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Developmental effects of PFOS, PFOA and GenX in a 3D human induced pluripotent stem cell differentiation model

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HIGHLIGHTS

- Developmental toxicity of PFOS/PFOS/GenX was tested in a human stem cell assay.
- PFOS and PFOA inhibited cardiomyocyte development with increasing concentrations.
- Gene expression of ISL1 and MYH7 was affected by PFOS and PFOA, respectively.
- Our results may hint at a mechanism, whereby PFAS disrupt embryonic development.

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ABSTRACT

Polyfluoroalkyl substances (PFASs), including perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), are persistent pollutants routinely found in human blood. PFASs have been associated with health issues such as decreased birth weight and impaired vaccination response in children. Substitutes to these PFASs, such as ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoate (GenX) have been introduced, although hazard information is limited. Human induced pluripotent stem cell (hiPSC) based models are valuable for studying these compounds, as they mimic human embryonic development. We used our recently developed PluriBeat assay to investigate PFOS, PFOA and GenX for effects on early embryonic development in vitro. In our assay hiPSCs go through the early stages of embryonic development in 3D cultures of embryoid bodies (EBs) that mimic the human blastocyst until they finally form beating cardiomyocytes. Both PFOS and PFOA had a strong effect on cardiomyocyte differentiation at non-cytotoxic concentrations, with PFOS being more potent than PFOA. Moreover, both compounds decreased EB size at the highest test concentrations. GenX induced a weak concentration-dependent effect on differentiation of one hiPSC line, but not of another. Transcriptional analysis of mRNA from the cardiomyocytes showed that PFOS increased expression of the early cardiac marker ISL1, whereas PFOA decreased expression of the cardiomyocyte marker MYH7. This suggest that PFOS and PFOA perturb cardiomyocyte differentiation by disrupting molecular pathways similar to those taking place in the developing embryo. Based on these findings, we conclude that our PluriBeat assay has the potential to become a valuable, sensitive model system for elucidating embryotoxic effects of PFASs in future.

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1. Introduction

Per- and polyfluoroalkylated substances (PFAS), such as
perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), belong to a large group of persistent organic pollutants characterized by fluorinated carbon chains of varying length and containing different functional groups. Widespread use of these chemicals has led to near ubiquitous presence in human blood and serum (Buck et al., 2011; Knutsen et al., 2018), as several PFASs remain in the human body for long periods of time, with half-lives of up to 5 years (Knutsen et al., 2018). Additionally, PFASs have been shown to cross the placenta (Gützkow et al., 2012), highlighting the importance of investigating the effects of PFASs on development and the embryo.

A number of epidemiological studies have linked PFOS and PFOA exposure in humans to reduced birth weight of newborns (Apelberg et al., 2007; Fei et al., 2007; Lenters et al., 2016; Maisonet et al., 2012; Washino et al., 2009; Wu et al., 2012), Besides effects on birth weight, other developmental effects such as reduced head circumference in newborns have been reported (Apelberg et al., 2007). Findings in animal models also show developmental effects of PFAS including stunted in utero growth after PFOS and PFOA exposure in rodents (Lau et al., 2003, 2006), reduced birth weight following exposure to PFOS (Yahia et al., 2008), and PFOA (Yahia et al., 2016), birth defects such as cleft palate and abnormal bone formation following exposure to PFOA (Yahia et al., 2010), and mitochondrial injury in the rat heart with PFOS exposure (Xia et al., 2011). However, the molecular mechanisms behind these effects remain largely unknown (Knutsen et al., 2018; Liew et al., 2018). These findings, in addition to other adverse effects, has led to the phase-out and restrictions of long-chain PFASs (UNEPA, 2018, 2020), including PFOS and PFOA, and the emergence of substitute PFASs with shorter perfluorinated chains (Scheringer et al., 2014), such as ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoate (HFPO-DA, also known as GenX) (Sun et al., 2016), for which an increasing number of studies are reporting adverse effects (Blake et al., 2020; Conley et al., 2021; Wen et al., 2020).

