



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

Kam Draskau, Monica; Spiller, Cassy M.; Boberg, Julie; Bowles, Josephine; Svingen, Terje

Published in:
Molecular Human Reproduction

Link to article, DOI:
[10.1093/molehr/gaaa001](https://doi.org/10.1093/molehr/gaaa001)

Publication date:
2020

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Kam Draskau, M., Spiller, C. M., Boberg, J., Bowles, J., & Svingen, T. (2020). Developmental biology meets toxicology: contributing reproductive mechanisms to build Adverse Outcome Pathways. *Molecular Human Reproduction*, 26(2), 111–116. <https://doi.org/10.1093/molehr/gaaa001>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

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

3
4
5 Monica Kam DRASKAU (ORCID: 0000-0001-6986-5991)¹, Cassy M. SPILLER (ORCID:
6 0000-0002-1343-8269)², Julie BOBERG (ORCID: 0000-0003-2255-1201)¹, Josephine
7 BOWLES (ORCID: 0000-0003-2867-7438)² and Terje SVINGEN (ORCID: 0000-0003-
8 4650-7651)^{1,3}

9
10 1. Division of Diet, Disease Prevention and Toxicology, National Food Institute, Technical
11 University of Denmark, Denmark

12
13 2. School of Biomedical Sciences, The University of Queensland, Australia
14

15
16
17 3. Corresponding Author:

18 Tel: +45 9351 8880

19 Email: tesv@food.dtu.dk
20
21
22

23 Running Title: Adverse Outcome Pathways for reproductive disease
24
25
26
27

28 **Abstract**

29

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

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

54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77

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).

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

93
94 To remedy the lack of applicable AOPs for human reproduction, we need to incorporate a
95 solid understanding of the mechanisms that underpin both normal and pathophysiological
96 pathways of human reproduction into the AOP framework. For success in this endeavour,
97 however, it will be necessary for professional molecular/developmental biologists and
98 toxicologists to work collaboratively. This is, of course, already taking place to some degree,
99 for example within the newly funded EU Framework Programmes for Research and
100 Innovation, FREIA (Female reproductive toxicity of EDCs: a human evidence-based
101 screening and identification: <http://www.freiaproject.eu/>). Nonetheless, it is obvious that
102 more can be done and we hope this review will encourage many fundamental biochemical

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

105

106 **The AOP concept at a glance**

107

108 A complete description of the AOP framework, from organisation to application, is outside of
109 scope for this review, but can be found elsewhere (Ankley & Edwards, 2018; Leist et al,
110 2017; Vinken et al, 2017). A simple overview is required, however, and given here. In short,
111 an AOP is a pragmatic description of the mechanistic basis of a toxicological effect which is,
112 at the same time, chemical-agnostic (i.e. non-committal to any specific chemical; Figure 1).
113 Typically, an AOP would start with a ‘molecular initiating event’ (MIE), which is the
114 (toxicity) trigger that initiates a series of ‘key events’ (KEs) linked by causally-plausible ‘key
115 event relationships’ (KERs), ultimately leading to an ‘adverse outcome’ (AO) (Villeneuve et
116 al, 2014; Vinken et al, 2017). This does not mean that any molecular or cellular event that is
117 known to be involved in normal development, or disease progression, should be included in
118 an AOP - far from it. As defined in OECD guidance documents, “AOPs define a series of
119 *measurable biological changes* that can be expected to occur if the perturbation is sufficiently
120 severe (i.e., in terms of potency, duration, frequency) to drive the pathway all the way to the
121 AO” (OECD, 2018). In turn, the AO should be “relevant to risk assessment or regulatory
122 decision-making”.

123

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

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

137

138 A key principle of the AOP framework, and something that must be kept in mind, is that
139 “AOPs are deliberate simplifications of normal biological pathways” (Ankley & Edwards,
140 2018). They do not aim to describe all the detailed processes taking place from initial
141 molecular interactions through to pathophysiological outcomes, but rather extract a subset of
142 key events with plausible causal relationships that are measurable and linked to apical effect
143 outcomes. The term apical endpoint refers to “an observable outcome in a whole organism,
144 such as a clinical sign or pathological state, that is indicative of a disease state that can result
145 from exposure to a toxicant” (Krewski et al, 2011). The word ‘testable’ is also very
146 important: it is essential that toxicologists are able to use properly validated assays if the data
147 is to be used for regulatory purposes.

