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11-Ketotestosterone Is a Major Androgen Produced in Human Gonads.

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11-ketotestosterone is a major androgen produced in human gonads

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29 **Disclosure statement**; all authors have nothing to disclose.

30

31 **Context**: 11-ketotestosterone (11-KT) is a novel class of active androgen. However, the detail of
32 its synthesis remains unknown for humans.
33
34 **Objective**: The objective of this study was to clarify the production and properties of 11-KT in
35 human.
36
37 **Design, Participants and Methods**: We investigated the 11-KT production in granulosa cell
38 tumor-derived KGN cells during their differentiation by the introduction of steroidogenic factor-
39 1. Expression of CYP11B1 and HSD11B2 (key enzymes involved in the synthesis of 11-KT from
40 testosterone) were investigated in human gonads. The production of 11-KT was also investigated
41 in human Leydig cells. Plasma concentrations of testosterone and 11-KT were measured in 10
42 women and 10 men of reproductive age. Investigation of its properties was performed using breast
43 cancer-derived MCF-7 cells.
44
45 **Results**: 11-KT production was induced in KGN cells during differentiation. CYP11B1 and
46 HSD11B2 were detected in human testicular Leydig cells and ovarian theca cells. Leydig cells
47 produced 11-KT, and relatively high levels of plasma 11-KT were measured in both men and
48 women. There was no sexual dimorphism in the plasma levels of 11-KT, even though testosterone

49 levels were more than 20-times higher in men than in women. It is noteworthy that the levels of

50 testosterone and 11-KT were similar in women. In a luciferase reporter system, 11-KT activated

51 human androgen receptor (AR)-mediated transactivation in KGN cells. Conversely, 11-KT did

52 not activate estrogen receptor-mediated transactivation in aromatase-expressed MCF-7 cells,

53 whereas testosterone did following conversion to estrogen. Consistent with observations in the

54 reporter assays, 11-KT did not affect the estrogen/estrogen receptor-mediated cell proliferation of

55 MCF-7 cells. Furthermore, it significantly inhibited cell proliferation when AR was transfected

56 into MCF-7 cells.

57

58 **Conclusions**: The current study indicates that 11-KT is produced in the gonads and represents a

59 major androgen in human. It can potentially serve as a non-aromatizable androgen.

60 ## Introduction

61 Androgens are sex steroid hormones that play a role in various physiological processes via
62 pathways involving the androgen receptor (AR) (1). Testosterone is the most important androgen,
63 and is produced from cholesterol in the gonads in a series of steps catalyzed by steroid P450
64 hydroxylases and hydroxysteroid dehydrogenase (2). Although testosterone itself strongly
65 activates AR-mediated transactivation, it is also converted to a more potent androgen, 5α-
66 dihydrotestosterone (DHT), by 5α-reductase in male peripheral tissues. The androgen/AR
67 pathway is essential for male physiology, reproduction and the development of sexual
68 characteristics (3). Although androgens are traditionally viewed as male hormones, androgen/AR
69 signaling is also important for optimal female reproduction and physiology (4,5). Because
70 androgen levels reduce with aging before menopause, postmenopausal women suffer various
71 symptoms by androgen insufficiency (5,6). On the other hand, androgen excess in women results
72 in a variety of pathological conditions, including polycystic ovary syndrome (PCOS) and
73 idiopathic hirsutism (7). Therefore, proper androgen signaling is important for the health of
74 women. In addition to androgenic actions, testosterone is also a precursor for estrogen. Aromatase
75 (CYP19A1) converts testosterone into the most potent estrogen, 17β-estradiol (E_2). It activates
76 two subtypes of estrogen receptors (ER), ERα and β, the expression patterns of which are tissue
77 and cell specific. Adrenal gland also produces testosterone and high concentrations of weak

78 androgens, such as dehydroepiandrosterone (DHEA), DHEA sulfate, androstenedione, 11β-
79 hydoxyandrostenedione (11-OHA), and 11β-hydroxytestosterone (11-OHT), which are also
80 precursors for stronger androgens in target tissues (8-10). In previous studies, we and others have
81 reported that 11-ketotestosterone (11-KT, International Union of Pure and Applied
82 Chemistry name is 17-Hydroxyandrost-4-ene-3,11-dione) is another class of active
83 androgen that can be converted from testosterone and other weaker precursors (9-11).
84 CYP11B1 and HSD11B2 play important roles in the 11-KT synthesis from testosterone (10,11).
85 It is well-known that these enzymes are involved in glucocorticoid synthesis and metabolism.
86 CYP11B1 catalyzes the final step of glucocorticoid production in the adrenal gland (12,13), while
87 HSD11B2 converts active glucocorticoids into inactive 11-ketosteroid forms, which are
88 abundantly expressed in the kidney and placenta (14). We have demonstrated that Cyp11b1 and
89 Hsd11b2 are expressed in murine gonads and are involved in gonadotropin-induced 11-KT
90 production (11). 11-KT can strongly activate mammalian AR-mediated transactivation. It was
91 also reported that 11-KT can be detectable in human blood samples (9). Thus, it is conceivable
92 that 11-KT is common androgen in mammals, although the detail of its synthesis remains
93 unknown. In the present study, we evaluated the production and properties of 11-KT in humans.
94

95 **Materials and Methods**

96 **Cell culture and transfection**

97 Human ovarian granulosa cell tumor-derived KGN cells (kindly donated by Dr. Toshihiko 98 Yanase, University of Fukuoka, Fukuoka, Japan) and MCF-7 cells were cultured in 99 DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS). Human 100 adrenocortical tumor-derived H295R cells were cultured in Opti-MEM supplemented with 2% 101 Nu-serum IV (BD Biosciences, Franklin Lakes, NJ, USA). Human Leydig cells were purchased 102 from ScienCell Research Laboratories (Carlsbad, CA), which are sourced within the USA under 103 protocols that have obtained Institutional Review Board (IRB) approval. They were cultured in 104 Leydig Cell Meidum (ScienCell Research Laboratories). The research protocol using human 105 materials was approved at the ethical committee of Asahikawa Medical University. H295R cells 106 were transfected using an Amaxa Nucleofector Technology system (Lonza, Cologne, Germany) 107 as described (15). KGN and MCF-7 cells were transfected using Lipofectamine LTX reagent (Life 108 Technologies, Inc., Carlsbad, CA, USA) and HilyMax (Dojindo Laboratories, Kumamoto, Japan). 109 One day before transfection, the cells were seeded on 24-well plates and cultured with phenol 110 red-free medium supplemented with 10% charcoal/dextran stripped (CD)-FBS. After 24 h 111 transfection, the cells were treated with vehicle (EtOH) or steroid hormones for 24 h. Luciferase 112 assays were performed as described previously (15,16). Each data point represents the mean of at 113 least four independent experiments.

114

115 **Adenovirus production and infection**

116 Adenovirus vectors for LacZ, GFP, and Steroidogenic factor-1 (SF-1) were prepared using the

117 Adeno-X Expression System 1 (Takara, Shiga, Japan), following the manufacturer's instructions

118 as described (16). Using these vectors, replication-defective recombinant adenoviruses were

119 propagated and titered in HEK 293 cells. Then, they were used to infect KGN cells at MOI 10.

120 GFP-expressing adenovirus used as a transduction control during infection. Transduction

121 efficiency and optimal concentrations of virus were determined by investigating GFP expression

122 using fluorescent microscopy. At 48 h post-infection, cells were processed for RNA or protein

123 extraction.

124

125 **DNA microarray**

126 Expression analysis by DNA microarray has been described elsewhere (17). Briefly, labeled

127 cRNA was prepared from KGN cells with adenovirus-mediated expression of LacZ, GFP, or SF-

128 1. After fragmentation of cRNA, hybridization was performed with a human U133 Plus 2.0

129 Affymetrix GeneChip (Affymetrix, Santa Clara, CA, USA). The arrays were scanned using a

130 Gene-Array scanner and the data were generated by Affymetrix Microarray Suite 4.0. Data were

131 analyzed using Subio Platform software (http://www.subio.jp/products/platform).