A limited number of PFASs has been studied extensively and these are increasingly being replaced by new less studied alternatives, such as GenX (Scheringer et al., 2014; Sun et al., 2016). Therefore, new tools are needed for testing of larger numbers of PFASs for potential disruptive effects and for identifying the replacement PFASs of greatest toxicological concern. Stem cell-based models have been used to study developmental toxicity of chemicals, such as the mouse embryonic stem cell (mESC) test, in which effects of potential developmental toxicants on mESC differentiation are evaluated (Seiler and Spielmann, 2011). In order to overcome species differences we previously established a developmental toxicity assay using human induced pluripotent stem cells (hiPSCs), called the PluriBeat assay for which there are no ethical concerns (Lauschke et al., 2020). This assay is based on 3D cultures of hiPSCs termed embryoid bodies (EBs) which model the human blastocyst and that can be differentiated into beating cardiomyocytes. During this time period, the cells undergo molecular events similar to those of the developing embryo (Lauschke et al., 2020).

We hypothesized that the adverse developmental effects including underlying molecular events of PFAS could be detected in the PluriBeat assay. Considering that organogenesis is perceived the most sensitive period of early development and that the heart is the first organ to form during development (Bulatovic et al., 2016), we hypothesized that embryotoxicity of PFAS would be evident in this model.

Thus, the aim of this study was to examine if and how PFOS, PFOA, and GenX might affect human embryonic development using the PluriBeat assay and to identify markers of these effects. Further, we wished to evaluate the potential of the assay for testing of a data-poor PFAS. PFOS and PFOA was selected as known model compounds, and HFPO-DA (GenX) was included because of its increasing use as a PFAS substitute for which there is limited knowledge.

2. Materials and methods

2.1. Test substances

Perfluorooctanesulfonic acid (PFOS) (98% purity, CAS# 2795-39-3) and perfluorooctanoic acid (PFOA) (98% purity, CAS# 335-67-1) were purchased from Sigma-Aldrich (MO, USA). Ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoate (GenX) (97% purity, CAS# 13252-13-6) was purchased from Synquest laboratories (FL, USA). Stock solutions of 200 mM was prepared in dimethyl sulfoxide (DMSO) from Sigma-Aldrich (MO, USA) for PFOS and PFOA, and in MilliQ water for GenX. Respective solvents controls were 0.1% for all treatments. Stocks were visually inspected to assure solubility of the substances and stored together with controls at −20 °C.

2.2. Cell culture

The hiPSC line BIONi010-C was obtained from Bioneer A/S (Hoersholm, Denmark). The hiPSC line IMR90-1 was purchased from WiCell (Madison, USA). Cells were cultured in mTESR1 medium (Stemcell Technologies, USA) with mTeSR1 5X supplement (Stemcell Technologies) in 6 cm Matrigel (BD Biosciences, NJ, USA) coated dishes at 37 °C with 5% CO2. Once a week, cells were passaged by washing with D-PBS (Sigma-Aldrich, MO, USA) and treating with 0.02% EDTA for 1–3 min, before being passed as small colonies to new dishes with a split ratio of 1:10–1:40.

2.3. Cell viability

BIONi010-C cells were seeded in mTESR1 medium into Matrigel coated flat bottom 96-well polystyrene plates (Thermo Fisher Scientific, MA, USA) with a density of 10 000 cells/well. After approximately 24 h, culture medium was changed and treatment with PFOS, PFOA, and GenX was initiated. Treatment concentrations of 25 μM, 50 μM, 100 μM, and 200 μM was selected based on a review of available literature on vitro cytotoxicity of PFAS, the vehicle concentration was kept constant across the plate at 0.1%. For PFOS and PFOA a total of 5 independent experiments (unique cell passages) each with 6 technical replicates per treatment were conducted, for GenX a total of 3 independent experiments were conducted each with 6 technical replicates per treatment. Treatment medium was exchanged approximately every 24 h for three days. At experiment termination, ATP levels were measured by adding 100 μL/well Cell Titer Glo 2D (Promega, WI, USA). Plates were placed on shaker for 2 min and left at room temperature for 10 min. The lysate was transferred to white 96-well culture plates (Perkin Elmer, MA, USA) and luminescence measured (Enspire, Perkin Elmer). Three independent experiments were conducted with each treatment tested in six technical replicates.

