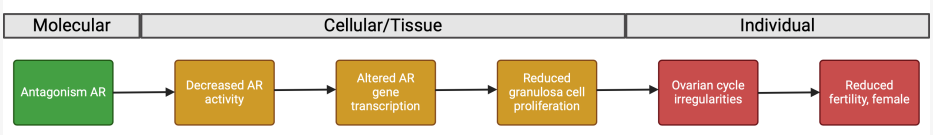
AOP ID and Title:

AOP 345: Androgen receptor (AR) antagonism leading to decreased fertility in females
Short Title: AR antagonism leading to decreased fertility

Graphical Representation



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Status

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Abstract

The proposed AOP (Figure 2) links AR antagonism (MIE26) to ovarian cycle irregularities (KE405) and reduced female fertility (KE406) via three key events: decreased AR activity (KE1614), altered AR gene transcription (KE286), and reduced granulosa cell proliferation (KE1800). Briefly, the binding of an antagonist to the AR prevents receptor activation and subsequent transcriptional regulation, ultimately disrupting expression of AR target genes necessary for follicle growth. This attenuates granulosa cell proliferation, leading to changes in the follicle population, which again disrupts the finely tuned ovarian cycle leading to subfertility.

The six KEs span a selected causal pathway between direct AR antagonism and reduced fertility in females. The first three KEs describe the essential component linking a chemical's direct interaction with the AR preventing normal ligand binding and receptor activation, leading to altered AR-regulated gene transcription in target cells and tissues in complex *in vivo* systems (Draskau et al 2024, accepted). The first two KEs may have broad taxonomic applicability, whereas KE286 serves as a placeholder KE for tissue/organ-specific changes in gene regulation; for this AOP the ovaries.

Decreased AR activity described in KE1614 can result from several upstream events, notably lower androgen levels, or as presented in this AOP, from AR antagonism (KE26). KE26 can be easily measured *in vitro* either by using reporter gene assays or by monitoring AR dimerization and nucleus translocation, both essential for the canonical AR pathway. KE1614 is not measured directly in mammals, but an assay in fish, the RADAR assay, is available.

Although AR can have both non-genomic and genomic actions, we have focussed on the canonical genomic actions in this AOP, including KE286 which refers to altered expression of AR-target genes. In principle, KE286 can describe the transcriptional changes in specific organs or tissues at specific life stages in response to AR antagonism, which will be specific for whichever AO it leads to. There is currently no standardized method for measuring this KE; however, standard methods such as reverse transcription-quantitative PCR (RT-qPCR) or RNA sequencing can be employed.

The fourth KE, 'reduced granulosa cell proliferation'(KE1800), represents an ovary-specific outcome of reduced AR signaling which integrates several known signaling pathways, such as PI3K/Akt, but also kit-ligand (*Kitl*) and growth differentiation factor 9 (*Gdf9*) that all may be under the control of the AR in the granulosa cells (Shiina et al., 2006). With the many pathways potentially involved in granulosa cell proliferation, they are challenging to measure in isolation, hence cell proliferation was considered the most pragmatic KE leading to disrupted pathway progression. KE1800 can be measured *in vitro* by proliferation assays using commercially available granulosa-like cell lines. Granulosa cell proliferation manifests as follicle growth, therefore counting and assessing the growth stage of follicles is the currently standardized method to measure this KE *in vivo*. Follicle growth can also be assessed with detection of proliferation markers *in situ*, albeit not currently included in test guidelines.

KE405 relates to ovarian cycle irregularities, encompassing variations in cycle length and/or ovulation problems (deferred ovulation or anovulation). These irregularities indicate disturbances in any parts of the Hypothalamic-Pituitary-Ovarian (HPO) axis, which regulates reproductive processes. Therefore, we have considered KE405 as an AO. It can be measured *in vivo* by estrous cycle monitoring, an endpoint in several guideline tests. Lastly, the AO on impaired female fertility refers to the capacity to conceive and is measured by calculating fertility rate based on born offspring numbers.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	26	Antagonism, Androgen receptor	Antagonism, Androgen receptor
	KE	1614	Decrease, androgen receptor activation	Decrease, AR activation
	KE	286	Altered, Transcription of genes by the androgen receptor	Altered, Transcription of genes by the AR
	KE	1800	Granulosa cell proliferation of gonadotropin-independent follicles, Reduced	Reduced granulosa cell proliferation
	AO	405	disrupted, ovarian cycle	disrupted, ovarian cycle
	AO	406	decreased, Fertility	decreased, Fertility

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Antagonism, Androgen receptor	adjacent	Decrease, androgen receptor activation	High	Moderate
Decrease, androgen receptor activation	adjacent	Altered, Transcription of genes by the androgen receptor	High	Moderate
Altered, Transcription of genes by the androgen receptor	adjacent	Granulosa cell proliferation of gonadotropin-independent follicles, Reduced	Moderate	Low
Granulosa cell proliferation of gonadotropin-independent follicles, Reduced	adjacent	disrupted, ovarian cycle	High	Low
disrupted, ovarian cycle	adjacent	decreased, Fertility	High	Low

Stressors

Name	Evidence
Flutamide	Moderate

Overall Assessment of the AOP

Weight of evidence assessment is conducted for the AOP overall to establish the confidence in the causal relationships between linked KEs. Using modified Bradford-Hill criteria, we subjectively rated the overall confidence in AOP345 as ‘moderate’, with the weakest link, relative to scientific evidence, being KER2273.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Sex Applicability

Sex	Evidence
Female	High

The domain of applicability of an AOP is defined by the most narrowly restricted of its KE(R)s. In this AOP, the early KEs have a broad domain of applicability that includes all ages and sexes within vertebrates, although they have been developed currently for mammalian species. The adverse outcomes of this AOP narrow the applicability domain to females of reproductive age with evidence currently from mainly rodent studies but also humans, non-human primates, and livestock animals.

Essentiality of the Key Events

Direct evidence for all included KEs is provided from studies where KE upstream is blocked and an effect on KE downstream is observed. However, one of the strongest pieces of evidence for this AOP comes from ARKO mouse models where all of the

downstream KEs can be observed. Global ARKO models demonstrate altered gene expression, whereas granulosa cell-specific ARKO models demonstrate reduced granulosa cell proliferation, ovarian cycle irregularities and subfertility (Sen & Hammes, 2010; Walters et al., 2012). The essentiality of all KEs was assessed as high (Table 1).

Weight of Evidence Summary

We have classified the strength of each KER based on the modified Bradford-Hill criteria, by rating their biological plausibility, empirical support and essentiality of downstream KEs as ‘high’, ‘moderate’, or ‘low’ according to the instructions in OECD’s Guidance Document for Developing and Assessing AOPs.

Table 1. The strength of each KER was assessed using the modified Bradford-Hill criteria. The biological plausibility for each KER, empirical support, and essentiality of the downstream KE were assessed and assigned as ‘high’ or ‘moderate’. No criterion was assigned as ‘low’ strength within the proposed AOP. Biological plausibility was deemed ‘high’ in cases of established mechanistic basis and ‘moderate’ when mechanistic understanding was incomplete. For essentiality, direct evidence exists for all included KEs where AR antagonists and ARKO models show that downstream KEs are impacted. Empirical support was deemed ‘high’ when there was consistent evidence using a wide range of stressors and as moderate in the case of a limited range of stressors.

Criteria	KER2130	KER2124	KER2273	KER3142	KER394
Biological Plausibility	HIGH	HIGH	MODERATE	HIGH	HIGH
Essentiality of downstream KEs	HIGH (KE1614)	HIGH (KE286)	HIGH (KE1800)	HIGH (KE405)	HIGH (KE406)
Empirical support	HIGH	HIGH	MODERATE	HIGH	HIGH

For the KERs in which systematic and semi-systematic literature search approaches were employed (KER2273 and KER3142 respectively), additional quality control was performed. The exposure studies used as empirical evidence for each KER were assessed for their quality using the online tool SciRAP (Science in Risk Assessment and Policy, scirap.org) (Molander et al., 2015). SciRAP provided predetermined criteria for reporting and methodological quality for *in vitro* and *in vivo* studies. In this case, a simple approach using the score outcome was used to assign studies to different reliability categories, as listed in Table 2. Studies with methodological scores of more than 80% were categorized as reliable without restriction. Studies with scores below that cutoff but above 65% were classified as reliable with restriction. In Table 2, the scores of all assessed studies within one KER have been averaged. The scores of individual studies can be found in Supplementary material. Based on the reliability category assigned from the SciRAP evaluation and the empirical support strength of the non-canonical knowledge KER2273, we concluded that the overall confidence in the KER was ‘moderate’.

Table 2. Average reporting and methodological quality score of exposure studies used as empirical evidence to support KERs. Based on the methodological score the overall reliability was assessed.

KER ID	Average reporting quality score	Average methodological quality score	Reliability category
2273	77	76	Reliable with restrictions
3142	71	81	Reliable without restrictions

Quantitative Consideration

The quantitative understanding of this AOP is limited, particularly regarding all KERs beyond the initial one, consequently categorizing it as low.

Considerations for Potential Applications of the AOP (optional)

Female reproductive disorders are on the rise and there is increasing evidence supporting a role for exposure to environmental chemicals, not least EDCs (Johansson et al., 2017). Despite this proposed causal relationship, there is still a lack of sensitive endpoints and understanding of causal mechanisms. This AOP addresses a knowledge gap as far as EDC identification is concerned, by providing an analytically constructed causal pathway linking disrupted androgen signaling with ovarian dysfunction and reduced fertility in females. Importantly, most KEs of the pathway include methods for effect measurements, which can support causal inference between *in vitro* data and adverse effects in an intact organism.

AOP345 also highlight gaps in knowledge and assay capacity, which can encourage the development of new approach methodologies (NAMs) to aid with chemical testing and regulation. Furthermore, it highlights the importance of ovarian follicle counts as an endpoint that currently is only optional in OECD test guidelines. Notably, however, follicle counting is a subjective, time-consuming and labor-intensive endpoint to measure, thus replacing it with a method assessing granulosa cell proliferation could be valuable. Such a method could potentially compliment estrus cycle monitoring, an endpoint that is potentially affected by different experimental set-ups, for example group size, study length and statistical analyses. AOP345 therefore offers a promising approach to address these methodological challenges. Finally, as quantitative understanding of this AOP continues to develop, it can provide a standardized methodology for assessing chemical effects and guide future regulatory decisions for the complex endpoint of female fertility.

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Appendix 1

List of MIEs in this AOP

Event: 26: Antagonism, Androgen receptor

Short Name: Antagonism, Androgen receptor

Key Event Component

Process	Object	Action
androgen receptor activity	androgen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	MolecularInitiatingEvent
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	MolecularInitiatingEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	MolecularInitiatingEvent
Aop:372 - Androgen receptor antagonism leading to testicular cancer	MolecularInitiatingEvent
Aop:477 - Androgen receptor (AR) antagonism leading to hypospadias in male offspring	MolecularInitiatingEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	MolecularInitiatingEvent
Aop:19 - Androgen receptor antagonism leading to adverse effects in the male foetus (mammals)	MolecularInitiatingEvent

Stressors

Name
Mercaptobenzole
Triticonazole
Flusilazole
Epoxiconazole
Prochloraz
Propiconazole
Tebuconazole
Flutamide
Cyproterone acetate
Vinclozolin

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence

Sex	Evidence
Mixed	High

Both the DNA-binding and ligand-binding domains of the AR are highly evolutionary conserved, whereas the transactivation domain show more divergence which may affect AR-mediated gene regulation across species (Davey & Grossmann, 2016). Despite certain inter-species differences, AR function mediated through gene expression is highly conserved, with mutations studies from both humans and rodents showing strong correlation for AR-dependent development and function (Walters et al, 2010).

