Characterization of NCI-H295R Cells as an In Vitro Model of Hyperaldosteronism

Authors

U. D. Lichtenauer[1], I. Shapiro[1], A. Osswald[1], S. Meurer[2], A. Kulle[3], M. Reincke[1], F. Riepe[3], F. Beuschlein[1]

Affiliations

[1] Endocrine Research Unit, Medizinische Klinik und Poliklinik IV, Klinikum der LMU, Munich, Germany
[2] Endocrine Laboratory, Medizinische Klinik und Poliklinik IV, Klinikum der LMU, Munich, Germany
[3] Pediatric Clinic, University Hospital Kiel, Kiel, Germany

Key words
- aldosterone producing cell line
- Conn's syndrome
- adrenal tumor

Abstract

In depth analysis of key molecular mechanisms involved in functional autonomy of aldosterone secretion is hampered by the lack of tumor cell lines that reflect functional characteristics of aldosterone producing adenomas. Herein, we describe the characteristics of the adrenocortical carcinoma cell line NCI-H295R and its suitability as a model of hyperaldosteronism in relation to different culture conditions. Steroid profiling revealed that NCI-H295R cells predominantly secrete cortisol, while aldosterone and other steroids are released at much lower concentrations. However, aldosterone output specifically increased in response to different stimuli such as ACTH and angiotensin II, and in particular to potassium in a dose dependent manner. NCI-H295R cells readily formed spheroids under specific culture conditions, a method widely used for the enrichment of progenitor cells. Unexpectedly, spheroid cells excelled with higher aldosterone concentration and higher expression levels of the steroidogenic enzymes StAR, 3βHSD, CYP17, SF-1, and the MC2-receptor. Further investigations revealed that this phenomenon is mainly attributed to epithelial growth factor (EGF) and particularly fibroblast growth factor (FGF), which are both essential ingredients in the spheroid culture medium. Aldosterone release under the combinatory influence of EGF and FGF was not higher than the effect of FGF alone. Spheroid growth per se, therefore, does not ensure an enrichment of less differentiated cell types in this cell line.

received 10.06.2012
accepted 20.08.2012

Bibliography
DOI http://dx.doi.org/10.1055/s-0032-1323810
Published online: 2012
Horm Metab Res

ISSN 0018-5043

Correspondence
F. Beuschlein, MD
Endocrine Research Unit
Medizinische Klinik und Poliklinik IV
Klinikum der Ludwig-Maximilian University
Ziemssenstraße 1
80336 Munich
Germany
Tel.: +49/89/5160 2110 (2116)
Fax: +49/89/5160 4467
felix.beuschlein@med.uni-muenchen.de

Introduction

Primary aldosteronism (PA) has been recognized as the most common cause of secondary hypertension, which is responsible for elevated blood pressure in 5–10% of all hypertensive patients [1]. While the classical hypokalemic form of PA is prevalent in less than 0.5% of patients with hypertension, more sensitive diagnostic screening approaches allow for the detection of the normokalemic variant of the disease which resulted in an increase of case detection and, thus, identification of PA as a relatively common disease [2].

On the contrary, the mechanisms accounting for autonomous aldosterone secretion are still only partly understood. All models currently being used in preclinical research have limitations. In vitro, the human NCI-H295R cell line is most commonly utilized [3,4], although it has been derived from an adrenocortical carcinoma and therefore inherently might not reflect the molecular and functional changes of true aldosterone producing adenomas [5]. Nevertheless, in contrast to other adrenocortical cell lines, such as the SW13 cells, these cells are known to be capable of aldosterone production to some extent. The aim of this study was to increase the knowledge about the characteristics of aldosterone production in NCI-H295R cells and their steroid profile under different culturing conditions.

In previous studies, the existence of subpopulations with distinct characteristics within a given tumor has been described. Some of these subpopulations seem to represent cells with progenitor or stem cell-like properties, and were claimed to be responsible for most of the tumor growth [6,7]. Interestingly, these subpopulations could also be found in clonally expanding cell populations, such as cell lines [8,9]. Therefore, besides analyzing the feasibility of NCI-H295R cells as an in vitro model for aldosterone excess, we investigated, whether a subpopulation of cells within the NCI-H295R adrenocortical carcinoma cell

line could be enriched by sphere formation, a process originally described to enrich for neural and other progenitor cells, including the adrenal medulla, and stem cell-like cancer cells [6, 8, 10–13].

