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Developmental biology meets toxicology: contributing reproductive mechanisms to build Adverse Outcome Pathways

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Running Title: Adverse Outcome Pathways for reproductive disease

Abstract

An Adverse Outcome Pathway (AOP) is a simplified description of the sequence of mechanistic events that lead to a particular toxicological effect, from initial trigger to adverse outcome. Although designed to inform regulatory risk assessors, the AOP framework also provides a platform for innovative collaborations between experts from relevant research fields and the regulatory community. The underpinning for any AOP is basic knowledge about molecular and developmental processes; such knowledge can only be attained by solid bioscientific research. Starting with this fundamental knowledge, the objective is to devise novel testing strategies that focus on key events in a causative pathway. It is anticipated that such a knowledge-based approach will ultimately alleviate many of the burdens associated with classical chemical testing strategies that typically involve large-scale animal toxicity regimens. This hails from the notion that a solid understanding of the underlying mechanisms will allow the development and use of alternative test methods, including both in-vitro and in-silico approaches. This review is specifically targeted at professionals working in bioscientific fields, such as developmental and reproductive biology, and aims to i) inform on the existence of the AOP framework and ii) encourage new cross-disciplinary collaborations. It is hoped that fundamental biological knowledge can thus be better exploited for applied purposes: firstly, an improved understanding of how our perpetual exposure to environmental chemicals is causing human reproductive disease and, secondly, new approaches to screen for harmful chemicals more efficiently. This is not an instructional manual on how to create AOPs; rather, we discuss how to harness fundamental knowledge from the biosciences to assist regulatory toxicologists in their efforts to protect humans against chemicals that harm human reproductive development and function.

Key words: reproduction; Adverse Outcome Pathway, AOP; mechanism-of-action; regulatory; toxicology; retinoic acid

Introduction

We no longer question whether environmental chemicals can perturb reproductive development and function; rather, we question how they do so, which chemicals are particularly problematic and under what circumstances they act. On the surface, these questions may seem simple enough but, in reality, they are extremely complex and the answers are elusive. In attempting to answer these questions, we often have to rely on a set of uncertainties and assumptions that, naturally, present their own set of challenges. Thus, the problem of adequately assessing the risk of specific chemicals to human health is considerable, and this is even before we begin to tackle questions of if and how we should regulate the use of such chemicals.

In the 21st century, regulatory toxicologists have increased their efforts to incorporate knowledge from molecular and developmental biology to facilitate more rapid and cost-efficient testing of chemicals in terms of their potential harmful effects on human health (Krewski et al, 2010). Conventionally, extensive animal experimentation is required to assess the effects of harmful substances; although in-vitro and in-silico approaches are used for overall risk assessments of chemicals, they are rarely, in and of themselves, sufficient for many regulatory purposes. To alleviate this limitation, the concept of Adverse Outcome Pathways (AOPs) was introduced around 2010. Initially, AOPs focused on environmental toxicology, but the approach was quickly broadened to include human toxicology (Ankley et al, 2010; Ankley & Edwards, 2018).

Before we discuss the AOP framework in more detail, we want to highlight how non-toxicologists, or more precisely, developmental and molecular biologists, can make a contribution. In essence, AOPs outline a sequence of biomolecular events that are required to produce a given toxicological effect (or adverse outcome). We contend that a deep mechanistic understanding of the physiological pathways leading to disease, whether this knowledge arises from in-silico, in-vitro or in-vivo studies, will provide important underlying detail to support the validity of AOPs. Despite the fact that a decade has elapsed since the introduction of the AOP concept, AOPs are still lacking for most of the reproductive adverse outcomes, or diseases, where exposure to environmental chemicals are believed to be a contributory cause. For example, at the time of writing this review, the AOPwiki (see below for discussion on public repositories) only contained one AOP pertaining to female (human) reproductive disease: 'Aromatase (Cyp19a1) reduction leading to impaired fertility in adult female'. As anyone in the field of female reproductive research would know, this single entry is likely to relate to only a small proportion of the female reproductive diseases that arise from, or are exacerbated by, exposure to harmful chemicals.

