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Differentiation of human induced pluripotent stem cells into testosterone-producing Leydig-like cells

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Abstract

Late-onset hypogonadism (LOH) syndrome due to a partial lack of testosterone, which is mainly secreted by Leydig cells in the testes, decreases the quality of life of older men. Leydig cell transplantation is expected to be a promising alternative to conventional testosterone replacement therapy (TRT) for LOH syndrome. We herein report a simple and robust protocol for directed differentiation of human induced pluripotent stem cells (hiPSCs) into Leydig-like cells by doxycycline-inducible overexpression of NR5A1 and treatment with a combination of 8-bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP) and forskolin. The differentiated cells expressed the steroidogenic enzyme genes StAR, CYP11A1, CYP17A1 and HSD3β2 and the specific markers of adult Leydig cells HSD17B3, INSL3 and LHCGR. Furthermore, we confirmed the secretion of functional testosterone from the cells into the culture supernatant by a testosterone-sensitive cell proliferation assay. These findings showed that the hiPSCs were able to be differentiated into Leydig-like cells, supporting the expectation that hiPSC-derived Leydig-like cells can be novel tools for treating LOH syndrome.

36 **Introduction**

37 Late-onset hypogonadism (LOH) syndrome is caused by a reduction in testicular testosterone
38 production in aging males and is characterized by various symptoms, such as fatigue, decreased
39 libido, erectile dysfunction, depression, anemia, and decreased muscle mass and bone density,
40 leading to a poor quality of life (1,2). In addition, low serum testosterone levels have been
41 reported to be associated with metabolic syndrome and diabetes mellitus (3,4). The decline in
42 testosterone levels in LOH syndrome has been thought to be due to a decrease and dysfunction
43 in Leydig cells in the testes (5), which are the primary source of testosterone in males.

44 LOH syndrome has been treated with testosterone replacement therapy (TRT), which
45 has been shown to ameliorate the symptoms attributed to LOH syndrome (1). TRT is presently
46 available in injectable, oral and ointment form, but the length of effectiveness of a single
47 testosterone administration is limited in these forms, making repeated administrations
48 necessary(6). Injectable testosterone needs to be replenished every two to four weeks, whereas
49 ointment form of testosterone requires fewer hospital visits but everyday application, and the
50 issue of secondary exposure of the coating formulation has been noted. Oral administration
51 requires dosing four times in a day, and users must be alert for various adverse effects(2,6).
52 Furthermore, presently available TRTs do not restore the physiological fluctuation patterns of
53 blood testosterone levels regulated by gonadotropin and gonadotropin-releasing hormone with

54 feedback mechanisms (2). Thus, novel therapeutic approaches as an alternative to TRT for LOH
55 syndrome are required.

56 Leydig cell transplantation may overcome the issues of the present TRTs for patients
57 with LOH syndrome. Once the transplanted Leydig cells engraft, the effectiveness might last
58 the entire life of the recipient, or at least much longer than a few weeks, which is the effective
59 duration of a single injection of a testosterone product. Furthermore, the transplanted Leydig
60 cells might secrete testosterone in a physiological manner. One group previously reported the
61 induction of Leydig-like cells from mouse embryonic stem (ES) cells by the forced expression
62 of NR5A1 (7) and another group reported direct reprogramming of mouse fibroblasts to
63 Leydig-like cells by three transcriptional factors: NR5A1, Dmrt1 and Gata4 (8). A protocol
64 reported by another group for the induction of Leydig cells from human induced pluripotent
65 stem cells (hiPSCs) without the overexpression of NR5A1 or any other factors consists of six
66 steps and it also requires the use of two different mediums with complex compositions, and the
67 resultant cells in the protocol showed only slight production of testosterone (9). Crucially, to
68 our knowledge, nobody has demonstrated the function of testosterone secreted by artificially
69 induced human Leydig cells as a potent transplantation therapy for LOH syndrome.

70 In the present study, we established a simple and robust differentiation protocol into
71 functional testosterone-producing Leydig-like cells from hiPSCs via doxycycline-inducible

72 overexpressing NR5A1 and showed not only marker gene expression but also the secretion of

73 functional testosterone from the resultant cells.

74

75 **Material and methods**

76 *hiPSCs and culture conditions*

77 In the present study, we used four iPSC clones: two male clones (3AB4 (10), 73E1) and two
78 female clones (201B7(11), 46C2-s4 (12)). The 73E1 clone was established from healthy donors'
79 peripheral blood mononuclear cells using CytoTune-iPSC 2.0 (ID Pharma Co., Ltd., Tsukuba,
80 Japan) according to a previously described method (12). The institutional review board of Kobe
81 University Graduate School of Medicine approved this study (No. 1722), and informed consent
82 was obtained from the donor.

83 The 73E1 clone showed typical human ES cell-like morphology (S-Fig. 1A), expressed
84 pluripotent stem cell markers OCT3/4, SOX2 and NANOG at the protein (S-Fig. 1B) and
85 mRNA levels (S-Fig. 1C). Removal of Sendai virus was confirmed by immunostaining (S-Fig.
86 1D) and reverse transcription polymerase chain reaction (RT-PCR) (S-Fig. 1E). An embryoid
87 body (EB)-mediated *in vitro* differentiation experiment showed that 73E1 had the ability to
88 differentiate into cells comprising all three germ layers (S-Fig. 1F). We cultured these iPSCs
89 according to a previously described method (12). In brief, the culture plates were precoated
90 with recombinant laminin-511 E8 fragments (iMatrix-511, Nippi, Tokyo, Japan; 0.5 μg/cm^2),
91 and the iPSCs were cultured in Stem Fit medium (Ajinomoto, Tokyo, Japan) at 37 °C with 5%
92 CO_2. The medium was changed every other day and passaged every seven days using 0.5x
93 TrypLE Select (TrypLE Select diluted 1:1 with 0.5 mM EDTA/PBS (-); Life Technologies,

94 Tokyo, Japan) and 10 μM of Rock inhibitor (Y-27632; WAKO, Osaka, Japan) for one day after
95 passage.
96
97 *Generation of a doxycycline-inducible NR5A1-hiPS cell line*
98 Using Gateway cloning, the NR5A1 cDNA was transferred to the PB-TAC-ERN (KW111)
99 destination vector to yield the transposon PB-TAC-ERN-NR5A1 (13,14). The PB-TAC-ERN-
100 NR5A1 vector was transfected together with PB transferase (PBase) into four independent
101 hiPSC clones using FuGENE HD (Roche, Swiss), according to the manufacturer's instructions.
102 A total of 100 μg/ml G418 (Nacalai Tesque, Kyoto, Japan) was added to media for 10 days to
103 generate NR5A1-hiPSCs containing genomic transposon integrations. The appropriate
104 NR5A1-hiPSC clones with high mCherry expression were selected. We then maintained
105 NR5A1-hiPSCs under the same conditions as the hiPSCs before vector introduction, except for
106 adding 100 μg/ml G418.
107
108 *Differentiation into Leydig-lineage*
109 For EB formation, 3×10^4 NR5A1-iPSCs were allowed to aggregate in maintenance medium
110 (DMEM high glucose [Nacalai Tesque], 15% KSR [Thermo Fisher Scientific, Tokyo, Japan],
111 doxycycline [1 μM; Tocris, Bristol, UK]) and plated into each well of a 96-well nonadherent
112 culture plate (96-well prime surface; Sumitomo Bakelite) for incubation in 5% CO_2 at 37 °C