Materials and Methods

Cell culture conditions

NCI-H295R monolayer cells and SW13 cells were grown at 37 °C and 5 % CO_2 in DMEM/F12 medium (Gibco Invitrogen, Carlsbad, CA, USA) containing 10 % FBS (HyClone, South Logan, UT, USA) and 2 mM L-glutamine (BioWhittaker Cambrex, East Rutherford, NJ, USA), and 10 µl/ml Pen/Strep (Gibco) as described previously [14]. Spheroid growth was induced by culturing the cells without serum in DMEM/F12 medium supplemented with B27, N2, 20 ng/ml hEGF, 20 ng/ml bFGF, and 10 µl/ml Pen/Strep (all Gibco). To measure growth factor effects on aldosterone release, either EGF or FGF was withheld from the medium in comparison to medium containing both factors, and in comparison to regular cell culture medium. For stimulation experiments, NCI-H295R monolayer cells were plated at a density of 50 000 cells in 24-well plates. After 72 h, the medium was replaced, and ACTH, angiotensin II, and potassium chloride (all Invitrogen) were added to 3 wells per stimulant at various concentrations (ACTH: 20 nM, angiotensin II: 20 nM, potassium chloride: 5.2, 7, 9, 12, 15, 20, and 40 mM total concentration).

Steroid profiling

Mineralocorticoids, glucocorticoids, and androgens in the supernatant of NCI-H295R cells grown for 24 h under standard cell culture conditions and after stimulation with 20 mM potassium, were measured in triplicates by ultra-pressure liquid chromatography tandem mass spectrometry (UPLC-MS/MS) according to a previously described method [15]. In brief, aliquots of samples, calibrators, and controls were extracted by solid-phase extraction (SPE) using Oasis MAX SPE system plates (Waters, Milford, MA, USA). Deuterium labeled steroids were used as internal standards. The UPLC-MS/MS system was used in the multiple reaction monitoring mode (MRM), and steroids were measured in the positive ion mode except aldosterone, which was measured in the negative mode. For each hormone 2 different MRM transitions were monitored. The limit of quantification was between 0.1 nmol/l for 17-hydroxyprogesterone and 2 nmol/l for cortisol. The intra- and interassay coefficient of variations for replicate quality controls for different concentrations range between 2.4 % and 9.7 %. Total run time for the assay was 5 min.

Aldosterone assay

Aldosterone in cell culture supernatants was measured by an inhouse immunofluorometric assay, which initially was developed for the measurement of aldosterone in saliva [16]. Samples were run in triplicates, and for comparative aldosterone release studies between spheroid and monolayer cells, results were normalized to total cellular protein. To exclude matrix effects, cell culture supernatants were extracted with dichloromethane and reconstituted in an artificial saliva matrix prepared according to the German Industry Norm (DIN 53 160) standards (4.2 g/l $NaHCO_3$, 0.5 g/l NaCl, 0.2 g/l K_2CO_3). The same matrix was also used to produce standards and to dilute samples with very high aldosterone concentrations.

Tissue staining and immunohistochemistry

NCI-H295R cells were grown on Coverslips (Becton Dickinson, Franklin Lakes, NJ, USA) for 5 days and fixed with PFA for at least 1 h. Spheroid cells were collected with a 100 µm mesh, fixed with PFA, dehydrated, and paraffin embedded. Tissue slides and coverslips were subsequently investigated by H & E staining. For immunohistochemical analysis, cover slips and tissue sections were rehydrated, blocked with 0.3 % H_2O_2 in methanol for 10 min, and incubated with blocking buffer for 15 min. P450SCC primary rabbit anti-human antibody (kindly provided by Dr. Walter Miller, UCSF, CA, USA) was incubated overnight at 4 °C after dilution in blocking buffer containing 3 % bovine serum albumin (Sigma, St. Louis, MO, USA), 5 % goat serum (Jackson Immuno Research Laboratories, West Grove, PA, USA), and 0.5 % Tween 20 (Calbiochem, San Diego, CA, USA). After rinsing for 15 min in PBS, secondary antibody (Goat anti-rabbit biotinylated IgG; Vector Laboratories, Burlingame, CA, USA) was applied for 30 min at room temperature. Bound primary antibody was detected using the VECTASTAINE Elite ABC Kit (Vector Laboratories) according to the manufacturer's protocol.

Real-time PCR

RNA from cell preparations was extracted using the Qiagen RNA mini kit (Qiagen, Valencia, CA, USA) following the instructions of the manufacturer. cDNA was transcribed using a reverse transcription kit (Promega, Mannheim, Germany) and 1.0 µg of total RNA.

