Perfluorohexanoic Acid-Induced Inhibition of Human Palate Cell Proliferation Through Upregulation of *miR-374a-5p*

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Article

Perfluorohexanoic Acid-Induced Inhibition of Human Palate Cell Proliferation Through Upregulation of *miR-374a-5p*

Hiroki Yoshioka [1,2,*,†], Hanane Horita [2,†], Kenichi Ogata [3], Hyogo Horiguchi [1] and Yosuke Tsukiboshi [4,*]

[1] Department of Hygiene, Kitasato University School of Medicine, 1-15-1 Kitasato, Minami-ku, Sagamihara, 252-0374, Japan
[2] Faculty of Pharmacy, Gifu University of Medical Science, 4-3-3 Nijigaoka, Kani, Gifu, 509-0293, Japan
[3] Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[4] Department of Neurotoxicology, Nagoya City University Graduate School of Medical Sciences,1 Kawasumi, Mizuho-cho Mizuho-ku, Nagoya, 467-8601 Japan
* Correspondence: yoshioka.hiroki@kitasato-u.ac.jp (H.Y.); y.moonstar2014@gmail.com (Y.T.); Tel.: +81-42-778-8111 (H.Y.); +81-52-853-8991 (Y.T.)
† These authors contributed equally to the work.

Abstract: *Per- and poly fluoroalkyl substances (PFAS) pose serious health concerns worldwide. Although the use of classical PFAS, such as perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), is regulated, the toxicological effects of alternative PFASs remain unknown.* Cleft palate is a congenital condition influenced by a combination of environmental and genetic factors. While PFOS has been linked to cleft palate, the effects of other PFAS compounds remain unexplored. *The aim of this study was to clarify the involvement of classical and alternative PFAS (PFHxA and PFHxS) in the proliferation of human embryonic palatal mesenchymal cells (HEPM).* Following PFAS treatment for 48 h, cell viability, apoptosis, and cell cycle-related proteins were tested. In addition, microRNA levels and its predicted target genes were measured, and a rescue experiment against PFHxS was conducted using a miR-374a-5p inhibitor. Among the four PFASs, *PFHxS decreased the number of cells with cyclin- and cyclin-dependent kinase reduction. In addition, PFHxS treatment upregulated* miR-374a-5p and *downregulated its downstream genes. Furthermore,* miR-374a-5p inhibitor alleviated the *PFHxS*-induced cell proliferation reduction. These findings indicate that miR-374a-5p plays a key role in the development of PFHxS-induced cleft palate and alternative PFAS may have a highly toxic effect on HEPM cells.

Keywords: *Perfluoroalkyl and Polyfluoroalkyl Substance*; cleft palate; microRNA; cell cycle arrest

1. Introduction

Gynecological disorders encompass a broad range of conditions that affect the female reproductive system, including the uterus, ovaries, fallopian tubes, uterine cervix, and vagina [1]. These disorders can significantly affect women's health, fertility, and quality of life and range from benign conditions such as polycystic ovary syndrome (PCOS), endometriosis, and uterine fibroids to malignant diseases such as cervical, ovarian, and endometrial cancers [1, 2]. Several studies have suggested that gynecological disorders may affect the fetus and the course of pregnancy [3, 4]. For example, patients with uterine fibroids have an increased risk of placental abnormalities and preterm birth [5]. Pregnant women with endometriosis have an increased risk of miscarriage and preterm birth [6]. Infants of women with PCOS have an increased risk of congenital anomalies, such as cleft lip (CL) with or without cleft palate (CP) (CL/P) [7].

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CL/P is a common congenital anomaly, *affecting approximately one in every 500–700 babies born in Asia, and is one of the most frequent birth defects worldwide. Studies have reported that 70% of CL/P cases are non-syndromic, with the remaining 30% linked to syndromic conditions [8, 9].* In humans, lip formation begins in the fourth week of gestation and is completed by approximately the seventh week. The development of the secondary palate begins around the sixth week of gestation and is typically completed by the twelfth week. Despite differences in the timing of lip and secondary palate formation, the underlying processes, such as mesenchymal cell proliferation, the fusion of two epithelial cells, and epithelial-mesenchymal transition (EMT), are similar. Disruption of these processes results in CL/P [10, 11].