132

133 **RT-PCR and quantitative (Q)-PCR**

134 Total RNA from the cultured cells was extracted using TriPure Isolation Reagent (Roche,

135 Carlsbad, CA, USA). Total RNA from human testis, ovary, adrenal, liver, and kidney was

136 purchased from Takara Bio Inc. (Shiga, Japan) and Biochain Institute Inc. (Newark, CA). RT-

137 PCR and Q-PCR was performed as described (16,18). The RT-PCR products were

138 electrophoresed on a 1.5% (w/v) agarose gel, and the resulting bands were visualized by staining

139 with ethidium bromide. In Q-PCR, each gene expression was measured by real-time PCR and

140 normalized with β-actin expression. The primers used for PCR are described in Supplemental

141 Table1. The primers used for other genes were as described (16,18).

142

143 **Western blotting analysis**

144 Extraction of total proteins from cultured cells and subsequent quantification were conducted

145 as described previously (16). Protein extract samples from each human tissue were purchased

146 from BioChain Institute Inc. (Hayward, CA, USA). Ovarian tissue donors were able to donate

147 healthy organs and were of reproductive age (aged 30-34 years). Each tissue sample was

148 collected under strict IRB ethical consenting practices. Equal amounts of protein (20 μg) were

149 resolved using 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes.

150 Western blot analyses of HSD11B2, CYP11B1, AR, β-actin and GAPDH were performed with

151 antibodies directed against HSD11B2 (H-145, Santa Cruz Biotechnology, Inc., Santa Cruz, CA,

152 USA), CYP11B1 (13), AR (N-20, Santa Cruz Biotechnology, Inc.), β-actin (C4, Santa Cruz

153 Biotechnology, Inc.) and GAPDH (6C5, Santa Cruz Biotechnology, Inc.), respectively.

154 Enhanced chemiluminescence western blot reagents (Bio-Rad Laboratories Inc., Hercules, CA,

155 USA) were used for detection.

156

157 **Plasmids**

158 The pcDNA3 expressing HSD11B2 was generated by cloning the open reading frame of

159 HSD11B2 into a pcDNA3 vector (Invitrogen, Carlsbad, CA). A Slp-ARU/Luc reporter and

160 pQCXIP/human AR were prepared as described (11,19). The ERE/Luc was constructed by

161 inserting the oligonucleotides into a pGL4.24 plasmid (Promega Corp., Madison, WI, USA).

162

163 **Immunohistochemistry**

164 Immunohistochemistry was performed as described previously (11,19). Sections of human testis

165 (from men aged 23 and 50 years) and ovary (from woman aged 19 and 34 years) were purchased

166 from US Biomax Inc. (Rockville, MD) and Biochain Institute Inc. (Newark, CA). Sections were

167 subjected to the antigen retrieval technique with Dako Target Retrieval Solution, pH 9 (Dako

168 Denmark A/S, Glostrup, Denmark) and treated with anti-CYP11B1 (13) and anti-HSD11B2 (20).

169 They were then developed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame,

170 CA).

171

172 **Human blood samples**

173 Blood samples were collected in collection tubes containing heparin from the median cubital

174 vein of two healthy women volunteers at University of Fukui Hospital in 2007. Plasma was

175 separated by centrifugation at 1000 x g for 5 min. Other plasma samples were purchased from

176 AllCells (Emeryville, CA) and ProMedDX (Norton, MA). All plasma samples were collected

177 under IRB approved collection protocols and subject informed consent. The donors were 10 men

178 (aged 32.1 ± 7.8 year) and 10 women (aged 31.7 ± 6.0 years). Plasma samples were stored at -

179 80 °C until assays. The research protocol for using human materials was approved by the Ethics

180 Committee of the University of Fukui.

181

182 **Measurements by enzyme immunoassays (EIA) and liquid chromatography-tandem mass**

183 **spectrometry (LC-MS/MS)**

184 Concentrations of testosterone, 11-KT and E2 in culture media of KGN, H295R and human

185 Leydig cells were determined by competitive EIA (11,19). Each sample was diluted with EIA

186 buffer, and analyzed using progesterone, testosterone, 11-KT and E_2 EIA Kit (Cayman Chemical
187 Company, Ann Arbor, MI) following the manufacturer's instructions in a microplate reader
188 (Molecular Device SpectraMax M5; Molecular Device, LLC, Sunnyvale, CA). On the other hand,
189 concentrations of steroid hormones in human plasma were measured by LC-MS/MS for optimal
190 quantification of the clinical samples. Processing of human plasma samples and quantification of
191 testosterone, 11-KT and E2 by LC-MS/MS are based on methods as described previously (9).
192
193 **Retrovirus preparation and infection**
194 The Phoenix packaging cell line was transiently transfected with the retroviral
195 plasmids using the FuGENE 6 reagent (Promega). The supernatant was concentrated
196 by centrifugation. The virus solution was stored at −80 °C until use. MCF-7 cells
197 were infected with the retrovirus in the presence of 8 μg/mL Polybrene (Sigma-
198 Aldrich) for 48 h. The cells were then replated and selected using puromycin.
199
200 **Proliferation assay**
201 MCF-7 cells or AR-introduced cells were seeded with DMEM/F-12 supplemented with 10% or
202 2% CD-FBS at 1×10^3 cells/well in 96-well plates. At 24 h after seeding, the cells were treated
203 with the media containing various concentrations of DHT, testosterone, 11-KT, or E_2. Six days

204 (parental cells) or nine days (AR-introduced cells) after incubation, cell proliferation was
205 evaluated using a CellTiter 96 Aqueous One Solution Kit (Promega) following the manufacturer's
206 instructions. To evaluate the effect of an aromatase inhibitor, fadrozole (Sigma, St, Louis, MO)
207 or an estrogen receptor antagonist, fulvestrant (Sigma), on the growth of MCF-7 cells, a
208 proliferation assay was performed with or without these agents.

209

210 **Statistics**

211 Data are presented as the mean ± SEM. Differences between groups ($P < 0.05$) were assessed
212 by the Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison tests using
213 EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (21),
214 which is a graphical user interface for R (The R Foundation for Statistical
215 Computing, Vienna, Austria).

216

217 **Results**

218 **HSD11B2 is induced by SF-1 and involved in the 11-KT production**

219 Human ovarian granulosa cell tumor-derived KGN cells have low steroidogenic capacity under
220 basal conditions. However, they can be transformed to produce a range of steroid hormones by
221 infection with an adenovirus expressing SF-1 and its co-activator (16). To examine changes in

222 gene expression in this transformation, we used a DNA microarray with GFP- or SF-1-introduced

223 KGN cells. SF-1 introduction induced a number of genes, including steroidogenic enzymes such

224 as CYP11A1, HSD3B2, CYP17A1, and CYP19A1 (Supplemental Table 2), which are known SF-

225 1 targets (22). In addition to these genes, HSD11B2 was a strong SF-1-inducible candidate gene

226 (Supplemental Table 2) and was almost undetectable in GFP-introduced cells (Figure 1 A and B).

227 Consistent with DNA microarray data, Q-PCR and immunoblotting analyses revealed that

228 introduction of SF-1 strongly induced HSD11B2 mRNA and proteins (Figure 1 A and B). In

229 previous studies, it was demonstrated that HSD11B2 is involved in 11-KT production from

230 testosterone with CYP11B1 (Supplemental Figure 1) in murine gonads (11,19) or from adrenal

231 androgens by *in vitro* analysis (10). The DNA microarray analyses suggest that CYP11B1 is an

232 SF-1-inducible gene, as well as other steroidogenic genes that are involved in testosterone and E_2

233 synthesis (Figure 1C, Supplemental Table 2, Supplemental Figure 1). In fact, the introduction of

234 SF-1 induced not only the production of testosterone and E_2, but also the CYP11B1 expression

235 and 11-KT production (Figure 1 C and D).