This KE is applicable for both sexes, across developmental stages into adulthood, in numerous cells and tissues and across mammalian taxa. It is, however, acknowledged that this KE most likely has a much broader domain of applicability extending to non-mammalian vertebrates. AOP developers are encouraged to add additional relevant knowledge to expand on the applicability to also include other vertebrates.

Key Event Description

The androgen receptor (AR) and its function

The AR is a ligand-activated transcription factor belonging to the steroid hormone nuclear receptor family I (Davey & Grossmann, 2016). The AR has three domains: the N-terminal domain, the DNA-binding domain and the ligand-binding domain, with the latter being most evolutionary conserved. Testosterone (T) and the more biologically active dihydrotestosterone (DHT) are endogenous ligands for the AR (MacLean et al, 1993; MacLeod et al, 2010; Schwartz et al, 2019). In teleost fishes, 11-ketotestosterone is the second main ligand (Schuppe et al, 2020). Human AR mutations and mouse knock-out models have established a pivotal role for the AR in masculinization and spermatogenesis (Walters et al, 2010). Apart from the essential role for AR in male reproductive development and function (Walters et al, 2010), the AR is also expressed in many other tissues and organs such as bone, muscles, ovaries, and the immune system (Rana et al, 2014).

AR antagonism as Key Event

The main function of the AR is to activate gene transcription in cells. Canonical signaling occurs by ligands (androgens) binding to AR in the cytoplasm which results in translocation to the cell nucleus, receptor dimerization and binding to specific regulatory DNA sequences (Heemers & Tindall, 2007). The gene targets regulated by AR activation depends on cell/tissue type and what stage of development activation occur, and is, for instance, dependent on available co-factors. Apart from the canonical signaling pathway, AR can also initiate cytoplasmic signaling pathways with other functions than the nuclear pathway,for instance rapid change in cell function by ion transport changes (Heinlein & Chang, 2002) and association with Src kinase to activate MAPK/ERK signaling and activation of the PI3K/Akt pathway (Leung & Sadar, 2017).

How it is Measured or Detected

AR antagonism can be measured in vitro by transient or stable transactivation assays to evaluate nuclear receptor activation. There is already a validated test guideline for AR (ant)agonism adopted by the OECD, Test No. 458: *Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals* (OECD, 2016). This test guideline contains three different methods. More information on limitations, advantages, protocols, and availability and description of cells are given in the test guideline.

Besides these validated methods, other transiently or stably transfected reporter cell lines are available as well as yeast based systems (Campana et al, 2015; Körner et al, 2004). AR nuclear translocation can be monitored by various assays (Campana et al 2015), for example by monitoring fluorescent rat AR movement in living cells (Tyagi et al 2020), with several human AR translocation assays being commercially available; e.g. Fluorescent AR Nuclear Translocation Assay (tGFP-hAR/HEK293) or Human Androgen NHR Cell Based Antagonist Translocation LeadHunter Assay.

Additional information on AR interaction can be obtained employing competitive AR binding assays (Freyberger et al 2010, Shaw et al 2018), which can also inform on relative potency of the compounds, though not on downstream effect of the AR binding.

The recently developed AR dimerization assay provides an assay with an improved ability to measure potential stressor-mediated disruption of dimerization/activation (Lee et al, 2021).

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List of Key Events in the AOP

Event: 1614: Decrease, androgen receptor activation

Short Name: Decrease, AR activation

Key Event Component

Process	Object	Action
androgen receptor activity	androgen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:288 - Inhibition of 17α-hydrolase/C 10,20-lyase (Cyp17A1) activity leads to birth reproductive defects (cryptorchidism) in male (mammals)	KeyEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	KeyEvent
Aop:372 - Androgen receptor antagonism leading to testicular cancer	KeyEvent
Aop:477 - Androgen receptor (AR) antagonism leading to hypospadias in male offspring	KeyEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	KeyEvent
Aop:111 - Decrease in androgen receptor activity leading to Leydig cell tumors (in rat)	MolecularInitiatingEvent
Aop:570 - Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:571 - 5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

This KE is considered broadly applicable across mammalian taxa as all mammals express the AR in numerous cells and tissues where it regulates gene transcription required for developmental processes and functions. It is, however, acknowledged that this KE most likely has a much broader domain of applicability extending to non-mammalian vertebrates. AOP developers are encouraged to add additional relevant knowledge to expand on the applicability to also include other vertebrates.

Key Event Description

This KE refers to decreased activation of the androgen receptor (AR) as occurring in complex biological systems such as tissues and organs in vivo. It is thus considered distinct from KEs describing either blocking of AR or decreased androgen synthesis.

The AR is a nuclear transcription factor with canonical AR activation regulated by the binding of the androgens such as testosterone or dihydrotestosterone (DHT). Thus, AR activity can be decreased by reduced levels of steroidal ligands (testosterone, DHT) or the presence of compounds interfering with ligand binding to the receptor (Davey & Grossmann, 2016; Gao et al., 2005).

In the inactive state, AR is sequestered in the cytoplasm of cells by molecular chaperones. In the classical (genomic) AR signaling pathway, AR activation causes dissociation of the chaperones, AR dimerization and translocation to the nucleus to modulate gene expression. AR binds to the androgen response element (ARE) (Davey & Grossmann, 2016; Gao et al., 2005). Notably, for transcriptional regulation the AR is closely associated with other co-factors that may differ between cells, tissues and life stages. In this way, the functional consequence of AR activation is cell- and tissue-specific. This dependency on co-factors such as the SRC proteins also means that stressors affecting recruitment of co-activators to AR can result in decreased AR activity (Heinlein & Chang, 2002).

Ligand-bound AR may also associate with cytoplasmic and membrane-bound proteins to initiate cytoplasmic signaling pathways with other functions than the nuclear pathway. Non-genomic AR signaling includes association with Src kinase to activate MAPK/ERK signaling and activation of the PI3K/Akt pathway. Decreased AR activity may therefore be a decrease in the genomic and/or non-genomic AR signaling pathways (Leung & Sadar, 2017).

How it is Measured or Detected

This KE specifically focuses on decreased *in vivo* activation, with most methods that can be used to measure AR activity carried out *in vitro*. They provide indirect information about the KE and are described in lower tier MIE/KEs (see for example MIE/KE-26 for AR antagonism, KE-1690 for decreased T levels and KE-1613 for decreased dihydrotestosterone levels). In this way, this KE is a placeholder for tissue-specific responses to AR activation or inactivation that will depend on the adverse outcome (AO) for which it is included.

In fish, The Rapid Androgen Disruption Activity Reporter (RADAR) assay included in OECD test guideline no. 251 can be used to measure genomic AR activity (OECD, 2022). Employing a spg1-gfp construct under control of the AR-binding promoter spiggin1 in medaka fish embryos, any stressor activating or inhibiting the androgen axis will be detected. This includes for instance stressors that agonize or antagonize AR, as well as stressors that modulate androgen synthesis or metabolism. Non-genomic AR activity cannot be detected by the RADAR assay (OECD, 2022). Similar assays may in the future be developed to measure AR activity in mammalian organisms.

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Event: 286: Altered, Transcription of genes by the androgen receptor

Short Name: Altered, Transcription of genes by the AR

Key Event Component

Process	Object	Action
regulation of gene expression	androgen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:19 - Androgen receptor antagonism leading to adverse effects in the male foetus (mammals)	KeyEvent
Aop:307 - Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:344 - Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	KeyEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	KeyEvent
Aop:305 - 5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:495 - Androgen receptor activation leading to prostate cancer	KeyEvent
Aop:306 - Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	KeyEvent
Aop:547 - Androgen receptor agonism leading to long anogenital distance in female offspring	KeyEvent
Aop:496 - Androgen receptor agonism leading to reproduction dysfunction □in zebrafish□	KeyEvent
Aop:372 - Androgen receptor antagonism leading to testicular cancer	KeyEvent
Aop:570 - Decreased testosterone synthesis leading to hypospadias in male (mammalian) offspring	KeyEvent
Aop:571 - 5α-reductase inhibition leading to hypospadias in male (mammalian) offspring	KeyEvent

Stressors

Name
Bicalutamide
Cyproterone acetate
Epoxiconazole
Flutamide
Flusilazole
Prochloraz
Propiconazole
Stressor:286 Tebuconazole
Triticonazole
Vinclozalin

Biological Context

Level of Biological Organization

Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

Both the DNA-binding and ligand-binding domains of the AR are highly evolutionary conserved, whereas the transactivation domain show more divergence, which may affect AR-mediated gene regulation across species (Davey and Grossmann 2016). Despite certain inter-species differences, AR function mediated through gene expression is highly conserved, with mutation studies from both humans and rodents showing strong correlation for AR-dependent development and function (Walters et al. 2010).

This KE is considered broadly applicable across mammalian taxa, sex and developmental stages, as all mammals express the AR in numerous cells and tissues where it regulates gene transcription required for developmental processes and function. It is, however, acknowledged that this KE most likely has a much broader domain of applicability extending to non-mammalian vertebrates. AOP developers are encouraged to add additional relevant knowledge to expand on the applicability to also include other vertebrates.

Key Event Description

This KE refers to transcription of genes by the androgen receptor (AR) as occurring in complex biological systems such as tissues and organs *in vivo*. Rather than measuring individual genes, this KE aims to capture patterns of effects at transcriptome level in specific target cells/tissues. In other words, it can be replaced by specific KEs for individual adverse outcomes as information becomes available, for example the transcriptional toxicity response in prostate tissue for AO: prostate cancer, perineum tissue for AO: reduced AGD, etc. AR regulates many genes that differ between tissues and life stages and, importantly, different gene transcripts within individual cells can go in either direction since AR can act as both transcriptional activator and suppressor. Thus, the 'directionality' of the KE cannot be either reduced or increased, but instead describe an altered transcriptome.

The Androgen Receptor and its function

The AR belongs to the steroid hormone nuclear receptor family. It is a ligand-activated transcription factor with three domains: the N-terminal domain, the DNA-binding domain, and the ligand-binding domain with the latter being the most evolutionary conserved (Davey and Grossmann 2016). Androgens (such as dihydrotestosterone and testosterone) are AR ligands andact by binding to the AR in androgen-responsive tissues (Davey and Grossmann 2016). Human AR mutations and mouse knockout models have established a fundamental role for AR in masculinization and spermatogenesis (Maclean et al.; Walters et al. 2010; Rana et al. 2014). The AR is also expressed in many other tissues such as bone, muscles, ovaries and within the immune system (Rana et al. 2014).