The etiology of CL/P is influenced by a combination of genetic and environmental factors [12, 13]. Multiple types of signaling pathway disruptions, resulting from gene deletions or mutations, have been implicated in the development of CL/P through the inhibition of mesenchymal cell proliferation, preventing the fusion of epithelial cells, and impairing EMT [14, 15]. For example, the WNT signaling pathway plays an important role in secondary palate formation through by regulating Paired box gene 9 [16-18]. A feedback loop between the WNT signaling pathway and Irf6 regulates morphological changes in palatal shelves [19, 20]. The bone morphogenetic protein (BMP) signaling plays a critical role in craniofacial development because it regulates key cellular processes including apoptosis, differentiation, and proliferation [21-23]. Msx1 knockout mice exhibit mouse CP through the downregulation of WNT and BMP signaling pathways [24, 25]. JARID2 exhibits high and specific expression in epithelial cells and is functionally linked to the BMP and transforming growth factor (TGF) β signaling [26, 27]. Conditional knock-out of Sirt6 increased mouse embryonic palatal mesenchyme (MEPM) cell proliferation through upregulation of TGF signaling pathway [28]. *A systematic review has identified more than 130 genes linked to the occurrence of CP in human [29], whereas more than 190 genes are linked to mouse CP [30]. Suzuki et al. identified 55 genes involved in mouse CL [31] and Yoshioka et al. reported that 173 genes are associated with human CL [32]. While recent studies have identified novel gene mutations linked to orofacial cleft, including CL/P [33], the mechanism by which CL/P is induced remains unknown.* Maternal exposure to environmental factors is known to increase the risk of CL/P [34], such as alcohol consumption [35], cigarette smoking [36], and the use of certain medications during pregnancy [37, 38]. Exposure to certain environmental factors can lead to CL/P by altering the expression of crucial developmental genes or by perturbing key signaling pathways involved in craniofacial development. Recent studies have identified new environmental factors, including PM2.5, as being associated with the development of CP *[39]; therefore, we need to understand how environmental factors are involved in the etiology of CL/P.*

Perfluoroalkyl and polyfluoroalkyl substances (PFAS) are a large group of synthetic chemicals that have been manufactured and utilized in various industries since the 1940s [40, 41]. There are more than 4700 PFAS compounds in the world [42]. The two most common PFAS compounds are perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), which were produced in large quantities during the 2000s and 2010s [43, 44]. The health effects of PFOS and PFOA on adults and fetuses have become serious concerns [45-47]. Although the manufacture and import of products containing PFOA and PFOS were banned in Japan in 2010, and 2021, respectively, these substances are still being detected because of their high persistence in the environment [48]. In recent years, perfluorohexanoic acid (PFHxA) and perfluorohexanesulfonic acid (PFHxS) have been employed as alternatives to PFOA and PFOS. However, their toxicological profiles are poorly understood. *Environmental exposure to PFAS in drinking water may increase the risk of CL/P [49, 50]. In experimental models, exposure to PFOS induces CP in mice [51, 52]. However, the molecular mechanisms underlying PFOS-induced CL/P have not yet been reported. Furthermore, it remains unclear whether other PFAS induce CL/P.* In this study, we investigated whether two classical PFAS (PFOA and PFOS) and two alternative PFAS (PFHxA and PFHxS) reduce the number of human embryonic palatal mesenchyme (HEPM) cells. *Furthermore, we explored the molecular mechanisms focusing on microRNAs (miRNAs).*

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2. Materials and Methods

2.1. Cell Culture

HEPM cells (JCRB9095) were obtained from the JCRB Cell Bank (Osaka, Japan) and cultured in Minimum Essential Medium Eagle-alpha modification (αMEM) supplemented with 10% fetal bovine serum, and penicillin/streptomycin mixture. HEPM cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

2.2. Cell Proliferation Assay

HEPM cells were seeded at a density of 5,000 cells per well in 96-well plates (n=6) and treated 24 h post-seeding with 0–100 μM of PFOA, PFOS, and PFHxS or with 0–2 μM of PFHxA. Following 24 h of treatment (PFOA, PFOS, PFHxA, or PFHxS), cell count was assessed using Alamar Blue, and fluorescence was measured at Ex540/Em600.

2.3. Apoptosis Assay

HEPM cells were seeded at a density of 10,000 cells per chamber and treated with either 100 μM PFHxS or vehicle control (0.1% DMSO). After 48 h of treatment, apoptotic cells were detected using ApoTracker Green [53][54]. The nuclei of the cells were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI).

