# Establishment of a novel human fetal adrenal culture model that supports de novo and manipulated steroidogenesis

# Establishment of a novel human fetal adrenal culture model that supports *de novo* and manipulated steroidogenesis

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**Short title:** Human fetal adrenal *ex vivo* culture model

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# Abstract

**Context:** Disorders affecting adrenal steroidogenesis promote an imbalance in the normally tightly controlled secretion of mineralocorticoids, glucocorticoids, and androgens. This may lead to differences/disorders of sex development in the fetus, as seen in virilized girls with congenital adrenal hyperplasia (CAH). Despite the important endocrine function of human fetal adrenals, neither normal nor dysregulated adrenal steroidogenesis is understood in detail.

**Objective:** Due to significant differences in adrenal steroidogenesis between human and model-species (except higher primates), we aimed to establish a human fetal adrenal model that enables examination of both *de novo* and manipulated adrenal steroidogenesis

**Design and Setting:** Human adrenal tissue from 54 1$^{st}$ trimester fetuses were cultured *ex vivo* as intact tissue fragments for 7- or 14-days.

**Main Outcome Measure(s):** Model validation included examination of post-culture tissue morphology, viability, apoptosis and quantification of steroid hormones secreted to the culture media measured by liquid chromatography-tandem mass spectrometry.

**Results:** The culture approach maintained cell viability, preserved cell populations of all fetal adrenal zones, and recapitulated *de novo* adrenal steroidogenesis based on continued secretion of steroidogenic intermediates, glucocorticoids, and androgens. Adrenocorticotropic hormone (ACTH) and ketoconazole treatment of *ex vivo* cultured human fetal adrenal tissue resulted in the stimulation of steroidogenesis and inhibition of androgen secretion, respectively, demonstrating a treatment-specific response.

**Conclusions:** Together these data indicate that *ex vivo* culture of human fetal adrenal tissue constitutes a novel approach to investigate local effects of pharmaceutical exposures or emerging therapeutic options targeting imbalanced steroidogenesis in adrenal disorders including CAH.

# Introduction

During fetal life, human adrenal glands are highly active endocrine organs producing mineralocorticoids, glucocorticoids, and androgens. In disorders such as congenital adrenal hyperplasia (CAH), mutations affecting steroidogenic enzyme function cause an imbalance of the normally tightly controlled secretion of adrenal steroids (1). The impaired enzyme function in CAH can occur at different steps in the adrenal steroidogenesis pathway, resulting in a unique combination of elevated precursors and deficient products that is strongly correlated to the clinical severity of the disorder (2). Excess of adrenal androgens during early development can affect sexual differentiation of the fetus and manifest as virilized genitalia in girls with CAH. Despite the importance of steroids secreted by human fetal adrenals (HFA) the development and endocrine function of these organs are still relatively unexplored.

The morphology of the HFA gland is unique, initially consisting of a definitive zone (DZ) and a fetal zone (FZ). The DZ, localized under the capsule of the organ, contains a thin layer of tightly packed basophilic cells, while the remaining approximately 80 % of the gland consists of the inner FZ with large cells containing lipid-filled vacuoles (3,4). DZ and FZ are distinguishable from about gestational week (GW) 7 while a third transitional zone (TZ) containing intermediate ultrastructural characteristics emerges between the DZ and FZ during early 2$^{nd}$ trimester (3,5). Together, these three zones form the HFA cortex that express essential steroidogenic enzymes in a distinct pattern and are responsible for the adrenal production of mineralocorticoids, glucocorticoids and androgens throughout fetal development (3,5-12). Cells in the fetal adrenal cortex originate from mesodermal adrenogenital primordium cells, but from around GW 7-8 migrating sympathoadrenal progenitor cells with a neural crest origin reach the adrenal primordium (4,13,14). These cells secrete catecholamines during fetal development and coalesce to form the inner medulla after birth (15). Interactions between the fetal adrenal steroid-producing cortical cells and the catecholamine-secreting chromaffin cells are crucial for cell differentiation, morphogenesis, and survival of the gland. Hence, dysregulated cross-talk between the fetal adrenal cortical and chromaffin cells can affect regulation of hormone synthesis and stress response, and can be involved in the pathogenesis of diseases that manifest in the adult adrenal glands (15-17). Thus, alterations in the tightly regulated HFA steroidogenesis, catecholamine secretion, or fetal adrenal cortical-medullary cell interaction may cause fetal endocrine dysfunction resulting in adrenal hypo- or hyperplasia (4,15.16).

The endocrine function of HFAs and underlying mechanisms of congenital adrenal diseases are difficult to investigate as morphology and steroid hormone profile of laboratory animal modes (except higher primates) differs from HFA glands (18,19). Several approaches have previously been used to study the endocrine function of the HFA gland, predominantly as primary cell cultures of DZ and FZ cells obtained from HFA tissue cultured separately or together (20-29). However, the lack of DZ-FZ and/or the cortical-chromaffin cell interactions in single-cell suspensions is a limitation to these models. Additionally, a couple of approaches to culture intact HFA tissue have previously been employed, but none of them have subsequently been used systematically to determine their potential use in studies investigating HFA steroidogenesis (9, 30,31). The use of a “hanging drop” approach has to our knowledge not previously been reported for culture of HFA tissue fragments. This approach is effective in preserving functional integrity and signaling activity as shown from differentiation of mouse embryoid bodies into different cell lineages (32,33) and has previously been used to study manipulations of specific signaling pathways and pharmaceutical exposures in human fetal testis and ovary (34-37). Therefore, in this study we aimed to test whether this culture approach could be applied to HFA tissue (aged GW 8-11) in order to develop a model that recapitulates normal and dysregulated HFA steroidogenesis and simultaneously support cell viability and tissue morphology.

# Material and Methods

## *Collection of human fetal adrenals*

The collection of human fetal adrenals in this study was approved by the Danish regional ethics committee (permit number H-1-2012-007). Women were recruited to participate in the study by medical staff working independently of the project and all women gave their informed written and oral consent. HFA tissue (GW 8-11) was isolated from material available following elective surgical termination of pregnancy at Copenhagen University Hospital (Rigshospitalet), Hvidovre Hospital, and Herlev Hospital, Denmark. None of the terminations were for reasons of fetal abnormality or pathology of pregnancy. Fetal samples were kept at 4 °C immediately after termination and during transportation to the laboratory. Fetal age was determined by scanning crown-rump length and by evaluation of foot length (38). Fetal adrenal tissue was dissected in ice-cold PBS and immediately set-up in *ex vivo* cultures. A total of 59 adrenals was used for *ex vivo* cultures,

corresponding to adrenals from 54 individual fetuses as both adrenals from the same fetus were occasionally used for culture. Additionally, 8 intact adrenals from independent fetuses were formalin fixed immediately after dissection and used as non-culture controls. Some of these adrenal control samples were also used as controls in a previous study (5).