Altered transcription of genes by the AR as a Key Event

Upon activation by ligand-binding, the AR translocates from the cytoplasm to the cell nucleus, dimerizes, binds to androgen response elements in the DNA to modulate gene transcription (Davey and Grossmann 2016). The transcriptional targets vary between cells and tissues, as well as with developmental stages and is also dependent on available co-regulators (Bevan and Parker 1999; Heemers and Tindall 2007). It should also be mentioned that the AR can work in other 'non-canonial' ways such as non-genomic signaling, and ligand-independent activation (Davey & Grossmann, 2016; Estrada et al, 2003; Jin et al, 2013).

A large number of known, and proposed, target genes of AR canonical signaling have been identified by analysis of gene expression following treatments with AR agonists (Bolton et al. 2007; Ngan et al. 2009, Jin et al. 2013).

How it is Measured or Detected

Altered transcription of genes by the AR can be measured by measuring the transcription level of known downstream target genes by RT-qPCR or other transcription analyses approaches, e.g. transcriptomics.

Since this KE aims to capture AR-mediated transcriptional patterns of effect, downstream bioinformatics analyses will typically be required to identify and compare effect footprints. Clusters of genes can be statistically associated with, for example, biological process terms or gene ontology terms relevant for AR-mediated signaling. Large transcriptomics data repositories can be used to compare transcriptional patterns between chemicals, tissues, and species (e.g. TOXsIgN (Darde et al, 2018a; Darde et al, 2018b), comparisons can be made to identified sets of AR 'biomarker' genes (e.g. as done in (Rooney et al, 2018)), and various methods can be used e.g. connectivity mapping (Keenan et al, 2019).

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Event: 1800: Granulosa cell proliferation of gonadotropin-independent follicles, Reduced

Short Name: Reduced granulosa cell proliferation

Key Event Component

Process	Object	Action
ovarian follicle development	granulosa cell	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term
eukaryotic cell

Organ term

Organ term
ovarian follicle

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
Monkey	Monkey	High	NCBI
Pig	Pig	High	NCBI
cow	Bos taurus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Female	High

Overview

Mechanisms controlling folliculogenesis are well conserved between mammalian species, including mice, farm animals and humans(Adhikari and Liu, 2009; McGee and Hsueh, 2000).

Key Event Description

Granulosa cell function

Granulosa cells of the ovary play an important structural and functional role during folliculogenesis. They form the ovarian follicle architecture and transmit molecular messages to the oocyte through gap junction channels, ensuring developmental competence(Kidder and Vanderhyden, 2010). Folliculogenesis can be roughly divided into two phases: gonadotropin-independent and gonadotropin-dependent by the requirement for the gonadotropin follicle-stimulating hormone (FSH) to grow(Hsueh et al., 2015). During the gonadotropin-independent growth phase, growth factors secreted by the follicle, e.g. growth differentiation factor-9 (GDF9) by the oocyte and anti-Müllerian hormone (AMH) by the granulosa cells control the necessary morphological changes of granulosa cells and their proliferation(Hsueh et al., 2015). The growth can be histologically observed as proliferation of the granulosa cells as the flat granulosa cells of primordial follicles become cuboidal and increase in numbers(Gougeon, 2010). The connection between granulosa cell numbers and follicle growth during gonadotropin-independent growth is well described (Gougeon and Chainy, 1987).

Reduced granulosa cell proliferation as Key Event

Genetically modified mouse models have demonstrated that granulosa cell proliferation is a prerequisite for normal follicle growth and fertility. For example, deletion of the oocyte-specific growth factor GDF9 that stimulates granulosa cells halt folliculogenesis at the primary follicle stage in mice: the granulosa cells fail to proliferate to generate secondary follicles, the oocytes degenerate, and the mice are sterile(Dong et al., 1996). Conversely, mice administered GDF9 have accelerated granulosa cell proliferation and higher numbers of primary and secondary follicles compared to non-treated ones(Vitt et al., 2000).

AMH is a growth factor secreted by granulosa cells during the gonadotropin-independent follicle growth stage, and it inhibits the activation of primordial follicles to keep the growing and dormant follicles in balance. In mice overexpressing AMH, follicle growth to antral stages is inhibited and the numbers of all developmental stages of follicles decline faster by age than in wildtype controls(Pankhurst et al., 2018). Exposure of human ovarian tissue to AMH in culture inhibits follicle growth(Carlsson et al., 2006).

How it is Measured or Detected

In vitro

Decreased granulosa cell proliferation can be measured in cell culture. There are commercially available human granulosa cell tumor lines, for instance KGN (#RCB1154) “Granulosa cell tumor”, available from the Riken cell Bank. This cell line is representative of undifferentiated granulosa cells at early stages of follicle development making it suitable to study interactions of primordial to early antral pathways independent from hormonal control from theca cells and hypothalamic-pituitary axis (Nishi et al., 2001).

Well-established assays to detect proliferation include methods to assess DNA synthesis (e.g. BrdU), cellular metabolism (e.g. MTT, XTT, ATP detection assays), and proliferation proteins (e.g. PCNA, Ki67, MCM-2)(Adan et al., 2016). The same methods can also be used in ovarian follicle or tissue culture.

In vivo

Granulosa cell proliferation manifests as increased numbers of granulosa cells within ovarian follicles(Gougeon and Chainy, 1987). Analysis of follicle growth is based on the numbers of granulosa cell layers which is also reflected in the diameter of the follicle(Gougeon and Chainy, 1987). Granulosa cell proliferation is inseparably connected to folliculogenesis, and therefore numbers of follicles in different developmental stages reflect the proliferation of granulosa cells. Granulosa cell proliferation can therefore be measured by counting

follicles in different stages (primordial, primary, secondary) or by measuring the follicle diameters. Changes in the proliferation of granulosa cells during the early follicle growth phase would lead to altered proportions of follicles in different stages. For example, inhibition of granulosa cell proliferation can lead to reduced numbers of secondary follicles(Dong et al., 1996; Pankhurst et al., 2018). Therefore, studying ratios between follicles in different developmental stages can reveal changes in the proliferation of granulosa cells. Follicle counts are already suggested endpoints in the Extended One-Generation Reproductive Toxicity Study; EOGRTS (OECD 443)(2018).

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List of Adverse Outcomes in this AOP

Event: 405: disrupted, ovarian cycle

Short Name: disrupted, ovarian cycle

Key Event Component

Process	Object	Action
ovulation cycle	ovarian follicle	disrupted

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:7 - Aromatase (Cyp19a1) reduction leading to impaired fertility in adult female	AdverseOutcome
Aop:398 - Decreased ALDH1A (RALDH) activity leading to decreased fertility via disrupted meiotic initiation of fetal oogonia	KeyEvent
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	AdverseOutcome

Biological Context

Level of Biological Organization

Level of Biological Organization

Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mice	Mus sp.	Low	NCBI
rat	Rattus norvegicus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	

Sex Applicability

Sex	Evidence
Female	High

The estrous cycle comprises the recurring physiologic changes that are induced by reproductive hormones in most mammalian females. Many of the mechanisms involved in the regulation of the reproductive axis are similar across species (particularly those mediated through the estrogen receptor), assessments of rodent estrous cyclicity can offer insight into potential adverse effects in humans (Goldman, Murr, & Cooper, 2007). While evaluations of vaginal cytology in the laboratory rodent can provide a valuable reflection of the integrity of the hypothalamic-pituitary-ovarian axis, other indices are more useful in humans to determine the functional status of the reproductive system (e.g. menses, basal body temperature, alterations in vaginal pH, cervical mucous viscosity, and blood hormone levels). Nevertheless, since many of the mechanisms involved in the regulation of the reproductive axis are similar across species (particularly those mediated through the estrogen receptor), assessments of rodent estrous cyclicity can offer insight into potential adverse effects in humans (Rasier, Toppari, Parent, & Bourguignon, 2006).

Key Event Description

Biological state

The female ovarian cycle is the result of a balanced cooperation between several organs and is determined by a complex interaction of hormones. Ovarian cycle irregularities include disturbances in the ovarian cycle (e.g. longer cycle, persistent estrus) and/or ovulation problems (deferred ovulation or anovulation). The estrous cycle (also oestrous cycle) comprises the recurring physiologic changes that are induced by reproductive hormones in females. Estrous cycles start after sexual maturity in females and are interrupted by anestrous phases or pregnancies. During this cycle numerous well defined and sequential alterations in reproductive tract histology, physiology and cytology occur, initiated and regulated by the hypothalamic-pituitary-ovarian (HPO) axis. The central feature of the mammalian estrous cycle is the periodic maturation of eggs that will be released at ovulation and luteinisation of the follicles after ovulation to form corpora lutea. Adapted from www.oecd.org/chemicalsafety/testing/43754807.pdf Biological compartments

The cyclic changes that occur in the female reproductive tract are initiated and regulated by the hypothalamic-pituitary-ovarian (HPO) axis. Although folliculogenesis occurs independently of hormonal stimulation up until the formation of early tertiary follicles, the gonadotrophins luteinising hormone (LH) and follicle stimulating hormone (FSH) are essential for the completion of follicular maturation and development of mature preovulatory (Graafian) follicles. The oestrous cycle consists of four stages: prooestrus, oestrus, metoestrus (or dioestrus 1) and dioestrus (or dioestrus 2) orchestrated by hormones. Levels of LH and FSH begin to increase just after dioestrus. Both hormones are secreted by the same secretory cells (gonadotrophs) in the pars distalis of the anterior pituitary (adenohypophysis). FSH stimulates the development of the zona granulosa and triggers expression of LH receptors by granulosa cells. LH initiates the synthesis and secretion of androstenedione and, to a lesser extent, testosterone by the theca interna; these androgens are utilised by granulosa cells as substrates in the synthesis of estrogen. Pituitary release of gonadotrophins thus drives follicular maturation and secretion of estrogen during prooestrus. Gonadotrophin secretion by the anterior pituitary is regulated by luteinising hormone-releasing hormone (LHRH), produced by the hypothalamus. LHRH is transported along the axons of hypothalamic neurones to the median eminence where it is secreted into the hypothalamic-hypophyseal portal system and transported to the anterior pituitary. The hypothalamus secretes LHRH in rhythmic pulses; this pulsatility is essential for the normal activation of gonadotrophs and subsequent release of LH and FSH. Adapted from www.oecd.org/chemicalsafety/testing/43754807.pdf

Follicles that produce estrogens have sequestered pituitary FSH which in turn stimulates the aromatase reaction. Such follicles can undergo normal development and ovulation and contain eggs that readily resume meiosis when released. In the absence of an active local aromatase (i.e., no follicle-stimulating hormone), the follicles and oocytes become atretic and regress without ovulating. If aromatase is present, the estrogen and follicle stimulating hormone can further develop the follicular cells for normal luteal function after ovulation takes place (Ryan, 1982).

General role in biology

A sequential progression of interrelated physiological and behavioural cycles underlines the female's successful production of young. In many but not all species the first and most basic of these is estrous cycle, which is itself a combination of cycles.

