Disruption of steroidogenesis: cell models for mechanistic investigations and as screening tools

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Abstract

In the modern world, humans are exposed during their whole life to a large number of synthetic chemicals. Some of these chemicals have the potential to disrupt endocrine functions and contribute to the development and/or progression of major diseases. Every year approximately 1000 novel chemicals, used in industrial production, agriculture, consumer products or as pharmaceuticals, are reaching the market, often with limited safety assessment regarding potential endocrine activities. Steroids are essential endocrine hormones, and the importance of the steroidogenesis pathway as a target for endocrine disrupting chemicals (EDCs) has been recognized by leading scientists and authorities. Cell lines have a prominent role in the initial stages of toxicity assessment, *i.e.* for mechanistic investigations and for the medium to high throughput analysis of chemicals for potential steroidogenesis disrupting activities. Nevertheless, the users have to be aware of the limitations of the existing cell models in order to apply them properly, and there is a great demand for improved cell-based testing systems and protocols. This review intends to provide an overview of the available cell lines for studying effects of chemicals on gonadal and adrenal steroidogenesis, their use and limitations, as well as the need for future improvements of cell-based testing systems and protocols.

1. Introduction

There is an increasing interest in the identification of chemicals that interfere with the endocrine system. The Endocrine Society defines an endocrine disrupting chemical (EDC) as an “exogenous chemical or mixture of chemicals that can interfere with any aspect of hormone action” [1]. It is important, in our opinion, to distinguish between transient influences followed by adaptation and disruption of endocrine functions leading to adverse health effects. This is considered by the European Union (EU) that defines an EDC as an “exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function” [2, 3]. The protection of human health and the environment is of high priority for major organizations and regulatory authorities. Regarding the large number of chemicals that need to be tested for potential endocrine disrupting effects, in programs such as REACH (Registration, Evaluation, Authorization and Restriction of Chemicals, http://ec.europa.eu/growth/sectors/chemicals/reach/index_en.htm), the EPA’s EDSP (Environmental Protection Agency’s Endocrine Disruptor Screening Program, <http://www.epa.gov/endo/>) or the FDA (U.S. Food and Drug Administration) guidelines for drug development (<http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/>), it is important to first evaluate the most relevant chemicals, *i.e.* chemicals with evidence of causing adverse effects and for which relevant exposure is known or can be expected. Besides chemicals used in industrial production, agriculture, electronics, and consumer products, the safety of pharmaceuticals and food constituents need to be assessed. Thus, a huge number of chemicals need to be tested for a wide range of possible adverse effects, including such caused by a disruption of steroid hormone action.

Amongst other endocrine hormones, steroids play crucial roles in the regulation of nearly all physiological processes. Several reports provided evidence for an association of disturbances of steroid hormone action caused by exogenous chemicals with developmental defects [4], infertility

and reproductive dysfunctions [5, 6], testicular, prostate and breast cancer [7-9], obesity and diabetes [10-12], immune disorders and neurobehavioral and learning dysfunctions [13, 14]. Further research is needed to identify other chemicals disrupting steroid hormone action, to evaluate the mechanisms by which such chemicals disrupt steroid hormone action, and to assess the critical exposure windows and concentrations that are relevant regarding development and progression of diseases.

For the initial endocrine safety testing of a large number of chemicals, improved *in silico* and *in vitro* assays are needed to facilitate the prioritization of chemicals for further toxicological investigations. Cell-based steroidogenesis assays represent a suitable starting point to assess disturbances of steroid biosynthesis, induced by direct inhibition of steroidogenic enzymes or by affecting their expression. The advantage of the cell-based models is that several enzymes and receptors required for the synthesis of steroids, as well as the signaling pathways regulating their activities, may be covered in a single assay. In addition to the identification of potentially hazardous chemicals, the cell-based steroidogenesis assays allow first mechanistic insights into the mode-of-action of EDCs; however, the users need to be aware of the limitations of the system applied in order to avoid drawing inappropriate conclusions and over-interpretation of results. This review focuses on the cell lines that are available to study steroidogenesis, their advantages and limitations, and the existing gaps for early safety testing of chemicals disrupting steroid homeostasis.

2. Steroidogenesis

Primary organs that are producing steroids from their precursor cholesterol include the adrenal glands and the gonads, with testes in males and ovaries in females. Additionally, in females the placenta produces high amounts of progesterone during pregnancy [15]. Other organs expressing steroidogenic enzymes include the brain [16, 17], the intestinal tract [18] and the skin [19].

However, the steroids produced in these tissues seem to be restricted to affect local rather than systemic levels, and the relevance of steroidogenesis in these tissues will not be discussed.

The major steroidogenic organs synthesize steroids *de novo* from cholesterol that is either produced directly by the cell from acetyl-CoA or taken up from dietary cholesterol bound to low-density lipoproteins (LDL) in the circulation (for a comprehensive review see [20]). Cholesterol can be esterified, stored in lipid droplets and be released by the activity of hormone-sensitive lipase. The rate-limiting step in adrenal and gonadal steroidogenesis is the uptake of cholesterol into the mitochondria. The steroidogenic acute regulatory protein (StAR) facilitates the transfer of cholesterol from the outer to the inner mitochondrial membrane, and its conversion to pregnenolone by the cytochrome P450 side chain cleavage enzyme (P450_{scc}, CYP11A1) in cooperation with adrenodoxin reductase that functions as an electron transfer protein of CYP11A1 [20]. Dependent on the organ, pregnenolone is then further converted by tissue- and cell type-specific enzymes into androgens, estrogens, glucocorticoids or mineralocorticoids.

The cortex of the adult human adrenals is responsible for the production of mineralocorticoids in the *zona glomerulosa*, glucocorticoids in the *zona fasciculata* and precursors of active androgens in the *zona reticularis* (Figure 1). The *zona reticularis* expresses high levels of CYP17A1 [21], which possesses 17 α -hydroxylase activity for the formation of 17 α -hydroxypregnenolone and 17,20-lyase activity for the subsequent formation of dehydroepiandrosterone (DHEA). The high expression of cytochrome b5, in the presence of cytochrome P450 reductase, allows efficient 17,20-lyase activity that is needed for the production of DHEA [20, 22]. Additionally, the *zona reticularis* expresses high levels of the steroid sulfotransferase SULT2A1 that is responsible for the formation of sulfated DHEA (DHEAS) [23], the most abundant steroid in human blood [24]. Importantly, 3 β -hydroxysteroid dehydrogenase 2 (3 β -HSD2) is expressed in the *zona reticularis* at very low levels, thus leading to only low amounts of Δ 4-androstene-3,17-dione (androstenedione) production [20]. Since 17 β -hydroxysteroid dehydrogenase type 3 (17 β -HSD3) is absent and 17 β -HSD5 (AKR1C3)

expressed at very low levels in the *zona reticularis* [21, 25], only very low levels of testosterone are produced by the adrenals [26, 27]. CYP21A2 is absent in the *zona reticularis*, thus no mineralocorticoids and glucocorticoids are formed in this layer [20].

