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The mammalian ovary: concerns about evaluation of prenatal environmental exposures.

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Declaration of interests

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Abstract

The number and volume of processed natural or synthetic chemical toxicants introduced on the market has soared over the past decades. Possible human environmental exposures to potentially adverse compounds have, therefore, increased, as has awareness regarding their potential hazard for reproduction. Concomitantly, numbers of couples seeking assisted reproduction has climbed sharply. Toxicant risk assessment represents a concern at both individual and population and socio-economic levels. Here, we review current methods used to assess impacts of prenatal environmental exposures on mammalian ovary development and female reproductive function. We highlight technical challenges that need to be overcome in a regulatory context and the necessity for the development of guidelines and policies to better characterise potentially deleterious substances for the female reproductive function.

Key words

Endocrine disrupting compounds, ovary, development, fetus, adverse outcome

Abbreviations

AOP: Adverse outcome pathway; DOHaD: Developmental Origins of Health and Disease; EDC: Endocrine disrupting compound; MIE: molecular initiating event; ODS: Ovarian Dysgenesis Syndrome; POI: Premature Ovarian insufficiency; PCOS: Polycystic Ovarian Syndrome; QSAR: Quantitative structure-activity relationship; REACH: Registration, Evaluation, Authorisation and restriction of Chemicals; TDS: Testicular Dysgenesis Syndrome. PCW: post-conceptional weeks; dpc: days post-conception, dpp: days post-partum.

Introduction

Since the 19th century, there have been significant increases in production of environmental pollutants and toxicants and, simultaneously, escalation in numbers of confirmed and potential toxicants to which we are exposed [1]. Nevertheless, concerns about environmental pollutants have been slow to emerge although that has improved over the past 40 years. This raised awareness has led authorities to direct more resource to research on risk assessment of exposure to such molecules, with endocrine disrupting compounds (EDCs) of particular concern [2]. The European Commission adopted a common law concerning the Registration, Evaluation, Authorisation and restriction of Chemicals (REACH) in 2006 [3]. This is even more crucial because, in addition to synthetic molecules, some naturally occurring substances are also potentially harmful for human health. Evidence that some sub-populations are more vulnerable to such exposures, especially during pregnancy, is supported by growing numbers of studies on the fetal exposome [4], with short- and long-lasting impacts on organs. Long-term effects of dysregulated development on adult function were first hypothesised for the association of low birth weight and chronic noncommunicable diseases in adulthood [5,6]. This gave rise to the concept of developmental origins of health and disease (DOHaD or "Barker Hypothesis") linking *in utero* exposures during critical developmental "windows" with post-natal disease. Increasing frequencies of male reproductive disorders over the past 70 years are considered symptomatic of a common underlying entity, testicular dysgenesis syndrome (TDS) [7]. Although less clear, partly due to the delay between *in utero* environmental exposures and observed adverse outcomes, a similar trend is proposed regarding female reproductive impairments. Symptoms like premature ovarian failure, delayed menarche and Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome, are gathered under an ovarian dysgenesis syndrome (ODS) umbrella [8].

The mammalian germ cell lineage arises early during fetal life from an extra-embryonic territory and migrates into the presumptive gonadal territory at the surface of the mesonephros. This coincides with the proliferation and differentiation of somatic cells into the nascent bipotential gonad. In the ovary, the crucial element of differentiation is the genesis of a stock of germ cells, by rounds of exponential proliferation, and their commitment into meiosis before their arrest at the diplotene stage of prophase I. Subsequently, germ cells are enclosed into a finite number of functional units, the primordial follicles. Female reproductive lifespan is determined by this ovarian "stockpile" (reserve) [9]. Importantly, although ovarian genesis is based on a sequence of morphogenetic processes starting during fetal life that is common to mammals, stage duration and overlap differ greatly

between species. One major difference is the endocrine environment around follicle formation, which is characterised by a sudden decrease in estrogen levels in rodents, but high levels of estrogens for species like humans. If the prenatal exposure window is associated with the establishment of the germ cell reserve, the somatic cells are similarly undergoing active differentiation into either the epithelial or interstitial cell lineages. Unlike the adult ovary, whose endocrine relationships within the pituitary-hypothalamic-gonadal axis are well characterised, the endocrine properties of the fetal ovary are less well known in most species.