148

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

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

168

169 **Regarding human relevance and adversity**

170

171 AOPs that describe events leading to an adverse effect outcome in humans must, obviously,
172 contain information relevant to humans. However, the assays or measurements to determine
173 end effects can be performed in other models relevant to the human system, such as rodent
174 models as well as human or human-derived cells or tissues. At present, human-relevant
175 toxicological testing relies heavily on rodent models, largely because of the powerful in-vivo
176 effect data this model permits for risk assessment and chemical regulation. The important
177 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

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

204

205 Nipple retention is another interesting effect endpoint, or AO, that does not immediately
206 seem to be of any human relevance. In laboratory mice and rats, the males lose their nipples
207 due to androgen action (Imperato-McGinley et al, 1986; Kratochwil, 1971; Wolf et al, 2002).
208 The females retain theirs, of course, so that they can feed their young. In male rodents that
209 have been exposed in utero to anti-androgenic compounds, nipples are retained. This is a
210 clear sign of feminisation in male offspring, on par with a short AGD, and hence is included
211 as a mandatory measurement in certain OECD test guidelines (TG 221/222, TG 443).

212 Although all humans normally ‘retain’ two nipples regardless of sex, nipple retention is still
213 considered a human-relevant measure in rodent studies as it is a readout of perturbed
214 androgen signaling, which in humans is associated with other adverse reproductive effects
215 (Schwartz et al, 2019).

216

217 **Contributing expert knowledge**

218

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

227

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

247

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

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

256

257 **Concluding Remarks**

258

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

276

277

278 **AUTHORS' ROLES**

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

282

283 **FUNDING**

284 MKD, JuB and TS received funding from the Danish Environmental Protection Agency as a
285 project under the Centre on Endocrine Disrupters (CEHOS). CS and JoB were funded by the
286 National Health and Medical Research Council of Australia and the Australian Research
287 Council (DP180103811).

288

289 **CONFLICT OF INTEREST**

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

292

293

294 **REFERENCES**

295 Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR,
296 Nichols JW, Russom CL, Schmieder PK, et al. Adverse outcome pathways: a conceptual
297 framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem.*
298 2010; **29**: 730-741.

299

300 Ankley GT, Edwards SW. The Adverse Outcome Pathway: A Multifaceted Framework
301 Supporting 21st Century Toxicology. *Curr Opin Toxicol.* 2018; **9**: 1-7.

302

303 Bowles J, Feng CW, Spiller C, Favidson TL, Jackson A, Koopman P. FGF9 suppresses
304 meiosis and promotes male germ cell fate in mice. *Dev Cell.* 2010; **19**: 440-449.

305

306 Bowles J, Feng CW, Ineson J, Miles K, Spiller CM, Harley VR, Sinclair AH, Koopman P.
307 Retinoic Acid Antagonizes Testis Development in Mice. *Cell Rep.* 2018; **24**: 1330-1341.

308

309 Hecker M, LaLone CA. Adverse Outcome Pathways: Moving from a Scientific Concept to an
310 Internationally Accepted Framework. *Environ Toxicol Chem.* 2019; **38**: 1152-1163
311

312 Imperato-McGinley J, Binienda Z, Gedney J, Vaughan ED Jr. Nipple differentiation in fetal
313 male rats treated with an inhibitor of the enzyme 5 alpha-reductase: definition of a selective
314 role for dihydrotestosterone. *Endocrinology.* 1986; **118**: 132-137.
315

316 Johansson HKL, Svingen T, Fowler PA, Vinggaard AM, Boberg J. Environmental influences
317 on ovarian dysgenesis - developmental windows sensitive to chemical exposures. *Nat Rev*
318 *Endocrinol.* 2017; **13**: 400-414.
319

320 Kratochwil K. In vitro analysis of the hormonal basis for the sexual dimorphism in the
321 embryonic development of the mouse mammary gland. *J Embryol Exp Morphol.* 1971; **25**:
322 141-153.
323

324 Krewski D, Acosta DJ, Andersen M, Anderson H, Bailar JC 3rd, Boekelheide K, Brent R,
325 Charnley G, Cheung VG, Green SJ, et al. Toxicity testing in the 21st century: a vision and a
326 strategy. *J Toxicol Environ Health B Crit Rev.* 2010; **13**: 51-138.
327

328 Leist M, Ghallab A, Graepel R, Marchan R, Hassan R, Bennekou SH, Limonciel A, Vinken
329 M, Schildknecht S, Waldmann T, et al. Adverse outcome pathways: opportunities, limitations
330 and open questions. *Arch Toxicol.* 2017; **91**: 3477-3505.
331

332 Missmer SA, Hankinson SE, Spiegelman D, Barbieri RL, Michels KB, Hunter DJ. In utero
333 exposures and the incidence of endometriosis. *Fertility and Sterility.* 2004; **82**: 1501-1508
334