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the

following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

The pattern of events in the estrous cycle may provide a useful indicator of the normality of reproductive neuroendocrine and ovarian function in the nonpregnant female. It also provides a means to interpret hormonal, histologic, and morphologic measurements relative to stage of the cycle, and can be useful to monitor the status of mated females. Regular cyclicity is one of the key parameters in assessment of female reproductive function in rodents. Parameters assessed for cyclicity: - Number of cycling females - Number of females with regular cycles - Number of cycles - Estrous cycle length - Percentage of time spent in the various estrous cycle stages Estrous cyclicity provides a method for evaluating the endocrine disrupting activity of each test chemical under physiologic conditions where endogenous concentrations of estrogen vary. Abnormal cycles were defined as one or more estrous cycles in the 21-day period with prolonged estrus (≥3 days) and/or prolonged metestrus or diestrus (≥4 days) within a given cycle (Goldman, Murr, & Cooper, 2007).

Estrous cycle normality can be monitored in the rat and mouse by observing the changes in the vaginal smear cytology. Visual observation of the vagina is the quickest method, requires no special equipment, and is best used when only proestrus or estrus stages need to be identified. For details see: (Westwood, 2008), (Byers, Wiles, Dunn, & Taft, 2012) and OECD guidelines (www.oecd.org).

The observation that animals do not ovulate while exhibiting estrous cycles indicates that estrous cyclicity alone may not be a sufficient surrogate of healthy function of ovaries; the measurements of serum hormones and particularly FSH can contribute to more sensitivity indicators of healthy function of ovaries (Davis, Maronpot, & Heindel, 1994).

Monitoring of oestrus cyclicity is included in OECD test guidelines (Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents, 2008) [1], (Test No. 416: Two-Generation Reproduction Toxicity, 2001)[2] and (Test No. 443: Extended One-Generation Reproductive Toxicity Study, 2012) [3]and in USA EPA OCSPP 890.1450.

In vitro testing

The follicle culture models were developed for the in-vitro production of mature oocytes and used to study the process of folliculogenesis and oogenesis in vitro (Cortvrindt & Smitz, 2002). These in vitro cultures demonstrate near-identical effects to those found in vivo, therefore might be able to acquire a place in fertility testing, replacing some in-vivo studies for ovarian function and female gamete quality testing (Stefansdottir, Fowler, Powles-Glover, Anderson, & Spears, 2014).

Regulatory Significance of the AO

Chemicals may be found to interfere with reproductive function in the female rat. This interference is commonly expressed as a change in normal morphology of the reproductive tract or a disturbance in the duration of particular phases of the estrous cycle. This key event lies within the scope of testing for endocrine disrupting activity of chemicals and therefore for testing of female reproductive and developmental toxicity. Monitoring of oestrus cyclicity is included in OECD test guidelines (Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents, 2008), (Test No. 416: Two-Generation Reproduction Toxicity, 2001) and (Test No. 443: Extended One-Generation Reproductive Toxicity Study, 2012) and in USA EPA OCSPP 890.1450. While an evaluation of the estrous cycle in laboratory rodents can be a useful measure of the integrity of the hypothalamic-pituitary-ovarian reproductive axis, it can also serve as a way of insuring that animals exhibiting abnormal cycling patterns are excluded from a study prior to exposure to a test compound. When incorporated as an adjunct to other endpoint measures, a determination of a female's cycling status can contribute important information about the nature of a toxicant insult to the reproductive system. In doing so, it can help to integrate the data into a more comprehensive mechanistic portrait of the effect, and in terms of risk assessment, may provide some indication of a toxicant's impact on human reproductive physiology. Significant evidence that the estrous cycle (or menstrual cycle in primates) has been disrupted should be considered an adverse effect (OECD, 2008). Included should be evidence of abnormal cycle length or pattern, ovulation failure, or abnormal menstruation.

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Event: 406: decreased, Fertility

Short Name: decreased, Fertility

Key Event Component

Process	Object	Action
fertility		decreased
fertilization	fertility	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:7 - Aromatase (Cyp19a1) reduction leading to impaired fertility in adult female	AdverseOutcome
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents	AdverseOutcome
Aop:18 - PPARα activation in utero leading to impaired fertility in males	AdverseOutcome
Aop:64 - Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility	AdverseOutcome
Aop:348 - Inhibition of 11β-Hydroxysteroid Dehydrogenase leading to decreased population trajectory	KeyEvent
Aop:349 - Inhibition of 11β-hydroxylase leading to decresed population trajectory	KeyEvent
Aop:396 - Deposition of ionizing energy leads to population decline via impaired meiosis	KeyEvent
Aop:398 - Decreased ALDH1A (RALDH) activity leading to decreased fertility via disrupted meiotic initiation of fetal oogonia	AdverseOutcome
Aop:492 - Glutathione conjugation leading to reproductive dysfunction via oxidative stress	AdverseOutcome
Aop:345 - Androgen receptor (AR) antagonism leading to decreased fertility in females	AdverseOutcome

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High
Juvenile	High
Adults	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Sex	Evidence

Plausible domain of applicability

***Taxonomic applicability*:** The impaired fertility may also have relevance for fish, mammals, amphibians, reptiles, birds and and invertebrates with sexual reproduction.

Life stage applicability: The impaired fertility can be measured at juveniles and adults.

Sex applicability: The impaired fertility can be measured in both male and female species.

Key Event Description

Biological state

capability to produce offspring

Biological compartments

System

General role in biology

Fertility is the capacity to conceive or induce conception. Impairment of fertility represents disorders of male or female reproductive functions or capacity.

How it is Measured or Detected

As a measure, fertility rate, is the number of offspring born per mating pair, individual or population.

Regulatory Significance of the AO

Under REACH, information on reproductive toxicity is required for chemicals with an annual production/importation volume of 10 metric tonnes or more. Standard information requirements include a screening study on reproduction toxicity (OECD TG 421/422) at Annex VIII (10-100 t.p.a), a prenatal developmental toxicity study (OECD 414) on a first species at Annex IX (100-1000 t.p.a), and from March 2015 the OECD 443(Extended One-Generation Reproductive Toxicity Study) is reproductive toxicity requirement instead of the two generation reproductive toxicity study (OECD TG 416). If not conducted already at Annex IX, a prenatal developmental toxicity study on a second species at Annex X (≥ 1000 t.p.a.).

Under the Biocidal Products Regulation (BPR), information is also required on reproductive toxicity for active substances as part of core data set and additional data set (EU 2012, ECHA 2013). As a core data set, prenatal developmental toxicity study (EU TM B.31) in rabbits as a first species and a two-generation reproduction toxicity study (EU TM B.31) are required. OECD TG 443 (Extended One-Generation Reproductive Toxicity Study) shall be considered as an alternative approach to the multi-generation study.) According to the Classification, Labelling and Packaging (CLP) regulation (EC, 200; Annex I: 3.7.1.1): a) “reproductive toxicity” includes adverse effects on sexual function and fertility in adult males and females, as well as developmental toxicity in the offspring; b) “effects on fertility” includes adverse effects on sexual function and fertility; and c) “developmental toxicity” includes adverse effects on development of the offspring.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2130: Antagonism, Androgen receptor leads to Decrease, AR activation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	High
Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	adjacent	High	
Androgen receptor (AR) antagonism leading to hypospadias in male offspring	adjacent	High	
Androgen receptor (AR) antagonism leading to decreased fertility in females	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

This KER is applicable to mammals as AR expression and activity is highly conserved (Davey & Grossmann, 2016). AR activity is important for sexual development and reproduction in both males and females (Prizant et al., 2014; Walters et al., 2010). AR function is required during development, puberty, and adulthood. It is, however, acknowledged that this KER most likely has a much broader domain of applicability extending to non-mammalian vertebrates. AOP developers are encouraged to add additional relevant knowledge to expand on the applicability to also include other vertebrates.

Key Event Relationship Description

The androgen receptor (AR) is a ligand-activated steroid hormone nuclear receptor (Davey & Grossmann, 2016). In its inactive state, the AR locates to the cytoplasm (Roy et al., 2001). When activated, the AR translocates to the nucleus, dimerizes, and, together with co-regulators, binds to specific DNA regulatory sequences to regulate gene transcription (Davey & Grossmann, 2016) (Lamont and Tindall, 2010). This is considered the canonical AR signaling pathway. The AR can also activate non-genomic signalling (Jin et al., 2013). However, this KER focuses on the canonical pathway.

The two main AR ligands are the androgens testosterone (T) and the more potent dihydrotestosterone (DHT). Androgens bind to the AR to mediate downstream androgenic responses, such as male development and masculinization (Rey, 2021; Walters et al., 2010). Antagonism of the AR would decrease AR activation and therefore the downstream AR-mediated effects.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is considered high.

The AR belongs to the steroid hormone nuclear receptor family. The AR has 3 main domains essential for its activity, the N-terminal domain, the ligand binding domain, and the DNA binding domain (Roy et al., 2001). Ligands, such as T and DHT, must bind to the ligand binding domain to activate AR allowing it to fulfill its role as a transcription factor. The binding of the ligand induces a change in AR conformation allowing it to translocate to the nucleus and congregate into a subnuclear compartment (Marcelli et al., 2006; Roy et al., 2001) homodimerize and bind to the DNA target sequences and regulate transcription of target genes. Regulation of AR target genes is greatly facilitated by numerous co-factors. Active AR signaling is essential for male reproduction and sexual development and is also crucial in several other tissues and organs such as ovaries, the immune system, bones, and muscles (Ogino et al., 2011; Prizant et al., 2014; Rey, 2021; William H. Walker, 2021).

AR antagonists can compete with or prevent in different ways AR ligand binding, thereby preventing AR activation. Antagonism of the AR can prevent translocation to the nucleus, compartmentalization, dimerization and DNA binding. Consequently, AR cannot regulate transcription of target genes and androgen signalling is disrupted. This can be observed using different AR activation assays such as AR dimerization, translocation, DNA binding or transcriptional activity assays (Brown et al., 2023; *OECD*, 2020).

Empirical Evidence

The empirical evidence for this KER is considered high

The effects of AR antagonism have been shown in many studies *in vivo* and *in vitro*.

Several stressors can act as antagonists of the AR and lead to decreased AR activation. Some of these are detailed in an AOP key event relationship report by (Pedersen et al., 2022) and shown below, exhibiting evidence of dose-concordance:

Stressors

- Cyproterone acetate: Using the AR-CALUX reporter assay in antagonism mode, cyproterone acetate showed an IC50 of 7.1 nM (Sonneveld, 2005)
- Epoxiconazole: Using transiently AR-transfected CHO cells, epoxiconazole showed a LOEC of 1.6 µM and an IC50 of 10 µM (Kjærstad et al., 2010).
- Flutamide: Using the AR-CALUX reporter assay in antagonism mode, flutamide showed an IC50 of 1.3 µM (Sonneveld, 2005).
- Flusilazole: Using hAR-EcoScreen Assay, triticonazole showed a LOEC for antagonisms of 0.8 µM and an IC50 of 2.8 (±0.1) µM (Draskau et al., 2019).
- Prochloraz: Using transiently AR-transfected CHO cells, prochloraz showed a LOEC of 6.3 µM and an IC50 of 13 µM (Kjærstad et al., 2010).
- Propiconazole: Using transiently AR-transfected CHO cells, propiconazole showed a LOEC of 12.5 µM and an IC50 of 18 µM (Kjærstad et al., 2010).
- Tebuconazole: Using transiently AR-transfected CHO cells, tebuconazole showed a LOEC of 3.1 µM and an IC50 of 8.1 µM (Kjærstad et al., 2010).
- Triticonazole: Using hAR-EcoScreen Assay, triticonazole showed a LOEC for antagonisms of 0.2 µM and an IC50 of 0.3 (±0.01) µM (Draskau et al., 2019).
- Vinclozolin: Using the AR-CALUX reporter assay in antagonism mode, vinclozolin showed an IC50of 1.0 µM(Sonneveld, 2005).”(Pedersen et al., 2022)

Other evidence:

Known AR antagonists are used for treatment of AR-sensitive cancers such as flutamide for prostate cancer (Mahler et al., 1998).

