Human-Relevant NAMs for Developmental Toxicity Testing

Subjects: Public, Environmental & Occupational Health

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Developmental toxicity testing urgently requires the implementation of human-relevant new approach methodologies (NAMs) that better recapitulate the peculiar nature of human physiology during pregnancy, especially the placenta and the maternal/fetal interface, which represent a key stage for human lifelong health. Fit-for-purpose NAMs for the placental–fetal interface are desirable to improve the biological knowledge of environmental exposure at the molecular level and to reduce the high cost, time and ethical impact of animal studies.

Keywords: new approach methodologies (NAMs) ; adverse outcome pathways (AOPs) ; endocrine disruptors

1. Human-Relevant NAMs for Placenta Toxicology

One aspect sometimes overlooked in developmental toxicity testing is that fetuses are exposed to chemicals through their mothers; thus, women's exposure, their metabolism and placental health are critical issues to be considered. In the Organization for Economic Co-operation and Development (OECD) test guidelines (TG) 414, no endpoint regarding placenta is included while its full functionality is essential for the correct fetus development.

The placenta is a transient organ with a relevant role as a barrier protecting the fetus and as an exchanging surface for nutrients, gases and metabolites [1]. However, many chemicals and/or their metabolites might pass this barrier, with potential health consequences for a safe gestation. Further, placenta is metabolically competent, thus playing an active role in metabolizing and delivering the chemicals from the mother to the exposed embryo/fetus. Importantly, placenta transfer may substantially differ among species, both for anatomical and metabolic reasons, making results obtained in animal models hardly transferrable to humans [2]. Indeed, compared to the hemodichorial and hemotrichorial placentas of rabbits and rodents, respectively, the human placenta is hemomonochorial and consists of the syncytiotrophoblast, which is in direct contact to the maternal blood, a reduced number of cytotrophoblast cells, stroma cells and the endothelium of the fetal capillaries [2][3]. Further, placenta has endocrine activity, thus representing itself a target organ for endocrine disrupting chemicals (EDCs) [1].

For these reasons, it is necessary to use and implement human placenta models that more closely recapitulate placenta physiology, therefore representing elective candidates for maternal and developmental toxicity testing.

1.1. Ex Vivo Dual Side Human Placenta Perfusion

The most advanced available model is the ex vivo human placenta perfusion, a unique model that allows biological and toxicological testing in an intact and vital human organ outside the body. The major aim of this method is to test the transfer of substances through the placenta, mostly from the mother to the fetus, but also vice versa. The placenta can be obtained immediately after delivery and installed in the perfusion system within an ischemia time of less than 30 min. Routinely, it can be maintained completely functional for 6 h, which can be assessed by numerous parameters [4]. The method was invented and established in the late 1960s [5]. Since then, especially the equipment, the standardization and the medium composition have improved.

Previous and recent original studies have focused on the transfer of drugs, but the transfer of a variety of environmental chemicals, including EDCs, has also been evaluated. A recent review summarized the studies performed so far grouping the chemicals on the basis of their feto–maternal ratio transfer [6]. Such a parameter is generally obtained comparing the transfer rate with an internal quality marker for valid perfusion experiments such as antipyrine or creatinine that are spiked into the maternal circuit and have a specific transfer kinetic to the fetal side. Compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), glucoronated bisphenol A (BPA), perfluorinated compounds (PFC), glyphosate,

medium-high molecular weight polybrominated diphenyl ethers (e.g., BDE-99 and -209) and some phthalate metabolites have been classified as having low transfer rate, whereas free BPA, BDE-47, genistein and, most of all, parabens, benzo[a]pyrene and acrylamide, have high transfer rates [6]. Being a metabolically competent organ, placenta may detoxify or even toxify compounds passing the barrier; thus, it is highly relevant to assess which metabolites and parental compounds have passed on the fetal side, for example, by metabolomics analysis.

Of course, a lower transfer rate does not mean the compound is less toxic; rather, it may affect placenta functionality. Noteworthy, the placenta perfusion model also allows assessing the influence of external factors on physiological functions of the placenta such as transport enzymes or hormone production [7][8].