2.4. Western Blot Analysis

HEPM cells were seeded at a density of 200,000 cells per well in 35 mm dish plates and treated with 100 μM PFHxS or 0.1% DMSO after 24 h of cell seeding. After 48 h of treatment, the cells were washed two times and 100 μL lysis buffer with a protease inhibitor cocktail was added and the cells were incubated at 4°C for five minutes. Subsequently, the cells were collected (20,000 ×*g* for 20 min at 4°C) [55, 56]. Protein samples (10 μg) were separated by 5-20 % gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride membranes. Western blotting was carried out with the antibodies listed below: anti-mouse monoclonal cyclin-D1 (CCND1) antibody (1:750 dilution), anti-mouse monoclonal CCNE (1:1,000 dilution), anti-mouse monoclonal BAX (1:1000 dilution), anti-mouse monoclonal cyclin-dependent kinase-2 (CDK2) antibody (1:1,000 dilution), anti-mouse monoclonal CDK4 antibody (1:1,000 dilution), anti-mouse monoclonal CDK6 antibody (1:2,000 dilution), anti-rabbit polyclonal cleaved CASPASE-3 antibody (1:3,000 dilution), and anti-mouse monoclonal β-actin antibodies (1:4,000 dilution). Peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) and anti-mouse IgG were used as secondary antibodies at a dilution of 1:10,000 dilution). Detection of immunoreactive bands was achieved using substrate.

2.5. Bromodeoxyuridine (BrdU) Incorporation Assay

HEPM cells were seeded at a density of 10,000 cells per chamber and treated with either 100 μM PFHxS or vehicle control (0.1% DMSO). After 48 h of treatment, the cells were incubated with 100 μg/mL BrdU for 1 h. The BrdU in the nucleous was detected using an anti-mouse monoclonal BrdU antibody (1:150 dilution) and CoraLite594-conjugated anti-mouse IgG (1:180 dilution). DAPI was used to counterstain the nuclei and the number of BrdU-positive cells was counted across eight fields.

2.6. Quantitative RT-PCR

HEPM cells were seeded at a density of 200,000 cells per well in 35 mm dish plates and treated with 100 μM PFHxS or 0.1% DMSO after 24 h of cell seeding. After 48 h of treatment, the cells were washed two times and total RNA was extracted from the cells using miRNA detectable Kit, following the procedure we previously reported [57, 58]. For miRNA detection, total RNA was reverse-transcribed using an miRNA Reverse Transcription Reaction Kit. MiRNA expression was assessed

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using an all-in-one miRNA qRT-PCR Detection Kit. Information of Probe and PCR conditions were detailed in earlier studies [53]. For normal gene detection, the protocol for reverse transcription and PCR conditions were detailed in earlier studies [55]. Target mRNA levels were normalized to the β-actin levels. The sequences of the primer sets used are as follows: human β-actin (NM_001101), sense 5′-ACCTTCTACAATGAGCTGCGTG-3′ and antisense 5′-TGGGGTGTTGAAGGTCTCAAAC-3′; human cysteine-rich secretory protein LCCL domain containing 1 (CRISPLD1; NM_031461), sense 5′-ATCACAGACAATGACATGCAGAG-3′ and antisense 5′-AGATCTTTCCAGCTCTACATCCC-3′; human fibroblast growth factor receptor 2 (FGFR2; NM_000141), sense 5′-ATGAGGATGACACCGATGGTG-3′ and antisense 5′-TTGACAGTGTTGGCCGCAG-3′; human jumonji and AT-rich interaction domain containing 2 (JARID2; NM_001267040), sense 5′-CTTCATCTTCATGCCAGTCGAC-3′ and antisense 5′-CAGGTTCCTTCTCCCGTGTTG-3′; human msh homeobox 1 (MSX1; NM_002448), sense 5′-GAAGATGCGCTCGTCAAAGC-3′ and antisense 5′-GGTTCGTCTTGTGTTTGCGGAG-3′; and human zinc finger protein 236 (ZNF236; NM_001306089), sense 5′-AGAGTGGCTAGTCTCAAAGCG-3′ and antisense 5′-CAGCTGACTCTGCAGAGTAAAC-3′.