2.4. PluriBeat assay

Cardiomyocyte differentiation was performed as described in Lauschke et al., 2020 (Lauschke et al., 2020). PFOS, PFOA, and GenX were tested using BIONi010-C cells (Bioneer Denmark), and GenX was in addition tested in IMR90-1 cells (WiCell, WI, USA). Media recipes are given in “Cell Medium Contents” in the supplemental material. Briefly, hiPSCs were harvested by removal of culture medium and addition of 1 mL Gibco™ TrypleET™ Select (Gibco, USA). After incubation for approximately 1 min, TrypLE was
removed and the cells harvested in mTeSR-ROCK as single cells. Cells were seeded with a density of 10000 cells/well in mTeSR-ROCK into 96-well Polystyrene Conical Bottom MicroWell™ Plate (Thermo Fisher Scientific). Subsequent optimization of the Pluri-Beat assay has established a cell seeding density of 5000 cells/well (Lauschke et al., 2020). Plates were centrifuged at 500 g for 5 min and incubated over night at 37 °C with 5% CO₂ to induce EB formation. The following day (D0), 80 µL medium were removed and 80 µL of D0-medium added. After this, medium was replaced at D1 with TS-medium, D2 with Wnt-medium, D3 with TS-medium and D6 with TS-medium by exchanging 80 µL old medium with 80 µL new medium with the exception of D6, where only 60 µL old medium was replaced.

Test substances were added every day from D1 until D6, with final concentrations of 3.13 µM, 6.25 µM, 12.5 µM, 25 µM, and 50 µM for PFOS, and 6.25 µM, 12.5 µM, 25 µM, 50 µM, 100 µM for PFOA and GenX, the solvent concentration was kept constant across the plate at 0.1%. Based on the viability assay, non-cytotoxic test concentrations were selected. Generally, each PFAS was tested with three biological replicates each with 32 EBs per treatment, for each cell line. For PFOS and PFOA one biological replicate had 48 technical replicates per treatment, and for GenX in the IMR90-1 cell line one biological replicate had 64 technical replicates per treatment. These deviating biological replicates were due to differential availability of cells at experimental start. As a proxy for a positive control in our assay, thalidomide was used (Lauschke et al., 2020). The experiments were terminated on D7 and EBs assessed as described in Lauschke et al., 2020 (Lauschke et al., 2020). Briefly, contractility was scored into three categories using a light microscope. Each well was observed for up to 15 s to categorize contractility. Beat score 2: Contraction of the entire circumference of the EB. Beat score 1: EB contracts, but without moving the entire circumference. Beat score 0: No visible contraction. No EB: Missing or disintegrated EB. A quality criteria for the assay was that a minimum of 90% of the control EBs had a beat score of 2.

Pictures were taken of all EBs using microscopy-camera (Olympus DP21, Olympus) mounted on a widefield light microscope (Leica DMIL LED, Leica Microsystems). A total of three independent experiments was performed for PFOS, PFOA and GenX in the BiONi010-C cell line and for GenX additional three independent experiments were conducted in the IMR90-1 cell line. In every experiment, beat score and EB size were evaluated, all EBs were harvested and pooled from each treatment on D7, which were stored in RLT buffer (RNeasy Mini Kit, Qiagen) containing 1% β-mercaptoethanol (Merck, Germany) at −80 °C for later gene expression analysis.