113 for six days without changing the medium. After six days' culture, four EBs were transferred
114 to a 24-well prime surface (Sumitomo Bakelite) with 500 μl/well differentiation medium
115 consisting of DMEM high glucose (Nacalai Tesque) with 10% FBS (Biowest, Nuaille, France),
116 Dox (1 μM) (Tocris), 8Br-cAMP (1 mM; Nacalai Tesque) and Forskolin (100 μM; abcam,
117 Cambridge, UK). All media were changed every seven days after day 14. Evaluations, such as
118 the marker gene expression and hormone secretion, in differentiated cells were performed on
119 day 21 (Figs. 2 and 3 and S-Figs. 2 and 3). In experiments to examine the duration of
120 testosterone production in Figure 4, the time frame was extended to 49 days.
121
122 *Steroid hormone measurement*
123 The cell culture supernatants were collected at each experimental time point for the quantitative
124 measurement of steroid hormones (testosterone, cortisol and aldosterone). Testosterone and
125 cortisol levels were measured with an electrochemiluminescence immunoassay (ECLIA), and
126 aldosterone levels were measured with an immunoradiometric assay (IMRA).
127
128 *RT-PCR*
129 Total RNA was extracted using TRIzol reagent (Life Technologies) and treated with a Turbo
130 DNA-free kit (Life Technologies) to remove genomic DNA contamination. Total RNA (500
131 ng) was reverse-transcribed to cDNA using a PrimeScript II 1st Strand Synthesis Kit (Takara,

132 Shiga, Japan) with oligo-dT primers according to the manufacturer's instructions. We used
133 water as a negative control for each reaction. The primer sequences of the RT-PCR are listed
134 in Table S1.
135
136 *Histological and immunocytochemical examinations*
137 Tissue sections of the induced Leydig-like cells and the human testis were stained with
138 Hematoxylin and Eosin (H&E). Immunostaining for hiPSCs and EB-mediated differentiated
139 cells was performed as previously described (12). The used primary antibodies included
140 OCT3/4 (BD, 611202, RRID: AB_398736), SOX2 (abcam, ab97959, RRID: AB_2341193),
141 NANOG (R&D Systems, AF1997, RRID: AB_355097), Sendai virus (MBL, PD029, RRID:
142 AB_10597564), β-III-tubulin (Chemicon, MAB1637, RRID: AB_2210524), α-SMA (DAKO,
143 M0851, RRID: AB_2223500) and SOX17 (R&D systems, AF1924, RRID: AB_355060). The
144 secondary antibodies (Alexa 594-conjugated anti-goat, -rabbit, -mouse IgG and Alexa Fluor
145 488-conjugated anti-mouse, anti-goat IgG) were obtained from Life technologies. Hoechst
146 (Life Technologies, 33342, RRID: AB_10626776) was used for nuclear staining.
147 For Figure 2D, differentiated NR5A1-3AB4 cells (Day 21) were coated with alginate
148 acid, fixed with formalin and embedded in paraffin. The paraffin sections were reacted with
149 Tyramide signal amplification (TSA) (Perkin-Elmer, Waltham, MA, USA) and stained with
150 polyclonal rabbit anti-human INSL3 antibody (Sigma-Aldrich, St. Louis, MO, USA; dilution

151 1:500; HAP028615, RRID: AB_2893117) and monoclonal rabbit anti-human STF-1/NR5A1

152 (Cell Signaling Technology, Danvers, MA, USA; dilution 1:500; #12800, RRID: AB_2798030).

153 Nuclei were stained with Hoechst.

154

155 *Western blotting*

156 The cells were lysed with the M-PER Mammalian Protein Extraction Reagent (Thermo Fisher

157 Scientific). The cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel

158 electrophoresis (SDS-PAGE). After the electrophoretic transfer of the proteins to the PVDF

159 membranes, immunoblotting with mouse anti- NR5A1/SF-1 (Cell Signaling Technology) and

160 mouse anti-β-actin (Sigma-Aldrich) followed by horseradish peroxidase (HRP)-conjugated

161 secondary antibodies at a 1:3000 dilution in TBS-T was performed. The LAS 3000 imaging

162 system (Fuji Film, Tokyo, Japan) was used to detect signals.

163

164 *Testosterone-sensitive cell proliferation assay*

165 LNCaP cells and PC3 cells, which are known to be testosterone-sensitive and testosterone-

166 insensitive, respectively (15), were seeded at a density of 3,000 cells per well into 96-well

167 culture plates in 3 kinds of medium. The first type was "supernatant of doxycycline (+)

168 differentiated cells", which contained 5 ng/ml of testosterone secreted from hiPSC-derived

169 Leydig cells. To prepare this medium, we measured the concentration of testosterone in the

170 supernatant of doxycycline (+) differentiated cells and added the supernatant to RPMI
171 containing 10% fetal bovine serum (FBS) to achieve a final testosterone concentration of 5
172 ng/ml. The second type was “medium + T”, which was RPMI containing 10% FBS with
173 commercially available synthetic testosterone (Sigma Aldrich) added to achieve a final
174 concentration of 5 ng/ml. The third type was “medium”, which was just RPMI containing 10%
175 FBS. The FBS used in this study originally contained a low level of testosterone, so even
176 “medium” contained 0.12-0.15 ng/ml of testosterone.

177 Three days later, we assessed the cell numbers using Cell Titer-Glo (Promega, Madison,
178 WI, USA). For the assay, we equilibrated the plate and its contents at room temperature for
179 approximately 30 minutes, added 100 µl Cell Titer-Glo per well to 96-well plates, and mixed
180 the contents for 2 minutes with an orbital shaker to induce cell lysis. We then allowed the plate
181 to incubate at room temperature for 10 minutes to stabilize the luminescent signal.

182

183 **Results**

184 *Generation of hiPSC lines expressing NR5A1 in a doxycycline-dependent manner*

185 To examine whether or not Leydig cells could be induced from hiPSCs via the forced
186 expression of NR5A1, we constructed the vector PB-TAC-ERN NR5A1, which expresses
187 NR5A1 in a doxycycline-dependent manner (Fig. 1A), and introduced it into four iPSC lines:
188 two male lines (3AB4, 73E1) and two female lines (201B7 and 46C2-s4). We established four
189 doxycycline-inducible NR5A1-hiPSC lines showing human ES cell-like morphologies (Fig.
190 1B and S-Fig. 2A) and expressing undifferentiated markers (OCT3/4, SOX2 and NANOG),
191 similarly to the parental iPSC line, which had not been transduced with the vector (Fig. 1C and
192 S-Fig. 2B). Most of the iPSC colonies became mCherry-positive, indicating exogenous NR5A1
193 expression, when doxycycline (1 μM) was added for one day (Fig. 1D and S-Fig. 2C), and the
194 expression of NR5A1 was confirmed at the mRNA level (Fig. 1E and S-Fig. 2D) and protein
195 level (Fig. 1F and S-Fig. 2E) by RT-PCR and Western blotting, respectively. We used HepG2
196 cell line as positive control, as these cells are known to highly express NR5A1 (16).

197

198 *Testosterone-producing Leydig-like cells were induced only from iPSCs that forcibly expressed*
199 *NR5A1*

200 To differentiate hiPSCs into Leydig cell lineage, we first established embryoid bodies (EBs)
201 and then performed suspension culture with 8-bromoadenosine-3',5'-cyclic monophosphate

202 (8-Br-cAMP) and Forskolin (FSK), which were reported to be effective for Leydig cell
203 induction in mice (7) with or without the overexpression of NR5A1 (Fig. 2A). On day 6,
204 mCherry-positive EBs were formed only when doxycycline was added (Fig. 2B left panels).
205 Next, four EBs were transferred to a 24-well low-attachment plate, and suspension culture was
206 performed with 8-Br-cAMP and FSK for 15 days. The medium was changed at day 14.
207 Consequently, the EBs gathered and formed a cohesive assembly in the culture with
208 doxycycline on day 21 (Fig. 2B right upper panel). In contrast, in the culture without
209 doxycycline, the EBs collapsed without aggregation (Fig. 2B right lower panel).