236 To verify the role of HSD11B2 during 11-KT production in human steroidogenic cells, it was

237 ectopically expressed in human adrenocortical H295R cells. H295R cells expressed CYP11B1

238 and other steroidogenic enzymes for testosterone synthesis (Supplemental Figure 2), whereas

239 endogeneous HSD11B2 was undetectable (Figure 2A). Even though H295R cells produce

240 testosterone at relatively high levels, its conversion to 11-KT was marginal (Figure 2 C and D).

241 Transient transfection of the HSD11B2 expression vector (Fig. 2B) markedly increased 11-KT

242 production compared with the control group (Figure 2D), whereas testosterone concentrations

243 were similar in both groups (Figure 2C). These results indicate that the expression of human

244 HSD11B2 could be an important factor for the production of 11-KT in steroidogenic cells.

245

246 **11-KT is produced in gonads and is one of the major androgens in human**

247 To elucidate the 11-KT synthesis pathway, we investigated the expression of CYP11B1 and

248 HSD11B2 in human gonads (Figure 3 A and B). Q-PCR and western blot analyses showed that

249 both genes were detectable in the testis and ovary at the mRNA and protein levels (Figure 3 A and

250 B). Immunohistochemical analysis showed that both proteins are localized on testicular Leydig

251 cells and ovarian theca cells, even though HSD11B2 is also detectable in some populations of

252 ovarian granulosa cells (Fig. 3C). These results strongly suggest that 11-KT is produced in

253 testicular Leydig cells and ovarian theca cells. To confirm this hypothesis, we investigated the

254 production of 11-KT in human Leydig cells. In support of the immnohistochemical analyses,

255 Leydig cells expressed CYP11B1 and HSD11B2 genes (Supplemental Figure 3A). They can

256 produce progesterone, testosterone and 11-KT under basal conditions (Fig. 3D, Supplemental

257 Figure 3B). cAMP-treatment moderately increased the production of these steroid hormones.

258 Then, we measured plasma concentrations of 11-KT, testosterone and E_2 in both sexes (Figure

259 3 E-G). Testosterone levels in men were about 22-fold higher than those in women, whereas 11-

260 KT levels were similar between the sexes (Figure 3 E and F). It is noteworthy in women that 11-

261 KT concentrations were similar to testosterone concentrations and about 5-fold higher than E2

262 concentrations (Figure 3H). Human AR-mediated transactivation was significantly increased by

263 11-KT at concentrations >10^{-9} M in KGN cells (Figure 4). This level is lower than that for the

264 induction by other androgens, although transactivation was increased to similar levels by DHT

265 and testosterone at 10^{-8} and 10^{-7} M, respectively. These results suggest the possibility that 11-KT

266 plays some roles in human as one of the major androgens.

267

268 **11-KT is not convertible to estrogenic hormones**

269 In female individuals, testosterone acts as both an androgen and a precursor for estrogen. To

270 determine whether 11-KT is a precursor for estrogen, we performed a luciferase assay using a

271 reporter plasmid containing the estrogen response element (ERE) in human breast cancer derived

272 MCF-7 cells, which endogenously express aromatase and ERα. In contrast to E_2, androgens had

273 no effect on luciferase activity at lower concentrations (10^{-11} and 10^{-10} M) (Figure 5A). However,

274 testosterone activated ER-dependent transcription at high concentrations and at 10^{-7} M

275 testosterone was effective as E_2. This activity was completely suppressed by an aromatase

276 inhibitor, fadrozole (Figure 5B). DHT weakly activated ER-mediated transactivation in an
277 aromatase-independent manner at 10^{-7} M. In contrast, 11-KT had no effect on ER-mediated
278 transactivation, even at 10^{-6} M (Figure 5A, data not shown). These results indicate that 11-KT is
279 a non-aromatizable androgen, and does not convert to a compound that activates ER-mediated
280 transactivation.
281 Then, we assessed the effect of 11-KT on cell proliferation in MCF-7 cells. The proliferation of
282 MCF-7 cells is highly dependent on estrogens. Consistent with previous studies, MCF-7 cells
283 were increased by $>10^{-11}$ M E_2 (Figure 6A). In support of the reporter assays result, testosterone
284 significantly stimulated cell proliferation at higher concentrations (Figure 6A), and fadrozole and
285 the ER antagonist fulvestrant completely inhibited testosterone-induced cell proliferation (Figure
286 6 B and C). Conversely, 11-KT had no effect on cell growth at any concentration, similar to DHT.
287 These results strongly suggest that 11-KT acts as a stable androgen in both AR- and aromatase-
288 positive cells. To further test this hypothesis, AR was stably transfected into MCF-7 cells (Figure
289 6D) and we measured the AR-mediated growth inhibition of each androgen. Consistent with
290 previous reports (23), DHT strongly suppressed cell proliferation in AR-introduced MCF-7 cells
291 (Figure 6E). Similarly, 11-KT also inhibited proliferation, though to a lesser extent than DHT. In
292 contrast, testosterone did not significantly inhibit cell growth.
293

294 **Discussion**

295 11-KT is one of the active androgens, which was originally characterized as a teleost-specific
296 hormone. However, we and others have since noted that it is also present in mammals (9-11).
297 Here, we found evidence that 11-KT is produced in human gonads and is one of the major
298 androgens. It can potentially act as a non-aromatizable androgen. Testicular Leydig cells and
299 ovarian theca cells expressed the enzymes for producing 11-KT from testosterone, CYP11B1 and
300 HSD11B2. In fact, Leydig cells produced 11-KT, autonomously.

301 HSD11B2 protects mineralocorticoid receptor (MR) from glucocorticoid by inactivating cortisol
302 to corticosterone in aldosterone-sensitive tissues (14). The gonads are target organs for
303 mineralocorticoids, which express MR (24,25). Mineralocorticoids can stimulate the production
304 of testosterone and progesterone in testicular Leydig cells (24) and ovarian granulosa cells (26),
305 respectively. Conversely, glucocorticoids inhibit testicular and ovarian steroidogenesis (27).
306 Because HSD11B2 proteins are expressed in these cells, it is reasonable to assume that gonadal
307 HSD11B2 plays a role in the protection of steroidogenic cells from the adverse effects of
308 glucocorticoids. In addition to this classical role, the involvement of HSD11B2 in 11-KT
309 production in Leydig cells and theca cells may represent another important role (10,11). Our
310 results strongly suggest that the gonads are main organs for 11-KT production in humans by the
311 expression of HSD11B2.

312 In mammals, CYP11B1 is thought to be an adrenal-specific enzyme that is essential for the
313 final step of glucocorticoid synthesis (12). However, it is also expressed in the testicular Leydig
314 cells and ovarian theca cells to a lesser extent. There is also evidence that CYP21 is expressed in
315 the gonads (25,28). Thus, a contribution of gonadal CYP11B1 to local glucocorticoid synthesis
316 cannot be ruled out, although there are no studies documenting high levels of cortisol in the
317 gonads. Rather, it is often reported that cortisol levels are lower in ovarian follicular fluid (FF)
318 than in the blood (29,30). Therefore, it is conceivable that the production of precursors for 11-KT
319 could be one of the most important functions of CYP11B1 in the gonads. Indeed, testosterone and
320 androstenedione are efficiently converted to the 11-KT precursors (11-OHT and 11-OHA) by
321 ectopic expression of human CYP11B1 with ferredoxin in COS-1 cells. (10). Consistent with this
322 *in vitro* study, high levels of these steroids are produced in the CYP11B1-abundant adrenal gland,
323 even though they are rarely converted to 11-keto products by low-level expression of HSD11B2
324 in the adrenal. However, it is probable that adrenal-derived 11-OHT and 11-OHA could be another
325 sources of human 11-KT by the conversion in HSD11B2 expressing organs, including gonads.
326 There was no sexual dimorphism of human plasma 11-KT levels, even though testosterone levels
327 were much higher in men. Similar phenomena were also observed in rodents (11). This indicates
328 that the dominance of 11-KT production in female is conserved between rodents and humans. In
329 mice, the abundant expression of ovarian HSD11B2 is likely responsible for this dominance (11).