25. Gene expression

Pooled samples were thawed at room temperature and total RNA isolated using Qiagen RNeasy micro kit (Qiagen, Hilden, Germany) with associated on-column DNase I treatment. The quantity and purity of extracted RNA was measured using a nano-drop spectrometer (Fisher Scientific). cDNA was amplified using random primer mix from Bio Lab (MA, USA), with anti-RNase from Invitrogen. cDNA synthesis was performed with Omniscript kit (Qiagen) using 300 ng RNA from EBs treated with PFOS, and 500 ng RNA from EBs treated with PFOA and GenX. 300 ng RNA was collected from EBs treated with GenX in the IMR90-1 cell line. TaqMan gene expression assays for the various stages of cardiomyocyte differentiation were selected based on the characterization performed by Lauschke et al., 2020. The selected transcripts highlight different stages and characteristics of the differentiation process starting with pluripotency markers; OCT3/4 (Hs04260367_g1), NANOG (Hs02387400_g1), SOX2 (Hs01053049_s1), to the formation of mesoderm; T (Brachyury, Hs00610080_m1), to early cardiomyocytes; ISL1 (Hs00158126_m1), MESP1 (Hs00251489_m1), NKX2.5 (Hs00231763_m1), and finally late cardiomyocytes TNN2 (Hs00943911_m1), MYH6 (Hs01014253_m1), and MYH7 (Hs0110632_m1). In addition, two known targets of PFAs were selected; PPARγ (Hs00231882_m1) and NR1I3 (Hs01101228_g1). RT-qPCR was run on a Quantstudio 7 Flex Real-Time PCR System (Applied Biosystems, MA, USA), 3 µL cDNA was diluted in each 11 µL reaction. Relative transcription levels were calculated using the 2−ΔΔCt method, normalizing RNA input with the geometric mean of the reference genes GAPDH (Hs02786624_g1) and β-actin (Hs01060665_g1).

26. Data processing & statistics

The beat score was categorized as 2: Beat, 1: Partial Beat, or 0: No Beat. Total beat score was divided by the total number of EBs. Statistical analysis of the beat score was done as described in Lauschke et al. (2020) using ordinal logistic regression in R with RStudio (RStudio inc., MA, USA), in which each EB was considered an individual unit. Experiments were performed in biological triplicates using between 16 and 48 EBs per treatment in each experiment resulting in n = 112 for PFOS and PFOA, and n = 96 for GenX in the BiONi010-C cell line and n = 80 in the IMR90-1 cell line. EB volume was calculated after measuring the diameter with Fiji image analysis software. Volume mean ± standard deviation was calculated based on 16 EBs (technical replicates) from each treatment per independent experiment. The means from the three independent experiments was subsequently pooled (n = 3). Treatments were tested against controls using one-way analysis of variance (ANOVA) followed by Dunnett’s test for multiple means comparison in GraphPad Prism 8 (GraphPad Software, USA). Gene expression data were evaluated as mean ± standard deviation. Treatments were tested against controls using one-way analysis of variance (ANOVA) followed by Dunnett’s test for multiple means comparison in GraphPad Prism 8.

3. Results

3.1. PFAs disrupt cardiomyocyte differentiation

Neither PFOS, PFOA nor GenX showed any statistically significant effects on viability of EBs at concentrations up to 200 µM, although a non-significant trend towards slight cytotoxicity was observed for GenX at the highest test concentrations (Fig. 1). Thus, we proceeded using a maximum concentration of 200 µM for PFOS and PFOA, with test concentrations of 12.5, 25, 50, 100, and 200 µM in the PluriBeat assay, whereas 6.25, 12.5, 25, 50 and 100 µM GenX was used due to minor indication of cytotoxicity at 200 µM.

Initial testing of PFOS and PFOA in the PluriBeat assay led to marked reductions of beat scores at all test concentrations (data not shown), and thus test concentrations were adjusted to include data points at the linear part of the concentration-response curves. For PFOS, the concentrations 3.13, 6.25, 12.50, 25, and 50 µM were used and for PFOA, 6.3, 12.5, 25, 50 and 100 µM.