The large volume of the perfusion circuits allows frequent sampling (e.g., every 15 min for up to 6 h) for analysis of pH, glucose consumption and lactate production as metabolic controls and β-hCG release, the main pregnancy hormone, as a measure for functionality of hormone production in the syncytiotrophoblast. The concentration of the test substance with the questionable placenta transfer, its metabolites or secondary mediators can be analyzed at the same or another frequency in the circuits. At the end of perfusion, the perfused tissue can be used for any kind of further biochemical or microscopical investigation assessing endpoints of placenta functionality.

The advantage of ex vivo human placenta perfusion is that it is very near to the in vivo functionality of the organ, which allows highly reliable results. Especially compared to animal experiments, the relevance for human medicine and toxicology is far higher, as placenta structure, size and functions vary immensely between species. However, it has to be taken into account that the model uses term placenta, which may differ from first or second trimester placenta as regards metabolism, endocrine activity and placental layers' development. A disadvantage of the model is the enormous time consumption to complete valid sets of experiments and the need of a well-trained team and well-established cooperation with the delivery room [9]. Indeed, ex vivo human placenta perfusion is still a challenging method that is difficult to apply and with a low rate of successful experiments, as the placentas often have defects not visible macroscopically and the performing scientists and technicians need an intensive training period.

1.2. Human Placenta Ex Vivo Explant Cultures

A level down, to using the whole organ and in between the common usage of cell lines or organoids, is the establishment of tissue cultures by explanting freshly harvested compartments of assembled cells still integrated in their in vivo microenvironment [10][11].

The main challenge in this regard is the long-time culturing in order to be accepted as an equivalent alternative to animal testing. As mentioned above, human placenta is the most readily available human organ and can be transferred to in vitro culture within minimal time after birth, to keep the in vivo situation stable without major changes in temperature, nutrition or microenvironment. In the past, the first placental explants to study transport mechanisms were prepared as 0.5 mm slices and cultured over two hours [12]. Experimental improvements helped to prolong the duration of viable cultivation and to maintain placental characteristics, such as hormone production and villous morphology, up to 48 or 72 h but rarely longer [13]. Automatized sampling and long-term culture of human ex vivo explant cultures in different toxicological applications can be afforded by using, for example, a microfluidic system and TissGrid® 3D culture scaffolds [14].

Compared to cell or organoid cultures, placental explants have the advantages of being original primary tissue containing different cell types morphologically assembled as in the in vivo microenvironment and consisting of the typical placental barrier formed by syncytiotrophoblasts, cytotrophoblast cells and the endothelium of the fetal capillaries. Moreover, all surrounding cell types, such as stroma and immune cells, including Hofbauer cells, are preserved and can be investigated in culture over a long time (up to 28 days), although the different cell types have different behavior and viability over time [15]. After a few days of regeneration, the syncytiotrophoblast re-establishes [16] and produces hormones such as estradiol, progesterone or β-hCG. By adding different substances or drugs, toxic effects can be observed much longer than by the ex vivo placenta perfusion. Still, the explant culture system is comparably cost-effective and a suitable system for translational research.

Further research is needed to evaluate the intra- and inter-individual variations of placentas and explants; however, these variations represent an advantage in toxicological studies since they reflect the variety among human individuals. Placenta explant studies offer the option of substituting animal experiments not only for testing acute toxic effects but also for mid- to long-term reactions.

2. Human-Relevant NAMs of Amniotic Membranes

The developing embryo/fetus, other than being protected and fed by placenta, is also surrounded by amniotic fluid and membrane, whose inclusion and consideration in developmental toxicity testing is mostly neglected. The human amniotic membrane is of embryonic origin, and it is the innermost of the fetal membranes. It is in direct contact with the amniotic fluid via one of its two cell populations, a layer of human amniotic membrane epithelial cells (hAEC). The second cell population, the human amniotic membrane mesenchymal stromal cells (hAMSC), is interspersed within a rich extracellular matrix layer [17]. Due to the continuous cross-talk with both placenta and fetus, exposure to chemicals of amniotic membranes may cause inflammation of fetal membranes with drastic effects on pregnancy and fetus development and with possible consequences later in life. Indeed, an aberrant release of cytokines can be toxic to organs such as brain, leading to preterm brain injury. Effects on isolated human and murine amnion epithelial cells have been described following exposure to: (1) tributyltin, which impairs the amniotic membrane through the disruption of tight junctions [18]; (2) polychlorinated biphenyl (PCB) 50 congener, which may lead to inflammation in fetal membranes [19]; (3) BDE-47 and -99, which may lead to induction of cellular senescence in vitro, possibly through oxidative stress [20].