In the *zona fasciculata* pregnenolone is converted to 17 α -hydroxypregnenolone by CYP17A1, and pregnenolone and 17 α -hydroxypregnenolone are converted to progesterone and 17 α -hydroxyprogesterone, respectively, by 3 β -HSD2. Most of the progesterone formed is also 17 α -hydroxylated. Further metabolism by CYP21A2 leads to 11-deoxycortisol and lower amounts of 11-deoxycorticosterone that are further converted by CYP11B1, which is specifically expressed in this zone, into cortisol and corticosterone, respectively [20, 28]. Cytochrome b5 is expressed at background levels in the *zona fasciculata* [21], resulting in very low CYP17A1 17,20-lyase activity and thus low amounts of DHEA formation [20]. The *zona fasciculata* expresses the melanocortin-2-receptor and is therefore responsive to adrenocorticotrophic hormone (ACTH) [20, 28].

The *zona glomerulosa* does not express CYP17A1, and pregnenolone is converted to progesterone by 3 β -HSD2 and further to 11-deoxycorticosterone by CYP21A2, and to corticosterone and aldosterone by CYP11B2. In the adrenals, CYP11B2 expression is restricted to the *zona glomerulosa* and the production of aldosterone is regulated by angiotensin II receptors [20].

The human fetal adrenals produce high amounts of DHEAS, which is abolished soon after birth where the adrenals mainly consist of a *zona glomerulosa* and a *zona fasciculata* and thus produce mineralocorticoids and glucocorticoids [29]. The *zona reticularis* actively starts producing adrenal androgens at adrenarche at around 6-8 years of age and reaching peak levels in the third decade of life, before declining gradually [30, 31].

In the testis, steroidogenesis is restricted to the Leydig cells. They convert pregnenolone by CYP17A1 into 17 α -hydroxypregnenolone and further to DHEA (Figure 2). Because of the high expression of 3 β -HSD2 and 17 β -HSD3 but the absence of SULT2A1, DHEA is not sulfated and therefore further converted to androstenediol, or by a lower extent to androstenedione, and

subsequently to testosterone in Leydig cells [20, 32]. Furthermore, CYP21A2, CYP11B1 and CYP11B2 are absent, thus no gluco- and mineralocorticoids are produced. Testicular steroidogenesis is under the control of human chorionic gonadotropin (hCG) and luteinizing hormone (LH).

In the ovaries, steroidogenesis is mediated by theca and granulosa cells. The granulosa cells are located in the avascular cellular compartment surrounding the oocyte, and the theca cells reside in the ovarian stroma; these cellular compartments are separated by the basal membrane. The theca and granulosa cells both express StAR and CYP11A1 [33]. Because granulosa cells do not express CYP17A1 [34], they can synthesize pregnenolone from cholesterol and they convert it further to progesterone in the *corpus luteum* (Figure 3) [20]. However, for the production of estrogens, pregnenolone needs to be secreted from the granulosa cells and taken up by the theca cells, or it is produced directly by the theca cells, to form DHEA. The theca cells express 3 β -HSD2 and convert DHEA into androstenedione [35]. Androstenedione is then delivered back to the granulosa cells for the aromatase-dependent production of estrogens [34]. Granulosa cells also express 17 β -HSD1, which is needed for the conversion of estrone into estradiol. There are cycle-dependent changes in ovarian steroidogenesis: in the luteal phase the luteinized granulosa cells are supplied with sufficient cholesterol, due to enhanced vascularization of the previously avascular compartment, and elevated LH levels enhance the expression of CYP11A1 and 3 β -HSD2, resulting in the synthesis of high amounts of progesterone [33]. In the follicular phase, follicle stimulating hormone (FSH) enhances the expression of aromatase and 17 β -HSD1 for the production of increased amounts of estradiol from theca cell-derived androstenedione. LH also activates LH receptors on theca cells to induce CYP17A1 expression, thereby enhancing androgen precursors for estrogen production by granulosa cells. Thus, a tight control of the cooperation of granulosa and theca cell function is essential for the appropriate regulation of estradiol synthesis.

3. Leydig cell models to investigate steroidogenesis

Three independent large epidemiological studies revealed a decline in male serum testosterone levels in the general population [36-38]. Obesity was identified as a contributing factor for some but not all observations [39]. Increasing evidence suggests that exposures to EDCs contribute to male reproductive diseases and that prevention of EDC exposures may reduce the burden of male reproductive health problems [40]. As an example, cryptorchidism is a typical impairment following exposure to antiandrogenic chemicals during male sexual development [41]. Evidence was provided that levels of polybrominated diphenyl ethers (PBDEs) in human breast milk are associated with congenital cryptorchidism, although a contribution of other environmental factors cannot be excluded [42]. PDBEs have been shown in *in vitro* studies to directly antagonize AR activity (IC₅₀ of approximately 5 μ M for the mixture DE-71 in an MDA-kb2 cell model expressing an AR-dependent luciferase reporter), and PDBEs additively and/or synergistically acted with other AR antagonistic compounds [43]. In *in vivo* studies PDBEs were shown to cause diminished growth of androgen-dependent tissues and a delay in puberty in the male rat following a pubertal exposure to 60 and 120 mg/kg/day of the DE-71 mixture [44]. Although such high exposure levels are unlikely to be reached in humans, the fact that humans are exposed to a multitude of compounds that may exert additive or synergistic effects emphasizes the need for the screening of chemicals for potential antiandrogenic effects. Because of the high public demand to reduce animal testing [45], improved cell-based assays are needed that allow the identification of chemicals disrupting the biosynthesis of steroids and the gaining of insights into the mode-of-action of such chemicals.

There are several immortalized rodent Leydig cell lines available for studying the regulation of steroidogenesis and to assess the impact of substances on steroid hormone production. However, to our knowledge, no human Leydig cell model is currently available that can be used for screening purposes and for toxicological studies. The available rodent Leydig cell lines have been derived from spontaneous tumors, upon experimental induction, or by *in vitro* immortalization. All of these

cell systems have their limitations, as some of the steroidogenic enzymes and regulatory pathways are expressed at very low levels, if at all, likely as a result of the selection of cell clones that rapidly proliferate and because of dedifferentiation and loss of initial phenotype during prolonged cultivation.

