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Mechanistic profiling of the cAMP-dependent steroidogenic pathway in the H295R endocrine disrupter screening system: New endpoints for toxicity testing

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ABSTRACT

The need for implementation of effects on steroid synthesis and hormone processing in screening batteries of endocrine disruptive compounds is widely acknowledged. In this perspective, hormone profiling in the H295R adrenocortical cell system is extensively examined and recently OECD validated (TG 456) as a replacement of the minced testis assay. To further elucidate the complete mechanisms and endocrine responsiveness of this cell system, microarray-based gene expression profiling of the cAMP response pathway, one of the major pathways in steroidogenesis regulation, was examined in H295R cells.

Next to the steroid synthesis pathway, a broader lipid metabolic pathway, including cholesterol uptake/biosynthesis, hormone metabolization and many hormone and nuclear receptors, are sensitive towards cAMP stimulation in this cell system. Moreover, these pathways were clearly dose and time responsive, indicating early regulation (10 h) of cholesterol uptake and mobilization genes and later expression (24–48 h) of cholesterol biosynthesis and steroid synthesis. Transcription network analysis suggested several important transcription factors that could be involved in regulation of the steroid hormone pathway, of which HNF4α, a broader lipid metabolism related transcription factor, might indicate some new transcription regulation patterns in this cell line.

Overall we can conclude that the time dependent gene expression patterns of the strongly coordinated cholesterol supply and steroidogenesis pathways in the H295R cell system seem to reflect well the *in vivo* ACTH/cAMP signalling cascade in adrenal cells. Moreover, the completeness of the steroidogenic related pathways in terms of gene expression sensitivity, indicates the H295R cell line as a promising cell line in omics-based endocrine disruption screening.

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1. Introduction

Since the development of the parent H295 cell line by Gazdar et al. (1990), this cell line and its derived adherent H295R cell line have been extensively studied as model system for adrenocortical function and adrenal toxicity (Bird et al., 1998; Harvey and Everett, 2003; Rainey et al., 1993; Staels et al., 1993). Due to its pluripotent nature, this cell system is capable of expressing the complete steroidogenesis pathway, including secretion of adrenal aldosterone, cortisol and dehydroepiandrosterone (DHEA) but also the gonad hormones testosterone and estradiol (Rainey et al., 1994; Staels et al., 1993). Moreover, the regulation of steroidogenesis and hormone secretion patterns reflect that of freshly isolated adrenal cells (Rainey et al., 2004). Nowadays, the H295R cell system is also a well documented screening system for endocrine disruptive compounds (EDCs) and H295R hormone profiling is recently OECD validated (OECD TG 456, July 2011) to replace the rodent based testis and ovary explant assays (Gracia et al., 2006; Hecker et al., 2006, 2007; Sanderson, 2006; Ullerås et al., 2008).

Although a lot of research has been performed in unravelling the mechanisms underlying the steroidogenic pathway in H295R cells, comprehensive gene expression profiling of this cell line with microarray techniques has not been extensively examined. By means of the recent transcriptomics techniques, not only key pathways, such as steroidogenesis, but also the interaction with other underlying pathways, metabolic pathways, nuclear hormone receptors and transporter molecules can be evaluated on a gene expression level (Moggs, 2005; Nikolsky et al., 2005). This pathway integrative approach, leading to a more systems biology-based understanding of toxic events is recently considered as the way forward in future toxicity testing (NRC, 2007). Nuclear hormone

Abbreviations: ACTH, adrenocorticotropic hormone; Ang II, angiotensin II; CREB, cAMP responsive element binding protein; DHEA, dehydroepiandrosterone; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDC(s), endocrine disrupting compound(s); GO, gene ontology; HNF4α, hepatocyte nuclear receptor factor 4 α; LDLR, low density lipoprotein receptor; Log_2 FC, Log_2 fold change; MIAME, minimal information about a microarray experiment; PKA, protein kinase A; SR-BI, scavenger receptor class B type 1 (SCARB1); VSN, variance stabilization normalization.