It is relevant to set up reliable in vitro models of human amniotic membranes to understand the pathophysiology of many pregnancy and fetal complications induced by exposure to chemicals. However, the cell source to use as a model is still a matter of discussion. In fact, it was recently discovered that the transformed epithelial FL cell line was misidentified and indeed is a HeLa derivative (https://www.atcc.org/products/ccl-62, accessed on 18 August 2022); notwithstanding, they were often used and reported in the scientific literature, also for toxicology studies [21][22]. While considering human stem cells, the pluripotency potential of human cells derived from fetal annexes and human induced pluripotent stem cells (iPSCs) has been compared, and differences at epigenetic level were highlighted [23]. Further, several attempts have been made to develop in vitro models represented by amniotic cells, explant cultures (floating or in transwell) [24], and strategies towards an amniotic membrane on a chip [25].

A substantial improvement has been obtained by separating the two relevant sub-regions of the human amniotic membrane, the placental and the reflected amnion, demonstrating that these areas respond to distinct sub-regional differences including metabolic and secretory parameters [26][27][28], thus possibly representing different targets displaying different outcomes following exposure to chemicals. Moreover, to overcome the loss of viability and functionality during cultivation of floating membrane biopsies, the system has been distended, obtaining an extension of the life span and the physiological function in vitro for up to 21 days [29]. This may represent the most promising amniotic membrane model whose implementation and adoption should be recommended for a useful in vitro tool to test the impact of environmental pollutants, such as EDCs or nanoparticles, on mitochondrial status, inflammatory status, senescence and apoptosis and parameters of membrane rupture, still poorly investigated.

3. Human-Relevant NAMs for Embryonic/Fetal Toxicology

The main goal for developmental toxicity testing is represented by the direct assessment of exposure effects of chemicals on the fetal/embryo development of organs in a precise window of gestation before birth. The OECD TG 414 only considers the evaluation of macroscopic endpoints, but the application of alternative new approach methodologies (NAMs) varying from morphological, to proliferation, metabolomics, proteomics, epigenomics and transcriptomics, till to functional endpoints related to the cell type and assay used will expand the range of endpoints, still complying with the relevant adverse outcomes of TG 414.

Conceptually, both models allow dissecting the direct effects of toxicants on the isolated developing organs avoiding any systemic effect and interaction with other organs during embryogenesis. Only an ex vivo whole embryo culture would retain the systemic interactions between organs as well as better resemble the way of toxicant exposure. Since an ex vivo human embryo system cannot be set up for evident ethical reasons, a zebrafish embryo teratogenicity assay has recently been established as an alternative method to predict human developmental toxicity as a whole organism [30].

3.1. In Vitro Cultures of ESCs and iPSCs and Derived 3D Organotypic Models

Human embryonic development covers the first 8 weeks of gestation. hESCs are pluripotent cells that can be isolated from the inner cell mass of the blastocyst before implantation, retaining unlimited ability of self-renewal and of differentiating into all embryonic tissues and extra-embryonic annexes. Since their isolation causes the destruction of the blastocyst, their use is strictly regulated and impaired by ethical and clinical concerns; moreover, in vitro cultures may result in teratomas. To overcome these restraints, iPSCs have been developed by reprogramming adult somatic cells to a pluripotent stage through retroviral transduction of specific genes according to the Yamanaka's protocols [31].

The majority of the studies adopting in vitro cell systems based on hESCs and hiPSCs have been focused on neuro- and cardio-toxicology [32][33][34]. For the developing brain and heart, where the complexity of the cellular composition and of the spatial organization of the organ can affect the functionality as well as the sensitivity to toxicant insults, developmental toxicology research has incredibly benefitted from the establishment of 3D organized structures called spheroids or organoids [33][34]. Three-dimensional gastruloids, aggregates of embryonic stem cells that recapitulate key aspects of gastrula-stage embryos, have emerged as a powerful tool to study early stages post-implantation development in vitro [35][36]. As gastrulation is the checkpoint for the body formation plan during embryogenesis [37], this new 3D model is rapidly affirming as a validated model to test developmental toxicity of chemical exposure [38].