Probably the most widely used immortalized Leydig cell line is MA-10 [46]. MA-10, the related MA-12, and the frequently used mLTC-1 are all derived from a C57Bl/6 Leydig cell tumor (designated M5480) [47]. These cell lines express LH receptors, and incubation with LH/hCG induces cAMP-dependent steroidogenesis. MA-10 cells also express mouse epidermal growth factor receptor (EGFR), which suppresses the hCG-induced steroidogenesis [48]. In both, MA-10 and mLTC-1, progesterone was the main steroid being produced, in line with the observation that the original tumor M5480 secreted progesterone but only very low amounts of testosterone, and the two cell lines displayed similar functional characteristics [47]. These observations suggest that 3β -hsd1 activity is dominant over Cyp17a1; therefore, pregnenolone is mainly converted into progesterone, with only minor amounts being further converted into androstenedione and testosterone. For these reasons, we propose that, using progesterone as a read-out, MA-10 and mLTC-1 cells can serve as suitable models to detect chemicals that affect the induction of steroidogenesis, the cAMP- and PKA-dependent signaling, or that directly inhibit the activities of StAR, Cyp11a1 or 3β -hsd1. Due to the generation of only low amounts of testosterone by these cell lines, it is difficult to quantitatively assess the effect of chemicals that disrupt Cyp17a1 or 17β -hsd3 activities. Nevertheless, the mRNA expression of key steroidogenic enzymes, including StAR, Cyp11a1 and 3β -hsd1, and to a lesser extent that of Cyp17a1 and 17β -hsd3, has been detected in MA-10 and mLTC-1 cells, and has been found to be affected upon exposure to chemical modulators [49-51].

MA-10 cells are applied by many investigators to study the impact of EDCs on the regulation of steroidogenesis; only a few will be mentioned in this review as examples. Recent studies on effects

of bisphenol A (BPA) and its analogues on steroidogenesis in MA-10 cells suggested that tetrabromobisphenol A (TBBPA) concentration-dependently increased testosterone production at concentrations of 3 μ M and higher, while bisphenol S (BPS) had no effect and BPA and bisphenol F (BPF) induced testosterone secretion only at very high concentrations (30 and 100 μ M, respectively) [49, 50]. Following incubation of the cells for 48 h in the presence of 10 μ M of TBBPA, BPF or BPS an increased production of progesterone, and in the case of TBBPA of 17 α -hydroxyprogesterone and androstenedione, was measured. Furthermore, incubation of cells with 10 μ M of BPF, BPS or TBBPA led to an elevated expression of 5 α -reductase 1, indicating an increased production of 5 α -androstenedione and dihydrotestosterone. Importantly, the authors provided evidence that the TBBPA-mediated increase in testosterone production may be due to an inhibition of the efflux of androgen precursors required for testosterone synthesis by the multidrug resistance proteins MRP1 and MRP4 [50]. These observations emphasize the need to include steroid transporters in the assessment of EDCs and provide a further explanation for the low amount of testosterone produced by MA-10 cells under basal conditions.

MA-10 cells were also used to study direct effects of monophthalates on testicular steroidogenesis [52]. The LH-induced production of cAMP and progesterone was significantly inhibited in MA-10 cells treated with 30 μ M of mono(2-ethylhexyl)phthalate (MEHP), whereas testosterone production was significantly lowered upon incubation of the cells with 1 μ M MEHP, 3 μ M monobutylphthalate (MBP), 10 μ M mono-n-octylphthalate (MnOP) or 3 μ M monobenzylphthalate (MBzP) but not in the presence of monoethylphthalate (MEP) or monomethylphthalate (MMP) [52]. At the high concentration of 100 μ M MEHP the mRNA expression levels of StAR, Cyp11A1 and Cyp17A1 were down regulated. Interestingly, in mLTC-1 cells (not induced by LH) the phthalates di-n-butyl phthalate (DBP), MBP, di(2-ethylhexyl)phthalate (DEHP) and MEHP seemed to increase testosterone production at low concentrations of 0.001 to 0.1 μ M but inhibited at high concentrations of 100 μ M. Interestingly, the mRNA expression levels of Cyp11A1, Cyp17 and 3 β -

HSD1 were decreased even at concentrations as low as 0.1 μ M [53, 54]. Also, the impact of the major metabolites of MEHP and DEHP on the expression of steroidogenic genes has been analyzed, suggesting that the metabolite 2-ethylhexanal might inhibit Leydig cell testosterone formation, although this effect was only observed at high concentrations of 100 μ M [55]. The human relevance of such high concentrations are questionable and further research using lower concentrations are needed. Also, it should be noted that progesterone and testosterone were measured by ELISA in this study. Furthermore, a possible effect of phthalates on the efflux of androgen precursors or on cholesterol flux in MA-10 or in mLTC-1 cells has not been investigated.

Other studies focused on initial steps of steroidogenesis. Incubation of MA-10 and mLTC-1 cells with an organochlorine compound mixture resulted in a decreased expression of StAR, CYP11A1 and the adrenodoxin reductase, enzymes crucial for the production of pregnenolone from cholesterol [56]. The cAMP- and hCG-induced production of progesterone tended to be lower at 1 μ g/ml and was significantly lower at 10 μ g/ml of organochlorine mixture. Also, the UV-filter chemical 2,2',4,4'-tetrahydroxybenzophenone (BP2), applied at 30 μ M, was found to alter the expression of StAR, 3 β -hsd and Cyp17a1 and had opposite effects on Leydig cell steroidogenesis than thyroid hormone signaling [57]. Moreover, MA-10 cells were employed to test pesticide formulations that are widely used in agriculture. The pesticide mixture Roundup, a broad-spectrum systemic herbicide containing glyphosate (N-phosphonomethyl-glycine), inhibited the cAMP analogue-induced progesterone production at subcytotoxic concentrations of 25 μ g/ml by decreasing the expression of the StAR protein [58]. Furthermore, the benzodiazepine midazolam was found to stimulate progesterone and testosterone production, measured by radio-immunoassay, at the subcytotoxic concentrations of 30 and 150 μ M in primary mouse Leydig cells and in MA-10 cells by an induction of the expression of the peripheral-type benzodiazepine receptor and StAR, probably via a pathway involving protein kinase A (PKA) and protein kinase C (PKC) [59]. Murine mLTC-1 Leydig cells were used to further investigate the reproductive toxicity of perfluorooctanic

acid (PFOA) that was observed in mice treated by gavage [60]. Exposure of mLTC-1 cells with 100 μ M PFOA decreased Cyp11a1 mRNA and protein expression and at 300 μ M PFOA progesterone production was significantly decreased. Also, StAR protein seemed to be decreased, likely as a result of oxidative stress caused by PFOA exposure [60, 61]. The mycotoxin zearalenone at concentrations of 5 μ M was suggested to affect steroidogenesis in mLTC-1 cells by disrupting lipid metabolism and inducing endoplasmic reticulum stress-mediated apoptosis [62, 63]. Furthermore, the polybrominated diphenyl ether BDE-47 at a concentration of 1 μ M was found to decrease progesterone production via cAMP-PKA-dependent downregulation of CYP11a1 [64]. Thus, a multitude of chemicals were shown to affect initial steps of steroidogenesis by different mechanisms. It should be noted that in order to judge on the human relevance of the findings described above further investigations are required, as in most of these *in vitro* studies the concentrations applied were either much higher than concentrations measured in humans or data on such concentrations are not yet available.