Real-time PCR was performed using the FastStart DNA MasterPlus SYBR Green I reaction mix in the LightCycler 1.5 (Roche, Mannheim, Germany). The cycling conditions for real-time PCR included a preincubation step at 95 °C for 10 min, followed by an amplification step, which consisted of 40–45 cycles at 95 °C for 10 s, annealing (the temperature was primer dependent as given below) for 6 s and an extension step at 72 °C, for which the time was calculated by the product length in bp divided by 25 (Roche, Indianapolis, IN, USA). Primer sequences as well as product lengths and annealing temperatures were as follows: P450SCC: 5′-GCAACGTGGAGTCGGTTTAT-3′ and 5′-TCCTCGAAGGACATCTTGCT-3′ (664 bp; 52 °C); StAR: 5′-CAGGACAATGGGGACAAAGT-3′ and 5′-ATGAGCGTGTGTACCAGTGC-3′ (608 bp; 63 °C); SF-1: 5′-TGCACTGCAGCTGGACCGCCAGGAGTT-3′ and 5′-AGGGCTCCTGGATCCCTAATGCAAGGA-3′ (390 bp; 58 °C); 3βHSD 5′-CTAAGTTACGCCCTCTTCTG-3′ and 5′-AATGTCTCCTTCAAGTACAGT-3′ (285 bp; 50 °C); Cyp 17 5′-CAA GCC AAG ATG AAT GCA GA-3′ and 5′- CAT AAA CCG ATC TGG CTG GT-3′ (438 bp; 50 °C); MC2-receptor 5′-CATGGGCTATCTCAAGCCAC-3′ and 5′- GAGATCTTCCTGGTGTGGGATC-3′ (360 bp; 55 °C); CYP11B2 synthase 5′-GGGAGCGTGGACACGACAGC-3′ and 5′-GGCATTGCGACCCAGCGAGT-3′ (303 bp; 60 °C). Melting curve analysis was performed between 65 and 98 °C (0.1 °C/s) to determine the Tm of the amplified product and in order to exclude undesired primer dimers. Furthermore, the products were run on a 1 % agarose gel to verify the size of the amplified product. Each sample was run at least in triplicate. Quantification was normalized using HPRT (hypoxanthine phosphoribosyltransferase) as a housekeeping/reference gene. cDNA of 8 normal adrenals were pooled in equal volumes to achieve a representative standard for the expression analysis part of this study.

Statistical analysis

All results are expressed as mean ± SD. If not stated otherwise, all statistical comparisons were analyzed by one-way ANOVA and