PFOS decreased the beat score, with the highest potency and efficacy causing a statistically significant decrease at 6.3 µM and a maximum efficacy of 100% at 50 µM (Fig. 2A). This was associated with a significant decrease in the EB size of 28% compared to controls at 50 µM (Fig. 2B). PFOA decreased the beat score significantly starting at 12.5 µM with a maximum efficacy of 18% at 100 µM (Fig. 2A). The reduced beat score was reflected by a significant decrease in EB size of 51% and 10% of controls at concentrations of 50 and 100 µM, respectively (Fig. 2B). GenX did not significantly affect the beat score or EB size in the BiONi010-C cell
line (Fig. 2A). It is known that biological variation between different hiPSC clones originating from different donors can arise (Lauschke et al., 2020) and we therefore decided to test GenX in an additional hiPSC cell line, the IMR90-1 cells. Here, we observed a weak concentration-dependent effect that was significant starting at 12.5 μM (Fig. 3A).

3.2. Transcriptional analysis revealed expression changes of cardiac markers

In order to understand how PFOS, PFOA and GenX affected differentiation of EBs in the PluriBeat assay, we assessed the expression of key genes characteristic of the different developmental stages during cardiomyocyte differentiation at assay termination.

Expression of the cardiac progenitor marker ISL1 was higher in EBs exposed to 25 μM PFOS and a similar trend was observed for PFOA, although not statistically significant (Fig. 4A). The expression of the cardiac marker MYH7 was significantly lower at 100 μM PFOA and a similar trend was observed for 25 μM PFOS, although not statistically significant (Fig. 4C). No other test genes including the general cardiac marker NKK2.5 was changed significantly (Fig. 4D). Similar trends were generally detected for PFOS and PFOA, with PFOS inducing more potent effects than PFOA. GenX did not affect gene expression in any of the tested cell lines (Fig. 4 A – D, supplemental figures Expression of cardiac and pluripotency markers following exposure to GenX in the IMR90-1 cell line).

4. Discussion

In humans, epidemiological studies have reported associations between PFAS exposure and reduced birth weight, an effect that is also observed in animal studies (Schrenk et al., 2020). Chemical exposure in general has been linked to decreased birth weight in a multitude of studies (Govarts et al., 2016; Kamai et al., 2019; Zheng et al., 2016). Even relatively low dose exposure to pesticide mixtures has been shown to decrease birth weight in rats (Hass et al., 2017). A low birth weight is an established risk-factor for developing disease later in life, as it correlates with increased risk of cardiovascular disease, coronary heart disease, hypertension and type 2 diabetes in adulthood (Knop et al., 2018). In addition, multiple studies have found a connection between reduced birth weight and congenital heart disease (Itsukaichi et al., 2011; Petrossian et al., 2015; Rosenthal, 1996), which affect more than 1% of all live births (Hoffman and Kaplan, 2002).

PFAS exposure may decrease birth weight by perturbing fetal growth (Govarts et al., 2016). The intrauterine environment plays an important role in fetal growth and development. Genetic factors can explain around 40% of birth weight variability (Warrington et al., 2019), whereas the remaining variability can be attributed to environmental factors, including chemical exposure (Zheng et al., 2016).