Another mouse Leydig cell line, designated TTE1, was derived from transgenic mice, upon immortalization using a temperature-sensitive simian virus 40 (SV40) large T-antigen [65]. These cells can be grown at 33°C and differentiated at a non-permissive temperature of 39°C. The cell model was used to study genes involved in Leydig cell differentiation characteristics, and the expression of the terminal enzyme of testosterone synthesis, 17 β -hsd3, was confirmed at least on the mRNA level [66]. TTE1 cells were only used in very few studies, so for example to investigate the impact of diethylstilbestrol on the expression of steroidogenic genes [67, 68]. Diethylstilbestrol at concentrations of 50 nM or higher decreased Cyp11a1 expression and, furthermore, diminished apoptotic cell death pathways and DNA repair capability, suggesting an increased carcinogenic potential of the exposed cells.

Mice transgenic for the SV40 T-antigen under the control of the inhibin- α promoter were used to establish the steroidogenic Leydig cell line BLT-1 [69]. BLT-1 cells responded well to LH and hCG

by increased cAMP levels and enhanced production of progesterone. As observed for MA-10 and mLTC-1, BLT-1 cells are only producing very low amounts of testosterone, as measured by enzyme immunoassay. Regarding investigations into EDCs, the BLT-1 derived cell clone BLTK1 was used to study several environmental toxicants [70]. BLTK1 cells seem to express all key steroidogenic proteins such as StAR, Cyp11a1, Cyp17a1, 3 β -hsd1, 17 β -hsd3 and 5 α -reductase 1. These cells were shown to respond to hCG and forskolin, which resulted in enhanced cAMP production and expression of steroidogenic genes. An elevated production of progesterone and testosterone was indicated by enzyme immuno assays measurements. The antifungals prochloraz (30 μ M) and triclosan (30 μ M) seemed to decrease the hCG-induced testosterone production, whereas MEHP (300 μ M) and atrazine (at concentrations of 30 μ M or higher) promoted basal testosterone formation but inhibited the hCG-dependent testosterone synthesis. Furthermore, the triazine herbicides atrazine, simazine, propazine and terbuthylazine were reported to enhance progesterone and testosterone production in BLTK1 cells at high concentrations (with significant effects observed at 100 μ M or higher), effects explained by the altered expression of steroidogenic genes [71]. However, in these studies very high concentrations of questionable human relevance were used.

The non-tumor mouse epithelial Leydig cell line TM3 was originally derived from the testis of an immature Balb/c mouse [72]. TM3 cells respond to LH, but not FSH, with an increased cAMP production [73]. Evidence was provided that LH and EGF are involved in the regulation of cyclin-dependent kinase 5 (Cdk5) expression and activity, and that this signaling pathway modulates hormonally stimulated testicular steroidogenesis [74]. Furthermore, LRH-1 was found to regulate Cyp19a1 expression via promoter II in multiple testis cell types [75]. Additionally, a role for hypoxia-inducible factor-1 α by mediating hypoxia-dependent changes on steroidogenesis by regulating the transcriptional expression of 3 β -hsd1 was reported [76]. The C1q and tumor necrosis factor-related protein (CTRP3) was found to induce testosterone production by increasing cAMP

and phosphorylation of cAMP response element-binding protein (CREB) by PKA and subsequently enhancing the expression of StAR and Cyp11a1 [77]. TM3 cells express V1 type arginine vasopressin receptors that seem to act independent of the adenylate cyclase system [78] and calcitonin receptors, which mediate calcium influx and stimulate cAMP formation and testosterone secretion [79]. They also express inhibin/activin β -A subunits and activin receptors II and IIB [80]. A role for the Src tyrosine kinase in the regulation of phosphodiesterase PDE4 activity and the production of cAMP was reported [81]. TM3 cells mainly produce progesterone, and only minor amounts of testosterone (own observations), suggesting that they can serve as a model to study early steps of the regulation of steroidogenesis and direct inhibition of the activities of StAR, Cyp11a1 and 3β -hsd1.

The TM3 mouse Leydig cell line is frequently used to study the impact of environmental pollutants on testicular toxicity and on alterations in steroidogenesis. A study on gap junctional intercellular communication in TM3 cells showed inhibitory effects by estradiol and diethylstilbestrol via an estrogen receptor (ER)-dependent mechanism [82]. Interestingly, similar effects were observed at 10 pM and 10 μ M concentrations for both diethylstilbestrol and estradiol, and these effects were fully reversed in the presence of an ER antagonist. Incubation of TM3 cells with diesel exhaust particles led to a reduced expression of ER α (at 0.1 μ g/ml particle concentration) and an induction of Cyp11a1 (at 1 μ g/ml) [83]. A transcriptomics analysis was performed on the impact of 1 and 5 mM methoxyacetic acid, the active metabolite of the industrial chemical ethylene glycol monomethyl ether, on TM3 cells revealing alterations in steroidogenesis, inflammation reactions and metabolic functions [84]. It needs to be noted that these concentrations are very high, and thus the human relevance is questionable. Two recent studies provided evidence for a protective role of the activation of the transcription factor Nrf2 towards the toxicity caused by the phthalate DBP, indicating the importance of the antioxidant defence system to protect Leydig cells from toxic chemicals [85, 86]. Studies on chemicals affecting testosterone production in TM3 cells are rather

uncertain, since these cells produce very low amounts. Furthermore, results on changes in testosterone production obtained using ELISA kits should be confirmed using quantification by GC-MS or LC-MS.

