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## **ACTH and prolactin synergistically and selectively regulate CYP17 expression and adrenal androgen production in human foetal adrenal organ cultures**

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1 **ACTH and prolactin synergistically and selectively regulate CYP17 expression**  
2 **and adrenal androgen production in human foetal adrenal organ cultures**

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39 **Short title:** Steroidogenesis in HFA organ cultures

40  
41 **Keywords:** human foetal adrenal gland, ACTH, prolactin, steroidogenesis, gene expression

42  
43 **Abbreviations:** ACT, adrenocortical tumours; ACTH, adrenocorticotrophic hormone; DHEA,  
44 dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DZ, definitive zone; FZ,  
45 foetal zone; GW, gestational weeks; HPS, Hematoxylin-Phloxine-Safran; IHC,  
46 immunohistochemistry; LC-MS/MS: liquid chromatography coupled to tandem mass  
47 spectrometry; PBS, phosphate-buffered saline; PRL, prolactin; PRLA, prolactin-secreting  
48 pituitary adenomas; PRLR, prolactin receptor; RT, room temperature; RT-qPCR, reverse  
49 transcription-quantitative polymerase chain reaction; TZ, transitional zone.

50 **Abstract**

51

52 **Objective:** The essential role of ACTH on the growth and function of the human foetal adrenal  
53 (HFA) has long been recognized. In addition, many studies have suggested a role of the  
54 pituitary hormone prolactin (PRL) in the regulation of the HFA, but the effects of this hormone  
55 on steroidogenesis and gene expression are still unknown. Our objective was to investigate  
56 the effect of ACTH and PRL on the steroidogenic capacities of the HFA.

57 **Design:** *In vitro/ ex vivo* experimental study.

58 **Methods:** We used a hanging drop *in vitro* organ culture system. First trimester HFA samples  
59 were cultured for 14 days in basal conditions or treated with ACTH, PRL or a combination of  
60 the two (3 to 11 replicates depending on the experiment). Steroids were measured by liquid  
61 chromatography/tandem mass spectrometry or immunoassay, gene expression by RT-qPCR,  
62 protein expression by immunoblot.

63 **Results:** ACTH significantly increased corticosterone, cortisol and cortisone production, both  
64 by itself and when used together with PRL. PRL stimulation by itself had no effect. Combined  
65 stimulation with ACTH+PRL synergistically and selectively increased adrenal androgen (DHEAS  
66 and  $\Delta$ 4-androstenedione) production and CYP17A1 expression in the HFA, while treatment  
67 with each single hormone had no significant effect on those steroids.

68 **Conclusions:** These results have important implications for our understanding of the  
69 hormonal cues regulating adrenal steroidogenesis in physiological and pathological conditions  
70 in the HFA during the first trimester and warrant further studies to characterize the molecular  
71 mechanisms of converging ACTH and PRL signalling to regulate *CYP17A1* expression.

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79 **Significance**

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81 Here we have shown that combined treatment of human foetal adrenal organ cultures with  
82 ACTH and prolactin significantly increased androgen production and CYP17 expression. These  
83 results show that ACTH and PRL may act in concert to regulate the function of the human  
84 foetal adrenal gland and may provide a mechanistic explanation to many studies showing  
85 increased circulating adrenal androgen levels in hyperprolactinemic patients. Our results have  
86 important implications for our understanding of the hormonal cues regulating adrenal  
87 steroidogenesis in physiological and pathological conditions during foetal and post-natal life.

