

# H295R expression of melanocortin 2 receptor accessory protein results in ACTH responsiveness

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## Abstract

The H295R adrenocortical cell line is widely used for molecular analysis of adrenal functions but is known to have only modest ACTH responsiveness. The lack of ACTH response was linked to a low expression of its receptor, melanocortin 2 receptor (MC2R). We hypothesized that increasing the MC2R accessory protein (MRAP), which is required to traffic MC2R from the endoplasmic reticulum to the cell surface, would increase ACTH responsiveness. Lentiviral particles containing human MRAP-open reading frame were generated and transduced in H295R cells. Using antibiotic resistance, 18 clones were isolated for characterization. The most ACTH-responsive steroidogenic clone, H295RA, was used for further experiments. Successful induction of MRAP and increased expression of MC2R in H295RA cells was confirmed by quantitative real-time RT-PCR and protein analysis. Treatment with ACTH significantly increased aldosterone, cortisol, and dehydroepiandrosterone production in H295RA cells. ACTH also significantly increased transcript levels for all of the steroidogenic enzymes required to produce aldosterone, cortisol, and dehydroepiandrosterone, as well as *MC2R* mRNA. Using liquid chromatography/tandem mass spectrometry, we further revealed that the main unconjugated steroids produced in H295RA cells were 11-deoxycortisol, cortisol, and androstenedione. Treatment of H295RA cells with ACTH also acutely increased cAMP production and cellular protein levels for total and phosphorylated steroidogenic acute regulatory protein. In summary, through genetic manipulation, we have developed an ACTH-responsive human adrenocortical cell line. The cell line will provide a powerful *in vitro* tool for molecular analysis of physiologic and pathologic conditions involving the hypothalamic–pituitary–adrenal axis.

## Key Words

- ▶ ACTH
- ▶ MC2R
- ▶ MRAP
- ▶ H295R
- ▶ cell line

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## Introduction

The adrenal cortex is composed of three functionally distinct regions: zona glomerulosa, zona fasciculata, and zona reticularis. The zona glomerulosa is regulated by circulating angiotensin II (Ang II), potassium, and

adrenocorticotrophic hormone (ACTH) and synthesizes mineralocorticoids. The zona fasciculata is regulated by ACTH and produces cortisol. The zona reticularis is mainly regulated by ACTH and produces 19 carbon (C<sub>19</sub>)

steroids including DHEA and DHEAS. The disruption of adrenal steroid production results in various disorders. *In vitro* cell culture models provide important information for understanding molecular and cellular mechanisms controlling both the normal and pathologic function of the adrenal cortex. The H295R adrenocortical cell line is widely used but is known to have only modest ACTH responsiveness (Bird *et al.* 1993, Rainey *et al.* 2004, Wang & Rainey 2012). Therefore, studies directed toward ACTH action have used primary cultures of adrenal cells (Xing *et al.* 2010, 2011) or Y-1 mouse adrenal cell line, which retains ACTH responsiveness (Schimmer 1979).

ACTH acts through melanocortin 2 receptor (MC2R) to induce the intracellular production of cAMP and stimulate steroidogenesis in the adrenal cortex. For functional expression of MC2R, MC2R accessory protein (MRAP) is required to traffic MC2R from the endoplasmic reticulum (ER) to the cell surface (Metherell *et al.* 2005, Webb *et al.* 2009). Mutations in *MRAP* gene cause familial glucocorticoid deficiency (FGD) type 2, which is an autosomal recessive disorder characterized by severe cortisol deficiency with high plasma ACTH levels (Metherell *et al.* 2005). In the Y-1 mouse cell line that endogenously express both MC2R and MRAP, knockdown of MRAP by shRNA resulted in a loss of response to ACTH, which was rescued by overexpression of the shRNA-insensitive human MRAP (Cooray *et al.* 2008). It has also been demonstrated that mRNA levels of both MC2R and MRAP are regulated by ACTH in human adrenal primary cultures (Xing *et al.* 2010). In the present study, we hypothesized that increasing the expression of MRAP in the H295R cell line would improve ACTH responsiveness.