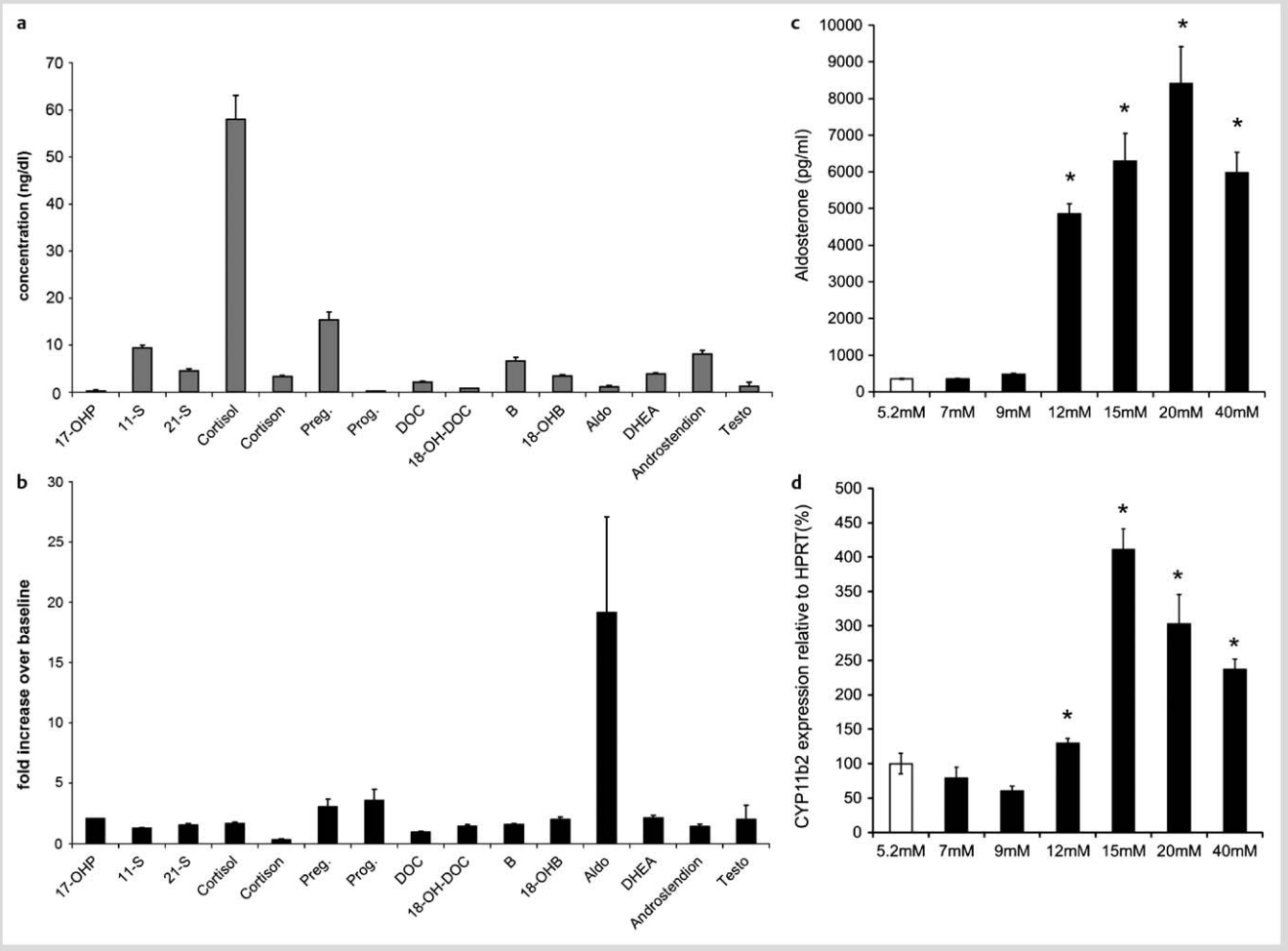

Fig. 1 Steroid profiling: At baseline, cortisol was the most abundantly secreted steroid hormone **a**. Potassium stimulation led to a predominant increase in aldosterone output while secretion of the other steroids remained largely unaltered **b**. 17OHP: 17-hydroxyprogesterone; 11 s: 11-deoxycorticosterone; 21 s: 21-deoxycortisol; Preg: pregnenolone; Prog: progesterone; DOC: deoxycorticosterone; 18-OH-DOC: 18-hydroxy-11-deoxycorticosterone; Aldo: aldosterone; DHEA: dehydroepiandrosterone; Testo: testosterone. Potassium induced an increase in aldosterone secretion **c** and CYP11B2 expression **d** in a dose dependent manner.

independent samples T test. Statistical significance was defined as $p < 0.05$ and is indicated as a star (*) in the figures.

Results

Steroid profile

At baseline, ultra-pressure liquid chromatography tandem mass spectrometry revealed that cortisol was the predominantly secreted hormone in the supernatant of NCI-H295R cells (58.0 ± 5.1 ng/dl), whereas the other steroid hormones were released on a much lower level in similar range (**Fig. 1a**). Interestingly, potassium stimulation resulted in a distinct shift in the steroid profile due to a selective and significant 19.2-fold increase of aldosterone secretion (**Fig. 1b**) while most other steroid metabolites were not significantly changed in a potassium dependent manner.

Aldosterone output of NCI-H295R cells at baseline and upon stimulation

Baseline aldosterone levels measured in the supernatant of NCI-H295R cells grown as monolayers after 24 h of incubation ranged between 328–716 pg/ml, and were found to be responsive to stimulation by angiotensin II (131 ± 1.2 % vs. 100 ± 6.2 %; $p < 0.01$), while incubation with ACTH increased aldosterone secretion only slightly (118 ± 6.4 %; $p = 0.17$; data not shown). However, the most pronounced aldosterone induction was observable upon potassium stimulation when aldosterone output increased from 352 ± 8.64 pg/ml at physiological potassium concentrations (5.2 mM) up to 8 407 ± 1 013 pg/ml at potassium concentrations of 20 mM in a dose dependent manner (**Fig. 1c**). Likewise, CYP11B2 expression gradually increased with increasing potassium concentrations up to a maximum of 411 ± 0.3 % at a potassium concentration of 15 mM (**Fig. 1d**). Further increase in potassium supplementation resulted in lower aldosterone concentrations and lower CYP11B2 expression levels, respectively, most likely due to potassium dependent cell death (**Fig. 1c, d**).

Spheroid versus monolayer growth

NCI-H295R cells were exposed to 2 different culture conditions: Regular serum containing medium and serum free medium supplemented with epithelial growth factor (EGF) and fibroblast growth factor (FGF). Adherent monolayer growth was observed (day 2 after plating), when cells were cultured in regular, serum containing medium, whereas they adopted spheroid-like structures after plating a minimum of 100 cells per well on a 24-well