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## 92 Introduction

93

94 In humans and in some primate species the structure of the cortical part of the adrenal gland  
95 presents peculiar characteristics during the foetal life compared to the adult<sup>1</sup>. Before birth,  
96 most of the gland volume is occupied by the foetal zone (FZ), which is made up by large,  
97 eosinophilic cells, surrounded by the outer definitive zone (DZ) harbouring small, proliferating  
98 basophilic cells, separated from the FZ by a transitional zone (TZ) with intermediate cytological  
99 characteristics<sup>1</sup>. After birth, rapid involution of the FZ takes place causing a dramatic fall in the  
100 weight of the adrenal gland, with progressive differentiation of the DZ to form the  
101 characteristic zones (*zona glomerulosa*, *fasciculata* and, later, *reticularis*) of the adult adrenal  
102 cortex. The different zones of the foetal adrenal have distinct steroidogenic capacities. The FZ  
103 expresses the cytochrome CYP17A1 at high levels and produces large quantities of  
104 dehydroepiandrosterone sulfate (DHEAS), which is largely metabolized into oestrogens in the  
105 placenta. *HSD3B2* is expressed in the DZ-TZ and allows for biosynthesis of cortisol<sup>2,3</sup>.

106 While the essential role of the pituitary hormone ACTH on the growth and function of  
107 the human foetal adrenal (HFA) has long been recognized, the effect of other factors is still a  
108 matter of debate. Winters *et al.* have suggested a role for prolactin (PRL) in the regulation of  
109 HFA function, based on the finding that PRL levels progressively increase, while ACTH levels  
110 decrease, in the foetus as gestation progresses and then rapidly decrease after birth<sup>4,5</sup>. PRL  
111 was also reported to increase secretion of DHEAS and cortisol by HFA *in vitro*<sup>6</sup> and to increase  
112 serum DHEA levels after infusion in the baboon foetus<sup>7</sup>. A role of PRL as a regulator for HFA  
113 growth and function is also consistent with its shared phylogenetic origin with growth  
114 hormone (GH)<sup>8</sup>, its stimulating effects on visceral size in several species<sup>9,10</sup> and with the high  
115 levels of the PRL receptor (PRLR) expressed in the HFA<sup>11,12</sup>. However, other authors have  
116 suggested that the HFA may become more sensitive to the action of ACTH during late  
117 pregnancy<sup>1</sup> and that factors other than PRL can regulate growth and function of the HFA<sup>13,14</sup>.

118 We recently showed that, among all adrenal steroids, DHEAS levels are selectively  
119 increased in patients with PRL-secreting pituitary adenomas (PRLA) compared with patients  
120 with hormonally inactive pituitary adenomas and that they significantly decrease after  
121 dopamine agonists therapy, suggesting a direct effect of PRL in the regulation of DHEAS  
122 production<sup>15</sup>. Considering the reported effects of PRL on steroid hormone secretion by the  
123 HFA, we set out to investigate the consequences of treatment with ACTH, PRL and of the

124 combined treatment by both pituitary hormones on steroidogenic gene expression and  
125 steroid production in HFA using a hanging drop organ culture system. Here we show that the  
126 combined treatment of HFA organ cultures with ACTH and PRL, but by neither hormone alone,  
127 significantly and selectively increased CYP17A1 mRNA and protein expression in HFA and  
128 adrenal androgen (DHEAS and  $\Delta$ 4-androstenedione) levels in the culture medium.

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130

## 131 **Materials and methods**

### 132 ***HFA organ culture***

133 HFA were collected under a protocol approved by the Agence de la Biomédecine (PFS 21-014)  
134 after obtaining informed consent from the women undergoing voluntary termination of  
135 pregnancy. We established hanging drop HFA organ cultures according to a published  
136 method<sup>16</sup>. Briefly, adrenal glands were dissected from foetuses aged 8-13 GW (Fig. 1A). They  
137 were cut into approximately 1 mm<sup>3</sup> pieces which were placed on the lid of an inverted Petri  
138 dish, each one inside a 40  $\mu$ l drop of DMEM/F12 (Gibco) containing 10% FCS, 1% ITS+  
139 (Corning), penicillin/streptomycin (Gibco), and cultured at 37°C in 5% CO<sub>2</sub> (Fig. 1B). 10 ml PBS  
140 were placed in the dish to avoid dehydration. Some samples were cultured in medium only,  
141 while other samples were treated with medium supplemented with ACTH (human 1-39,  
142 #A0423 Sigma-Aldrich; 1 nM), recombinant human PRL (#682-PL-050, R&D Systems; 500  
143 ng/ml) or both (Fig. 1C). The ACTH concentration we used is the same employed by Melau *et*  
144 *al.* in their study<sup>16</sup>, while the PRL concentration we used is in the upper range of its plasma  
145 levels during pregnancy. The culture lasted for 14 days, with medium replaced every 2-3 days  
146 and pooled during the second week for steroid measurement. For BrdU labelling, some  
147 fragments were incubated in medium containing 10  $\mu$ g/ml BrdU (#B5002, Sigma-Aldrich) for  
148 6 hours before harvesting. At the end of culture, the HFA fragments were either snap-frozen  
149 in liquid nitrogen and kept at -80°C until RNA and protein extraction or formalin-fixed and  
150 paraffin embedded for immunohistochemistry (IHC). In total, 11 independent replicates of  
151 HFA cultures (7 from male foetuses, 4 from female foetuses) were established.