### *Ex vivo human fetal adrenal tissue cultures*

Fetal adrenals were cultured *ex vivo* in "hanging drops" as described previously for human fetal gonad tissue (34,36,37). In brief, all adrenals were divided into approximately 1 $mm^3$ fragments prior to culture set-up. Fetal adrenal tissue fragments were cultured in 40 µl medium for 7-14 days at 37°C under 5% $CO_2$ with complete medium change every 48 hours. Media from all replicates of the same sample and treatment were collected and pooled throughout the culture period of 7- or 14-days. Medium composition was: MEMα media supplemented with 1× MEM non-essential amino acids, 2 mM sodium pyruvate, 2 mM L-glutamine, 1× Insulin, Transferrin and Selenium (ITS) supplement, 1× Penicillin/Streptomycin, 10 % Fetal Bovine Serum (FBS). All cell media and supplements were from Gibco (Naerum, Denmark), except ITS (Sigma-Aldrich, Broendby, Denmark). To manipulate steroidogenesis, fetal adrenals were cultured in medium containing either 1 nM ACTH dissolved in sterile $H_2O$, or 10 µM ketoconazole dissolved in dimethyl sulfoxide vehicle (DMSO, 0.1 %), all reagents were from Sigma-Aldrich (Broendby, Denmark). ACTH concentration was chosen based on previous studies reporting stimulation of fetal adrenal steroidogenesis at various concentrations below 3 nM (21,30,39). Ketoconazole concentration was chosen based on inhibiting effects on steroidogenesis observed in human fetal testis *ex vivo* cultures (40) and *ex vivo* cultured human fetal testis was treated with the same stock of ketoconazole as a positive control. To ensure an equal relative ratio of DZ vs FZ cells were equal prior to the experimental vehicle vs. treatment set-up, the adrenal gland was initially divided into two halves prior to division into 1 $mm^3$ fragments. Afterward, all fragments from one half of the gland was cultured in vehicle control media and all fragments from the other half in treatment media, corresponding to 1-7 fragments per treatment depending on the initial size of the HFA. Thus, one sample was always divided into an equal number of vehicle control and treatment tissue fragments.

## *Immunohistochemistry*

Fetal adrenal tissue was fixed in formalin either immediately after dissection (used as non-culture controls) or at the end of the *ex vivo* culture period. The fixed adrenals were dehydrated, paraffin embedded and sectioned (4 µm) using standard procedures. Serial sections of each sample were used for Immunohistochemistry (IHC) as previously described for formalin-fixed samples (5). In brief, tissue sections were subjected to heat-induced antigen retrieval buffer in a pressure cooker, and endogenous peroxidase was blocked with 3 % (v/v) $H_2O_2$ in MeOH for 30 minutes. Between each step, sections were washed in Tris-buffered saline. Sections were incubated in 5% BSA (w/v) in horse serum (20% v/v) ImmPRESS (Vector Laboratories, Burlingame, California) for 30 minutes to minimize cross-reactivity. Primary antibodies were incubated overnight at 4$^{o}$C followed by 1 hour at room temperature. Sections were incubated for 30 minutes with the appropriate ImmPRESS HPR (peroxidase, Vector Laboratories, Burlingame, California) secondary antibody diluted in normal serum. Primary antibodies, dilutions and retrieval buffers are listed in Table 1. Visualization was performed using ImmPACT AEC peroxidase substrate (Vector Laboratories, Burlingame, California). Negative controls were included and processed with the primary antibody replaced by the dilution buffer alone, none of which showed staining. Sections were counterstained with Mayer's hematoxylin before mounting with Aquatex (Merck, Damstadt, Germany). Sections were initially evaluated on a Nikon Microphot-FXA microscope and then by scanning slides on a NanoZoomer 2.0 HT (Hamamatsu Photonics, Herrsching am Ammersee, Germany) followed by analysis using the software NDPview software, version 1.2.36 (Hamamatsu Photonics, Herrsching am Ammersee, Germany).

## *BrdU incorporation assay*

BrdU incorporation was used to determine the presence of proliferating cells just prior to the end of the *ex vivo* culture period as previously described (41). In brief, BrdU labeling reagent (Life Technologies, Naerum, Denmark) was diluted in culture media and tissue fragments were placed in BrdU containing media for 6 hours followed by fixation and paraffin embedding as described above. BrdU was visualized by IHC using a BrdU antibody (Table 1) as described in the IHC section, with positively stained cells considered as proliferating.

### *Immunohistochemical quantification*

IHC staining was used to quantitatively evaluate fetal adrenal tissue viability, zonation and expression of steroidogenic enzymes after culture. The number of $BrdU^+$ (proliferating) and cleaved PARP ($cPARP^+$, apoptotic) cells per $mm^2$ tissue were determined. IHC staining of CYP17A1 (steroidogenic enzyme), SPARC (FZ marker) and NCAM-1 (DZ marker) expression were quantitatively assessed by measuring the area of stained cells per total tissue fragment area. For each sample, the entire tissue section was quantified. All tissue fragments originating from the same HFA gland were analyzed as replicates of one sample. Tissue area was measured using NDPview software, version 1.2.36 (Hamamatsu Photonics, Herrsching am Ammersee, Germany).

### *Steroid hormone measurements in culture media*

Analysis of steroid hormone levels in culture media collected from the *ex vivo* culture experiments were performed on pooled media samples from tissue fragments originating from the same sample and treatment group collected during the entire culture period. Steroid hormone levels were measured using a method established to quantify steroid metabolites in serum by isotype-dilution TurboFlow-LC-MS/MS as previously described (42). The method was modified for measurement in culture media. This clinically validated analysis package included estrone sulfate, testosterone, androstenedione, DHEAS, cortisone, cortisol, 11-deoxycortisol, 17-hydroxyprogesterone, corticosterone, and progesterone. All measured metabolites except estrone sulfate are reported in this study. In brief samples were analyzed in four batches during summer/autumn of 2019, all including standards for calibration curves prepared in culture media, and for method control; two blanks (water), two un-spiked culture media controls, two time two media culture controls spiked with a mixture of native steroid standards in low and high concentration levels. For several of the samples, the concentrations of dehydroepiandrosterone-sulfate (DHEAS), cortisol and cortisone were out of the ordinary measurement range and were therefore diluted 1:10 with media and analyzed in a repeated batch. For the four analytical batches included in this study the relative standard deviation (RSD) were < 10% for all steroids in culture media controls spiked in low level, except for estrone sulfate and DHEAS (< 17%), and < 4.2% for all steroids in controls spiked in high level.

### *Statistics*

Differences in percentages of immunopositive cells and steroid hormone concentrations were analyzed using the Mann Whitney (two-tailed) u-test for comparison of 7- and 14-days cultures. Differences between treatment and vehicle controls from the same HFA were analyzed using the Wilcoxon signed rank test (two tailed). All statistical analysis was preformed using GraphPad Prism software. Asterisks indicate statistical significance with *P<0.05, **P<0.01, ***P<0.001. Each sample value represents the mean value of all tissue fragments originating from one sample and treatment group, and the number of replicates in each experiment is described in the figure legends.

