1 **RESEARCH ARTICLE**

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3 **KISSPEPTIN IS A NOVEL REGULATOR OF HUMAN FETAL ADRENOCORTICAL**

4 **DEVELOPMENT AND FUNCTION – A FINDING WITH IMPORTANT IMPLICATIONS FOR**

5 **THE HUMAN FETO-PLACENTAL UNIT**

6

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20 **Short title:** Control of the feto-placental unit by kisspeptin

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Abstract (247 words)

Context: The human fetal adrenal (HFA) is an integral component of the feto-placental unit and important for the maintenance of pregnancy. Low kisspeptin levels during pregnancy are associated with miscarriage and kisspeptin / its receptor (Kiss1R) are expressed in the HFA. However, the role of kisspeptin in fetal adrenal function remains unknown.

Objective: To determine the role of kisspeptin in the developing human fetal adrenal (HFA).

Design: Experiments using H295R and primary HFA cells as *in vitro* models of the fetal adrenal. Association of plasma kisspeptin levels with HFA size in a longitudinal clinical study.

Setting: Academic research center and tertiary fetal medicine unit.

Participants: Thirty-three healthy pregnant women were recruited at their 12-week routine antenatal ultrasound scan.

Main outcome measures: The spatio-temporal expression of *Kiss1R* in the HFA. The production of DHEAS from HFA cells following kisspeptin treatment, alone or in combination with ACTH or CRH. Fetal adrenal volume **(**FAV) and kisspeptin levels at 4 antenatal visits (~20, 28, 34 and 38 weeks gestation).

Results: *Kiss1R* was present in the HFA from 8 weeks post conception to term and was shown for the first time within the inner fetal zone. Kisspeptin significantly increased DHEAS production in H295R and second trimester HFA cells. Serial measurements of kisspeptin confirmed a correlation with FAV growth in the second trimester, independent of sex or estimated fetal weight.

Conclusions: Kisspeptin plays a key role in the regulation of the HFA and thus the feto-placental unit particularly in the second trimester of pregnancy.

Precis (200 characters): Kisspeptin is a novel regulator of human fetal adrenocortical development and function. This suggests an important role for kisspeptin in the human feto-placental unit and maintenance of pregnancy.

Abbreviations: *Kiss1,* kisspeptin gene; *Kiss1R,* kisspeptin receptor gene*;* Kiss1R*,* kisspeptin receptor, HFA, human fetal adrenal, FZ, fetal zone; DZ, definitive zone; TZ, transitional zone; DHEAS, dehydroepiandrosterone; DHEA, dehydroepiandrosterone sulfate; *SULT2A1,* Sulfotransferase Family 2A Member 1 gene (DHEA sulphotransferase), wpc; weeks post-conception.

Introduction

Kisspeptins are a family of peptide hormones encoded by the *Kiss1* gene and are the endogenous ligands for the G-protein coupled receptor, Kiss1R in humans (1,2). Kiss1R is widely expressed in a number of human tissues including the hypothalamus and pituitary (1-3). In the latter, kisspeptin-Kiss1R signaling has a vital role in the secretion of GnRH at puberty (4,5). Kisspeptin and Kiss1R are robustly expressed in the syncytiotrophoblast cells of the placenta and may have an important role in the regulation of trophoblast invasion into the maternal uterine wall during placentation (1,6,7). In males and non-pregnant females, circulating kisspeptin levels are very low. Maternal circulating kisspeptin increases from around 8 weeks gestation by ~940 and >7000 fold in the second and third trimesters, respectively (8). At ~5 days post-partum, the levels fall to pre-pregnancy levels, implicating the placenta as the source of kisspeptin (8). Several studies suggest that low circulating maternal kisspeptin is associated with intra-uterine growth restriction and pre-eclampsia (9,10). Additionally, trophoblast expression of kisspeptin and its receptor in the first trimester is lower in women with recurrent miscarriage compared to normal pregnancies (11). Recently, a large prospective study demonstrated that plasma kisspeptin at the antenatal booking visit was reduced in women who later had miscarriages compared to those with normal pregnancies (12). Therefore there is compelling evidence to suggest that decreased kisspeptin may be a novel biomarker of placental dysfunction in pregnancy and may also identify asymptomatic pregnant women at greater risk of miscarriage. However, the mechanisms underlying these associations are unknown.

The human fetal adrenal (HFA) cortex plays a critical role in the feto-placental unit and a pivotal role in the endocrine control of pregnancy and parturition (13-15). The developing HFA is composed of 3 zones, the centrally located fetal zone (FZ), the outer definitive zone (DZ) and the transitional zone (TZ) between the FZ and DZ (13,16). At ~8-10 weeks gestation (6-8 weeks post conception, wpc), there is rapid HFA growth due to enlargement of the FZ which accounts for most of the fetal adrenal mass by mid-gestation (16–20 weeks gestation, 14-18wpc) (13,16,17). Except for transiently in the first trimester, FZ cells do not express the enzyme, 3βHSD, required for glucocorticoid and mineralocorticoid production. Therefore HFA steroidogenesis is characterized by early transient cortisol production, which is then suppressed until late gestation (16,17). The principal steroid output from the FZ in humans is DHEA, which is sulphated to

96   DHEAS by SULT2A1 before secretion (13). The placenta cannot produce estrogens *de novo* as it lacks the

97   cytochrome P450 CYP17 enzyme (18). Therefore the functional role of the HFA is to produce steroid

98   precursors, which are converted to estrogens by the placenta (19). Placental estrogens are critical for

99   intrauterine homeostasis, fetal maturation and the activation of parturition (14,15,19). Consistent with this,

100   a disproportionate enlargement of the HFA gland FZ may accurately predict impending pre-term birth

101   (20,21). Soon after birth, the HFA undergoes rapid involution with rapid disappearance of the FZ and a

102   decrease in androgen secretion (22,23).

103

104   HFA development and function is complex and poorly understood. Although placental CRH and fetal

105   pituitary ACTH play important roles, other locally produced or placenta-derived factors must also be

106   involved (13). Interestingly, there is 50-fold higher expression of *Kiss1R* in the HFA compared to the adult

107   adrenal and *Kiss1R* expression has been confirmed in the DZ and TZ of the HFA (24). Additionally,

108   kisspeptin stimulates aldosterone production in cultured human adrenocortical H295R cells (24). Therefore,

109   the HFA may be an important target for the high levels of circulating kisspeptin in pregnancy.

110

111   We investigated the hypothesis that kisspeptin is a novel regulator of human adrenocortical development

112   and function, acting directly on the HFA to regulate fetal adrenal steroidogenesis via its receptor, Kiss1R.

113

114

Materials and Methods

Ethical approval

These studies were approved by the Brighton and Sussex Research Ethics Committee (REC reference: 12/LO/1755). For the Finnish tissue samples, ethical approval was obtained from the Ethics Committee of the Pohjois-Savo Health Care District, Finland and a permit to study human autopsy tissues and resection material was obtained from the Finnish National Authority for Medicolegal Affairs.

Adrenal tissues

Tissue blocks from human fetuses following either therapeutic termination of pregnancy, miscarriage, stillbirth or intrauterine death were obtained at autopsies performed at Kuopio University Hospital, Finland. Cryosections of frozen HFA tissue were obtained from the MRC-Wellcome Trust Human Developmental Biology Resource (HDBR) (London and Newcastle). Maternal informed consent and approval from the local National Health Authority Ethics Committees was obtained. The age of the fetal samples was estimated as previously described (25) and the embryos were staged using the Carnegie Staging classification system (26). All samples had a normal male or female karyotype (46,XY or 46,XX, respectively).

Immunofluorescence studies

Fresh HFA tissue, collected in ice-cold PBS (Sigma), was fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and embedded in Optimal Cutting Temperature compound (OCT) (Fisher Scientific, Loughborough, UK). Sections (10μm) were cut using a cryostat (Leica GM 1510S). Haematoxylin and eosin (H and E) staining was performed using standard procedures. For immunofluorescence studies, sections were blocked for 30 minutes with 10% normal goat serum in PBS and then incubated with primary antibody (or control solution) overnight at room temperature followed by 2 hours incubation with donkey anti-rabbit secondary antibody (Jackson Immunoresearch). For negative controls, the primary antibody was omitted or pre-incubated with its peptide antigen (1μg/ml) at room temperature with agitation for 30 minutes. Unbound antibody was removed by PBS-triton washes. Nuclei were stained with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride, 1μg/ml, Sigma Aldrich) and cover slips were secured with

144 fluorescent mounting media (Dako). Slides were visualized and images taken using the Zeiss LSM510 laser

145 scanning confocal microscope and the ZEN 2011 Light edition software. For details of antibodies see

146 Supplemental Table 1.

147

148 *Cell culture*

149 Ian Mason, University of Edinburgh donated the human adrenocortical carcinoma cell line (H295R; CRL-

150 2128). Primary HFA cells were derived from tissue samples obtained from the HDBR which were

151 dissected and disaggregated by incubation at 37°C in serum-free DMEM/F12 Ham (1:1 ratio) media