2.7. Rescue Experiments

HEPM cells were seeded at a density of 5,000 cells per well in 96-well plates or a density of 200,000 cells per well in 35 mm dish plates. After 6 h, the cells were treated with 3 or 60 pmol of *miR-374a-5p* inhibitor or 3 or 60 pmol of control miRNA inhibitor using FuGENE SI Transfection Reagent, following the manufacturer's protocol. Twenty-four hours after transfection, the cells were treated with 100 μM PFHxS or vehicle control (0.1% DMSO) for 48 h. Cell viability and gene expression levels were assessed using the methods described above.

2.8. Statistical Analysis

Comparisons between two groups were performed using Student's t-test, whereas comparisons among multiple groups were conducted using Tukey's test. All statistical analyses were performed using SPSS Statistics for Windows (version 27.0). A p-value of <0.05 was considered statistically significant.

3. Results

3.1. PFHxS Reduced Cell Viability in HEPM Cells

Initially, a cell viability assay was conducted to evaluate whether the four PFAS compounds reduced HEPM cell viability. Among the four PFAS, three PFAS (PFOA, PFOS, and PFHxA) did not alter the cell number (Fig. 1a, 1b, 1c). By contrast, treatment with 100 μM PFHxS significantly reduced the viability of HEPM cells (40% reduction, Fig. 1d). Therefore, we conducted further experiments using 100 μM PFHxS.

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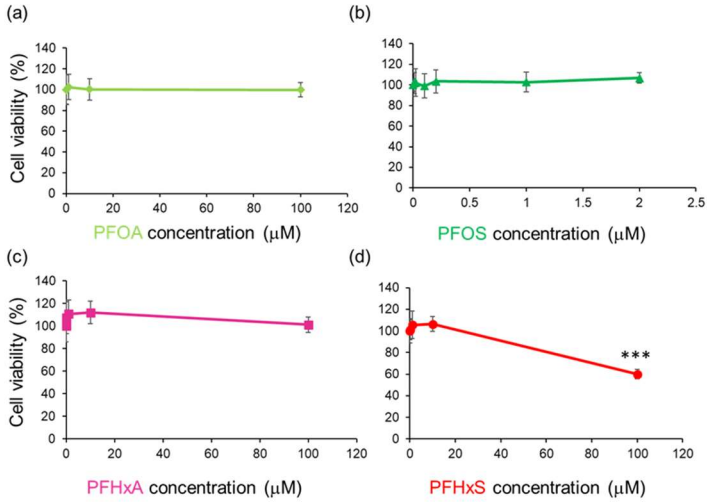

Figure 1. Effect of four PFAS compounds on HEPM cell proliferation after 48 hours of treatment. Panels (A), (B), (C), and (D) show PFOA, PFOS, PFHxA, and PFHxS, respectively. ***p<0.001 versus control (Tukey's test) (n=6).

3.2. PFHxS Reduced Cell Viability Through G1 Cell Cycle Arrest

It has been reported in several investigations that chemical-induced impaired proliferation of mesenchymal palatal cells can lead to apoptosis and cell cycle arrest [59-61]. Therefore, we examined whether PFHxS treatment induces apoptosis and/or cell cycle arrest in HEPM cells. As shown in Fig. 2a, treatment with PFHxS did not lead to apoptosis, whereas the positive control treatment did, suggesting that PFHxS did not affect HEPM cell apoptosis. In support of this result, the expression of cleaved CASPASE-3 and BAX, markers of apoptosis, was not detected by treatment with PFHxS (Fig. 2b).

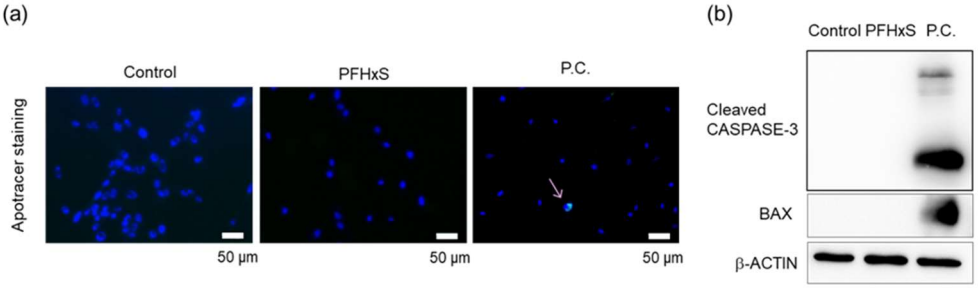

Figure 2. No Association between PFHxS-Induced cell proliferation and apoptosis.