## Results

### *Ex vivo culture preserves HFA tissue morphology and cell viability*

To examine whether the *ex vivo* culture approach preserved tissue morphology, HFA fragments were analyzed after 7- and 14-days of culture (Figure 1a). The overall morphology of the cultured HFA fragments was maintained preserving both the DZ and FZ after both culture periods (Figure 1b). However, division of the adrenal tissue into fragments (~1 $mm^3$) and subsequent *ex vivo* culture did slightly alter the shape of the tissue fragments which appeared more rounded. Thus, post-culture DZ cells were not restricted to the outer edge of the tissue and FZ cells were not always restricted to the middle of the tissue fragments, but the specific cell types always remained within zones after culture. Cell proliferation and apoptosis were examined after 7- and 14-days of culture, proliferation by BrdU incorporation and apoptosis by expression of cleaved PARP (cPARP) (Figure 1c). The applied *ex vivo* culture approach supported continued cell proliferation as determined by BrdU (Figure 1c) and Ki67 expression (Supplementary figure 1 (43)). Thus, both proliferating DZ and FZ cells were detected, with DZ cells being the most frequently proliferating, which is in accordance with observations in the non-culture control HFA tissue. Quantification of $BrdU^+$ cells demonstrated no difference in the number of proliferating ($BrdU^+$ cells) between 7- and 14-days culture (Figure 1d). Overall, only few $cPARP^+$ cells were detected in both cultured and non-culture control HFA tissue (Figure 1c, Supplementary figure 1(43)). A small increase in the prevalence of $cPARP^+$ cells/$mm^2$ was observed in cultured tissue compared with non-culture control HFA tissue, but the number of $cPARP^+$ cells/$mm^2$ decreased with length of culture period (3.8-fold,

222 $p<0.05$) when HFA tissue cultured for 14-days were compared to 7-days (Figure 1d). Together these results
223 suggest that the applied *ex vivo* culture approach supports HFA tissue for up to 14-days of culture with overall
224 preserved morphology, continued cell proliferation and a low level of apoptosis in the tissue.
225

226 ## *Steroidogenic activity is maintained after ex vivo culture of HFA tissue*

227 The endocrine function of *ex vivo* cultured HFA tissue was evaluated by steroidogenic protein expression and
228 secretion of steroid hormones. The examined steroidogenic enzyme expression was maintained after *ex vivo*
229 culture for 7- and 14-days. Thus, abundant expression of CYP17A1 and CYP11A1 were observed in FZ cells
230 in accordance with expression found in non-culture control samples (Figure 2a, Supplementary figure 2a (44)).
231 Overall, the zonation of the *ex vivo* cultured tissue was preserved containing both FZ cells ($SPARC^+$, (21)) and
232 DZ cells ($NCAM\text{-}1^+$, (45)) (Figure 2a). However, few areas of the cultured tissue fragments appeared negative
233 for DZ or FZ markers resulting in a 1.5-fold decrease ($p<0.05$) in tissue positive for the HFA zonation markers
234 compared with non-culture controls (Figure 2b). This reduction did not appear to be dependent on the culture
235 period as no differences were observed between CYP17A1, SPARC or NCAM-1 expression between tissue
236 fragments cultured for 7- and 14-days (Supplementary figure 2b (44)). Additionally, analysis of the ratio
237 CYP17A1/SPARC expression suggested that CYP17A1 expression was similar to SPARC expression in the
238 *ex vivo* cultured tissue as well as in the non-cultured controls (Figure 2c). To clarify whether the cells negative
239 for both SPARC and NCAM-1 maintained expression of other proteins, the expression of steroidogenic factor
240 1 (SF-1) was examined. In non-culture controls and cultured HFA tissue SF-1 was expressed in DZ cells and
241 FZ cells (Supplementary figure 3 (46)), but no SF-1 expression was detected in areas of *ex vivo* cultured tissue
242 negative for SPARC and NCAM-1 (Supplementary figure 3b, dotted lines (46)). Some of the SF1-negative
243 cells morphologically resembled capsule cells, expected to be SF-1 negative, while others morphologically
244 resembled adrenal cortical cells suggesting that this sub-population of cells may have differentiated.
245 Importantly, the general steroidogenic activity of HFA tissue was maintained in *ex vivo* culture. Hence, the
246 secretion of androgens, glucocorticoids, and steroidogenic intermediates was consistent with no differences
247 in culture media concentrations between 7- and 14-days cultures (Figure 2d-f). The cultured HFA tissue
248 produced high amounts of both androgens and glucocorticoids. As anticipated DHEAS were by far the most
249 abundant of all measured metabolites (>20,000 nmol/L). The remaining steroid hormones measured in this
250 study followed the distribution of the intra-adrenal steroid hormones previously reported (47), with cortisol

(>1000 nmol/L) measured in the second highest concentrations (after DHEAS). In line with this, testosterone (<5 nmol/L) levels were the lowest among the measured androgens but were still detectable in all samples. In addition to the observed steroidogenic active fetal adrenal cortical cells, proliferating migrating neural crest cells were also frequently observed in the tissue after 14-days culture (marked by NCAM-1 (48), Figure 3), resembling the cell populations of non-cultured HFA controls.

### *Steroidogenic activity can be both stimulated and inhibited in HFA tissue cultured ex vivo*

HFA tissue fragments were treated with ACTH (1 nM) or ketoconazole (10 µM) to examine whether the steroidogenic activity could be induced and inhibited in this *ex vivo* culture model (Figure 4a). The stimulating effect of ACTH and inhibitory effect of ketoconazole on steroid hormone production was assessed after 7- and 14-days of culture. ACTH-treatment increased androgen, glucocorticoid, and steroidogenic intermediates metabolite concentrations significantly compared with vehicle control treated samples after both culture periods (Figure 4b, d). After 14 days of culture, ACTH treatment resulted in increased androgen levels, including androstenedione (10.8-fold, $p<0.01$), testosterone (9.4-fold, $p<0.05$) and DHEAS (2.5-fold, $p<0.01$). Also, glucocorticoid levels were increased after 14 days ACTH stimulation, including 17-hydroxyprogesterone (4.5-fold, $p<0.01$), 11-deoxycortisol (12.7-fold, $p<0.01$), cortisol (6.9-fold, $p<0.01$) and cortisone (1.7-fold, $p<0.01$). The steroidogenic intermediates progesterone (2-fold, $p<0,05$) and corticosterone (4.9-fold, $p<0.01$) levels were also increased compared with vehicle controls. Treatment with ketoconazole resulted in decreased secretion of androstenedione (1.8-fold, $p<0.05$) and DHEAS (4.2-fold, $p<0.01$) after culture for 14 days, although this was only significant for DHEAS (1.7-fold, $p<0.05$) after 7 days of culture. Additionally, levels of 11-deoxycortisol (4.3-fold, $p<0.01$), 17-hydroxyprogesterone (2.3-fold, $p<0.05$) and progesterone (11.2-fold, $p<0.01$) were all increased compared with vehicle controls as a result of ketoconazole treatment (Figure 4c,e). Overall, the most pronounced effects on steroid hormone production were seen after 14 days of culture for both ACTH and ketoconazole treatments.

The effects of ACTH and ketoconazole treatment on steroidogenic protein expression and HFA tissue zonation were subsequently evaluated after 7- and 14-days of culture. Treatment with ACTH altered the composition of HFA tissue, including an increased FZ-area ($SPARC^+$ and $CYP17A1^+$, both 3.5-fold increase, $p<0.05$ and $p<0.01$, respectively) as well as a decreased DZ-area ($NCAM\text{-}1^+$, 1.9-fold, $p<0.05$), compared with vehicle

controls after 14 days of culture (Figure 5). In contrast, no effect on FZ-area (SPARC$^+$, CYP17A1$^+$) or DZ-area (NCAM-1$^+$) was observed after 7- or 14-days culture with ketoconazole treatment (Supplementary figure 4 (49), Figure 5).

### *Manipulation of steroidogenic activity does not affect morphology and cell viability of ex vivo cultured HFA tissue*

Treatment with ACTH and ketoconazole did not alter the overall morphology of the *ex vivo* cultured HFA tissue, which still contained all cell populations present in 1$^{st}$ trimester HFA tissue at the end of the culture period. Effects of treatment on cell proliferation (BrdU) and apoptosis (cPARP) were examined after 7- and 14-days culture (Figure 6a,b). No significant effects were detected on the number of proliferating (BrdU$^+$) or apoptotic (cPARP$^+$) cells after treatment with ACTH or ketoconazole compared with vehicle controls (Figure 6c-d). Proliferating cells were more frequently observed in DZ cells in *ex vivo* cultured samples regardless of treatment but were also observed in FZ and neural crest cells in accordance with non-culture controls (Supplementary figure 1 and Figure 3, respectively).

