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Improved androgen specificity of AR-EcoScreen by CRISPR based glucocorticoid receptor knockout

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ABSTRACT

The AR-EcoScreen is a widely used reporter assay for the detection of androgens and anti-androgens. Endogenous expression of glucocorticoid receptors and their affinity for the androgen responsive element that drives reporter expression, however, makes the reporter cells sensitive to interference by glucocorticoids and less specific for (anti-)androgens. To create a glucocorticoid insensitive derivative of the AR-EcoScreen, CRISPR/Cas9 genome editing was used to develop glucocorticoid receptor knockout mutants by targeting various sites in the glucocorticoid gene. Two mutant cell lines were further characterized and validated against the unmodified AR-EcoScreen with a set of 19 environmentally relevant chemicals and a series of environmental passive sampler extracts with (anti-)androgenic activity. Sequencing of the targeted sites revealed premature stop codons following frame-shift mutations, leading to an absence of functional glucocorticoid receptor expression. The introduced mutations rendered cell lines insensitive to glucocorticoid activation and caused no significant difference in the responsiveness towards (anti-)androgens, compared to the unmodified AR-EcoScreen cells, allowing the selective, GR-independent, determination of (anti-)androgenicity in environmental passive sampler extracts. The increase in selectivity for (anti-)androgens improves reliability of the AR-EcoScreen and will provide higher accuracy in determining (anti-)androgenic potential when applied in toxicity screening and environmental monitoring of both single compounds and mixtures.

1. Introduction

Androgenicity is an important endocrine disruption endpoint in toxicological and environmental screening. Brominated flame retardants (Hamers et al., 2006), pesticides (Kojima et al., 2003b), pharmaceuticals (Runnalls et al., 2010), food packaging constituents (Satoh et al., 2004) and various industrial chemicals (Araki et al., 2005a, 2005b) have previously been identified as (anti-)androgens. Such (anti-)androgens are present in the environment as (persistent) pollutants, mainly due to the release of particles or leachate from consumer products (Wooten and Smith, 2013), runoff water from agricultural activity (Durhan et al., 2006) or urban or industrial wastewater (Luvizutto et al., 2015; Rodriguez-Mozaz et al., 2004). Exposure can result from contact with house dust (Meeker et al., 2009), consuming contaminated food or drink (Carreño et al., 2007) or via air (Allen et al., 2007). Xenobiotic (anti-)androgens can potentially disrupt signaling of endogenous androgens in both humans and wild-life. The ability to selectively detect (anti-)androgenic potential is essential for accurate characterization of the endocrine disruptive potency of individual compounds and complex mixtures as found in environmental samples.

The AR-EcoScreen is a cell-based reporter assay for the detection of (anti-)androgenic activity. The assay, developed in 2004 (Satoh et al., 2004), uses Chinese hamster ovary (CHO) cells, stably transfected with a human androgen receptor (hAR) and a 6× androgen response element (ARE) (5′-AGTACGnnnTGTTCT-3′) from the C3 gene (Kojima

Abbreviations: AR, androgen receptor; ARE, androgen responsive element; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; CHO, Chinese hamster ovary; DBD, DNA binding domain; DSB, double stranded breaks; FACS, fluorescent-activated cell sorting; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; HDR, homology directed repair; NHEJ, non-homologous end-joining; NR3C1, GR gene; NTD, N-terminal domain; PAM, Protospacer adjacent motif; LBD, ligand binding domain; PS, passive sampler; SD, speedisk; sgRNA, single guide RNA; SNP, single nucleotide polymorphism; SR, silicone rubber; TU, toxic units; WWTP, waste water treatment plant

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et al., 2003a) regulating expression of a luciferase reporter gene. Luciferase is expressed in a dose-dependent manner and its resulting activity can be measured quantitatively by a luminometer after a 24 h exposure period. In contrast to a number of other cell-based AR reporter assays, the CHO cells used in the AR-EcoScreen lack metabolizing capacity for steroid hormones that might otherwise eliminate androgenic potency of tested compounds (Satoh et al., 2004). Recently, an OECD guideline has been established for application of the AR-EcoScreen in the testing of chemicals (*Test No. 458*, 2016).

The AR luciferase reporter system shows high sensitivity towards androgens. However, luciferase can also be induced by glucocorticoid receptors (GR) activated by glucocorticoids (GC) like cortisol or the synthetic GC dexamethasone (Satoh et al., 2005). The GR, endogenously expressed in CHO cells from the NR3C1 gene (Lee et al., 1981), shares homology with the AR and cross-talk between the two nuclear hormone receptors is known (Yen et al., 1997). The DNA binding domain (DBD) is well conserved between the AR and GR (Claessens et al., 1996; Schoenmakers et al., 2000). As a result the DNA binding sites to which the activated receptors bind share similarities. Consequently, the GR has affinity for the ARE which causes a non-AR-specific reporter activation by GCs, which may lead to misclassification of compounds as being androgenic when luciferase is actually induced through activated GRs in the AR-EcoScreen. Likewise, the androgenic potency of environmental samples can be overestimated if GCs are present. By preventing the expression of functional GR proteins, through introduction of mutations on the GR gene in the CHO genome, responsiveness to GCs can be reduced to undetectable levels. Various genome editing methods have been developed for introducing mutations with the most recent being the CRISPR (clustered regularly interspaced short palindrome repeats)/Cas9 editing (Mali et al., 2013).