210 To examine the expression of Leydig cell marker genes in induced cells with
211 doxycycline on day 21, we performed semi-quantitative RT-PCR (Fig. 2C). The results showed
212 that doxycycline (+)-induced cells expressed not only the steroidogenic enzyme genes STAR
213 (17), CYP11A1, CYP17A1 and HSD3B2 (13), which are necessary for synthesizing steroid
214 hormones from cholesterol (18,19), but also the specific marker genes of adult Leydig cells:
215 HSD17B3 (9), INSL3 (9,20,21) and LHCGR (9). The protein expression of NR5A1 and INSL3
216 was confirmed by immunohistochemistry (Fig. 2D). Induced Leydig-like cells showed round
217 nuclei, nucleoli within the nuclei, and aggregated chromatin at the margins of the nucleus, and
218 the cytoplasm is stained eosinophilic. These findings were similar to those of authentic Leydig
219 cells in human testes (Fig. 2E). In addition, the secretion of testosterone was observed in the
220 supernatant only when doxycycline was added (Fig. 2F). A small amount of testosterone in

221 FBS was also detected in the medium used as a negative control (0.12-0.15 ng/ml), but since
222 there was 50- to 460-fold difference from the level of testosterone secreted from Leydig-like
223 cells into the culture supernatant, we regarded it as having a negligible effect on our study and
224 decided to use the same FBS.

225 To examine whether or not testosterone produced from the induced Leydig-like cells
226 was functional, we assessed the effect of the testosterone on the proliferation of two prostate
227 cancer cell lines: LNCaP cells and PC3, which are known to be testosterone-sensitive and
228 testosterone-insensitive, respectively (15, 22). The results showed that testosterone produced
229 by induced Leydig-like cells had the ability to increase the number of LNCaP cells just as much
230 as commercially available synthetic testosterone of the same concentration (Fig. 2G left
231 panel).In contrast, when PC3 cells were cultured using the same protocol, only a slight effect
232 of the supernatant, probably due to some proliferation-promoting factors other than
233 testosterone, was noted (Fig. 2G right panel). As the magnitude of the proliferation-promoting
234 effect of the supernatant on LNCap was larger than PC3, the proliferation-promoting effect of
235 the culture supernatant on LNCaP may be mainly attributed to testosterone. These data
236 therefore indicated that the testosterone secreted by the iPSC-derived Leydig-like cells was
237 functional.

238

239

240 *Adrenal steroid hormone-producing cells were concurrently induced*

241 As NR5A1 is known to also be involved in the differentiation into adrenal steroid hormone-
242 producing cells (23), we examined the expression of adrenal cortex marker genes by RT-PCR
243 for the purpose of investigating the differentiation of adrenal cortex cells in iPSC-derived cells
244 which had been induced to differentiate by the Leydig cell lineage differentiation protocol. We
245 observed the expression of CYP21A2, CYP11B1 and CYP11B2, which are the adrenal cortex-
246 specific steroid hormones metabolizing enzymes for producing aldosterone and cortisol (24),
247 as well as ACTH receptor gene (ACTHR) (S-Fig. 3A). The concentrations of aldosterone and
248 cortisol in the culture supernatant were higher than those in the fresh medium, indicating the
249 production of these hormones by the iPSC-derivatives (S-Fig. 3B). Taken together, our present
250 differentiation protocol for Leydig cells induced not only Leydig cells but also cortex cells
251 (aldosterone- and cortisol-producing cells).

252

253 *Leydig-like cells were not induced from female iPSCs*

254 Leydig cells exist in male but not in female bodies. We therefore examined whether or not
255 Leydig-like cells could be induced from female-derived iPSCs under the same conditions
256 where Leydig-like cells were induced from male-derived iPSCs. At day 6, EBs formed from
257 both female and male iPSCs with no obvious differences (Fig. 2B left panel, S-Fig. 4A upper
258 panels). However, after additional induction with two molecules for 15 days, the shape of the

259 EB aggregates tended to break up a little when created from female iPSCs, especially in
260 NR5A1-201B7 (S-Fig. 4A lower panel). In the culture supernatant, male iPSC-derived cells
261 (NR5A1-3AB4 and NR5A1-73E1) showed higher testosterone levels (minimum concentration
262 of 4.26 ng/ml) than female iPSC-derived cells (NR5A1-46C2-s4 and NR5A1-201B7;
263 maximum concentration of only 3.9 ng/ml) (Fig. 3A). A semiquantitative RT-PCR analysis
264 showed the expression of steroidogenic genes (STAR, CYP11A1, CYP17A1, HSD3B2) and
265 LHCGR in cells differentiated from both male and female iPSCs (Fig. 3B). In contrast,
266 HSD17B3 and INSL3, specific marker genes of adult Leydig cells, were expressed only in the
267 cells derived from male iPSCs (Fig. 3B).

268 Regarding female steroid hormone-producing cells, one report showed that the forced
269 expression of NR5A1 in female skin, skeletal muscle, fat and other somatic stem cells induced
270 differentiation into theca cells, granulosa cells and corpus luteum, which comprise the ovary
271 (Japanese Patent Publication No. 2007071416). Theca cells, which are testosterone-secreting
272 cells in women, were reported to express INSL3 (25), but the expression of INSL3 was not
273 detected in the female iPSC-derived cells in this study (Fig. 3B), suggesting that theca cells
274 were not induced in the present study.

275 Similarly to male iPSC-derived cells, female iPSC-derived cells expressed adrenal
276 cortex marker genes (Fig. 3B). Cortisol and aldosterone were observed in the supernatant of
277 the female iPSC-derived cells (S-Fig. 4B).

278 Taken together, these findings show that Leydig-like cells were not induced from
279 female iPSCs, and the slight testosterone secretion noted might have been derived from adrenal
280 cortex-like cells that unintentionally arose with our differentiation procedure.
281
282 *Induced Leydig-like cells can secrete testosterone for at least four weeks*
283 Finally, to examine how long testosterone secretion is maintained in the Leydig-like cells
284 induced by our protocol, the concentration of testosterone in the culture supernatant was
285 measured every other week from day 21 to day 49 after the start of doxycycline addition to
286 NR5A1-3AB4. While the peak testosterone levels varied among experiments up to day 35, the
287 levels gradually decreased after day 35 in all six experiments (Figure 4A). The testosterone
288 levels on day 49 ranged from 4.04 to 28.2 ng/ml, and these values were higher than those
289 detected when female-derived iPSCs were cultured in our Leydig-cell differentiation induction
290 protocol (0.2-3.63 ng/ml, shown in Figure 3A). Based on these results, Leydig-like cells
291 induced by our protocol seem able to continue to produce testosterone for at least 4 weeks
292 (from day 21 to day 49), although the testosterone levels at days 42 and 49 were significantly
293 lower than those at day 21 (Fig. 4B).

294

295

296 **Discussion**

297 We succeeded in differentiating human iPSCs into Leydig-like cells with a simple protocol by
298 expressing NR5A1. Since our experimental system regulates the expression of NR5A1 in a
299 doxycycline-dependent manner, NR5A1-iPSCs can be maintained and expanded and
300 cryopreserved in the absence of doxycycline, and differentiation can be started promptly at any
301 time by adding doxycycline. Our protocol consists of two steps with basic medium, FBS, KSR
302 and two molecular compounds and requires medium be changed just once a week. In contrast,
303 the protocol reported by Chen et al. (9) is more complex than the present protocol; namely, it
304 consists of six steps using a basic medium, BSA, FBS, HS and 13 molecular compounds and
305 it also requires that the medium be changed every two days. In addition, whereas Leydig-
306 lineage differentiated cells from stem cells in some previous reports (7,26,27) required the *in*
307 *vivo* testis environment after transplantation for maturation to testosterone-producing cells, we
308 were able to create cells capable of producing testosterone *in vitro*. Furthermore, we
309 differentiated Leydig cells from two iPSC lines in this study, and both showed the expression
310 of Leydig cell markers and secretion of testosterone. Previous reports about Leydig-like cell
311 differentiation from mouse ESCs by Yang et al. (8) and from hiPSCs by Chen et al. (9) showed
312 experiments using only one cell line, and reproducibility of the differentiation processes was

313 not clarified. We repeated experiments multiple times and showed lack of consistency in
314 testosterone levels from experiment to experiment. The present results of multiple experiments
315 shown here demonstrated the robustness of our protocol and its issues to be addressed.

