PAPER

Maternal exposure to an n-3 polyunsaturated fatty acid diet decreases mammary cancer risk of female offspring in adulthood

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Maternal exposure to dietary factors during pregnancy influences the risk of many adult-onset diseases in the later life of offspring. Here, we investigated the effects of maternal n-3 polyunsaturated fatty acid (PUFA) diet on breast cancer risk of female offspring. Pregnant C57BL/6J mice were fed a normal diet (control group), or a high-fat diet rich in safflower oil (SO), fish oil (FO) or flaxseed oil (FSO) ($n = 10$) throughout gestation and lactation. Their female offspring were fed an AIN-93G diet from weaning. Tumor incidences in offspring induced by 7,12-dimethylbenz[α]anthracene (DMBA) were higher in high-fat groups than in the control group, and were lower in FO and FSO groups than in the SO group. The plasma concentrations of 17β-estradiol (E2), in both pregnant dams and offspring, were significantly lower in FO and FSO groups compared with the SO group. The FO and FSO offspring showed delayed puberty onset, and their mammary glands contained decreased numbers of epithelial terminal end buds (TEBs, targets for malignant transformation) compared with SO offspring. Reduced cell proliferation and increased apoptosis in FO and FSO offspring were observed compared with SO offspring. In line with these changes, maternal exposure to FO promoted the expression of long noncoding RNA (lncRNA) in p53 and apoptosis signaling pathways and inhibited that in NF-κB and Jak-STAT signaling pathways, while FSO promoted the expression of lncRNA in p53 signaling pathways and inhibited that in NF-κB, Jak-STAT and MAPK signaling pathways. In conclusion, maternal exposure to a high-fat diet rich in n-3 PUFAs, both marine- and plant-based, has a protective effect on mammary tumor risk of female offspring in later life.

Introduction

Breast cancer is one of the most frequently diagnosed malignancies and is the leading cause of death from cancer in women.[1] Several indicators are known to be its risk factors, such as sex, family history, lifestyle, and age at menarche and menopause.[2] In recent decades, epidemiological studies have demonstrated the important role that a healthy diet plays in the prevention of breast cancer.[3,4] As the initial mammary buds and primitive mammary epithelial tree start to form during fetal life,[5] and the development of mammary gland structure, such as terminal end buds (TEBs) known as tumor initiation sites, can be altered by maternal dietary intake during pregnancy,[6] the risk of breast cancer may be decreased

not only by protective dietary intake in adulthood, but also by the nutrient environment during the perinatal period.

Among the various nutrient factors, dietary fat is the most focused factor closely associated with risk of cancer.[7,8] Although the effect of individual fatty acids on breast cancer risk has been poorly investigated, n-3 polyunsaturated fatty acids (PUFAs) have been proven to be the most promising subtype to weaken carcinogenesis and decrease the risk of breast cancer. Experimental studies in rodent models[9,10] and *in vitro*[11] have provided consistent evidence supporting the anti-cancer role of n-3 PUFAs. While, in human studies, even though a number of results have also associated n-3 PUFAs with a reduced risk of breast cancer,[12,13] there are still several studies indicating that n-3 PUFAs show null[14,15] or even positive[16] associations with breast cancer risk. The inconclusive results in human studies may be partly explained by overlooking the difference in maternal dietary fat during pregnancy and lactation, which is a critical period influencing the susceptibility of offspring to breast cancer in later life.[17] It is therefore important and interesting to explore whether a diet rich in n-3

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PUFAs during pregnancy and lactation could decrease the risk of offspring developing breast cancer later in life.

Most studies that have investigated the effects of n-3 fatty acids on breast cancer have used eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) as the test fatty acids. Fish oils are such good sources of these fatty acids that they are frequently incorporated into experimental diets[18] or supplemented by gavage.[19] However, the double bond in the n-3 position is originally produced by plants rather than animals, and is commonly found in plant products such as α-linolenic acid (ALA). ALA can be metabolized without change or be elongated and desaturated to EPA or DHA when consumed by animals.[20] Flaxseed oil, containing more than 50% ALA, is a good dietary source of n-3 fatty acids. However, it is unknown whether it is comparable with fish oil when it comes to the influence on breast cancer risk of offspring.

In the present study, we tested the hypothesis that maternal exposure to high-fat diets supplemented with n-3 PUFAs during gestation and lactation may weaken the high-fat diet-induced increased risk of female mice offspring developing mammary tumors and compared the effect of different types of n-3 fatty acids. In addition, plausible mechanisms for modulating the multistage carcinogenesis process by n-3 PUFAs were investigated to explain the link between maternal diets and breast cancer risk in offspring.