We hypothesized that PFAS might be able to reduce beating of EBs in our PluriBeat assay due to embryotoxic effects, as we have previously shown for thalidomide and epoxiconazole (Lauschke et al., 2020). This study showed that PFOS, PFOA, and to a less extent GenX disrupt hiPSC-derived cardiomyocyte differentiation at non-cytotoxic concentrations. Our data suggest that PFASs may disturb early organogenesis by affecting key events starting after the formation of the mesoderm to the development into cardiomyocytes, which we consider a surrogate marker for disturbances of early embryonic development. To our knowledge, this is the first study reporting on the effects of PFOS, PFOA and GenX on cardiac differentiation using a human based 3D in vitro model. 3D culture offers multiple advantages over normal 2D cultures commonly used for in vitro toxicity testing in that their cell-cell interaction, morphology, and access to nutrients is more similar to that of the human body (Edmondson et al., 2014). 3D culture may therefore more accurately represent the in vivo physiology, allowing for more complex signaling to occur (Duval et al., 2017). The advantage of using a human cell line is illustrated by the marked species differences between mouse and human ESCs when looking at timing of expression of various genes related to embryonic
In order to discriminate a treatment-induced inhibited response on beat score from a general cytotoxic effect, we conducted cell viability experiments by measuring ATP levels in 2D cultures of hiPSCs. This was inspired by the well-known mouse Embryonic Stem cell Test (EST), in which cytotoxicity is measured in 2D cultures of fibroblasts (Spielmann et al., 1997). However, the 2D culture can only be considered a proxy for the 3D culture as the sensitivity of the cells and the intracellular concentration of test compounds may deviate between the two culture types. Moreover, another parameter apart from ATP could be included as well to better cover mechanisms of cytotoxicity and to reduce uncertainty in determining general cytotoxicity.

The effects seen for PFOS and PFOA in the PluriBeat assay were not observed for GenX in the first cell line that was tested. However, GenX elicited a small concentration-dependent response starting at 25 μM when tested using hiPSC of a different origin (Fig. 3). This may reflect the natural variation between hiPSC lines originating from different donors with different genetic backgrounds (Fossati et al., 2016; Narsinh et al., 2011). This highlights the importance of testing negative chemicals in more than one cell line to avoid false negatives. By testing chemicals in several hiPSC lines isolated from different donors, a better approximation of the variation between individual responses can be obtained. Thus, a panel of several hiPSC clones for chemical testing could in the future give a better representation of the human population than what has previously been possible with many classical cell or animal models.

In order to evaluate the biological relevance of our findings, we have compared the effective PFAS concentrations in vitro with PFAS levels that are present in mothers of newborns with reduced birth weight. PFOS blood concentrations in these mothers have been reported in the range of 5–35 ng/mL (Apelberg et al., 2007; Fei et al., 2007; Lenters et al., 2016; Maisonet et al., 2012). However, in occupationally exposed workers at a fluorocatalytic plant in China the median concentration of PFOS was measured to be as high as 118,000 ng/mL with a median level of 1,725 ng/mL (Fu et al., 2016). For comparison the lowest PFOS concentration tested in our study (3.1 μM) corresponds to 1,563 ng/mL, with a lowest observed adverse effect level (LOAEL) at 3,126 ng/mL. For PFOA, the reported blood concentrations range associated with birth weight

**Fig. 2. Beat score and EB size following exposure to PFOS, PFOA, and GenX.** A) Total beat score divided by the total number of EBs. The beat score was categorized as 2: Beat, 1: Partial Beat, or 0: No Beat. EBs were exposed to PFASs at non-cytotoxic concentrations for 6 days in 3 independent experiments each with either 32 or 48 technical replicates (mean ± SD, n = 96 for GenX and n = 112 for PFOS and PFOA). (*) indicates statistically significant responses p < 0.05 determined by ordinal logistic regression. B) EBs were exposed to PFASs at non-cytotoxic concentrations for 6 days in 3 independent experiments with 16 replicates within each experiment. Mean volume was calculated in each independent experiment and normalized to the control, each independent experiment is presented as a boxplot with each EB shown as a data point, n = 3. (*) indicates statistically significant responses of p < 0.05, determined using ANOVA followed by Dunnett’s post hoc test when comparing the pooled means of each experiment to the control.
directly regulated by cardiac progenitor cells (Moretti et al., 2010).


Kruskal-Wallis followed by Dunn PFOA. Expression of ISL1 each independent experiment is presented as a boxplot with each EB shown as a data point, n for 6 days in 3 independent experiments with 16 replicates within each experiment. Mean volume was calculated in each independent experiment and normalized to the control, (Fig. 4) following exposure to PFOS, and a similar trend was seen for ISL1 marker genes for pluripotency, the mesoderm, as well as early and the differentiating cardiomyocytes, we analyzed the expression of ISL1 at protein and gene expression level (Yang et al., 2020). Yang et al. (2020) found that PFOS affected WNT, IGF, FGF, and BMP signaling in developing cardiomyocytes and observed a decreased expression of the cardiac markers NKX2.5, MYH6, and MYL7 at protein and gene expression level (Yang et al., 2020).

