Human adrenal corticocarcinoma NCI-H295R cells produce more androgens than NCI-H295A cells and differ in 3β-hydroxysteroid dehydrogenase type 2 and 17,20 lyase activities

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Abstract

The human adrenal cortex produces mineralocorticoids, glucocorticoids, and androgens in a species-specific, hormonally regulated, zone-specific, and developmentally characteristic fashion. Most molecular studies of adrenal steroidogenesis use human adrenocortical NCI-H295A and NCI-H295R cells as a model because appropriate animal models do not exist. NCI-H295A and NCI-H295R cells originate from the same adrenocortical carcinoma which produced predominantly androgens but also smaller amounts of mineralocorticoids and glucocorticoids. Research data obtained from either NCI-H295A or NCI-H295R cells are generally compared, although for the same experiments no direct comparison between the two cell lines has been performed. Therefore, we compared the steroid profile and the expression pattern of important genes involved in steroidogenesis in both cell lines. We found that steroidogenesis differs profoundly. NCI-H295A cells produce more mineralocorticoids, whereas NCI-H295R cells produce more androgens. Expression of the 3β-hydroxysteroid dehydrogenase (HSD3B2), cytochrome b5, and sulfonyltransferase genes is higher in NCI-H295A cells, whereas expression of the cytochrome P450c17 (CYP17), 21-hydroxylase (CYP21), and P450 oxidoreductase genes does not differ between the cell lines. We found lower 3β-hydroxysteroid dehydrogenase type 2 but higher 17,20-lyase activity in NCI-H295R cells explaining the 'androgenic' steroid profile for these cells and resembling the zona reticularis of the human adrenal cortex. Both cell lines were found to express the ACTH receptor at low levels consistent with low stimulation by ACTH. By contrast, both cell lines were readily stimulated by 8Br-cAMP. The angiotensin type 1 receptor was highly expressed in NCI-H295R than NCI-H295A cells and angiotensin II stimulated steroidogenesis in NCI-H295R but not NCI-H295A cells. Our data suggest that comparative studies between NCI-H295A and NCI-H295R cells may help find important regulators of mineralocorticoid or androgen biosynthesis.
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Introduction

Human androgens are predominantly produced in the gonads (ovaries and testes) and in the adrenal cortex. The adult adrenal cortex consists of three morphologically and biochemically distinct zones (Miller 1988, Rainey *et al.* 2004): the outer zona glomerulosa (ZG), which produces mineralocorticoids for the regulation of water and salt homeostasis; the middle zona fasciculata (ZF), which produces glucocorticoids controlling body fuel metabolism and stress response; and the inner zona reticularis (ZR), which produces adrenal androgens for which the specific role and regulation are unknown. Generally, androgens are essential for the normal sexual development in males during fetal life and puberty. In adulthood, they are essential for sexual behavior, fertility, and thus maintenance of the human species.

All steroid hormones are produced from cholesterol (Fig. 1). The transfer of cholesterol to the mitochondria for the first and rate limiting step of steroidogenesis, the conversion of cholesterol to pregnenolone (Preg), is supported by the steroidogenic acute regulatory protein (StAR). Enzymes involved in adrenal steroidogenesis are P450 side-chain cleavage (*CYP11A1*), 3β-hydroxysteroid dehydrogenase type 2 (3βHSDII; *HSD3B2*), 21-hydroxylase (P450c21; *CYP21A2*), 11β-hydroxylase (P450c11β; *CYP11B1*), and aldosterone synthase (P450c11AS; *CYP11B2*) for the production of mineralocorticoids in the ZG. Additionally, the 17α-hydroxylase activity of cytochrome P450c17 (*CYP17*) is needed for the production of glucocorticoids in the ZF. Finally, both the 17α-hydroxylase and 17,20-lyase activities of P450c17 and the activity of the sulfonyltransferase (*SULT2A1*) are required to produce the C19 steroids dehydroepiandrosterone (DHEA) and its sulfate (DHEAS). P450c17 plays a key role in the zone-specific production of steroids. Presence of the 17α-hydroxylase activity of P450c17 alone in the ZF results in the production of cortisol, while presence of both 17α-hydroxylase and 17,20-lyase

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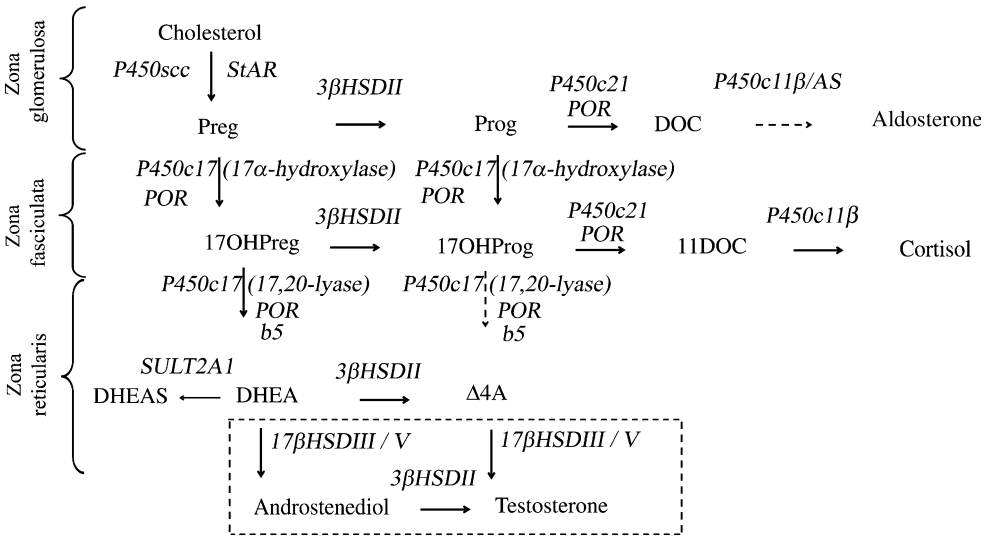

Figure 1 Adrenal steroidogenesis. The initial and rate-limiting step of steroidogenesis is the conversion of cholesterol to Preg by the P450scc enzyme. Preg may undergo 17α-hydroxylation to 17OHPreg by the 17α-hydroxylase activity of the P450c17 enzyme or may be converted to Prog by the 3βHSDII enzyme. Prog may be converted to deoxycorticosterone (DOC) and aldosterone by sequential conversion by the P450c21, P450c11β, and P450c11AS enzymes, which are all present in the human adrenal ZG. Alternatively, 17OHPreg may be converted stepwise to 17OHProg, 11DOC and cortisol by 3βHSDII, P450c21, and P450c11β in the human adrenal ZF. Moreover, 17OHPreg may be converted to DHEA by the 17,20-lyase activity of P450c17 followed by conversion to DHEAS by SULT2A1, or to Δ4A by 3βHSDII; all present in the human adrenal ZR. POR is the obligate electron transfer cofactor for P450c21 and P450c17, while b5 fosters 17,20-lyase activity through allosteric mechanisms. In human adrenal H295 cells (as in the human testes), Δ4A may be converted to testosterone by 17βHSDIII/V. Dashed box shows reactions which are not described for the normal human adrenal cortex.

activities in the ZR results in the production of androgens (Miller *et al.* 1997). The 17,20 lyase activity of P450c17 in the ZR is fostered by the expression of the flavoprotein NADPH cytochrome P450 oxidoreductase (POR) and cytochrome b5 (b5) in the ZR, as well as by phosphorylation of P450c17 (Lin *et al.* 1993, Zhang *et al.* 1995, Auchus *et al.* 1998, Lam *et al.* 2001, Rainey *et al.* 2002, Pandey *et al.* 2003).