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receptors, for instance, are not only crucial transcription factors in expression regulation of many downstream responses, but are also important cellular targets of xenobiotics, such as EDCs (Janošek et al., 2006). In this way, transcriptomic experiments can provide fundamental information of the basic regulatory pathways in the H295R cells, and in addition further explore the value of this cell system for mode of action evaluation of compounds in an endocrine disruptive screening context. The angiotensin-signalling cascade, important in adrenal steroid hormone synthesis, has been evaluated in H295R cells using a microarray approach by Romero et al. (2004, 2007) and Nogueira et al. (2009). Angiotensin II (Ang II)-induction experiments showed that several steroid related genes and transcription factors remain sensitive in this cell system. Both groups evaluated the early transcription regulation in H295R cells by adrenal steroid secretion stimulators of the Ang II and ACTH/cAMP pathway, however focused predominantly on Ang II-induced transcription profiles (Nogueira et al., 2009; Romero et al., 2007). Though the ACTH/cAMP signalling cascade has a major role in regulation of steroid biosynthesis in adrenal cells, so far a broad and comprehensive pathway-driven analysis of the ACTH/cAMP stimulated H295R cells has not yet been performed.

In this study, a microarray based approach was used to further unravel the cAMP signalling cascade in the adrenocortical H295R cell line. For this purpose, a H295R dedicated microarray was developed based on transcription profiles of 5 model compounds (8Br-cAMP, atrazine, genistein, ketoconazole, lindane). This cell line specific custom array was then further used for ACTH/cAMP stimulated pathway analysis. By using the cAMP-analogue, 8Br-cAMP, internal cAMP mediated responses are reflected and dose and time dependency of the affected pathways can be assessed. cAMP analogues have often been used for mechanistic research in the H295 and H295R cell line and appear strong inducers of the steroidogenic pathway (Rainey et al., 1993; Samandari et al., 2007; Staels et al., 1993; Zhang et al., 2005). Next to the expected steroidogenic pathway, other pathways such as cholesterol uptake and biosynthesis, steroid metabolism and regulation of important nuclear and hormone receptors will be evaluated at the gene expression level to further explore the variety of endocrine endpoints and pathways present in this cell system.

2. Materials and methods

2.1. Routine cell culture and experimental set-up

The human adrenocortical carcinoma cell line, H295R, was obtained from the American Type Culture Collection (CRL-2128, ATCC, Manassas, VA, USA) and cultured as a monolayer in T-75 Nunc culture flasks in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12), supplemented with 1% ITS-G (6.25 μg/ml insulin, 6.25 μg/ml transferrin and 6.25 ng/ml selenium), antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin) and 2% UltroserSF serum. Cells were grown in mycoplasma free conditions at 37 °C in a 5% CO_2 atmosphere. Twice a week, at 70–80% confluence, cells were detached with 0.25% trypsin/EDTA and split 1/3. All cell culture reagents were obtained from Gibco (Invitrogen LT, Merelbeke, Belgium), except for the steroid-free UltroserSF serum (Biosepra, Pall Life Sciences, Cergy-Pontoise Cedex, France).

For experiments, H295R cells were seeded in T-25 Nunc culture flasks at a density of 2×10^6 cells/flask and cultured for 48 h (70% confluence). Then, medium was refreshed and 8Br-cAMP (Sigma), dissolved in ultrapure milliQ water, was added. Three concentrations (50 μM, 100 μM, 300 μM) and three time points (10 h, 24 h, 48 h) were evaluated. A 24 h exposure period was selected for the dose response analysis; a 300 μM concentration was selected for the time response analysis. Control cells were treated with an equal volume of milliQ water (solvent control). After exposure, RNA was extracted using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. RNA quantity and quality were evaluated using NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, DE) and denaturing gel electrophoresis.