Although promising, these iPSC-based assays have not yet become the gold standard for developmental toxicity testing. Indeed, assessment of the adverse impact of new chemicals on embryogenesis by these approaches is not yet recommended by regulatory agencies. Reports from the International Council for Harmonization's guideline on Detection of Toxicity to Reproduction for Human Pharmaceuticals generally recommend the use of in vitro assays for developmental toxicity assessment but with no endorsement of any specific stem cell assay [39]. Thus, validation of the application of these models for developmental toxicity testing is still far from being achieved, a condition that makes stakeholders, including regulatory agencies and chemical companies, not yet able to adopt any specific stem cell-based assays as part of the decision-making process in developmental toxicity assessment.

The best in vitro assays using iPSCs should define the inhibitory impact of the chemical exposure on viability, differentiation or cell functions as a sign of developmental toxicity. Many types of developmental toxicity assays have been reported until now for ESC and iPSCs, based on the ability to differentiate towards a variety of cell types, including neurons and osteoblasts [40]. Morphological and molecular techniques to measure the adverse impact on cell differentiation are used to constantly improve the sensitivity and specificity of the test.

One of the typical morphological endpoints for developmental neurotoxicity (DNT) is the formation of rosette structures that are an in vitro indicator of the successful closure of the neural tube during development [41]. Moreover, demyelination represents a relevant functional DNT endpoint, as axon myelination in the central nervous system starts early during the first months of embryogenesis and is one of the key events of brain development [42]. Some in vitro human neural models have been developed for DNT studies, as comprehensively reviewed elsewhere [43][44]. The most challenging aspect for these models is to reproduce in vitro cell population heterogeneity (neurons, astrocytes, oligodendrocytes and microglia) in a 3D cytoarchitecture that can mimic the brain's physiological activity and in the case of DNT studies, the specific developmental stage of exposure. hiPSCs can be functionally differentiated into cell lineages organized in organoids comparable to brain development in utero [45]. Oligodendrocyte spheroids and oligodendrocyte precursors have been obtained from iPSCs for specifically studying the myelination process [45][46][47][48], while 3D models have been generated from hESCs reproducing in vitro brain structures consisting of mixed populations of neurons, astrocytes and oligodendrocytes with immature myelin sheaths [49].

An innovative 3D model of human brain (mini brains) has recently been developed from human iPSCs [50]. These organoids, named BrainSpheres, can recapitulate several morphologic, architectural and functional aspects of the human brain as they are formed by a mixed population of neurons, astrocytes and oligodendrocytes with high level of myelinization and spontaneous electrical activity; for these characteristics, they are extensively used for DNT studies and neurodegenerative diseases [51][52][53]. This model is particularly interesting as it can also be co-cultured in a transwell system with the endothelial cell barrier to test the differential penetration ability of the toxicants [54]. Moreover, it has recently been used to perform the first developmental toxicology screening for reference compounds on the myelination process of oligodendrocyte differentiation, based on an extensive search of the neurodevelopmental toxicity literature [53].

The PluriBeat assay has been set up to test cardiotoxicity by developing 3D cultures of human iPSCs (hiPSCs), termed embryoid bodies, which model the human blastocyst and can be differentiated into beating cardiomyocytes undergoing molecular events similar to those of the developing embryo [55]. More recently, in vitro mixed cell systems for assessing chemical toxicity on mesoderm and endoderm-derived organs during embryo development have been established [40] as highlighted in the last update of the DevTox database [56], extending the horizon of in vitro developmental toxicity testing. For an updated and exhaustive analysis of the methods and type of tests adopted, see Mennen et al., 2022 [40].