## Materials and methods

### H295RA cell culture establishment

The human adrenocortical cell line H295R was cultured in DME/F12 medium (Invitrogen), 10% Cosmic Calf serum (Hyclone, Logan, UT, USA), and antibiotics. The expression of MRAP was enhanced in H295R cells using a pLX304-Blast-V5 vector with a MRAP open reading frame (GE Dharmacon, Lafayette, CO, USA, OHS6085-213581101, Accession #BC062721). Lentiviral particles containing the MRAP open reading frame were produced and transduced into H295R cells with a multiplicity of infection of 5.0 using 6 µg/ml of polybrene (EMD Millipore Corporation, Billerica, MA, USA). Transduced cells were then selected by 1 µg/ml of blasticidin

(Life Technologies). The concentration of blasticidin was determined according to the blasticidin selectivity test with H295R cells. After the antibiotic selection, cells were plated at cloning density and isolated using cloning cylinders (Sigma–Aldrich). From 18 isolated clones, the most ACTH-responsive steroidogenic clone (H295RA) was used for this study. The parental H295R cells were used for comparison experiments. For experiments, cells were plated in 24-well plates at a density of 200 000 per well and incubated at 37 °C for 2 days. One day before the experiment, cells were changed to a low serum experimental medium (DME/F12 medium with 1% Cosmic Calf serum and antibiotics). The next morning, cells were treated in the same low serum or serum-free experimental medium for the indicated times. Serum-free medium was used for cAMP measurement to avoid interference of Cosmic Calf serum to the assay.

### Steroid immunoassays

Aldosterone, cortisol, and DHEA were measured in cell culture medium using ELISA. ELISA kits for cortisol and DHEA were purchased from Alpco (Sakem, NH, USA). MAB against aldosterone was kindly provided by Dr C E Gomez-Sanchez, University of Mississippi Medical Center (Gomez-Sanchez *et al.* 1987). The results were normalized to protein amounts and shown as fold change over basal condition.

### Steroid quantitation by liquid chromatography-tandem mass spectrometry

Unlabeled and deuterium-labeled steroid standards were obtained from Sigma–Aldrich, Steraloids, Cerilliant (Round Rock, TX, USA), C/D/N Isotopes (Pointe-Claire, Quebec, Canada), or Cambridge Isotope Laboratories (Tewksbury, MA, USA), or they were synthesized (shown in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article for unpublished steroids). Preparation and measurement of  $\Delta^4$  steroids were performed as previously described (Turcu *et al.* 2015). Steroid sulfates were extracted with 1 ml of 1:1 chloroform:2-butanol from a 50 µl serum aliquot after mixing with 200 µl 1 M ammonium sulfate. The dried extracts were reconstituted with 200 µl of methanol/deionized water (1:1) and transferred to a 0.25 ml vial insert. Steroid quantitation was performed as previously described (Turcu *et al.* 2015). [Supplementary Table 1](#) gives retention times and precursor/product ion pairs for the targeted steroids not previously published.

## RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from cells using RNeasy mini kits (Qiagen). The purity and integrity of the RNA were checked spectroscopically using a Nano Drop spectrometer (Nano Drop Technologies, Wilmington, DE, USA). Total RNA was reverse transcribed using the High-capacity cDNA Archive kit (Applied Biosystems). PCR were performed in the ABI StepOnePlus Real-Time PCR systems (Applied Biosystems). Primer and probe mixtures for the amplification of the *MRAP* (Hs1588793\_m1), *MC2R* (Hs00300820\_s1), and peptidylprolyl isomerase A (*PPIA*, Hs99999901\_m1) target sequences were purchased from Applied Biosystems. Primer and probe sets for cholesterol side-chain cleavage (*CYP11A1*), steroidogenic acute regulatory protein (*StAR*), 3 $\beta$ -hydroxysteroid dehydrogenase type II (*HSD3B2*), 17 $\alpha$ -hydroxylase-17, 20-lyase (*CYP17*), 21-hydroxylase (*CYP21*), 11 $\beta$ -hydroxylase (*CYP11B1*), and aldosterone synthase (*CYP11B2*) were prepared as described previously (Sirrianni *et al.* 2005, Ye *et al.* 2009). *PPIA* transcript was used as a reference gene for sample normalization. The delta-delta threshold cycle ( $\Delta\Delta C_t$ ) method was used to calculate fold changes in mRNA expression (Livak & Schmittgen 2001).