(A) HEPM cells treated with 100 μM PFHxS for 48 h and stained with Apotracker. Nuclei were stained with DAPI. P.C.; positive control ($CuCl_2$). Scale bar, 50 μm.

(B) HEPM cells treated with 100 μM PFHxS for 48 h were subjected to western blotting. β-Actin serving as reference control. P.C.; positive control ($CuCl_2$).

Subsequently, cell cycle progression was assessed through a BrdU incorporation assay, revealing a significant reduction in the number of BrdU-positive cells following PFHxS treatment (Fig. 3a). To investigate the molecular mechanisms of PFHxS-induced cell cycle arrest (G1- cell cycle arrest), we analyzed the of cyclins and CDKs protein levels using western blotting. PFHxS treatment downregualted the protein levels of CCND1, CDK4, and CDK6, whereas those of CCNE and CDK4 remained unchanged (Fig. 3b). These results indicated that PFHxS induced G1 cell cycle arrest by inhibiting the CCND1/CDK4 and CCND1/CDK6 pathways in HEPM cells.

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(A) HEPM cells were stained for BrdU (green) after 48-hour treatment with 100 μM PFHxS. Nuclei were stained with DAPI (blue). Scale bar, 50 μm. BrdU-positive cell rates are shown. Values are expressed as mean ± standard deviation (SD). $^{**}p<0.01$ (Student's t-test) (n=8).

(B) HEPM cells treated with 100 μM PFHxS for 48 h were subjected to western blotting. β-Actin serving as reference control.

3.3. PFHxS Upregulates miR-374a-5p

Several literatures have demonstrated a link between miRNAs and etiology of CL/P [11, 62]. Suzuki and Li *et al.* reported several miRNAs (*miR-133b, miR-140-5p, miR-374a-5p, miR-381-3p*, and *miR-4680-3p*) associated with human CP-related genes through a combination of approaches, including systematic reviews, bioinformatics analyses, cell proliferation assays, and qPCR [29, 63]. In the present study, we evaluated the expression levels of five miRNAs *(miR-133b, miR-140-5p, miR-374a-5p, miR-381-3p*, and *miR-4680-3p*) using qPCR. PFHxS treatment significantly upregulated *miR-374a-5p* expression, while no changes were observed in the other four miRNAs (Fig. 4).

Expression levels of *miR-133b*, *miR-140-5p*, *miR-374a-5p*, *miR-381-3p*, and *miR-4680-3p* were measured using quantitative RT-PCR after treatment of HEPM cells with 100 μM PFHxS for 48 h. Data are shown as the mean ± SD. $^{**}p<0.01$ (Student's t-test) (n=3).

3.4. Blocking miR-374a-5p Alleviates PFHxS-Cediated Cell Proliferation Inhibition

To elucidate the role of miR-374a-5p, HEPM cells were transfected with a miR-374a-5p inhibitor to determine whether miR-374a-5p could reverse the PFHxS-induced inhibition of cell proliferation. Under our experimental conditions, expression of miR-374a-5p expression level was reduced by over 85% following transfection with its inhibitor (Fig. 5a). Subsequently, after transfection with miR-374a-5p inhibitor, HEPM cells were treated with PFHxS. We found that miR-374a-5p inhibitor partially restored cell viability, that was inhibited by PFHxS (Fig. 5b). Furthermore, treatment with an miR-374a-5p inhibitor significantly upregulated the gene expression level of CRISPLD1 and FGFR2. The PFHxS-induced downregulation of these genes was significantly alleviated by miR-374a-5p inhibition in HEPM cells (Fig. 5c). These findings imply that miR-374a-5p contributes to PFHxS-induced suppression of HEPM cell proliferation.

(A) Expression level of *miR-374a-5p* were measured using quantitative RT-PCR after after transfection of HEPM cells with *miR-374a-5p* inhibitor for 24 h. Data are shown as the mean ± SD. $^{***}p<0.001$ (Tukey's test) (n=3).

(B) HEPM cell proliferation after 48 h of treatment with 100 μM PFHxS, the miR-374a-5p inhibitor, or their combination. Data are shown as the mean ± SD. $^{**}p<0.01$ and $^{***}p<0.001$ (Tukey's test) (n=6).