In this review, we update on the study of prenatal chemical toxicant exposure effects on early steps of ovarian differentiation. We identify the technical blocks in current experimental strategies in order to highlight research challenges, especially in a regulatory context. Indeed, the endpoints currently used to unravel these questions remain mainly highly focused on the germ cell lineage and on toxicity without questioning their potential endocrine disrupting activity. These questions are crucial to identify pollutants or pollutant categories that exert the most adverse endocrine disrupting effects on the developing ovary. This is essential to categorise for regulators those EDCs for which *in utero* exposure presents risks for female fertility.

Methodological approaches

Epidemiological studies first highlighted plausible associations between *in utero* exposures and female reproductive alterations, such as for diethylstilbestrol [10]. Nevertheless, the delay between exposure and adverse outcomes, and additional exposures after birth, makes the establishment of cause-effect links very complex. To overcome confounding factors, *in vivo* animal studies conducted under controlled conditions and *in vitro/ex vivo* studies were used to address possible long- and short- term effects, respectively. A common limitation of rodent studies is due to the experimental constraints on exposure routes which do not necessarily correspond to the environmental exposure of the mother or differ in the routes of exposure.

In vitro/ex vivo studies allow dissection of short-term effects at cellular and molecular levels. However, while some cell lines can be considered as proxies for the human germ cell lineage, such as embryonic stem cells [11], there is a lack of validated cell lines for the human fetal ovarian somatic cell lineages. Therefore, organotypic cultures of fetal organs in rodents or humans have been used for decades to address direct and short-term effects of chemicals [12]. However, *ex vivo* cultures isolate the ovary from the other organs of the

body, not only those from the reproductive axis, but also organs involved in the absorption, distribution, metabolism, and elimination of chemicals. These processes, determining factors in real life, are, therefore, not necessarily investigated. To the best of our knowledge, multi-organ cultures have not yet been used for fetal ovary studies. This is a method gap since the approach could rebuild the systemic complexity of ovarian function [13], and take into account the relationships between organs, including the hypothalamo-pituitary ovarian axis (active in second trimester human fetuses). This approach would also be highly relevant to take into account the bio-transformation of chemicals by both placenta and fetal liver, and the endocrine relationships between organs, including the adrenals.

Unlike large mammals and humans, who are exposed to a wide range of environmental pollutants, longitudinal studies are possible in small animal models bred in highly controlled environments. Tight control of the environment is possible with human multi-organ chip microfluidic culture technology [14], yet very long-term experiments are currently unachievable. While fetal exposome studies are crucial to understand the complexity of exposures, there remain issues around the more relevant dose to study: classic toxicological studies with doses ranging from low to unrealistically high doses *vs* environmentally relevant doses to identify realistic targets. The question of the optimum dose/s to test comes when addressing chemical effects from suspected EDCs. Exposomics also opens new avenues in experimental strategy design, shifting research in the field from exposure to single component to “real-life” mixtures. However, to achieve environmental realism, choosing the most relevant mixtures, and concentrations of each component, remains a major challenge. Adding to the complexity is the unfortunate fact that experimental designs of cocktails may induce complex responses within which the role/s, if any, of each single environmental pollutant is almost impossible to assess. From a broader point of view, the choice of exposure/s and relevant pathophysiological endpoints, do not meet regulatory requirements. Regulatory tests are required to pinpoint highly sensitive biomarkers of exposure and future adverse outcomes. Overall, this raises the importance of cross-sectional studies to address the potential repercussions of fetal environmental exposures on ovarian development and future function.

Classification of environmental pollutant according to their effects: the Adverse Outcome Pathway (AOP) challenge

At the cellular level, several morphogenetic consequences of endocrine disruption have been described, including alteration of: (i) cell determination and differentiation, (ii) development and growth of the organ, (iii) cell, and more specifically germ cell proliferation.