152

### 153 ***Steroids measurement***

154 Progesterone, 17-hydroxyprogesterone, 11-deoxycortisol, corticosterone, cortisone, cortisol,  
155  $\Delta$ 4-androstenedione and testosterone were measured by liquid chromatography using an  
156 Ultra Performance Liquid Chromatography 1290 Infinity II system (Agilent Technologies)  
157 coupled to a 6495C tandem mass spectrometer (Agilent Technologies), as described<sup>17</sup>. DHEAS  
158 was measured by immunoassay (Cobas 801e, Roche). The concentration of steroids in each  
159 pooled sample was normalized by dividing the concentration value by the weight of the  
160 corresponding adrenal fragment. Data for ACTH-, PRL- and ACTH+PRL-treated samples are  
161 expressed as fold differences compared to samples cultured in medium only. Steroid  
162 concentration values were calculated from 4 to 11 biological replicates for each condition.

163

#### 164 ***RNA isolation, dosage, quality assessment and treatment by DNase***

165 Total RNA from HFA fragments cultured in hanging drops was extracted by phenol/guanidine-  
166 based lysis followed by silica membrane purification. Briefly, HFA fragments were transferred  
167 to Lysing Matrix D – containing tubes (MP Biomedicals) for homogenization with 700  $\mu$ l of  
168 QIAzol Lysis Reagent (#79306 Qiagen). Samples were processed in the Fast Prep-24 Classic  
169 bead beating grinder and lysis system (MP Biomedicals) for 40 seconds (sec) at a speed setting  
170 of 6.0 and then incubated on the benchtop at room temperature (RT) for 5 minutes (min) to  
171 promote nucleoprotein complexes dissociation. After the addition of 140  $\mu$ l of chloroform  
172 samples were vigorously shaken, then incubated at RT for further 2-3 min and centrifuged for  
173 15 min at 12,000g at 4°C. The upper aqueous phase was then transferred to a new collection  
174 tube, 1.5 volumes of 100% ethanol were added and the solution was mixed thoroughly by  
175 pipetting up and down several times. Each sample was then transferred to a RNeasy Mini spin  
176 column and processed using the miRNeasy Mini kit (#217004 Qiagen) according to the  
177 manufacturer's instructions. For the final elution, 25  $\mu$ l of RNase-free water per sample was  
178 employed. RNA concentrations were determined by a NanoDrop 2000 instrument (Thermo  
179 Fisher Scientific) and their integrity was controlled by running an aliquot of each sample on a  
180 1% agarose gel stained with ethidium bromide. Samples were then subjected to DNase  
181 treatment using the DNA-free DNA Removal kit (#AM1906 Invitrogen) according to the  
182 manufacturer's instructions.

183

#### 184 ***Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)***

185 A total of 100 ng of total RNA was reverse transcribed using SuperScript IV Reverse  
186 Transcriptase (#18090010 Invitrogen). RT-qPCR was performed using the SYBR Green I dye  
187 assay on a LightCycler 480 (Roche Applied Science) instrument using cyclophilin (*PPIA*) as a  
188 reference transcript. Primer sequences are reported in Table 1. Gene expression was  
189 calculated from 9 biological replicates for each condition, each one processed in duplicate.