152 (DMEM/F12, Invitrogen) with 100 units/ml penicillin and 100µg/ml streptomycin (Pen/Strep,

153 ThermoFisher Scientific) and 2mg/ml collagenase (Sigma Aldrich), for 90 minutes. Cells were then plated

154 and grown as below. H295R and HFA cells were grown in DMEM/F12, with 2% Ultroser G (BioSepra),

155 1% ITS (1mg/ml insulin, 0.55mg/ml transferrin and 0.5µg/ml sodium selenite, Sigma Aldrich) and

156 Pen/Strep (27).

157

158 *cDNA synthesis and quantitative RT-PCR (qPCR)*

159 Total RNA was extracted from cultured H295R and HFA cells and human placental tissue using the

160 RNeasy kit (QIAGEN). HFA RNA and adult adrenal RNA were obtained from the HDBR tissues and a

161 Human Total RNA Master Panel II (Clontech), respectively and converted to cDNA using M-MLV

162 Reverse Transcriptase according to manufacturer's instructions (Promega). Quantitative RT-PCR was

163 performed in triplicate (each sample) on a Stratagene Mx3000P thermocycler using KAPA SYBR fast

164 quantitative PCR master mix with 200 nM forward and reverse primers (sequences available on request)

165 Data were analyzed using MxPro software (Stratagene, Stockport, UK). The Ct value was quantified by

166 interpolating the quantity from a standard curve made from a gel-purified amplicon. Data were normalized

167 to *GAPDH* expression and presented as a proportional increase or decrease from the calibrator (placenta or

168 adult adrenal normalized to a value of 1 for comparison).

169

170 *Cell treatments*

171 H295R and HFA cells were seeded into 6-well plates (Greiner Bio-One) and grown until 60-70%

172 confluency then incubated in 1ml serum-free media overnight. 1ml fresh serum-free media alone

173 (untreated) or containing one of the following was added for 24h: 100nM Kisspeptin, 10nM CRH, 10nM

174 ACTH or 10µM forskolin (Sigma Aldrich). CRH and ACTH directly stimulate DHEAS production in HFA

175 cells (15,28). Forskolin treatment directs steroid production towards the androgen pathway in the H295R

176 adrenocortical tumour cell line (27). DHEAS was measured in the cell media by enzyme linked

177 immunosorbent assay (ELISA) (Demeditec Diagnostics, Kiel) and the assay quality was checked for some

178 experiments by liquid chromatography–tandem mass spectrometry (LC-MS/MS). LC-MS/MS is the gold

179 standard method for quantifying steroid production, however mass spectrometry results were unavailable

180 for all the time points of interest. The kisspeptin concentration used was supraphysiological (100x maternal

181 circulating concentrations). This was decided on the basis of previously published data (24) and dose

182 response studies which showed a significant increase in DHEAS compared to untreated H295R cells with

183 10nM ($p<0.01$) and 100nM ($p<0.05$). Only 100nM kisspeptin produced a significant increase in DHEAS

184 compared to no treatment in 8-10wpc ($p<0.05$) and 15-20 HFA ($p<0.0001$) cells (Supplemental Figs. 1A, B

185 and C, respectively).

186

187 *Protein assay*

188 The cell lysate protein concentrations were determined by Bradford assay (29). Absorbance of each well at

189 595 nm (OD595) was determined using the Perkin Elmer Wallace Victor2 1420 Microplate reader. The

190 protein content of each sample was determined as previously described (29).

191

192 *Immunoblotting*

193 *Kiss1R* expression was assessed by immunoblotting following treatment of cells with kisspeptin. Cells were

194 lysed in 200 µl RIPA buffer (Sigma-Aldrich) containing protease inhibitor (complete, Mini, EDTA-free

195 Protease Inhibitor Cocktail tablets, Roche). Samples were heated to 95°C for 5 minutes in Laemmli buffer

196 (Sigma-Aldrich) and loaded on 4%–12% SDS-PAGE gels (Invitrogen). Size separated proteins were then

197 transferred to a nitrocellulose membrane and the blots were immunolabeled overnight with the anti-Kiss1R

198 antibody at 1:500 dilution or mouse monoclonal β-actin at 1:10,000 dilution (Sigma) as a loading control.

199 Visualization of the proteins was performed using Alexa-fluor 680 and 800 secondary antibodies

200 (Invitrogen) at a 1:5000 dilution and the Li-CoR Odyssey system.

201

202 *DHEAS quantification*

203 Cell media were collected from treated cells and DHEAS measurements were obtained using either the

204 DHEAS ELISA Kit (Demeditec Diagnostics, Kiel) or LC-MS/MS utilizing an optimized protocol

205 (Supplemental Methods and Supplemental Tables 2-4). Experiments were performed in triplicate and

206 repeated at least three times. The DHEAS results were corrected to the protein content of the attached cells,

207 quantified by Bradford assay.

208

209 *Clinical study design and recruitment*

210 A prospective observational study of patients with singleton, uncomplicated pregnancies was undertaken.

211 Women attending their routine antenatal ultrasound scan (USS) at ~12 weeks gestation at the Royal

212 London Hospital, London between February 2013 and April 2014 were recruited. Informed consent was

213 obtained and translators were provided where necessary. Gestational age was established during the USS

214 evaluation. Exclusion criteria included: multiple pregnancy, coexistent maternal medical conditions

215 (hypertension, preeclampsia, diabetes, thyroid, adrenal or renal disease), congenital fetal abnormalities or

216 chromosomal anomalies, maternal infection (including HIV), maternal alcohol abuse, heavy smoking (>10

217 cigarettes daily prior to pregnancy; >5 cigarettes daily during pregnancy) and maternal exposure to

218 psychotropic medications. Serial measurements of fetal adrenal size were performed at the time of the

219 routine anomaly scan (~20 weeks gestation, visit 1) and at 3 other time points: ~28, 34 and 38 weeks

220 gestation (visits 1-3, respectively) (Table 1). Maternal plasma samples for kisspeptin were taken at the

221 same time points as the USS and subjects were followed up until the outcome of pregnancy was known.

222

223 *Subject details and pregnancy outcome*

224 Thirty-three pregnant women of mean age 26.8 ± 6.1 yrs were recruited. The median gravidity and parity of

225 the women were 1 (range 1-4) and 0 (range 0-2), respectively. The patient demographics are detailed in

226 Table 1 and Supplemental Table 5. Two subjects (21 and 23) moved area before completion of the study

227 (after visits 2 and 3, respectively). Two babies were born prematurely at 26 and 33 weeks gestation (18 and

228 30, respectively) with normal birth weights of 950 and 1340g (BW SDS 0.84 and -1.95), respectively. Two

229 infants (6 and 22) born at 39.3 and 37.1 weeks gestation were small for gestational age, BW 2420 and

230   1720g (BW SDS -2.42 and -3.30), respectively. The remaining subjects had term deliveries, mean gestation

231   39.96 ± 1.18 (range 37.14 - 42.14) and BW SDS -0.71 ± 0.87 (-3.30 - 0.57).

232

233   *Fetal adrenal volume calculation*

234   Fetal USS were performed by two fetal medicine doctors (RA, SM) independently using the Voluson 730

235   and E8 systems (Voluson Expert; Milawaukee, WI). Two-dimensional measurements were taken in the

236   transverse, coronal and sagittal planes to obtain the length, width and depth of the total adrenal gland and

237   the fetal zone. Three dimensional ultrasonography was performed to obtain the adrenal gland volume

238   which was calculated using VOCAL (Virtual Organ Computer-aided AnaLysis, 4D view; General

239   Electrical Medical Systems) software (30). Adrenal volume data were missing in several patients who

240   failed to attend USS appointments (Supplemental Table 5).

241

242   *Measurement of plasma kisspeptin*

243   Samples were stored at -20°C for between 6 and 18 months prior to kisspeptin measurements. Plasma

244   kisspeptin immunoreactivity was measured at Imperial College, London using the established in-house

245   assay (31,32).

246

247   *Data analysis and statistics*

248   *In-vitro* data were evaluated using a paired two-tailed Student's t test or one-way ANOVA followed by a

249   post-hoc Tukey comparisons test (GraphPad Prism 6, San Diego, CA). All experiments were performed in

250   triplicate and represent 3 or 4 independent experiments; error bars depict the standard deviation of each

251   individual experiment. Non-parametric continuous variables (FAV and kisspeptin levels) were analyzed by

252   a Kruskal–Wallis test with Dunn-Bonferroni post hoc multiple comparison test correction. Continuous

253   parametric variables (FAV in male and female fetuses) were compared using student t-test. R-values are

254   Pearson's correlation coefficient (SPSS V.23 Armonk, New York, USA: IBM Corp.). P values <0.05 were

255   statistically significant.