316 The differentiated Leydig-like cells were able to continue to secrete testosterone for
317 four weeks after the end of differentiation (Fig. 4A). In previous reports, testosterone levels in
318 the culture supernatant of Leydig-like cells from mouse ESCs, mesenchymal stem cells (MSCs),
319 mouse fibroblasts and hiPSCs had only been measured at one time point: at day 8 (7), day 7
320 (28), day 10 (8) and day 30 (9), respectively. This is therefore the first report showing
321 testosterone production for a long time after the completion of the differentiation processes.
322 The induced Leydig-like cells generated in this study showed a gradually reduced testosterone
323 production in long-term culture and seemed to not grow according to morphological
324 observation (data not shown). Our current technology cannot determine whether the amount of
325 testosterone secretion by individual cells decreased over time or the number of cells decreased.
326 Consistently, Chen et al. reported that the hiPSC-derived cells gradually withered after day 25,
327 and Yang et al. also mentioned that their mouse ESCs-derived Leydig-like cells did not
328 proliferate (7). Long term culture of not only these cells artificially induced *in vitro* but also
329 primary Leydig cells has been difficult, and there is no report of the establishment of any
330 protocol for long-term culture of primary human Leydig cells, to our knowledge. Future studies

331 should search for novel culture conditions that will enable us to maintain and expand Leydig
332 cells in order to realize Leydig cell transplantation therapy.

333 Another issue to address is the lack of consistency in testosterone levels from
334 experiment to experiment. We should identify and control factors that cause inconsistency in
335 the differentiation process in order to establish more robust differentiation protocols for the
336 generation of Leydig cells in future studies. Furthermore, we were unable to observe
337 responsiveness of testosterone secretion by iPSC-derived Leydig-like cells following LH
338 treatment in this study. LH responsiveness is an essential attribute of cells for ensuring
339 testosterone secretion in a physiological manner when cells are transplanted into patients. We
340 need to perform pre-clinical transplantation experiments, such as the transplantation of iPSC-
341 derived Leydig-like cells into immune-deficient animals, or the transplantation of cells
342 encapsulated in alginate into immune-competent animals in order to evaluate the engraftment
343 and function of the cells *in vivo*. Alternatively, we should transplant the resultant cells into
344 mouse testis from which Leydig cells have been ablated by administering ethane
345 diethylstilbestrol (DES) and assess whether or not the cells can colonize the testis and sustain
346 a proper steroidogenic function.

347 In the present study, the resultant cell cluster of our experiments included both Leydig
348 cells that produced testosterone and adrenal cortex cells that produced cortisol and aldosterone.
349 In previous studies, steroid hormone (cortisol and aldosterone)-producing cells were

350 successfully induced from MSCs (28-30), ESCs (31,32) and hiPSCs (23). Previous reports
351 concerning the successful induction of Leydig cells (7-9,33) made no mention of whether or
352 not adrenal cells were concurrently induced. Since NR5A1 is expressed in intermediate
353 mesoderm-derived cells that form genital ridges and play a central role in the development of
354 the adrenal glands and gonads, it is reasonable that the forced expression of NR5A1 induces
355 both the adrenal glands and gonads (34-37). How component cells of a genital ridge
356 differentiate into Leydig cells and adrenal cells during the differentiation process *in vitro* as
357 well as the developmental process *in vivo* remains unclear, and the differentiation induction
358 methods specific to Leydig cells and adrenal cells have yet to be established. A time-course
359 analysis of a single-cell transcriptome during our Leydig-lineage differentiation system might
360 have enabled us to identify specific markers upregulated in the early stages and reveal the
361 molecular mechanisms underlying Leydig cell specification.

362 In cell transplantation therapy, immune rejection and tumorigenicity are key issues. To
363 our knowledge, no report has yet described the transplantation of iPSC-derived Leydig-cells
364 into humans, although rats have reportedly undergone transplantation of human iPSC-derived
365 Leydig cells (9) and mouse ES-derived Leydig precursor cells (7), and engraftment of the cells
366 in the testes has been shown. However, these reports did not mention teratoma formation. Since
367 the testis is an immune privileged site, transplantation into the testis can be a rational strategy
368 to overcome immune rejection, but it raises some concerns about tumorigenesis. Therefore, as

369 is done for islet transplantation (38), encapsulating the iPSC-derived Leydig cells in a
370 semipermeable device and then transplanting them into either the subcutaneous or peritoneal
371 space may be a viable strategy for protecting cells from the recipient's immune system and,
372 moreover, for preventing invasion and metastasis in cases in which oncogenic transformation
373 of the cells occurs. In the present study, we successfully generated functional testosterone-
374 producing Leydig-like cells from hiPSCs. Several issues mentioned above remain at present;
375 however, if they can be resolved, Leydig-like cells produced from iPSCs may be able to be
376 applied to cell transplantation therapy for LOH syndrome.
377

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509

510 **Figure legends**

511 **Figure 1. Generation of hiPSC lines expressing NR5A1 in a doxycycline-dependent**

512 **manner.**

513 A) Construction of the doxycycline-inducible NR5A1 expressing piggyBac vector.

514 Expression of NR5A1 in response to doxycycline was indirectly monitored by co-incident

515 mCherry expression.

516 B) iPSCs (NR5A1-3AB4) expressing NR5A1 in a doxycycline-dependent manner maintained

517 an undifferentiated morphology in the absence of doxycycline. The scale bars are shown

518 in the images.

519 C) Expression of undifferentiated markers (OCT3/4, SOX2 and NANOG) was examined by

520 RT-PCR in NR5A1-3AB4 and its parental iPSC line 3AB4. GAPDH was used as a loading

521 control. Water was used as a negative control.

522 D) When NR5A1-3AB4 was treated with doxycycline (1 ng/ml), mCherry-positive cells were

523 observed (left panels). Scale bar; 200 μm

524 E) Expression of NR5A1 mRNA in NR5A1-3AB4 were examined by RT-PCR with or

525 without treatment of doxycycline (1 ng/ml) for one day. HepG2 was used as a positive

526 control of NR5A1 expression (16).

527 F) Expression of NR5A1 protein in NR5A1-3AB4 were examined by Western Blot with or
528 without treatment of doxycycline (1 ng/ml) for one day. β-actin was used as an internal
529 control.
530
531 **Figure 2. Differentiation induction of NR5A1-hiPSCs into Leydig cell lineage.**
532 A) Schematic diagram of the induction of NR5A1-hiPSCs into Leydig cell lineage. To
533 overexpress NR5A1 continuously, 1 ng/ml doxycycline was added throughout the period.
534 Medium was changed at the timing indicated by black arrows.
535 B) Cell morphologies of EB on day 6 (left panels) and on day 21 (right panels) were shown.
536 Under doxycycline (+) conditions, the EBs were positive for mCherry, and the
537 differentiated cells aggregated on day 21, whereas under doxycycline (-) conditions, the
538 EBs were mCherry-negative, and the differentiated cells did not aggregate.
539 C) Semi-quantitative RT-PCR showed that doxycycline (+) differentiated NR5A1-3AB4
540 expressed NR5A1, steroid hormone-metabolizing genes (STAR, CYP11A1, CYP17A1
541 and HSD3B2) and adult Leydig cell marker genes (HSD17B3, INSL3 and LHCGR). Testis
542 and adrenal RNA were used as positive controls for the markers genes.
543 D) Immunohistochemistry indicated that NR5A1-3AB4 differentiated with doxycycline (+)
544 expressed NR5A1 and INSL3 at day 35. White arrows indicate INSL3 and NR5A1 double-
545 positive cells. Scale bar: 50 μm