## Protein extraction and western analysis

Cells were lysed in mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA). The protein content of samples was determined by the bicinchoninic acid protein assay using the micro bicinchoninic acid protocol (Thermo Scientific). For western analysis, equal amounts of protein samples were incubated with reducing buffer and boiled in lithium dodecyl sulfate sample buffer. The reaction mix was separated on bis-tris gel and transferred to polyvinylidene difluoride membranes (Invitrogen). The membranes were incubated with V5 antibody (mouse, 1:1000; Thermo Scientific, MA5-15253), *MRAP* antibody (mouse, 1  $\mu$ g/ml; Abcam, Cambridge, MA, USA, ab168120), steroidogenic acute regulatory protein (*StAR*) antibody (rabbit, 1: 2500; kindly provided by Dr D B Hales, Southern Illinois University School of Medicine) (Hales *et al.* 2000), phosho-*StAR* (P-*StAR*) antibody (rabbit, 1: 1000, kindly provided by Dr D M Stocco, Texas Tech University Health Sciences Center) (Manna *et al.* 2006), or  $\beta$ -actin antibody (mouse, 1:10,000; A5441, Sigma–Aldrich) (Table 1) followed by secondary antibody incubation at room temperature. Signals were detected with enhanced electrochemiluminescence kit (Thermo Scientific). The signal density of each protein band was analyzed for

**Table 1** List of primary antibodies used in the present study

Target	Manufacturer or individual providing the antibody	Species raised	Dilution
MRAP	Abcam, ab168120	Mouse	1 $\mu$ g/ml (WB)
	Thermo Scientific, MA5-15253	Mouse	1:1000 (WB)
MC2R	Santa Cruz, sc-13107	Rabbit	1:50 (ICC)
StAR	Dr D B Hales (Southern Illinois University School of Medicine)	Rabbit	1:2500 (WB)
P- <i>StAR</i>	Dr D M Stocco (Texas Tech University Health Science Center)	Rabbit	1:1000 (WB)
$\beta$ -actin	Sigma–Aldrich, A5441	Mouse	1:10000 (WB)

WB, western blotting; ICC, immunocytochemistry.

quantification using Image J software (US National Institute of Health, Bethesda, MD, USA).

## cAMP assay

cAMP was assayed in cell culture medium treated with or without 10 nM ACTH for 3 h by using cAMP Direct EIA kit (Arbor Assays, Ann Arbor, MI, USA). Acetylated protocol was applied for the assay following the manufacture's instructions. Results were normalized to protein concentration and shown as fold change over basal condition.

## Immunocytochemistry

Cells were grown on the glass slides covered with poly-D-lysine. Cells were washed with PBS and then fixed with 100% methanol for 20 min. After washing, slides were incubated with MC2R antibody (rabbit, 1:50; H-70, sc-13107, Santa Cruz) (Table 1) overnight at 4  $^{\circ}$ C followed by secondary antibody incubation (goat-anti rabbit IgG Alexa Fluor 488, Jackson Immunoresearch, Inc., West Grove, PA, USA). Fluorescence was imaged using an Olympus Fluo-View 500 confocal microscope system.

## Statistical analysis

Results are given as means  $\pm$  s.e.m. unless noted otherwise. The data were analyzed and compared with control values using the *t* test with the SigmaPlot 12.5 software package (Systat Software, Inc., San Jose, CA, USA). One-way ANOVA methods were applied for samples with variables totaling three or more groups. Results were considered to be significantly different when the  $P < 0.05$ .