Materials and methods

Mouse experimental procedure

A total of 40 C57BL/6J female mice aged 7 weeks were purchased from SLAC Laboratory Animal Corporation Ltd (Shanghai, China). All were housed in a 12 h light/dark cycle room with constant temperature (20 °C) and humidity (above 60%). After being exposed to the AIN-93G diet for the first 7 days, they were mated with C57BL/6J male mice (2 females and 1 male per cage).

Upon mating, the female mice were randomized into 4 groups of 10 mice each. These were the Control, Safflower Oil (SO), Fish Oil (FO), and Flaxseed Oil (FSO) groups. Each was fed the corresponding diet for the duration of gestation and lactation, during which the diet was replaced daily to limit oxidation of various fatty acid species. After dam weaning, only female offspring were kept for further experiments.

To avoid the litter effect, the offspring were cross-fostered on postnatal day (PND) 2. The mean litter-size was balanced to 8–10 pups. Body weight and food intake were recorded weekly. After weaning on PND 21, all pups were fed a standard AIN-93G diet. All animal procedures were performed in accordance with the appropriate institutional and federal regulations and approved by the Zhejiang University Animal Care and Use Committee (Approval Number 2015018).

Diets

The experimental diets, prepared commercially by Trophic Animal Feed High-tech Co., Ltd (Jiangsu, China), included a

Table 1 Dietary composition of experimental diets

	Control diet AIN-93G	High-fat diet		
		Safflower oil	Fish oil	Flaxseed oil
% of total energy				
Protein	20	20	20	20
Carbohydrate	64	37	37	37
Fat	16	43	43	43
kcal g^{-1}	4.0	4.7	4.7	4.7
Ingredient (g kg^{-1} diet)				
Casein	200	233	233	233
L-Cysteine	3	3.5	3.5	3.5
Corn starch	398	85	85	85
Maltodextrin 10	132	117	117	117
Sucrose	100	201	201	201
Cellulose, BW200	50	58	58	58
Soybean oil	70			
Safflower oil		236	158	158
Fish oil			78	
Flaxseed oil				78
Mineral mix S10022G	35	53	53	53
Vitamin mix V10037	10	12	12	12
Choline bitartrate	2.5	2.3	2.3	2.3
% of total fatty acid				
Palmitic acid	5.2	6.6	5.8	5.9
Stearic acid	1.5	2.1	2.4	2.3
Oleic acid	9.3	9.9	13.4	13.9
Linoleic acid	18.4	76.5	8.7	21.6
Linolenic acid	59.8	2.4	25.3	44.5
Eicosapentaenoic acid			7.4	
Docosahexaenoic acid			5.1	

control diet (AIN-93G, 16% energy from fat), a high-fat diet rich in n-6 PUFA (safflower oil) and a high-fat diet rich in n-3 PUFA (safflower oil: fish oil/flaxseed oil = 2 : 1, w/w). The high fat diets were modified based on the AIN-93G diet, with 43% energy from fat. The caloric densities of the control and high-fat diets were 4.0 kcal g^{-1} and 4.7 kcal g^{-1}, respectively. The dietary components and fatty acid compositions of each diet are shown in Table 1.

Mammary tumorigenesis

Mammary tumors were induced in 49-day-old female offspring by administering 7,12-dimethylbenz(α)anthracene (DMBA, Sigma, 10 mg L^{-1} dissolved in olive oil) as a dose of 65 mg per kg body weight by oral gavage once a week for 4 weeks. Mice were checked weekly for mammary tumors by palpation, starting from week 5 and continuing to week 20 after DMBA administration. The end point for data analysis included: (i) tumor incidence (the number of mice with tumor); (ii) tumor multiplicity (the number of tumors in each mouse); (iii) latency to tumor appearance (just determined in mice with tumor); and (iv) tumor volume (final tumor size). Tumor volume was calculated using the formula: $V = 1/6\pi ABC$, where A, B and C represent the final length, width and height of each tumor at the end of experiment. During the follow-up, the mice would be euthanized ahead of time if the detectable tumor burden approximated 10% of body weight as required by our insti-

tution. All surviving animals were disposed in week 20 post-DMBA.

Mammary gland morphology

The harvested fourth abdominal mammary glands, obtained from female offspring on PND 21 (weaning) and 49 (sexual maturity), were processed for morphological analysis as whole mounts. Briefly, they were stretched onto a microscopic slide and placed overnight into Carnoy's Fixative Solution. After washing with gradient ethanol, they were stained with a carmine aluminium solution.[21] The stained mammary glands were examined under an Olympus dissecting microscope. The number of TEBs, alveolar buds (ABs) and alveolus (As) in each mammary gland were counted in a double-blind manner. These structures have been well characterized by Russo.[22] After this counting, the differentiation degree of mammary gland was calculated as the number of (ABs + As)/number of TEBs, which was inversely associated with breast cancer risk. In addition, the relative density of epithelial trees was evaluated using the Optimas Image Analysis software based on images of the glands, which were taken from the same part of the individual glands with an area of 3 × 3 mm.