330 In addition, the testicular expression of Hsd11b1 was much higher than in the ovary (11). Because
331 this 11β-HSD isoform preferentially catalyzes the reduction of 11-ketosteroid (31), the difference
332 in its expression between sexes also results in ovarian dominance of 11-KT production. Such
333 sexual differences in the expression of both HSD11B1 (unpublished data) and HSD11B2 were
334 unclear in human gonads. In addition to the level of expression, the activity of 11β-HSD enzymes
335 is regulated by a number of factors (32,33). It is interesting that human ovarian FF contains the
336 selective inhibitor for HSD11B1 (34). Additionally, it was also reported that ovarian FF contains
337 much higher concentrations of 11-OHA than plasma levels, which is converted from cortisol in
338 granulosa cells (35). This might be another pathway for ovarian 11-KT production. Further studies
339 are needed to evaluate the sexual dimorphism of 11-KT production in humans.

340 DHT, testosterone, and 11-KT can strongly activate AR-mediated transactivation, even though
341 there are some differences, especially at lower levels. Testosterone acts not only as an androgen,
342 but also as a precursor for estrogens. It can be converted to E_2 in aromatase-expressing cells, and
343 induces estrogen-dependent phenomena. Conversely, it is probable that 11-KT is not converted to
344 estrogen by aromatase. Although DHT is also a non-aromatizable androgen, it can be converted
345 to 3β-diol by HSD17B7, which can bind to ERs (36). Therefore, 11-KT likely represents
346 extremely difficult androgen to convert to an estrogenic steroid. In addition, plasma 11-KT levels
347 are similar to testosterone levels in women at reproductive age, and in menopausal women (9).

348 Thus, it is possible that 11-KT play important roles in female, especially in AR- and aromatase-

349 expressing tissues such as ovary and breast. In a previous study, we demonstrated using mice that

350 testosterone and 11-KT are elevated at ovulatory LH/hCG-stimulation, and are involved in the

351 expression of ovulation-related genes, such as Cyclooxygenase-2 and Amphiregulin, in granulosa

352 cells (19). This issue could be a subject of further investigation in human, because preovulatory

353 follicles express CYP19A1 gene at high levels (37).

354 In summary, we demonstrated that 11-KT is a major androgen and produced in gonads. Because

355 androgens are essential for reproduction and physiology, their excess and deficiency often induce

356 pathogenesis. Then, it is possible that 11-KT could be responsible for, and the novel target of

357 therapies against, such diseases. In addition, it might provide novel insights for elucidating

358 ambiguous AR-mediated phenomena.

359

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367

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491

492 **Figure Legends**

493 **Figure 1.** SF-1 induces HSD11B2 expression and 11-KT production in KGN cells. KGN cells

494 were infected with adenoviruses expressing GFP or SF-1. Induction of HSD11B2 mRNA (A) and

495 protein (B) by SF-1. A, mRNA expression of HSD11B2 gene was analyzed by Q-PCR and

496 normalized to β-actin expression. Data represent the mean ± SEM of at least three independent

497 experiments. B, Western blot analyses were performed with antibodies against HSD11B2 and

498 GAPDH using the same lysates. C, Induction of the enzymes for the synthesis of testosterone, 11-

499 KT and E2 was confirmed by Q-PCR. Data represent the mean ± SEM of at least three

500 independent experiments. D, 11-KT, testosterone and E_2 levels in each group were measured by

501 EIA. Data represent the mean ± SEM of at least three independent experiments. Differences

502 between groups are indicated by $^{**}P<0.01$.

503 **Figure 2.** 11-KT production was induced by the ectopic expression of HSD11B2 in H295R cells.

504 A, Expression of HSD11B2 in H295R cells. Expression of HSD11B2 was analyzed by Q-PCR

505 and normalized to β-actin expression. The Kidney was used as positive controls. Q-PCR data

506 represent the mean ± SEM of at least three independent experiments. B-D, H295R cells were

507 transfected with the empty vector or the expression vector for HSD11B2. B, Protein extracts from

508 cells in each treatment were subjected to SDS-PAGE and western blot was performed using each

509 antibody. C–D, Testosterone (C) and 11-KT (D) levels in each group were measured by EIA. Data

510 represent the mean ± SEM of at least three independent experiments. Differences between groups

511 are indicated by **P<0.01.

512 **Figure 3.** Expression of 11-KT synthetic enzymes in human gonads and plasma concentrations

513 of 11-KT. A-B, Expression of CYP11B1 and HSD11B2 in human testis and ovary. A, mRNA

514 levels of each gene analyzed by Q-PCR and normalized to β-actin expression. The adrenal gland

515 and kidney were used as a positive control for each analysis. Q-PCR data represent the mean ±

516 SEM of at least three independent samples. B, Western blot analyses were performed with

517 antibodies against CYP11B1, HSD11B2, and GAPDH using lysates from each human tissue (20

518 μg protein). Western blot analysis is representative of the two experiments. C, Localization of

519 CYP11B1 and HSD11B2 proteins in human gonads. Positive staining both for CYP11B1 and

520 HSD11B2 was observed in testicular Leydig cells and ovarian theca cells. No staining was

521 observed in control sections incubated with nonimmune serum. An, Antrum. D, Production of

522 testosterone and 11-KT by human Leydig cells with or without 8br-cAMP for 48 h. Each androgen

523 levels in each group were measured by EIA. Data represent the mean ± SEM of at least three

524 independent experiments. E-H, Levels of plasma testosterone, 11-KT and E_2 in humans. Plasma

525 testosterone (E), 11-KT (F) and E_2 (G) levels in men and women were measured by LC-MS/MS.

526 In the box and whisker plots, boxes show 75th and 25th percentiles. Horizontal lines in the boxes

527 represent the medians. Whiskers show the lowest values and the highest values. Differences

528 between groups are indicated by *P<0.05 and **P<0.01. H, Comparison of plasma testosterone,

529 11-KT and E_2 levels within each sex. Data represent the mean ± SEM (n=10 men, n=10 women).

530 **Figure 4.** Activation of human AR-mediated transcription by DHT, testosterone, and 11-KT.
531 KGN cells were transfected with the ARE-Luc vector and the human AR-expression vector. At
532 24 h post-transfection, cells were incubated with or without increasing concentrations of each
533 androgen for 24 h. Data represent the mean ± SEM of at least four independent experiments.

534 **Figure 5.** Comparison of ER-mediated transactivation by E_2, DHT, testosterone, and 11-KT in
535 MCF-7 cells. MCF-7 cells were transfected with the ERE-Luc reporter vector. At 24 h post-
536 transfection, increasing concentrations of each steroid hormones were added with (A) or without
537 (B) the aromatase inhibitor fadrozole for 24 h. Data represent the mean ± SEM of at least four
538 independent experiments.

539 **Figure 6.** The effects of each androgen on the proliferation in parental and AR-introduced
540 MCF-7 cells. A-C, The effects of each steroid hormone on the proliferation of parental MCF-7
541 cells. A, Cells were cultured with or without each steroid hormones at various concentrations for
542 6 days. Data represent the mean ± SEM of three independent experiments. Values marked by
543 different letters are significantly different ($P<0.05$). B–C, Effects of the aromatase inhibitor
544 fadrozole (B) and the ER-antagonist fulvestrant (C) at 1 μM on testosterone-induced (10^{-7} M) cell
545 proliferation. Data represent the mean ± SEM of three independent experiments. Values marked
546 by different letters are significantly different ($P<0.05$). D-E, Cell growth inhibition by androgens

547 in AR-introduced MCF-7 cells. D, Western blot analyses were performed with the antibodies

548 against AR and GAPDH using lysates derived from parental and AR-introduced MCF-7 cells. E,

549 The effects of each androgen on the inhibition of cell growth in AR-introduced MCF-7 cells. Cells

550 were cultured with or without each androgen at 10^{-7} M for 9 days. Data represent the mean ± SEM

551 of five independent experiments. Values marked by different letters are significantly different

552 ($P<0.05$).