A pivotal aspect that should be considered when working with differentiated cells from iPSCs or embryonic stem cells (ESCs) is that there could be a temporal and developmental gap between the differentiated cell system and the in vivo developmental window sensitive to adverse effects exerted by the chemicals to be tested. Indeed, the protocols of reprogramming and differentiation are currently not sophisticated enough to allow differentiation to a temporal extent of embryogenesis [31][57][58]. In addition, it is current opinion that each organ and axis, in particular the endocrine ones, have

a specific temporal window of sensitivity to chemicals that accounts for any difference in the toxic effects measured. These sensitive windows also differ between humans and other animal models [59] and may also be affected by genetic sex as influenced by sex hormone production. Thus, in vitro differentiated ESCs or iPSCs may not reflect the sensitivity stage of development, thus potentially affecting the validity of the obtained toxicity results.

3.2. Primary Fetal Cell Culture Models

Fetal primary cell cultures from specific tissues or organs have been obtained from abortive material (weeks 9–12 of gestation) and can be cultured in vitro in monolayer for a longer period compared to 3D organoids without losing their properties (functional activities, differentiation potential, stem and proliferative properties), their phenotypic, as well as their genetic profile. Since the cells are isolated from waste abortive material, their use raises no major ethical concerns; thus, they could represent promising and valuable in vitro systems for developmental toxicity testing. However, until now, they have mainly been used in pharmacological studies and not yet validated for toxicity screening at a regulatory level.

Several different fetal cell populations derived from the developing central nervous system and neural crest have been characterized so far. Neuroblast cell populations (FNC-B4) have been derived from a fetal olfactory epithelium and extensively characterized in in vitro cultures to respond to sex steroids, kisspeptin and odorants by secreting gonadotropin-releasing hormone in both physiologic and pathophysiologic conditions [60][61][62][63][64]. They markedly respond to estrogens, androgens and thyroid hormones [65][66][67], thus making them a potentially relevant endocrine model to assess developmental effects of EDCs, as recently shown for the widespread persistent organic pollutant Benzo[a]pyrene [68]. To study cholinergic response in human developing central nervous system, a population of immature cholinergic neurons has been characterized from the Nucleus Basalis of Meynert in the basal forebrain [69].

A neuroendocrine population has been isolated from the trunk region of the neural crest of human fetuses at the edge of the forming neural tube. Their secretory and expression profile confirmed they retained in vitro the ability to differentiate toward the sympatho-adrenal phenotype, reflecting their in vivo destiny to migrate and populate the medullary catecholamine-secreting core of the adrenal [70].

Inducible inflammatory response and active cytokine secretory activity have been characterized in fetal cardiomyocytes [71] and skeletal muscle cells [72].

Finally, the establishment of human smooth muscle cell cultures from fetal male external genitalia enabled people to study the androgen and estrogen effects on cell proliferation and contractility [73], being also implied to assess adverse effects of PCBs as risk factors of developmental external genitalia diseases such as hypo- and epispadias [74].

To complete the scenario of the current available in vitro primary human fetal cell models that may be applied to developmental toxicological studies, a cell model of adipose fetal precursor retaining in vitro the adipogenic potential versus brown adipocytes was established [75]. This model could represent a unique system to investigate the developmental basis of metabolic pathologies such as obesity, lipodystrophy and diabetes.

These cell models, together with the one obtained from fetal adrenals [76], enrich the fetal adrenal cell systems to be used for investigating in vitro the possible prenatal alterations that may result in the development of chronic pathologies later in adult life [77].

3.3. Organotypic Fetal Models

Organotypic culture models can also be obtained by ex vivo maintenance of tissue or organ explants obtained during embryo development or can consist of 3D spheroids/organoids cultured in vitro and derived from spatial aggregation of cell populations derived from human fetal material. In both cases, organs or cells can be isolated from abortive material discarded in elective legal termination of pregnancy during the first trimester of pregnancy.

In the 3D organization, cell–cell spatial interactions are maintained, thus representing more realistically the physiological conditions compared to conventional cell cultures and being more suitable to test the effects of toxicants on embryo/fetus development. Valuable examples of these models have recently been obtained for developing human adrenals starting from fetal explants [78], or as in vitro-induced organoids derived from primary cells isolated from fetal adrenals [76]. Both 3D models can be cultured in vitro in static conditions (no microfluidic system) up to 10–15 days and maintain functional endocrine activity without gross degeneration, before necrotic processes start. Of note, the organoid (called adrenoid) spontaneously formed from the mixed cell population obtained from dissociation of the fetal adrenal display a spatial

organization and a functional activity (steroidogenic and catecholamine production) resembling the adrenal organ of origin [76].