Many human-made or natural chemicals displaying estrogenic properties have been studied in animal models. However, the mechanisms of action on the ovary are often hypothesised rather than demonstrated. Transcriptional studies following prenatal exposure to estrogenic compounds have begun to unravel their mechanisms of action [15-19]. Endocrine disruption is a first-line readout of exogenous compound effects on the fetal testis. This is poorly investigated in the ovary where alterations in prenatal endocrine activity are rarely studied [20]. While this is understandable in rodents, the steroidogenic capabilities of the human fetal ovary are well known [21].

At the subcellular level, exogenous compounds can trigger effects such as the formation of reactive oxygen species, alterations of lipid and protein structures, and DNA damage [22,23]. The classical readout of such damage is DNA quality, including both its integrity and epigenetic alterations (defined as inheritable modifications without alterations of the genetic sequence). Epigenetic regulation is crucial in physiological processes and several studies show xenobiotic-induced epigenetic alterations in laboratory models [24,25]. Importantly, epigenetic marks play roles in regulating expression of crucial genes at specific timepoints of development. For the germ cell lineage this is vital since these modifications could be transmitted to the next generation, leading to intergenerational or even transgenerational effects. While epigenetic effects rely on several mechanisms, such as DNA methylation, histone modifications and interfering non-coding RNA, DNA methylation alterations by environmental exposures remains the most studied [26]. Germ cell meiotic commitment and progression are key morphological events that are sensitive to xenobiotics, especially estrogenic compounds (e.g. BPA)* [27,28]. Discrepancies between studies may be explained by differences in the routes of exposure and by interspecies difference in patterns of expression of estrogen receptor variants [29-31]. The challenge of epigenetic alterations in the fetal ovary is the interference by xenobiotics with methylation status or physiological demethylation/remethylation processes that takes place during fetal development [32]. This is also true for DNA damage and repair [33,34].

The germ cell lineage, a vector of long-term effects on subsequent generations, is often in the crosshairs of these studies, the somatic cell lineage being mostly left in the shade. The lack of interest in the study of epigenetic alterations on the somatic lineage is unfortunate considering the tight relationship between somatic and germ cell lineages, and therefore the possible indirect effects of the alteration of one cell lineage on events such as primordial follicle formation. Overall, the single cell ‘omics revolution has, unfortunately, had limited contact with fetal ovarian toxicological studies.

Classification of pollutants according to their molecular mechanisms of action is extremely delicate and requires large-scale studies at the transcript and/or the protein levels. These studies are essential for AOP description. The principle of AOP is to define a series of events initiated by a molecular initiating event (MIE) that ultimately leads to adverse effects in the function of a given organ and can be induced by multiple exogenous compounds. Surprisingly, to date, very few AOPs describe the ovary [35] and the best established in shown in Fig. 1 (e.g. diisobutyl phthalate). One reason is that mechanisms of action of xenobiotics on the fetal ovary are poorly understood. Animal models have provided many correlations between fetal exposures to toxicants and long-term adverse effects on the ovary, such as early decrease of the follicle reserve. Indeed, premature ovarian insufficiency (POI, e.g. phthalates, polychlorinated biphenyls and organochlorine pesticides) [36], polycystic ovarian syndrome (PCOS, e.g. perfluorooctanoic acid), infertility (e..g phthalates, organophosphate pesticides), delayed or precocious puberty are commonly investigated in rodents in relation to fetal exposures [37-39], unlike ovarian cancer and endometriosis (e,g. polybrominated diphenyl ethers).

Classification of environmental pollutant according to their chemistry: the QSAR challenge

Typically, xenobiotics triggering an impact on the ovary are categorised as either estrogenic or antiandrogenic, and these properties were used to design experimental strategies for mixtures [40]. *In silico* quantitative structure-activity relationship (QSAR) models were recently introduced as a way to identify chemicals most likely to be harmful based on their similarities [41-44] with chemical structures of characterised compounds. While well accepted that estrogenic compounds display various estrogenic potencies (e.g. Bisphenol A) [45], this is not always taken into account for compounds with other activities. In addition, the question of the dose is crucial because one compound can display multiple characteristics, and properties can vary along concentration ranges. Classification of chemicals according to their chemical family, their structure, or their known targets, is hazardous when challenging their expected effect on a complex organ such as the developing ovary. A further complication is that receptor endowments can vary between different cell types and stages of development. All these parameters must be taken into account in study design and interpretation. So far no ovarian chemical risk has been identified directly from QSAR.