Serum estradiol

Blood was collected by cardiac puncture from pregnant dams on gestation day 17, and from their offspring on PND 21 and 49. Serum was separated and kept at −80 °C until use. Total circulating E2 levels were measured with a mice double-antibody kit (Shanghai Langdun Bioengineering Institute, Shanghai, China) according to the manufacturer's instructions. To match estrus cycle, the estrus stage of pregnant dams and offspring on PND49 was determined with vaginal smear before collecting blood. The animals in proestrus were excluded, because E2 levels are at their peak in this stage but relatively steady in the other estrus stages. The smears in proestrus were characterized by a large number of nucleated cells. The estrus stage of offspring on PND 21 was not determined, as they had not undergone puberty and had not started the estrus cycle yet.

Puberty onset

Starting from PND 28, the female offspring were checked daily for vaginal opening to establish their age of puberty onset, which is a marker of sexual maturation in rodents and a risk factor for breast cancer.[23]

Proliferation and apoptosis of offspring's mammary glands

Proliferation and apoptosis indexes were assessed in the fifth abdominal mammary glands obtained from female offspring on PND 21 and 49. The mammary tissues were embedded in paraffin and processed for either proliferation or apoptosis, using Ki67 immunohistochemistry[24] and TUNEL staining,[25] respectively (n = 7–8 per group and age). DAKO K4002 (Agilent) and IOSL S2100 kits (Chemicon) were used for proliferation and apoptosis according to the manufacturer's instructions. The indexes were determined by calculating the number of cells that were positive stained among at least 1000 TEB cells after histological evaluation. Slides were all subjected to blind evaluation by two independent investigators.

LncRNA and mRNA genomics and KEGG enrichment analysis

The lncRNA and mRNA profiling was sequenced at the Novogene Bioinformatics Institute (Beijing, China) with Illumina Hiseq 2500.[26] A total amount of 3 μg RNA per mammary gland of 7-week-old offspring was used as input material for the RNA sample preparation. Quantification of both lncRNA and mRNA expression levels was estimated by calculating the FPKMs of the transcripts with Cuffdiff (v2.1.1).[27] Differential expression in the digital transcript among the three high-fat groups was determined with a model based on the negative binomial distribution. Transcripts with a P-adjust <0.05 were regarded as differentially expressed. As lncRNA exerts its function always by regulating the expression of mRNA, we subsequently predicted the target gene (mRNA) of lncRNA with WGCNA.[28] To make sure the differentially expressed lncRNA really brings changes to mRNA expression, it was the intersection of target mRNA and differentially expressed mRNA that was selected as the final mRNA for further KEGG enrichment analysis. The KOBAS software was used to test the statistical enrichment of the final genes in KEGG pathways.

Statistical analysis

Data were presented as mean ± standard deviation (SD) and analyzed using Duncan's multiple range test. Significantly different means at the 5% level are followed by different letters. Differences in the day of puberty onset and tumor incidence among the four groups were compared using the Kaplan–Meier survival curves followed by the log-rank test. All the tests were performed using the SPSS 23.0 software.

Results

Pregnancy outcome

Food consumption, success rate of pregnancy, litter size and offspring's gender distribution were similar in different dietary groups (Table 2). Body weight gains of pregnant mice were heavier in high-fat groups than in the control group. Female offspring in the FO group were heavier than the other three groups, and the difference remained even though the pups reached maturity.

Mammary tumorigenesis

Incidences of mammary tumors were significantly higher in offspring exposed to the high-fat diets than in offspring exposed to the control diet during gestation and lactation (P < 0.03) (Fig. 1). Among the high-fat groups, FO (P = 0.013) and FSO (P = 0.029) diets decreased the mammary tumor incidence demonstrably compared with the SO diet, from 91% incidence to 65% and 75%, respectively. No difference was noted between the FO and FSO groups. Besides, an increase in tumor

Table 2 Variables in mother and offspring mice exposed to different dietary fatty acids during pregnancy and lactation