Figure 1

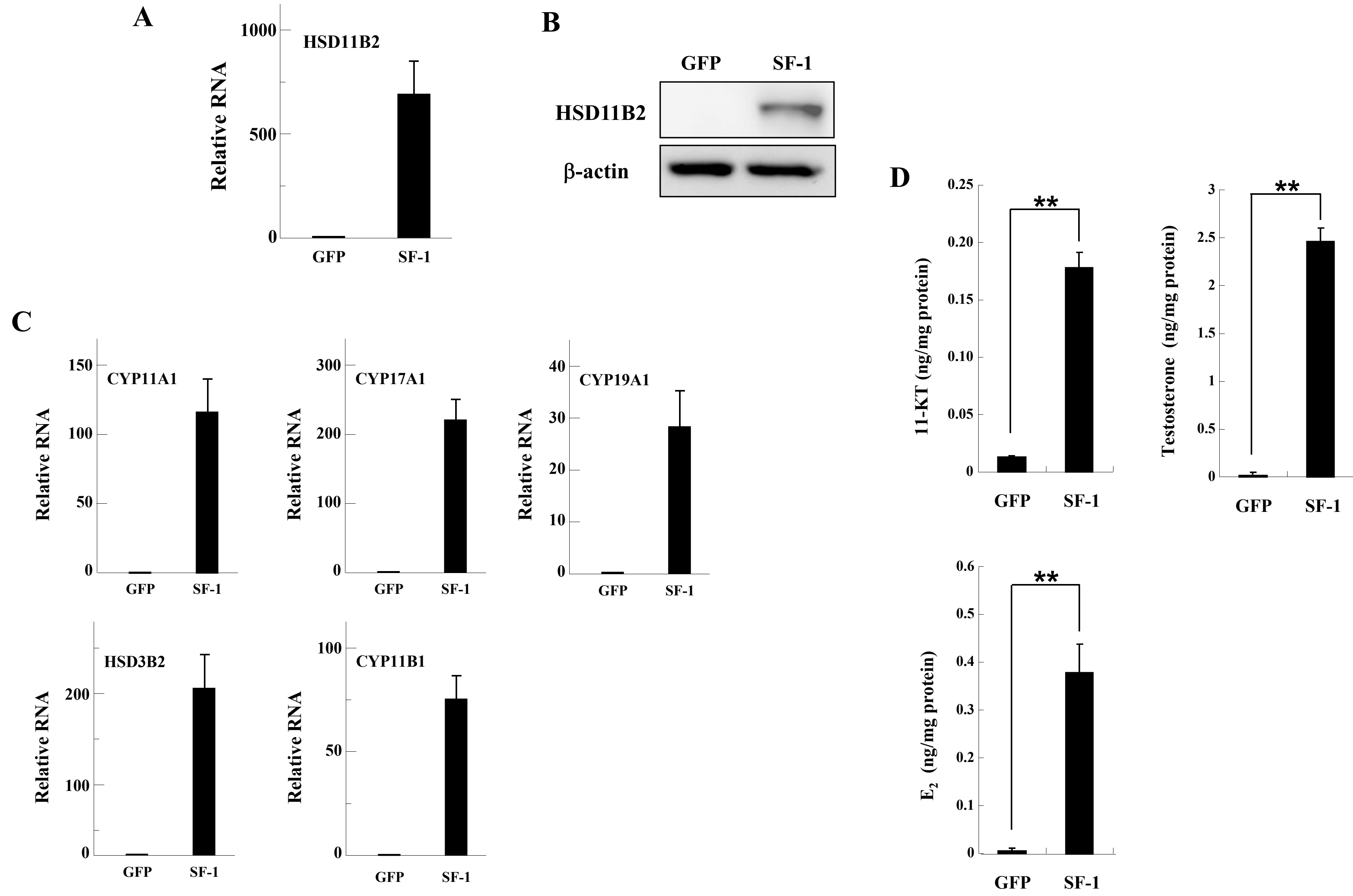


Figure 2

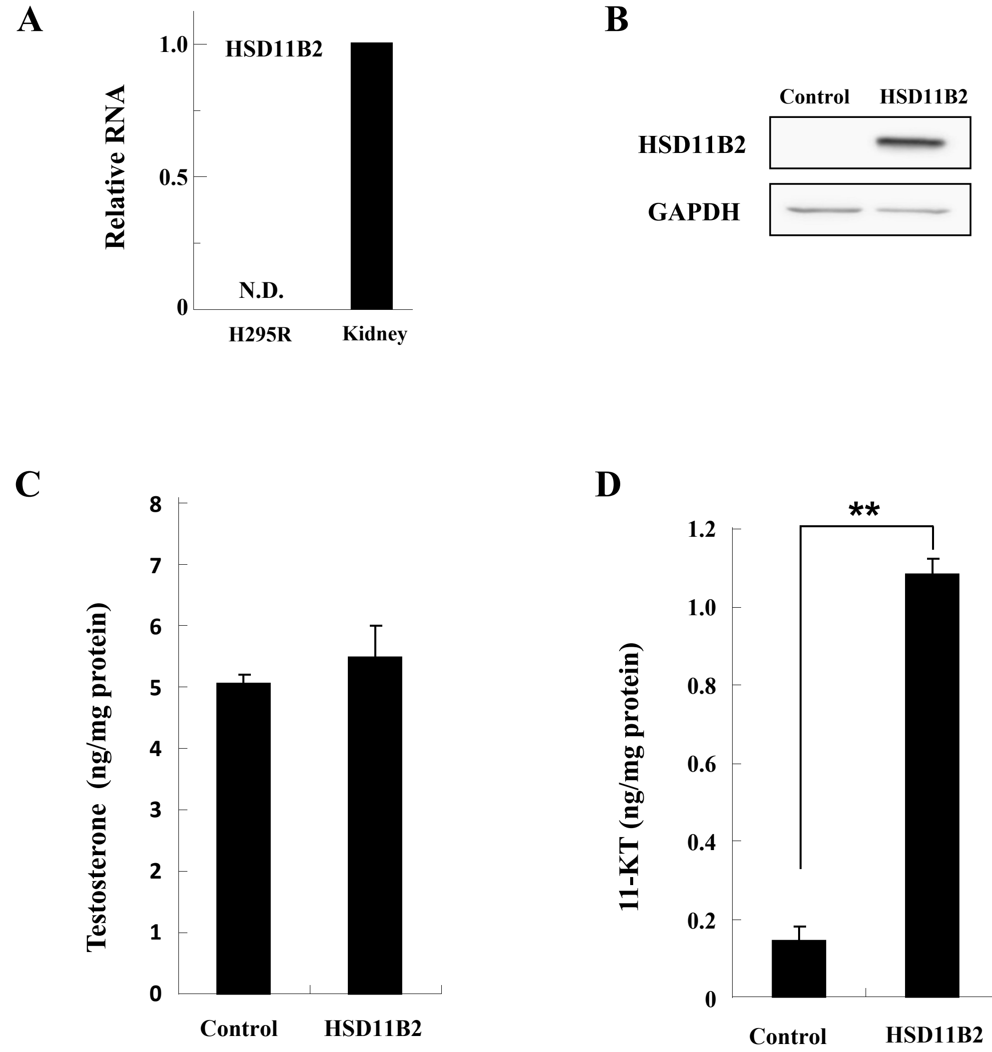
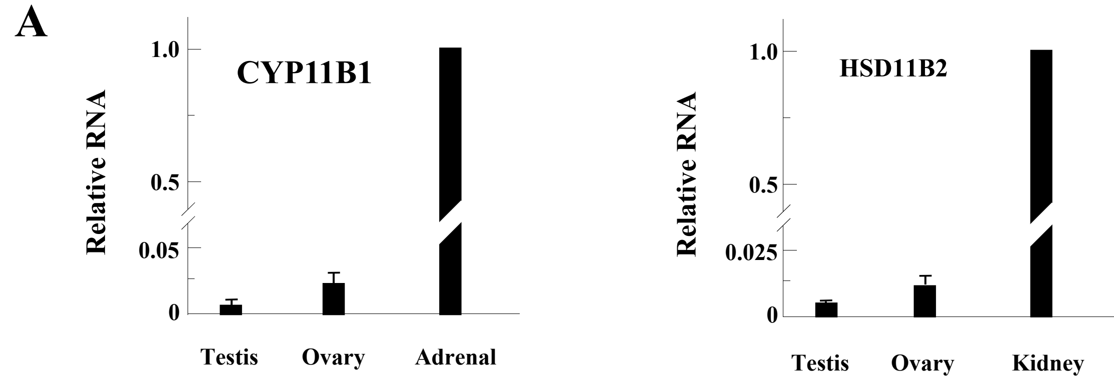
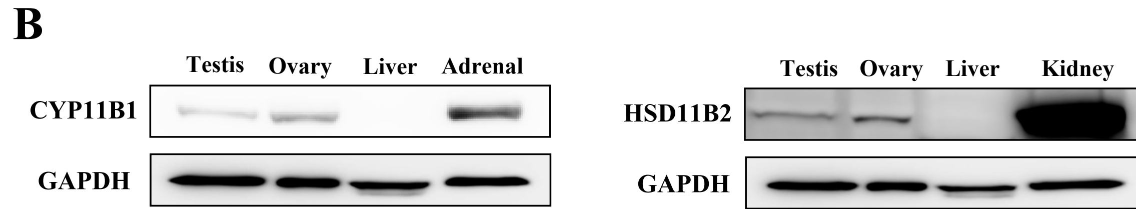
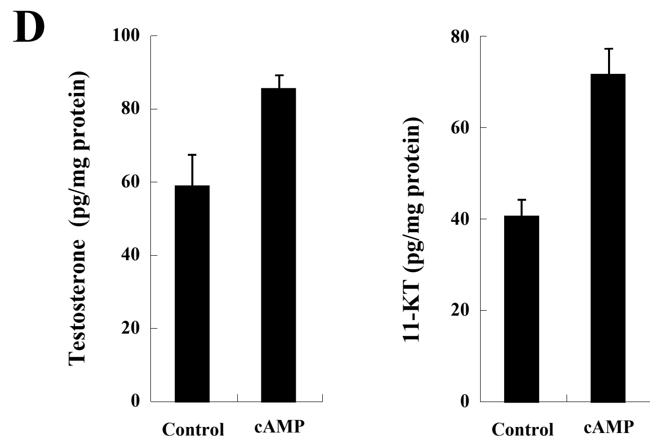