Classification of environmental pollutant according to windows of sensitivity

In rodents, four specific time windows of heightened ovary sensitivity to disruption by exogenous insults have been identified: (i) gonadal sex determination (e.g. paracetamol, tamoxifen), (ii) meiotic division (e.g. bisphenol A, atrazine), (iii) follicle assembly (e.g. polycyclic aromatic hydrocarbons, genistein) and (iv) the first wave of follicle recruitment (e.g. benzo[a]pyrene, Di (2-ethylhexyl) phthalate) [46]. While almost synchronous for all germ cells in small laboratory rodents, morphogenetic processes of ovarian differentiation overlap in larger mammals and humans. Asynchronicity of processes make the precise identification of sensitive windows difficult and they may more likely correspond to morphogenetic events rather than developmental time periods (e.g. humans, sheep, monkey). Follicles form in rodent models, like mice and rats, shortly after birth when the circulating levels of estrogens drop to nadir, while in humans, and large mammal models, they assemble in the womb in a high (human) or relatively high (sheep) estrogenic endocrine environment (Fig. 2). Therefore, exposure to estrogenic compounds via the mother do not necessarily target identical processes. A critical challenge, especially in the case of endocrine disruption, is to determine whether a given pollutant will have similar effects on specific process in different species. This has to be taken into account in the choice of the appropriate time window/s according to the suspected process/es targeted by the chemical/s of interest.

Discussion

The weight of evidence that female reproductive disorders partly result from deleterious environmental exposures, such as to EDCs, during prenatal life has been building. These effects represent challenges, not only in terms of technical approaches, but also in data interpretation. Nevertheless, if a decrease in the germ cell “stockpile” may lead to reduced future fertility, we have to be cautious regarding this assertion. Indeed, depending on the severity of the depletion of germ cells, compensatory mechanisms cannot be excluded [47,48]. An additional difficulty is the range of key factors subsequently affecting fertility, for instance, the quality as well as number of oocytes and follicles are key components of female reproductive function. The complexity of studying female reproductive function comes from the fact that it does not rely only on ovarian function and germ cell/follicle reserves, but also on relationships between ovarian cell types and their functions (e.g. steroidogenesis) and with organs such as the hypothalamo-pituitary-ovary axis, liver and adrenal gland, as well as with the placenta in fetal life.

An additional dimension of the challenges of studying exposure of the fetal ovary to EDCs is the complexity of mechanisms involved: will the EDC be toxic for the germ cell lineage, alter

fetal endocrine function, disturb somatic cell lineage programming, dysregulate formation of follicles and generate epigenetic alterations? Thus, endocrine disruption could be a direct result of exposure, or an indirect, long-lasting, adverse effect on the endocrine cell lineage. Fetal ovarian cultures present difficulties beyond the small number of endpoints currently in the experimental toolbox. These include relatively small organ size and the limited number of conditions that can be addressed simultaneously, which, together with the current outdated toxicological guidelines, are relatively insensitive to detect disturbances that might lead to long-term effects. These will limit the number of fetal ovarian studies of *in utero* exposure to pollutants, including EDCs, published, especially in the human, exacerbating the problem by adding publication bias. A consequence is the potential misinterpretation of the level of risk a particular toxicant might pose to the female fetus and her future fertility.

Footnote to go after and close to line 185

*For ease of reading, (e.g. text) is used to provide examples of endocrine diruptors associated with the process under discussion.

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Annotations

[1] This review highlights not only the complexity of the chemical exposome but also the challenges in assessing its effects in an integrative way.

[4] In this review, the authors provide an overview of the prenatal chemical exposome. They pinpoint the knowledge gaps that are still to be overcome in order to better characterise the exposome and thus link it with fetal outcomes.