Uncertainties and Inconsistencies

Known antiandrogenic compounds like hydroxyflutamide have been shown to act as agonists when the AR carries certain mutations, therefore contributing to uncertainties (Yeh et al., 1997). Additionally, the levels of endogenous androgens (e.g., testosterone or dihydrotestosterone) and the variability in the presence and function of AR co-activators may modulate the effect of AR antagonism.

Quantitative Understanding of the Linkage

Response-response relationship

The quantitative relationship between AR antagonism and AR activation will depend on the type of antagonist.

Time-scale

Nuclear translocation in HeLa cells transfected with AR-GFP show a response within 2 hours after ligand exposure (Marcelli et al., 2006; Szafran et al., 2008). Another assay focusing on AR binding to promoters in LNCaP cells has shown that after ligand binding, AR is able to translocate and bind to the DNA sequences within 15min showing the speed of AR activation (Kang et al., 2002).

Known Feedforward/Feedback loops influencing this KER

AR antagonism can lead to increased AR transcript stability and levels as a compensatory mechanism in prostate cancer cells (Dart et al., 2020). In turn, in presence of increased AR levels, AR antagonists can exhibit agonistic activity (Chen et al., 2003).

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Relationship: 2124: Decrease, AR activation leads to Altered, Transcription of genes by the AR

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor (AR) antagonism leading to nipple retention (NR) in male (mammalian) offspring	adjacent	Moderate	Moderate
Androgen receptor (AR) antagonism leading to decreased fertility in females	adjacent	High	Moderate
5α-reductase inhibition leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	High	
Androgen receptor (AR) antagonism leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	Moderate	
Decreased testosterone synthesis leading to short anogenital distance (AGD) in male (mammalian) offspring	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

This KER is applicable for both sexes, across developmental stages into adulthood, in numerous cells and tissues and across mammalian taxa. It is, however, acknowledged that this KER most likely has a much broader domain of applicability extending to non-mammalian vertebrates. AOP developers are encouraged to add additional relevant knowledge to expand on the applicability to also include other vertebrates.

Key Event Relationship Description

The androgen receptor (AR) is a ligand-dependent nuclear transcription factor that upon activation translocates to the nucleus, dimerizes, and binds androgen response elements (AREs) to modulate transcription of target genes (Lamont and Tindall, 2010, Roy et al. 2001). Decreased activation of the AR affects its transcription factor activity, therefore leading to altered AR-target gene expression. This KER refers to decreased AR activation and altered gene expression occurring in complex systems, such as *in vivo* and the specific effect on transcription of AR target genes will depend on species, life stage, tissue, cell type etc.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is considered high

The AR is a ligand-activated transcription factor part of the steroid hormone nuclear receptor family. Non-activated AR is found in the cytoplasm as a multiprotein complex with heat-shock proteins, immunophilins and, other chaperones (Roy et al. 2001). Upon activation through ligand binding, the AR dissociates from the protein complex, translocates to the nucleus and homodimerizes. Facilitated by co-regulators, AR can bind to DNA regions containing AREs and initiate transcription of target genes, that thus will be different in e.g. different tissues, life-stages, species etc.

Through mapping of AREs and ChIP sequencing studies, several AR target genes have been identified, mainly studied in prostate cells (Jin, Kim, and Yu 2013). Different co-regulators and ligands lead to altered expression of different sets of genes (Jin et al. 2013; Kanno et al. 2022). Alternative splicing of the AR can lead to different AR variants that also affects which genes are transcribed (Jin et al. 2013).

Apart from this canonical signaling pathway, the AR can suppress gene expression, indirectly regulate miRNA transcription, and have non-genomic effects by rapid activation of second messenger pathways in either presence or absence of a ligand (Jin et al. 2013).

Empirical Evidence

The empirical evidence for this KER is considered high

In humans, altered gene expression profiling in individuals with androgen insensitivity syndrome (AIS) can provide supporting empirical evidence (Holterhus et al. 2003; Peng et al. 2021). In rodent AR knockout (KO) models, gene expression profiling studies and gene-targeted approaches have provided information on differentially expressed genes in several organ systems including male and female reproductive, endocrine, muscular, cardiovascular and nervous systems (Denolet et al. 2006; Fan et al. 2005; Holterhus et al. 2003; Ikeda et al. 2005; Karlsson et al. 2016; MacLean et al. 2008; Rana et al. 2011; Russell et al. 2012; Shiina et al. 2006; Wang et al. 2006; Welsh et al. 2012; Willems et al. 2010; Yu et al. 2008, 2012; Zhang et al. 2006; Zhou et al. 2011).

Exposure to known antiandrogens has been shown to alter transcriptional profiles, for example of neonatal pig ovaries (Knapczyk-Stwora et al. 2019).

Dose concordance has also been observed for instance in zebrafish embryos; a dose of 50 µg/L of the AR antagonist flutamide resulted in 674 differentially expressed genes at 96 h post fertilization whereas 500 µg/L flutamide resulted in 2871 differentially expressed genes (Ayobahan et al., 2023).

Uncertainties and Inconsistencies

AR action has been reported to occur also without ligand binding. However, not much is known about the extent and biological implications of such non-canonical, ligand-independent AR activation (Bennesch and Picard 2015).

Quantitative Understanding of the Linkage

Response-response relationship

There is not enough data to define a quantitative relationship between AR activation and alteration of AR target gene transcription, and such a relationship will differ between biological systems (species, tissue, cell type, life stage etc).

Time-scale

AR and promoter interactions occur within 15 minutes of ligand binding, RNA polymerase II and coactivator recruitment are proposed to occur transiently with cycles of approximately 90 minutes in LNCaP cells (Kang et al. 2002). RNA polymerase II elongation rates in mammalian cells have been shown to range between 1.3 and 4.3 kb/min (Maiuri et al. 2011). Therefore, depending on the cell type and the half-life of the AR target gene transcripts, changes are to be expected within hours.

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Age	AR expression in aging male rats	Tissue-specific alterations in AR activity with aging	(Supakar et al. 1993; Wu, Lin, and Gore 2009)
Genotype	Number of CAG repeats in the first exon of AR	Decreased AR activation with increased number of CAGs	(Tut et al. 1997; Chamberlain et al. 1994)

Known Feedforward/Feedback loops influencing this KER

AR has been hypothesized to auto-regulate its mRNA and protein levels(Mora and Mahesh 1999).

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Relationship: 2273: Altered, Transcription of genes by the AR leads to Reduced granulosa cell proliferation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor (AR) antagonism leading to decreased fertility in females	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Low	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
Pig	Pig	High	NCBI

Term	Scientific Term	Evidence	Links
cow	Bos taurus	Moderate	NCBI
Monkey	Monkey	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Female	High

This KER refers to females, as granulosa cells reside in the ovary. This KER might apply to all mammals as AR expression and activity are highly conserved (Davey & Grossmann, 2016). However, it is currently restricted to the mammalian species that a direct connection has been established between the adjacent KEs, which are human, mouse, rat, pig, cow and monkey. The life stage applicability is defined as 'During development and in adulthood' as primordial follicles are activated to grow at all ages, even prior to menarche(Peters et al., 1978).

Key Event Relationship Description

Decreased transcription of genes that are downstream of AR activation leads to reduced granulosa cell proliferation of the early-stage gonadotropin-independent ovarian follicles. Therefore, the follicle growth to the antral stage is decreased.

Evidence Supporting this KER

Biological Plausibility

AR is a ligand-activated nuclear transcription factor expressed in the ovaries across mammalian species, including humans(Gervásio et al., 2014). During the gonadotropin independent follicular stage, AR activation is hypothesized to promote follicle growth, whereas in later stages it has been shown to inhibit growth and induce apoptosis(Franks and Hardy, 2018; Harlow et al., 1988). In humans, both mRNA and protein of AR are present in the oocyte, stroma cells, theca cells, but most prominently in granulosa cells of small antral follicles(Gervásio et al., 2014; Jeppesen et al., 2012).

In the mouse ovary, AR mRNA and protein are present in the oocyte, theca, and granulosa cells(Gill et al., 2004; Hirai et al., 1994; Szoltys and Slomczynska, 2000; Tetsuka and Hillier, 1996; Tetsuka et al., 1995) . In the cow and sheep ovary, AR mRNA is present in granulosa and theca cells, and most prominently in granulosa of antral and early antral follicles(Hampton et al., 2004; Juengel et al., 2006). In the pig ovary, AR mRNA is mainly expressed in the granulosa cells until the antral stage(Cárdenas and Pope, 2002; Slomczynska et al., 2001). In the monkey ovary, AR mRNA and protein are present in theca, but mainly granulosa cells of antral and early antral follicles(Hillier et al., 1997; Weil et al., 1998).

In human follicles, the expression of the *AR* transcript is observed after the primordial stage and is most pronounced during the small antral stage(Rice et al., 2007). Throughout early folliculogenesis, AR expression controls transcription of genes involved in promoting growth and differentiation of granulosa cells and formation of antrum(Gervásio et al., 2014). Genes under the control of AR that are involved in these processes include Kit ligand (*KITL*), Bone morphogenetic protein 15 (*BMP15*), and Hepatocyte growth factor (*HGF*)(Astapova et al., 2019; Prizant et al., 2014).

In the monkey ovary, high levels of *AR* mRNA correlates with high levels of granulosa cell proliferation(Vendola et al., 1998; Weil et al., 1998) Increased AR activation is associated with increased follicle growth and increased granulosa cell proliferation in small antral rat follicles, supporting the important role for AR during this developmental stage(Lim et al., 2017a).

AR may mediate early follicle growth through FSHR, supported by studies correlating mRNA levels of AR and FSHR in granulosa cells of small antral follicles(Nielsen et al., 2011, Weil et al., 1999). In mice, FSH-mediated *in vitro* follicle growth is increased by androgens, suggesting that androgens through AR may act synergistically with FSHR, which in turn increases follicle growth to antral follicles(Sen et al., 2014).

It is also hypothesized that FSHR activation through AR leads to increased AMH expression in granulosa cells of primary to small antral follicles(Lin et al., 2021). In turn, elevated levels of AMH lead to inhibition of FSH-induced aromatase activity, resulting in higher androgen levels that inhibit further follicular growth.

AR activation has been associated with Insulin-like Growth Factor 1 (IGF1) and Insulin-like Growth Factor 1 Receptor (IGFR1) and other key factors of the IGF signaling pathway, which is essential for granulosa growth and differentiation(Baumgarten et al., 2014), (Vendola et al., 1999). In human granulosa cells of primordial and primary follicles, AR and IGF-related factors are highly enriched at the transcriptional level(Steffensen et al., 2018).