Variables	Control diet AIN-93G	High-fat diet		
		Safflower oil	Fish oil	Flaxseed oil
Mother				
Body weight before pregnancy, g	17.8 ± 0.6	17.9 ± 0.7	18.0 ± 0.5	17.9 ± 0.7
Body weight gain during pregnancy, g	9.4 ± 1.3^a	11.5 ± 1.9^b	12.1 ± 2.4^b	11.2 ± 1.5^b
Food consumption during pregnancy, kcal day^{-1}	10.4 ± 1.2	10.8 ± 0.8	10.3 ± 1.2	10.8 ± 0.8
Successful pregnancies, %	80.0	80.0	80.0	80.0
Offspring				
Number of pups/litter	8.4 ± 1.2	8.2 ± 1.4	8.7 ± 0.9	8.4 ± 1.1
Female/male ratio	1.0 ± 0.2	1.1 ± 0.4	0.9 ± 0.5	1.2 ± 0.4
Female pup weight				
Postnatal day 1	1.2 ± 0.2^a	1.3 ± 0.1^b	1.4 ± 0.2^c	1.3 ± 0.2^b
Postnatal day 21	8.4 ± 1.2^a	8.9 ± 0.5^a	10.3 ± 0.7^b	8.5 ± 0.5^a
Postnatal day 49	16.5 ± 0.4^a	16.9 ± 0.7^a	18.3 ± 0.6^b	16.8 ± 1.1^a

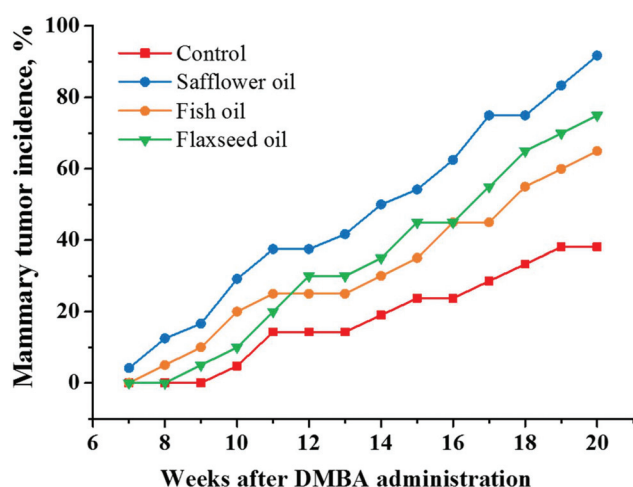The values are expressed as means ± SD ($n = 12$ in each group).

Fig. 1 The proportion of DMBA-induced mammary tumors (%) in female offspring mice exposed to control ($n = 21$), safflower oil ($n = 24$), fish oil ($n = 20$) or flaxseed oil ($n = 20$) diet *via* their mother during gestation and lactation. Tumor incidence was significantly lower in the low-fat group than that in the high-fat group ($P < 0.03$) and in the fish oil ($P = 0.013$) and the flaxseed oil ($P = 0.029$) group than that in the safflower oil group.

volume was observed in SO pups, compared with the other three groups. However, the other parameters of tumorigenesis, tumor latency and multiplicity were not affected by maternal dietary exposures (Table 3).

Mammary gland morphology

Maternal exposure to different fatty acids influenced the mammary gland morphology of female offspring, mainly by affecting the number of TEBs, which are the targets of carcinogen-induced malignant transformation. Although all offspring were fed post-weaning according to the AIN-93G diet, those exposed *in utero* to high-fat diets displayed more TEBs on both PND 21 and PND 49 than control offspring (Fig. 2). Maternal FO and FSO diets decreased the number of TEBs in female

Table 3 The latency for the appearance of a tumor after DMBA exposure, the number of tumors per animal (tumor multiplicity), and the mean volume of tumors in DMBA-treated mice that were exposed to different fat diets *in utero*

Treatment	Tumor latency, weeks	Tumor multiplicity, no. of tumors per mouse	Tumor volume, mm^3	n
Control ($n = 21$)	14.1 ± 1.2	1.1 ± 0.4	67.3 ± 21.4	8
Safflower oil ($n = 24$)	13.6 ± 0.9	1.3 ± 0.5	$122.0 \pm 53.9^*$	22
Fish oil ($n = 20$)	14.2 ± 1.1	1.1 ± 0.3	78.2 ± 22.6	13
Flaxseed oil ($n = 20$)	14.5 ± 0.9	1.2 ± 0.4	84.1 ± 18.4	15

n = number of mice with tumors; DMBA = 7,12-dimethylbenz[α] anthracene; * indicates statistical significance at the $P < 0.05$ level.
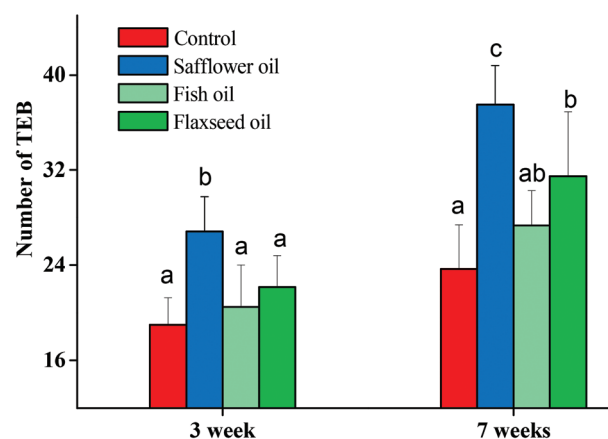