The effect of steroidogenic inhibition on steroid hormone production in basal conditions and under Adreno Corticotropic Hormone stimulation has been studied on fetal adrenal organotypic culture [79].

A 3D model derived from commercially available fetal human neural progenitor cells (hNPCs) induced in vitro to microsphere has been successfully used to assess the endocrine disrupting effects of PBDEs on oligodendrocyte differentiation [80].

Testis and ovary organotypic cultures have also been established to assess the effect of manipulation of fibroblast growth factor 9 signaling on sex-specific gonadal differentiation, demonstrating that this grow factor is fundamental for the correct developing program for both the germ cell and the steroidogenic compartment [81].

3.4. Remarks

These in vitro experimental approaches, which reduce the complex biological system of the embryo/fetus development to simplified cell or tissue systems, display the advantage of facilitating quantitative measurement of the specific responses to chemical perturbations [82]. However, they have the disadvantage of lacking the cellular/organ interaction and spatial dynamics playing a relevant role in the potential effects of toxicant exposure. Thus, integration of these types of data and their elaboration by computer modeling is mandatory in order to reconstruct such a complexity (as described further on). Further, a single type of assay and readout in vitro, based on a limited representation of embryogenesis and fetus development, would not be able to detect a whole range of chemicals and their mixtures that probably target different embryological steps with different mechanisms. Therefore, the standardization and validation of readouts and assays is pivotal, as well as the careful selection of multiple types of assays to be combined, in particular if developed considering available Adverse Outcome Pathways (AOPs) in an Integrated Approach to Testing and Assessment (IATA) framework. The interpretation of the results obtained by a testing system that is independent from the whole-embryo model and relies on focusing on specific parts should be carefully evaluated to be extrapolated to the in vivo situation.

4. Immortalization of Cellular Models in Developmental Toxicology Testing

Among the characteristics identifying the different cellular models described thus far, there are cell growth and cultivation lifetime, which are critical issues when several developmental toxicity testing are carried out.

As described above, primary cells can be isolated from various tissues of the organisms, and protocols have been established to cultivate many different cell types in vitro. These cells closely reflect the properties of cells in vivo. However, in primary cells, cell growth is tightly controlled by the tumor suppressor genes p53 and the retinoblastoma protein (pRB) [83] and further influenced by the progressive telomere shortening occurring during every replication cycle [84]. Therefore, cell numbers required for industrial or high throughput applications are difficult to obtain using cellular assays based on primary cells. Although primary fetal cells tend to display a higher proliferation capacity compared to their adult counterparts [85], their use in industrial settings may raise ethical concerns, therefore limiting a widespread use.

Standard cell lines do provide the ease of use needed for screening tens of thousands of compounds in a high-throughput manner, due to their unlimited cell proliferation which is achieved by inactivating the above-mentioned safeguard mechanisms. Immortalization can be achieved spontaneously also in primary cells by specific cultivation regimen [86]; however, this method is cumbersome and is only applicable to certain cell types and very inefficiently. The introduction of viral oncogenes such as, e.g., SV40 large T antigen [87], human papilloma virus E6 and E7 genes [88] or adenoviral E1A and E1B genes [89], increased the immortalization efficiency dramatically; however, the drawback is that these genes very often induce genomic instability and thus lead to an altered karyotype in the resulting cell lines. A milder immortalization regimen was achieved when the catalytic subunit of the human telomerase was introduced into human primary cells [90]. In this case, the recombinant hTert maintains the ends of the telomeres, which would otherwise shorten with every DNA replication/cell doubling. As this progressive telomere shortening is circumvented by the ectopic expression of hTert, the primary cells become immortal and, most importantly, maintain functionality [91][92] and often also chromosomal integrity. Although very promising, especially for the expansion of fetal cells [93], the immortalization of hTert regimen is not universally applicable. It rather seems that, especially for adult or for fully differentiated cell types, the activities of additional oncogenes are required [94][95].