AR activation affects the level of connexins, proteins that form gap junctions between granulosa cells and the oocyte and hence regulate intracellular communication; a prerequisite for folliculogenesis(Kamal et al., 2020).

In humans, the importance of AR in follicular growth becomes evident with the beneficial effects of androgens in assisted reproductive technology outcomes(Bosdou et al., 2012; Casson et al., 2000; Fábregues et al., 2009; Kim et al., 2011, 2014; Nagels et al., 2015; Noventa et al., 2019; Petya Andreeva, Ivelina Oprova, Luboslava Valkova, Petya Chaveeva, Ivanka Dimova, 2020). Although the mechanism remains elusive, it has been suggested that androgen priming of women seeking fertility treatment promotes follicle growth resulting in an increase in the FSH-sensitive follicle pool(Hu et al., 2017). Gene expression studies in human small antral follicles reveal significant association of AR and FSHR levels, suggesting that the increase in follicle growth could be mediated through regulating AR transcription in granulosa cells(Hu et al., 2017; Nielsen et al., 2011). Epidemiological studies have shown that upon androgen pretreatment, increase in the number of antral follicles and mean follicular diameter were observed(Balasch et al., 2006; Kim et al., 2011).

This increase supports the hypothesis that androgen receptor signaling is important for early follicle growth. Studies observing no effects upon androgen pre-treatment claim that dose and duration of the selected androgen might lead to contradicting results(Yeung et al., 2014).

Hypoandrogenism provides further evidence for an important role for androgen actions in human follicle development. Lower levels of DHEA or testosterone have been associated with women that have diminished ovarian reserve or premature ovarian aging(Gleicher et al., 2013). In the case of untreated primary adrenal insufficiency, the androgen deficient patient exhibit significantly reduced fertility(Erichsen et al., 2010).

Conclusions on the androgen significance can also be drawn from clinical evidence where women are exposed to an androgen excess. Hyperandrogenism in the case of congenital adrenal hyperplasia and exogenous androgen treatments in trans males lead to polycystic ovaries(Walters and Handelsman, 2018). This indicated that the androgens stimulate early follicle growth and inhibit further maturation(Walters and Handelsman, 2018). In polycystic ovarian syndrome, a syndrome characterized by accumulation of small antral follicles in the ovarian cortex, a plausible cause for this morphology is hyperandrogenaemia(Balen et al., 2003; Lebbe and Woodruff, 2013).

Empirical Evidence

Androgen Receptor Knock Out (ARKO) mouse model

Granulosa-specific ARKO model	Relevant observations	Reference
GCARKO^{Ex2}	Premature ovarian failure, subfertility, longer estrous cycles, slower *in vitro* follicle growth compared to wild type	(Sen & Hammes,
GCARKO^{Ex3}	Subfertility, longer estrous cycles, decreased number of preantral and antral follicles compared to wild type, trend of lower ovarian expression of kitl, ifgr1 and fshr compared to wild type (not statistically significant)	(Kirsty A. Walters et

In vitro*/*Ex vivo

Study type	Species	Compound	Effect Dose	Duration	Method	Result	Re
Isolated secondary follicles in culture	Mouse	Hydroxyflutamide, Bicalutamide	50µM	8d, 12d	Follicle diameter measured	Reduced follicular growth	(Lenie & S
Isolated late secondary follicles in culture	Mouse	Bicalutamide	10µM, 32µM	6d	Follicle diameter measured	Reduced follicular growth (dose-depended)	(Murray, Allison, &
Isolated secondary follicles in culture	Mouse	Hydroxyflutamide	1µM, 10µM	4d	Follicle diameter measured	Reduced androgen-induced follicular growth	(Wang et
Isolated secondary follicles in culture	Mouse	Flutamide	20µM	2d, 3d	Follicle diameter and area measured	Reduced androgen-induced follicular growth	(Laird et a
Isolated secondary follicles in culture	Mouse	Enzalutamide	1µM	2d, 4d, 6d	Follicle diameter measured and number of antral follicles counted	Reduced follicular growth and antrum formation	(Lebbe et
Fetal ovaries in culture	Mouse	Vinclozolin	10µM, 50µM, 100µM, 200µM	7d of 17d	Follicle diameter measured	Reduced follicular growth	(González Barreñada Enriquez, 2020)
Isolated late secondary follicles in culture	Rat	Flutamide	10µM	2d, 4d	Follicle diameter measured and expressed as follicular volume	Reduced GDF9 and INSL3-induced follicular growth	(Xue, Kim 2014)
Isolated late secondary follicles in culture	Rat	Flutamide	10µM	2d, 4d	Follicle diameter measured and expressed as follicular volume	Reduced NR4A1-induced follicular growth	(Xue, Liu Tsang, 20
Isolated late secondary follicles in culture	Rat	Flutamide	10µM	4d	Follicle diameter measured and expressed as follicular volume	Reduced GDF9-induced follicle growth	(Orisaka, Kotsuji, &
Ovarian cortex pieces in culture	Cow	Flutamide	0.1µM, 1µM, 10µM	10d	Follicle counting and classification (histology)	Reduced number of secondary follicles compared to testosterone group (dose-dependent)	(Yang & F

Isolated granulosa cells from antral follicles	Pig	Hydroxyflutamide	5µM	1d	Incorporation of [^{3}H]-thymidine	Reduced granulosa cell proliferation	(T. E. Hicl Gilchrist, Armstrong
Isolated mural granulosa cells from small antral follicles	Pig	Hydroxyflutamide	0.1µM, 1µM	1h of 24h	Incorporation of [^{3}H]-thymidine	Reduced granulosa cell proliferation	(T. E. H 2005)
Ovarian cortex pieces in culture	Pig	Cyproterone acetate	0.001 µM, 0.0001 µM	7d	Follicle counting and classification (histology)	Reduced primordial follicle activation	(Magama Moniruzza Miyano, 2

In vivo

Study type	Species	Compound	Dose	Duration	Method	Result	R
Fetal exposure	Pig	Flutamide	50 mg/kg body weight/day	7d	Follicle counting and classification (histology)	Reduced numbers of primary follicles and increased of primordial	(Kna Grze Kozic Slom 2013
Neonatal exposure	Pig	Flutamide	50 mg/kg body weight/day	10d	Immunohistochemistry (PCNA)	Reduced number of follicles with proliferating granulosa cells	(Kna et al.
Neonatal exposure	Pig	Flutamide	50 mg/kg body weight/day	10d	RNAseq of whole ovary	Altered expression of genes involved in cell proliferation	(Kna et al.
Adult exposure	Rat	Flutamide	One-time 100 pg	48h after injection	Follicle counting and classification (histology)	Reduced number of all stages of follicles	(Kum Das,

Quality assessment of the studies was performed and can be found at:QA of Empirical Evidence

Uncertainties and Inconsistencies

Genomic and non-genomic effects are not distinguished in the studies included in the KER analysis. Hence, it cannot be concluded that all observations are solely due to directly perturbed transcription. However, since AR transcribes genes necessary for early folliculogenesis (*KITLG, BMP15, HGF*), it is reasonable to assume that genomic mechanisms are involved.

Other uncertainties to consider: different anti-androgenic compounds have different effects on the AR (e.g. different IC_{50}, C_{max}); compounds that are anti-androgenic may also affect other mechanisms/modalities; downstream effects of perturbed AR transcriptional function might depend on the duration of exposure as well as the developmental stage of the follicles. In humans, effects can be inconclusive since a part of the population can have androgen-related conditions such as polycystic ovary syndrome (PCOS)(Gleicher et al., 2011).

Quantitative Understanding of the Linkage

Response-response relationship

The nature of the response-response relationship between decreased AR activation and reduced granulosa cell proliferation in the early stage of follicular development is not clear. Some of the aforementioned studies claim the effects were dose-dependent; however, the limited number of concentrations tested prevents a solid conclusion(Murray et al., 1998; Wang et al., 2001; Yang and Fortune, 2006). Therefore, at present, the quantitative understanding of this KER is rated ‘low’.

Time-scale

Studies included in establishing this KER exhibit observed changes *in vitro* at 24h in pig granulosa cells, and *in vivo* studies after 48h in rats(Hickey et al., 2004, 2005; Kumari et al., 1978). The conclusion that can presently be drawn is that the approximate timescale of the changes in KEdownstream relative to changes in KEupstream is less than 48h. However, the species differences between the time scales of folliculogenesis need to be taken into consideration in order for human extrapolations to be made.

Known modulating factors

The E3 ubiquitin ligase protein Ring Finger Protein 6 (RNF6) regulates AR levels in granulosa cells through polyubiquitination and AR transcriptional activity for *KITLG* expression in small antral follicles(Lim et al., 2017b, 2017a).

Epidermal growth factor receptor (EGFR) may mediate the androgen-induced granulosa cell proliferation(Franks and Hardy, 2018).

Sixteen different mutations of the *AR* gene (Xq11.2-q12) that cause androgen insensitivity syndrome have been identified(Jiang et al., 2020).

The number of CAG repeats on the N-terminal domain of the*AR* has been associated with effects on fertility and ovarian

reserve(Hickey et al., 2002; Lledo et al., 2014).

Known Feedforward/Feedback loops influencing this KER

Activated AR can transcriptionally regulate its own expression through a negative feedback loop(Gelmann, 2002). However, in granulosa cells of monkey ovaries, AR was shown to have the opposite effect, thus creating an autocrine positive feedback(Weil et al., 1998). More studies are needed to understand when AR regulation of its own expression is positive or negative.

During the early stages of folliculogenesis, mainly from the secondary to the small antral stage, activated AR can induce FSH activities in granulosa cells and promote granulosa cell differentiation and follicle maturation, even though follicles are still not gonadotropin-dependent(Gleicher et al., 2011). These activities include changes in androgen metabolism due to altered expression of steroidogenic enzymes. Therefore, it has been suggested that androgens can bind to the AR to establish a loop between activated AR and FSH action(Gleicher et al., 2011; Lenie and Smitz, 2009).

A positive feedback loop connecting activated AR and AMH through FSHR is also hypothesized. Activated AR could lead to increased expression of AMH through FSHR activation, resulting in inhibition of FSH-induced aromatase, ultimately increasing levels of androgens and AR activation(Dewailly et al., 2016). Typically, elevated levels of androgens, for instance in transgender males and PCOS patients, correlate with increased levels of AMH, however, contradictory results exist connecting elevated androgen or FSH levels to reduced AMH(Caanen et al., 2015; Li et al., 2011).

There is also evidence of an intra-follicular feedback loop that regulates steroidogenesis during the secondary follicle stage, causing downregulation of androgen synthesis upon exogenous androgen exposure and upregulation upon androgen receptor antagonism(Lebbe et al., 2017).

As mentioned, genes involved in early folliculogenesis like*KITLG* and *HGF* are under the control of AR. Those two genes have been shown to create a positive feedback loop in mice, by increasing the expression levels of each other(Guglielmo et al., 2011).