To remedy the lack of applicable AOPs for human reproduction, we need to incorporate a solid understanding of the mechanisms that underpin both normal and pathophysiological pathways of human reproduction into the AOP framework. For success in this endeavour, however, it will be necessary for professional molecular/developmental biologists and toxicologists to work collaboratively. This is, of course, already taking place to some degree, for example within the newly funded EU Framework Programmes for Research and Innovation, FREIA (Female reproductive toxicity of EDCs: a human evidence-based screening and identification: http://www.freiaproject.eu/). Nonetheless, it is obvious that more can be done and we hope this review will encourage many fundamental biochemical

and biomedical scientists to reexamine how the invaluable knowledge they generate about mammalian molecular reproduction, could inform on human-relevant AOP frameworks.

The AOP concept at a glance

A complete description of the AOP framework, from organisation to application, is outside of scope for this review, but can be found elsewhere (Ankley & Edwards, 2018; Leist et al, 2017; Vinken et al, 2017). A simple overview is required, however, and given here. In short, an AOP is a pragmatic description of the mechanistic basis of a toxicological effect which is, at the same time, chemical-agnostic (i.e. non-committal to any specific chemical; Figure 1). Typically, an AOP would start with a 'molecular initiating event' (MIE), which is the (toxicity) trigger that initiates a series of 'key events' (KEs) linked by causally-plausible 'key event relationships' (KERs), ultimately leading to an 'adverse outcome' (AO) (Villeneuve et al, 2014; Vinken et al, 2017). This does not mean that any molecular or cellular event that is known to be involved in normal development, or disease progression, should be included in an AOP - far from it. As defined in OECD guidance documents, "AOPs define a series of *measurable biological changes* that can be expected to occur if the perturbation is sufficiently severe (i.e., in terms of potency, duration, frequency) to drive the pathway all the way to the AO" (OECD, 2018). In turn, the AO should be "relevant to risk assessment or regulatory decision-making".

KEs should be both measurable and essential for progression towards an AO. Furthermore, they are not necessarily unique to one AOP, but often shared between different AOPs. In this way, KEs are reusable and can link different AOPs together to create AOP networks. KERs, on the other hand, describe causal links between one KE and another (or to an AO), and

allow extrapolations from an upstream event or prediction of a downstream event based on sound scientific evidence of causality. In other words, KERs must be biologically plausible to allow for the use of downstream data from lower in the pathway hierarchy in a regulatory context. If we know that KE1 leads to AO1 then, theoretically, it should be sufficient to show that a chemical causes KE1 to occur in order to predict that AO1 will manifest without actually having to prove the progression with extensive animal testing. To establish encyclopedic AOPs, we are reliant on sound knowledge from the biological sciences to identify more KEs, to establish better methods to measure KEs, and to support or reject the biological-plausibility of any given KER.

A key principle of the AOP framework, and something that must be kept in mind, is that "AOPs are deliberate simplifications of normal biological pathways" (Ankley & Edwards, 2018). They do not aim to describe all the detailed processes taking place from initial molecular interactions through to pathophysiological outcomes, but rather extract a subset of key events with plausible causal relationships that are measurable and linked to apical effect outcomes. The term apical endpoint refers to "an observable outcome in a whole organism, such as a clinical sign or pathological state, that is indicative of a disease state that can result from exposure to a toxicant" (Krewski et al, 2011). The word 'testable' is also very important: it is essential that toxicologists are able to use properly validated assays if the data is to be used for regulatory purposes.