256

257   **Results**

258

259 *Expression of Kiss1R in the developing human fetal adrenal (HFA) cortex*

260 Immunohistochemical analysis demonstrated high expression of Kiss1R throughout the HFA cortex in all

261 the gestational ages examined (8 wpc to term) (Fig. 1A, panels a-d). Co-localization studies using CD56

262 and SULT2A1 as markers of the DZ/TZ and FZ, respectively, verify Kiss1R expression in all 3 zones of

263 the HFA (Fig. 1B). Steroidogenic cells are identified by SF1 immunoreactivity throughout the HFA and

264 there is co-localization of SF1 and Kiss1R (Supplemental Fig. 1D). Densely packed nuclei below the

265 capsule and the larger cells in the center of the adrenal cortex have the appearance of DZ and FZ cells

266 respectively, the TZ lies between these 2 zones (Fig. 1C).

267

268 *Quantitative assessment of Kiss1R expression in the developing HFA*

269 *Kiss1R* and *SULT2A1* are highly expressed in the human placenta and adrenal gland respectively.

270 Quantitative reverse-transcriptase PCR (qPCR) performed on 1st and 2nd trimester HFA cDNA (8-10wpc,

271 11-14wpc, 15-20wpc) showed an increase in both *Kiss1R* (Fig. 1D) and *SULT2A1* (Fig. 1E) mRNA

272 expression with increasing gestational age. *Kiss1R* mRNA was significantly higher in 11-14wpc HFA (7.9-

273 fold, $p<0.05$) and 15-20 wpc HFA (22.3-fold, $p<0.0001$) than placenta (Fig. 1D). At 8-10wpc *Kiss1R*

274 mRNA was 3.7-fold higher in HFA than in human placenta, although this was not significant (Fig. 1D).

275 There was also a significant increase in *Kiss1R* expression from 8-10wpc to 15-20wpc (5.9 fold, $p<0.0001$)

276 and 11-14wpc to 15-20wpc (2.8-fold, $p<0.001$). The 2-fold increase in *Kiss1R* expression between 8-10wpc

277 and 11-14wpc was not significant.

278

279 *SULT2A1* mRNA was significantly higher in 11-14wpc (7.3-fold, $p<0.05$) and 15-20 wpc HFA (9.4-fold,

280 $p<0.01$) than in human adult adrenal (Fig. 1E). At 15-20wpc SULT2A1 mRNA was 2.6-fold higher in HFA

281 than at 8-10wpc ($p<0.05$). At 8-10wpc *SULT2A1* mRNA was 3.6-fold higher in HFA than in adult adrenal,

282 although this result was not significant (Fig. 1E). The 2.0- and 1.3-fold increase in *SULT2A1* expression

283 from 8-10wpc to 11-14wpc and 11-14wpc to 15-20wpc, respectively, were also not significant.

284

285 *The effect of kisspeptin treatment on Kiss1R mRNA and protein expression in H295R and HFA cells*

286 Treatment of H295R adrenocortical cells with kisspeptin resulted in a significant (60%) decrease in *Kiss1R*

287 mRNA expression (Fig. 2A; $p<0.05$). A significant decrease (34%) in *Kiss1R* mRNA expression was also

observed in 15-20 wpc HFA cells (Fig. 2C; p<0.05) but not 8-10 wpc HFA cells (16% decrease, Fig. 2B) in response to kisspeptin treatment. Kisspeptin treatment also resulted in a significant decrease in Kiss1R protein levels in H295R adrenocortical cells (53.5% decrease from baseline; p<0.05) (Fig. 2D). Kiss1R protein expression was decreased in 8-10 week HFA cells (8.3% reduction; Fig. 2E) and 15-20 week HFA cells (8.9% reduction; Fig. 2F) in response to kisspeptin treatment, however these differences were not significant.

The effect of kisspeptin and known adrenal regulators on DHEAS production (ELISA) in H295R and HFA cells

Kisspeptin significantly increased DHEAS secretion from H295R, 8-10wpc and 15-20wpc HFA cells 3.7-fold (p<0.05, Fig. 3A), 2.5-fold (p<0.05; Fig. 3B) and 4.0-fold (p<0.05; Fig 3C) compared to untreated cells, respectively. DHEAS production from 8-10wpc and 15-20wpc HFA cells following kisspeptin treatment was similar to that produced by ACTH (3.5-fold (p<0.01) and 4.1-fold (p<0.05) compared to untreated cells, respectively). Kisspeptin with forskolin increased DHEAS secretion 8.6-fold from H295R cells compared to untreated cells (p<0.0001), which was 2.3-fold (p<0.01) and 1.4 fold (p<0.05) higher than kisspeptin or forskolin alone, respectively (Fig. 3A). Kisspeptin with ACTH also increased DHEAS production 4.5-fold (p<0.001; Fig. 3B) and 9.1-fold (p<0.0001, Fig. 3C) from 8-10wpc and 15-20wpc HFA cells compared to untreated cells, respectively. This was 1.7-fold (p<0.05; Fig. 3B) and 2.3-fold (p<0.01; Fig. 3C) higher than with kisspeptin alone in 8-10wpc HFA cells and 15-20wpc HFA cells, respectively and 2.2-fold (p<0.01; Fig. 3C) higher than with ACTH alone in 15-20wpc HFA cells.

DHEAS production by H295R, 8-10wpc and 15-20wpc HFA cells following CRH treatment was similar to that produced by ACTH and kisspeptin treatment alone (4.0-fold (p<0.01), 4.0-fold (p<0.001) and 2.5-fold (p<0.05) compared to untreated cells, respectively) (Figs 3D-F). Compared to kisspeptin and CRH alone, treatment of H295R cells with a combination of kisspeptin and CRH resulted in a significant decrease (2.6-fold; p<0.01 and 2.8-fold; p<0.01) in DHEAS production, respectively (Fig. 3D). The same pattern was observed in 8-10wpc HFA cells (3.1-fold (p<0.01) and 4.7-fold (p<0.001) decrease of DHEAS compared to kisspeptin and CRH treatment alone, respectively) (Fig 3E) and 15-20wpc HFA cells (3.5-fold (p<0.001)

316 and 2.2-fold (p<0.05) decrease of DHEAS compared to kisspeptin and CRH treatment alone, respectively)

317 (Fig 3F).

318

319 *The effect of kisspeptin on DHEAS production (LC-MS/MS) in H295R and HFA cells*

320 DHEAS levels measured by LC-MS/MS increased 8.3-fold (H295R; p<0.05) and 93.2-fold (8-10wpc HFA

321 cells; p<0.05) following kisspeptin treatment (Supplemental Figs. 1E & F and Supplemental Tables 4A &

322 B). These DHEAS increases are 2.2 and 34.5 fold higher compared to the ELISA assay, respectively. The

323 lower calculated fold change observed by the ELISA analysis compared to the LC-MS/MS assay in 8-

324 10wpc HFA cells can be attributed to the higher baseline values. This is likely to be due to interference in

325 the ELISA assay at baseline from other steroid sulfates generated by the HFA tissue.

326

327 *Fetal adrenal volume and kisspeptin levels in uncomplicated singleton pregnancies*

328 Fetal adrenal volumes (FAV) increase steadily during pregnancy (Fig. 4A & Supplemental Table 5).

329 Median FAVs were 0.19 cm^3 (IQR 0.08-0.48; n=31), 0.52 cm^3 (0.26-1.53; n=32), 1.52 cm^3 (0.94-2.40;

330 n=28) and 2.16 cm^3 (1.17-7.87; n=23) at antenatal visits 1-4, respectively. The range of FAVs increase as

331 gestation advances but there are significant increases in FAV between visits 1 and 2 (p<0.01), visits 1 and 3

332 (p<0.001), visits 1 and 4 (p<0.001) and visits 2 and 4 (p<0.01). Median kisspeptin levels were 2822 pmol/L

333 (IQR 1913-0.48; n=33), 3953 pmol/L (2823-5615; n=31), 4545 pmol/L (3182-6182; n=30) and 3711

334 pmol/L (2546-4937; n=26) at antenatal visits 1-4, respectively (Fig. 4B). There is considerable overlap of

335 kisspeptin levels as gestation advances but significant increases are noted between visits 1 and 2 (p<0.05),

336 visits 1 and 3 (p<0.001), visits 1 and 4 (p<0.001) and visits 2 and 4 (p<0.01) (Fig. 4B).

337

338 *Relationship between fetal adrenal volume and plasma kisspeptin in singleton pregnancies*

339 To corroborate the *in-vitro* data, we assessed the association of the maternal kisspeptin levels with the

340 subsequent FAV increment at the four different time points. The mean increase in FAV between antenatal

341 visits 1 and 2 correlated with the kisspeptin level at visit 1 (r=0.41, p=0.026) (Fig. 4C), suggesting that the

342 kisspeptin levels between 19-20 weeks gestation (17-18 wpc) may influence FAV increase between 19-28

343 weeks gestation (17-26 wpc). There was no significant difference in the mean rise of FAV between the 1st

344 and 2nd antenatal visits in male 0.83 ± 0.94 (n=18) and female 1.28 ± 1.38 (n=12) infants, respectively

345 (p=0.30, 95% CI: -0.42 to 1.31). There was no significant correlation between FAV and estimated fetal

346 weight (efw) between the 1st and 2nd antenatal visits (r -0.166; p=0.36). Therefore, the significant

347 correlations between maternal kisspeptin and FAV were independent of fetal sex and efw, suggesting that

348 kisspeptin may be important for FA development in mid-pregnancy. There was no significant correlation

349 between the maternal kisspeptin levels and the subsequent FAV increment at the other antenatal time

350 points.