546 E) HE staining of differentiated NR5A1-3AB4 cells with doxycycline (+) (left panel) and
547 human testis tissue (right panel)
548 F) Testosterone was secreted into the culture supernatant only in the differentiated NR5A1-
549 3AB4 cells with doxycycline treatment. Data of six independent differentiation
550 experiments were shown. Statistical analyses were evaluated using an unpaired Student's
551 *t*-test. $p<0.05$ was considered statistically significant *($p<0.05$).
552 G) Culture supernatant of doxycycline (+) differentiated NR5A1-3AB4 enhanced LNCaP
553 cells proliferation just as much as commercially available synthetic testosterone of the same
554 concentration (T) (left panel), and only slightly enhanced PC3 cells proliferation (right
555 panel). Cell proliferation was examined using Cell Titer-Glo® in three independent
556 differentiation induction experiments. Statistical analyses were performed using an
557 Student's t-test. $p<0.05$ was considered statistically significant. *($p < 0.05$); n.s.: not
558 significant
559 **Figure 3. Only the cells differentiated from male-derived NR5A1-hiPSCs with**
560 **doxycycline secreted testosterone and expressed ALC markers**
561 A) Testosterone levels were measured in culture supernatants of doxycycline (+)-
562 differentiated cells from male-derived NR5A1-hiPSCs (3AB4 shown in blue, number of
563 independent differentiation experiments: n=37; 73E1 shown in red, n=7) and female-
564 derived NR5A1-hiPSCs (201B7 shown in yellow: n=7, 46C2 shown in grey: n=3).

565 B) Expression of steroidogenic genes (STAR, CYP11A1, CYP17A1 and HSD3B2), adult
566 Leydig cell marker genes (HSD17B3, INSL3 and LHCGR) and adrenal cell marker genes
567 (CYP21A2, CYP11B1, CYP21B2, ACTHR) were examined in two doxycycline (+)-
568 differentiated female NR5A1-hiPSCs (201B7, 46C2-s4) and two male NR5A1-hiPSCs
569 (73E1, 3AB4) by semi-quantitative RT-PCR. GAPDH was used as an internal control.
570
571 **Figure 4. Time course of testosterone secretion in doxycycline (+)-differentiated NR5A1-**
572 **3AB4 cells.**
573 (A) The concentration of testosterone in the culture supernatant was measured from Day 21 to
574 Day 49 in six independent experiments.
575 (B) Mean ± SD of the testosterone concentration at each type point. T-test; *$p<0.05$; n.s.: not
576 significant.
577

578 **S-Figure 1. The characterization of newly established hiPSC line 73E1.**

579 A) Phase contrast images of hiPSC line 73E1. The scale bars are shown in the images.

580 B) Immunofluorescent staining of the hES cell markers OCT3/4 (green, upper panels), SOX2

581 (red, middle panels) and NANOG (red, lower panels) in 73E1. Nuclei were stained with

582 Hoechst 33342 (blue). Scale bars, 100 μm

583 C) Expression of undifferentiated markers (OCT3/4, SOX2 and NANOG) was examined by

584 RT-PCR in 73E1 and the conventional hiPSC clone 201B7.

585 D) & E) Removal of Sendai virus (SeV) vector from 73E1 clone was checked by

586 immunofluorescence (D, scale bars: 100 μm) and RT-PCR (E). 46A1 is a SeV (+) hiPSC

587 clone (12), and 201B7 is a SeV (-) clone reprogrammed using retroviral vectors.

588 F) *In vitro* differentiation via embryoid body formation of 73E1. The images show

589 immunofluorescent staining for β-III-tubulin (ectodermal marker, shown in upper panels),

590 α-smooth muscle actin (α-SMA; mesodermal marker, shown in middle panels) and SOX17

591 (endodermal marker, shown in lower panels). The nuclei were stained with Hoechst 33342

592 (blue). Scale bars, 50 μm.

593

594 **S-Figure 2. Established NR5A1-hiPSCs expressed NR5A1 in response to doxycycline.**

595 A) Phase contrast images of hiPSC lines: NR5A1-201B7, NR5A1-46C2-s4 and NR5A1-

596 73E1. The scale bars are shown in the images.

597 B) Expression of undifferentiated markers (OCT3/4, SOX2 and NANOG) was examined

598 by RT-PCR in NR5A1-hiPSCs before treatment with doxycycline.

599 C) NR5A1-hiPSCs became mCherry-positive 1 day after 1 ng/ml Dox was add at day 6

600 (upper panels), whereas no mCherry-positive cells were seen without doxycycline

601 (lower panels).

602 D) & E) Upregulation of NR5A1 was confirmed in NR5A1-iPSCs with or without

603 doxycycline (1 ng/ml) treatment for 24 h by RT-PCR (C) and Western blotting (D).

604 GAPDH and β-actin were used as internal controls in (C) and (D), respectively. The

605 HepG2 hepatocarcinoma cell line was used as a positive control for NR5A1 expression

606 (16).

607 **S-Figure 3. Immunohistochemistry of INSL3 at the Leydig cells of human testis.**

608 Human Leydig cells in the testis expressed INSL3, indicating that the antibody worked.

609 We used human testis tissue as a positive control and staining with isotype IgG of human

610 testis tissue as a negative control for INSL3 staining. Scale bar: 50 μm.

611

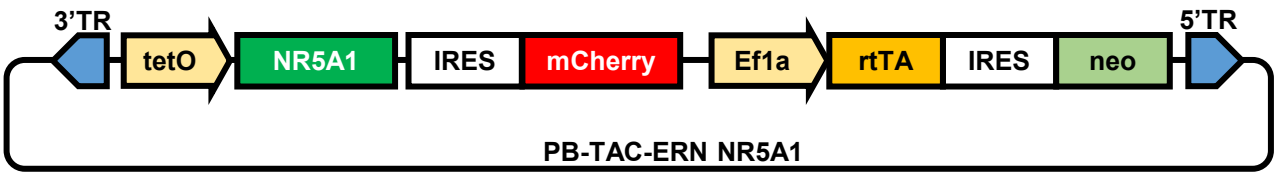
612 **S-Figure 4. Doxycycline (+)-differentiated NR5A1-3AB4 cells included the cells that have**

613 **differentiated into the adrenal lineage**

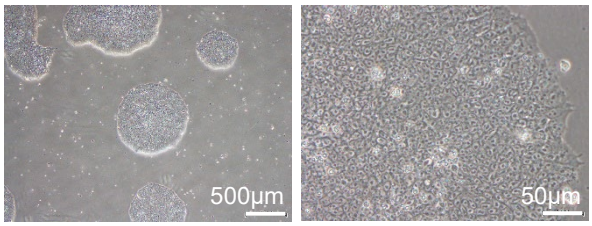
614 A) RT-PCR data showed that the differentiated NR5A1-3AB4 cells with doxycycline (at Day
615 21) included cells expressing adrenal marker genes (CYP21A1, CYP11B1, CYP11B2 and
616 ACTHR). GAPDH was used as an internal control.
617 B) Differentiated NR5A1-3AB4 cells with doxycycline (at Day 21) secreted cortisol and
618 aldosterone in culture supernatant.
619
620 **S-Figure 5. The differences between differentiated male and female iPSCs**
621 A) Morphologies of male-iPSC differentiated cells (NR5A1-73E1) and female-iPSC-
622 derived differentiated cells (NR5A1-201B7 and NR5A1-46C2-s4) with doxycycline at
623 Day 6 (upper panels) and Day 21 (lower panels).
624 B) Doxycycline (+)-differentiated NR5A1-iPSC-derived cells (73E1, 46C2-4s and 201B7)
625 secreted aldosterone and cortisol into the culture supernatant. Fresh medium was used
626 as a control. As the medium was composed of FBS, which originally contains
627 aldosterone and cortisol, a small amount of aldosterone and cortisol were detected in the
628 control sample.
629