I-10 clonal Leydig cells were originally obtained from a spontaneous mouse testicular tumor [87]. Like other mouse Leydig cell lines described above, I-10 were reported to mainly produce progesterone, which was stimulated by cAMP [88], although not as efficient as in MA-10 and mLTC-1 cells. I-10 Leydig cancer cells were scarcely used for the assessment of EDCs. A study on PCBs showed enhanced CYP19a1 expression in mouse I-10 Leydig and human H295R adrenal cells following incubation for 24 h with the high concentration of 10 μ M PCB126 [89]. Interestingly, this effect was blunted in hCG and cAMP analogue-treated cells, and the authors proposed a role for AhR in these effects. Similarly, the mouse Leydig tumor cell line K28 was applied only in a few studies, including the investigation of the time-dependent induction of StAR mRNA expression and progesterone production by 9-cis and all-trans of retinoic acid (increases at concentrations greater than 10 nM) [90], the impact of LH on the expression of Nur77 (NR4A1) [91], the effect of 1 μ M BPA under serum-free conditions for 24 h on the induction of Nur77 expression and the production of progesterone [92], as well as the stimulating effects of 30 μ M cadmium chloride on CREB protein phosphorylation and StAR expression [93].

The rat Leydig tumor interstitial cell line R2C displays high StAR expression and produces high amounts of progesterone [94-96]. The high expression of StAR, Cyp11a1 and 3 β -hsd1 was confirmed by RT-PCR and Western blot [97] and the production of progesterone was detected by ELISA and RIA measurements [98, 99]. The expression of Cyp17a1 and the production of testosterone have been reported [97-99]; however, a general problem with antibody-based quantification of proteins and steroids remains the often limited specificity of the antibodies used [100]. Thus, testosterone production by R2C cells should be confirmed using quantification by GC-MS or LC-MS. An interesting property of R2C cells is that they are insensitive to cAMP regulation

and do not require trophic stimulation to produce progesterone, which might be explained by a constitutively activated downstream signaling pathway [95, 101]. Because of the constitutive production of progesterone, these cells are suitable to test chemicals that directly inhibit the activity of StAR, Cyp11a1 or 3 β -hsd1. On the other side, this cell line is not suitable to study chemicals affecting the induction of steroidogenesis due to the lack of sensitivity of the involved signaling pathways.

R2C cells were used in a comparative study with MA-10 cells to assess effects of various phthalates on testosterone production measured by ELISA [98]. The phthalates MBP and MEHP significantly inhibited testosterone synthesis at concentrations of 1 and 3 μ M, with IC₅₀ values of 3 and 6 μ M respectively. Phthalates with shorter alkyl side chains were found to be less active or inactive. Interestingly, R2C cells express substantial levels of Cyp19a1, and this cell line has been applied to characterize aromatase inhibitors [102, 103]. A study on effects of BPA (concentrations of 0.1 to 10 nM) on steroidogenesis suggested an up regulation of Cyp19a1 protein expression and activity, whereas testosterone synthesis was decreased [104]. Testosterone was measured by ELISA. Using R2C cells the anabolic androgenic steroids nandrolone and stanozolol (at 1 μ M concentration) were shown to increase Cyp19a1 expression as well as estradiol production [105]. Further, these authors provided evidence for an additive effect of androgens and IGF-1 on R2C cell proliferation and aromatase expression. In contradiction, a recent study showed that treatment of R2C cells with the androgen mibolerone up regulated the transcription factor DAX-1 and inhibited the expression and activity of Cyp19a1, in line with observations in old Fischer rats with spontaneous Leydig cell tumors where AR and DAX-1 were down regulated and Cyp19a1 was up regulated [106]. The reason for the discrepancies of the above studies remains unclear and requires further research.

A major limitation for mechanistic investigations into the regulation of steroidogenesis in Leydig cells and the assessment of the impact of potential EDCs is the fact that currently no human Leydig cell model is available. There are considerable species differences in the functions of Leydig cells.

For example, it has been shown that the expression level of LH receptors is an order of magnitude higher in rat compared with human Leydig cells, and that rat Leydig cells respond with hyperplasia to hCG, whereas human Leydig cells become hypertrophic [107-110]. Furthermore, rat Leydig cells express gonadotropin-releasing hormone, whereas mouse and human Leydig cells do not [111, 112].

Additionally, several studies demonstrated species-specific inhibition of testicular steroidogenesis by EDCs. Using organotypic primary culture systems, the phthalate MEHP at a concentration of 10 μ M was shown to decrease testosterone production in rat but not in human fetal testis explants [113, 114]. Further support for species-specific effects of phthalates was provided by studies where rat and human fetal testes were xenografted into a host mouse or rat [115, 116]. Treatment with di-n-butylphthalate (500 mg/kg per day for four days) inhibited steroidogenesis in animals with rat but not human xenografts. Also, diethylstilbestrol did not affect human fetal testicular steroidogenesis in the xenograft model [117] and in human fetal testis explants, in contrast to rat and mouse testis cultures [118, 119], a difference explained by the fact that ER α is expressed in rat and mouse but not in human fetal Leydig cells [117]. Moreover, the anti-diabetic drug metformin inhibited testosterone production at an order of magnitude higher concentrations in human compared with mouse testis explants [120]. In contrast, it was shown that BPA inhibited testosterone synthesis at 100 times lower concentrations in human compared with rat and mouse fetal testis explants [118]. These studies demonstrate important species-specific differences in the susceptibility of human, rat and mouse testes to xenobiotics and further emphasize the need to establish a human Leydig cell model for the investigations into the molecular mechanisms of steroidogenesis disruption.

4. Cell-based systems to study effects of EDCs on ovarian steroidogenesis

In the industrialized countries, there is an increasing incidence of reproductive disorders such as polycystic ovary syndrome (PCOS) [121], which is characterized by chronic anovulation and

hyperandrogenism and results in hirsutism, infertility and menstrual disturbances. As with male infertility, there is evidence for the contribution of EDCs from consumer products or environmental pollutants to the increasing incidence of female reproductive disorders (for a recent comprehensive review see [122]). Several EDCs and potential EDCs have been detected in human samples, including follicular fluid, from the general population [123-127]. Exposure to EDCs likely contributes to sub-fecundity, ovarian failure and infertility, and affects reproductive behavior. Exposure to EDCs may contribute to ovulatory dysfunction by decreasing estradiol biosynthesis in granulosa cells or as abortifacients by disrupting progesterone production in luteal cells [128].