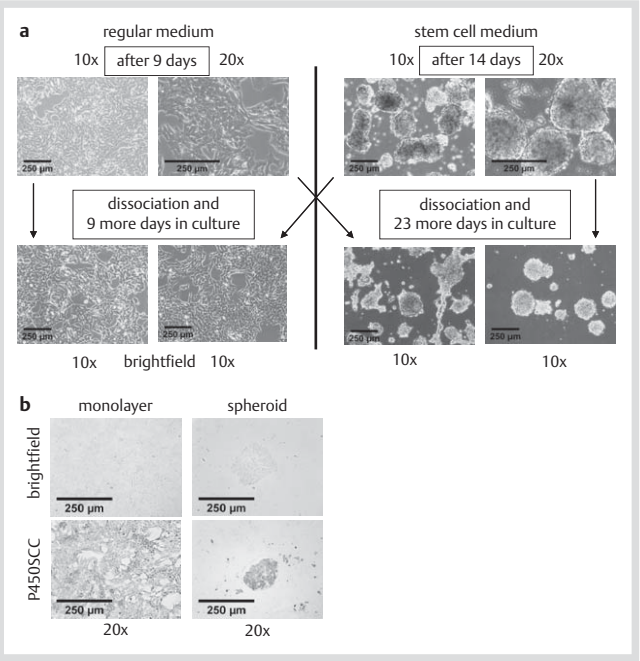

Fig. 2 Spheroid vs. monolayer growth: NCI-H295R cells readily grew as monolayer in their standard medium, or as spheroids in serum free medium containing growth factors (**a**+**b**, upper panels). After dissociation into single cells, spheroids can be regrown as spheroids or as adherent cells, depending on cell culture conditions (**a**, lower panel). Both types of cells stained positive for p450SCC (**b**, lower panel).

plate in EGF and FGF containing serum free medium for a minimum of 3 days (**Fig. 2a**, upper panels; **Fig. 2b**, upper panels). NCI-H295R spheroids could be trypsinized and dissociated into single cell suspension and continued to grow as monolayer culture, or re-formed spheroids at a much slower rate (23 more days vs. 9 days), depending on the culture conditions they were exposed to (**Fig. 2a**, lower panel). Without dissociation, spheroid growth decreased over time and seized after approximately 2 weeks (data not shown).

Fixed and embedded spheroids and monolayer cells clearly stained positive for P450SCC in immunohistochemical analyses (**Fig. 2b**, lower panels) indicating persistence of steroidogenic properties independent of culture conditions.

Steroidogenic capacity of spheroids

Baseline aldosterone levels secreted by spheroids (S) and normalized to total cellular protein were significantly higher in comparison to monolayer (M) cells (3409±644.9 vs. 615.7±11.9 pg/ml/mg/ml total protein; $p=0.01$; **Fig. 3a**). Real-time PCR analyses of NCI-H295R cells grown as monolayers or spheroids revealed that expression levels of steroidogenic enzymes – normalized to HPRT – were approximately 100 times lower in NCI-H295R cells in comparison to pooled normal human adrenals (N): 3β-HSD, 0.36±0.02%; $p<0.01$ (S) and 0.01±0.0%; $p<0.01$ (M) vs. 100±19.5% (N), p450SCC: 6.9±0.15%; $p<0.01$ (S) and 6.29±0.19%; $p<0.01$ (M) vs. 100±3.14% (N); StAR: 7.65±1.08%; $p<0.01$ (S) and 0.91±0.24%; $p<0.01$ (M) vs. 100±9.29% (N); Sf-1: 22.65±1.11%; $p<0.01$ (S) and 15.0±0.3%; $p<0.01$ (M) vs. 100±1.95% (N); Cyp17: 0.19±0.01%; $p<0.01$ (S) and 0.14±0.01%; $p<0.01$ (M) vs. 100±1.54% (N); MC2-R: 45.8±0.33%; $p<0.01$ (S) and 0.8±0.01%; $p<0.01$ (M) vs. 100±2.17% (N). Notably, with the exception of P450SCC, expression levels of all investigated genes were either equal or higher in spheroids in comparison to monolayer cells. In contrast, no expression of any steroidogenic enzyme analyzed was detectable in SW13 cells, which is in agreement with their known lack of steroidogenic properties (**Fig. 3b**).

To investigate the rationale behind the different steroidogenic properties of NCI-H295R cells grown as monolayer vs. spheroids, monolayer cells were incubated with the same concentrations of growth factors as used for spheroid growth induction. While the addition of EGF alone already led to a significant increase of aldosterone release (2529±255.4 vs. 1206±44.4; $p=0.01$), FGF treatment had an even greater effect, with aldosterone levels similar to those observed for spheroid cells (4779±435.4; $p<0.01$). The combination of EGF and FGF did not result in a response greater than FGF treatment alone (4219±22.1; $p=0.35$). Vice versa, when spheroid cells were switched to regular monolayer culture medium without growth factors, aldosterone concentrations in the supernatant dropped to a level similar to that of monolayer cells (1013±31.4; $p=0.04$; **Fig. 4**). To exclude differences in aldosterone concentrations from different amount of cells, results were related to total cellular protein levels for all conditions mentioned.