## Results

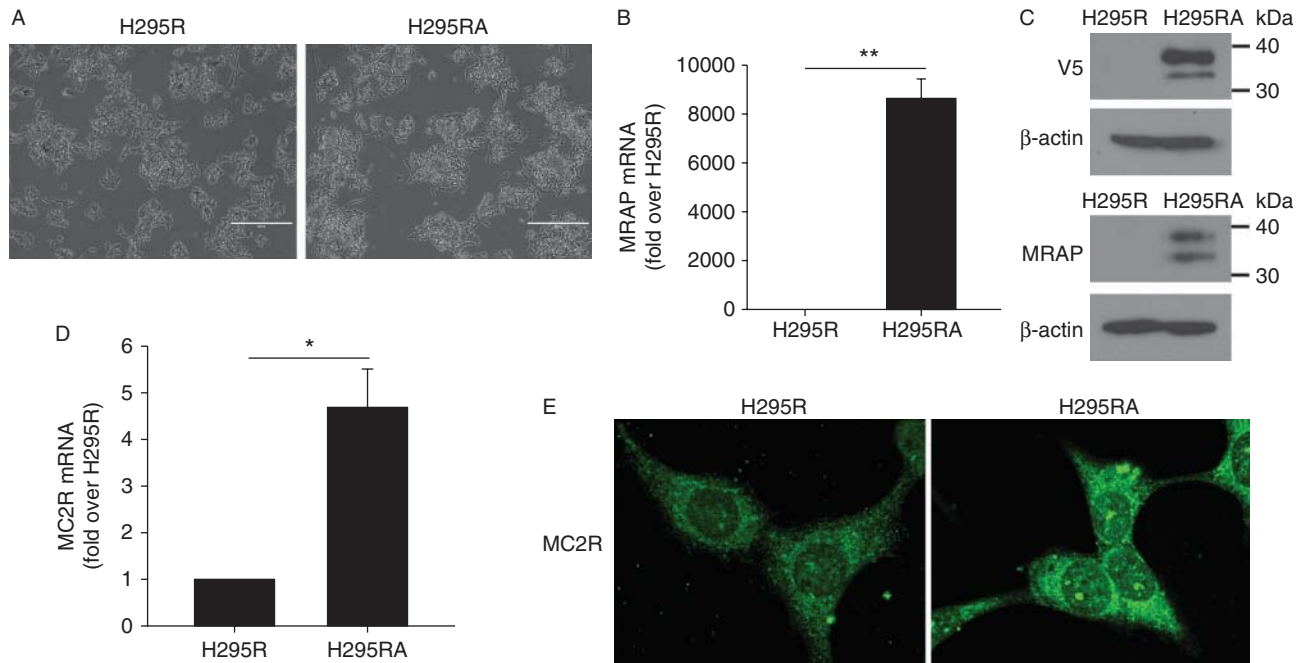
### Expression of MRAP in H295RA cells

Phase contrast photomicrographs of the parental H295R cells and H295RA cells are shown in Fig. 1A. Successful induction of MRAP was assessed by quantitative PCR (qPCR) and western blotting. *MRAP* mRNA was significantly upregulated in H295RA cells compared to regular H295R cells (8638-fold vs H295R cells,  $P < 0.001$ ; Fig. 1B). H295RA MRAP tagged protein was detected between 30 and 40 kDa in western analysis using a V5 antibody (Fig. 1C, upper panel). Western analysis with an MRAP antibody showed similar results as seen with the V5 antibody, detecting double bands only in H295RA cell lysate between 30 and 40 kDa (Fig. 1C, lower panel). Neither endogenous nor tagged MRAP was detected in the parental H295R cells (Fig. 1C). We further compared basal *MC2R* expression between H295R and H295RA cells. In qPCR, *MC2R* mRNA expression was significantly higher in H295RA cells than in H295R cells (4.7-fold vs H295R cells,  $P = 0.01$ ; Fig. 1D). Confocal microscopic images showed

increased *MC2R* protein expression in H295 RA cells in immunocytochemistry (Fig. 1E).

### Steroidogenic ability of the H295RA cell line

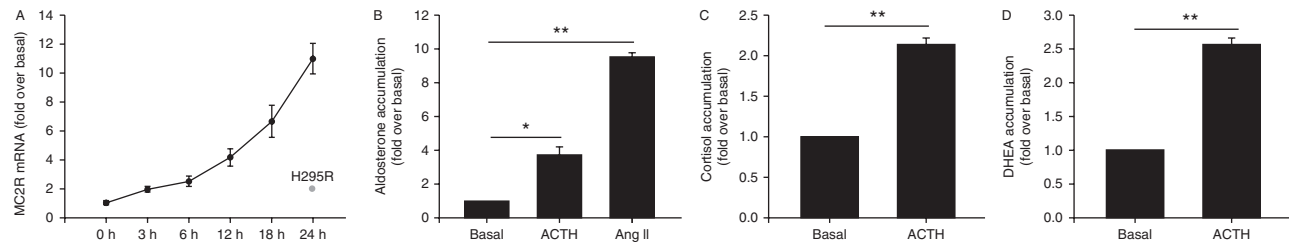
As observed in the previous results using human adrenal primary cells (Xing *et al.* 2010), treatment with ACTH for 24 h significantly increased the *MC2R* mRNA in H295RA adrenal cells compared to that observed in untreated cells ( $12.0 \pm 1.5$ -fold;  $P < 0.001$ ) (Fig. 2A). On the other hand, the parental H295R cells showed only a modest increase in *MC2R* mRNA after ACTH treatment ( $2.0 \pm 0.2$  fold vs basal). H295RA cells also significantly increased aldosterone production ( $3.8 \pm 0.5$ -fold vs basal;  $P = 0.001$ ), cortisol ( $2.1 \pm 0.08$ -fold vs basal;  $P < 0.001$ ), and DHEA accumulation ( $2.6 \pm 0.08$ -fold vs basal;  $P < 0.001$ ) after treatment with ACTH for 24 h (Fig. 2B, C, and D). Of note, the H295RA cells also retained Ang II activation of aldosterone production ( $9.6 \pm 0.2$ -fold vs basal;  $P < 0.001$ ; Fig. 2B). Compared to H295RA cells, the parental H295R cells showed little or no increase in these steroids after 24 h