In vivo testing of EDCs for reproductive toxicity is mostly conducted in rodents, with fertility as a primary endpoint [129]. Alteration in serum steroid levels may indicate an adverse effect but it may also represent an adaptive response, thus often not providing sufficient information on the toxicity of a given chemical. Also, changes in circulating steroid levels may be due to a direct effect on steroidogenesis or an altered feedback regulatory system. *Ex vivo* tissue samples, *e.g.* whole ovaries or isolated individual follicles, can be used to study follicular development, ovulation and steroidogenesis, and assays using such samples can provide results on multiple fertility-related endpoints [130]. In order to allow high throughput analyses and to gain mechanistic insight into the action of EDCs, cultured cells are advantageous. Isolated primary theca and granulosa cells can be applied for functional studies, and they retain the normal responses and steroidogenic pathways [131]. Porcine and bovine primary cells can be isolated from ovaries obtained from the slaughterhouse or from ovaries of rodent animal models; however, there are significant species-specific differences in the steroidogenic pathways, which need to be taken into account when trying to extrapolate results to the human system. Human granulosa cells are mostly obtained from women undergoing *in vitro* fertilization; however, these cells are usually subjected to supraphysiological concentrations of hCG and FSH, and these cells can only be cultivated for a relatively short time [132]. For these reasons, there is a great demand for suitable human theca and granulosa cell lines

to investigate a large number of individual EDCs at various concentrations and incubation time as well as mixtures of EDCs. The establishment of a theca cell line was not successful so far; in contrast, several granulosa cell lines are available for investigating effects of chemicals on steroidogenesis.

Granulosa cell lines are useful to study the impact of potential EDCs on progesterone synthesis as well as on the aromatase- and 17 β -HSD1-dependent production of estradiol upon incubation of these cells with androstenedione. There is a large number of human ovarian cancer cell lines available (for a recent review see [133]). Most of them express CYP19A1 and 17 β -HSD1 and their proliferation is stimulated by estrogens. Additionally, immortalized granulosa cell lines from various animal species and of human origin have been described [134].

Among the rodent granulosa cell lines, KK-1, GRM01 and GRM02 were found to produce progesterone, and retain responsiveness to cAMP, FSH and LH/hCG [134]. KK-1 cells were derived from mice bearing an SV40 T-antigen driven by the inhibin- α promoter, and treatment of these cells with hCG, forskolin and FSH increased cAMP 10-fold, 40-fold and 2.6-fold, respectively, indicating enhanced steroidogenesis [135]. KK-1 cells were shown to express Cyp19a1 and 17 β -hsd1 and convert androstenedione to estradiol. The KK-1 cell line was used, for example, to study effects of phthalates on the stimulation of steroidogenesis [136]. The phthalate MEHP at high concentrations of 20-100 μ M stimulated basal steroid production in KK-1 granulosa cells, a finding confirmed in mLTC-1 Leydig cells. The expression of StAR and cAMP-mediated signaling did not seem to be affected, and the authors suggested that MEHP may stimulate steroidogenesis by enhanced cholesterol supply. Thus, KK-1 represents a mouse cell system to study granulosa steroidogenesis. GRM01 and GRM02 granulosa cell lines were established by transfection of murine granulosa cells with v-myc [137]. Both cell lines retained steroidogenic activity and were shown to express 3 β -hsd2 and 17 β -hsd1 [138]. GRM01 was able to produce both progesterone and estradiol *de novo*, whereas GRM02 produced progesterone but not estradiol [139].

However, aromatase activity was also demonstrated in GRM02 upon the addition of androstenedione or testosterone to the culture medium. Steroid production was induced in both GRM01 and GRM02 by LH/hCG, FSH, forskolin and cAMP analogs. Both cell lines also express inhibin- α , which has a role in feedback regulation by inhibiting pituitary FSH secretion. They represent alternative murine cell models to study the impact of potential EDCs that act as direct inhibitors of enzymes involved in progesterone or estradiol production or of the signaling pathways involved in steroidogenesis in granulosa cells.

There are several human granulosa cell lines that are useful for the investigation of endogenous regulators of steroidogenesis as well as pathways involved in metabolic regulation, the regulation of cell proliferation and apoptotic pathways. The cell lines HGP53, HO23, HGL5, HTOG and SVOG were primarily used to study signaling pathways, which are involved in the regulation of steroidogenesis and effects on apoptosis as well as cell proliferation [140-145]. The immortalized human granulosa cell line COV434, initially isolated from a primary granulosa cell tumor, was shown to express FSH receptor and CYP19A1. In FSH supplemented medium COV434 was able to produce estradiol from androstenedione [146]. FSH and forskolin both stimulated steroidogenesis by induction of cAMP. Pharmacological inhibition of the FSH receptor was found to inhibit COV434 cell proliferation [147]. Furthermore, incubation of COV434 cells with soy isoflavones, considered to act as phytoestrogens, promoted cell proliferation [148]. Incubation with 5 to 50 μ M genistein led to increased expression of ER α and enhanced cell proliferation by repressing proapoptotic genes. The human relevance of these observations remain uncertain because of the low bioavailability of oral intake of isoflavones.

The most widely used human ovarian granulosa-like tumor cell line is KGN. Progesterone production as well as CYP19A1- and 17 β -HSD1-dependent estradiol formation from androstenedione supplemented culture medium was found to be induced by FSH *via* induction of IGF-1 in KGN granulosa cells [149]. Additionally, several endogenous regulators, such as

steroidogenic factor-1 (SF-1) [150], liver receptor homolog-1 (LRH-1) [151], AMP-kinase (AMPK)/sirtuin-1 (SIRT1) [152], oocyte-derived growth differentiation factor and bone morphogenic protein 15 [153], the Notch signaling pathway [154] and the Hippo pathway [155], were shown to affect progesterone production and CYP19A1- and 17 β -HSD1-dependent estradiol synthesis.

Several investigators used the KGN granulosa cell line to address the impact of xenobiotics on steroid synthesis. Bisphenol-A (BPA) was found to activate peroxisome proliferator-activated receptor (PPAR) γ and inhibit the FSH-stimulated insulin-like growth factor-1 (IGF1)-dependent induction of CYP19A1 expression and estradiol synthesis in KGN cells and in primary granulosa cells [156]. A significant blunting of the FSH-induced CYP19A1 expression was seen at 40 μ M whereas estradiol production was reduced after treatment with 80 μ M of BPA. The BPA concentrations applied are very high and human relevance of this findings remains uncertain. Another study found that BPA concentration-dependently down regulated CYP19A1 expression in KGN cells as well as in human fetal osteoblastic cells, with significant effects seen at 5 μ M [157]. Additionally, DEHP (5 μ M) and TCDD (10 nM) were found to inhibit the FSH-induced estradiol synthesis and to enhance the AhR expression in a PPAR-dependent manner [158]. Another study found that atrazine and simazine at 10 μ M enhanced the stimulatory effect of transfected SF-1 on aromatase mRNA expression and activity in KGN cells [159]. Recently, the pesticide simazine was found to shorten anogenital distance and to decrease whole body, ovarian and uterine weights in offspring of pregnant mice treated with 5-500 μ g/kg of this pesticide [160]. Simazine at a concentration of 1 nM diminished the viability and proliferation of KGN granulosa cells. Interestingly, a U-shaped curve was observed, whereby concentrations of 100-1000 nM no longer inhibited cell viability and proliferation.