335 OECD. User's handbook supplement to the guidance document for developing and assessing
336 AOPs. OECD Publishing. 2018; Vol. ENV/JM/MONO(2016)12.
337

338 Krewski D, Westphal M, Al-Zoughool M, Croteau MC, Andersen ME. New directions in
339 toxicity testing. *Annu Rev Public Health.* 2011; **32**: 161-178.
340

341 Schwartz CL, Christiansen S, Vinggaard AM, Axelstad M, Hass U, Svingen T. Anogenital
342 distance as a toxicological or clinical marker for fetal androgen action and risk for
343 reproductive disorders. *Arch Toxicol.* 2019; **93**: 253-272.
344

345 Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an
346 increasingly common developmental disorder with environmental aspects. *Hum Reprod.*
347 *2001; 16*: 972-978.
348

349 Villeneuve DL, Crump D, Garcia-Reyero N, Hecker M, Hutchinson TH, LaLone CA,
350 Landesmann B, Lettieri T, Munn S, Nepelska M. Adverse outcome pathway (AOP)
351 development I: strategies and principles. *Toxicol Sci.* 2014; **142**: 312-320.
352

353 Vinken M, Knapen D, Vergauwen L, Hengstler JG, Angrish M, Whelan M. Adverse outcome
354 pathways: a concise introduction for toxicologists. *Arch Toxicol.* 2017; **91**: 3697-3707.
355

356 Wolf CJ, Hotchkiss A, Ostby JS, LeBlanc GA, Gray LE Jr. Effects of prenatal testosterone
357 propionate on the sexual development of male and female rats: a dose-response study.
358 *Toxicol Sci.* 2002; **65**: 71-86.

359
360
361
362
363
364
365

366

367

368

369

370

371

372

373

374

375

376

377

378

379

380

381

382

383

384

385

386

387

388

389

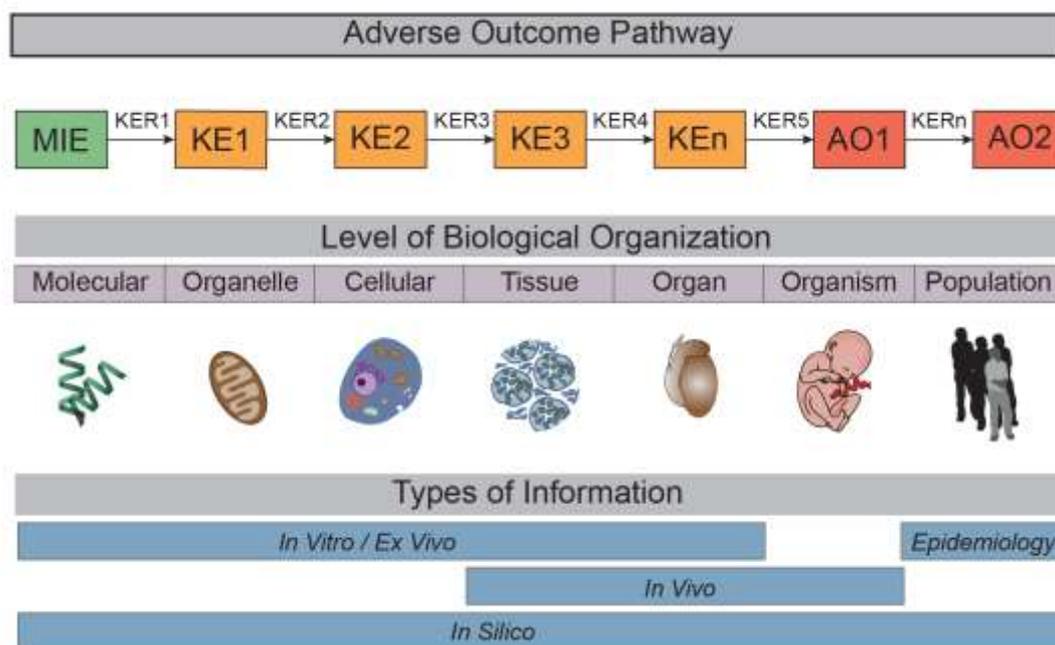
390

391

392

Yu J, Berga SL, Zou W, Taylor RN. Interleukin-1 β Inhibits Estrogen Receptor- α , Progesterone Receptors A and B and Biomarkers of Human Endometrial Stromal Cell Differentiation: Implications for Endometriosis. *Mol Hum Reprod.* 2019; In Press.

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



403
 404
 405
 406
 407
 408
 409

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