Fig. 2 The number of TEBs on 3-week and 7-week old female offspring of mothers fed a control, safflower oil, fish oil or flaxseed oil diet during gestation and lactation ($n = 6–8$ per group). The different letters indicate statistical significance ($P < 0.05$) using a Duncan test.

offspring compared with SO offspring. Although a smaller increase in TEBs number was observed in FSO offspring than FO offspring, the difference was not significant. There was no

difference among the four groups in terms of differentiation degree and relative density of epithelial trees in the mammary glands.

E2 levels in pregnant mother and offspring

For pregnant mothers, the serum levels of E2 on gestation day 17 were much higher in high-fat dietary dams than in control dams (Fig. 3). Among the high-fat groups, FO and FSO diets significantly decreased the E2 levels in pregnant mothers when compared with the SO group. There was no difference between FO and FSO. For the offspring, E2 levels dropped sharply compared with their mothers. It is consistent with the clearance of estrogens from neonates. Among offspring of the same age, both 21-day-old and 49-day-old, the variation of E2 levels of different groups displayed similar tendencies to those of their mothers. That is to say, the influence of different dietary exposures on E2 levels of pregnant mothers was transmitted to offspring, irrespective of whether or not their lifespan reached 3 weeks or 7 weeks. Thus, maternal exposure to high-fat diet rich in n-3 PUFAs during the fetal period significantly decreased the E2 levels in adulthood compared with n-6 PUFA high-fat diet, even though they were still higher than those in the low-fat control group.

Vaginal opening

Vaginal opening is considered to be a sign of puberty initiation, which is an influential factor in the development of breast cancer. Fig. 4 showed that vaginal opening occurred earlier in the offspring exposed to high-fat diets in the fetal period than in the control diet offspring. Puberty onset in FSO and FO offspring occurred earlier than in SO offspring. The FO diet delayed the timing of puberty onset compared with the FSO group, but the differences didn't reach statistical significance. These data suggest that maternal exposure to high-fat diets advanced the timing of the offspring's puberty onset, while n-3 PUFAs can mitigate the advancing effect of high-fat diets.
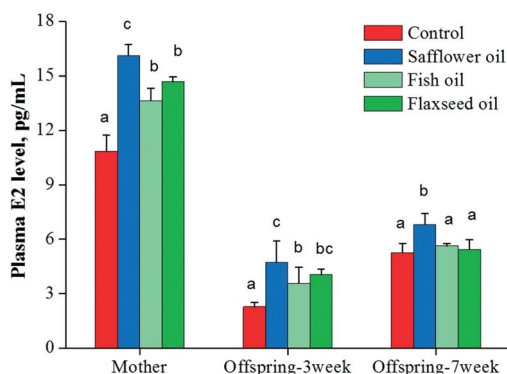
Fig. 4 The vaginal opening (%) between postnatal days 32 and 38 of female offspring mice exposed to control ($n = 30$), safflower oil ($n = 34$), fish oil ($n = 32$) or flaxseed oil ($n = 32$) diet *via* their mother during gestation and lactation. Puberty onset occurred significantly earlier in high-fat groups than in the low-fat control group ($P < 0.05$). Fish oil ($P = 0.028$) and flaxseed oil ($P = 0.046$) significantly delayed puberty onset compared with the safflower oil group.

Proliferation and apoptosis of the offspring's mammary glands

In TEB structures of mammary gland sections from both 3- and 7-week-old offspring, the levels of cell proliferation were decreased significantly in the FO and FSO groups compared with the SO group (Fig. 5). Cell proliferation in the FO group of 3-week-old pups was lower than in the FSO group and was comparable to the control group. Proliferation in 7-week-old pups did not significantly differ among animals exposed to FO, FSO and control diets.