To illustrate how AOPs can be extracted from current biomolecular knowledge, we have depicted a complex developmental pathway, or network, describing the effects of ectopic retinoic acid during testis development. The effects of ectopic retinoic acid was modeled in vivo in mice and the effects on multiple aspects of testicular development, including germ

and somatic cell differentiation as well as efferent duct development, was recently described (Bowles et al, 2018). Based on this careful molecular analysis of retinoic acid exposure, we have extracted a putative AOP network for the same pathway (Figure 2). As depicted, ectopic retinoic acid can have consequences for both the germ line and somatic niche, with the former ultimately affecting sperm production and fertility, and the latter affecting general masculinisation of the developing fetus. Such a somatic effect is typically associated with anti-androgenic chemicals that either perturb testosterone synthesis or block of androgen receptor action, leading to feminisation effects in the male fetus. An example of a defect in general masculinisation is short anogenital distance (AGD; Schwartz et al, 2019), as described in the next section. With this, it is apparent how a conceptual AOP framework can highlight other potential molecular initiating events and ultimately be linked with yet more AOP networks through shared KEs or AO. Thus, by consideration of this example (Figure 2), it is also evident that there are many molecular and cellular events taking place in vivo that do not belong in a simplified AOP, whilst those events that are extracted are both essential for progression to an AO and are measurable, albeit in test assays that are yet to be validated.

Regarding human relevance and adversity

AOPs that describe events leading to an adverse effect outcome in humans must, obviously, contain information relevant to humans. However, the assays or measurements to determine end effects can be performed in other models relevant to the human system, such as rodent models as well as human or human-derived cells or tissues. At present, human-relevant toxicological testing relies heavily on rodent models, largely because of the powerful in-vivo effect data this model permits for risk assessment and chemical regulation. The important point is that in an AOP for human health effects, the AOs and KEs should be relevant for

178 human pathophysiology, a requirement that is constantly questioned and analysed. Thus, any
179 new information derived from non-human animals or tissues must be thoroughly verified as
180 applicable to humans before inclusion in encyclopedic AOPs for human health effects. This
181 does not mean that the exact same KEs or AOs that are identified in animal models must also
182 occur in the human, if they are to be relevant to human toxicology. In fact, there are two
183 elements to consider when dealing with human relevance in this context: firstly, we need to
184 consider what is meant by 'adverse' and, secondly, we need to consider whether or not the
185 AO needs to also manifest in humans. To exemplify this, we will consider two AOs included
186 in OECD test guidelines for potential endocrine disrupting chemicals, namely AGD and
187 'nipple retention'.
188
189 The AGD refers to the distance between anus and the external genitalia and is a readout of
190 fetal androgen action. In humans and other mammals, AGD is about twice as long in
191 newborn males than females and is directly related to fetal androgen levels. Males require
192 androgen signalling (chiefly through testosterone synthesis and action) to masculinise during
193 fetal life and a failure to do so results in feminisation phenotypes, including a shorter AGDi
194 (i.e. the AGD-index, which is a more accurate measure as it takes into account body
195 weight/size of the animal/person). This occurs in humans as well as rodents, and is
196 considered an AO and thus included in several OECD test guidelines (TG 414, TG 421/422,
197 TG 441) using in-vivo rodent experiments (Schwartz et al, 2019). However, it is debatable
198 whether or not a short AGD should be defined as 'adverse' in humans. A 10% shorter AGD
199 than the mean average of the population is, of course, not an adverse outcome for the one
200 individual in a medical sense, but since there is a strong association between a short AGD
201 and other male reproductive disorders and, more importantly, because a short AGD is a clear

sign of undervirilisation in humans as well as rodents, a short AGD in rodents is included as an AO for risk assessment purposes.

Nipple retention is another interesting effect endpoint, or AO, that does not immediately seem to be of any human relevance. In laboratory mice and rats, the males lose their nipples due to androgen action (Imperato-McGinley et al, 1986; Kratochwil, 1971; Wolf et al, 2002). The females retain theirs, of course, so that they can feed their young. In male rodents that have been exposed in utero to anti-androgenic compounds, nipples are retained. This is a clear sign of feminisation in male offspring, on par with a short AGD, and hence is included as a mandatory measurement in certain OECD test guidelines (TG 221/222, TG 443). Although all humans normally 'retain' two nipples regardless of sex, nipple retention is still considered a human-relevant measure in rodent studies as it is a readout of perturbed androgen signaling, which in humans is associated with other adverse reproductive effects (Schwartz et al, 2019).