**Figure 1**

Expression of MRAP and *MC2R* in H295R and H295RA cells. (A) Phase contrast photomicrographs of H295R and H295RA cells. Scale bar, 400  $\mu$ m. (B) Expression of *MRAP* mRNA. The experiments were performed in quadruplicate and results are given as means  $\pm$  s.e.m. of data from three independent experiments. Statistical analysis was performed using the *t* test.  $**P < 0.001$ . (C) Western analysis of MRAP protein expression using V5 antibody and MRAP antibody. Results are representative of those

obtained from three independent experiments.  $\beta$ -actin was used as a loading control. (D) Expression of *MC2R* mRNA. The experiments were performed in quadruplicate and results are given as means  $\pm$  s.e.m. of data from three independent experiments. Statistical analysis was performed using the *t* test.  $*P = 0.01$ . (E) Representative confocal microscopic images of H295R and H295RA cells in *MC2R* immunocytochemistry.

**Figure 2**

Steroidogenic responsiveness of H295RA cells to agonist treatment. (A) Expression of *MC2R* mRNA after treatment of 10 nM ACTH. The experiments were performed in quadruplicate and results represent the mean  $\pm$  s.d. of data from a single experiment. Statistical analysis was performed from three independent experiments of basal vs 24 h treatment using *t* test ( $P < 0.001$  vs basal). The labeled solid gray circle indicates the fold increase vs basal condition in the parental H295R cells after 24 h treatment with 10 nM ACTH. (B, aldosterone; C, cortisol; D, DHEA) Steroid

accumulation in cell culture medium following treatment with 10 nM ACTH or 10 nM angiotensin II (Ang II) for 24 h. Experiments were performed in quadruplicate. Results were normalized to protein concentration and shown as fold change over basal conditions. Results represent means  $\pm$  s.e.m. of data from three independent experiments. Statistical analyses were performed using one-way ANOVA followed by Dunnett's *post hoc* test for aldosterone and *t* test for cortisol and DHEA. \* $P = 0.001$  vs basal, \*\* $P < 0.001$  vs basal.

treatment with ACTH (aldosterone,  $1.6 \pm 0.1$ -fold vs basal; cortisol,  $1.2 \pm 0.01$ -fold vs basal; DHEA,  $1.0 \pm 0.06$ -fold vs basal). We further analyzed the steroid profile of the H295RA cells using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Steroids produced by the H295R and H295RA cells at baseline and after ACTH stimulation are summarized in Table 2. Similar to our previous study of H295R steroid profiles (Xing *et al.* 2011, Rege *et al.* 2015), the main unconjugated steroids produced in H295RA cells were 11-deoxycortisol, androstenedione, and cortisol, while the mass of aldosterone was low under basal conditions. After ACTH stimulation for 24 h, H295RA cells showed a significant increase in  $17\alpha$ -hydroxyprogesterone, 11-deoxycorticosterone,

corticosterone, 11-deoxycortisol, pregnenolone sulfate, and  $17\alpha$ -hydroxypregnenolone sulfate as well as aldosterone and cortisol ( $P < 0.05$ ; Table 2).

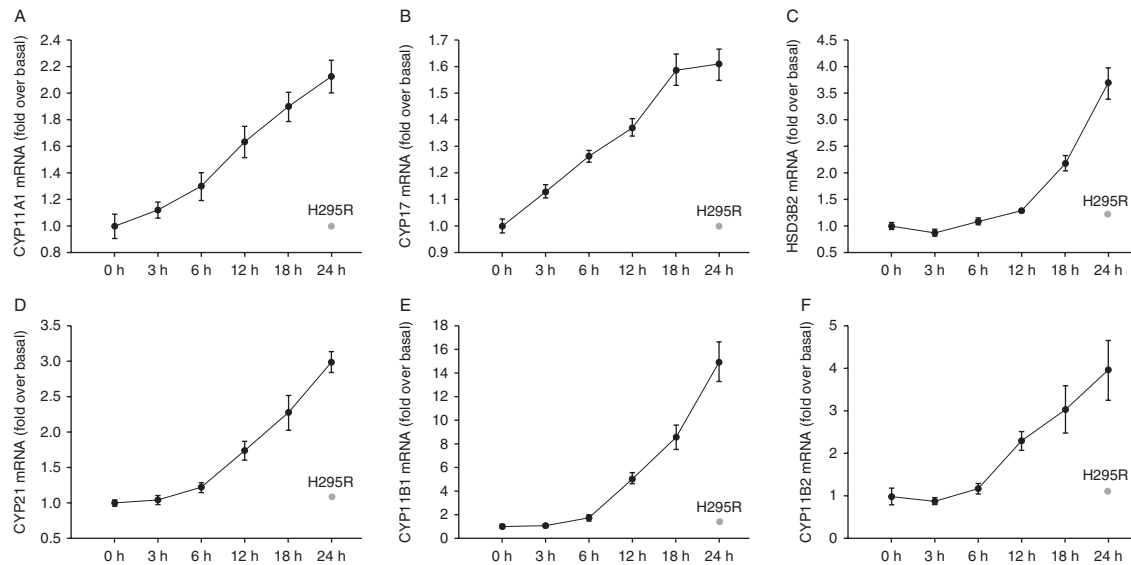
#### mRNA expression of steroidogenic enzymes in H295RA cell line