## Discussion

In this study, an *ex vivo* tissue culture approach for HFA tissue has been established and validated. Thus, we have shown that HFA tissue fragments can be cultured for 2 weeks with continued support of adrenal morphology, cell proliferation and steroidogenic activity. Additionally, we demonstrated that this culture approach allows inhibition and stimulation of the HFA tissues steroidogenic activity.

The *ex vivo* culture approach established in this study allows culture of HFA tissue for 14 days with continuing proliferation with presence of only few apoptotic cells. Studies from the late 1970s reported that HFA tissue fragments could be cultured *ex vivo* (0.5 $cm^3$ on steel grids) for up to 14 days, however no details about morphology or cell viability of the cultured tissue were reported (30,31). Also, long-term culture of HFA organoids from primary cell cultures were recently reported, but necrotic core formation was observed after 10 days of culture (48). Thus, tissue fragments of approximately 1 $mm^3$ used in this study seems to allow diffusion of oxygen into the core of the fragments protecting the tissue from necrosis. In addition to continued

proliferation, the *ex vivo* culture approach used here preserved 1st trimester HFA morphology maintaining both FZ, DZ and migrating neuroendocrine cells. Cross-talk between the different cell populations in HFA have been shown to be important to ensure normal development of the adrenal gland (15,16). Hence, patients with CAH due to 21-hydroxylase deficiency do not only have disrupted steroidogenesis, but the adrenal morphology is also affected with poorly defined zonation and incomplete formation of the adrenal medulla, showing small islands of chromaffin cells within the adrenal cortex (50). Accordingly, infants with CAH have reduced adrenomedullary function (51). Thus, conserved cross-talk between the different adrenal cell-types are central in the establishment of a culture model that aims to recapitulate normal and dysregulated HFA development and function.

A small sub-population of cells in the cultured tissue fragment lost the expression of both FZ and DZ zonation markers causing a slight reduction of the FZ-area in the cultured tissue compared with non-culture controls. At least some of the difference in the quantification of zonation-area was due to the variation in orientation of the paraffin embedded cultured tissue fragments. This variance made zonation-area quantification more dependent on tissue orientation in *ex vivo* cultured tissue fragments compared with non-culture controls where each tissue section represented a cross-section of an intact fetal adrenal gland. Therefore, zone-specific IHC results should be considered as semi-quantitative. However, few small tissue areas lost expression of both zonation markers and SF-1 which could indicate that this sub-population of cells are capsule cells with high proliferation rate and/or adrenal cortical cells that have differentiated. The small affected areas did not increase with a longer culture period suggesting that the *ex vivo* culture approach supports all cell populations up to 14 days. Overall, the presented HFA *ex vivo* culture approach preserved cell viability and an overall tissue zonation that consisted of both neuroendocrine and steroidogenic cells.

The *ex vivo* culture approach described in this study supports the endocrine function of the HFA tissue. The secreted steroid hormone profiles of androgens, glucocorticoids and steroidogenic intermediates from the cultured tissue is in accordance with the intra-adrenal metabolites previously measured in un-cultured 1st trimester HFA samples (5). Additionally, the high DHEAS levels secreted from the cultured HFA tissue in this study is in accordance with DHEAS being the most abundant adrenal hormone produced during fetal life (52). The continued production of androgens and glucocorticoids reported in this study contrasts with previous long-

term HFA tissue cultures which showed a rapid time-dependent decrease in the production of both DHEAS and cortisol (30). Except for DHEAS and cortisol, the unstimulated steroid hormone production has not been reported in any of the previously described tissue culture models. Thus, the presented HFA *ex vivo* approach maintains a high steroidogenic activity which is a unique advance of this experimental approach while also preserving FZ-specific abundant expression of steroidogenic enzymes. The *ex vivo* approach could therefore be used to investigate the endocrine activity of the HFA gland in future studies that relate to understanding HFA physiology in health and disease. This could include determination of *de novo* synthesis of HFA backdoor androgen secretion as HFA tissue is able to convert steroid metabolites into backdoor pathway androgens (53) which are suspected to play a crucial role in several congenital diseases including CAH (54-56).

Notably, we demonstrated that steroidogenic activity in *ex vivo* cultured HFA tissue can be stimulated and inhibited resulting in altered profiles of classic steroid hormones. Thus, this provides proof-of-principle that this culture approach can be used in future studies to examine effects on adrenal function following specific treatments. ACTH stimulated the secretion of all measured metabolites, which is in accordance with previous studies showing a stimulating effect on both cortisol, DHEAS, androstenedione and testosterone (9,30,31,39). Interestingly, stimulation of steroid hormone secretion was accompanied by an increased FZ area and CYP17A1 expression after culture for 14 days. This suggests that *ex vivo* cultured tissue responds in a similar manner to HFA tissue *in vivo* as enlargement of the steroidogenic adrenal cortex is known to be a consequence of hyperstimulated adrenals (57). Increased *CYP17A1* expression have also previously been reported in response to ACTH (26). The enlarged FZ in ACTH stimulated tissue found in the present study after both 7- and 14-days treatment could also explain the tendency towards a decreased number of proliferating cells since DZ cells are known to have a higher proliferation rate than FZ cells (25).

Effects of the well-known inhibitor of steroidogenic enzymes, ketoconazole which affects multiple steps of adrenal steroidogenesis was also examined in the HFA *ex vivo* model. Ketoconazole has different IC50 values for each steroidogenic enzyme (58) and therefore some metabolites are expected to be upregulated while metabolites downstream of these enzymes with the lowest IC50 values are expected to be downregulated in a system where steroidogenesis is not completely inhibited. The observed inhibition of the HFA androgens androstenedione and DHEAS following ketoconazole treatment for 14 days was therefore expected, as was

the increased levels of substrates for affected enzymes such as progesterone and 17-hydroxyprogesterone. The overall lack of ketoconazole-mediated effects on corticosteroid levels was unexpected, especially the lack of effect on cortisol secretion when compared with vehicle control samples. However, the increased concentration of 11-deoxycortisol indicates that if the system had been pushed further either by initial ACTH stimulation or higher ketoconazole concentrations an effect on cortisol might have been detectable. The higher prevalence of 3βHSD2$^{+}$ cells/tissue area in 1$^{st}$ trimester compared with 2$^{nd}$ trimester HFA tissues and similar intra-adrenal concentrations of corticosteroids (5) could suggest an elevated capacity for glucocorticoid synthesis and subsequently a lower sensitivity towards inhibition of corticosteroid biosynthesis in 1$^{st}$ trimester adrenal tissue. Accordingly, no effects were observed in the *ex vivo* cultured HFA tissue following treatment with 1 µM ketoconazole (Supplementary figure 5 (59)) and the used dose of 10 µM ketoconazole in this study is lower than expected *in vivo* ketoconazole serum concentrations in patients, as ketoconazole normally is administrated in doses between 400-1200 mg/day (60) corresponding to ~12-25 µM serum-ketoconazole detected in patients 4-6 h after drug intake (61). Treatment effects of ketoconazole have previously been tested in several primary cultures of human adrenocortical tissue and human adrenocortical carcinoma cell lines, however the reported effects on steroid hormone production have been inconsistent depending on cell type and dose (47,62-63). Thus, it is difficult to compare the effects of ketoconazole on the *ex vivo* cultured HFA tissue composed of a heterogeneous cell population with previous studies. In this study, ketoconazole treatment did not affect FZ-area (SPARC expression), expression of CYP17A1 in the cultured tissue or cell viability compared with vehicle controls. This is in line with a previous study which found ketoconazole concentrations below 10 µM did not reduce cell viability of human adrenal carcinoma cells (61). Together these data suggest that signaling pathways involved in the regulation of adrenal steroidogenesis are kept intact in the *ex vivo* cultured tissue which retains the capacity to respond to both ACTH stimulation and ketoconazole inhibition for up to 14 days. This specific steroidogenic response in the presented HFA *ex vivo* cultured tissue suggest that this model can be used to investigate local effects of pharmaceutical exposures or emerging therapeutic treatments that may impact adrenal development or function during pregnancy. Additionally, manipulations of specific steroidogenic enzymes and cofactors in this model can serve as the foundation of a human CAH *ex vivo* culture model allowing mechanistic studies of the dysregulated steroidogenesis in this rare disease.