351

352 **Discussion**

353

354 Circulating kisspeptin levels increase dramatically during pregnancy (8) and may have an important role in

355 placentation by regulating placental invasion into the maternal uterine wall (1,3,6). Circulating kisspeptin

356 levels are reduced in women with intrauterine growth retardation and preeclampsia (9,33) and low maternal

357 levels in early pregnancy have been associated with greater miscarriage risk (12). Therefore, kisspeptin

358 may be a novel endocrine marker of functional placental tissue and low placental kisspeptin may be

359 associated with serious obstetric complications. The role of kisspeptin in pregnancy and the mechanisms

360 underlying these associations are unclear.

361

362 Kisspeptin and its receptor, Kiss1R, are expressed in the central nervous system, pancreas, adipose tissue,

363 testes and spleen (2,3). One other group has assessed the expression and localization of Kiss1R in the

364 human fetal adrenal gland (HFA) (24). This study showed robust expression of *Kiss1R* mRNA in HFA

365 tissue and Kiss1R protein expression in the definitive and the transitional zones (DZ and TZ) of 14-36

366 weeks gestation HFA tissues by immunohistochemistry. For the first time, we confirmed Kiss1R protein

367 expression in the HFA cortex from 8 weeks post conception (wpc; 10 weeks gestation) to term by

368 immunofluorescence. Interestingly, it was identified throughout the adrenal cortex, with expression in the

369 inner FZ as well as the outer DZ and TZ. The reason for this discrepancy is unclear but may be accounted

370 for by the different methodology and antibody used. Additionally, our immunofluorescence and in-vitro

371 data concord as presumably, kisspeptin stimulates DHEAS production from FZ cells. Consistent with this,

372 SULT2A1 (DHEA sulphotransferase) is localized to the FZ and converts DHEA to DHEAS. This suggests

373 that the FZ may represent an important target for kisspeptin during pregnancy.

374

Quantitative evaluation shows that *Kiss1R* mRNA expression increases significantly in mid-gestation (11-20 wpc); therefore this may be a critical time point for the action of kisspeptin on the FZ. The production of DHEAS begins at ~8-10 weeks gestation (6-8wpc) but increases considerably during the second and third trimesters (13). The increase in *Kiss1R* expression in mid-gestation (11-20 wpc) is paralleled by an increase in *SULT2A1* mRNA expression. Taken together, these data suggest that the FZ is an important target for kisspeptin, particularly in the second trimester.

381

Kiss1R is a G-protein coupled receptor (GPCR). Kisspeptin-KISS1R signaling is best characterized in GnRH neurons and in these cells, KISS1R undergoes both kisspeptin-triggered and kisspeptin-independent signaling, internalization and recycling (34). This ensures a dynamic population of functional cell-surface receptors and tight regulation of the biochemical response. In HFA cells, kisspeptin treatment resulted in a significant decrease in *Kiss1R* mRNA expression in adrenocortical tumor (H295R) and second trimester HFA cells. This was paralleled by a significant decrease in Kiss1R protein levels in H295R cells. This data is novel and we hypothesise that high circulating kisspeptin levels may down-regulate *Kiss1R* expression in the HFA to regulate signaling and therefore ensure tight control of steroidogenesis throughout pregnancy. This process of desensitization is a recognized phenomenon of many other GPCRs (35) as well as kisspeptin (36).

392

ACTH secreted from the fetal pituitary is a crucial regulator of FA growth partly mediated by peptide growth factors in an autocrine or paracrine fashion (13,24). Placental CRH and estrogens may also play important roles in the development of the FA. However, the rapid growth and steroid output of the FZ during the second trimester are not paralleled by an increase in ACTH and in humans, CRH levels peak near parturition. (13). This suggests that other pregnancy-specific factors regulate FZ growth and function, particularly in the second trimester.

399

It is established that ACTH and CRH directly promote DHEAS production from FA cells (15,37) but the regulation of androgen production from FZ cells is not fully understood. One previous study has shown that kisspeptin can increase aldosterone production from H295R cells (24). Its role in the production of other

403   steroidogenic hormones by the HFA has not previously been examined. Our ELISA data confirm that

404   kisspeptin can significantly increase DHEAS production from H295R and 8-10 / 15-20wpc (10-22 weeks

405   gestation) HFA cells. Analysis by LC-MS/MS showed a similar response for H295R. For 8-10 wpc HFA

406   cells, the baseline values were much higher by ELISA, so that the calculated fold change was much higher.

407   This is likely to be due to interference in the ELISA assay from other steroid sulfates generated by this

408   tissue. Paradoxically, DHEAS values post kisspeptin are lower by ELISA than by LC-MS/MS, which

409   supports the concept that kisspeptin specifically stimulates DHEAS production. The kisspeptin effect was

410   comparable to stimulation with ACTH or CRH alone. Furthermore, kisspeptin in combination with ACTH

411   appears to augment this effect. In contrast, CRH in combination with kisspeptin significantly decreased

412   DHEAS production. Thus kisspeptin may work in concert with CRH and ACTH to regulate HFA function

413   and therefore the balance of estrogens during pregnancy. The placenta is the primary source of estrogen

414   and the concentration of estrogen increases with progressing gestational age. The timing of these

415   interactions may be critical as ACTH levels remain fairly steady throughout pregnancy and circulating

416   kisspeptin levels rise steadily between the first and third trimesters. Consequently as pregnancy progresses,

417   kisspeptin may work in tandem with ACTH to enhance DHEAS, the production of estrogens and the

418   maintenance of pregnancy. In support of this hypothesis, low levels of kisspeptin, particularly in early

419   pregnancy, are associated with greater miscarriage risk (12).

420

421   CRH is postulated to play critical roles in fetal maturation and the onset of parturition. Consequently, the

422   levels of CRH increase as pregnancy progresses and peak from 35 weeks gestation corresponding with a

423   fall in the level of cortisol binding protein. Abnormal activation of the fetal HPA and enlargement of the

424   FAV has been associated with impending preterm birth (20,21). It is feasible that kisspeptin modulates the

425   effects of CRH in mid gestation however, its role in late pregnancy when CRH is critical, warrants further

426   investigation.

427

428   3-D ultrasonography (3DUS) is an established and accurate method of assessing fetal organ volumes. More

429   recently it has been reported as a reliable technique to measure HFA volume, with good intra- and inter-

430   observer repeatability (30,38). One cross-sectional study reports correlations between FAV and estimated

431   fetal weight (efw) and FAV and gestational age (GA) (39). Interestingly, our data suggest that FAV was

432   independent of both GA and efw. This discrepancy may be explained by the fact that our data was

433   longitudinal and is therefore is more likely to show true correlations. Chang et al observed larger FAVs

434   than those obtained in the current study (39) and two other groups report slightly lower FAVs at

435   comparable GAs (20,38). These differences may be attributed to different methodologies and inclusion

436   criteria employed. Additionally, all three studies report cross sectional rather than longitudinal data.

437   Importantly, the FAV measurements in the current study are in agreement with data obtained from a

438   detailed postmortem study (40).

439

440   Circulating kisspeptin concentrations increase dramatically during pregnancy and its levels reflect the

441   amount of viable placental tissue (8). Consequently a decline in the levels may be associated with increased

442   miscarriage and preeclampsia (10,12). This was also demonstrated in twin pregnacies where the death of

443   one twin was associated with lower kisspeptin levels (12). Serial measurements of plasma kisspeptin in

444   pregnant women have not previously been undertaken but cross-sectional data suggest that the levels

445   increase as pregnancy progresses (8). We report a significant increase in circulating kisspeptin in pregnant

446   females between 20 and 28 weeks gestation which correlates with the second trimester rise in FAV. This

447   increase in FAV is independent of sex and efw. It also coincides with the *in vitro* data, which shows a

448   significant increase in *Kiss1R* mRNA expression in second trimester (13-22 weeks) HFA cells and DHEAS

449   production from mid-trimester (10-22 gestation) HFA cells following kisspeptin treatment.

450

451   In summary, kisspeptin-Kiss1R signaling may be a key regulator of HFA development and steroidogenesis

452   and therefore an integral component of the feto-placental unit. As well as being critical in the regulation of

453   placentation in early pregnancy, it may have a key physiological role in intrauterine homeostasis and the

454   maintenance of pregnancy, particularly in the second trimester. Therefore, our data suggests a novel

455   functional role for kisspeptin *in utero*.