190

### 191 ***Immunoblotting***

192 Proteins were extracted from HFA samples with RIPA buffer and proteins quantified by the  
193 Lowry assay. Immunoblots were performed as previously described<sup>18</sup>. Primary antibodies  
194 were rabbit anti-PARP (#9542, Cell Signaling Technology), rabbit monoclonal anti-CYP17A1  
195 (#ab134910, 1:1000; Abcam) and mouse monoclonal anti-GAPDH (#CB1001, 1:1000; Sigma-  
196 Aldrich) antibodies. Secondary antibodies were HRP-conjugated anti-rabbit (#NA-934, 1:5000;  
197 GE Healthcare) and anti-mouse (#NA-931, 1:5000; GE Healthcare) antibodies. Immunoblot  
198 band intensities were quantified by the Image J software (<https://imagej.net/ij>). The ratio  
199 between the full-length (116 kDa) and cleaved (89 kDa) PARP band intensities was calculated  
200 for each sample and then normalized by the corresponding GAPDH signals. CYP17A1 band  
201 intensities for each sample were normalized by the corresponding GAPDH signals. Protein  
202 expression was calculated from 3 biological replicates for each condition.

203

### 204 ***IHC***

205 After formalin fixation and paraffin embedding, HFA fragments were cut in 3 µm- thick  
206 sections and some sections stained with HPS (Hematoxylin-Phloxine-Safran). IHC was  
207 performed employing the streptavidin-biotin and peroxidase method on an automated  
208 immunohistochemical system (Benchmark ULTRA, Ventana) according to the manufacturer's  
209 instructions and using the reagents supplied with the kit<sup>19</sup>. Briefly, after deparaffinization and  
210 rehydration, sections were subjected to antigen retrieval (Heat-Induced Epitope Retrieval  
211 [HIER] method, 8 min at 100 °C, pH 6). Tissue sections were treated with H<sub>2</sub>O<sub>2</sub> to block  
212 endogenous peroxidase, followed by an additional washing procedure with the  
213 manufacturer's buffer, and pretreated by ULTRA CC1 (Ventana). Slides were then incubated  
214 with the following antibodies: anti-NCAM/CD56 (#NCL-L-CD56-504, 1:100; Leica Biosystems),  
215 anti-SPARC (#AF941, 1:1200; R&D Systems), anti-SF-1 (#ab217317, 1:2000; Abcam), anti-Ki67  
216 (#M7240, 1:100; Agilent) and anti-BrdU (#MCA2483, 1:100; Bio-Rad). After washing, tissues



217 were incubated with biotinylated antibodies, followed by the streptavidin-biotin complex, the  
218 amplification reagent, and the streptavidin-peroxidase conjugate. Tissue staining was  
219 visualized with a DAB substrate chromogen solution (ultraView DAB Detection Kit, Ventana).  
220 Counterstaining was performed using hematoxylin and Bluing Reagent (Ventana).

221

### 222 ***Statistical analyses***

223 They were performed using the Prism software, version 9.4 (GraphPad), considering a *p*-value  
224 <.05 as the threshold for significance. One-way ANOVA with Dunnett's multiple comparisons  
225 test was used to calculate the significance of differences in steroid concentrations and  
226 CYP17A1 protein expression following the hormonal treatments. RT-qPCR data were analysed  
227 using REST (Relative Expression Software Tool; [https://www.gene-](https://www.gene-quantification.de/rest.html)  
228 [quantification.de/rest.html](https://www.gene-quantification.de/rest.html))<sup>20</sup>.