Currently, most studies on EDCs affecting ovarian steroidogenesis are conducted using tumor cell lines of granulosa origin, where several pathways may be altered compared with normal granulosa

cells. This limitation needs to be considered in the interpretation of results. Also, most cell lines are cultivated in medium containing high glucose concentrations and fetal bovine serum as well as under hyperoxia, a situation clearly distinct from that of the physiological context and likely to affect metabolic pathways and steroid production. Another limitation is that currently no suitable human theca cell line is available. Since the production of steroids by the ovaries requires a tight cooperation of granulosa and theca cells, ideally a co-culture system of granulosa and theca cells should be applied for the investigation of ovarian steroidogenesis.

5. Adrenal cell models to investigate disruption of steroidogenesis

The adrenal glands play an essential role in the regulation of electrolyte and energy homeostasis [161]. An over production of glucocorticoids by the adrenal glands ultimately causes Cushing's syndrome, which is characterized by increased visceral adipose tissue, insulin resistance, skin and skeletal muscle atrophy, and impaired wound healing. In contrast, insufficient glucocorticoid production causes Addison's disease, characterized by hypotension, fatigue, muscle weakness, loss of body weight and depression. The clinical observations emphasize the importance of including the assessment of chemicals applied to humans (drugs, chemicals in food and personal care products) or released at high amounts into the environment for potential adrenal toxicity. In this respect, fatal adrenal insufficiency, due to unexpected severe adverse drug effects [162-165], is a known clinical problem that has been recognized by the FDA [166]. In contrast to the investigations of the safety of chemicals regarding reproductive and developmental endpoints, with a major focus on the disruption of sex steroid hormone action, the adrenal gland has been neglected in EDCs regulatory testing strategy, as recently discussed by Harvey [167]. However, there are several chemicals, *e.g.* drugs, chemicals contained in consumer products and environmental pollutants, that were shown to cause adrenal toxicity (for reviews see [163, 167, 168]), further emphasizing the necessity of testing chemicals for potential adrenal toxicity.

Nevertheless, regarding the use of cell-based testing systems, there is a widely used human adrenal cell line, *i.e.* H295R. The OECD (Organization for Economic Cooperation and Development) published a guideline for the testing of chemicals using this cell line [169]. The H295R cell line was derived from the NCI-H295 cell line that was established from an adrenocortical carcinoma of a female patient [170]. The use of NCI-H295 cells was limited by the slow proliferation and the fact that they formed cell clusters in culture. Using GC-MS analysis and radio-immuno assays (RIA) NCI-H295 cells were shown to produce about 30 different steroids [170-174]. Importantly, this cell line was shown to express most of the major steroidogenic genes; it also expresses CYP11B2 and has the ability to produce aldosterone, mainly upon stimulation with angiotensin II or potassium. The parental NCI-H295 cells were used to derive the H295A cells [175] as well as the H295R cells [173]. H295R cells can further be distinguished as H295R-S1, H295R-S2 and H295R-S3 clones, depending on the cultivation conditions. H295R-S1 are cultivated in a medium containing Nu-serum, H295R-S2 in a medium with the serum substitute Ultrosor-G and H295R-S3 in a medium containing Cosmic calf serum [176]. Furthermore, three additional clones were derived from NCI-H295, namely HAC13, HAC15 and HAC50 [176-178]. The NCI-H295 derived clonal cell lines all grow as adherent monolayers but show significant differences in the expression of steroidogenic enzymes, the response to endogenous regulators and the amounts of steroids synthesized, emphasizing the importance of the culture medium composition. Nevertheless, the NCI-H295 cell lines respond to angiotensin II and potassium by increased aldosterone production; however, their response to ACTH is either absent or very weak [179]. Besides the NCI-H295 clonal cell lines, no other human adrenal cell line with substantial steroidogenic properties has been reported to date.

Based on the secreted steroids and mRNA analyses the NCI-H295 clonal cell lines appeared to express all of the adrenocortical enzymes that were present in the original tumor including StAR, 3 β -HSD2, CYP11A1, CYP17A1, CYP21A1, CYP11B1, CYP11B2, 3 β -hydroxysteroid sulfotransferase and low levels of CYP19A1 [173, 178]. The expression pattern and steroids

produced indicates that these cells represent characteristics of the different adrenal zones. It needs to be noted that the basal production of cortisol and aldosterone in H295R cells is low, indicating a low expression of CYP11B1 and CYP11B2 in the absence of inducers. However, treatment with endogenous regulators can enhance some zone-specific effects. Forskolin and cAMP analogues enhance the production of adrenal androgens (DHEA, DHEAS, androstenedione) and glucocorticoids (cortisol, 11-deoxycortisol, corticosterone), whereas angiotensin II, the primary regulator of the renin-angiotensin-aldosterone system, and potassium induce the production of aldosterone in H295R cells [171, 172, 180, 181]. It was shown that H295R cells mediate angiotensin II effects through angiotensin receptor 1 (AT1) [172, 179, 182-184]. In contrast, H295A do not express substantial levels of AT1 and lack sensitivity to angiotensin II [185]. NCI-H295 clonal cell lines show weak or absent response to ACTH due to the very low expression of melanocortin 2 receptor (MC2R) [177]. Interestingly, in H295R cells ACTH induced a transient increase in aldosterone but not in cortisol production. Thus, depending on whether the cells are used in the basal state or upon stimulation with various effective agonists, the adrenal cell lines may be used to study the effect of EDCs on the functions of the different adrenocortical zones.

Besides the human NCI-H295 clonal cell lines, mouse adrenal cell lines have been used in several studies on adrenal steroidogenesis. The mouse Y-1 cells were reported to exhibit characteristics of both *zona fasciculata* and *zona glomerulosa*, and they are able to produce corticosterone and aldosterone [173, 186-188]. Y-1 cells were shown to respond to ACTH with increased expression of steroidogenic genes and enhanced corticosterone production; however, the stimulatory effect was rather modest compared with that of isolated primary mouse adrenal cells [179, 189]. Later, two other cell lines, designated ATC1 and ATC7-L, established from adrenal tumors of two transgenic mice expressing the SV40 large T-antigen under the control of the *akr1b7* promoter, have been described [190]. Both cell lines exhibited a typical phenotype of the *zona fasciculata*. They produced high amounts of corticosterone and retained responsiveness to ACTH. Incubation of these

cells with ACTH increased SF-1 and decreased DAX-1 expression, providing an explanation for the observed stimulation of corticosterone production. Thus, ATC1 and ATC7-L represent useful cell models to study *zona fasciculata* specific function.

