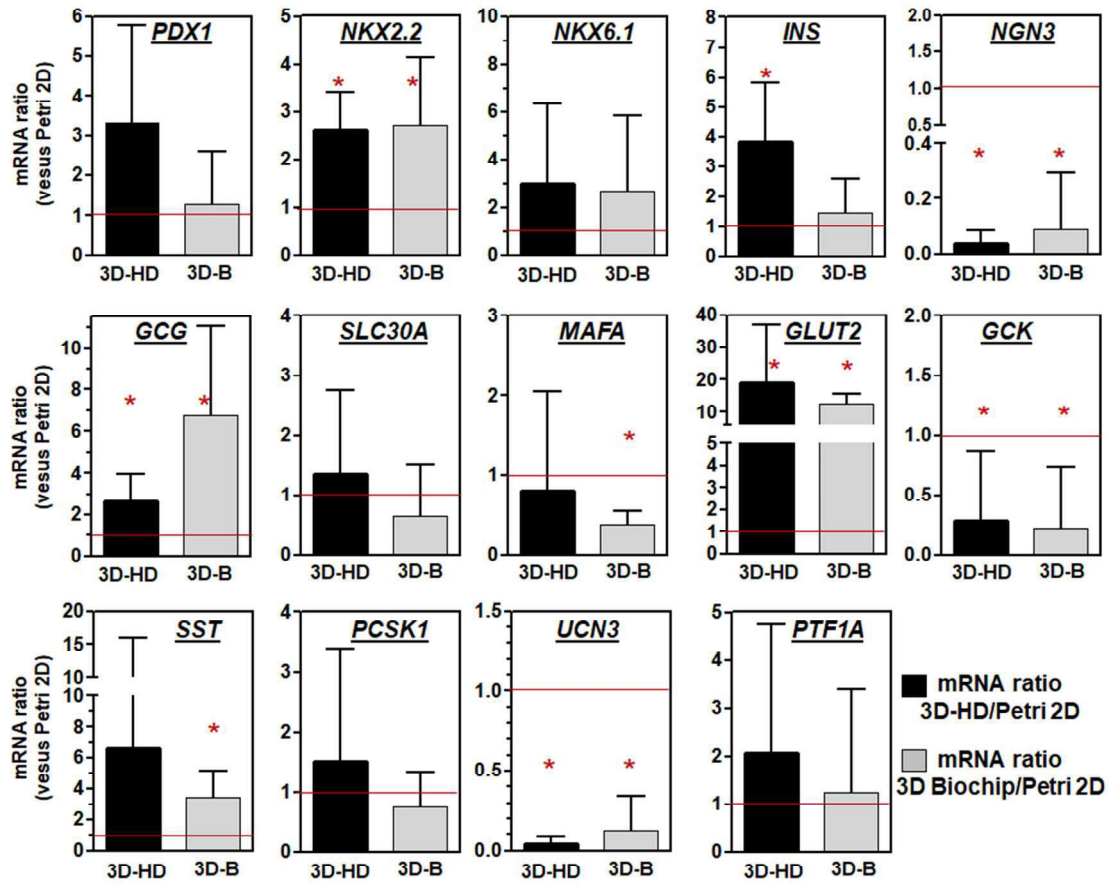


Fig.7

Figure 8

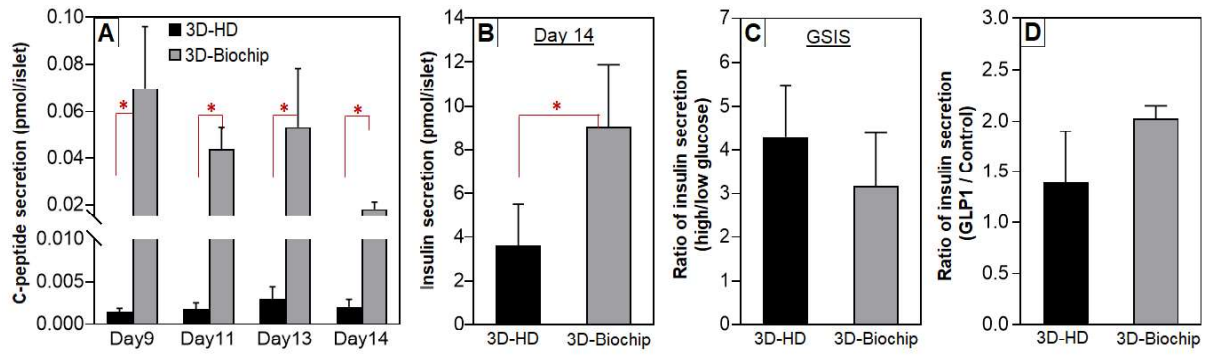


Fig.8