456

461

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Figure and Table Legends

578   **Figure. 1. Expression of Kiss1R in the developing human fetal adrenal (HFA) cortex. A.**

579   Immunofluorescence studies of the HFA from 8wpc to term (panel a. 8wpc, b.11wpc, c. 33wpc, d & e.

580   38wpc). Localization of Kiss1R (red) in the definitive zone, DZ, transitional zone, TZ and throughout the

581   fetal zone, FZ (panels a-d). No immunoreactivity is detected in the negative control with antigen (Kiss1R)

582   pre-incubation (Kiss1R-con, panel e), demonstrating specificity of the Kiss1R antibody. The adrenal cortex

583   is surrounded by an outer mesenchymal capsule (dashed line). Scale bar: 100μm. **B.** Co-localization studies

584   of HFA at 12wpc (panels a-c). CD56 (green) is expressed in the outer DZ/TZ (panels a & b) and SULT2A1

585   (red) in the inner FZ (panel b). Kiss1R (red) is seen throughout the cortex (FZ and DZ) (panel a). No

586   immunoreactivity detected in the negative controls where the primary antibodies (CD56 and SULT2A1)

587   were omitted (panel c, CD56-con, SULT2A1-con). The capsule is shown (dashed line). Scale bar: 100μm.

588   **C.** H&E staining of HFA 33wpc. A thin capsule (c) surrounds the outer definitive zone (DZ). The DZ of

589   the cortex is the most superficial layer with closely packed, darker stained cells. The deeper layer with the

590   more eosinophilic appearance is the fetal zone (FZ). The transitional zone (TZ) lies between the outer DZ

591   and inner FZ. Scale bar: 1mm (panel a), 100μm (panel b). **D.** *Kiss1R* qPCR was performed on cDNA

592   obtained from 3 HFA samples from each stage of development (8-10wpc, 11-14wpc, 15-20wpc). Placental

593   cDNA was used as a positive control. Data points represent the mean +/- SD from 4 independent

594   experiments performed in triplicate. **E.** *SULT2A1* qPCR was performed on cDNA obtained from 3 HFA

595   samples from each stage of development (8-10wpc, 11-14wpc, 15-20wpc). Adult adrenal cDNA was used

596   as a positive control. Data points represent the mean +/- SD from 3 independent experiments performed in

597   triplicate. D&E. Data are normalized to *GAPDH* expression and presented as a proportional increase or

598   decrease from the calibrator (placenta and adult adrenal, normalized to a value of 1 for comparison).

599   *$p<0.05$; **$p<0.01$ ***$p<0.001$; ****$p<0.0001$.

600

601   **Figure. 2 The effect of kisspeptin treatment on *Kiss1R* mRNA and protein expression in H295R and**

602   **primary fetal adrenocortical cells.**

603   Treatment with 100nM kisspeptin for 24 hrs significantly reduced *Kiss1R* mRNA expression in H295R

604   cells (A). This was also evident in HFA 15-20wpc (C) but not 8-10wpc (B). Data are normalized to

GAPDH expression and presented as a proportional increase or decrease from the control (unstimulated cells, normalized to a value of 100 for comparison). Data points represent the mean +/- SD from 3 independent experiments performed in triplicate. **D-F.** Densitrometric analysis of Western blots showed a significant reduction in Kiss1R protein following treatment of H295R cells with 100nM kisspeptin for 24 hours (D) but not 8-10wpc HFA cells (E) or 15-20wpc (F). Data points represent the mean +/- SD from 3 independent experiments performed in triplicate. β-actin was used as a loading control. *$p < 0.05$.

Figure. 3 The effect of kisspeptin and known adrenal regulators on DHEAS production (ELISA) in H295R and primary fetal adrenocortical cells.

A-F. DHEAS production (ELISA) by H295R and HFA cells following kisspeptin and CRH treatments. H295R cells were incubated for 24 hours with 100nM kisspeptin and 10μM forskolin individually or together (A). 8-10wpc HFA cells (B) and 15-20 wpc HFA cells (C) were incubated for 24 hours with 100nM kisspeptin and 10nM ACTH individually or together. D-F DHEAS production (ELISA) by H295R and HFA cells following kisspeptin and CRH treatments. H295R cells (D), 8-10wpc HFA cells (E) and HFA cells 15-20wpc (F) were incubated for 24 hours with 100nM kisspeptin or 10nM CRH individually or together. Data points are mean +/- SD from 3 independent experiments run in triplicate and expressed as the fold over basal level (normalized to a value of 1). (-), no treatment; (+), treatment added. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Figure 4. Human fetal adrenal gland volumes and kisspeptin levels in the maternal circulation during pregnancy

A and B Box and whisker plots (A) of fetal adrenal gland volumes (FAV; cm^3) and (B) Maternal serum kisspeptin levels at the 4 antenatal visits (visit 1, 19-20 weeks; visit 2, 26-28 weeks; visit 3, 34-35 weeks; visit 4, 37-40 weeks). Box plots show the median, upper and lower quartiles and interquartile range (IQR). Open circles, outliers; star, extreme outliers. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. C and D Scatter Plots showing (C) The correlation between the kisspeptin level at the 1st visit and the increase in FAV between the 1st and 2nd antenatal visits. r, pearson coefficient.

634   **Table 1.** Details of the patients, the timing of the antenatal assessments and pregnancy outcome

635

Patient characteristic	Mean ± SD	Range	N
Age (years)	26.8 ± 6.1	17.0 - 37.0	33
BMI	25.4 ± 5.3	18.8 - 41.1	33
Gestational age (weeks)			
Visit 1	20.35 ± 0.68	19.29 - 22.57	31
Visit 2	28.07 ± 0.75	26.29 - 29.86	32
Visit 3	34.42 ± 0.75	33.00 - 36.14	28
Visit 4	38.09 ± 0.62	36.00 - 39.71	22
Gestation at delivery (weeks)	39.33 ± 2.83	26.86 - 42.14	31
Birth weight (grams)	3006 ± 650	950 - 3680	31
Birth weight SDS	-0.698 ± 0.913	-3.30 - 0.840	31