Discussion

Despite a number of potential drawbacks inherent to cell line dependent model systems, adrenocortical NCI-H295R cells are well established for their maintained steroid capabilities. Herein, we specifically analyzed the suitability of these cells as an in vitro model of autonomous aldosterone excess. Recently, endocrine properties of different strains, or subtypes of NCI-H295R cells have been described [17]. These studies provided evidence that phenotypic variability and shifts through clonal selection have to be taken into account for the interpretation of experimental results. In addition, as shown herein, cell culture conditions that might induce stem cell like properties have to be considered as modifiers of secretory abilities.

At baseline conditions, cortisol was the most abundantly secreted hormone, which was expected and reflects the physiological ratio of steroid hormones from the adrenal cortex. Interestingly, potassium stimulation led to a relative decrease in cortisol output. Overall, potassium induced effects on adrenal glucocorticoid secretion are not well defined and clearly influenced by species-, cell- and context- (*in vivo* vs. *in vitro*) dependent mechanisms with examples of potassium induced increased [18], decreased [19], or unchanged [20] glucocorticoid levels.

While aldosterone output could be stimulated to some extent by ACTH and angiotensin II, incubation with high levels of potassium was followed by a pronounced, specific and exclusive shift towards mineralocorticoid production, as exemplified in a multisteroid analysis. Accordingly, CYP11B2 expression as the rate limiting step of aldosterone production increased with rising potassium concentrations. However, the stimulatory response set in on supra-physiological potassium concentrations, a common finding in cell line experiments. Concentrations higher than 15–20 mM led to a decrease in CYP11B2 expression and aldosterone concentration, most likely due to hyperkalemia induced cell death, as the cells had a different appearance and partially detached from the bottom of the dish.

Since spheroid formation has been recognized as a tool to enrich neural precursors and stem cell like cancer cells, even in the context of mainly clonally expanding cells [8,9,12], we aimed to investigate, whether spheroid growth could be induced also