In conclusion, we have established an *ex vivo* culture approach for HFA tissue that allows culture for two weeks with preserved adrenal morphology, cell viability, and a continued high steroidogenic activity. Investigations of treatment effects in the HFA *ex vivo* cultures have demonstrated a specific response to both stimulation and inhibition. Therefore, this model may be used to study both normal and dysregulated HFA steroidogenic activity and function, which will be of particular relevance for understanding development and management of adrenal disorders in humans.

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## Abbreviations

ACTH: Adrenocorticotropic hormone; CAH: Congenital adrenal hyperplasia; cPARP: Cleaved PARP; DZ: Definitive zone; DHEAS: Dehydroepiandrosteron-sulfate; FZ: Fetal zone; GW: Gestational week; HFA: Human fetal adrenals; IHC: Immunohistochemistry; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; RSD: Relative standard deviation; SEM: Standard error of the mean; TZ: Transitional zone.

## Data availability

All data generated or analyzed during this study are included in this published article or in the data repositories listed in references.

## Author Contributions

C.M. and A. Jørgensen conceived and designed the experiments. C.M., J.E.N., and H.F. performed the experiments. S.P., L.L., L.L.T., K.J.H., and M.S. provided study material. C.M., J.E.N., H.F., R.T.M., A. Juul, and A. Jørgensen analyzed the data. C.M. and A. Jørgensen wrote the manuscript. All authors read and approved the submitted version of the manuscript.

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# Legends for figures and tables

**Figure 1. Morphology and cell viability in human fetal adrenals cultured *ex vivo.*** (a) Experimental overview of the *ex vivo* culture approach. (b-c) Morphology and expression pattern of BrdU (proliferation marker) and cPARP (apoptosis marker) investigated on serial sections of fetal adrenal tissue cultured *ex vivo* for 7- or 14-days. Counterstaining with Mayer's hematoxylin; scale bar corresponds to 100 µm. Age of fetal samples shown (at the start of experiment): 7 days culture (b+c) GW 10+1; 14 days culture (b+c) GW 9+2. (d) Quantification of proliferating ($BrdU^+$) and apoptotic ($cPARP^+$) cells per $mm^2$ tissue. Values represent mean ± SEM, n = 7 fetuses for both culture periods. Values from the individual fetal samples are shown and represents 3-20 analyzed tissue fragments per fetal sample depending on the initial size of the adrenal gland. Significant difference between 7- and 14-days cultures were analyzed using Mann Whitney (two-tailed) u-test, *$p<0.05$.

**Figure 2. Steroidogenic activity of human fetal adrenals cultured *ex vivo*.** (a) Expression of CYP17A1, SPARC (fetal zone marker), and NCAM-1 (definitive zone marker) investigated on serial sections of intact 1st trimester adrenal non-culture controls and fetal adrenal tissue cultured *ex vivo* for 7- or 14-days. Counterstaining with Mayer's hematoxylin; scale bar corresponds to 100 µm. Age of fetal samples shown (at the start of experiment): non-culture control GW 11; 7 days culture GW 10+1, 14 days culture GW 10+4. (b) Quantification of fetal- or definitive-zone tissue-area per $mm^2$ tissue was determined as the percentage of SPARC and NCAM-1 expression, respectively. (c) Quantification of fetal-zone CYP17A1 expression was determined as the ratio of CYP17A1/SPARC expression per $mm^2$ tissue. Values represent mean ± SEM, non-culture controls: n = 8 fetuses, *ex vivo* cultured tissue: n = 7 fetuses for both groups. Values from the individual fetal samples are shown and represents 3-20 analyzed tissue fragments per fetal sample depending in the initial size of the adrenal gland. Significant difference compared with non-culture controls were analyzed using Mann Whitney (two-tailed) u-test, **$p<0.01$. (d-f) Quantification of secreted adrenal androgen, glucocorticoid, and steroidogenic intermediates metabolites measured by LC-MS/MS (nmol/L) in culture media collected throughout the experimental period of 7- or 14-days. Values represent mean ± SEM; 7 days cultured tissue, n = 7 fetuses; 14 days culture tissue, n = 11 fetuses, with media pooled from 3-20 tissue fragments per adrenal

622 depending on the initial size of the adrenal gland. Significant difference between 7- and 14-days cultures were
623 analyzed using Mann Whitney (two-tailed) u-test.
624
625 **Figure 3. Migrating neural crest cells are maintained in *ex vivo* cultured tissue.** Migrating neural crest
626 cells were frequently observed in *ex vivo* cultured tissue similar to non-cultured control tissue. (a) Expression
627 of NCAM-1 (definitive zone and neural crest cell marker) and CYP17A1 (fetal zone specific) in an intact 1st
628 trimester adrenal non-culture controls. (b) In addition to immunohistochemical staining of NCAM-1 and
629 CYP17A1, BrdU (proliferation marker) and cPARP (apoptosis marker) were investigated on serial sections of
630 fetal adrenal tissue cultured *ex vivo* for 14 days. Arrows indicate BrdU+ neural crest cells. Counterstaining with
631 Mayer's hematoxylin; scale bar corresponds to 50 µm. Age of fetal samples shown (at the start of experiment):
632 non-culture control GW 10+6; 14 days culture GW 11+4.
633
634 **Figure 4. Manipulation of steroidogenesis in human fetal adrenals cultured *ex vivo*.** (a) Experimental
635 overview of *ex vivo* cultured human fetal adrenals treated with ACTH (1 nM) or ketoconazole (10 µM) for 7- or
636 14-days. Quantification of secreted adrenal androgen, glucocorticoid, and steroidogenic intermediates
637 metabolites after ACTH or ketoconazole treatment. Secretion of adrenal steroid metabolites was measured by
638 LC-MS/MS in the culture media collected throughout the experimental period of 7- (b,c) or 14-days (d,e).
639 Quantification of secreted metabolite concentrations presented as a ratio relative to vehicle control samples.
640 Values represent mean ± SEM, 7 days cultures: ACTH, n = 8 fetuses; ketoconazole, n = 10 fetuses; 14 days
641 cultures: ACTH, n = 9 fetuses; ketoconazole, n = 9 fetuses, with media pooled from 1-7 tissue fragments per
642 treatment depending on the initial size of half of the adrenal gland. Significant difference compared with vehicle
643 controls from the same HFA were analyzed using Wilcoxon matched-pairs signed rank test (two-tailed) t-test.,
644 *p<0.05, **p<0.01.
645
646 **Figure 5. Expression of CYP17A1 and zonation markers after manipulation of steroidogenesis in**
647 **human fetal adrenals cultured 14 days *ex vivo*.** (a) Effects of ACTH (1 nM) and ketoconazole (10 µM)
648 treatment were examined by immunohistochemical staining for CYP17A1, SPARC (fetal zone marker), and
649 NCAM-1 (definitive zone marker) on serial sections of human fetal adrenals cultured *ex vivo* for 14-days.
650 Counterstaining with Mayer's hematoxylin; scale bar corresponds to 100 µm. Age of fetal samples shown (at