To evaluate the adrenal differentiated function in H295RA cells, the expression of mRNA-encoding enzymes involved in steroid hormone biosynthesis was measured (Fig. 3A, B, C, D, E, and F; Supplementary Figure 1, see section on supplementary data given at the end of this article). Treatment with 10 nM ACTH for 24 h increased all of the mRNA of steroidogenic enzymes required for aldosterone,

**Table 2** Steroids produced under basal and ACTH treatments in H295R and H295RA cells. H295R cells and H295RA cells were incubated with or without ACTH (10 nM) for 24 h. Concentrations of steroids were measured using LC-MS/MS and normalized with cellular protein content. Results represent the mean  $\pm$  s.d. from three independent experiments. *P* values were calculated using *t* test

Steroid (nmol/mg protein)	H295R			H295RA		
	Basal	ACTH	Fold change	Basal	ACTH	Fold change
<b>21-carbon steroids</b>						
17OHP	22.0 $\pm$ 1.6	25.7 $\pm$ 1.0*	1.2	23.7 $\pm$ 3.2	40.6 $\pm$ 3.9*	1.7
11-DOC	9.5 $\pm$ 2.1	14.6 $\pm$ 1.0*	1.6	11.4 $\pm$ 1.2	73.3 $\pm$ 18.8*	6.4
Corticosterone	11.2 $\pm$ 2.0	15.9 $\pm$ 1.1*	1.4	17.2 $\pm$ 5.1	82.8 $\pm$ 27.2*	4.8
Aldosterone	0.22 $\pm$ 0.08	0.32 $\pm$ 0.13	1.7	0.33 $\pm$ 0.16	0.77 $\pm$ 0.078*	2.9
11-Deoxycortisol	204.1 $\pm$ 3.6	275.7 $\pm$ 9.8*	1.4	245.9 $\pm$ 27.7	717.7 $\pm$ 191.3*	2.9
Cortisol	41.2 $\pm$ 5.2	54.5 $\pm$ 2.3*	1.3	83.4 $\pm$ 23.9	132.9 $\pm$ 15.8*	1.6
<b>19-carbon steroids</b>						
Androstenedione	99.3 $\pm$ 13.1	102.8 $\pm$ 7.7	1.1	110.2 $\pm$ 20.8	157.4 $\pm$ 28.3	1.4
<b>Steroid sulfates</b>						
Preg-S	196.9 $\pm$ 12.6	255.1 $\pm$ 23.8*	1.3	86.6 $\pm$ 15.8	251.3 $\pm$ 44.8*	3.3
17OHPreg-S	18.6 $\pm$ 1.9	23.5 $\pm$ 3.9	1.3	7.7 $\pm$ 0.8	16.0 $\pm$ 0.6*	2.3
DHEAS	48.5 $\pm$ 12.7	41.0 $\pm$ 5.1	0.9	19.0 $\pm$ 3.5	22.1 $\pm$ 2.4	1.3

\* $P < 0.05$  vs basal. 17OHP,  $17\alpha$ -hydroxyprogesterone; 11-DOC, 11-deoxycorticosterone; Preg-S, pregnenolone sulfate; 17OHPreg-S,  $17\alpha$ -hydroxypregnenolone sulfate.