636

637   N= total number of subjects assessed

638
639

640

Figure 1

A

B

C

D

E
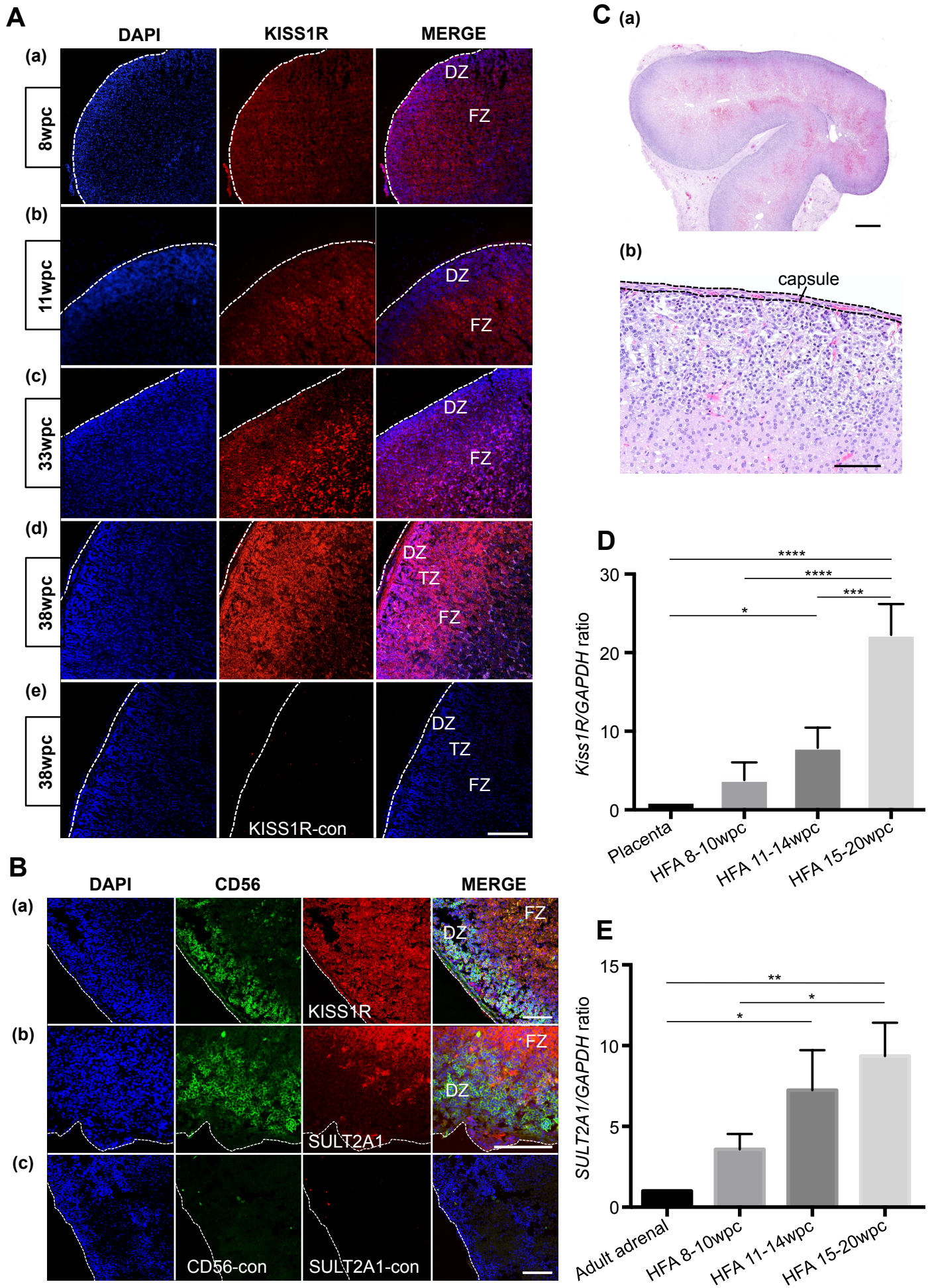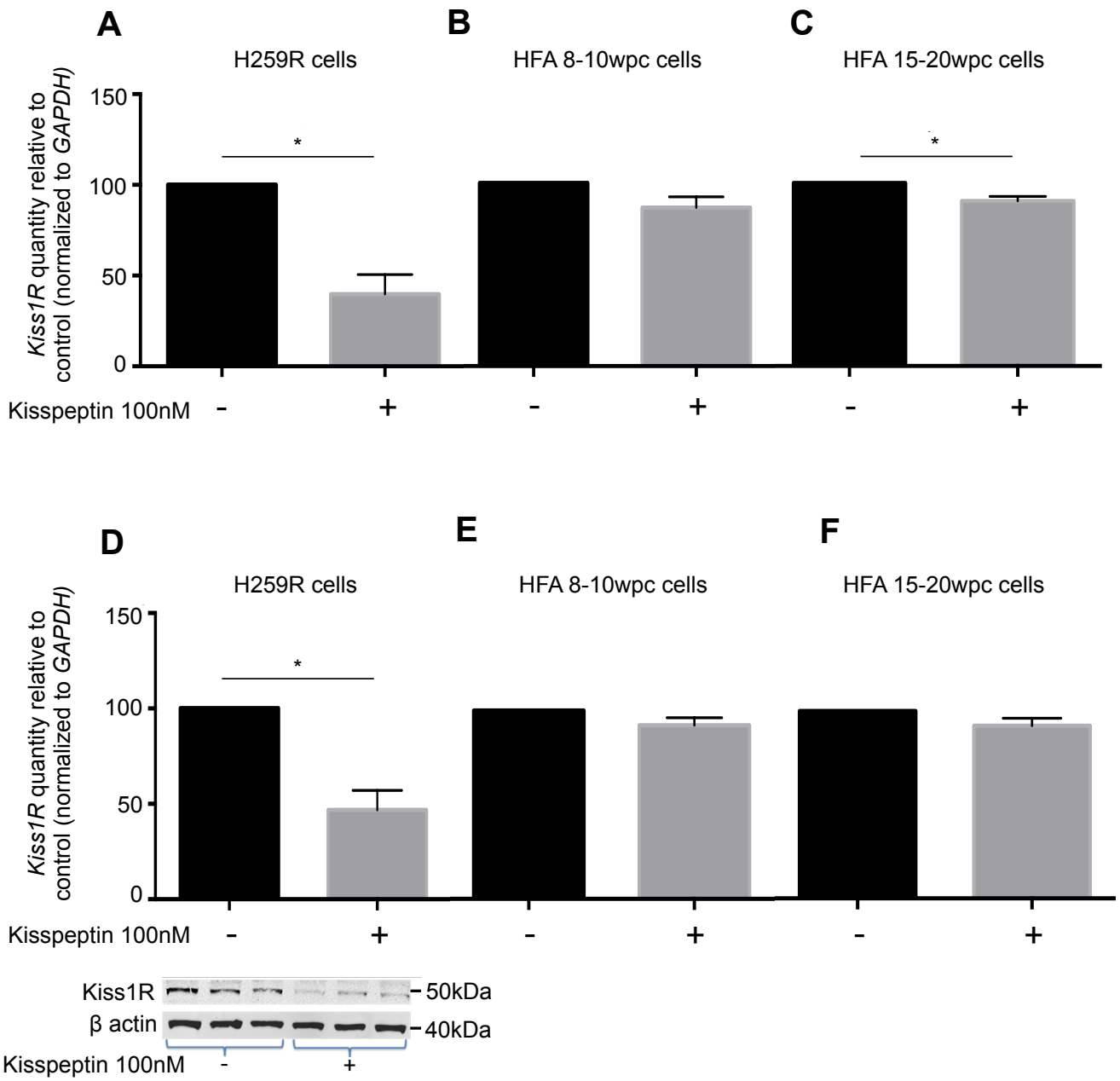
Figure 2

Figure 3

Figure. 4

A

B

C

r = 0.41
p = 0.026

Supplemental Figure 1

D

	DAPI	SF1	KISS1R	MERGE
(a)				
(b)				
(c)				