229

230

## 231 **Results**

232

### 233 ***ACTH and PRL synergistically and selectively modulate steroid production in HFA organ*** 234 ***cultures***

235 Hanging drop organ cultures are able to maintain *in vitro* the differentiated function of HFA,  
236 recapitulating the expression of zonal markers and the production of a variety of steroids<sup>16</sup>.  
237 We took advantage of this system to investigate the effects of ACTH, PRL and their  
238 combination on steroid production and steroidogenic gene expression. We cultured the HFA  
239 fragments with medium alone, medium supplemented with ACTH (1 nM), PRL (500 ng/ml) or  
240 their combination for 14 days (Fig. 1C). HFA organ cultures express the steroidogenic  
241 transcription factor SF-1, markers of both the DZ (NCAM/CD56) and the FZ (SPARC), and  
242 scattered proliferating cells (Ki67/BrdU positive) (Fig. 1D). Conditioned media (replaced every  
243 2-3 days) were pooled during the second week of culture and steroids concentrations were  
244 measured by LC MS/MS or immunoassay (DHEAS). A diagram of steroidogenesis in the HFA is  
245 shown in Fig. 2A. DHEAS was by far the most abundantly produced steroid by the HFA organ  
246 cultures with concentrations reaching hundreds of micromolar, while the levels of the other  
247 steroids measured varied from the nanomolar to the micromolar (Fig. 2B). The different  
248 treatments had no effect on the density of BrdU-positive (proliferating) cells and levels of

249 cleaved PARP (apoptosis marker) in the HFA cultures (Fig. S1). Among all the steroids  
250 measured, treatment with either ACTH alone or the ACTH+PRL combination significantly  
251 increased the levels of corticosterone, cortisol and cortisone, while only the combined ACTH-  
252 PRL treatment significantly increased the production of the androgens DHEAS and  $\Delta$ 4-  
253 androstenedione by the HFA organ cultures (Fig. 2C). Treatment with PRL alone had no  
254 significant effect on the levels of any of those steroids. In addition, treatment with ACTH, PRL  
255 and the ACTH+PRL combination had no significant effect on progesterone, 17-  
256 hydroxyprogesterone, 11-deoxycortisol and testosterone levels (Fig. S2).

257

### 258 ***Gene expression regulation by ACTH and PRL in HFA organ cultures***

259 To clarify the mechanisms underlying the differential regulation of steroid production by ACTH  
260 and by the ACTH+PRL combined treatment, we measured the expression of transcripts  
261 encoding proteins involved in steroidogenesis, adrenocortical function and PRL signalling in  
262 the HFA fragments recovered after the end of cultures under the different conditions. ACTH  
263 treatment significantly increased the expression of *CYP11B1*, a key gene implicated in the  
264 synthesis of glucocorticoids<sup>21</sup> (Fig. 3). Conversely, PRL treatment increased the expression of  
265 its known target gene *CISH*<sup>22</sup> (Fig. 3). Remarkably, the ACTH+PRL combined treatment  
266 selectively increased the expression of *CYP17A1*, encoding the CYP17A1 enzyme which  
267 catalyses the final step in the biosynthesis of the adrenal androgens DHEAS and  $\Delta$ 4-  
268 androstenedione (Fig. 2A, 3).

269

### 270 ***The CYP17A1 protein is selectively upregulated by combined ACTH and PRL treatment in HFA*** 271 ***organ cultures***

272 Consistent with the RNA results, the CYP17A1 protein was selectively upregulated by the  
273 combined ACTH+PRL treatment and not by treatments with ACTH or PRL alone (Fig. 4).

274

275

## 276 **Discussion**

277 A number of studies have described the association of hyperprolactinemia with increased  
278 adrenal androgen levels and an increased adrenocortical response to ACTH in patients with  
279 high circulating PRL levels<sup>12,23-29</sup>. We have also reported that in patients with PRLA DHEAS was