Figure 3







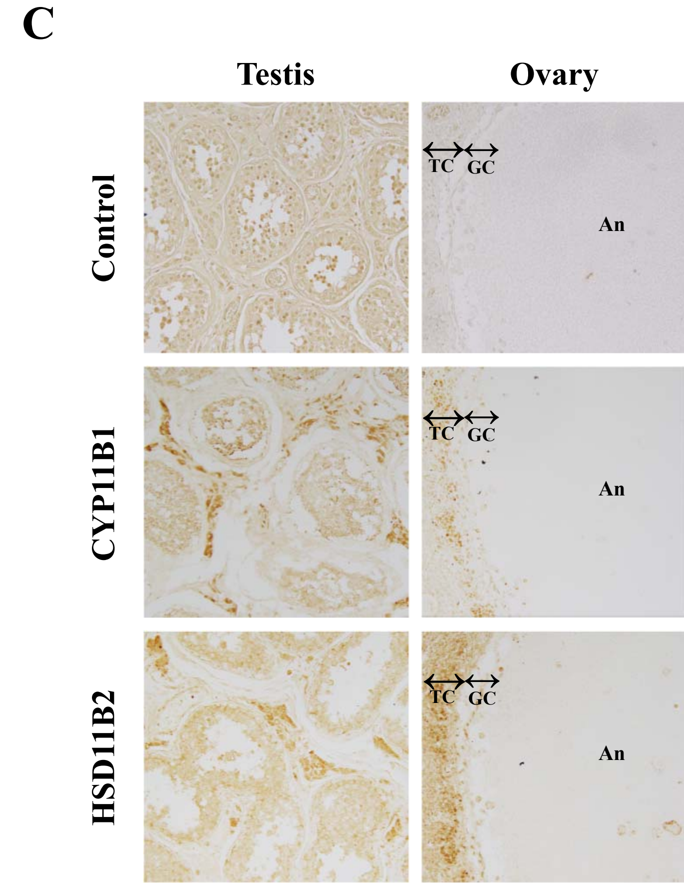
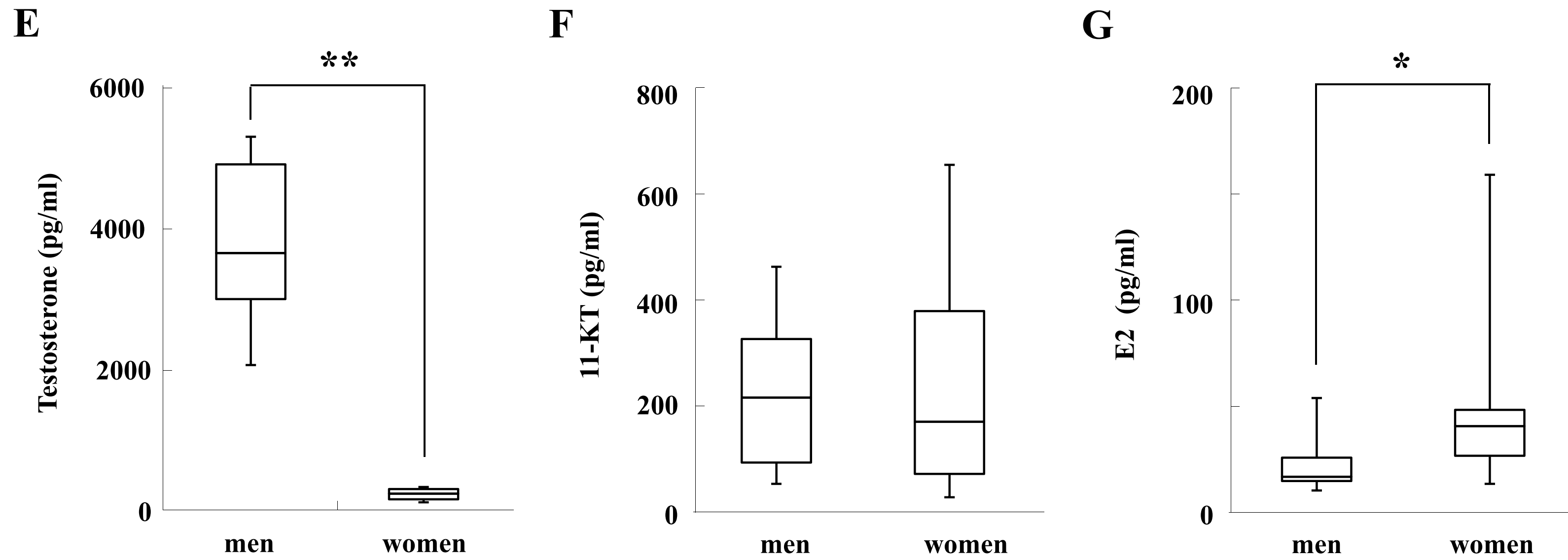


Figure 3 (continued)