A

H259R cells

E

H259R cells

B

HFA 8-10wpc cells

F

HFA 8-10wpc cells

C

HFA 15-20wpc cells
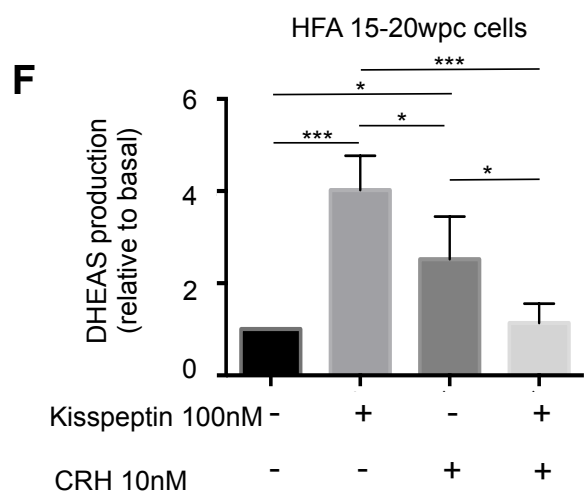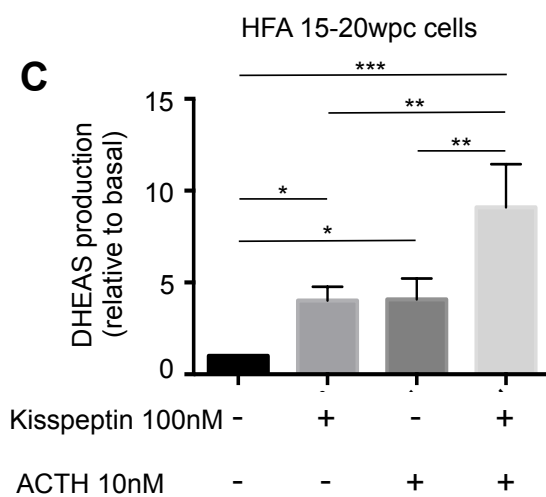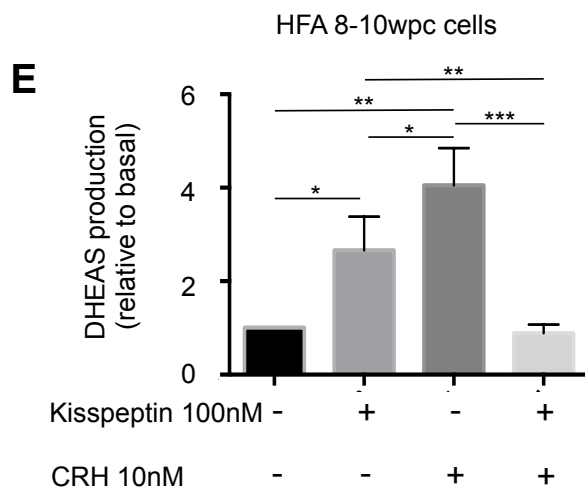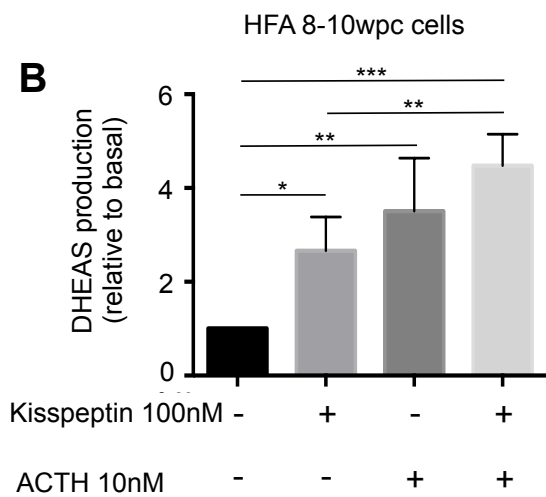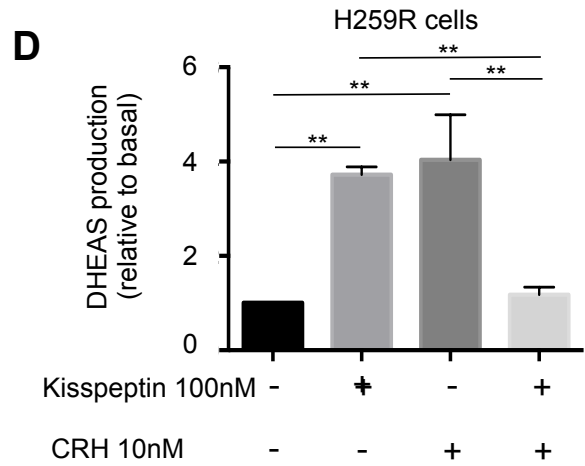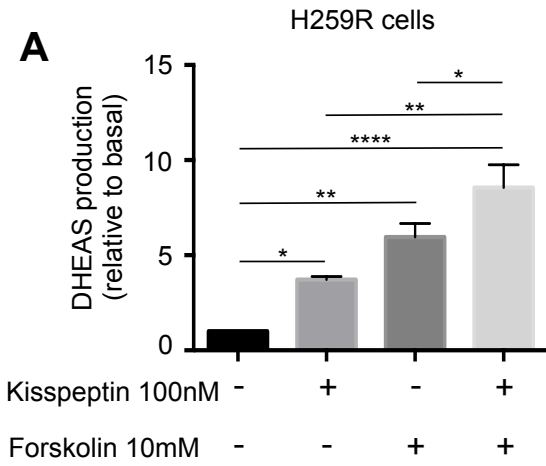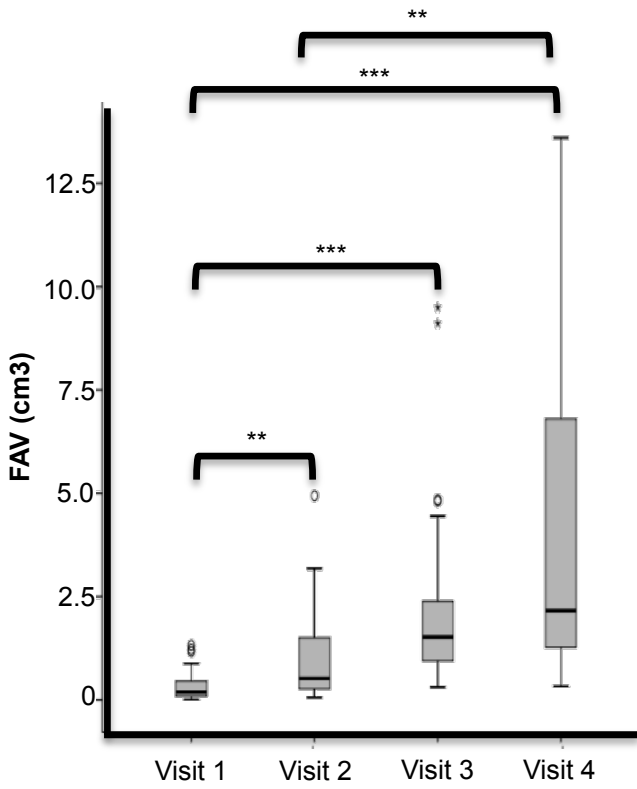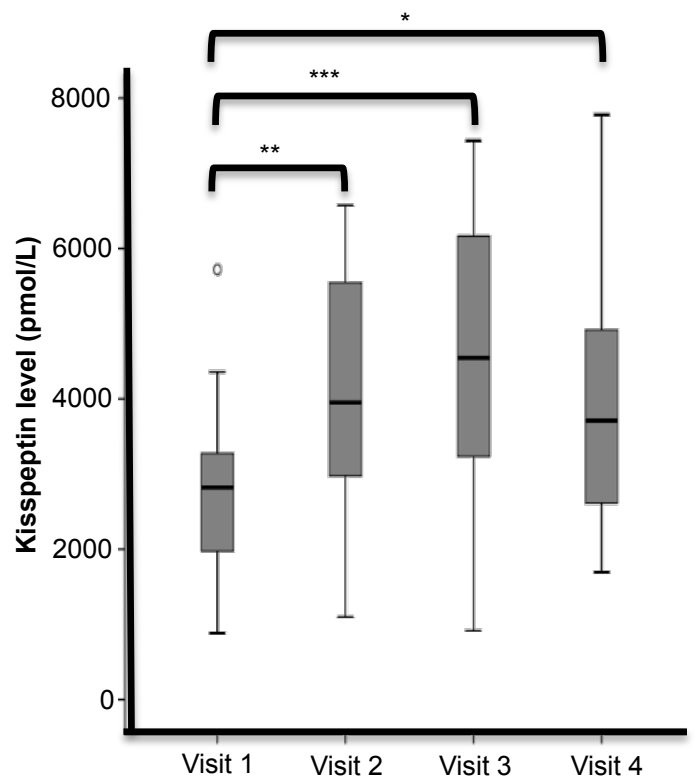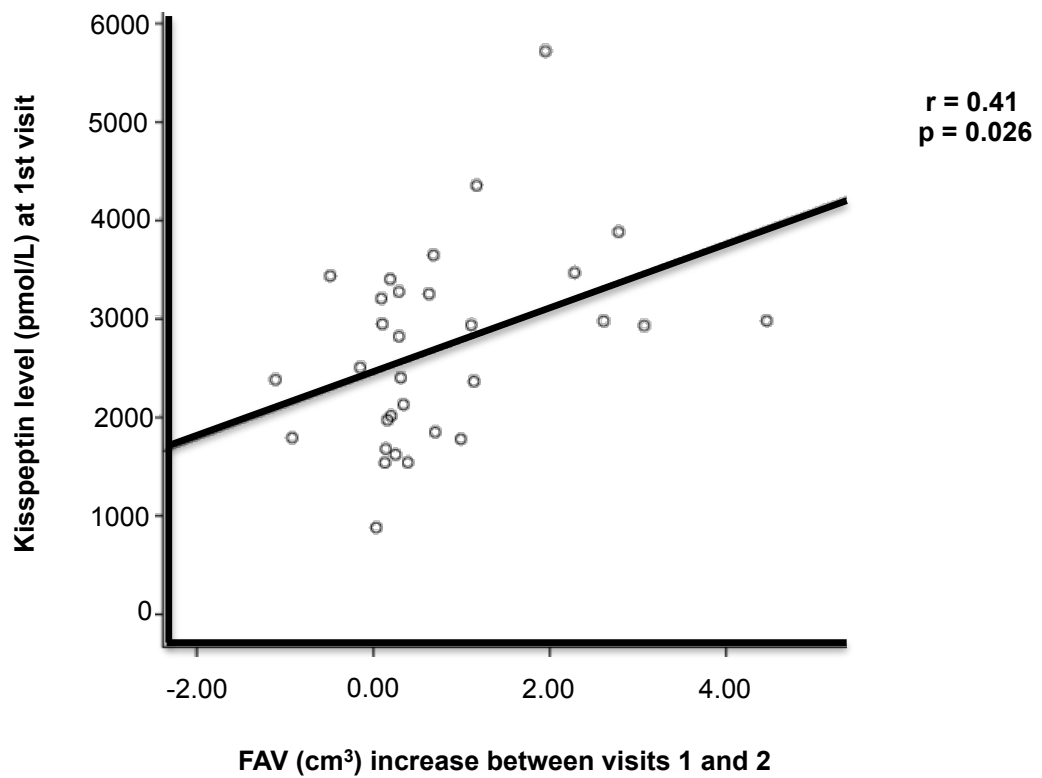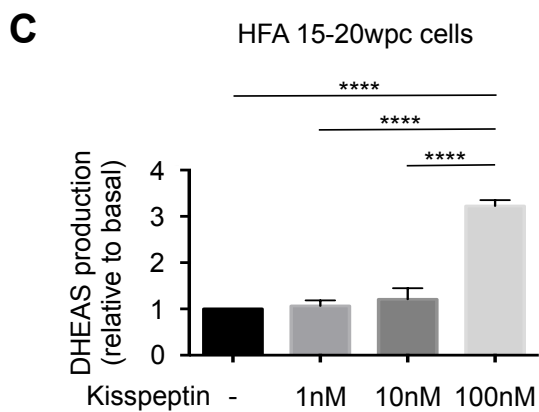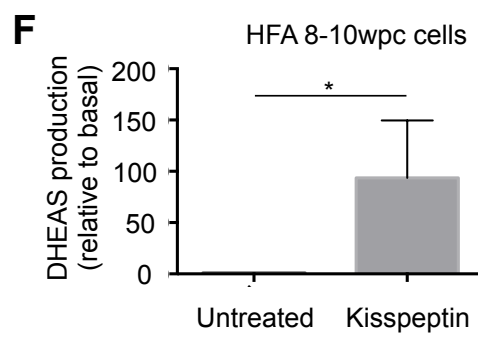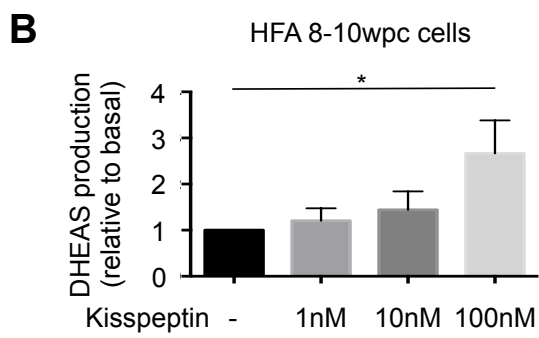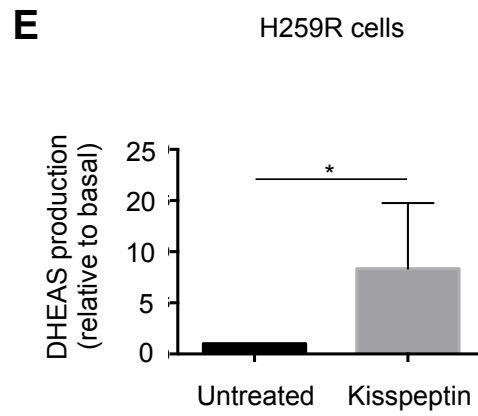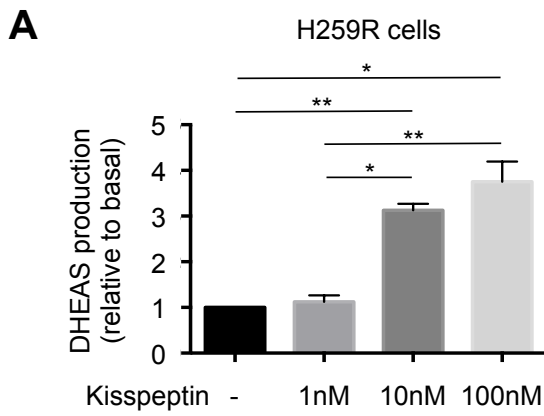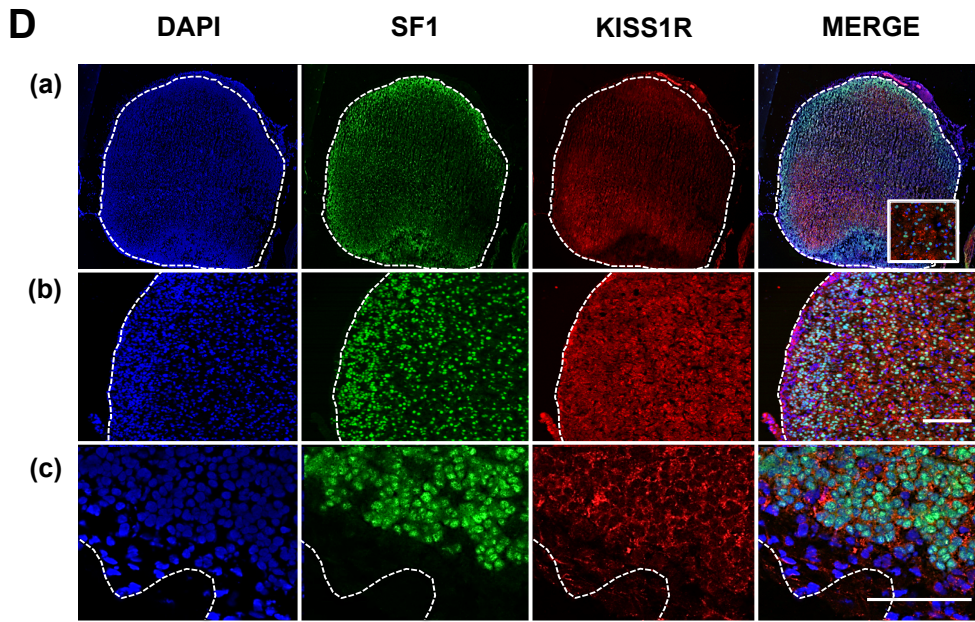
Supplemental Table 1. Details of antibodies used for immunofluorescence studies