Steroid hormone biosynthesis is regulated in a species-specific, developmentally characteristic, tissue-specific, and a cAMP-dependent fashion (Waterman & Keeney 1996, Sewer & Waterman 2001, Fluck & Miller 2004), but not all regulation is understood in great details. The adrenocorticotropic hormone (ACTH)/cAMP pathway plays a major role in the regulation of steroid hormone biosynthesis (John *et al.* 1986*b*, Sewer & Waterman 2003, Stocco *et al.* 2005). Upon release from the pituitary gland, ACTH stimulates the G-protein-coupled melanocortin 2 receptor (MC2R) which is located almost exclusively in the adrenal cortex (Forti *et al.* 2006). Activation of MC2R triggers the production of cAMP, which in turn activates protein kinase A and possibly crosstalk with other signaling pathways (Sewer & Waterman 2002). For adrenal steroidogenesis, it is known that ACTH/cAMP stimulation increases gene transcription of most steroidogenic enzymes (John *et al.* 1986*a*, Sewer & Waterman 2003, Stocco *et al.* 2005). Yet the exact downstream signalling cascade remains elusive as most genes encoding steroidogenic enzymes do not harbor typical cAMP response elements (CRE) in their promoters. Aldosterone synthesis may be influenced by ACTH, but it is mainly regulated by the renin–angiotensin system as well as by the concentration of potassium (Goodman 2003). Angiotensin II (AngII) stimulates type 1 membrane receptors (AT1) of adrenal cells which act through inositol trisphosphate, diacylglycerol, as well as calcium to promote the formation of Preg from cholesterol and the synthesis of aldosterone through stimulation of P450c11AS.

The fetal and adult human adrenals differ principally. The fetal adrenal consists of a definitive zone (the later adult adrenal) and a larger fetal zone, principally producing DHEA and DHEAS. After birth, the fetal zone disappears by the age of 6 months and the adrenal cortex first consists of the ZF and ZG. During 'adrenarche' by 3–4 years of age, the ZR appears histologically and starts to produce androgens by 6–8 years of age (Dhom 1973*a*,*b*, Cutler *et al.* 1978, Havelock *et al.* 2004, Campbell 2006). The role and the regulation of human adrenal androgen biosynthesis in general and adrenarche in particular are still poorly understood. Animal models may not be suitable for studies, because first of all, adrenal steroidogenesis varies fundamentally from species to species and secondly, adrenarche occurs only in higher-order primates (Havelock *et al.* 2004). Thus, most investigators use human cell models for their studies, i.e. NCI-H295A (A) and NCI-H295R (R) cells.

Human adrenocortical carcinoma A and R cells are two human adrenocortical cell lines that derive from the same adrenocortical tumor cell line NCI-H295 (H295; Gazdar

et al. 1990, Staels *et al.* 1993, Rainey *et al.* 2004). H295 cells were originally cultured from an adrenocortical carcinoma in a 48-year-old black female (Gazdar *et al.* 1990). H295 cells express all genes for the steroidogenic enzymes found in all three layers of an adult adrenal cortex (Fig. 1; Staels *et al.* 1993). Using different growth conditions, the two substrains A and R cells were produced in two different laboratories (Gazdar *et al.* 1990, Rodriguez *et al.* 1997). In contrast to the parental H295 cells, both A and R cells grow as a tight monolayer. Similar to H295 cells, A and R cells express all genes involved in adrenal steroidogenesis (Staels *et al.* 1993, Rodriguez *et al.* 1997, Dardis & Miller 2003). However, all investigators who study adrenal steroidogenesis use either A or R cells and compare their research data directly assuming that the two cell lines are identical. So far, no data are available comparing the two cell systems in the same experiments. Hence, we asked the question whether the two commonly used adrenal cell models are identical; and if they were different, what differences could be observed especially with respect to androgen production. To compare the two cell lines we studied their growth pattern, the steroid profiles and the expression pattern of genes involved in steroidogenesis. As we found major differences in androgen production between the two cell lines, we suggest that comparative studies of the two cell systems will give new insight into the regulation of adrenal androgen biosynthesis.

Materials and Methods

Materials

We purchased 8-bromo-cAMP (8Br-cAMP) and AngII from Fluka (Sigma–Aldrich). Synacthen^{R} (ACTH; beta$^{1-24}$-corticotropin) was obtained from Novartis (Basel, Switzerland). Radio-labeled [^{3}H]-Preg (14 Ci/mmol) and [^{3}H]-DHEA (63 Ci/mmol) were bought from Perkin–Elmer (Boston, MA, USA). Radio-labeled [^{3}H]-17α-hydroxypregnenolone (17OHPreg; 50 Ci/mmol) was procured from American Radiolabel Chemicals Inc. (St. Louis, MO, USA). Trilostane was extracted in absolute ethanol (EtOH) from tablets commercially available as Modrenal® (Bioenvision, NY, USA).

Cell cultures

Human adrenal A cells were kindly provided by Prof. W L Miller and R cells were purchased from the American Type Culture Collection (number CRL-2128). Standard medium for A cells is RPMI-1640 (Gibco BRL Invitrogen) supplemented with 2% fetal calf serum, 0·1% selenium/insulin/transferrin (SIT), and penicillin/streptomycin (antibiotics; Rodriguez *et al.* 1997). Standard medium for R cells is Dulbecco's modified Eagle's/Ham's F12 (DMEM/F12; Gibco BRL Invitrogen) supplemented with 5% NuI serum, 0·1% SIT, and antibiotics (all from Gibco BRL).

Cell proliferation

A and R cells were cultured in six-well plates (750×10^{3} cells/well). After 72 h, cells were washed with PBS, trypsinized, and collected in PBS for counting with a hemocytometer.

Steroid profiling

A and R cells were grown in six-well plates (750×10^{3} cells/well). Steroid metabolism was labeled by adding [^{3}H]-Preg, [^{3}H]-17OHPreg, or [^{3}H]-DHEA (~200 000 c.p.m./well), all dissolved in EtOH. To block the 3βHSD activity, cells were treated with 1 μM trilostane for 4 h prior to labeling steroidogenesis (Mahesh & Brann 1992). Steroids were extracted from the media as previously described (Auchus *et al.* 1998) and separated on thin layer chromatography (TLC) plates (Whatman, Bottmingen, Switzerland) using chloroform:ethylacetate in the ratio of 3:1 as the running system. TLC plates were then exposed on screens and visualized on a Storm PhosphoImager (GE Healthcare, Wankesha, WI, USA). Radioactive spots were densitometrically quantified using either the ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA) or the PDQuest Software (Bio-Rad). Steroid conversion was assessed by calculating percentage of radioactivity measured from a specific steroid hot spot in relation to the total radioactivity added to the reaction. To compare enzyme activities unstimulated versus stimulated, the conversion ratio of substrate to product was estimated.

RNA extraction and semi-quantitative RT-PCR

Total RNA from A and R cells was extracted using the Nucleospin RNA kit from Macherey Nagel (Oensingen, Switzerland). Normal human adrenal tissues were obtained from patients undergoing nephrectomy/adrenalectomy for kidney disorders with the permission of the local ethical committee. RNA from the human adrenal tissues was extracted using the Trizol method as described by the manufacturer (Gibco BRL). Extracted RNA was reverse transcribed to cDNA with the Improm RNA Transcriptase kit (Promega) using oligo dT primers or random hexamers (0·5 μg/1 μg RNA). Synthesized cDNA was amplified by Go-Taq polymerase (Promega) and specific primers (Table 1) in a final volume of 25–50 μl. PCR conditions were 45 s at 95 °C, 45 s at 59 °C, 45 s at 72 °C for 28–35 cycles. Aliquots of RT-PCR products were electrophoresed on a 1·5% agarose gel, visualized with ethidium bromide, then scanned and quantified using the Alpha Imager 3400 (Alpha Innotech, San Leandro, CA, USA). The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18s ribosomal RNA (18SrRNA) served as internal controls. Primers were purchased from Microsynth (Balgach, Switzerland).