In addition to these universally acting immortalization or expansion genes, reports have also shown that specific genes preferably expand or immortalize only restricted cell types such as, e.g., v-myc for macrophages [96], Id1 for keratinocytes

[97], Bcl-xl and Bcl6 for B-cells [98], to name just a few.

Based on these observations, a completely new approach was established which was inspired by that adopted by Yamanaka to identify the iPSC-generating genes [31]. In this setting, a small library of genes was used to identify the most promising gene combination for the immortalization of different cell types. Thereby, not only gene combinations could be identified that lead to the immortalization of the respective cell types, but also these novel established cell lines maintain the cell physiology of the primary cells they were derived from [99]. This approach was used to generate novel physiologically relevant cell systems from human and animal species such as endothelial cells [100], alveolar and airway epithelial cells [101][102][103], osteoblasts [104], mesenchymal stem cells [105], placental cells [106] and thyrocytes [107].

As mentioned above, other approaches such as the in vitro differentiation of iPSCs/ESCs into the desired cell type, or the most recent advancement of organoids, allow the generation of physiologically relevant cells in sufficient quantities. The technologies, advances and potentials have been nicely and comprehensively reviewed elsewhere [108][109]. The major disadvantage of these approaches is that the generation of large numbers of differentiated iPSCs and organoids is extremely expensive, time-consuming, requires sophisticated protocols and thus represents a significant hurdle for the use of such cell systems in routine applications such as developmental toxicity screenings.

5. In Silico Models for Developmental Toxicity

In silico modeling is a board term that comprises several methods relying on computer simulations to estimate or predict different kind of properties. Among them, quantitative structure–activity relationships (QSAR) and read-across are often used to estimate toxicological properties of chemicals from their molecular structure or similar analogues, respectively [110]. Some attempts have been made to use these techniques as a kind of full replacement of the test itself, as it was performed for other quite complex endpoints such as carcinogenicity, so the apical effect is often modeled in such a way that they can be considered non-testing methods [111].

There are currently some QSAR models addressing developmental and reproductive toxicity that have been summarized in a recent review [112]. In general, these models depend on the availability of collections of data for a specific endpoint. In the case of the experimental data, according to the OECD TG 414, the number of substances evaluated is quite limited, thus reducing the effectiveness and capability to develop robust and reliable models. The most common strategy adopted to overcome the data limitation is to develop classification models, meaning that original data are often labelled as toxicant or non-toxicant. This is because it is easier to obtain classification rather than quantitative-predicting models and allows pulling together different data sources. At the same time, the utility of models predicting a toxic or non-toxic label is more limited compared to the original ambition of replacing an experimental test since this kind of information can be useful for screening purposes but not enough to be used for regulatory purposes (such as for the general adaptations allowed in Regulation, Evaluation, Authorization and Restriction of Chemicals (REACH) legislation for dossier preparation).

One of the available classifiers for developmental toxicity is the CAESAR one [113], based on a collection of data for about 200 substances that have been evaluated by experts and labelled as positive or negative according to a risk–benefit analysis on their use during pregnancy. The model is based on a set of molecular descriptors and a random forest as algorithm, and it is publicly available within the VEGAHUB platform (www.vegahub.eu, accessed on 18 August 2022). A quite similar model is also available within the USEPA TEST platform (https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test, accessed on 18 August 2022).

An analogous study was performed by Wu et al. [114] at Procter & Gamble, and the developed model was made available through VEGA and the OECD QSAR Toolbox (https://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm, accessed on 18 August 2022) as a profiler. It covers not only developmental but also reproductive toxicity (DART). The authors collected and critically assessed evidence from the literature about DART effects of chemicals. Compared to CAESAR, which is a machine-learning model, this model is an expert system, since it implements a large collection of rules that have been developed by experts by analyzing the common moieties of toxic compounds and associating them to known mechanisms of developmental and reproductive toxicity such as binders of the retinoic acid receptor. The model considers some tens of skeletons, each of them representing a family of toxicants and, for each skeleton, a number of identified substituents counting more than 180,000 possible substances generated by the different alternative substituents. In the VEGA implementation, a substance is assigned as positive for developmental and/or reproductive toxicity, while the Toolbox profiler flags the compound as associated or not to previously known toxicants.