To investigate the underlying cause of the observed effects in the differentiating cardiomyocytes, we analyzed the expression of marker genes for pluripotency, the mesoderm, as well as early and late cardiomyocyte differentiation (Lauschke et al., 2020). We observed a significant 3-fold increase in the expression of ISL1 (Fig. 4) following exposure to PFOS, and a similar trend was seen for PFOA. Expression of ISL1 signifies the multipotent properties of cardiac progenitor cells (Moretti et al., 2010). ISL1 is thought to be directly regulated by β-catenin of the canonical WNT-signaling pathway (Lin et al., 2007). Isl1 knockout mice exhibit cardiac defects (Gao et al., 2019) and variations in ISL1 are linked to greater risk of congenital heart disease in humans (Stevens et al., 2010). A higher expression of ISL1 on the last day of differentiation indicates that PFOS may delay or hinder the full differentiation of cardiac progenitors into cardiomyocytes. Moreover, we observed a decreased expression of the cardiomyocyte marker MYH7 for both PFOS and PFOA, though only statistically significant for PFOA, supporting the notion that PFOS and PFOA disrupt full cardiomyocyte differentiation.

Only a few studies of PFOS-induced effects in stem cells have been conducted so far. Cheng et al. (2013) have reported on the effects of PFOS in 3D cultures of differentiating cardiomyocytes, using mouse ESCs. They found that PFOS affected cardiomyocyte contraction at 30 μM, as opposed to 6.3 μM observed in our study (Cheng et al., 2013). Cheng et al. also reported PFOS-induced apoptosis during the cardiac differentiation process (Cheng et al., 2013), which could explain why we see a decreased size of EBs for PFOS and PFOA. In other words, the functional output of the PluriBeat assay seems to be more sensitive for PFOS-driven effects than the mouse ESC-based assay. This higher sensitivity is further suggested with our observation that the PluriBeat assay was affected from 6.3 μM, whereas 60 μM PFOS was required to decreased the percentage of beating cardiomyocytes in a human ESC 2D culture (Yang et al., 2020). Thus, both human origina and the 3D culture method may contribute to improved sensitivity of the PluriBeat assay.

There are a number of signaling pathways downstream of ISL1, including Wnt-, BMP-, Notch-, Hedgehog-, and Calcineurin-signaling (Lin et al., 2012) that could potentially be affected by PFAS. Yang et al. (2020) found that PFOS affected WNT, IGF, FGF, and BMP signaling in developing cardiomyocytes and observed a decreased expression of the cardiac markers NKX2.5, MYH6, and MYL7 at protein and gene expression level (Yang et al., 2020).
Decreased expression of the cardiac marker MYH6 correlates well with increased expression of ISL1, as MYH6 is generally found in cardiomyocytes. We observed similar trends for PFOS although we did not observe changes in the expression of NKX2.5, which may be due to a lower test concentration in our study. Thus, future work should also focus on elucidating the complex spatiotemporal expression pattern of key genes in order to fully appreciate how chemical substances disrupt differentiation.

5. Conclusions

In this study we show that PFOS and PFOA, and their substitute
GenX impaired differentiation of human cardiomyocytes, suggesting that PFAAs may disrupt early embryonic development. GenX affected differentiation to a much lesser degree than PFOS and PFOA. PFOS increased expression of ISL1, and PFOA decreased expression of MYH7, suggesting that these compounds affect cardiomyocyte differentiation similarly at concentrations close to human relevant levels. Further studies should focus on understanding how PFAAs affect signaling pathways important for early development of the embryo, and how PFAS exposure early in life may contribute to disease later in life.

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**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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