Figure 1

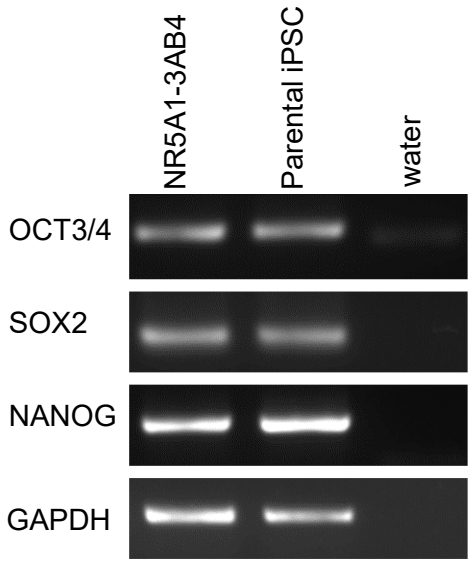
A



B



C



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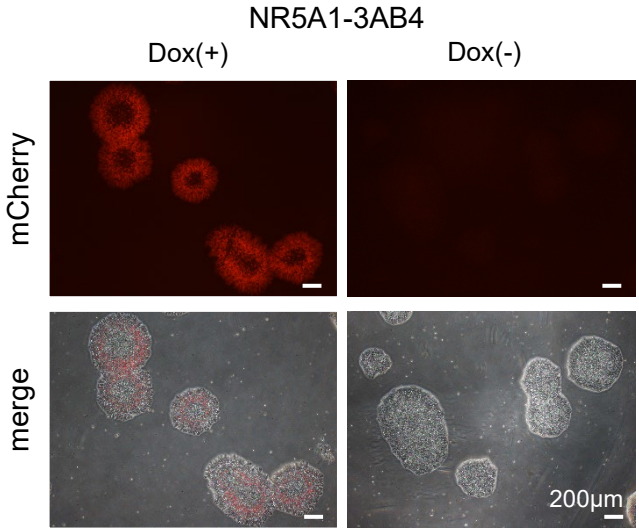
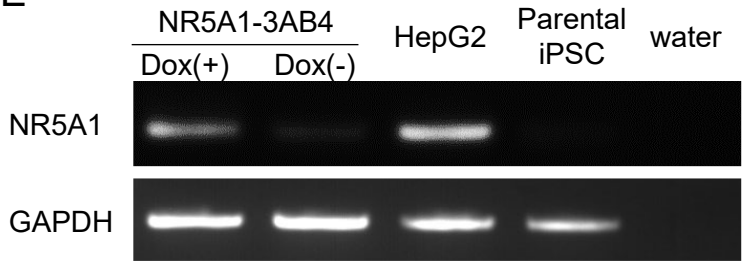


E



F

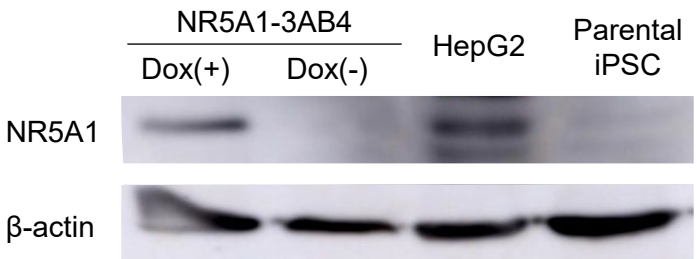
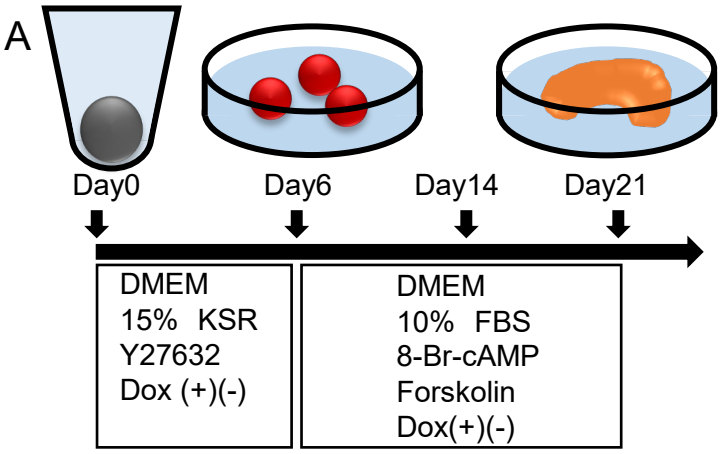
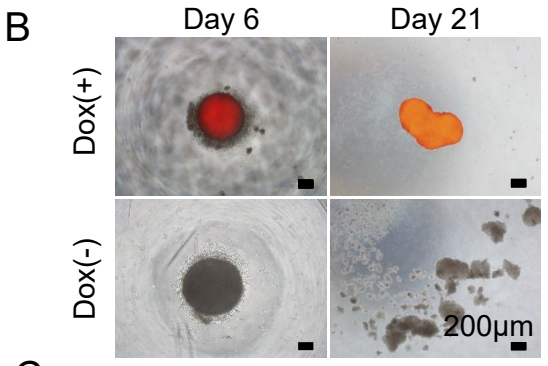
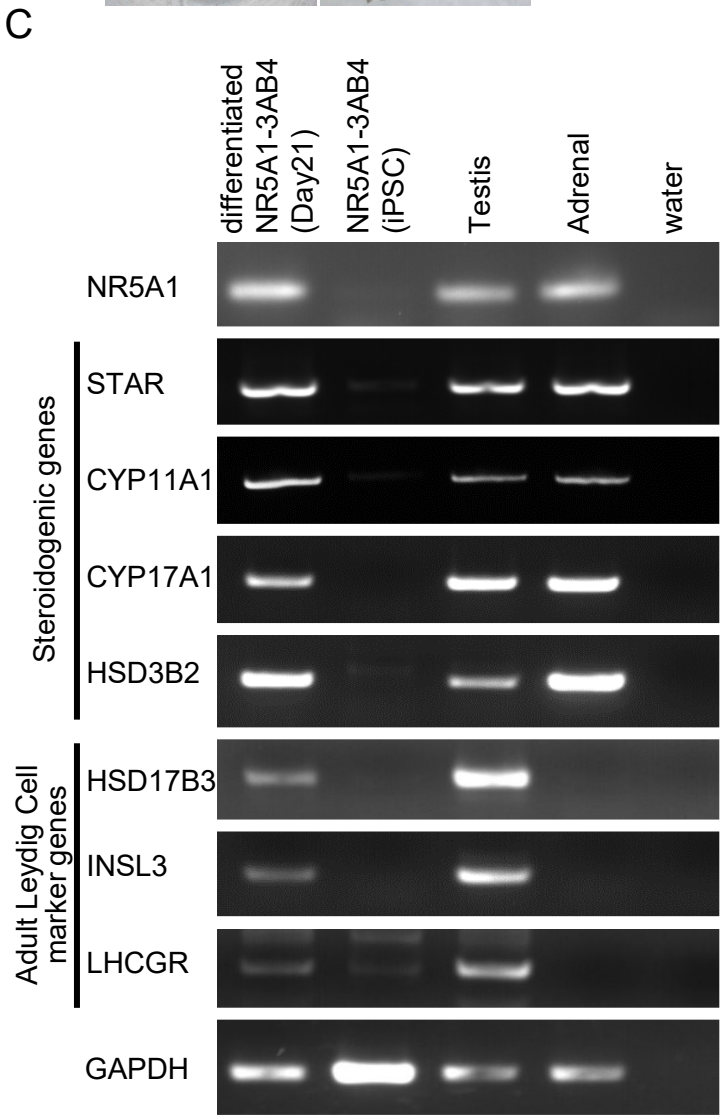
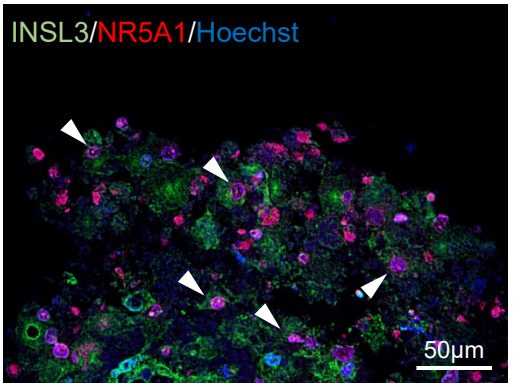