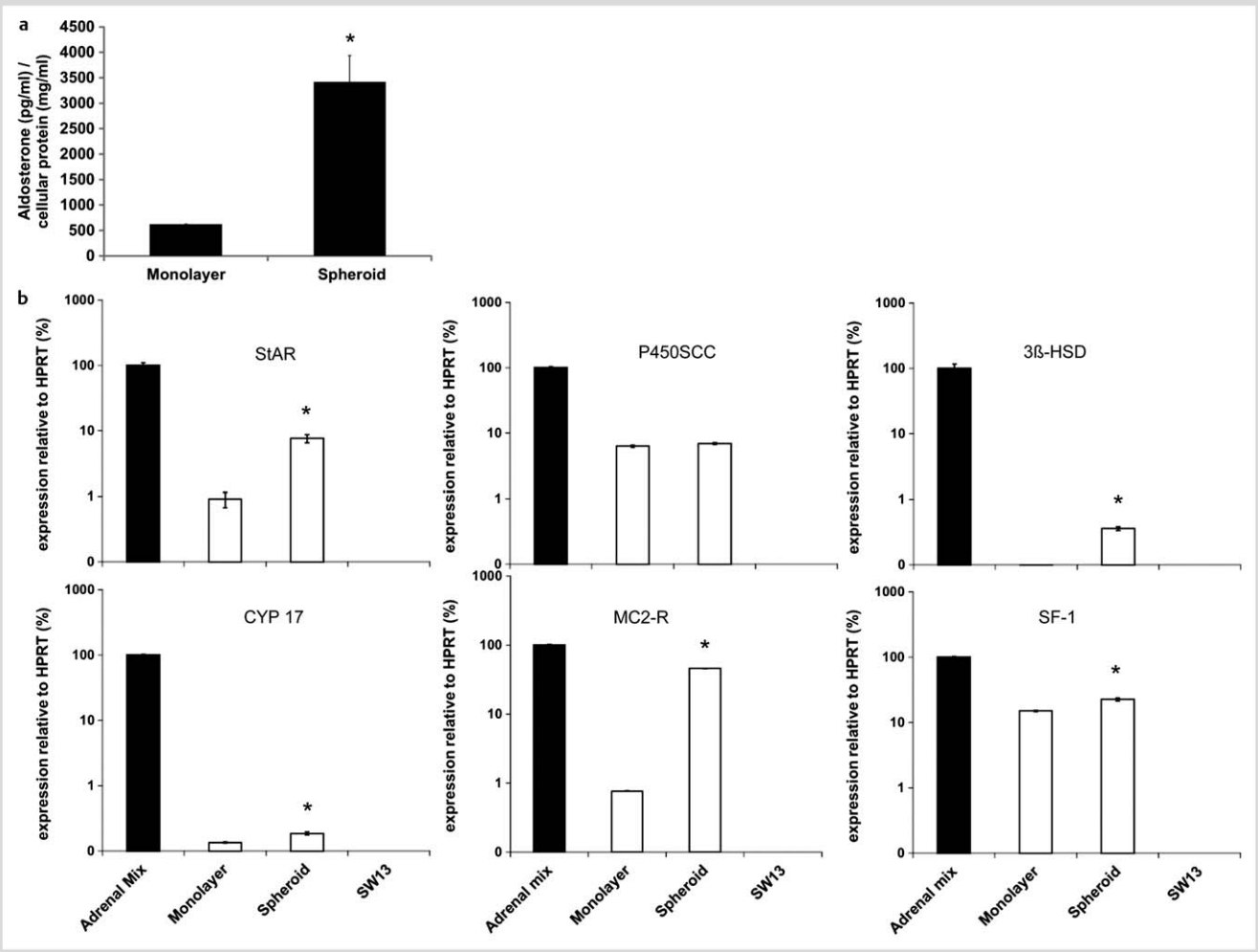

Fig. 3 Expression profile: Baseline aldosterone output was significantly higher in the supernatant of spheroid cells in comparison to monolayer cells **a**. mRNA expression levels of StAR, P450SCC, 3β-HSD, CYP17, SF-1, and the MC2-receptor in NCI-H295R and SW13 cells were measured in monolayer cells and spheroids by real-time PCR analysis and compared to a pool of normal adrenals **b**.

from adrenocortical cells. In fact, in the presence of EGF and FGF in a serum-free medium NCI-H295R cells readily formed cell clusters, which progressively adopted the typical spheroid appearance. After dissociation and re-plating, the cells regrew as spheroids or as adherent cells, depending on the cell culture conditions they were exposed to. While we expected a less differentiated phenotype, surprisingly, spheroids seemed to retain the ability to produce steroids, as they expressed even higher levels of most of the steroidogenic enzymes tested, had greater aldosterone output, and grew slower than monolayer cells. Therefore, as confirmed by immunohistochemistry, RT-PCR and hormone measurements, spheroids from NCI-H295R cells seem to enrich for a rather differentiated phenotype.

In further experiments we analyzed potential direct effects of different culture conditions on spheroid and monolayer cell growth. Interestingly, when monolayer cells were exposed to the growth factors of spheroid medium, especially to FGF, their responsiveness to potassium significantly increased to a similar level as observed in spheroid cells. Vice versa, spheroid cells cultured in monolayer medium were characterized by aldosterone concentrations in the supernatant similar to that of monolayer cells. Taken together, most of the observed differences in aldosterone release between monolayer and spheroid cells seem to be attributable to FGF in the spheroid cell culture medium. This

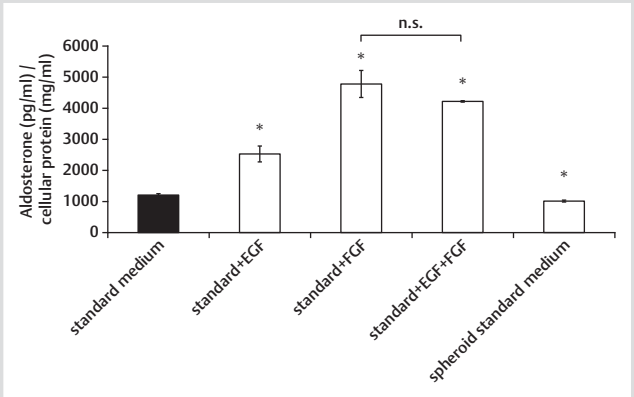

Fig. 4 Influence of growth factors on aldosterone output: Monolayer cells were incubated with different combinations of the growth factors EGF and FGF. The last column represents the aldosterone concentration secreted from spheroids grown in the culture medium of monolayer cells.

further confirmed the results of the expression analyses, that spheroid growth per se, does not necessarily ensure an enrichment of progenitor or stem cell like tumor cells in all cell types.

In conclusion, we have demonstrated the ability of NCI-H295R cells to specifically respond to different stimuli of the mineralocorticoid regulatory system, especially potassium, with a specific increase in aldosterone output. In addition, the cells easily grew spheroids, however, adopted surprisingly a rather differentiated phenotype, which is most likely related to FGF exposure. The highest aldosterone concentrations achieved were still approximately 50 times lower than those measured within the first 24 h of primary cells from aldosterone producing adenomas (data not shown). This finding underlines the need to continue the search for alternative, potentially more potent cell lines, preferentially from an aldosterone producing adenoma.

Conflict of Interest

The authors have nothing to disclose and have no conflict of interest.