Table 1 List of primers used for conventional PCR and SYBR green real-time PCR assays

	Oligonucleotide sequences	**NCBI accession number** (5′ start site for S, 3′ for AS)
Name		
HSD17B3	S: 5′-GAGATCGAGCGGACTACAGG-3′	BC034281 (303)
	AS: 5′-TACTCAGCTTCCAAGGCGTT-3′	BC034281 (643)
HSD17B5	S: 5′-CCAGTTGACTGCAGAGGACA-3′	NM_003739 (927)
	AS: 5′-TGTCTACCAGAAGCCCTGTG-3′	NM_003739 (1080)
POR	S: 5′-TTCTGTTTTCGCTCATCGTG-3′	BC034277 (110)
	AS: 5′-GGACTTCTACGACTGGCTGC-3′	BC034277 (493)
SULT2A1	S: 5′-TTCGTGATAAGGGATGAAGATGTA-3′	NM_003167 (167)
	AS: 5′-GAGATTCTCTGCCTGATGCA-3′	NM_003167 (252)
b5	S: 5′-TGCACCACAAGGTGTACGAT-3′	NM_148923 (200)
	AS: 5′-AGGATGTCGGGCACTCTACA-3′	NM_148923 (321)
CYP21A2	S: 5′-AGAGGGATCACATCGTGGAG-3′	BC125182 (791)
	AS: 5′-CTCTGGACAGCTCCTGGAAG-3′	BC125182 (931)
18SrRNA	S: 5′-CTCAACACGGGAAACCTCAC-3′	NR_003286 (1247)
	AS: 5′-AGACAAATCGCTCCACCAAC-3′	NR_003286 (1364)
GAPDH	S: 5′-GAGTCAACGGATTTGGTCGT-3′	BC083511 (72)
	AS: 5′-CTGAGAACGGGAAGCTTGTC-3′	BC083511 (256)
MC2R	S: 5′-GACCGCTACATCACCATCTT-3′	NM_000529 (556)
	AS: 5′-CCCTGGTTTTAGAATCCA-3′	NM_000529 (1099)
AT1	S: 5′-TCAATGCTCAAGCCAGTGAC-3′	NT_005612 (1283129)
	AS: 5′-AGTGGCTGCTCAGTCCTGTT-3′	NT_005612 (1283321)

Real-time PCR

The mRNA levels of several genes involved in steroid production were quantitatively analyzed by real-time PCR using an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Briefly, PCRs were performed in 96-well plates (Abgene, Epsom, UK) using cDNA prepared as described above (50 ng/20 μl), TaqMan Universal PCR Mix and 1 μl specific primers and probes obtained as assay-on-demand gene expression products (Applied Biosystems, www.appliedbiosystems.com). Alternatively, ABsolute QPCR SYBR Green Mix (ABgene) and specific primers were used (Table 1). GAPDH or 18SrRNA served as internal controls (reference genes). Correction for internal control, ΔC_t, was calculated as $C_{\text{tgene of interest}} - C_{\text{treference gene}}$. Further, expression of each gene was calculated by the formula $2^{-\Delta C_t}$ (Livak & Schmittgen 2001).

Aldosterone, cortisol, and free testosterone measurements

Aldosterone measurement was done by competitive RIA on a gamma counter (Diagnostic Products Corporation, Los Angeles, CA, USA). Cortisol was measured by the competitive immunoassay Modular Analytics E170 (Roche Diagnostics). Measurement of the free testosterone was carried out by the Coat-A-Count solid phase ^{125}I RIA as described by the manufacturer (Diagnostic Products Corporation).

Plasmid constructs and luciferase reporter assays

Transient transfection of A and R cells with promoter constructs fused to a luciferase reporter gene was used to characterize promoter activities of CYP17, HSD3B2, b5, and SULT2A1. Constructs of the long (−3·7 kb) and short basal promoters (−227 bp) of CYP17 in pMG3 vector, and of the HSD3B2 and the b5 promoters were described previously (Lin *et al.* 2001, Huang *et al.* 2005, Kempna *et al.* 2006). The reporter construct pCREluc, which is a luciferase expression vector under the control of 16 CRE, has been described earlier (Vaisse *et al.* 2000). For the construction of the −1400/+50 SULT2A1 promoter, the following primers were used: sense 5′-CTA*GCTAGC*TAGCTTTTGC-TCCTGCCCTGCTG-3′ and antisense 5′-CCG*CTC-GAG*CTGCGTGGTGTGAGGGTTTC-3′, the fragment was cloned into the pGL3 basic vector using *XhoI* and *NheI* restriction sites. For transient transfection, A and R cells were sub-cultured into 24-well plates at a density of about 250 000 cells/well. Prior to lipofectamin transfections, growth medium was replaced by the transfection medium DMEM/F12 containing 10% NuI serum supplemented with 0·1% SIT. Transfection was carried out for 6 h at 37 °C using 1·6 μl Lipofectamin2000 (Invitrogen), 10 ng *Renilla* luciferase in pRL-TK vector, and 0·5 μg specific *Firefly* luciferase reporter plasmids per well. After transfection, medium was replaced by growth medium. Some cells were stimulated with 100 nM ACTH or 0·5 mM 8Br-cAMP for 24 h. Finally, cells were lysed and assayed for Dual luciferase activities as described by the manufacturer (Promega). Co-transfection with *Renilla* luciferase was used to control for the transfection efficiency. As an additional control, cells were transfected with the empty vectors.

Statistical analysis

Data represent the mean of at least three independent experiments. Experimental variation is given as S.E.M. For statistics, to test whether data were parametric, *F*-test was

performed. As we found that the variance was not significantly different between the datasets, we used parametric statistical testing, namely the unpaired two-tailed Student's *t*-test (Prism 4.00, GraphPad software Inc., SanDiego, CA, USA). Statistical significance: $\star P \leq 0{\cdot}05$ and $\star\star P \leq 0{\cdot}01$.

Results

R cells produce more androgens than A cells

Human adrenal A and R cells are originally cultured in RPMI-1640 and DMEM/F12 media respectively. To compare the effect of different growth media on cell proliferation, both cell lines were grown in either of the two growth media. A and R cells showed higher proliferation in DMEM/F12 growth medium compared with RPMI-1640 medium. However, no difference between the two cell lines was observed (Fig. 2A). Hence, we decided to use DMEM/F12 medium as growth medium for both A and R cell lines for all further comparative experiments.

In order to characterize A and R cells, the steroid profile was assessed using [^{3}H]-Preg as a substrate for labeling the steroidogenesis. Interestingly, we found profound differences in the steroid profile of A and R cells (Fig. 2B and C). A cells mainly produced 17α-hydroxyprogesterone (17OHProg) and 11-deoxycortisol (11DOC), which are precursors of glucocorticoids. In contrast, R cells produced not only 17OHPreg, 17OHProg, and 11DOC, but also DHEA and androstenedione (Δ4A), which are the adrenal androgens (Fig. 1). In addition, little progesterone (Prog) was detected in both cell lines, more in A than R cells. These results suggested that both adrenal cell lines produce similar amounts of mineralocorticoids and glucocorticoids, but that R cells produce markedly more adrenal androgens.

To confirm the observed differences in steroidogenesis of A and R cells, we measured the amount of mineralocorticoids, glucocorticoids, and androgens in the culture medium of both cell lines. A cells produced more aldosterone than R cells, while cortisol production was similar between the two cell lines