[13] The integrated microfluidic platform set up by the authors supports, not only follicle maturation and differentiation, but also dynamic hormonal secretion through an extended period of time. This model represents significant technical progress that can be used for the integrative understanding of the female reproductive physiology and also as a more representative model for pharmacology and toxicology studies.

[18] This study provides a mechanistic insights into the understanding of premature ovarian failure following cyclophosphamide exposure and demonstrate that it occurs through alteration of the steroid biosynthesis pathway.

[30] The authors review the epigenetic effects of several endocrine disruptors on different components of the reproductive system. These could explain the multigenerational, and even transgenerational effects, that are observed following exposures.

[33] From a molecular point of view, the authors review the mechanisms underlying the DNA repair system which is such crucial for the oocyte quality. It provides a clear overview on the possible targets that need to be to investigated when addressing xenobiotic effects, especially when they are known to be associated with multigenerational effects.

[35] Suggesting new avenues to investigate causes of female reproductive disorders, this review pinpoints the urgent need for an intensified work towards characterising female reproductive adverse outcomes pathways. Such pathways are an essential basis of robust policy formulation.

Figure legends

Figure 1 : AOP7 from the AOPwiki was mainly established from rodent data and epidemiological studies, illustrating the importance of cross-sectional studies for the development of such AOPs.

This well established AOP presents the peroxisome proliferator activated receptor gamma (PPARγ) activation as the molecular event that leads to ovarian cycle irregularity and impaired fertility in adult females, and describes the key events leading to this adverse outcome. The ovarian cycle irregularity that ultimately causes impaired fertility following the initiating PPARγ activation, is dependent upon a reduction inf aromatase levels that lead to lowered circulating estradiol (E2) levels. Adapted from the AOPwiki at https ://aopwiki.org/aops/7.

Newly formulated putative AOPs for endocrine disruption of the ovary are given in reference [35] as part of the FREIA project outputs (http://freiaproject.eu/wp/).