(C) Expression level of five predicted genes were evaluated by quantitative RT-PCR following transfection of HEPM cells with the *miR-374a-5p* inhibitor and/or treatment with 100 μM PFHxS for 48 h. Data are shown as the mean ± SD. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ (Tukey's test) (n=4).

4. Discussion

In the present study, we examined the effects of four types of PFAS on HEPM cells. Among the four PFAS, PFHxS treatment significantly reduced cell number (Fig. 1d) and the protein levels of CCND1, CDK4, and CDK6 (Figure 3b). A recent report suggested that PFHxS reduces Ccnd1 levels in zebrafish [64], supporting the possibility that PFHxS induces cell cycle arrest (Figure 3a, 3b). PFHxS treatment upregulated *miR-374a-5p* expression (Figure 4) and decreased its downstream genes expression level (Figure 5c). Importantly, transfection with a *miR-374a-5p* inhibitor partially alleviated PFHxS-induced cell inhibition (Figure 5b).

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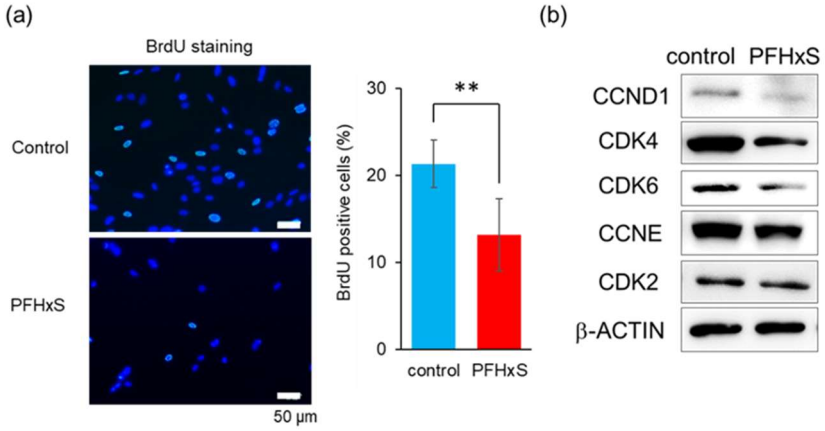

Figure 3. Association between PFHxS-induced proliferation reduction and G1 cell cycle arrest.

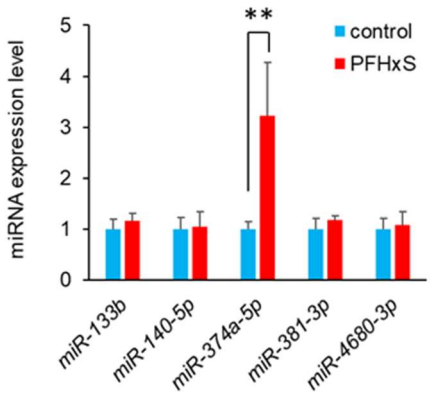

Figure 4. PFHxS upregulated miR-374a-5p.

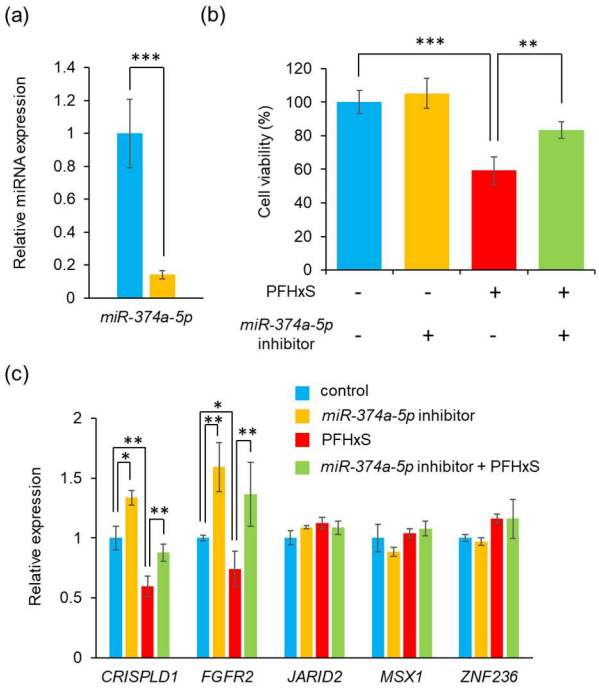

Figure 5. Inhibition of *miR-374a-5p* alleviated PFHxS-induced cell proliferation inhibition.