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Relationship: 3142: Reduced granulosa cell proliferation leads to disrupted, ovarian cycle

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor (AR) antagonism leading to decreased fertility in females	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Female	High

This KER refers to females, as it refers to the primary component of the female reproductive system, the ovary and it includes all mammals. Although follicle activation and growth can occur at all ages, the ovarian cycle can only be completed upon sexual maturation, therefore the life stage applicability is defined as ‘Adult, reproductively mature’. The supporting empirical evidence includes rodent studies and biological plausibility additionally includes humans. However, the taxonomic applicability can be expanded to other mammals as follicle growth is the basic principle driving the ovarian cycle in all mammalian species.

Key Event Relationship Description

This KER connects reduced granulosa cell proliferation of early-stage follicles (gonadotropin-independent, up to antral stage) to ovarian cycle irregularities, which include disturbances in the ovarian cycle (e.g. longer cycle) and/or ovulation problems (deferred ovulation or anovulation). Reduced granulosa cell proliferation manifests as reduced follicle growth, which can be observed by follicle counting or staging.

Evidence Supporting this KER

Biological Plausibility

Ovarian follicles are composed of a centrally located immature oocyte surrounded by supporting somatic cells where granulosa cells make up the inner layer most closely associated with the oocyte. In humans, it is important to emphasize that all follicles are formed during fetal life and this pool of primordial follicles makes up the ovarian reserve. Once primordial follicles are activated, they can grow into primary follicles as granulosa cells proliferate and change in morphology. This transition is driven by factors produced by either the granulosa cells or the oocytes, notably Kit ligand (KITL). As granulosa cells continue to proliferate, the follicles become secondary, a process regulated by factors including Growth Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15). As more layers of granulosa cells are established, the follicle forms an antral cavity. Up to this stage, the growth and survival of the follicles is not dependent on gonadotropins, and thus do not involve the hypothalamus-pituitary-gonadal signaling axis, which is activated during puberty.

For later stages of follicular maturation and ovulation, the gonadotrophins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are essential. They are secreted from the anterior pituitary gland upon stimulation from the gonadotropin-releasing hormone (GnRH) from the hypothalamus. LH stimulates androgen production in theca cells of late-stage ovarian follicles, and is used by the neighbor granulosa cells to produce estrogens upon FSH stimulation. The hormones produced by these late-stage follicles and the corpora lutea formed by ovulated follicles, control the release of hormones from hypothalamus and anterior pituitary. The ovarian cycle results from the cyclic changes that occur in the female reproductive tract and are initiated and regulated by the hypothalamic-pituitary-ovarian (HPO) axis.

All stages of follicles can be found in the ovaries of reproductively active adult mammals, with the majority of the follicles being primordial. Only a minority of the primordial follicles ever complete folliculogenesis, with the majority dying by atresia. The granulosa cells are key determinants of follicle fate. Therefore, disruption of granulosa cell proliferation can halt follicular growth. By reducing the number of available earlier-stage gonadotropin-independent follicles, this will also affect the pool of more mature follicles. Consequently, fewer steroid hormones that regulate the ovarian cycle will be produced, leading to disturbances such as longer cycles. Additionally, less mature follicles will be available for ovulation, leading to e.g. deferred ovulation or anovulation. Biological evidence in humans can be provided by the irregular menstrual cycles observed in perimenopausal individuals (O'Connor et al., 2001).

Empirical Evidence

Selected studies demonstrating an effect on early follicles by reducing their population and leading to effects on estrus cyclicity were selected as empirical evidence for the KER. An effect was observed by histologically identifying, staging, and counting the follicles in all of the included studies. Studies that observed a decrease in primordial follicle counts were excluded, as this indicates an effect on the follicular reserve, rather than on follicle growth.

Table 1. *In vivo* rodent exposure studies demonstrating reduced granulosa cell proliferation leading to ovarian cycle irregularities.

Species	Exposure	Dose	Duration and Age	Reduced granulosa proliferation	Ovarian cycle irregularities	Reference
Wistar rats	2,4,6-trinitrotoluene (TNT)	40, 80 g	Single explosion (6 w.o.)	Preantral proportions	Longer diestrus	(Lin et al., 2023)
ICR mice	Perfluorohexane sulfonate (PFHxS)	5 mg/kg/day intragastrical	42 days (8 w.o.)	Secondary and antral follicles	Longer diestrus	(Yin et al., 2021)
ICR mice	Pueraria murifica	100 mg/kg/day in water	8 weeks (~10 w.o.)	Primary, secondary, Graffian	Longer estrus	(Jaroenporn et al., 2007)
Sprague Dawley albino rats	Triclocarban	0.5 mg/l/day in water	GD 5 to PND 21	Primary, secondary, antral Reduced Ki67 on granulosa	Longer estrus	(Mandour et al., 2021)
Sprague Dawley rats	Di-(2-ethylhexyl)-phthalate (DEHP)	600 mg/kg/alternate day oral gavage	60 days (6 w.o.)	Primary, secondary	Longer estrus cycle	(Xu et al., 2010)
CD-1 mice	Phthalate mixture	20, 200 µg/kg/day oral dosing	GD 10 to birth	Preantral percentage in F3	Longer metestrus, diestrus in F3	(Brehm et al., 2020)
CD-1 mice	Di-(2-ethylhexyl)-phthalate (DEHP)	200 µg/kg/day 500mg/kg/day oral dosing	GD 11 to birth	Preantral percentage in F3	Shorter prestrus, longer metestrus/diestrus	(Brehm et al., 2018)
ICR mice	Perfluorooctane sulfonate (PFOS)	0.1 mg/kg/day in water	4 months (12 w.o.)	Antral	Longer diestrus	(Feng et al., 2015)
Sprague Dawley rats	Perfluorooctanoate (PFOA), perfluorooctane sulfonate (PFOS)	0.1 mg/kg/day subcutaneous injection	5 days (PND 1–5 or PND 26–30)	Primary and secondary	Longer diestrus, acyclicity	(Du et al., 2019)
Sprague Dawley rats	3,3′,4,4′,5-Pentachlorobiphenyl(PCB126)	250 ng/kg/day 7.5 µg/kg/day oral dosing	Day 13-19 post-conception	Antral	Longer diestrus	(Muto et al., 2003)

Wistar rats	1-bromopropane	400 ppm inhalation 8h/day	12 weeks (10 w.o.)	Primary, secondary, antral	Longer diestrus	(Yamada et al., 2003)

Table 2. *In vivo* rodent model demonstrating reduced granulosa cell proliferation leading to ovarian cycle irregularities.

Model	Reduced granulosa proliferation	Ovarian cycle irregularities	Reference
DHT-treated NOD/ShiLtJ mice (polycystic ovarian syndrome model)	Preantral, small antral	Longer metestrus/diestrus, shorter proestrus	(Binder et al., 2023)
Maternal high-fat diet Sprague-Dawley rats	Secondary	Irregular cycle	(Zhou et al., 2019)
Akt1$^{-/-}$ mice	Early antral, antral, Graffian Reduced BrdU incorporation in granulosa of secondary	Longer diestrus	(Brown et al., 2010)
GCARKO^{Ex2}	Antral	Longer estrus cycle	(Sen & Hammes, 2010)
GCARKO^{Ex3}	Preantral, antral	Longer estrus cycle	(Walters et al., 2012)

Uncertainties and Inconsistencies

The studies included as empirical evidence report decreased numbers of growing follicles of early stages. However, some of the studies also report increased atresia, which could imply that the observations of altered follicle numbers are a result of stage-specific atresia rather than decreased follicular growth.

During our evidence collection, we identified studies that observed changes in follicle numbers with no effects on the ovarian cycle (Guerra et al., 2011; Ulker et al., 2020) An additional uncertainty is that estrus cyclicity is an endpoint potentially affected by different experimental set-ups, for example, group size, study length and statistical analyses (Goldman et al., 2007). Lastly, ovarian cycle irregularities indicate disturbances in any parts of the Hypothalamic-Pituitary-Ovarian (HPO) axis, which regulates reproductive processes. Therefore, direct effects on hypothalamus and pituitary can lead to uncertainties.

Quantitative Understanding of the Linkage

Response-response relationship

The nature of the response-response relationship between reduced granulosa cell proliferation and ovarian cycle irregularities is not clear, therefore, at present, the quantitative understanding of this KER is rated ‘low’.

Time-scale

Studies included in establishing this KER exhibit observed changes upon a singular acute exposure, in the case of the TNT study presented in the empirical evidence (Lin et al., 2023).

Known Feedforward/Feedback loops influencing this KER

During the ovarian cycle, there is a feedback loop between the hypothalamus, anterior pituitary gland and the ovary. In brief, the hypothalamus produces gonadotropin-releasing hormone (GnRH), stimulating the anterior pituitary to produce gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that in turn stimulate growth of ovarian follicles and the production of androgens in theca cells and estrogens in granulosa (Jamnongjit & Hammes, 2006). The estrogens produced by the granulosa along with the progesterone from the corpus luteum feedback to hypothalamus and anterior pituitary gland, to control their release.

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Relationship: 394: disrupted, ovarian cycle leads to decreased, Fertility

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aromatase (Cyp19a1) reduction leading to impaired fertility in adult female	non-adjacent	Moderate	
Decreased ALDH1A (RALDH) activity leading to decreased fertility via disrupted meiotic initiation of fetal oogonia	adjacent	High	Low
Androgen receptor (AR) antagonism leading to decreased fertility in females	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human, mouse, rat	human, mouse, rat	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Female	High

In many instances, human female reproductive toxicity of an agent is suspected based on studies performed in experimental animals. The neuroendocrinology, steroid biochemistry, and other physiologic events in the females of most small experimental species often used (mouse, rat, hamster) are similar in their susceptibility to disruption by toxicants (Massaro, 1997).

Although the assessment of the human ovarian cycle may have a variety of biomarkers distinct from those in rats, many of the underlying endocrine mechanisms associated with successful follicular development, ovulation, pregnancy, and parturition are homologous between the two (for review see (Bretveld et al., 2006). For this reason, a toxicant-induced perturbation of ovarian cycles in female rats suggest that a compound may function as a reproductive toxicant in human females.

Mice

- environmental air pollution (Mohallem et al., 2005)
- phthalates (DEHP)
- abortion rate of 100% in F0 dams in the 500-mg/kg/day was observed, in F1 females found that the total number of F2 embryos (exposed to DEHP only as germ cells) was not impaired. However, in the 0.05- and 5-mg DEHP groups, 28% and 29%, respectively, of the blastocysts were degenerated, compared with 8% of controls (Schmidt et al., 2012).
- Lamb et al. studied fertility effects of DEHP in mice (both sexes) and found that DEHP caused dose-dependent decreases in fertility. DBP exposure resulted in a reduction in the numbers of litters per pair and of live pups per litter and in the proportion of pups born alive at the 1.0% amount, but not at lower dose levels. A crossover mating trial demonstrated that female mice, but not males, were affected by DBP, as shown by significant decreases in the percentage of fertile pairs, the number of live pups per litter, the proportion of pups born alive, and live pup weight. DHP in the diet resulted in dose-related adverse effects on the numbers of litters per pair and of live pups per litter and proportion of pups born alive at 0.3, 0.6, and 1.2% DHP in the diet. A crossover mating study demonstrated that both sexes were affected. DEHP (at 0.1 and 0.3%) caused dose-dependent decreases in fertility and in the number and the proportion of pups born alive. A crossover mating trial showed that both sexes were affected by exposure to DEHP. These data demonstrate the ability of the continuous breeding protocol to discriminate the qualitative and quantitative reproductive effects of the more and less active congeners as well as the large differences in reproductive toxicity attributable to subtle changes in the alkyl substitution of phthalate esters (Lamb et al., 1987).