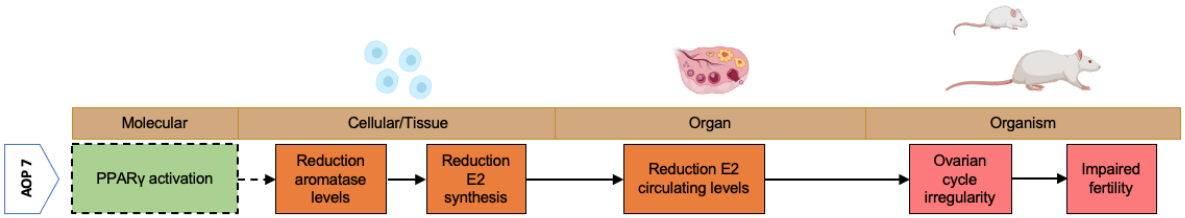


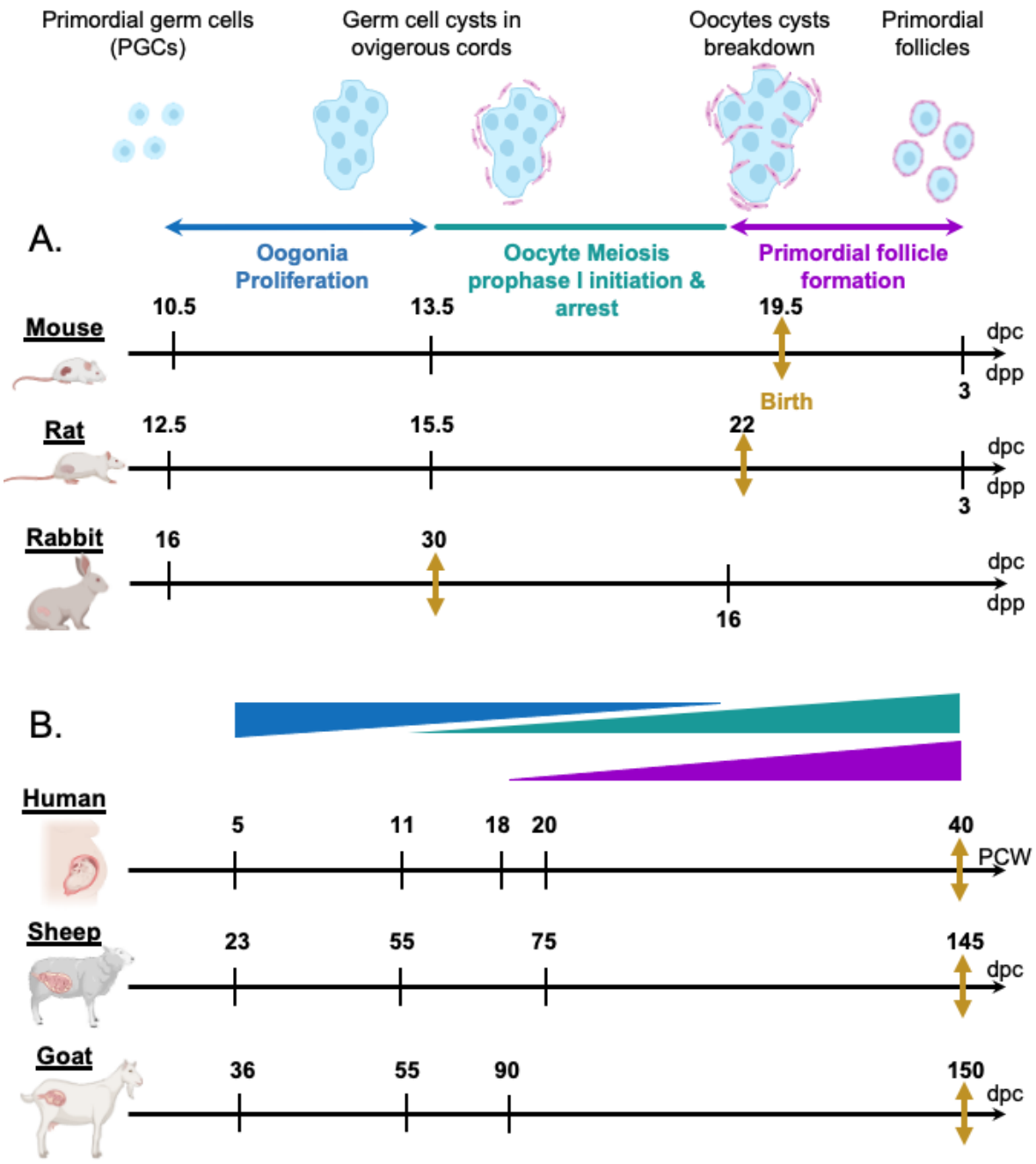
Figure 2: Comparison of the sequence of morphogenetic events occurring during fetal and/or neonatal development in the ovaries of different mammalian species.

Representative timelines of germ cells cyst formation by rounds of incomplete mitosis, meiotic onset and arrest in diplotene stage of prophase I and primordial follicle formation in rodents (mouse and rat), rabbit (A), and human, sheep and goat (B).

Although the overall sequence of morphogenetic events culminating in the formation of primordial follicles is similar in mammals, timing of specific events differs between species. Indeed, after differentiating in an extra-embryonic territory (5 dpc in mouse, 9 dpc in rabbit), primordial germ cells settle the genital ridge at about 10.5 dpc in mice, 12.5 dpc in rat, 5 DW in humans, 23 dpc in sheep and before 36 dpc in goat. By 16 dpc, most germ cells have entered the gonad in rabbit. During their migration and after they enter the gonad, germ cells undergo a series of incomplete mitotic divisions allowing germ cells cysts formation. As a sign of ovarian differentiation, germ cells then cease mitosis to enter in a synchronous manner in meiosis I at 13.5 dpc in mouse, 16.5 dpc in rat and around birth in rabbit. Finally, after meiosis, oocytes cysts breakdown to form primordial follicle pool, from shortly before birth in mouse, after birth in rat and from 16 dpp in rabbit (Fig 1A). While rodents and rabbit germ cells enter synchronously in the key steps of their differentiation, in human, sheep and goat several germ cells population coexist at a given timepoint. Indeed, while some germ cells keep proliferating until 20 DW in humans and 90 dpc in sheep, others enter meiosis as early as 11 DW and 55 dpc in humans and goat, respectively. In sheep, meiosis I onset occurs at 55 dpc and these cells are increasingly prevalent by 75 dpc. The first primordial follicles are observed at mid gestation (around 16 DW) in human, 75 dpc in sheep and 90 dpc in goat. Besides, while in humans mainly primordial and anecdotic primary follicles can be found in the ovary at birth, in sheep and goat, folliculogenesis occurs during fetal life. (in sheep, first primary and first antral follicles observed at 100 dpc and 135 dpc, respectively)

PCW: post-conceptional weeks; dpc: days post-conception, dpp: days post-partum.