2.2. Microarray analysis

Since the majority of human genes are not expressed in a single cell type, development of custom microarrays, based on gene expression profiles of a diverse set of compounds, allows for a dedicated analysis of cell line-specific genes. To select the broad endocrine related pathways present in this cell system, H295R cells were exposed to 5 model compounds with documented effects on adrenal endocrine function: 8Br-cAMP (300 μM, Staels et al., 1993), ketoconazole (10 μM, Johansson et al., 2002), genistein (10 μM, Ohno et al., 2002), atrazine (100 μM, Sanderson et al., 2000) and lindane (10 μM, Oskarsson et al., 2006). Ketoconazole, genistein and atrazine are also reference compounds for the validation of the H295R steroidogenesis assay (Hecker et al., 2008). Gene expression profiles were examined on a 'Whole Human Genome'-array (44K, 60-mer oligonucleotides, Kronick, 2004) (Agilent, Diegem, Belgium). A set of 1085 differentially expressed genes with an absolute expression value of the Log_2-transformed fold change or $|Log_2\ FC| > 1.0$, were selected (data not shown). This set was further completed with toxicologically relevant genes related to KEGG pathways and gene ontology classes important in endocrine processes, such as androgen/estrogen pathway (61), steroid hormone metabolism/receptors (37), cholesterol/lipid synthesis/metabolism (14), regulators of nuclear receptor transcription (54), retinoic acid metabolism (15), insulin pathway (44), xenobiotic metabolism (28) and adrenal/H295R specific genes derived from extensive literature search (51). In total, 1505 genes and 395 controls were *in situ* printed on the Agilent 8x1.9K format and further used as a dedicated platform for H295R microarray experiments (Gene Expression Omnibus (GEO) platform submission GPL9718, www.ncbi.nlm.nih.gov/geo).

2.3. Microarray design

RNA was amplified and labelled using the Low RNA Input Linear Amplification Kit (LRILAK, Agilent), according to the manufacturer's protocol. In brief, starting from 500 ng of total RNA, poly-A RNA was reversed transcribed using a poly dT-T7 primer. The resulting cDNA was immediately used for one round of amplification by T7 *in vitro* transcription reaction in the presence of Cyanine 3-CTP or Cyanine 5-CTP. The amplified and labelled RNA probes were purified separately with RNeasy purification columns (Qiagen). Amplification yield and incorporation efficiency of the probes were verified by measuring the RNA concentration at 280 nm, Cy3 incorporation at 550 nm and Cy5 incorporation at 650 nm using a Nanodrop spectrophotometer. Three hundred nanograms of Cy3 and Cy5 labelled cRNA were co-hybridized on a 1.9 K H295R custom array for 17 h at 60 °C in a continuous rotation hybridization oven. Arrays were washed according to manufacturer's protocols and scanned using a Genetix Personal 4100A scanner.

As hybridization design we preferred a common reference design in which we used a pool of blanks of different independent experiments as a reference sample on each array. Each condition was applied in triplicates (biological replicates), with intrinsic dye swaps. Statistical analysis of custom microarray data was performed with Bioconductor packages using a BioArray Software Environment database (BASE 1.2.17; http://www.islab.ua.ac.be/base) which is MIAME-compliant (Brazma et al., 2001). Variance stabilization normalization (VSN) was performed on all arrays followed by a ratio-based linear model (Limma) to detect significant differentially expressed genes of 8Br-cAMP exposed versus blank conditions (Smyth, 2005). Microarray design details, raw data files and normalized data are submitted to the NCBI GEO database (GSE32926, www.ncbi.nlm.nih.gov/geo). Genes were regarded as significant differentially expressed considering a *p*-value ≤ 0.05 and $|Log_2\ FC| \geq 0.8$. This cut-off value of 0.8 is based on the technical variation of labelling and hybridization process determined by independent self-hybridization experiments (mean ± 3SDs). A list of all genes that were differentially expressed in at least one of the conditions tested ($p \leq 0.05$; $|Log_2\ FC| \geq 0.8$), compared to the blank condition, is provided in Supplementary material (Table S1).

2.4. Pathway analysis

For biological interpretation of the differentially expressed genes, the software tool MetaCore^{TM} (http://www.genego.com; GeneGo, St. Joseph, MI) was used. To evaluate the enrichment of Gene Ontology (GO) categories, the 'statistically different GO processes' tool was applied. Secondly, significant enriched GO processes, indicated with $-Log(pvalue) \geq 1.3$, were selected and transcription regulation networks were build to derive potentially involved transcription factors. In this way, a list of transcription factors with their significance level (*p*-value) and *z*-score for the respective GO category is obtained. The *z*-score is MetaCore-specific and indicates the saturation of the transcription factor subnetwork with genes from the experiment; the *p*-value calculations are based on a hypergeometric distribution indicating a significance level of $p \leq 0.05$. The transcription factors and the respective differentially expressed genes can be visually shown in a subnetwork. This visualization tool predicts a network of genes that directly interacts with the transcription factor, indicating possible transcription regulation (Ekins et al., 2005). A 'direct interactions' algorithm was used, which means that only differentially expressed genes were included in the gene networks. Genes that were up- or down-regulated are visualized on the network as network nodes with red or blue circles, respectively.

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