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Cyclins and CDKs are essential for cell cycle progression [65]. When cells exit the G0 phase and re-enter the cell cycle, CDK4 and CDK6 form active complexes with CCND and other proteins, including the phosphorylated retinoblastoma protein. This interaction facilitates the transition from the G1 phase to the S phase [66]. Moreover, the members of the retinoblastoma protein family are phosphorylated and inactivated by the CDK2–CCNE complex [67], leading to the release of transcription factors, such as E2F, which further drives the G1/S transition. The overexpression of CCND1 represents a central mechanism contributing to therapeutic resistance across multiple cancer types [68, 69]. Cdk2 knockout mice has been shown to reduce neural progenitor cell viability [70]. CCNE overexpression also contributes to accelerated G1 phase progression in cancer patients [71]. Targeting these mechanisms, CDK4/6 inhibitors namely abemaciclib, palbociclib, and ribociclib are approved therapies for the epidermal growth factor receptor 2-negative breast cancer [72, 73]. Western blot analysis revealed that PFHxS treatment decreased the protein expression of CCND1, CDK4, and CDK6 (Figure 3b). These findings suggest that the PFHxS-induced reduction in cell viability is likely due to G1 phase arrest mediated by the suppression of CCND1/CDK4 and CCND1/CDK6 complexes in HEPM cells.

miRNAs, which are small non-coding RNAs measuring approximately 20–24 nucleotides, are well known for their role as post-transcriptional negative regulators of gene expression. The discovery of the first miRNA in 1993 [74, 75] was a landmark event, marking the onset of a new era in RNA biology. These evolutionarily conserved molecules are widely distributed across diverse organisms, and recent research suggests that over 2,500 distinct miRNAs have been identified in the human genome [76]. Recent studies have highlighted the role of miRNAs in the regulation of CL/P [77]. Specifically, the *miR-17–92* cluster regulates cell proliferation and cell cycle progression in palatal mesenchymal cells [78]. Mutations in this cluster are associated with severe craniofacial abnormalities [79]. Furthermore, polymorphisms in pre-*miR-146a* have been shown to influence the expression of *tumor necrosis factor receptor-associated factor 6*, thereby contributing to the pathogenesis of CP [80]. Li and Suzuki *et al.* found that the five miRNAs (*miR-133b, miR-140-5p, miR-374a-5p, miR-381-3p*, and *miR-4680-3p*) mimic inhibited HEPM cell proliferation [29, 63]. Several mouse miRNAs such as *miR-27a-3p, miR-27b-3p, miR-124-3p, miR-129-5p, miR-214-3p, miR-340-5p*, and *miR-486b-5p* mimic reduces the number of MEPM cells [38, 81-83]. Transfection with *miR-497-5p* and *miR-655-3p* mimic significantly reduced the viability of cultured human lip fibroblast cells [84]. PFHxS significantly induced the expression of *miR-374a-5p* in HEPM cells (Figure 4). Moreover, application of a specific inhibitor targeting *miR-374a-5p* partially mitigated PFHxS-induced inhibition of HEPM cell proliferation (Figure 5b), indicating that *miR-374a-5p* plays a crucial role in the toxicity associated with PFHxS treatment. Human *miR-374a-5p* is located on the X chromosome and *miR-374a-5p* are involved in cell proliferation [85]. *miR-374a-5p* is associated with WNT/b-catenin singling pathway [86] and overexpression of *miR-374a-5p* significantly reduced cell viability in human non-small lung carcinoma cell lines A549 and H1299 [87]. Among the five predicted downstream genes (*CRISPLD1, FGFR2, JARID2, MSX1, and ZNF236*) of *miR-374a-5p* (Supplementary Figure S1), inhibition of *miR-374a-5p* significantly upregulated the expression of *CRISPLD1* and *FGFR2* in HEPM cells (Figure 5c). These findings indicated that *CRISPLD1* and *FGFR2* play a crucial role in palatal development.