651 the start of experiment): vehicle control 9+5; ACTH GW 9+2; ketoconazole GW 10+1. (b-c) Expression of
652 CYP17A1, SPARC and NCAM-1 per $mm^2$ were quantified as a ratio relative to vehicle control samples. Values
653 represent mean ± SEM, ACTH treated samples, n = 8 fetuses; ketoconazole treated samples n = 6 fetuses.
654 Values from the individual fetal samples are shown and represents 1-7 analyzed tissue fragments per sample
655 depending on the initial size of half of the adrenal gland. Significant difference compared with vehicle controls
656 from the same HFA were analyzed using Wilcoxon matched-pairs signed rank test (two-tailed), *p<0.05,
657 **p<0.01.
658
659 **Figure 6. Morphology and cell viability after manipulation of steroidogenesis in human fetal adrenals**
660 **cultured *ex vivo*.** Morphology and expression pattern of BrdU (proliferation marker) and cPARP (apoptosis
661 marker) investigated on serial sections of fetal adrenal tissue cultured *ex vivo* for 7- (a) or 14-days (b).
662 Counterstaining with Mayer's hematoxylin; scale bar corresponds to 100 µm. Age of fetal samples shown (at
663 the start of experiment): 7 days cultures: vehicle control GW 11+1; ACTH GW 9+4; ketoconazole GW 10+6,
664 14 days cultures: vehicle control GW 10+2; ACTH GW 9+2; ketoconazole GW 10+1. (c-d) Quantification of
665 proliferating ($BrdU^+$) and apoptotic ($cPARP^+$) cells per $mm^2$ determined as a ratio relative to vehicle control.
666 Values represent mean ± SEM, 7 days cultures: ACTH, n = 5 fetuses; ketoconazole, n = 10 fetuses; 14 days
667 cultures: ACTH, n = 8 fetuses; ketoconazole, n = 6 fetuses. Values from the individual fetal samples are shown
668 and represents 1-7 analyzed tissue fragments per sample depending on the initial size of half of the adrenal
669 gland. Significant difference compared with vehicle controls from the same HFA were analyzed using Wilcoxon
670 matched-pairs signed rank test (two-tailed).
671
672 **Table 1.** Antibodies, dilutions and retrieval buffers used.

| **Antibody** | **Dilution** | **Retrieval buffer** | **Species** | **Supplier** | **Number** |
|---|---|---|---|---|---|
| CYP11A1 | 1:10.000 | TEG | Rabbit | Sigma | HPA016436 |
| CYP17A1 | 1:1500 | CIT | Rabbit | Abcam | Ab134910 |
| SPARC | 1:2000 | CIT | Goat | R&D Systems | AF941 |
| NCAM-1 | 1:1500 | CIT | Mouse | BD Pharmingen | 565237 |
| BrdU | 1:100 | CIT | Mouse | Dako | M0744 |

| | | | | | |
|---|---|---|---|---|---|
| cPARP | 1:500 | CIT | Rabbit | Cell Signaling | 5625 |
| Ki67 | 1:5000 | TEG | Mouse | Dako | M7240 |
| SF-1 | 1:10.000 | CIT | Rabbit | Abcam | Ab217317 |

673

674 *Antigen retrieval was conducted by pressure cooking of the sections in indicated retrieval buffer for 30 min. in*

675 *a decloaking chamber. TEG buffer: 10 mM Tris, 0.5 mM EGTA, pH 9.0; Citrate (CIT) buffer: 10 mM, pH 6.0.*