Contributing expert knowledge

The AOP framework is ultimately a tool for regulators to use when assessing whether or not a given substance is of risk to human health (or environment for ecotoxicological AOPs). It is important to remember, however, that AOPs themselves are chemical-agnostic. That is, they are biological pathways explaining causal relationships from molecular initiating events through to disease manifestations. The chemical universe is only juxtaposed onto the AOP framework to assess the likelihood of disease manifesting, given prior knowledge about a chemicals ability to affect one or more KEs in the pathway. In other words, AOPs are complex developmental pathways stripped bare for regulatory use.

As we have exemplified in Figure 2, there is no supposition as to the mechanism that leads to the MIE that is inhibition of Cyp26b1. Rather, we simply show ectopic retinoic acid as a first KE and link this to effects, or downstream KEs, that our fundamental developmental biology studies have shown are likely to eventuate; and further, that activation of Dax1 expression as a second MIE. We could make similar examples for various human diseases with likely links to chemical exposures. For example one could envision incorporating new molecular insights from a recent study on the involvement of interleukins and estrogen signaling in stromal cell differentiation (Yu et al, 2019) into AOPs dealing with human endometriosis. The obvious link here to toxicology is that there is extensive evidence that diethylstilbestrol causes endometriosis in women (Missmer et al, 2004), but disethylstilbestrol (or ectopic estrogen signaling) is also linked to other female reproductive disorders (Johansson et al, 2017). Beyond this example, there are numerous AOs, or disease outcomes, that would benefit from further development. For example, with respect to human reproduction, conditions such as premature ovarian failure, precocious puberty, infertility, gynecological cancers and early menopause in girls/women or hypospadias, undescended testicles, reduced sperm count, infertility and testis cancers in boys/men, have all been associated with early-life exposure to environmental chemicals (Johansson et al, 2017; Skakkebaek et al, 2001). This list could easily be expanded to also include more detailed efforts to pinpoint shared KEs in the evolving AOP networks.

There are currently various online sources for contributing and accessing AOP knowledge, as summarised by others (Hecker & LaLone, 2019). Within this web-based, OECD-sponsored, AOP knowledge base (AOP-KB), the most advanced is the AOPwiki module that, at the time of writing, hosts about 300 AOPs at various stages of development. AOPwiki is a crowd-

sourcing resource hosted by the Society for the Advancement of Adverse Outcome Pathways (SAAOP) and is the primary entry point for the AOP-KB (AOPwiki.org). Being open source, it is available to all to propose and develop AOPs, but any proposed AOP will undergo rigorous, tiered peer-review before final endorsement.

Concluding Remarks

The AOP framework has established itself as a useful tool for toxicologists and regulators of potentially harmful substances. In order for this system to provide maximum community benefit, it needs to continue to expand and be used more by researchers, regulators and the industry going forward. There are, however, several challenges remaining before the AOP approach can deliver on all its promises, not least the reduction or elimination of animal studies and their replacement by in-vitro and in-silico methods. Of relevance for this review, and of interest to readers of the journal of *Molecular Human Reproductio*n, is the need for new and robust mechanistic data describing key molecular and cellular events that lead to apical adverse health outcomes in humans. Such knowledge will help us grow the AOP network for human reproductive development as it pertains to reproductive disease caused by environmental stressors, not least endocrine disrupting chemicals. This ‘call to action’ does not propose that developmental and molecular biologists suddenly start developing AOPs and focusing on the chemical universe. Rather, we intend to provide such biologists with relevant knowledge so that they can better understand how they can help ensure the quality and appropriateness of AOPs as they are being developed for regulation of harmful substances. We hope that cross-disciplinary collaboration will ultimately lead to a healthier and safer environment for all.

AUTHORS' ROLES

All authors contributed to THE drafting of figures and writing of the manuscript. MKD and TS created the final figures. All authors helped revise the manuscript and approved the final version.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests, neither financial nor non-financial, with work presented in this study.