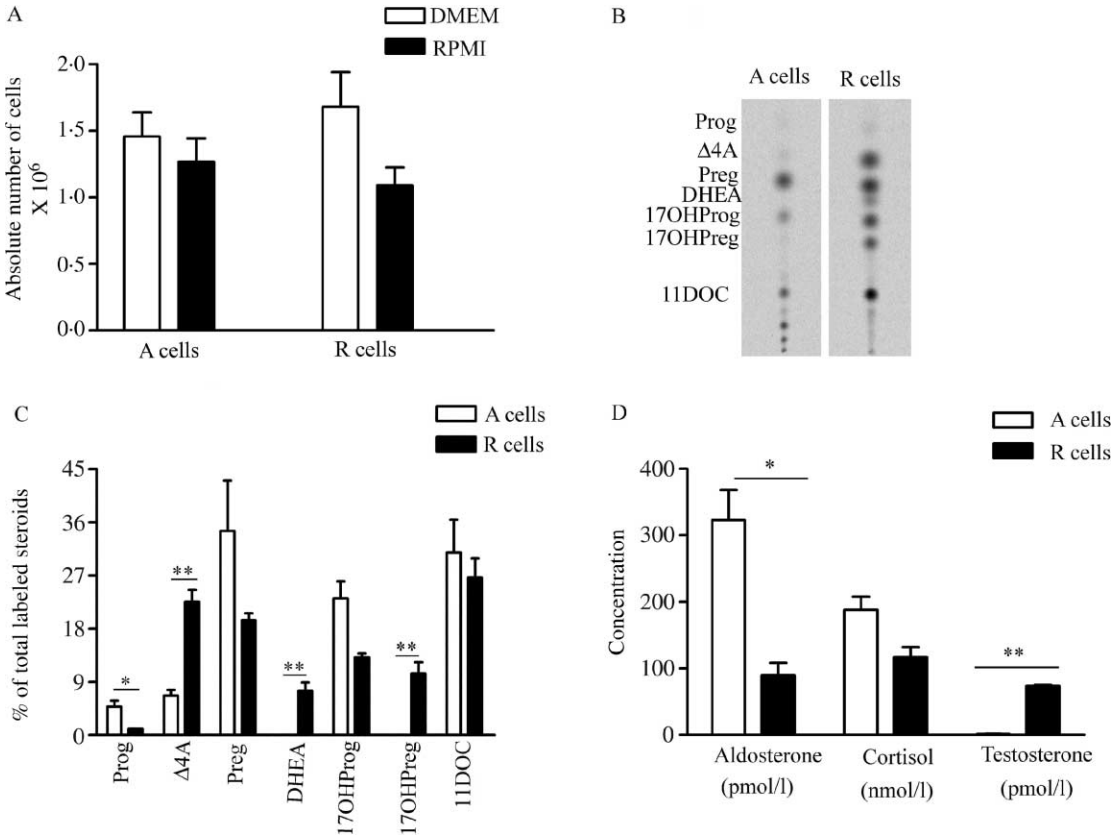

Figure 2 A and R cells have different steroid profiles even under the same growth conditions. (A) Proliferation of A and R cells. Equal numbers of cells were grown in either DMEM/F12 or RPMI-1640 medium for 72 h. Thereafter, cells were counted with a hemocytometer. Each counting was performed in duplicates. No difference was observed ($P=0{\cdot}61$ for A cells grown in DMEM versus RPMI; $P=0{\cdot}16$ for R cells in DMEM versus RPMI). (B and C) Steroid profiles of A and R cells. Cells were grown in DMEM/F12 medium. Steroidogenesis was labeled with [^{3}H]-Preg (~220 000 c.p.m./well) as substrate for 90 min. Steroids were extracted from the growth medium, separated on TLC plates, and then visualized. (B) Representative TLC. (C) Quantification of specific steroids produced by A and R cells assessed as percentage of total labeled steroids. (D) Production of mineralocorticoids, glucocorticoids, and androgens. Equal numbers of A and R cells were grown in DMEM/F12 medium supplemented with serum. After 50 h, supernatants of the cells were collected and concentrations of aldosterone, cortisol, and testosterone were determined. All data represent the mean ± S.E.M. of three independent experiments. $*P<0{\cdot}05$, $**P<0{\cdot}01$.

(Fig. 2D). Moreover, testosterone was only measurable in the culture medium of R cells (Fig. 2D). Thus, it seems that A cells produce more mineralocorticoids, whereas R cells produce more androgens. Hence, high mineralocorticoid production in A cells may be due to high 3βHSDII activity and low P450c17 activities, as this combination of enzyme activities favors the conversion of Preg to Prog towards the mineralocorticoid pathway (Fig. 1). In contrast, high activities of 17α-hydroxylase and 17,20-lyase are essential for the production of androgens (Fig. 1). Therefore, higher androgen production in R cells may be due to higher activity of P450c17 and lower activity of 3βHSDII in these cells.

The expression of genes encoding steroidogenic enzymes may differ among human adrenocortical cell lines

Being predominantly interested in differences in the androgen production between the two adrenal cell lines, we investigated the expression of the HSD3B2, CYP21A2, CYP17, SULT2A1, microsomal b5, and POR genes at the mRNA level performing qualitative RT-PCR. We found that all these genes were expressed in both A and R cells (data not shown). In order to compare the expression of the genes involved in the synthesis of androgens quantitatively, we performed real-time PCR experiments. A cells expressed higher levels of HSD3B2, b5, and SULT2A1 when compared with R cells (Fig. 3A and B). However, no differences in the expression of the CYP21A2, CYP17, and POR genes were observed (Fig. 3A and B). Higher expression of the HSD3B2 gene in A cells together with similar CYP21A2 gene expression in A and R cells support our hypothesis that 3βHSDII activity may be higher in A cells than in R cells. However, the observed gene expression data do not suggest higher P450c17 activities in R cells for which one would expect not only lower expression of HSD3B2, but also higher expression of CYP17, b5, and POR (Fig. 1).

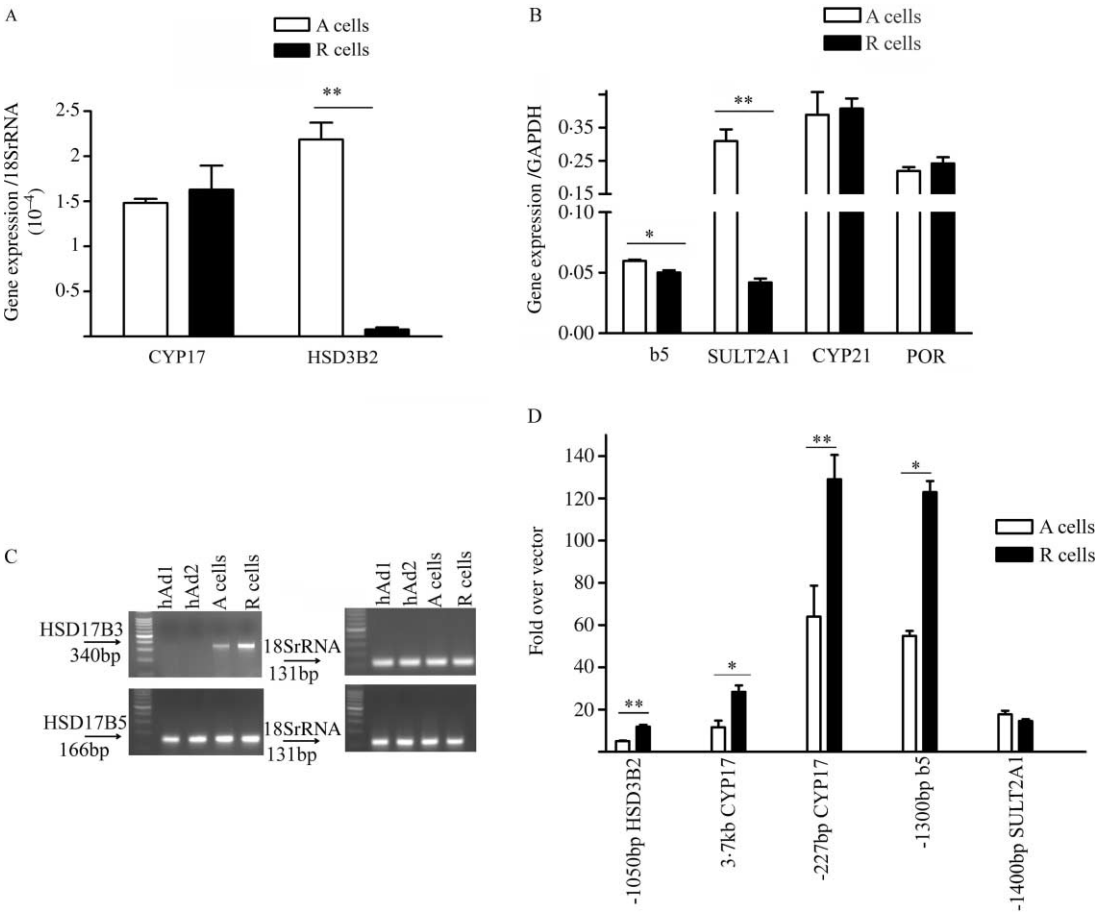

Figure 3 Expression and promoter activity of genes involved in steroidogenesis. (A and B) Quantitative assessment of gene expression in A and R cells using Taqman (A) or SYBR green (B) real-time PCR assays. For Taqman 18SrRNA and for SYBR green GAPDH served as internal control. Data represent the mean±S.E.M. of 4–6 independent experiments. *$P<0·05$, **$P<0·01$. (C) Semiquantitative assessment of the expression of the HSD17B3 and HSD17B5 genes in two different human adrenal tissues (hAd1 and hAd2), and in A and R cells (internal control 18SrRNA). Agarose gel depicting one representative experiment, confirmed three times independently. (D) Activities of different promoter luciferase reporter constructs of steroidogenic genes in A and R Cells. Cells were transfected with promoter constructs as indicated. PRL-TK served as an internal control for transfection efficiency, and transfection of empty vectors served as an additional control. Data represent the mean±S.E.M. of 4–6 independent experiments. *$P<0·05$, **$P<0·01$.