Figure 1



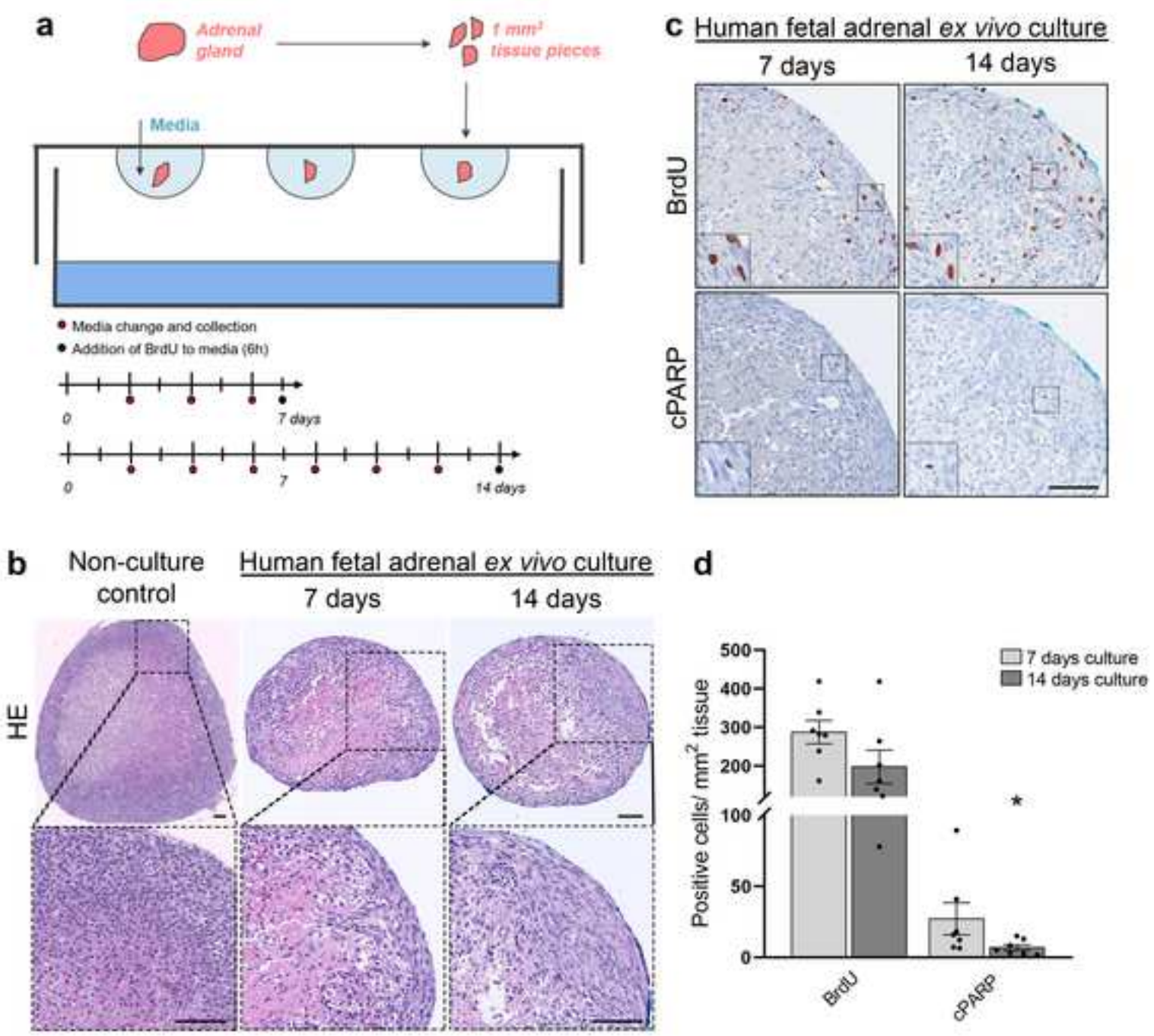

Figure 2

Click here to access/download;Figure;jc.2020-02323R1_Figure 2.tif

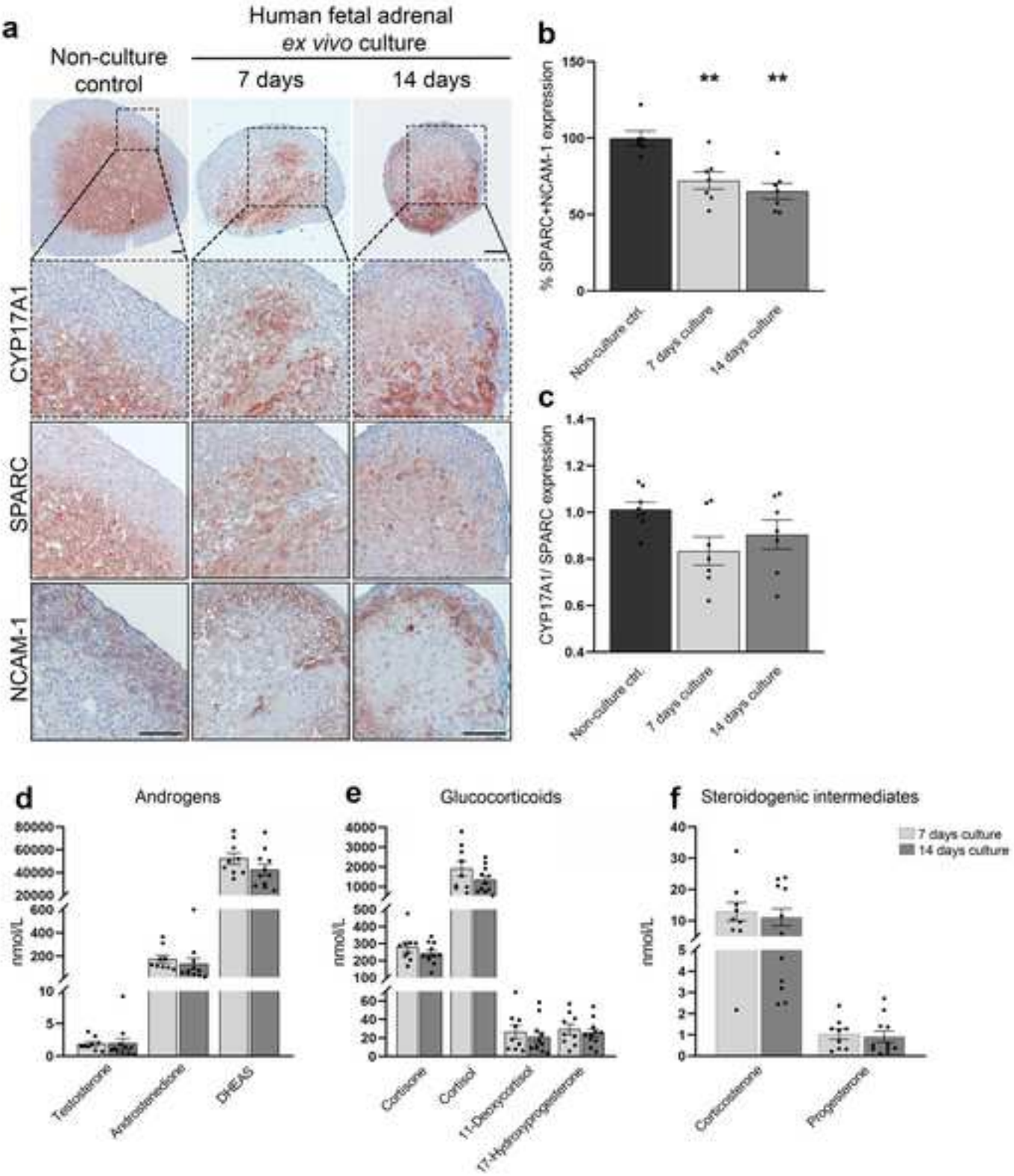