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Figure 1: Generic Adverse Outcome Pathway (AOP) for human toxicology. An AOP begins with a 'Molecular Initiating Event (MIE), induced by a chemical stressor. This leads to subsequent 'Key Event' (KEs) that are necessary for progress of the effect upwards in terms of biological organisation, as well as measurable, by some means. The pathway ultimately culminates in an 'Adverse Outcome' (AO), or effect endpoint, that should be relevant to risk assessment or regulatory toxicology. All KEs or AOs in the pathway are linked by 'Key Event Relationships', which should be biologically-plausible relationships. The types of 'measurable' events can vary from in-silico modelling through in-vitro/ex-vivo assays to in-vivo data and epidemiological studies.

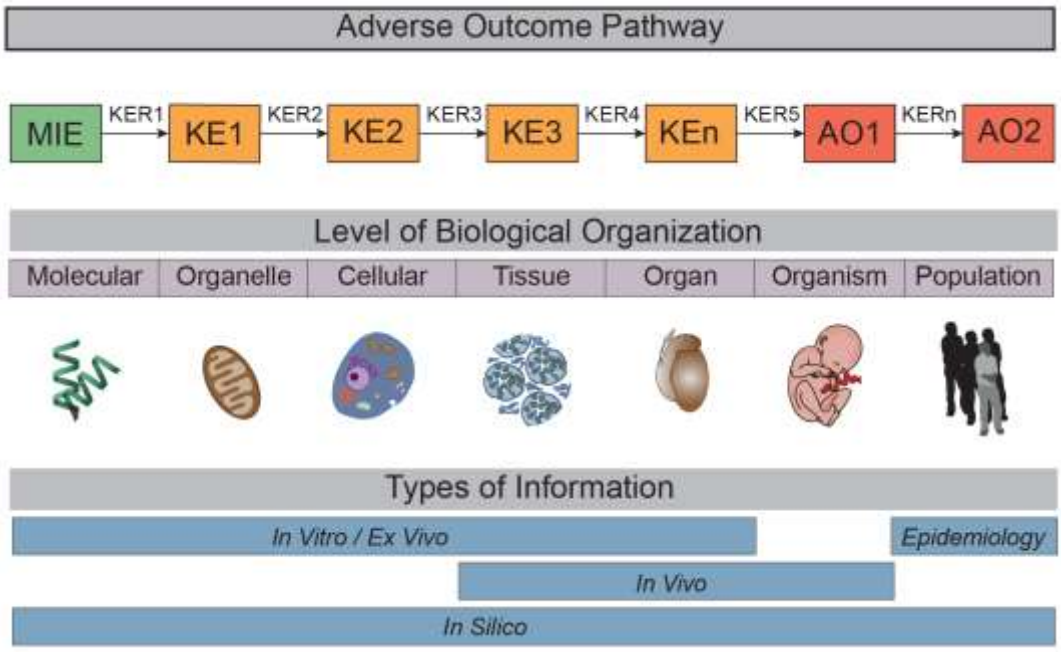
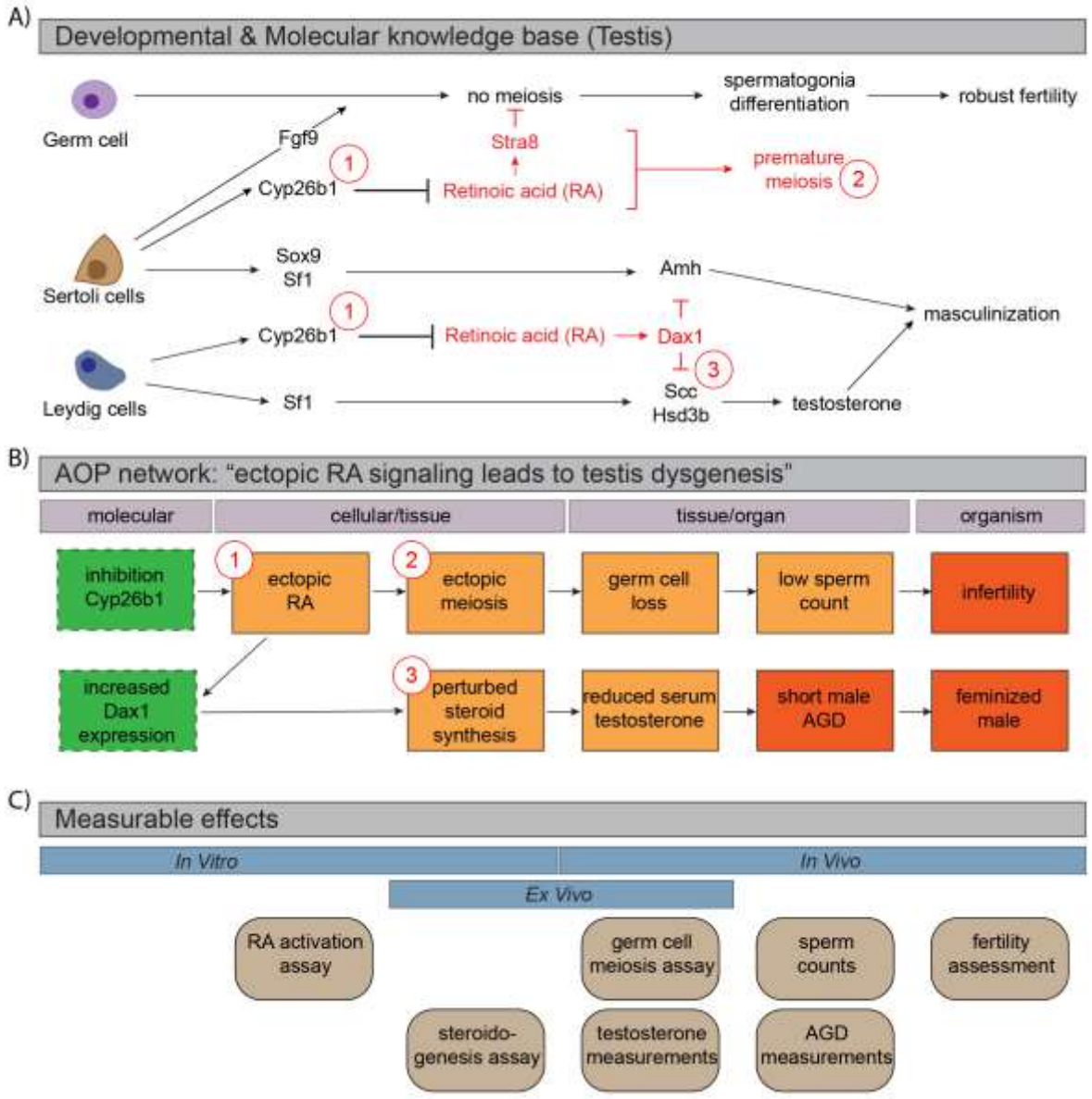


Figure 2: The extraction of basic scientific knowledge for the construction of Adverse Outcome Pathways: example, ectopic retinoic acid leading to reproductive disorders. A) Through decades of fundamental bioscientific research, we have gathered knowledge about how normal male reproductive development occurs and how perturbation of certain key molecular events can cause disease. Recent studies indicate that RA must be degraded in the developing testis and, if this does not occur, there are negative repercussions for the development of Sertoli, fetal Leydig and germ cells. Abnormal steps are shown in red (Bowles et al, 2010; Bowles et al, 2018). B) This fundamental knowledge uncovering key molecular pathways can be mined to create simplified mechanistic pathways to identify molecular initiating events (MIEs; green boxes) that are informative for regulatory toxicology. Notably, the extracted key events (KEs; orange boxes) should be both necessary for the progression to an adverse outcome (AO; red boxes) and C) measurable in some way, be it through in-silico modelling, in-vitro cell assays, ex-vivo organ cultures or in-vivo animal experimentation. Essentially, this means that the depth and breath of the fundamental network determines the usefulness and applicability of the resulting AOP.



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