H

	Men	Women
testosterone (pg/ml)	4548±625	225±26
11-KT (pg/ml)	234±47	250±66
E_2 (pg/ml)	23±4.3	54±14.2

Figure 4

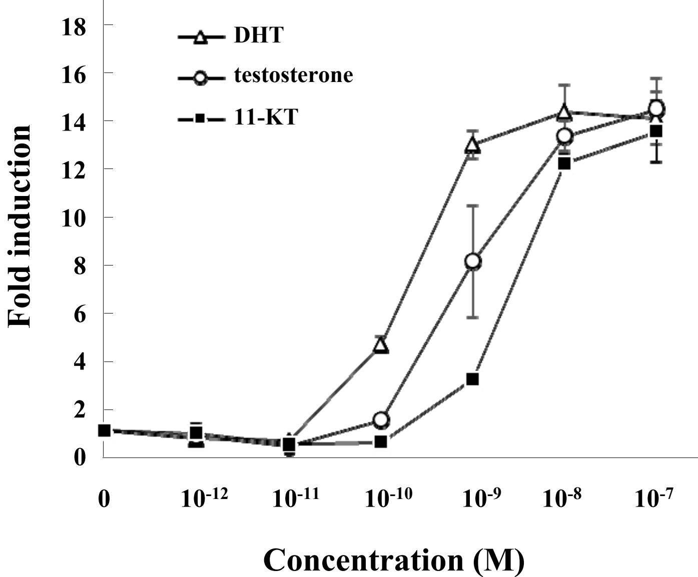
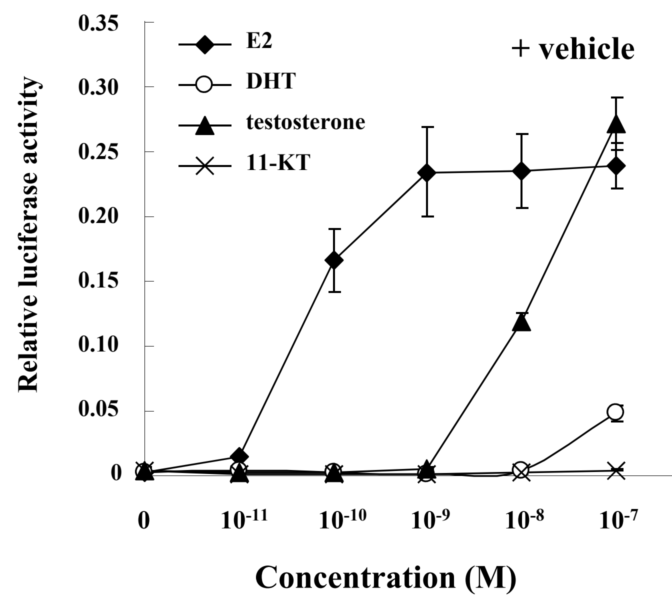


Figure 5



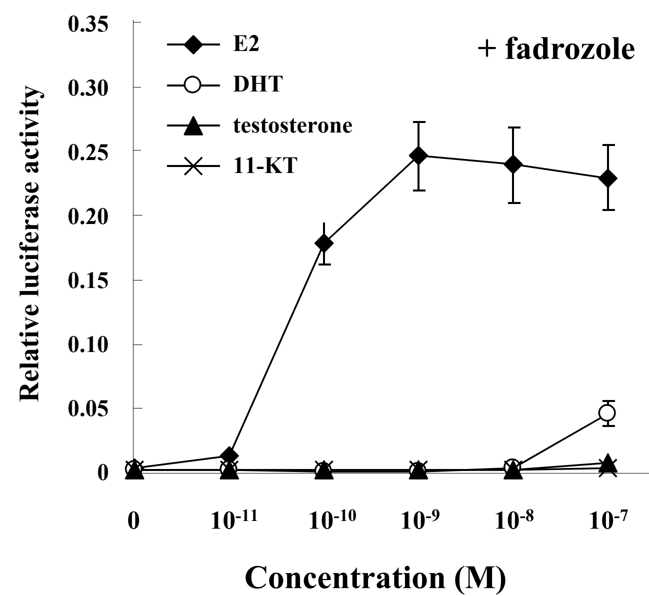
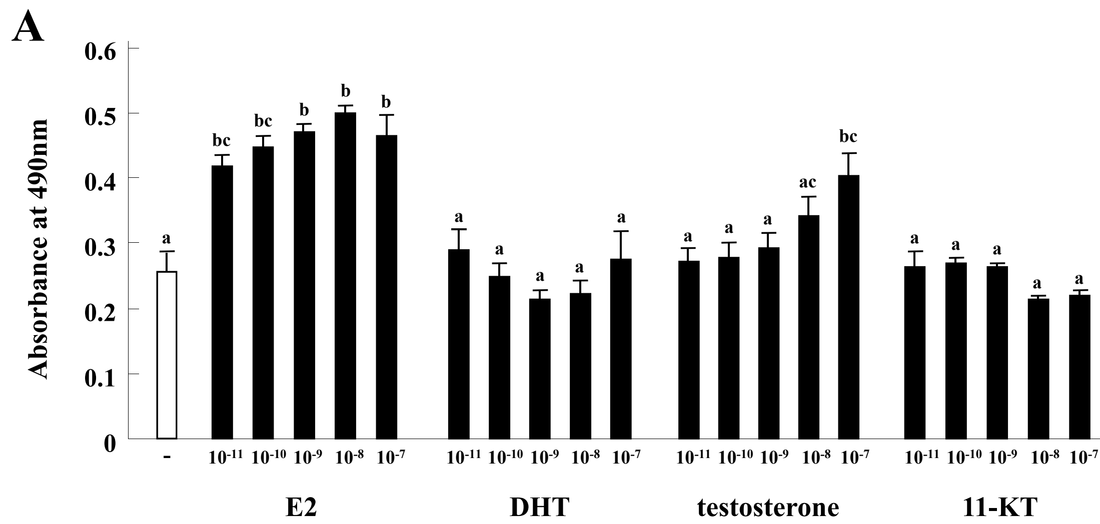


Figure 6

A



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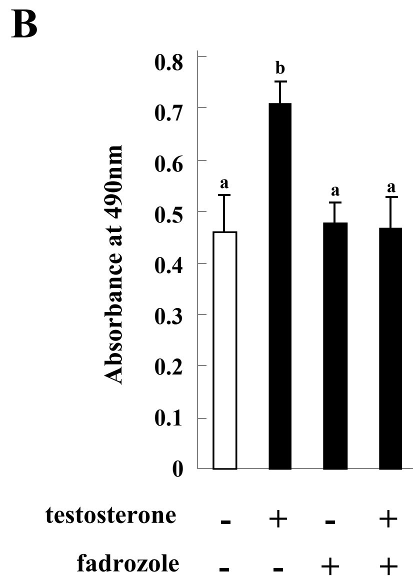

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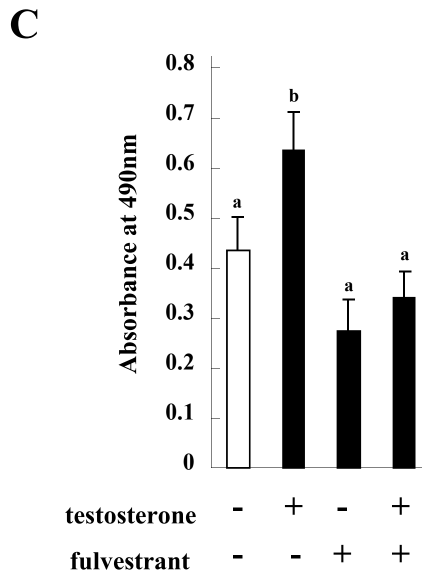

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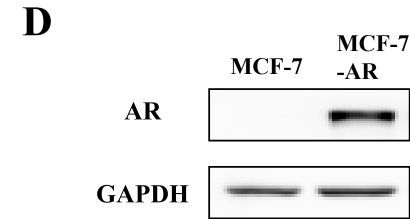