Rat phthalates (DEHP)

- female rats exposed to a high dose of DEHP (3,000 mg/kg/day) had irregular estrous cycles and a slight decline in pregnancy rate (Takai et al., 2009). At 1,000 mg/kg bw/day over a period of 4 weeks did not disturb female fertility or early embryo development.
- There was significant evidence that 5, 15, 50, and 400 mg /kg/day females differed from the control females in the relative amount of time spent in oestrous stages, however no changes were revealed in the number of females with regular cycles, cycle length, number of cycles, and in number of cycling females across the dose groups as compared to the control females The litter size (number of live pups) produced by the P0 generation was significantly reduced in the 400 mg/kg/day dose group (Blystone et al., 2010).

Human

Studies showing a correlation between decreased fertility and;

- professional activity (Olsen, 1994)
- phthalates (DEHP) In occupationally exposed women to high concentration of phthalates exhibit hypoestrogenic anovulary cycles and was associated with decreased pregnancy rate and higher miscarriage rates (Aldyreva,M.V.,Klimove,T.S.,Iziumova,A.S.,Timofeevskaia,L.A., 1975).
- smoking (Hull, North, Taylor, Farrow, & Ford, 2000)
- the use of certain drugs or radiation exposure (Dobson & Felton, 1983)

For the taxonomic applicability see also the Table 1.

Key Event Relationship Description

The ovarian cycle irregularities impact on reproductive capacity of the females that may result in impaired fertility:

1. Irregular cycles may reflect impaired ovulation. Extended vaginal estrus usually indicates that the female cannot spontaneously achieve the ovulatory surge of LH (Huang and Meites, 1975). The persistence of regular vaginal cycles after treatment does not necessarily indicate that ovulation occurred, because luteal tissue may form in follicles that have not ruptured. However, that effect should be reflected in reduced fertility. Conversely, subtle alterations of cyclicity can occur at

doses below those that alter fertility (Gray et al., 1989).

2. Persistent or constant vaginal cornification (or vaginal estrus) may result from one or several effects. Typically, in the adult, if the vaginal epithelium becomes cornified and remains so in response to toxicant exposure, it is the result of the agent's estrogenic properties (i.e., DES or methoxychlor), or the ability of the agent to block ovulation. In the latter case, the follicle persists and endogenous estrogen levels bring about the persistent vaginal cornification. Histologically, the ovaries in persistent estrus will be atrophied following exposure to estrogenic substances. In contrast, the ovaries of females in which ovulation has been blocked because of altered gonadotropin secretion will contain several large follicles and no corpora lutea. Females in constant estrus may be sexually receptive regardless of the mechanism responsible for this altered ovarian condition. However, if ovulation has been blocked by the treatment, an LH surge may be induced by mating (Brown-Grant et al., 1973; Smith, E.R. and Davidson, 1974) and a pregnancy or pseudopregnancy may ensue. The fertility of such matings is reduced (Cooper et al., 1994).

3. Significant delays in ovulation can result in increased embryonic abnormalities and pregnancy loss (Fugo and Butcher, 1966; Cooper et al., 1994).

4. Persistent diestrus indicates temporary or permanent cessation of follicular development and ovulation, and thus at least temporary infertility.

5. Prolonged vaginal diestrus, or anestrus, may be indicative of agents (e.g., polyaromatic hydrocarbons) that interfere with follicular development or deplete the pool of primordial follicles (Mattison and Nightingale, 1980) or agents such as atrazine that interrupt gonadotropin support of the ovary (Cooper et al., 1996). Pseudopregnancy is another altered endocrine state reflected by persistent diestrus. The ovaries of anestrous females are atrophic, with few primary follicles and an unstimulated uterus (Huang and Meites, 1975). Serum estradiol and progesterone are abnormally low.

6. Lengthening of the cycle may be a result of increased duration of either estrus or diestrus.

Evidence Supporting this KER

Biological Plausibility

In females, normal reproductive function involves the appropriate interaction of central nervous system, anterior pituitary, oviducts, uterus, cervix and ovaries. During the reproductive years the ovary is the central organ in this axis. The functional unit within the ovary is the follicle which is composed of theca; granulosa cells and the oocyte. The somatic compartment synthesizes and secrets hormones (steroids and growth factors) necessary for the orchestration of the inter-relationship between the other parts of the reproductive tract and the central nervous system. Oestrus cycle is under strict hormonal control, therefore perturbations of hormonal balance lead to perturbations of normal cyclicity (change in number of cycles or duration of each phase) and/or ovulation problems leading to impaired female reproductive function. However, there are other mechanisms that might result in impaired fertility (e.g cellular maturation in ovary).

Empirical Evidence

Many chemicals are found to interfere with reproductive function in the female. This interference is commonly expressed as a change in normal morphology of the reproductive tract or in ovarian cycle irregularities (disturbance in the duration of particular phases of the estrous cycle and/or ovulation problems). Monitoring estrous cyclicity provides a means to identify alterations in reproductive functions which are mediated through nonestrogenic as well as estrogenic mechanisms (Blasberg, Langan, & Clark, 1997), (Clark, Blasberg, & Brandling-Bennett, 1998). Adverse alteration in the nonpregnant female reproductive system have been observed at dose levels below those that result in reduced fertility or produce other overt effects on pregnancy or pregnancy outcomes. A disruption of cycling caused by xenobiotic treatment can induce a persistent estrus, a persistent diestrus, an irregular pattern with cycles of extended duration and ovulation problems. Common classes of chemicals have been shown to cause cycle irregularities in rats, humans, and non-human primates. Examples include the polychlorinated biphenyls (PCBs) and dioxins, which are associated with such irregularities in rats and humans (e.g (Li, Johnson, & Rozman, 1995) (Meerts et al., 2004), (Chao, Wang, Lin, Lee, & Päpke, 2007) and various agricultural pesticides, including herbicides, fungicides, and fumigants for review see (Bhattacharya & Keating, 2012),(Bretveld, Thomas, Scheepers, Zielhuis, & Roeleveld, 2006).

Compound class	**Species**	**AO:ovarian cycle irregularities**	**AO:Impaired fertility**	reference
Phthalates (DEHP)	rat	5-400 mg/kg/day females differed from the control in the relative amount of time spent in oestrous stages	number of live pups (P0) reduced (400 mg/kg/day)	(Blystone et al., 2010)
Phthalates (DEHP)	rat	irregular estrous cycles (3,000 mg/kg/day)	slight decline in pregnancy rate (3,000 mg/kg/day)	(Takai et al., 2009)
Phthalates (DEHP)	mice		dose-dependent decreases in fertility	(Lamb, Chapin, Teague, Lawton, & Reel, 1987)
Phthalates (DEHP)	mice	No change	abortion rate of 100% in F0 dams (500-mg/kg/day)	(Schmidt, Schaedlich, Fiandanese, Pocar, & Fischer, 2012).
Phthalates (DEHP)	sheep	dose-dependent effect on the duration of the estrous cycles shortening of the ovulatory cycles due mainly to a reduction in the size and lifespan of CL		(Herreros, Gonzalez-Bulnes, et al., 2013)
Phthalates (DEHP)	sheep	No effect on ovulatory efficiency		(Herreros, Encinas, et al., 2013)
Phthalates (DEHP)	rat	No changes in F0, increase of cycle by 0.4 day in F1 at 10,000ppm	18% and 21% decrease in live pups/litter F0 at 7500ppm and 10,000ppm respectively, no viable litters (F1 10,000 ppm ~643.95mg/kg/day)	(NTP, 2005)
Phthalates (DEHP)	rat	Deficit in growing follicles and corpora lutea	4-fold increase in females with stillborn pups in F0 at 9000ppm 2.1-fold Postimplantation loss in F0 at 9000ppm	(Schilling, K., Deckardt. K., Gembardt, Chr., and Hildebrand, 1999)
Phthalates (DEHP)	rat	prolong the estrous cycle, anovulation		(Davis, Maronpot, & Heindel, 1994)
Phthalates			Reduced fertility and fecundity	(Wolf et al., 1999)

Organochlorine (methoxychlor)	rat	Decreased number of cycles, extended diestrus and estrus		(Laws, 2000)
Organotins tributyltin chloride (TBTCl)	rat	At 125 ppm vaginal opening and impaired estrous cyclicity		(Ogata et al., 2001)

Table 1 Summary the empirical evidence supporting the KER.

It is known that exposure to 17-β-estradiol can disrupt the normal 4- to 5-day estrous cycle in adult female rats by inducing an extended period of diestrus consistent with pseudopregnancy within 5–7 days after the exposure (Gilmore & McDonald, 1969). This is due to the estrogen-dependent increase in prolactin that rescues ovarian corpora lutea and the subsequent synthesis and release of progesterone (Cooper, R. L., and Goldman, 1999). Significant evidence that the estrous cycle (or menstrual cycle in primates) has been disrupted should be considered an adverse effect (OECD, 2008).

Uncertainties and Inconsistencies

Chemicals may be found to interfere with reproductive function in the female. This interference is commonly expressed as a change in normal morphology of the reproductive tract or a disturbance in the duration of particular phases of the estrous cycle. However, menstrual cyclicity is affected by many parameters such as age, nutritional status, stress, exercise level, certain drugs, and the use of contraceptive measures that alter endocrine feedback. In nonpregnant females, repetitive occurrence of the four stages of the estrous cycle at regular, normal intervals suggests that neuroendocrine control of the cycle and ovarian responses to that control are normal. Even normal, control animals can show irregular cycles. However, a significant alteration compared with controls in the interval between occurrence of estrus for a treatment group is cause for concern. Generally, the cycle will be lengthened or the animals will become acyclic. Therefore changes in cyclicity should be interpreted with caution and not judged adverse without a comprehensive consideration of additional relevant endpoints in a weight-of-evidence approach.

Inconsistencies

Two generation studies by Tyl et al with Butyl benzyl phthalate (BBP) did not observe effects in F0 females on any parameters of estrous cycling, mating, or gestation. However, F1 females carrying F2 litters at and reduced number of total and live pups/litter at birth, with no effects on pre- or postnatal survival (Tyl et al., 2004).

Quantitative Understanding of the Linkage

Response-response relationship

A systematic review and meta-analysis, has made a correlation between menstrual cycle length and outcomes including fecundity defined by antral follicle count or anti-mullerian hormone (AMH) levels (Younis et al., 2020). This study can be used to provide quantitative information for this KER for humans. A short menstrual cycle length (21-27 days) was correlated with lower AMH levels and antral follicle counts.

Time-scale

Ovarian cycle irregularities encompass disturbances in the ovarian cycle and/or ovulation issues. In cases of anovulation, the time scale is immediate, whereas other irregularities depend on the duration of folliculogenesis and the menstrual or estrous cycle specific to each species.

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