**Figure 3**

mRNA expression of steroidogenic enzymes after treatment with ACTH in H295RA cells. H295RA cells were treated with 10 nM ACTH. mRNA levels for each enzyme were determined using qPCR: (A) cholesterol side-chain cleavage (*CYP11A1*); (B) 17 $\alpha$ -hydroxylase-17, 20-lyase (*CYP17*); (C) 3 $\beta$ -hydroxysteroid dehydrogenase type II (*HSD3B2*); (D) 21-hydroxylase (*CYP21*); (E) 11 $\beta$ -hydroxylase (*CYP11B1*); and (F) aldosterone synthase

(*CYP11B2*). The experiments were performed in quadruplicate and results represent the mean  $\pm$  s.d. of data from a single experiment. Statistical analysis was performed from three independent experiments of basal vs 24 h treatment using *t* test ( $P < 0.001$  vs basal). The labeled solid gray circles indicate the fold increase vs basal condition in the parental H295R cells after 24 h treatment with 10 nM ACTH.

cortisol, and DHEA production, namely, *CYP11A1*, *StAR*, *CYP17*, *HSD3B2*, *CYP21*, *CYP11B1*, and *CYP11B2* (means of fold increase vs basal  $\pm$  s.e.m. after 24 h treatment with ACTH were  $2.0 \pm 0.07$  ( $P < 0.001$  vs basal),  $1.6 \pm 0.08$  ( $P = 0.002$  vs basal),  $1.6 \pm 0.04$  ( $P < 0.001$  vs basal),  $3.6 \pm 0.2$  ( $P < 0.001$  vs basal),  $3.0 \pm 0.08$  ( $P < 0.001$  vs basal),  $13.7 \pm 0.9$  ( $P < 0.001$  vs basal), and  $3.9 \pm 0.07$  ( $P < 0.001$  vs basal), respectively), whereas only small effects were observed in the parental H295R cells (each fold increase vs basal condition after 24 h treatment with ACTH is indicated as gray solid circle in Fig. 3A, B, C, D, E, and F; Supplementary Figure 1).

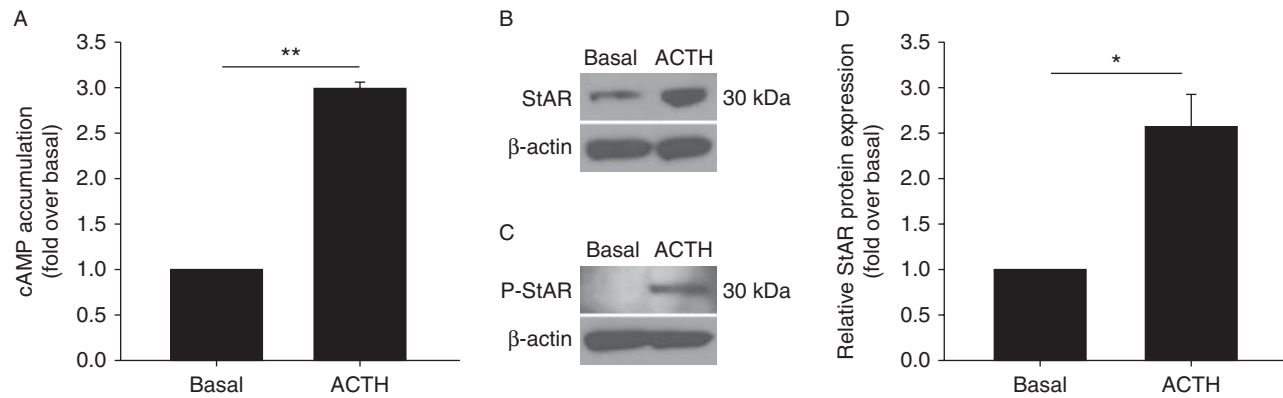
### Acute response to ACTH in H295RA cell line

To evaluate the acute response to ACTH in H295RA cells, we investigated cAMP production and StAR expression after 10 nM ACTH treatment in H295RA cells. Treatment with ACTH significantly increased cAMP accumulation in cell culture medium after 3 h treatment with ACTH ( $3.0 \pm 0.07$ -fold vs basal;  $P < 0.001$ ; Fig. 4A), whereas only a small increase was observed in the parental H295R cells ( $1.2 \pm 0.03$ -fold). In western analysis, 6 h treatment with ACTH significantly increased StAR expression in H295RA cells ( $2.5 \pm 0.4$ -fold vs basal; mean  $\pm$  s.d.,  $P < 0.01$ ) as well as phosphorylation of StAR at serine 195 (Fig. 4B, C, and D).