Significantly higher levels of apoptosis were observed in FO and FSO offspring compared with SO pups, at both 3 and 7 weeks of age (Fig. 6). Of the two types of n-3 PUFAs, the FSO group presented a higher apoptosis index in 3-week-old offspring, while in 7-week-old offspring, the FO group index was higher. Interestingly, cell apoptosis in the SO group was lower than in the control group in pups aged 3 weeks, while this result was reversed in pups aged 7 weeks. This may be

Fig. 3 The plasma levels (mean $\pm$ SD) of total 17β-estradiol (E2) in pregnant mice that were fed with control, safflower oil, fish oil or flaxseed oil diet throughout gestation and lactation ($n = 5$–7 per group), and their 3-week-old or 7-week-old offspring ($n = 6$–8 per group) that were kept on AIN-93G diet immediately after weaning. The different letters indicate statistical significance ($P < 0.05$) among groups.
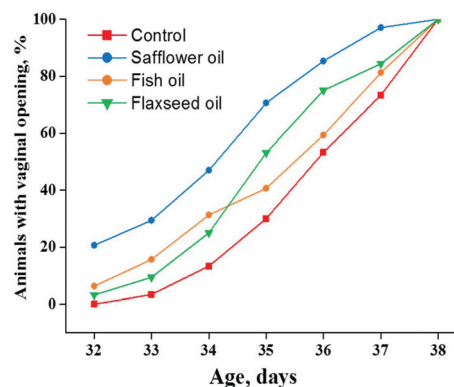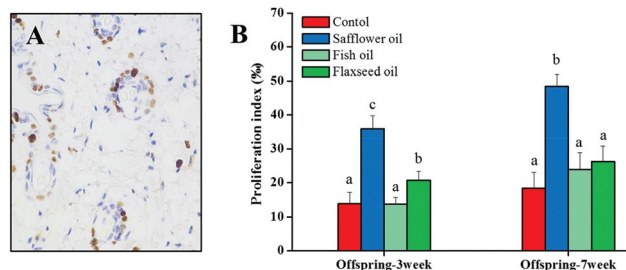
Fig. 5 Effects of experimental diets on mammary gland cell proliferation in the terminal end bud structure of mammary gland sections from female offspring mice of the control, safflower oil, fish oil or flaxseed oil dietary groups at 3 and 7 weeks of age ($n = 6$–8 per group). The proliferation indexes (‰) are expressed as the mean $\pm$ SD. The different letters indicate statistical significance ($P < 0.05$) among groups.
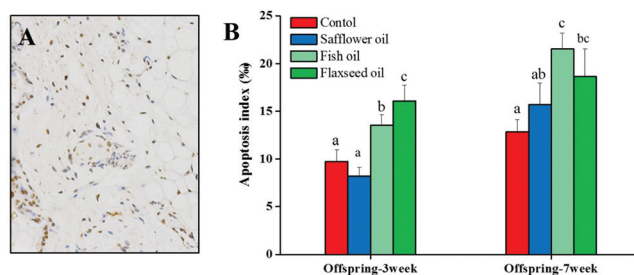
Fig. 6 Effects of experimental diets on mammary gland cell apoptosis in mammary gland sections from female offspring mice of the control, safflower oil, fish oil or flaxseed oil dietary groups at 3 and 7 weeks of age (n = 6−8 per group). The apoptosis indexes (‰) are expressed as the mean $\pm$ SD. The different letters indicate statistical significance ($P <$ 0.05) among groups.

because of the regulatory effect on the excessive proliferation index in 7-week-old offspring.

LncRNA and mRNA genomics and KEGG enrichment analysis

Transcriptional profiles of lncRNA and mRNA in the mammary gland showed distinct differences among the high-fat dietary groups. In the FO group, 467 lncRNA transcripts (194 up-regulated and 273 down-regulated) and 1590 mRNA transcripts (937 up-regulated and 653 down-regulated) were differentially expressed compared to the SO group. In the FSO group, 261 lncRNA transcripts (110 up-regulated and 151 down-regulated) and 1552 mRNA transcripts (1035 up-regulated and 517 down-regulated) were differentially expressed compared to the SO group. To further ascertain the function of differentially expressed genes, KEGG pathway enrichment analysis was performed. The results showed that, in FO offspring, the up-regulated genes were enriched in p53 and apoptosis signaling pathways and the down-regulated genes were enriched in NF-κB and Jak-STAT signaling pathways. In FSO offspring, the up-regulated genes that were enriched in p53 signaling pathways and the down-regulated genes that were enriched in NF-κB, MAPK and Jak-STAT signaling pathways were inhibited (Fig. 7).