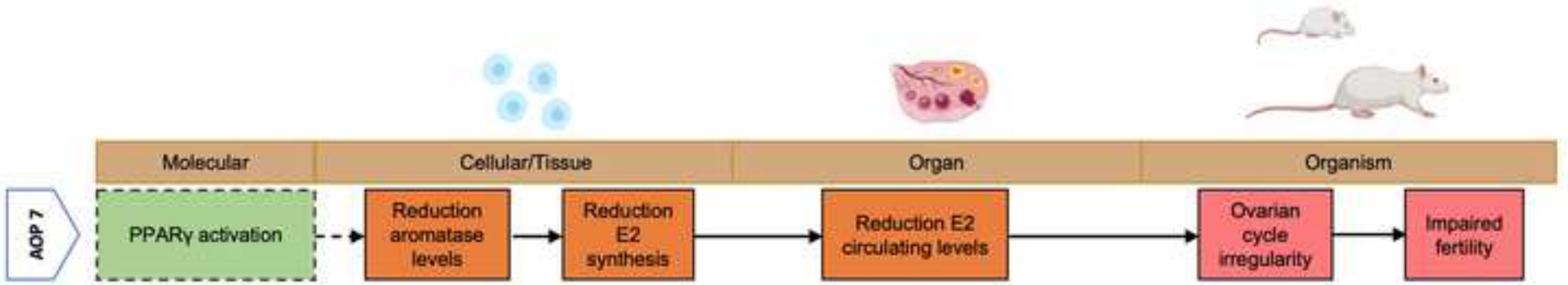
(Drawings were edited from BioRender.com)


Primordial germ cells (PGCs)
Germ cell cysts in ovigerous cords
Oocytes cysts breakdown
Primordial follicles
A.
Oogonia Proliferation
Oocyte Meiosis prophase I initiation & arrest
Primordial follicle formation
Mouse
10.5
13.5
19.5
Birth
3
dpc
dpp
Rat
12.5
15.5
22
3
dpc
dpp
Rabbit
16
30
16
dpc
dpp
B.
Human
5
11
18
20
40
PCW
Sheep
23
55
75
145
dpc
Goat
36
55
90
150
dpc