References

1 *Schirpenbach C*, *Reincke M*. Primary aldosteronism: current knowledge and controversies in Conn's syndrome. Nat Clin Pract Endocrinol Metab 2007; 3: 220–227
2 *Reincke M*, *Beuschlein F*, *Bidlingmaier M*, *Funder JW*, *Bornstein SR*. Progress in primary aldosteronism. Horm Metab Res 2010; 42: 371–373
3 *Rainey WE*, *Saner K*, *Schimmer BP*. Adrenocortical cell lines. Mol Cell Endocrinol 2004; 228: 23–38
4 *Bird IM*, *Hanley NA*, *Word RA*, *Mathis JM*, *McCarthy JL*, *Mason JI*, *Rainey WE*. Human NCI-H295 adrenocortical carcinoma cells: a model for angiotensin-II-responsive aldosterone secretion. Endocrinology 1993; 133: 1555–1561
5 *Gazdar AF*, *Oie HK*, *Shackleton CH*, *Chen TR*, *Triche TJ*, *Myers CE*, *Chrousos GP*, *Brennan MF*, *Stein CA*, *La Rocca RV*. Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. Cancer Res 1990; 50: 5488–5496
6 *Singh SK*, *Clarke ID*, *Terasaki M*, *Bonn VE*, *Hawkins C*, *Squire J*, *Dirks PB*. Identification of a cancer stem cell in human brain tumors. Cancer Res 2003; 63: 5821–5828
7 *Al-Hajj M*, *Wicha MS*, *Benito-Hernandez A*, *Morrison SJ*, *Clarke MF*. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003; 100: 3983–3988
8 *Hirschmann-Jax C*, *Foster AE*, *Wulf GG*, *Nuchtern JG*, *Jax TW*, *Gobel U*, *Goodell MA*, *Brenner MK*. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci USA 2004; 101: 14228–14233
9 *Lichtenauer UD*, *Shapiro I*, *Geiger K*, *Quinkler M*, *Fassnacht M*, *Nitschke R*, *Ruckauer KD*, *Beuschlein F*. Side population does not define stem cell-like cancer cells in the adrenocortical carcinoma cell line NCI h295R. Endocrinology 2008; 149: 1314–1322
10 *Carpenter MK*, *Cui X*, *Hu ZY*, *Jackson J*, *Sherman S*, *Seiger A*, *Wahlberg LU*. In vitro expansion of a multipotent population of human neural progenitor cells. Exp Neurol 1999; 158: 265–278
11 *Chung KF*, *Sicard F*, *Vukicevic V*, *Hermann A*, *Storch A*, *Huttner WB*, *Bornstein SR*, *Ehrhart-Bornstein M*. Isolation of neural crest derived chromaffin progenitors from adult adrenal medulla. Stem Cells 2009; 27: 2602–2613
12 *Dontu G*, *Al-Hajj M*, *Abdallah WM*, *Clarke MF*, *Wicha MS*. Stem cells in normal breast development and breast cancer. Cell Prolif 2003; 36 (Suppl 1): 59–72
13 *Uchida N*, *Buck DW*, *He D*, *Reitsma MJ*, *Masek M*, *Phan TV*, *Tsukamoto AS*, *Gage FH*, *Weissman IL*. Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci USA 2000; 97: 14720–14725
14 *Zwermann O*, *Schulte DM*, *Reincke M*, *Beuschlein F*. ACTH 1-24 inhibits proliferation of adrenocortical tumors in vivo. Eur J Endocrinol 2005; 153: 435–444
15 *Kulle AE*, *Riepe FG*, *Melchior D*, *Hiort O*, *Holterhus PM*. A novel ultrapressure liquid chromatography tandem mass spectrometry method for the simultaneous determination of androstenedione, testosterone, and dihydrotestosterone in pediatric blood samples: age- and sex-specific reference data. J Clin Endocrinol Metab 2010; 95: 2399–2409
16 *Manolopoulou J*, *Mulatero P*, *Maser-Gluth C*, *Rossignol P*, *Spyroglou A*, *Vakrilova Y*, *Petersenn S*, *Zwermann O*, *Plouin PF*, *Reincke M*, *Bidlingmaier M*. Saliva as a medium for aldosterone measurement in repeated sampling studies. Steroids 2009; 74: 853–858
17 *Wang T*, *Rowland JG*, *Parmar J*, *Nesterova M*, *Seki T*, *Rainey WE*. Comparison of aldosterone production among human adrenocortical cell lines. Horm Metab Res 2012; 44: 245–250
18 *Matthews EK*, *Saffran M*. Ionic dependence of adrenal steroidogenesis and ACTH-induced changes in the membrane potential of adrenocortical cells. J Physiol 1973; 234: 43–64
19 *Saruta T*, *Kondo K*, *Saito I*, *Misumi J*, *Nakamura R*, *Kidokoro S*, *Matsuki S*. Effects of low salt plus upright posture, angiotensin II, ACTH, and potassium upon plasma renin activity, aldosterone, and cortisol (author's transl). Nihon Naibunpi Gakkai Zasshi 1975; 51: 740–744
20 *Boyd JE*, *Palmore WP*, *Mulrow PJ*. Role of potassium in the control of aldosterone secretion in the rat. Endocrinology 1971; 88: 556–565