Discussion

The present study has demonstrated that maternal exposure to high-fat diets was associated with increased breast cancer risk of female offspring, and n-3 PUFAs, both marine- and plant-based types, could attenuate multiple aspects of the effect. Our results again confirm previous studies that have demonstrated that maternal fish oil supplementation during pregnancy, both by incorporation into dietary[18] and oral gavage,[19] can decrease the incidence of mammary tumors in offspring. In addition, we also found that the flaxseed oil n-3 PUFA diet during pregnancy and lactation was as effective as the fish oil n-3 PUFA diet in decreasing the breast cancer risk of offspring. Even though neither of them could decrease the risk to the same
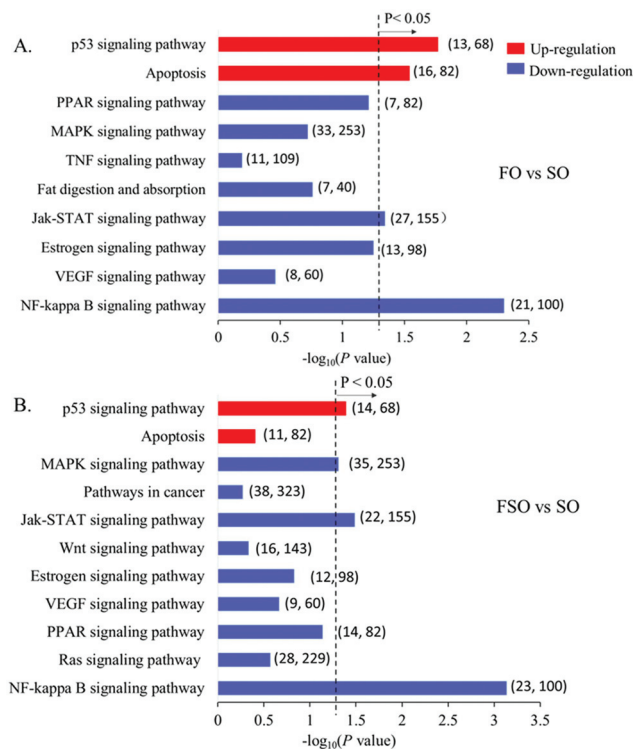

Fig. 7 Analysis of cancer-associated KEGG pathways enriched by differentially expressed genes between FO and SO groups (A), and between FSO and SO groups (B). FO: fish oil; FSO: flaxseed oil; SO: safflower oil. The numbers in brackets represent the number of significantly differentially expressed genes and the number of all background genes in the corresponding pathway, respectively. The pathways enriched by up-regulated genes were colored red, and the pathways enriched by down-regulated genes were colored blue. The pathways showing P values < 0.05 were considered to be statistically significantly different.

level as that in the low-fat control group, they decreased the incidence of DMBA-induced mammary tumorigenesis by 26% and 16% in FO and FSO offspring, respectively, compared with SO offspring. These findings are in agreement with a previous rodent study which showed beneficial effects of n-3 fatty acids in canola oil (10% ALA), compared with corn oil, in mitigating mammary tumor development.[29]

Potential mechanisms of action may be attributed to the changes of plasma E2 concentrations, which were significantly reduced in FO and FSO dietary mothers and their female offspring aged 3 or 7 weeks than those in the SO group. It has been reported in cohort studies that the incidence of breast cancer was 2-fold higher in the daughters of mothers who took synthetic estrogen diethylstilbestrol during pregnancy.[30] Additionally, animal studies also showed that estrogens, *via* injection[31] and diet,[32] were both linked to increased breast cancer risk in offspring. From the lowered E2 levels in FO and FSO offspring, it is evident that they showed delayed puberty onset, which is mostly mediated by hormones. In turn, the delayed puberty further lowered the risk of breast cancer through decreasing the cumulative time of exposure to ovarian

estrogens. Meanwhile, these observations are entirely in agreement with the finding in humans that early menarche is associated with increased breast cancer risk.[33]

Pathways that may also participate in mitigating the impact of different fatty acid exposures in fetus life on later breast cancer risk are suggested to include the changes in mammary gland morphology.[21] Our results indicate that n-3 PUFAs were related to modifications in mammary gland development: the number of TEBs and epithelial cell proliferation were decreased and, also, epithelial cell apoptosis was elevated. TEBs, considered to be the only target site for neoplastic transformation in carcinogen-treated rodents, were positively correlated with breast cancer susceptibility.[24] After TEBs regress to terminal ducts, the glands are no longer susceptible to carcinogenesis induced by chemicals or radiation.[34,35] In addition, it has been reported that *in utero* exposure to excess estrogens results in an elevated number of TEB structures;[36,37] it is thus possible that one of the processes causing a reduced level of breast cancer risk in n-3 PUFA offspring is firstly a decrease in estrogens, which further leads to a decreased number of targets for malignant transformation. Furthermore, a lower level of proliferating cells in the mammary gland of n-3 PUFA offspring decreased the breast cancer risk as it brought a decreased rate of genetic instability and DNA adduct formation. Meanwhile, apoptosis also plays an important role in determining the susceptibility to breast cancer, as it prevents the genetic or epigenetic changes that the damaged cells have encountered from being transmitted to daughter cells. Thus, the lower proliferation and the higher apoptosis observed in n-3 PUFA pups together led to a reduced number of TEBs as well as a decreased possibility of DNA damage, by which the carcinogenic process could be initiated.