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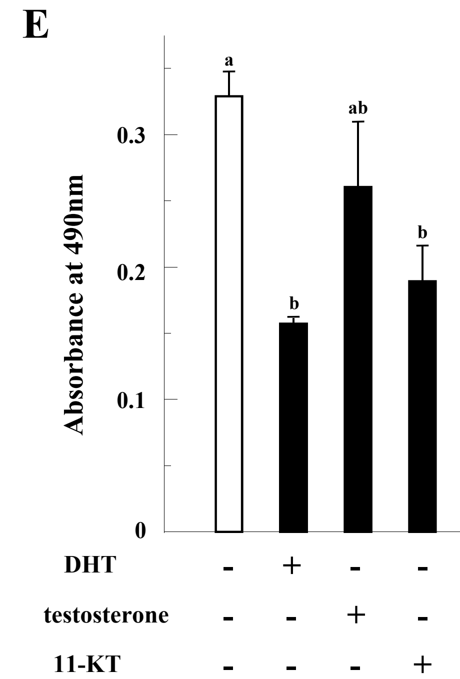
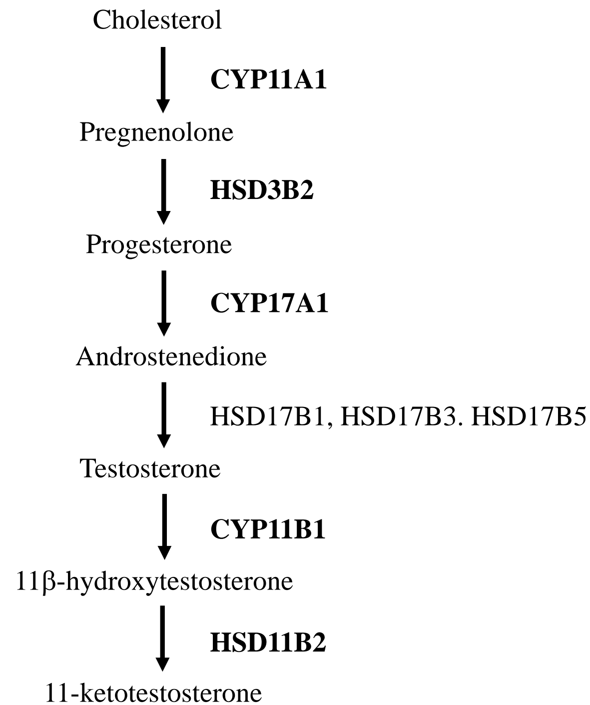


Supplemental Table 1. Primers used in each experiment.

PCR primers	Forward primers	Reverse primers
RT-PCR		
HSD17B5	F-atggcagtgtgaagagaga	R-tctagcaatttactccggttga
qPCR		
CYP17A1	F-gtggagaccaccacctctgt	R-gctgaaacccacattctggt
CYP11B1	F-tgaatccagaagtgctgtcg	R-gcagcaccttcttcttcagg
HSD11B2	F-gacctgaccaaaccagaaggaga	R-ccgcatcagcaactacttca
CYP19A1	F-atgaagctctgcaggccc	R-tcaacacgtccacatagccc

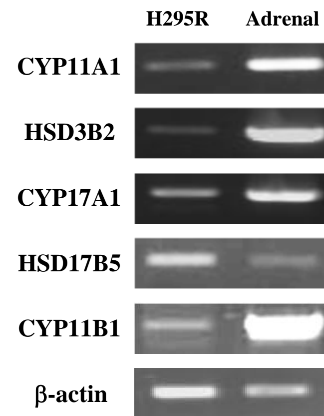
Supplemental table 2. Genes induced by Overexpression of SF-1 in Human Ovarian Granulosa Cell Tumor-derived KGN Cells.

Gene Symbol	Gene Name	Fold Change
PLIN1	perilipin	1061.9
GSTA1	glutathione S-transferase alpha 1	554.0
AADAC	arylacetamide deacetylase (esterase)	551.0
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	429.5
HSD3B2	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	397.8
AOC3	amine oxidase, copper containing 3 (vascular adhesion protein 1)	341.8
LBP	lipopolysaccharide binding protein	283.5
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4	281.7
DLK1	delta-like 1 homolog (Drosophila)	196.5
CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide 1	153.6
ITIH3	inter-alpha (globulin) inhibitor H3	131.1
KCNK3	potassium channel, subfamily K, member 3	112.4
GSTA3	glutathione S-transferase alpha 3	109.9
HES6	hairy and enhancer of split 6 (Drosophila)	99.9
LBP	lipopolysaccharide binding protein	94.9
RASGEF1A	RasGEF domain family, member 1A	94.8
APOA1	apolipoprotein A-I	80.3
CXCL14	chemokine (C-X-C motif) ligand 14	72.0
RBP4	retinol binding protein 4, plasma	56.6
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1	51.6
CYP11B1	cytochrome P450, family 11, subfamily B, polypeptide 1	46.9
SLC13A2	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2	39.2
LOC731779	hypothetical protein LOC731779	34.5
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1	33.4

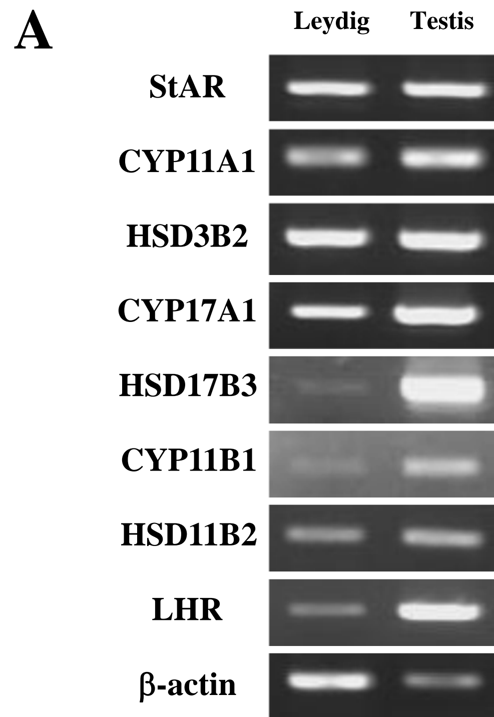


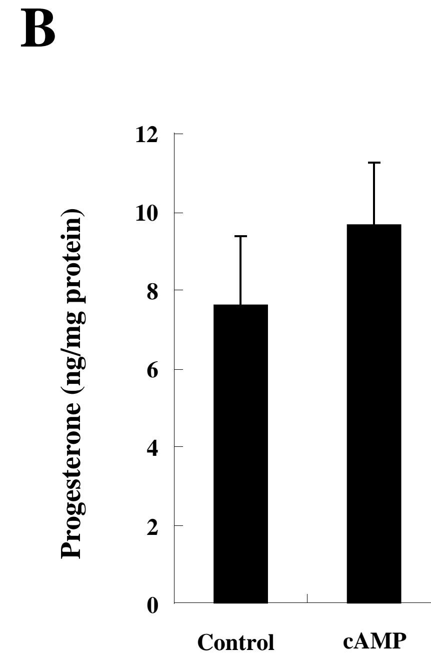
Supplemental Figure 1.

The enzymes and pathways for the synthesis of 11-KT. The enzymes, indicated by bold letters, represent genes that can be induced by SF-1 introduction in DNA microarray analyses.



Supplemental Figure 2.
Expression of the enzymes for the synthesis of 11-OHT in H295R and Adrenal. mRNA expression of each gene was analyzed by RT-PCR.





Supplemental Figure 3.

A, Expression of StAR, the enzymes for the synthesis of 11-KT and LH receptor in Leydig cells and testis. mRNA expression of each gene was analyzed by RT-PCR. B, Production of progesterone by Leydig cells with or without cAMP were measured by EIA.