Ab	Antigen and fluorophore	Species	Company (product number)	Dilution
1°	SF1	Mouse monoclonal	Invitrogen (434200)	1 in 200
2°	AF488-Green	Goat anti-mouse	Invitrogen (A11029)	1 in 1000
1°	Kiss1R	Rabbit polyclonal	Alomone (AKR-001)	1 in 100
2°	CY3-Red	Donkey anti-rabbit	Jackson Immunoresearch (711-165-152)	1 in 1000
1°	SULT2A1	Rabbit polyclonal	Abcam (38416)	1 in 200
2°	CY3-Red	Donkey anti-rabbit	Jackson Immunoresearch (711-165-152)	1 in 1000
1°	CD56-conjugated AF488-Green	Mouse monoclonal	Invitrogen MHCD5620	1 in 1000

Ab, antibody; 1°, primary antibody; 2°, secondary antibody.

Supplemental Methods

DHEAS measurements using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

500µl of precipitation reagent (a cocktail of internal standards in acetonitrile, including 16,16 d_2 DHA sulfate) was added to the samples, calibrators and internal quality controls (500µl). The quality controls were created in-house from a standard solution of DHA sulfate diluted in charcoal-stripped serum. Samples were vortexed (30 seconds) and centrifuged (13 000rpm) for 5 minutes. The supernatant was transferred into glass tubes. Bicarbonate solution (200µl, 8% aq, v/v) and 1ml of ethyl acetate was added and the tubes were vortexed (30 seconds) and centrifuged (13 000 rpm) for 5 minutes. The organic layer was transferred into a glass tube and evaporated to dryness under nitrogen gas and reconstituted in 125µl of freshly prepared reconstitution solution (Mobile phase A: Mobile phase B, 65:35 (v/v)). 100µl was injected onto the liquid chromatography (LC) system. Using the TSQ Vantage (ThermoFisher) in MS/MS positive APCI mode, *m/z* transitions 271.1 to 105 & 91 were monitored. DHEA was also quantified in the same runs and remained near or below detection limits, indicating that there was no significant desulfation during sample processing. Mass spectrometry parameters are listed in Supplemental Table 2 and method validation data is listed in Supplemental Table 3. Comparison of ELISA and LC-MS/MS DHEAS concentrations are given in Supplemental Table 4.

Liquid chromatography conditions

Eluents:	Mobile Phase A:	Water with 0.1 % formic acid
	Mobile Phase B:	Methanol with 0.1 % formic acid
Flow rate:		0.4ml/min
Column:		Accucore RP-MS Column (100 x 2.1mm. 2.6 µm)
Column temperature:		40 ºC (maintained by Hot Pocket. ThermoScientific

Supplemental Table 2. Mass spectrometry parameters

Vaporizer temperature	500°C
Capillary temperature	400°C
Discharge current (mA)	5.0
Sheath gas	20
Aux gas	5
Collision gas pressure (mTorr)	1.5
Q1 (FWHM):	0.40
Q3 (FWHM):	0.70
Scan time	0.05 seconds

Supplemental Table 3. DHEAS assay validation data

A. Precision and accuracy

Concentration nmol/L (n=6)	102	204	1697	8148
Intra-assay				
Mean	99.8	187.3	1840	8080
Accuracy %	97.9	92	108	99
CV%	5.3	3.0	3.8	1.3
Inter-assay				
Mean	102.3	201.7	1789.7	7700.8
Accuracy %	100.3	98.9	105.5	94.5
CV%	8.1	3.7	5.6	4.7

Lower limit of quantification is 35 nmol/L. No carry over or carry under was detected. No Ion suppression/enhancement of DHEAS-d2 signal was noted

B. Reagent stability

Concentration nmol/L (n=3)	204	1697	5729
Post extraction 1 week at 4°C			
Mean	201.4	1646	5720
Accuracy %	96.7	97.0	99.8
Post extraction 1 week at room temperature			
Mean	202.4	1570	5539.3
Accuracy %	99.2	92.5	96.7
3 freeze-thaw cycles			
Mean	203.2	1633	5581
Accuracy %	99.6	96.2	97.4

Supplemental Table 4. ELISA vs LC-MS/MS data

A. H295R cells

	DHEAS concentration ng/ml	DHEAS concentration ng/ml	DHEAS concentration ng/ml	Mean DHEAS concentration ng/ml	Mean fold change*
Un (ELISA)	105.9	141.8	110.4	119.4	**1.0**
KP (ELISA) **Fold increase**	393.16 3.7	551.5 3.8	392.9 3.5	445.9	**3.7**
Un (LCMS)	129.8	168.0	12.5	103.4	**1.0**
KP (LCMS) **Fold increase**	603.6 4.6	775.3 4.6	196.9 15.7	525.3	**8.3**

B. 8-10wpc HFA cells

	DHEAS concentration ng/ml 8wpc HFA	DHEAS concentration ng/ml 9wpc HFA	DHEAS concentration ng/ml 10wpc HFA	Mean DHEAS concentration ng/ml 8-10wpc HFA	Mean fold change*
Un (ELISA)	138.9	85.6	100	108.2	**1.0**
KP (ELISA) **Fold increase**	255.8 1.8	270.1 3.2	301.2 3.0	275.7	**2.7**
Un (LCMS)	2.92	4.8	2.32	3.3	**1.0**
KP (LCMS) **Fold increase**	86.5 29.6	552.8 115.2	312.5 134.7	317.2	**93.2**

Un, unstimulated cells; KP, cells treated with 100nM kisspeptin. * Fold change calculated relative to untreated samples which are normalized to 1.0.