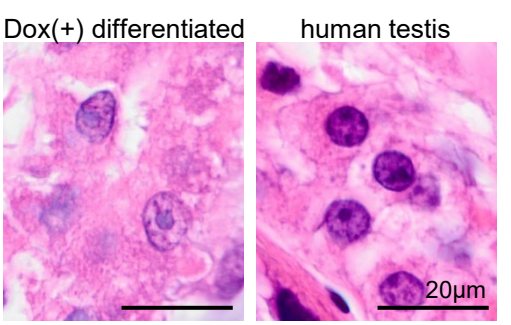
Figure 2

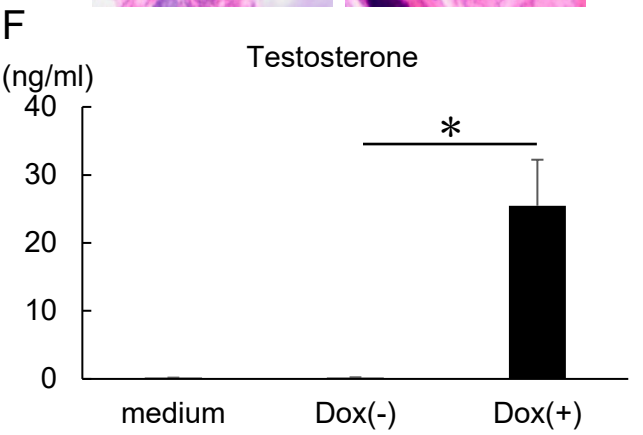

A
Day0
Day6
Day14
Day21
DMEM
15% KSR
Y27632
Dox (+)(-)
DMEM
10% FBS
8-Br-cAMP
Forskolin
Dox(+)(-)


B
Day 6
Day 21
Dox(+)
Dox(-)
200μm


C
differentiated NR5A1-3AB4 (Day21)
NR5A1-3AB4 (iPSC)
Testis
Adrenal
water
NR5A1
Steroidogenic genes
STAR
CYP11A1
CYP17A1
HSD3B2
Adult Leydig Cell marker genes
HSD17B3
INSL3
LHCGR
GAPDH


D
INSL3/NR5A1/Hoechst
50μm


E
Dox(+) differentiated
human testis
20μm


F
Testosterone
(ng/ml)
40
30
20
10
0
*
medium
Dox(-)
Dox(+)

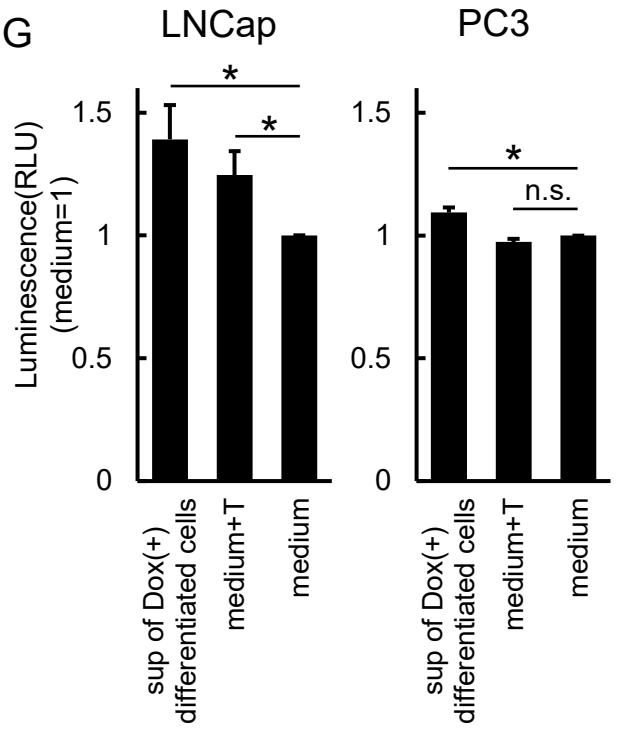
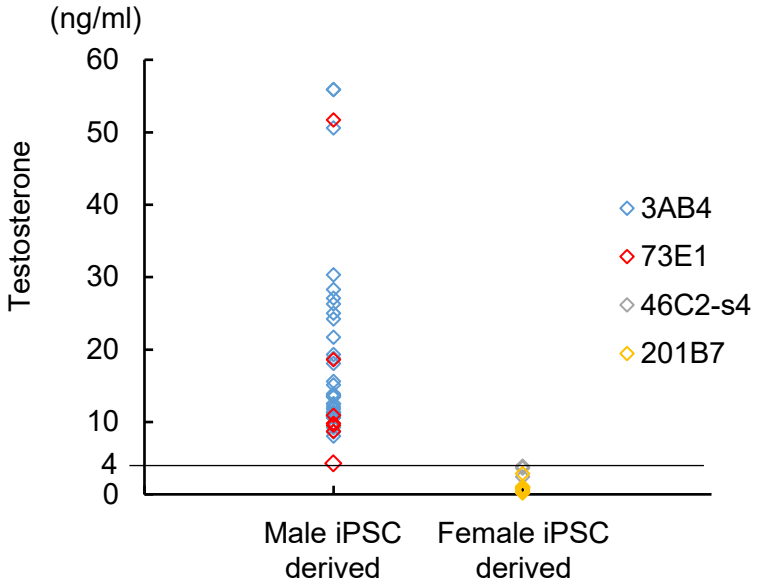

G
LNCap
PC3
Luminescence(RLU) (medium=1)
1.5
1
0.5
0
*
*
n.s.
sup of Dox(+) differentiated cells
medium+T
medium

Figure 3

A


(ng/ml)
Testosterone
60
50
40
30
20
10
4
0
3AB4
73E1
46C2-s4
201B7
Male iPSC derived
Female iPSC derived