Being the dataset at the basis of this model skewed for a large presence of toxic compounds, the model is often over-conservative, and the reliability associated to non-toxic predictions (as indicated by the applicability domain) is often low due to the poor representativeness of non-toxic compounds. Therefore, the user should consider this model as a profiler such as in the Toolbox, indicating a possible activity or common clustering features and not for a precise prediction.

Other authors developed their models more linked to animal studies, not necessarily related to OECD TG 414 but also considering higher-tier tests. Classification models have been developed according to the following types of effects: male reproductive toxicity, female reproductive toxicity, fetal dysmorphogenesis, functional toxicity, mortality, growth and newborn behavioral toxicity [115][116]. Authors used the MC4PC algorithm, also called MultiCASE, depending on the versions, identifying fragments (also called toxicophores) associated to the toxic effect. These MC4PC models have lower sensitivity and higher specificity; thus, they behave opposite compared to the Procter & Gamble model. These kinds of models can provide information closer to the animal experiment but still missing dose response information useful for risk assessment.

More recently, interesting modeling efforts explored the combination of chemical information (hundreds of chemical fragments) with biological activity extracted from nearly 2000 toxicological high-throughput screening assays extracted from PubChem and ToxCast [117]. The inputs were used to group assays based on their chemical–mechanistic relationships and identified two clusters where the in vitro assays were enriched with developmental toxic compounds.

Some studies compared the results from different models and discussed in detail the use and interpretation of the results [118][119][120] sometimes applied to specific chemical classes. Overall, as a rule of thumb, the integration of different in silico models may improve the results [121][122][123].

Some available interesting models link developmental toxicity and endocrine disruption [124][125][126], whereas others are specific for endocrine disruption, but these contents will not address them here. Read-across approaches are also used to assess the potential developmental toxicity of chemicals by comparing structure and toxicological features of one or few substances being quite similar to the target compound, thus obtaining a final assessment on the basis of the weight-of-evidence [127]. This latter method was more frequently employed to fulfill standard requirements for developmental toxicity in the dossiers submitted until 2019 for REACH, being chosen for 30% of the studies (while less than 1% used QSAR information) according to the European Chemicals Agency (ECHA) report for the use of alternatives to animal testing [128]. A limitation of read-across is that it relies on already existing data, somehow limiting its applicability.

A one-to-one replacement of an animal testing with a NAM is often not feasible for very complex endpoints. Therefore, an interesting contribution of in silico methods is in their support to experimental in vitro NAMs to help in covering the gaps compared to in vivo experiments. Indeed, QSAR models and other computational models such as PBK (Pharmacologically Based Kinetic) models can be used to better inform on the distribution process in a living organism and to cover those processes not fully represented in the in vitro system. For instance, QSAR models investigated the placental permeability [129][130] or the transfer of chemicals from the mother to the fetus [131][132][133]. Several studies focused on the computational assessment of metabolism, or other ADME properties were recently reviewed in [134][135].

As regards computational modeling applied to cellular models, an interesting open-source platform of cell–cell interaction modeling has recently been created by NIH and Environmental Protection Agency's (EPA) (http://www.compucell3d.org, accessed on 18 August 2022). Different computer dynamic simulations of processes occurring during different phases of embryo development have been obtained using this platform, including urethral fusion during sexual diversification of the genital tubercle [136] and fusion of the secondary palatal processes [137]. Applying information derived from toxicant exposure to these models would be precious for a further implementation of in silico modeling and predictions of developmental toxicology.

Recently, a predictive virtual embryo represents the ultimate effort of computational modeling to represent human embryogenesis and assess the effects of perturbations resulting from toxicant exposures on human development (https://www.epa.gov/chemical-research/virtual-tissue-models-predicting-how-chemicals-impact-development, accessed on 18 August 2022) [137].

Overall, in silico methods can assist different steps of developmental toxicity testing, from the classification and identification of stressors to the simulation of in vitro or in vivo experiments, thus representing highly valuable tools to be integrated in developmental toxicity NAMs and IATA approaches.

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