Figure 3



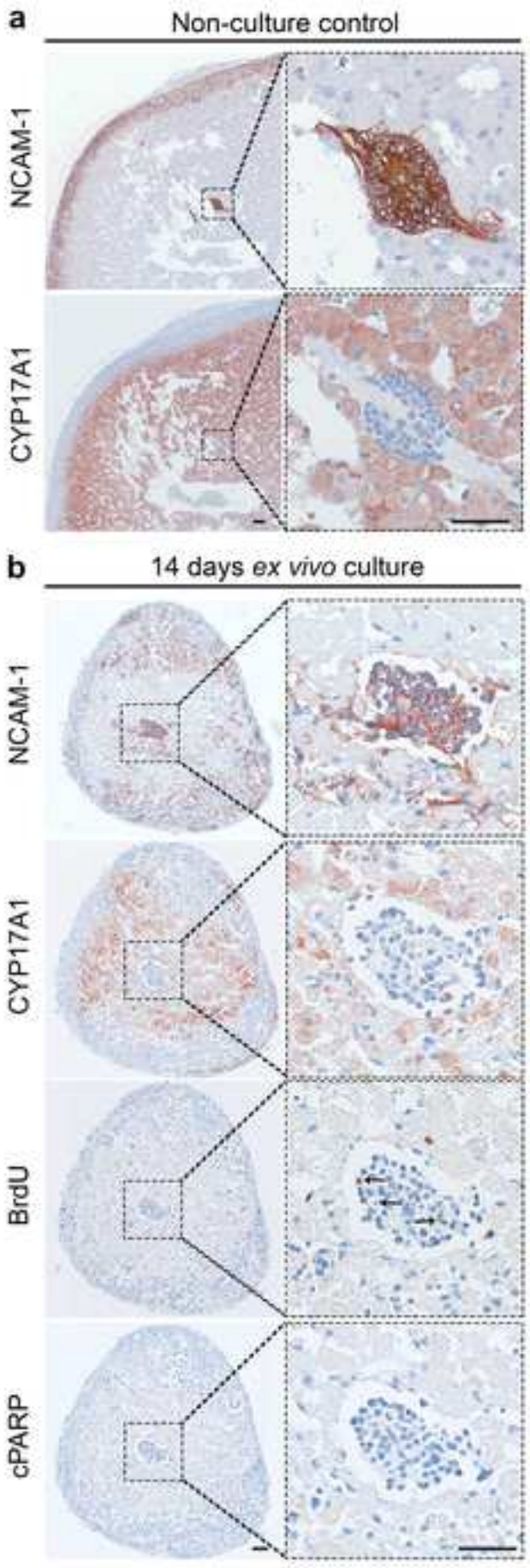

Figure 4



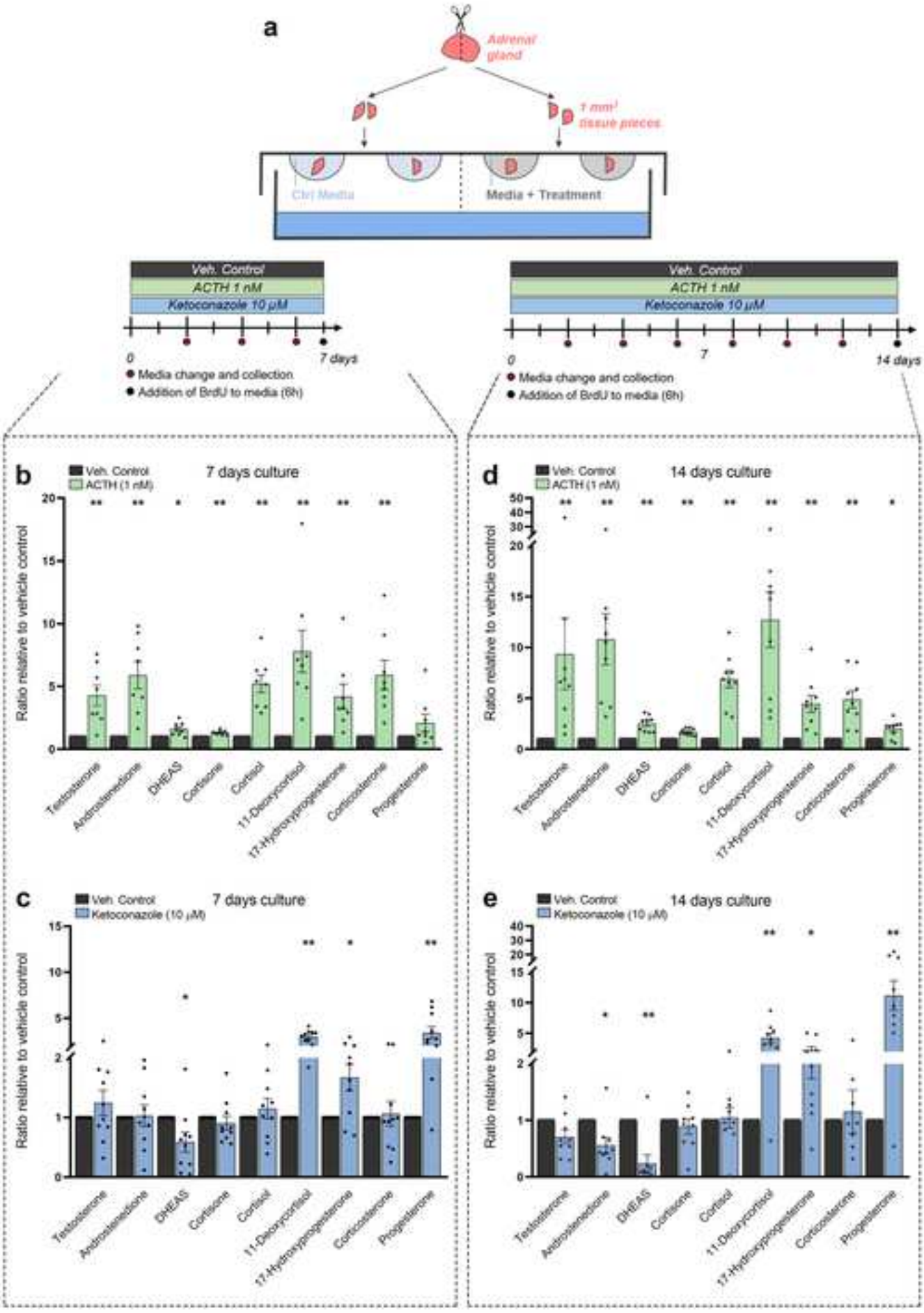

Figure 5



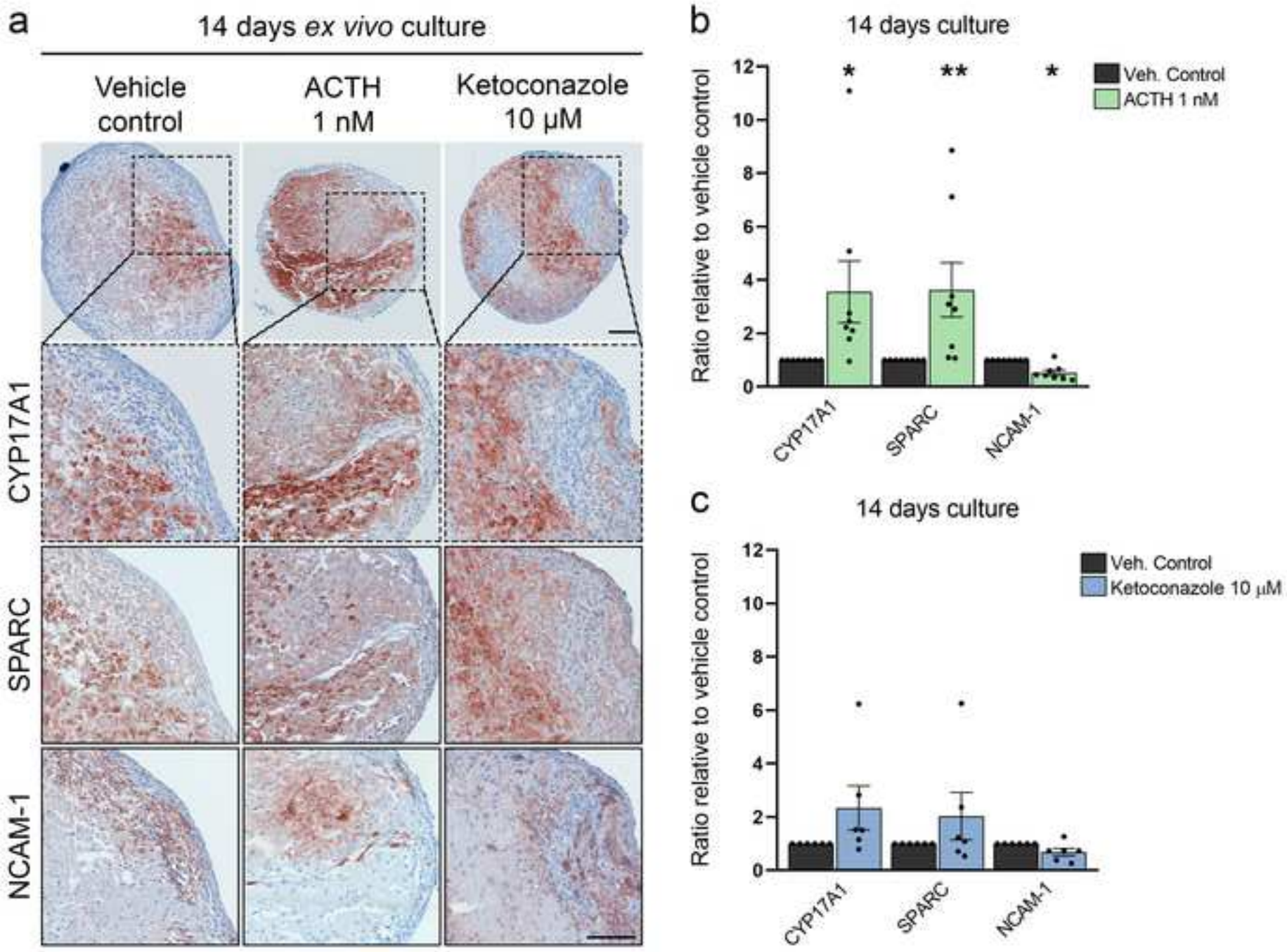

Figure 6