Human adrenal tumors often produce androgens (Geissler *et al.* 1994, Miller 2002, Allolio *et al.* 2004). We were able to detect testosterone in the cell culture medium of R cells but not A cells. Activities of the 17α-hydroxysteroid dehydrogenase type III (17βHSDIII; *HSD17B3*) and/or 17α-hydroxysteroid dehydrogenase type V (17βHSDV; *HSD17B5*) enzymes are required for the production of testosterone from Δ4A (Geissler *et al.* 1994, Dufort *et al.* 1999). Therefore, we studied the expression of the HSD17B3 and HSD17B5 genes in A and R cells and in human adrenal tissues (Fig. 3C). We confirmed the absence of the HSD17B3 gene expression in two independent tissue samples of normal human adrenals. However, we found that both human adrenal cell lines express the HSD17B3 gene. Slightly higher expression of the HSD17B3 gene in R cells may partially explain the higher levels of testosterone measured in cell culture medium of R cells. Expression of the HSD17B5 gene was detected in two independent human adrenal tissues as well as in both A and R cells (Fig. 3C). As the expression of HSD17B5 was similar between the cell lines, the activity of 17βHSDV may not explain the observed difference in testosterone production.

To compare transcriptional activation of different steroid genes in both cell lines, A and R cells were transiently transfected with specific promoter-luciferase reporter constructs. We found significantly higher activities of the −1050 bp HSD3B2, −227 bp CYP17, −3·7 kb CYP17 and −1300 bp b5 promoter constructs in R cells (Fig. 3D). The activity of the −1400 bp SULT2A1 promoter reporter was similar in both cell lines (Fig. 3D). From our previous observations from the gene expression patterns (Figs 2 and 3A–C), we would have suggested similar activity of the CYP17 promoter in A and R cells, but higher activities of HSD3B2, b5, and SULT2A1 promoters in A cells. Yet data obtained from transient transfection of reporter constructs in cells should be interpreted with caution for many reasons such as specific choice of the promoter region or the vectors used (Kempna *et al.* 2006).

Activities of 3βHSDII and P450c17 in A and R cells

High expression of the HSD3B2 gene in A cells at the mRNA level suggests high activity of 3βHSDII. To confirm this hypothesis, we performed functional studies. Cells were incubated with [^{3}H]-DHEA, a substrate for 3βHSDII that is converted to Δ4A (Fig. 1). Our studies revealed twofold higher activity of the 3βHSDII enzyme in A cells than in R cells (Fig. 4A). This finding is consistent with the gene expression studies (Fig. 3A). Thus, A cells express more HSD3B2 than R cells resulting in a higher 3βHSDII activity, which may lead steroid production towards the mineralocorticoid pathway and result in lower androgen production.

Further, functional studies were performed to compare the 17α-hydroxylase and 17,20-lyase activities of P450c17 in A and R cells. To assess P450c17 enzyme activity specifically, 3βHSD was blocked by trilostane. Cells were incubated with [^{3}H]-Preg to study both the 17α-hydroxylase and 17,20-lyase activities, or with [^{3}H]-17OHPreg to study the 17,20-lyase activity alone (Fig. 1). Using [^{3}H]-Preg as a substrate, we did not observe differences in 17α-hydroxylase or 17,20-lyase activities between A and R cells (Fig. 4B). In contrast, using [^{3}H]-17OHPreg as a direct substrate for the 17,20-lyase, we found significantly higher 17,20-lyase activity in R cells when compared with A cells (Fig. 4C). This difference in 17,20-lyase activity was not observed using [^{3}H]-Preg as a substrate because of the two steps in the reaction and the shorter incubation time (90 vs 480 min). Similar CYP17 gene expression and 17α-hydroxylase activity for A and R cells is in line with equal production of glucocorticoids in both cell lines. Furthermore, higher 17,20-lyase activity in R cells explains higher androgen production in R cells.

Expression of AT1 and MC2R, and the ACTH/cAMP signaling

Expression of the AT1 receptor is described in R cells but not investigated in A cells (Rainey *et al.* 2004). We found that the AT1 receptor is expressed in both cell lines but at lower levels in A cells (Fig. 5A). R cells are known to express low levels of the ACTH receptor (MC2R; Mountjoy *et al.* 1994), while no data are available for A cells. Thus, we studied MC2R expression in A and R cells at the mRNA level. Low expression of the MC2R gene was observed in both cell lines compared with the abundantly expressed POR gene or the housekeeping gene 18SrRNA (Fig. 5A). Further, we investigated the effect of ACTH and 8Br-cAMP stimulation on A and R cells. The classic ACTH/cAMP signaling pathway exerts its effect through PKA activation resulting in phosphorylation of CRE-binding proteins, which will then bind to CRE in targeted genes. Therefore, cells were transiently transfected with a pCREluc construct to assess transcriptional activation by ACTH or 8Br-cAMP stimulation in both cell lines. Eight Br-cAMP stimulated pCREluc activity in both cell lines about 9- to 15-fold (Fig. 5B). Generally, the activation of the pCREluc reporter tended to be higher in R cells but this difference did not reach statistical significance. By contrast, only a two- to threefold stimulation was observed by ACTH stimulation in both cell lines (Fig. 5B). This result may be explained by low expression of the MC2R in A and R cells. Alternatively, studies by others suggested that poor stimulation by ACTH may be due to impaired trafficking of the receptor from the endoplasmic reticulum to the cell surface (Noon *et al.* 2002).

Steroid production in A and R cells after stimulation with ACTH, 8Br-cAMP, and AngII

Previous studies have shown that H295 cells are unable to respond to ACTH or AngII stimulation (Staels *et al.* 1993). By contrast, steroidogenesis of R cells is reported to be regulated by AngII (Bird *et al.* 1993, 1994), potassium (Rainey *et al.* 1994, Pezzi *et al.* 1996, Hilbers *et al.* 1999), and ACTH (Rainey *et al.* 1993, Staels *et al.* 1993, Mountjoy *et al.* 1994). These investigators observed that the responsiveness of R cells is highly dependent on growth conditions (Rainey *et al.* 2004). However, no data are available comparing A and