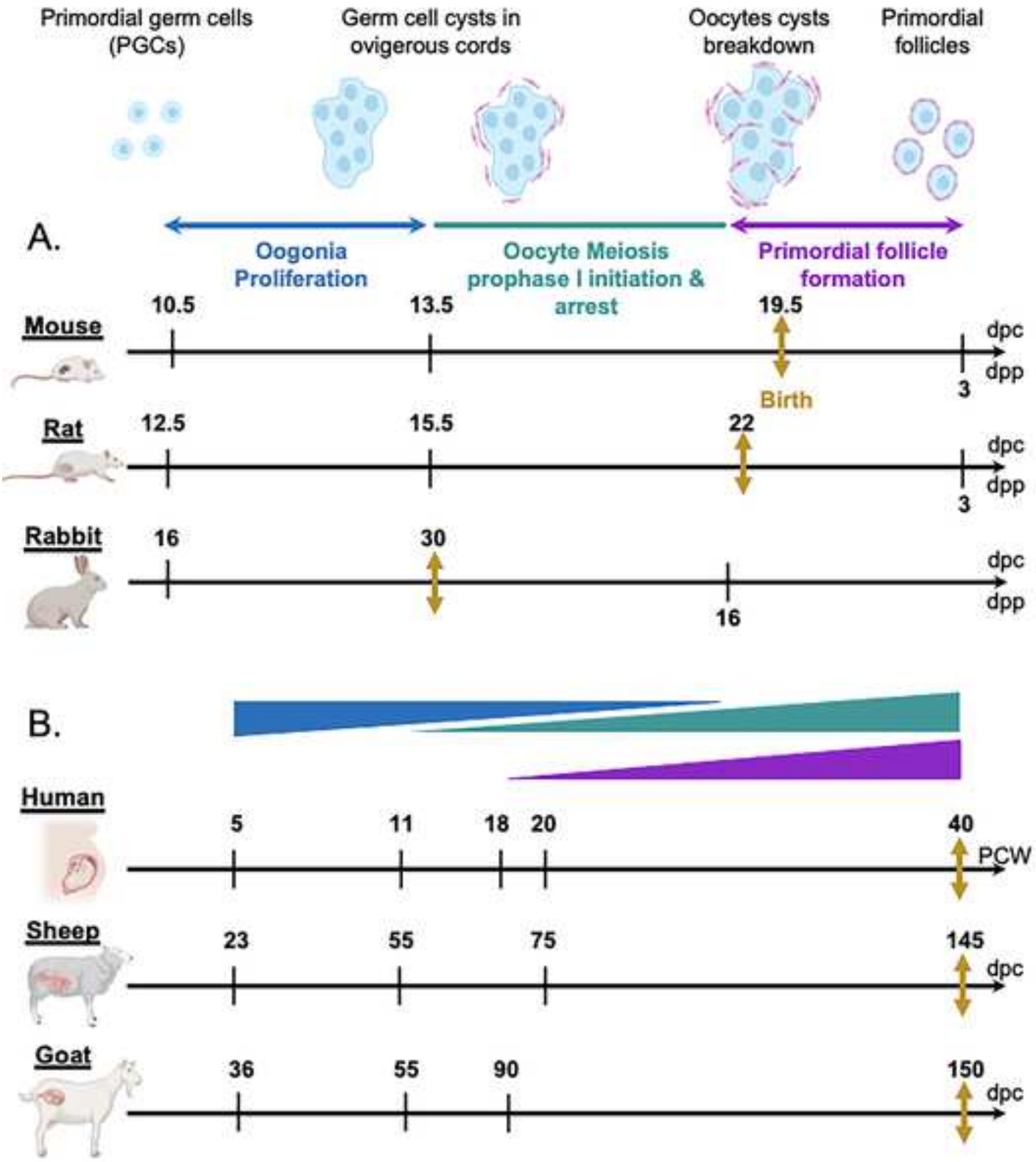
503
504

Figure

Click here to access/download;Figure;Fig1.jpg



Figure



The mammalian ovary: concerns about evaluation of prenatal environmental exposures.

Response to reviewers' and editors comments (shown in bold with our responses shown in plain text.

COMMENTS FROM THE EDITORS AND REVIEWERS:

Many thanks for the submission, which I arranged to have reviewed while the problems with upload were being sorted out. This is a very interesting review, covering an important topic well within what is, I appreciate, a short format.

Comments from the reviewer are immediately below (since obtained earlier).
Please also note the typographical error on line 229 ('i9n')

Thank you.

Reviewer comments:

The review by Lecante et al. is on an interesting and novel topic. Below are specific comments and suggestions for improvement.

We are grateful to the reviewer and editors for their assessments and interest in the manuscript, which we appreciate.

1. Line 41 should be re-written because it is not proper grammar to end a sentence with a preposition (i.e., to).

Sentence revised as suggested, now lines 41-43.

2. The authors should not capitalize words that are not proper names of persons, places, and things. For example, testicular dysgenesis syndrome should not be capitalized.

Capitalisation removed throughout, where appropriate, as suggested.

3. The authors should provide specific examples of EDCs that have been classified by AOP, QSAR, and windows of sensitivity.

We have indicated example EDCs throughout as suggested.

4. The authors should consider a schematic depicting the different pathways described in the review.

The accepted ovary AOP has been included and direct reference to a series of putative AOPs made. This is in Fig. 1. The previous Fig. 1 has been changed into Fig. 2.