CRISPLD1 is a member of a highly conserved cysteine-rich secretory protein family. This molecule has been implicated in facial morphogenesis, the folate metabolic pathway, and the cellular stress response of chondrocytes upon interleukin-1α stimulation [88]. Single-nucleotide polymorphisms in CRISPLD1/CRISPLD2 have been associated with variations in folate pathway-related genes that contribute to the susceptibility to CL/P [89]. CRISPLD1 inhibition by siRNA was significantly reduced in the human stomach carcinoma cell line HGC-27 through modulation of the PI3K-AKT signaling pathway [90]. FGFR2, including its isoforms FGFR2b and FGFR2c, functions as a receptor for fibroblast growth factors and mediates the RAS/ERK and PI3K/AKT signaling pathways [91, 92]. FGFR2 has been reported to regulates cyclin dependent pathways particularly modulation of CCND1 expression [93]. Moreover, CDK4/6 activity has been linked to FGFR2 signaling through the modulation of the MAPK pathway [94]. FGFR2 is essential for palate morphogenesis and plays an important role in CL/P. In Fgfr2 knockout mice, CP was observed,

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accompanied by a reduction in cell proliferation within both the palatal epithelium and mesenchyme [95]. Given that these genes are involved in multiple signaling pathways associated with cell proliferation, the identified miRNA–mRNA networks may play a critical role in palate development by modulating these pathways.

This study has some limitations. (1) Owing to regulatory restrictions on the purchase of PFOS, we were unable to test concentrations higher than 2 μM in our cell viability assays. Further studies are required to directly compare the toxic effects of PFOS and PFHxS under equivalent experimental conditions. (2) The present study was conducted using an *in vitro* model. To better understand the *in vivo* relevance of our findings, it is important to investigate whether PFHxS exposure induces CP in mouse models. However, since the miRNA-gene datasets differ between human and mouse, it remains unknown whether PFHxS induces mesenchymal cell proliferation inhibition through modulation of miRNAs . (3) Our investigation focused on specific miRNAs identified in previous studies by other research groups. To gain a more comprehensive understanding of the miRNA response to PFHxS, future studies should employ miRNA-seq to identify additional miRNAs that may be affected. Further investigations are needed in the future.

5. Conclusions

In conclusion, our study demonstrates that PFHxS inhibits cell proliferation by modulating the *miR-374a-5p-CRISPLD1* and *FGFR2*-CCND1/CDK4/CDK6 pathways (Figure 6). To the best of our knowledge, this is the first report to associate PFHxS with miRNAs and inhibit palatal cell growth. Although further research is required to elucidate the specific mechanisms through which *miR-374a-5p* regulates the G1 phase, our findings provide valuable insights into the potential roles of environmental factors in the etiology of CP.

Figure 6. Proposed mechanism of PFHxS-induced HEPM cell proliferation inhibition.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Identification of putative miR-374a-5p target genes and binding sites associated with cleft palate

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, H.Y. and Y.T.; methodology, H.Y.; software, H.Y. and H.H. (Hanane Horita); validation, H.Y., H.H. (Hanane Horita) and K.O.; formal analysis, H.Y. and H.H. (Hanane Horita); validation, H.Y., H.H. (Hanane Horita) and K.O; investigation, H.Y. and H.H. (Hanane Horita); resources, H.Y., H.H. (Hyogo Horiguchi), and Y.T. ; data curation, H.Y. and H.H. (Hanane Horita); writing—original draft preparation, H.Y.; writing—review and editing, H.H. (Hanane Horita), H.H. (Hyogo Horiguchi), and Y.T.; visualization, H.Y., H.H. (Hanane Horita) and K.O..; supervision, H.Y. and Y.T.; project administration, H.Y.; funding acquisition, H.Y. and Y.T.

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Abbreviations

The following abbreviations are used in this manuscript:

BMP	bone morphogenetic protein
CCND1	Cyclin D1
CDK	cyclin-dependent kinases
CL	Cleft lip
CP	Cleft palate
CL/P	Cleft lip with or without cleft palate
CRISPLD1	cysteine-rich secretory protein LCCL domain containing 1
DAPI	4′,6-diamidine-2′-phenylindole dihydrochloride
EMT	epithelial-mesenchymal transition
FGFR	fibroblast growth factor receptor
HEPM	human embryonic palatal mesenchymal
JARID2	jumonji and AT-rich interaction domain containing 2
IgG	immunoglobulin G
miRNA	microRNA
MSX1	msh homeobox 1
PCOS	polycystic ovary syndrome
PFAS	perfluoroalkyl substances
PFHxA	perfluorohexanoic acid
PFHxS	perfluorohexanesulfonic acid
PFOA	perfluorooctanoic acid
PFOS	perfluorooctanesulfonic acid
TGF	transforming growth factor
ZNF236	zinc finger protein 236

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