280 selectively upregulated while the circulating levels of all the other adrenal steroids were not  
281 significantly modified both in basal conditions and after a short-term stimulation with ACTH<sup>15</sup>.  
282 Altogether, these data represent strong clinical evidence for an important role of PRL in the  
283 regulation of the production of androgenic steroids by the adrenal cortex. Unfortunately, it is  
284 not possible to study the regulatory mechanisms of adrenal steroidogenesis by PRL *in vitro*  
285 using the NCI-H295R cell line or the other currently available adrenocortical cell lines since  
286 they do not express the PRLR at both the transcript and the protein level (our unpublished  
287 observations). In addition, the results of early experiments showing an effect of purified PRL  
288 on adrenal steroidogenesis *in vitro* may have been confounded by the contamination of the  
289 PRL preparations with other pituitary hormones, including ACTH<sup>30</sup>. For these reasons, in the  
290 present study we made use of first trimester HFA organ cultures as a model to investigate the  
291 effects of ACTH, PRL and their combination on steroid production and gene expression in the  
292 adrenal, using synthetic ACTH peptide and recombinant PRL protein. Here we have shown  
293 that in the HFA organ culture system PRL, together with ACTH, specifically and selectively  
294 stimulates the production of the androgens DHEAS and  $\Delta$ 4-androstenedione, while  
295 stimulation with ACTH alone was sufficient to stimulate glucocorticoid (corticosterone and  
296 cortisol) production and *CYP11B1* expression<sup>21</sup>, without additive effects of PRL. This action on  
297 steroidogenesis is consistent with the selective upregulation of *CYP17A1* mRNA and protein  
298 by ACTH+PRL combined treatment. These data are also consistent with the *in vivo* studies  
299 cited before showing that PRL has a selective stimulatory effect on adrenal androgen  
300 production<sup>12,23-29</sup>. However, our data differ from the results reported in the Melau *et al.*  
301 study<sup>16</sup>, which showed that ACTH alone was able to increase the levels of all measured steroids  
302 (including DHEAS and  $\Delta$ 4-androstenedione) in HFA organ cultures using the same hanging  
303 drop system we used in our study. We do not know the reasons of those discrepancies, but  
304 we can speculate that they can be, at least in part, attributed to different potencies of the  
305 ACTH used in the two series of experiments (we used human ACTH 1-39, while the type of  
306 ACTH peptide used is not specified in ref. 16) and/or to the presence of different  
307 concentrations of PRL in the foetal bovine serum used for the cultures.

308 Our study has important limitations since our experiments were performed in HFA  
309 cultures from first trimester fetuses and during a relatively limited time (14 days) of culture  
310 *in vitro*. Since circulating PRL levels greatly increase in the foetus from the second trimester

311 onward, it would be very interesting to study the chronic effects of PRL on foetal adrenal gland  
312 growth and steroidogenesis during the last part of the foetal development. Concerning this  
313 point, experiments in the baboon foetus at midgestation have shown that both ACTH and PRL  
314 can stimulate DHEA production in the foetal adrenal<sup>7</sup>. However, we have to consider that  
315 during the first trimester placental lactogen produced by the placenta, a potent agonist of the  
316 PRLR, may exert the same effects as PRL on the HFA.

317 We have previously formulated the hypothesis that ACTH and PRL may act in concert  
318 to regulate DZ and FZ growth and function in the HFA. In particular, we have suggested that  
319 the dramatic fall in foetal PRL levels which takes place after birth may trigger FZ regression,  
320 while the DZ can expand and differentiate under the control of ACTH<sup>12</sup>. Another potential  
321 effect of PRL would be to function as a growth factor for adrenocortical tumours (ACT)  
322 originating from the HFA. ACT in young children are derived from HFA “breaking bad” because  
323 of germline mutations in the *TP53* tumour suppressor gene with consequent loss of  
324 heterozygosity or other genetic alterations<sup>31</sup>. We can speculate that PRL may favour growth  
325 and hormonal secretion of ACT, being implicated in a self-sustaining circuit including  
326 peripheral conversion of androgens produced by the tumour into oestrogen, which in turn  
327 stimulates PRL production in the pituitary lactotrophs<sup>12</sup>. The action of PRL as a growth factor  
328 for ACT would be consistent with its effects on a variety of tumour types<sup>32</sup>. Furthermore, the  
329 effects of PRL on adrenal androgen production suggest that it may also have a role in  
330 adrenarche, the physiological process consisting in the development of axillary and pubic hair  
331 which is related to the increase in adrenal androgen production normally beginning in  
332 childhood, concomitant with the development of the adrenal *zona reticularis*<sup>33</sup>. Remarkably,  
333 congenital pituitary disorders causing selective PRL/GH deficiency, without concomitant ACTH  
334 deficiency, are associated with absence or delay in adrenarche<sup>34,35</sup>. On the other hand,  
335 premature adrenarche has been reported in the case of a boy with PRLA<sup>36</sup>. In addition, genetic  
336 variants in the *PRLR* gene were described to be associated to circulating DHEAS levels in  
337 children<sup>37</sup>. All these data in different physiopathological contexts converge towards a strong  
338 association between PRL and adrenal androgen production. The results presented in our study  
339 have important implications for our understanding of the hormonal cues regulating adrenal  
340 steroidogenesis in physiological and pathological conditions and warrant further studies to