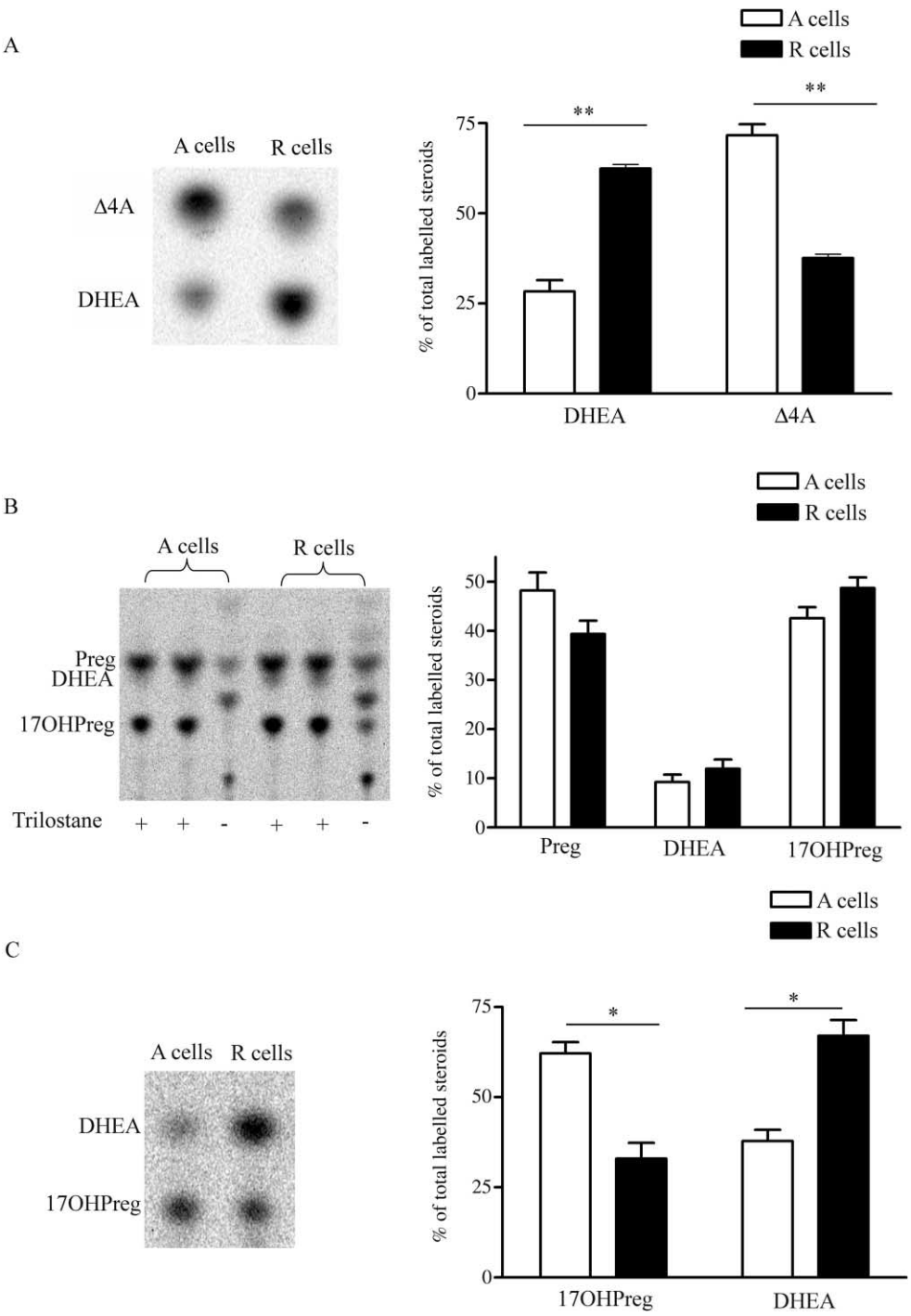

Figure 4 P450c17 and 3βHSDII activities. (A) Activity of 3βHSDII in A versus R cells. Cells were incubated with [^{3}H]-DHEA for 60 min. Steroids were then extracted from the growth medium and separated on TLC plates. Representative TLC plate and quantitative analysis of three independent experiments. (B and C) Activities of 17α-hydroxylase and 17,20-lyase. Cells were incubated with [^{3}H]-Preg (B) or [^{3}H]-17OHPreg (C) (~220 000 c.p.m./well) for 90 or 480 min to assess both the 17α-hydroxylase and 17,20-lyase activities (B) or the 17,20-lyase activity alone (C). Trilostane was used to block 3βHSD activity. Representative TLCs and quantitative analysis. Data represent the mean±S.E.M. of three independent experiments. *$P<0{\cdot}05$, **$P<0{\cdot}01$.

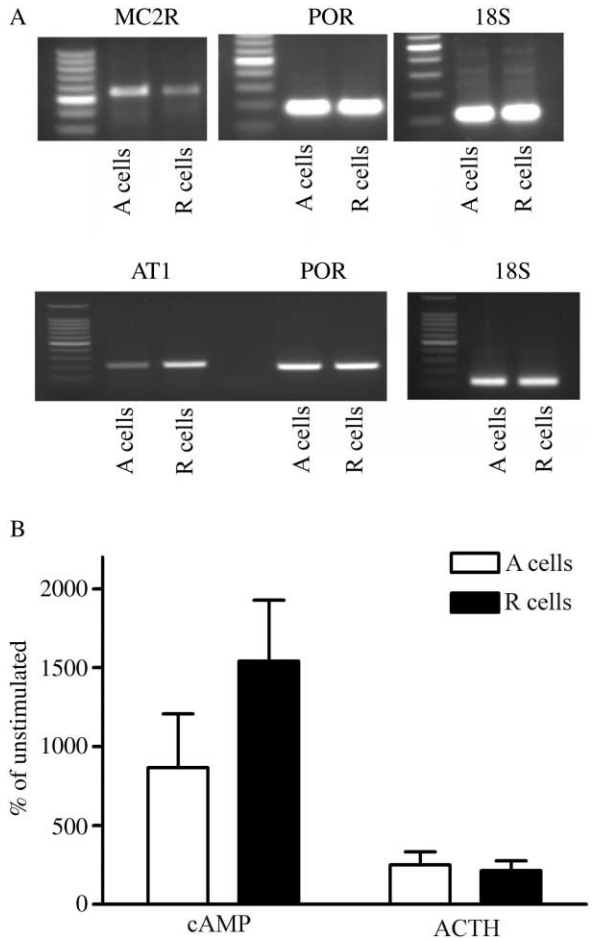

Figure 5 Expression of MC2R and AT1, and ACTH/cAMP stimulation. (A) Agarose gel showing the expression of the MC2R and the AT1 gene in A and R cells after 35 cycles of PCR. For comparison, expression of POR is depicted. Expression of 18SrRNA served as internal control. Shown is one representative experiment, confirmed three times independently. (B) Activity of the pCREluc reporter in A and R cells after stimulation with either 0·5 mM 8Br-cAMP or 100 nM ACTH for 24 h. Cells were transiently transfected with the pCREluc reporter, and luciferase activity was assessed by the Dual luciferase assay (Promega). No difference was observed ($P=0·39$ for cAMP stimulation in A versus R cells; $P=0·25$ for ACTH stimulation in A versus R cells). Data represent the mean±S.E.M. of 3–4 independent experiments. $^{*}P<0·05$, $^{**}P<0·01$.

R cells under the exactly same growth and stimulation conditions. Therefore, we stimulated both cell lines under standardized growth conditions with either ACTH, 8Br-cAMP, or AngII, and assessed aldosterone, cortisol, and testosterone production, as well as steroid profiles. We found that ACTH stimulation did not change the production of aldosterone, cortisol, and testosterone in A and R cells (Fig. 6A). By contrast, stimulation with 8Br-cAMP stimulated the production of all measured steroids significantly (Fig. 6B). While this stimulatory effect of cAMP was moderate for R cells, it was more than tenfold for A cells. AngII stimulation did not change steroid production in A cells, but increased aldosterone and cortisol production in R cells (Fig. 6C). Testosterone production seemed to decrease after AngII stimulation, but this change did not reach statistical significance ($P=0·08$). All these effects of ACTH, cAMP, and AngII on steroidogenesis in A and R cells were confirmed by steroid profiling experiments (Fig. 7A). While ACTH stimulation did not change the steroid profile of A and R cells (data not shown), 8Br-cAMP stimulated steroidogenesis in both (Fig. 7B). Substrate to product calculations from these data suggested a stimulatory effect of cAMP on the enzyme activities of P450c21 (A cells, 250%; R cells, 130% of unstimulated) and P450c17 (A cells, 190%; R cells, 190% of unstimulated). AngII stimulation did not change the steroid profile of A cells, but increased 11DOC and decreased 17OHPreg as well as DHEA production in R cells (Fig. 7C). Accordingly, substrate to product calculations suggested a stimulatory effect of AngII on the activities of P450c21 (130% of unstimulated) and 3βHSDII (150% of unstimulated) but an inhibitory effect on P450c17 activity (70% of unstimulated).

Discussion

Most molecular studies of human adrenal steroidogenesis use human adrenocortical A and R cells as cellular models for the adrenal cortex. As both cell lines originate from the adrenocortical H295 tumor cell line (Gazdar *et al.* 1990, Staels *et al.* 1993), it is generally believed that those three cell lines have the same characteristics concerning steroidogenesis. Several investigators reported expression of key steroidogenic genes for all three cell lines and described the steroid profile of these cell lines (Fig. 1). However, no data of a direct comparison between these cell lines were available. We show in this study that human adrenocortical A and R cells differ significantly in their steroidogenesis. Overall, A cells produce more mineralocorticoids, while R cells produce more adrenal androgens (DHEA, Δ4A) and testosterone. Interestingly, we found equal cortisol production for both A and R cells. The cell line-specific 'signatures' are mainly explained by higher expression and activity of 3βHSDII in A cells and lower expression and activity of 3βHSDII in combination with higher activity of 17,20 lyase in R cells. These findings underline the importance of the balance between 3βHSDII and P450c17 activities for steroidogenesis. First, the 17α-hydroxylase and 17,20 lyase activities of P450c17 are the qualitative regulators of steroidogenesis, which are essential for glucocorticoid and androgen production (Miller 1988). To favor androgen production over glucocorticoid production, the 17,20 lyase activity of P450c17 is enhanced by cytochrome b5, POR, and phosphorylation (Lin *et al.* 1993, Zhang *et al.* 1995, Pandey *et al.* 2003, Pandey & Miller 2005). Second, 3βHSDII and P450c17 have common substrates and are therefore competitors (Fig. 1). In previous competition experiments using recombinantly produced proteins expressed in yeast, we showed that decreased 3βHSDII activity in relation to P450c17 activities will shift steroidogenesis towards enhanced