The CRISPR/Cas9 technique is a novel, scarless genome editing technique that can introduce mutations at a specific location without leaving remnants of the editing machinery. CRISPR genome editing was adapted from a prokaryotic immune system which defends against viruses by inducing double stranded breaks (DSBs) at specific sites on viral DNA (Barrangou et al., 2007). The principle behind this immune system is that a single-guide RNA (sgRNA), which is complementary to the viral DNA sequence, associates with Cas9 nuclease. The sgRNA is expressed from viral DNA sequences incorporated into the prokaryote's own genome following previous encounters with the virus. The sgRNA subsequently directs the Cas9 nuclease towards its complementary site on the viral DNA where Cas9 nuclease activity will create a DSB. Binding of Cas9 to DNA requires the presence of an NGG protospacer adjacent motif (PAM) sequence, which is located three nucleotides downstream of the position where the DSB will occur.

In mammalian cells, this system can be used to induce DSBs at very specific sites in order to induce mutations or allow insertion of DNA. The CRISPR/Cas9 machinery is expressed from plasmid DNA after transient transfection. Ligation of a target sequence into the plasmid guides the CRISPR/Cas9 complex to the specific location of the operator's choice to introduce a dsDNA break. This break can be repaired by either non-homologous end joining (NHEJ) or homology directed repair (HDR). HDR is used to knock-in genes or DNA sequences with 3′- and 5′-end sequences homologous to sequences found at either side of the break. NHEJ, however, is an error-prone DNA repair mechanism which can introduce random mutations during imprecise repair of the break site. Deletion or insertion mutations in exons can cause disruptive frame-shifts that lead to non-functional proteins or truncated/extended proteins with altered activity. In-frame mutations, like single nucleotide polymorphisms (SNPs), can lead to changes in the protein sequence and alter activity in a more subtle manner.

To develop a mutant AR-EcoScreen cell line with functional knockout of the GR (i.e. lacking GC responsiveness), the CRISPR/Cas9 genome editing technique was used to introduce either SNPs or knockout mutations in the NR3C1 (GR) gene. Five SNPs in the NR3C1 gene related to a human or in vitro GC resistant phenotype were selected at residues that shared homology between human and Chinese hamster. The mutations, which were targeted at different functional domains, were analyzed by sequencing the targeted site. Mutant clones were screened for GC responsiveness and selected mutant cell lines were validated against the unmodified AR-EcoScreen cells by comparing their response to (1) a selection of (anti-)androgens and glucocorticoid dexamethasone described in literature, (2) a mixture of anti-androgens, and (3) a collection of extracts from passive samplers deployed at river water and wastewater treatment plant (WWTP) effluent water. The responses were analyzed for significant differences between the cell lines.

The obtained functional GR knockout in the well-established AR-EcoScreen provides more selective determination of (anti-)androgenic potency of compounds and (environmental) mixtures while maintaining the sensitivity for (anti-)androgenic compounds of the original AR-EcoScreen. When applied in toxicological screening, compounds can be accurately classified for their androgenic potential, while the concurrent presence of GCs in (environmental) mixtures will not lead to an over- or underestimation of the androgenic potency.

2. Materials and methods

2.1. Materials

DMEM/F12 with glutamax medium, DMEM/F12, phenol-free, L-glutamine medium and fetal bovine serum were obtained from Gibco (Eggenstein, Germany). Penicillin/streptomycin, hygromycin, zeocin, ampicillin, ATP, co-enzyme A and DNA oligonucleotides were obtained from Sigma (Zwijndrecht, The Netherlands). Luciferin was obtained from Promega (Fitchburg, WI, USA). All restriction enzymes and Proteinase-K were obtained from New England Biolabs (Ipswich, MA, USA). All plasmid isolations were performed using a Plasmid DNA Miniprep kit (Invitrogen, Carlsbad, CA) and all PCR-clean ups were performed with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Lipofectamine 2000 was obtained from Life technologies (Waltham, MA, USA). (Anti-)androgens used in validation of the cell lines were obtained from various suppliers (see Supporting information Table S1) and were dissolved in DMSO (Acros, Geel, Belgium). The pSpCas9(BB)-2A-GFP (PX458) plasmid was a kind gift from Dr. Feng Zhang (Addgene plasmid # 48138).

2.2. CRISPR/Cas9 plasmid construction

Five different CRISPR/Cas9 plasmids were constructed by introducing sgRNA coding sequences to the pSpCas9(BB)-2A-GFP (PX458) plasmid (Ran et al., 2013). Target sequences were designed against the Chinese hamster genome (RefSeq Assembly ID GCF_000223135.1). The dsDNA coding the sgRNA was formed by annealing single stranded DNA sequences (see Supporting information Table S2) at equimolar concentrations in annealing buffer (10 mM TRIS (pH 7.5–8.0), 50 mM NaCl and 1 mM EDTA). The reaction was performed in a PCR Thermocycler with a linear temperature gradient from 95 °C to 4 °C over a period of 2.5 h. The resulting fragment with overhanging ends was ligated to the *Bbs*I restriction product of the PX458 plasmid in a 3:1 ratio. Clones of successfully transformed *E. coli* (DH5α) cells (Invitrogen, Carlsbad, CA, USA) carrying the targeting sequence containing PX458 plasmid were selected by ampicillin resistance and the correct insert was validated by PCR (see Supporting information Table S2).

2.3. Transfection and cell isolation

AR-EcoScreen cells were maintained as described by Satoh et al. (2004). Eighteen hours prior to transfection, 70–90% confluent cells were trypsinized, diluted in DMEM/F12 culture medium (with 10% fetal bovine serum and 1% penicillin/streptomycin) to 200,000 cells/mL and seeded in 200 µL aliquots on 24-well plates. In the morning,