There is general agreement that the lifelong changes in the transcriptome affected by environmental alterations in the fetus time are likely to have been epigenetically induced. As the epigenetic signature in fetal cells is established during early development and it can interpret the information of the genetic code by means that do not involve mutations. LncRNA, as an important branch of epigenetics, has been shown to play key roles in many fundamental processes associated with mammary tumorigenesis, including apoptosis, cell cycle regulation and DNA damage response.[38,39] LncRNA exhibited distinct expression patterns between human cancers and normal tissues.[40] The present study showed that lncRNA expressions showed dramatic differences among mammary glands from FO, FSO and SO offspring. The common pathways enriched by differentially expressed genes between the FO/FSO and SO groups were p53, NF-κB, and Jak-STAT signaling pathways. These pathways are important for breast cancer susceptibility, as they can regulate various cancer-associated processes, such as cell proliferation, apoptosis and differentiation.[41–43] These results were consistent with a previous report that neonatal exposure to the synthetic estrogen diethylstilbestrol had a lifelong influence on gene expression in mammary glands, and the most significant changes involved the NF-κB signaling pathway.[44] NF-κB was proved to be related to breast cancer pro-

gression,[45] anti-estrogen resistance[46] and poor prognosis.[47] Activation of the Jak-STAT signaling pathway was regulated accordingly during different periods of mammary gland development.[48] The higher level of p53 protein in the mice exposed to pregnancy-mimicking hormonal manipulation might be part of the mechanism that prevented the high estrogen levels during pregnancy from inducing DNA damage and genetic alterations.[49] These results indicated that p53, NF-κB, and Jak-STAT signaling pathways were all important for regulating the differentiation of mammary gland and breast cancer susceptibility. Even though relatively little was known about long-term changes in the lncRNA profile of the mammary glands in animals exposed to n-3 PUFAs *in utero*, our study proved that the lifelong effect of maternal dietary fat on mammary cancer may be *via* influencing the expression of the related genes in these pathways.

Mice in this study were carefully monitored for food intake and body weight. Food consumption estimated by food disappearance from the feeding apparatus was similar among the three high-fat groups, but body weight gains in FO pregnant dams as well as their corresponding offspring were slightly greater than in the other two groups. Given that high birth weight is associated with increased breast cancer risk,[50] we concluded that the slightly greater weight of FO offspring might have lessened the benefits of n-3 PUFAs. Thus, the inhibitory effects of FO diet might have been even more dramatic if body weight was controlled to exacting levels.

The present study had several limitations. Firstly, mammary stem cells are always the most strategic target for epigenetic modification and the number of stem cells can determine a gland's susceptibility to malignant transformation.[51,52] Thus it may be an important mechanism that n-3 PUFAs *in utero* decrease later breast cancer risk by decreasing the total number of stem cells or influencing related gene expression profiles. However, information about the stem cell was neglected in this study. Secondly, pregnant mice in the study were exposed to diets with either adequate (FO and FSO groups) or deficient (SO group) amounts of n-3 PUFAs. However, there are two potential interpretations for the study results. One is the presence of n-3 PUFAs in FO and FSO groups that inhibit tumor outcomes. It is also possible that the absence of n-3 PUFAs in the –SO group resulted in an increase in tumor risk. Thus, it will be important to determine whether it is the former or the latter in future studies, with dose dependent methods. Nevertheless, the present study has powerfully demonstrated that high-fat maternal diets increase the offspring's breast cancer risk, while n-3 PUFAs in high-fat diets play an important role in weakening the risk.

Taken together, these findings support the hypothesis that maternal consumption of n-3 PUFAs alleviated the high-fat diet-induced increased risk of female offspring developing mammary cancer, and ALA had a comparably protective effect with EPA and DHA. Importantly, this study has, for the first time, proved that the protective effects of n-3 PUFAs were associated with up-regulated lncRNA in the p53 signaling pathway, and down-regulated lncRNA in NF-κB and Jak-STAT

signaling pathways. These data also have important public health implications, as they raise the possibility that prevention of breast cancer could be started from the fetus period by modifying the dietary fatty acid intake of pregnant women.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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