### Discussion

The H295R cell line is widely used to study steroid hormone production and to define the molecular mechanisms regulating transcription of steroidogenic enzymes (Rainey *et al.* 2004, Wang & Rainey 2012). Although the H295R cells increase aldosterone production in response to Ang II and potassium treatment (Bird *et al.* 1993, Clyne *et al.* 1997, Wang *et al.* 2012), the cells have only moderate responsiveness to ACTH. Therefore, most experiments designed to examine the cAMP-dependent pathway require the addition of either forskolin or cAMP analogues (Rainey *et al.* 1993, 2004). The low response to ACTH has been a drawback of this cell model and linked to low level of MC2R in the H295R cells (Mountjoy *et al.* 1994). Clonal selection of H295R cells, specifically with focus on clones with increased ACTH response, has produced HAC15 cells, but these cells do not have the responsiveness seen in primary cultures (Parmar *et al.* 2008, Xing *et al.* 2010, 2011, Wang & Rainey 2012).

The existence of an MC2R accessory factor had been suspected because heterologous expression of MC2R does not yield a functional receptor in most cell types (Noon *et al.* 2002). In 2005, MRAP was identified to cause FGD type 2 (Metherell *et al.* 2005). MRAP was shown to directly interact with MC2R and is essential for the trafficking of

**Figure 4**

Acute response to ACTH in H295RA cells. (A) cAMP response to 10 nM ACTH. Experiments were performed in triplicate. Results were normalized to protein concentration and shown as fold change over basal condition. Results represent means  $\pm$  s.e.m. of data from three independent experiments. Statistical analysis was performed using *t* test. Protein expression of

StAR (B) and phospho-StAR at serine 195 (P-StAR) (C) after 6 h treatment with ACTH. Results were representative of those obtained from at least three individual experiments.  $\beta$ -actin was used as a loading control. (D) Quantified protein expression of StAR. Results represent the mean  $\pm$  s.d. Statistical analyses were performed using *t* test. \**P* < 0.01, \*\**P* < 0.001.

MC2R from the ER to the cell surface (Metherell *et al.* 2005). The MRAP gene is encoding a protein of 172 residues and predicted molecular weight of 19 kDa. MRAP forms a stable antiparallel homodimer at the plasma membrane and in the ER and shows glycosylation of the protein (Sebag & Hinkle 2007, Cooray *et al.* 2008). In the present study, both anti-V5 antibody and anti-MRAP antibody detected the bands in H295RA cells between 30 and 40 kDa on western blotting. The discrepancy in protein size might be due to dimerization and posttranslational modification of the protein. In fact, a previous study demonstrated that endogenously expressed Y-1 adrenal cell MRAP was immunodetected as a band of more than 30 kDa despite the predicted molecular mass of 14 kDa (Cooray *et al.* 2008).

H295RA cells showed higher basal MC2R expression levels and greater response to ACTH stimulation compared to the parental H295R cells in terms of steroidogenesis and mRNA expression of steroidogenic enzymes. As observed in a previous report using primary cultures of human adrenal cells (Xing *et al.* 2010), the mRNA level of MC2R was upregulated by ACTH stimulation. It is not clear why H295RA cells exhibited higher basal levels of MC2R compared to the parental cell; however, based on the above the presence of low levels of ACTH in the serum used for growth medium may be responsible. LC-MS/MS analysis revealed that the most abundant unconjugated steroid hormones produced under basal conditions by the H295RA cells were 11-deoxycortisol, followed by androstenedione and cortisol. These results are consistent with previous LC-MS/MS steroid profiles reported in H295R cells (Xing *et al.* 2011). The reasons for the relatively mild

increase in cortisol after ACTH stimulation in our studies may relate to the low basal expression of CYP11B1 and slow ACTH-induction of the enzyme. Whereas the aldosterone production in H295RA cells was very low under basal conditions, the cells showed significant increase in aldosterone after ACTH stimulation. Nonetheless, the concentration of aldosterone produced after ACTH treatment is still much lower than that of cortisol (Table 2). Our previous studies have shown that aldosterone regulation is predominantly regulated by the Ang II pathway as opposed to ACTH/cAMP signaling (Denner *et al.* 1996). H295RA cells also acutely increased cAMP production and the cellular protein level of total StAR as well as phosphorylation of StAR at serine 195, which is known to approximately double the StAR activity (Arakane *et al.* 1997). These findings strengthen the functional evidence on the ACTH responsiveness of the H295RA cells.

In conclusion, we have developed an ACTH-responsive cell line, H295RA, from ACTH nonresponsive cells. The cell line will provide an improved *in vitro* model for molecular analysis of physiologic and pathologic actions involving hypothalamic–pituitary–adrenal axis.

#### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-15-0230>.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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