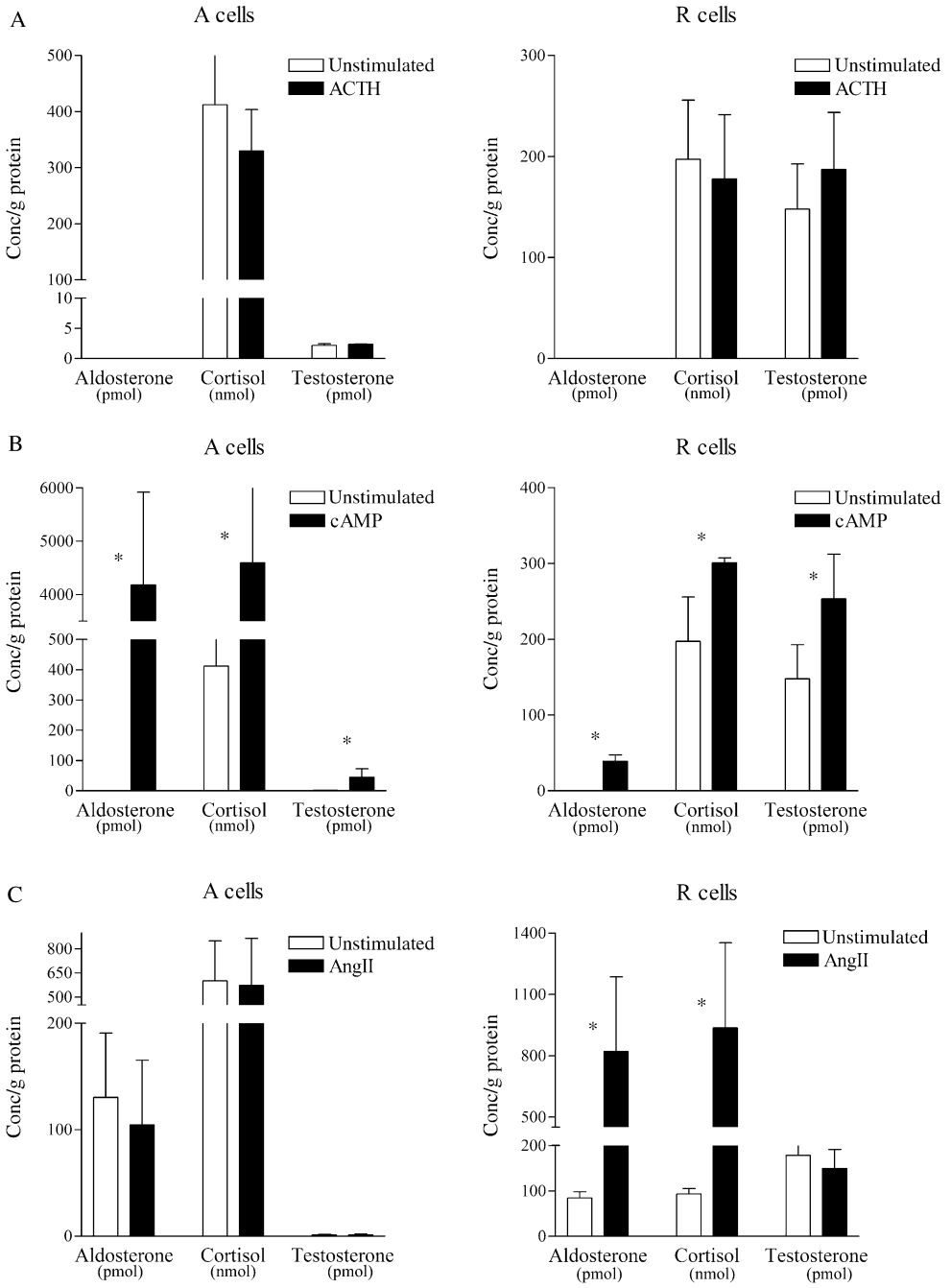

Figure 6 Production of mineralocorticoids, glucocorticoids, and androgens after stimulation with ACTH, 8Br-cAMP, and AngII. Equal numbers of A and R cells were grown in DMEM/F12 medium, initially with serum for 24 h, then serum-free for 24 h before stimulation. After stimulation supernatants of the cells were collected and concentrations of aldosterone, cortisol, and testosterone were measured. (A) Summary of the results for A cells and R cells 24 h after stimulation with 100 nM ACTH. (B) Summary of the results 24 h after stimulation with 0·5 mM 8Br-cAMP. (C) Summary of the results 72 h after stimulation with 100 nM AngII. Note that aldosterone was only detected after 72 h incubation in serum-free medium. All data represent mean ± S.E.M. of three independent experiments. *$P<0{\cdot}05$.