B

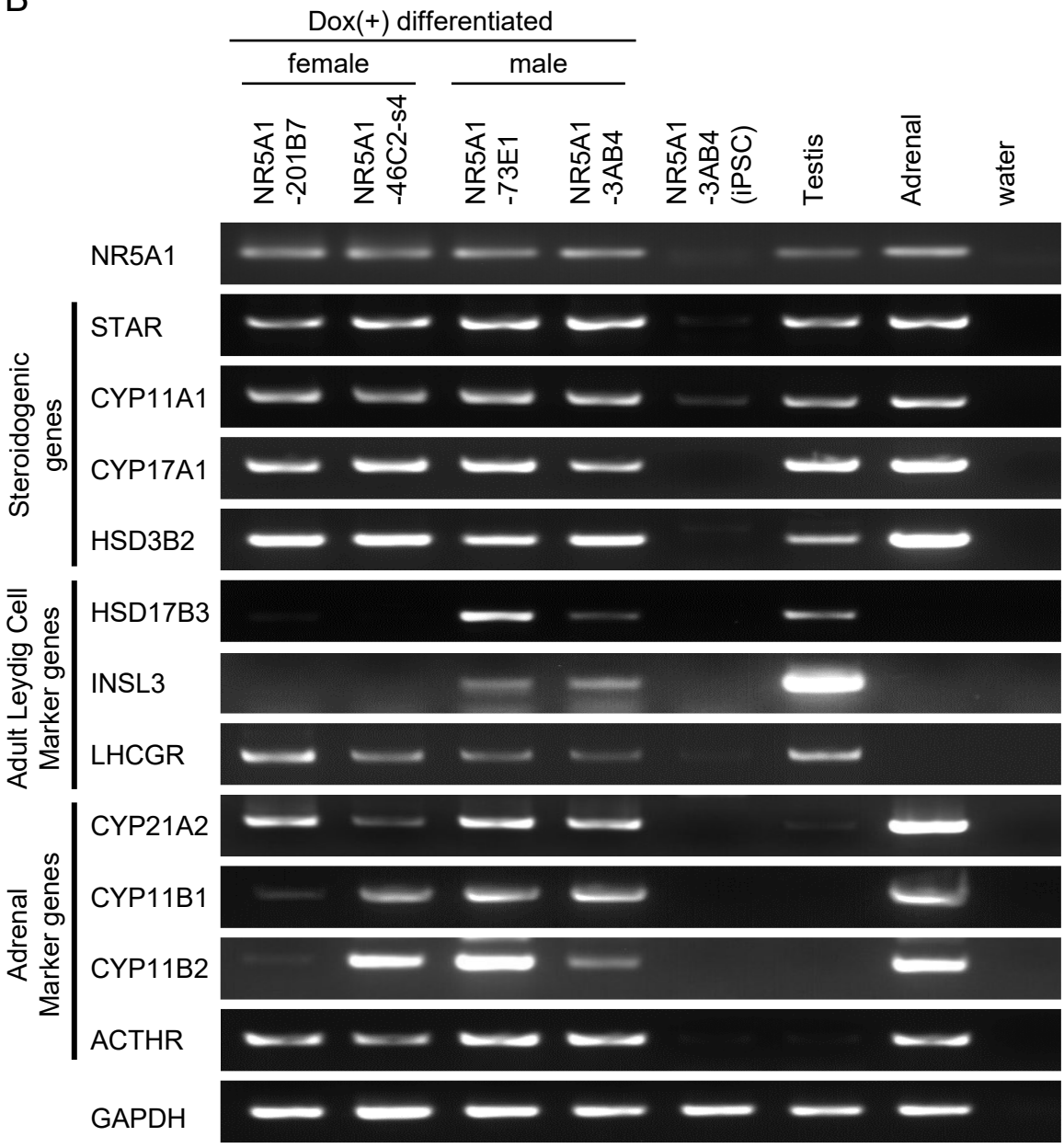
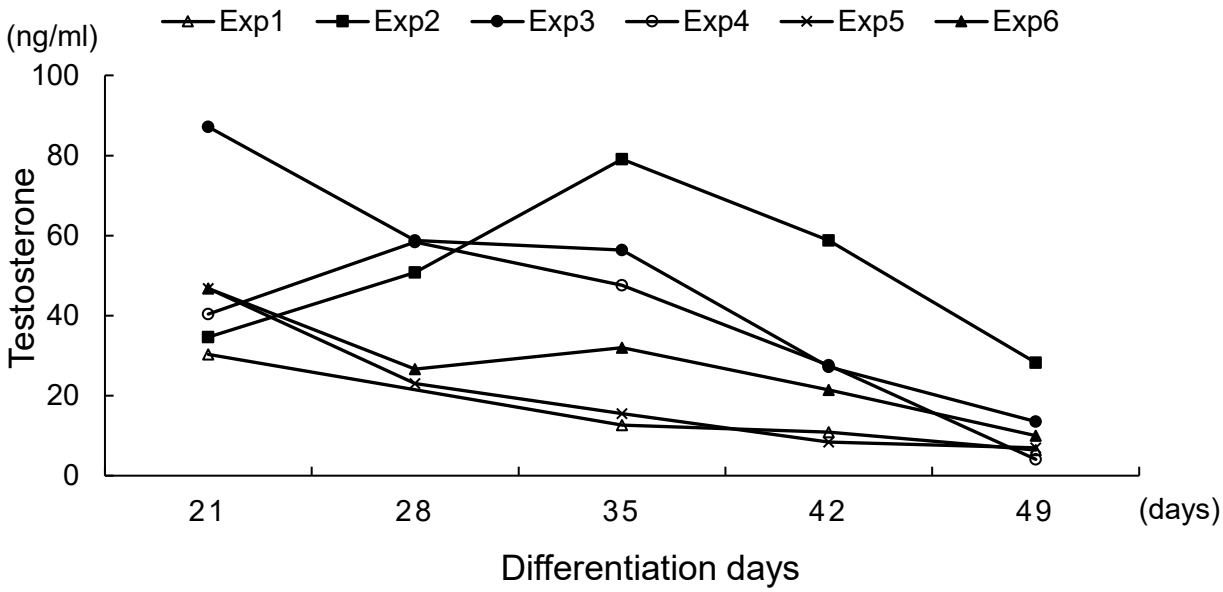
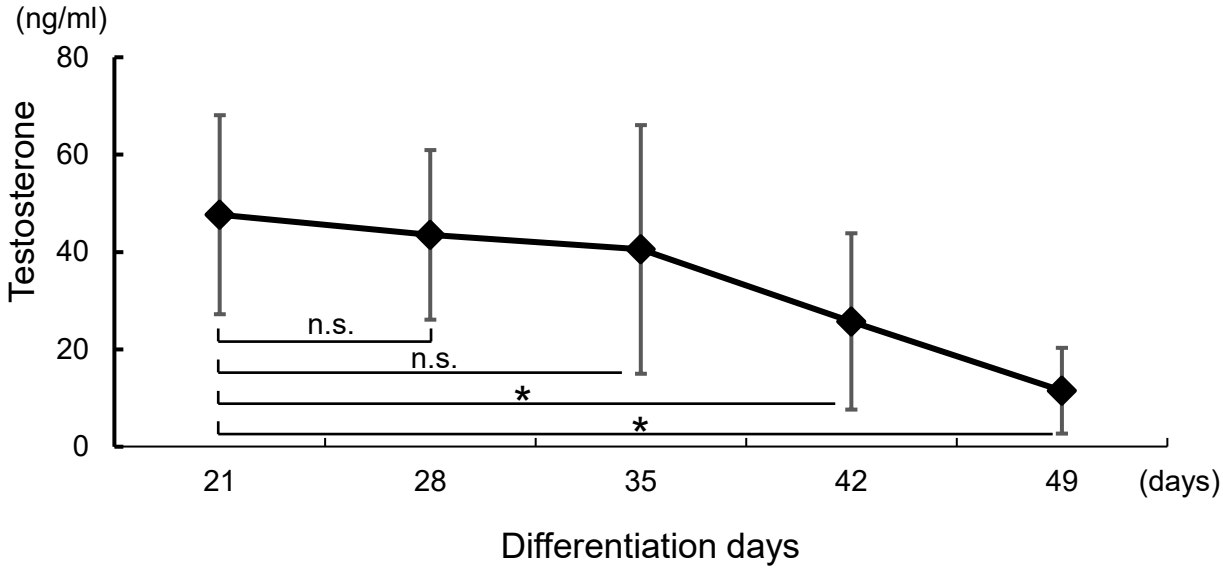

Dox(+) differentiated
female
male
NR5A1 -201B7
NR5A1 -46C2-s4
NR5A1 -73E1
NR5A1 -3AB4
NR5A1 -3AB4 (iPSC)
Testis
Adrenal
water
NR5A1
Steroidogenic genes
STAR
CYP11A1
CYP17A1
HSD3B2
Adult Leydig Cell Marker genes
HSD17B3
INSL3
LHCGR
Adrenal Marker genes
CYP21A2
CYP11B1
CYP11B2
ACTHR
GAPDH

Figure 4

A


Exp1
Exp2
Exp3
Exp4
Exp5
Exp6
(ng/ml)
100
80
60
40
20
0
Testosterone
21
28
35
42
49
(days)
Differentiation days

B


(ng/ml)
80
60
40
20
0
Testosterone
n.s.
n.s.
*
*
21
28
35
42
49
(days)
Differentiation days