In contrast to the testicular and ovarian cell models, there is a human adrenal cell model (H295R) that has been recognized by the regulators for toxicity screening and resulted in an OECD test guideline for the evaluation of EDCs [169]. Therefore, a large number of studies applied the H295R cell model for the assessment of chemicals that cause disturbances of steroidogenesis, including pharmaceuticals, consumer products, food constituents and environmental pollutants [191-198], and it is out of the scope of this review to cover the findings of these studies. Currently, the OECD guideline only focuses on the use of H295R cells in their basal state and on the production of estradiol and testosterone as endpoints [199], two hormones not typically produced by the adrenals. Thus, there are limitations of the current protocol as well as in the use of the H295R cells and the exploitation of this cell model could be significantly extended. Interestingly, the measurement of the main adrenal steroids, *i.e.* adrenal androgens, glucocorticoids and mineralocorticoids, is currently not covered by the OECD guideline and an extended protocol to include the quantification of DHEA, cortisol and aldosterone needs to be validated [200, 201]. Other important steroids such as progesterone, 17 α -hydroxyprogesterone, 11-deoxycorticosterone and 11-deoxycortisol should also be determined simultaneously with the major adrenal steroids in order to obtain a broader picture of disturbances caused by a given chemical.

Since in their basal state H295R cells produce only low amounts of cortisol and aldosterone, the cells should be used in the basal state to detect chemicals that induce steroidogenesis and upon treatment with specific agonists such as ACTH, angiotensin II and potassium [202] in order to detect chemicals that inhibit steroidogenesis. For the latter, the time point of adding a chemical is important. The pre-incubation or simultaneous addition of a chemical with an inducer may allow to identify chemicals that disrupt regulatory pathways of steroidogenesis. Incubation of a chemical

following stimulation of the cells will allow to identify compounds that directly inhibit steroidogenic enzymes. Thus, different protocols need to be applied depending on the mode-of-action of a given compound. Also, inclusion of appropriate reference compounds (positive and negative controls) and time course analysis of the steroid production would aid the interpretation of the data. Another important issue is the inclusion of measurements of the steroid concentrations in the complete medium at the time of the start of the experiment, as the amounts of these steroids are influenced by the composition of the serum used. Furthermore, the availability of LC-MS based methods allows to simultaneously quantify several steroid hormones and specific steroid pattern analysis can be performed for reference compounds and individual EDCs [203-206]. In many recent studies antibody-based detection methods have still been used for quantification of steroids. These methods often are lacking specificity due to cross-reactivity of the antibodies. Thus, GC-MS and LC-MS methods should not only allow more accurate quantification but allow the simultaneous assessment of multiple steroids.

A major limitation of the H295R cell system is the insensitivity towards ACTH. Thus, the establishment of an additional human adrenal cell line is required. Regarding ACTH response, murine ATC1 and ATC7-L cells may represent useful alternatives for testing until a suitable human cell system is available; however, species-specific differences in signaling pathways need to be taken into account.

7. Conclusions and outlook

Cell-based steroidogenesis models are highly valuable for mechanistic studies of chemicals disrupting steroidogenesis and allow an initial medium to high throughput assessment of the potential endocrine toxicity of chemicals. In contrast, to adrenal steroidogenesis, there is no commonly used cell line or standardized procedure to assess effects of chemicals on steroidogenesis in Leydig cells and ovarian cells. Future efforts should therefore aim at establishing a human

Leydig cell line with the capability to respond to LH and produce testosterone. To investigate ovarian steroidogenesis, a human theca cell model is needed and, ideally, a theca granulosa co-culture cell system responding to FSH should ideally be established, with the capability for *de novo* steroid synthesis up to the final step of estradiol production. Moreover, there is a need for an ACTH-sensitive human adrenal cell line.

In order to extend and improve the current cell-based testing protocols for studying chemicals that disrupt steroidogenesis and to facilitate the comparison of results from different laboratories, several general issues should be considered: 1) the description of experiments using steroidogenic cell lines should include passage number, cell density, incubation time and the composition of the complete medium used, including glucose concentration, possible use of antibiotics, amount of serum as well as the amount of steroids contained in the complete medium. Cells should only be used within certain passage numbers to guarantee comparable steroidogenic activity and responsiveness of the involved signaling pathways; 2) Ideally, the same positive and negative controls should be included in every experiment to verify the responsiveness of the cell batch used; 3) The cells should be used in the basal state as well as upon stimulation with specific inducers. Ideally, the same inducers, concentrations and conditions should be applied in different laboratories and experiments to allow a direct comparison of the results. The chemicals to be tested should be added prior to stimulation or simultaneously with the inducer in order to investigate whether the response to an inducer is blunted or potentiated, as well as following stimulation in order to detect direct effects on steroidogenic enzymes; 4) The quantification of steroid metabolites should be performed by GC-MS or LC-MS to assure specificity of the results. The major steroids should be quantified rather than a single steroid; and 5) another key issue remains the experimental concentration of a given chemical to be tested. A drawback of cell-based studies is the short duration of the incubation compared with humans who might be exposed for a long period of time. Also, often human exposure data is not available and concentrations of a given compound can vary

significantly from its tissue concentration. Usually concentrations chosen for *in vitro* experiments are higher than those observed in humans. Nevertheless, it has to be distinguished between studies aiming at providing mechanistic information and studies for risk assessment. For the latter, it is crucial to choose concentrations that realistically can be reached after occupational exposure or in case of environmental toxicants after exposure in the general population. As suggested by Teeguarden and Hanson-Drury toxicity study exposures should be directly compared to human exposure if such data are available and qualification of a study as “low dose” in the absence of reliable human exposure data should be avoided [207].

Thus, there is still considerable room for improvement of the currently available cellular testing systems and the protocols for measurements of chemical-induced disturbances of steroidogenesis.

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Figure legends

Figure 1: Schematic overview of adrenal steroidogenesis. Major steroids produced are indicated in bold and by solid lines, minor metabolites are indicated by dashed lines.

Figure 2: Schematic overview of steroidogenesis in Leydig cells.

Figure 3: Schematic overview of ovarian steroidogenesis.

Figure 1

Adrenal Gland

Zona Reticularis

Zona Fasciculata

Zona Glomerulosa

Figure 2

Leydig Cell

Pregnenolone

17 α -Hydroxypregnenolone

DHEA $\xrightarrow{3\beta\text{-HSD2}}$ **Androstenedione** $\xrightarrow{\text{CYP19A1}}$ Estrone

Androstenediol $\xrightarrow{3\beta\text{-HSD2}}$ **Testosterone** $\xrightarrow{\text{CYP19A1}}$ Estradiol

Figure 3