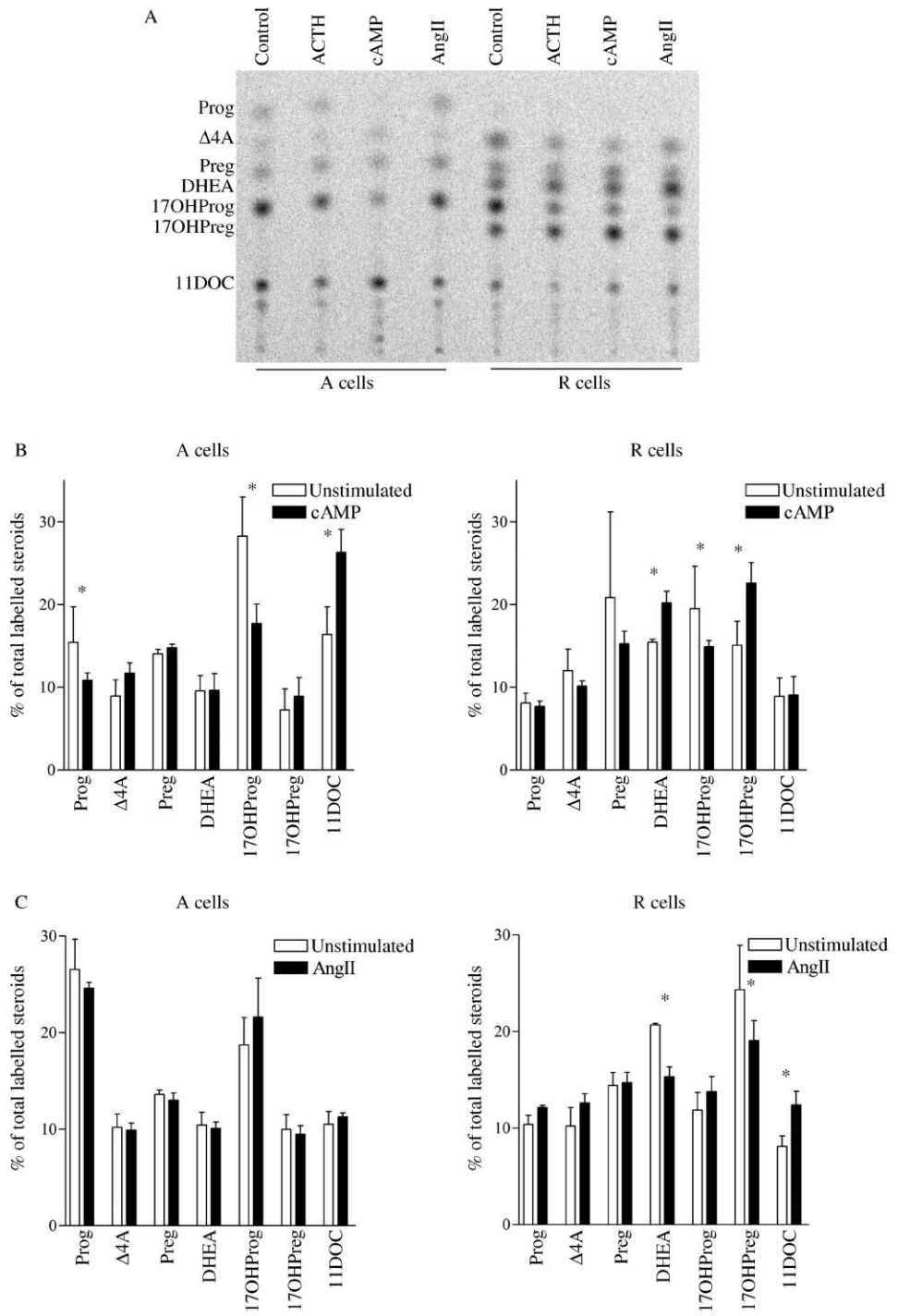

Figure 7 Steroid profiles after stimulation with ACTH, 8Br-cAMP, and AngII. Equal numbers of A and R cells were grown in DMEM/F12 medium, initially with serum for 24 h, then serum-free. Cells were stimulated and steroidogenesis was labeled with [^{3}H]-Preg (~220 000 c.p.m./well). Steroids were extracted from the supernatants, separated on TLC plates, and then visualized. (A) Representative TLC. (B and C) Quantification of specific steroids produced by A and R cells assessed as percentage of total labeled steroids after stimulation with 0·5 mM 8Br-cAMP for 24 h (B) or 100 nM AngII for 72 h (C). Data for ACTH after 24-h stimulation are not shown as no change was observed upon stimulation for both cell lines. Data represent mean±S.E.M. of 2–3 independent experiments, each performed in duplicates. *$P<0·05$.

Δ4A production from Preg, thus favoring the sex steroid over the mineralocorticoid pathway (Fluck *et al.* 2005). Thus, higher androgen production in R cells despite equal expression of P450c17 for A and R cells may be explained – at least in part – by lower expression of HSD3B2 in R cells. This concept of higher P450c17/3βHSDII ratio stressing androgen production was suggested previously by Conley & Bird (1997) and supported by the observation that adrenarche is characterized by a drop in HSD3B2 expression (Gell *et al.* 1996, 1998).

Physiologically, human adrenal mineralocorticoid production is acutely regulated by AngII and glucocorticoid production by ACTH. Both A and R cells do not express high levels of the ACTH receptor. Accordingly, we found that steroidogenesis of both cell lines may not be stimulated by ACTH. By contrast, stimulation with cAMP directly increased steroid production in both cell lines indicating that the cAMP signaling is intact. We found higher expression of the AT1 receptor in R cells and AngII was able to stimulate steroidogenesis in R cells but not A cells, although basally A cells produced more aldosterone than R cells. This suggests that the regulation of AngII on aldosterone production is disrupted in A cells. However, from our data we cannot say at what level this signaling is disturbed.

Our finding that A and R cells are different may have two major implications. First, research data obtained from studies using one cell line may not apply to the other cell line. Second, studying the difference between A and R cells in further detail may help us gain important information on how androgen production is regulated.

To understand the exact regulation of androgen biosynthesis including the concerted activities of 3βHSDII and P450c17 is important, but little is known. Recent studies have suggested that the expression and activity of 3βHSDII from weeks 7 to 12 of fetal life is essential for safeguarding the sexual development of a female fetus (Goto *et al.* 2006). Outside this fetal time window, the human fetal adrenals lack 3βHSDII activity until about 23 weeks, gestation (Narasaka *et al.* 2001), and therefore produce high amounts of DHEAS. After birth, adrenal androgen production is shut down with the change from the fetal to the postnatal adrenal cortex, which consists of the ZG and ZF producing relatively constant amounts of mineralocorticoids and glucocorticoids throughout life. In contrast, adrenal androgen production starts after the development of the ZR (adrenarche), reaches a sex-specific plateau around age 30 years, and decreases constantly thereafter (adrenopause; Rainey *et al.* 2002). Immunohistochemical studies of the human adrenal cortex revealed that during adrenarche, the ZR shows specific changes in the expression of b5, SULT2A1, and HSD3B2 (Suzuki *et al.* 2000). SULT2A1 and b5 are markedly enhanced and HSD3B2 is grossly diminished after adrenarche. In addition, epidemiologic data suggest that girls with premature exaggerated adrenarche have a significantly higher risk to develop the polycystic ovary syndrome (PCOS), the most common hyperandrogenic disorder affecting 5–10% of women (Escobar-Morreale *et al.* 2005). Elevated serum androgens in PCOS have been shown to originate from both the ovaries and the adrenals (Lachelin *et al.* 1982, Barnes *et al.* 1989, Ehrmann *et al.* 1992). Studies of the steroid metabolism of ovarian theca cells isolated from PCOS women demonstrated enhanced 3βHSDII and P450c17 activities in PCOS compared with normal cells (Nelson *et al.* 1999, 2001). Additionally, northern blot and quantitative real-time PCR analyses revealed an increase in the abundance of the mRNAs for CYP17, HSD3B2, and CYP11A1 in PCOS compared with normal cells, but no difference for StAR and 17βHSDV was observed (Nelson *et al.* 1999, 2001). In addition, there is experimental evidence that enhanced expression of b5 together with abundance in POR as well as phosphorylation of P450c17 lead to an increase in 17,20 lyase activity (Lin *et al.* 1993, Zhang *et al.* 1995, Pandey *et al.* 2003, Pandey & Miller 2005). Considering all these research data describing characteristics of androgen production, our comparison between A and R cells suggests that R cells are more 'androgenic' than A cells. The profile of R cells may be compared with the steroid profile of adrenarche for low expression and activity of 3βHSDII and high activity of 17,20 lyase. However, R cells do not have higher expression of b5 or SULT2A1 as found in the ZR after adrenarche. Similarly, R cells share the steroid profile of PCOS with higher activity of 17,20 lyase; but in contrast to PCOS expression of HSD3B2 and P450c17 is not enhanced when compared with A cells.

A and R cells are tumor cells. The original H295 cell line was isolated from an adrenocortical carcinoma of a middle-aged woman suffering from elevated serum aldosterone, cortisol, and androgens (Gazdar *et al.* 1990). The normal human adrenal cortex seems not to produce relevant amounts of testosterone (Miller 2002). However, 50% of human adrenocortical tumors are hormonally active and about half of them produce testosterone (Allolio *et al.* 2004). Original H295 cells are reported to secrete large quantities of adrenal androgens and smaller amounts of testosterone (Gazdar *et al.* 1990). Therefore, it has been suggested that the major steroid pathway in H295 cells is androgen formation, although mineralocorticoids and glucocorticoids were also detected (Gazdar *et al.* 1990). However, the steroid profile of H295 was reported to be influenced by serum conditions (Gazdar *et al.* 1990). Thus, the observed differences between A and R cells may have been caused by different growth conditions and handling in several laboratories over time. Both cell lines were subcultured and manipulated to grow adherently in different laboratories under different conditions (Gazdar *et al.* 1990, Rodriguez *et al.* 1997). From our studies using the same growth medium for all experiments, we can exclude that factors present in the growth medium were responsible for the observed differences. In our experience, passage number may play a role for both cell lines beyond 30–35 passages. R cells seem to resemble their parental H295 cells more than A cells. For both H295 and R cells, testosterone production has been reported (Gazdar *et al.* 1990, Danesi *et al.* 1996, Hecker *et al.* 2006). We were able to detect testosterone in the growth medium of R cells but not A cells. Two enzymes 17βHSDIII and 17βHSDV are involved in the

synthesis of testosterone from Δ4A or DHEA (Fig. 1; Miller 2002). Normally, the HSD17B3 gene is only expressed in the human testis (Geissler *et al.* 1994, Miller 2002), whereas the HSD17B5 gene is expressed in several human tissues such as the testes and the adrenals (Dufort *et al.* 1999). To date no data was available regarding the expression of these genes in A and R cells. We show that both HSD17B3 and HSD17B5 genes are expressed in A as well as R cells. Therefore, the difference in testosterone production between A and R cells may be mainly explained by lower 3βHSDII and higher 17,20 lyase activity in R cells leading to an increase in substrate availability for 17βHSDIII/V.

In conclusion, human adrenocortical R cells produce more androgens than A cells even though they derive from the same tumor. Thus, studying the differences between these two cell lines may serve as an excellent model to find key regulators of androgen production.

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