341 characterize the molecular mechanisms of converging ACTH and PRL signalling to regulate  
342 *CYP17A1* expression.

343

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348

### 349 **Disclosure**

350 All authors have nothing to disclose.

351

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355

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484 **Figure legends**

485

486 **Figure 1. HFA organ culture and diagram of the experiments.** (A) Foetal organs after  
487 dissection. \*testis; °adrenal gland; +kidney. (B) Images of HFA organ cultures in hanging drops.  
488 (C) Diagram of the experimental plan. After dissection, HFA were cut in about 1 mm<sup>3</sup> pieces,  
489 which were cultured in hanging drops of medium alone, medium containing ACTH (1 nM), PRL  
490 (500 ng/ml) or ACTH+PRL. The culture lasted for 14 days, with medium replaced every 2-3  
491 days and pooled during the second week for steroid measurement. For BrdU labelling, some  
492 fragments were incubated in medium containing 10 µg/ml BrdU for 6 hours before harvesting.  
493 (D) HPS staining - NCAM/CD56, SPARC, SF-1, KI67 and BrdU IHC of one HFA fragment cultured  
494 in medium only after 14 days of culture. Scale bars: 500 µm.

495

496 **Figure 2. Steroid production in HFA organ culture and its modulation by ACTH, PRL and**  
497 **combined ACTH+PRL treatment.** (A) Diagram of steroidogenesis in the HFA. FZ, foetal zone.  
498 Illustration from ref. 38. (B) Levels of different steroids in the medium of HFA fragments  
499 cultured in basal conditions. The means, the range of values and the individual data points are  
500 indicated for each steroid. Because of the large variability of the different concentrations of  
501 each steroid, values are shown in log scale. *n*=7-11. (C) Modulation of corticosterone, cortisol,  
502 DHEAS and Δ4-androstenedione production by ACTH (1 nM; blue), PRL (500 ng/ml; orange)  
503 and ACTH+PRL (red) combination in HFA organ cultures. Values were normalized by the weight  
504 of each cultured HFA fragment and expressed as fold of basal levels. The means, the range of  
505 values and the individual data points of the stimulation fold are indicated for each treatment.  
506 *n*=10. \**p*<.05, one-way ANOVA with Dunnett's multiple comparisons test.

507

508 **Figure 3. Modulation of gene expression by treatments in HFA organ cultures.** Effect of ACTH  
509 (1 nM; blue), PRL (500 ng/ml; orange) and combined ACTH+PRL (red) treatment on gene  
510 expression in HFA cultures. Expression of the indicated mRNAs was measured by RT-qPCR and  
511 expressed as fold modulation compared to basal levels. The means and the standard errors of  
512 the stimulation fold are indicated for each treatment. *n*=9. \**p*<.05, REST analysis.

513

514 **Figure 4. Modulation of P450 17A1 protein expression by treatments in HFA organ cultures.**  
515 The expression of CYP17 was measured by immunoblot and normalized by GAPDH levels in  
516 each sample. Basal expression (grey); ACTH (1 nM; blue), PRL (500 ng/ml; orange) and  
517 combined ACTH+PRL (red) treatments. The means, the range of values and the individual data  
518 points are indicated for each treatment. *n*=3. \**p*<.05, one-way ANOVA with Dunnett's multiple  
519 comparisons test.