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Maternal n-3 Polyunsaturated Fatty Acids Supplementation Potentiates Brown Adipose Tissue Development And Reduces The Risk Of Childhood Obesity

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MATERNAL N-3 POLYUNSATURATED FATTY ACID SUPPLEMENTATION POTENTIATES BROWN ADIPOSE TISSUE DEVELOPMENT AND REDUCES THE RISKS OF CHILDHOOD OBESITY

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Brown adipose tissue (BAT) is a crucial regulator in energy expenditure by dissipating energy in the form of heat through mitochondrial uncoupling protein 1 (UCP1). Previously, we demonstrated that n-3 PUFA (Omega-3 Polyunsaturated Fatty Acids) increase brown adipogenesis by miRNA-mediated epigenetic mechanisms. Given that BAT is formed at the late stage of pregnancy, we hypothesized prenatal exposure of n-3 PUFA through maternal nutrition potentiates BAT development. To test this hypothesis, Female C57BL/6 mice were fed a diet containing n-3 PUFA (3%) derived from fish oil (FO), or an isocaloric diet devoid of n-3 PUFA (Cont), and same diet was maintained throughout the pregnancy and lactation. Results showed that maternal FO was effectively delivered to the offspring significantly reducing n-6/n-3 ratio, altered blood lipid profile, and upregulated brown adipose tissue specific genes and proteins. The elevated levels of brown adipose tissue signature gene profiles, i.e., Uncoupling protein 1 (UCP1), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), in weaned pups with maternal FO intake were correlated with higher expression of GPR120 and the functional cluster of brown-specific miRNAs including miR30b and miR193b/365. Intriguingly, augmented BAT development in offspring by maternal FO intake was also associated with 1) increased epigenetic marks at H3K27Ac and H3K9me2, and decreased histone deacetylase 1

(HDAC1), and 2) upregulation of Drosha, which is a miRNA-editing enzyme for pre-miRNA production. More importantly, benefits of maternal FO intake remained later in life showing that pups that received maternal FO presented a lower body weight at weaning, an increase in energy expenditure and higher core body temperature against acute cold exposure in later life. Taken together, our results indicated that prenatal exposure to n-3 PUFA potentiates the offspring's BAT development, at least partly via synergistic epigenetic modifications in histone modifications and miRNA production, which may confer long-lasting metabolic benefits to offspring.

Keywords: Brown adipose tissue, n-3 PUFA, Fish oil, Thermogenesis, Maternal nutrition

This work is dedicated to

My beloved parents Cuimei Wu and Chunsheng Fan for their endless support and love.

All other family members for being supportive for me as the first in the family to pursue graduate school abroad in the United States.

And all the C57BL/6 experimental mice, their lives contributed to the true science and are indispensable for my research.

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CHAPTER 1. LITERATURE REVIEW

The epidemic of obesity is on the rise all around the world according to the global estimates made by World Health Organization (1). As is known, the development of obesity is caused by an energy imbalance, resulting in the storage of excess energy in the form of triglycerides. Obesity is commonly concurrent with insulin resistance and hyperinsulinemia, which lead to the development of type 2 diabetes mellitus (2) (3). Brown adipose tissue is a non-shivering thermogenic organ and it can generate heat to maintain thermal homeostasis by uncoupling oxidative phosphorylation from ATP production via mitochondrial uncoupling protein 1 (UCP1) (4). The classical brown adipocytes are found in the interscapular brown adipose tissue which exists when offsprings are born and the beige adipocytes are primarily found in subcutaneous white adipose tissue after adrenergic stimulation, which are produced by external stimuli, cold or dietary factors. (5). Due to the high efficiency in oxidizing glucose and fatty acids, and the recent discovery of its existence in adult human (6), brown adipose tissue has a promising therapeutic potential to combat obesity.

Adaptive thermogenesis is an energy-demanding process in which futile uncoupled-respiration releases the mitochondrial proton gradient as heat. It is regulated by a range of intrinsic and extrinsic factors including daily diet (7). Diet plays a first-line role in regulating energy balance and adiposity. Beyond the obvious strategy of calorie restriction, emerging evidence supports the thermogenic function of several food-derived components. Dietary intervention is more likely a safe approach to modulate brown/beige energetics compared to targeted activation of the β3-adrenergic receptor (ADRB3) by pharmacological interventions due to the increased risk of cardiovascular side effects (8).

Nearly a dozen dietary polyphenolic compounds have been identified as candidate molecules to stimulate brown/beige activation (7,9). For example, capsaicin (10), green tea catechins (11-13), resveratrol (14,15), quercetin (16,17), flavan-3-ols (18), and berberine (19) are reported to possess brown stimulatory property. However, low bioavailability of dietary polyphenols poses a challenge in launching a readily translatable anti-obesity approach through diet-induced thermogenesis.

1.1 N-3 polyunsaturated fatty acid

Dietary fat, as a vital macronutrient, participates in numerous biological activities in mammals. Based on the carbon numbers, fatty acids can be categorized as short chain (2-6C), medium chain (6-12C), long chain fatty acids (13-22C), and very long chain fatty acids (more than 22C). Based on the number of double bond, fatty acids can also be categorized as saturated, monounsaturated, and polyunsaturated fatty acids (20). Based on the position of the first double bond near the methyl terminus, long chain polyunsaturated fatty acids can be defined as n-3, n-6, and n-9 (21). Particularly, omega-3 polyunsaturated fatty acids (n-3 PUFA) are essential fatty acids abundant in fish source and fish oil, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Another omega 3 fatty acid, α-linolenic acid (ALA), also exists in green leafy vegetables, nuts, soybeans, and some seeds (20). Since n-3 PUFA cannot be synthesized in the body and the conversion rate from ALA to EPA and DHA is limited and inefficient, it is necessary to supply adequate amount from diet and supplementation (20). Recently, fish oil (n-3 PUFA) supplementation, mainly EPA and DHA, has been demonstrated to exert health beneficial effects against inflammatory, hyperlipidemic (22), autoimmune, and cardiovascular disorders (23). Also, it is regarded as a healthy addition to the diet of diabetic patients (24).

Particularly, previous researches have reported that eicosapentaenoic acid (EPA) reduces high-fat (HF)-diet-induced obesity and insulin resistance in mice (25). Also, accumulating evidences have supported that eicosapentaenoic acid (EPA), major n-3 PUFA contained in fish oil, can promote brown thermogenic adipogenesis and adaptive thermogenesis (7,25-28). However, the underlying mechanisms remain to be elucidated.

1.2 Proposed mechanism regarding n-3 PUFA and thermogenesis

1.2.1 Thermogenic function of n-3 PUFA through GPR120 activation

G-protein-coupled receptor 120 (GPR120) is a functional receptor/sensor of n-3 fatty acids (FA) (29). Upon binding of n-3 PUFA, GPR120 activates the heterodimeric Gαq subunit and subsequently induces diverse cellular responses via several second messengers such as $[Ca2+]_i$, cAMP, and diacylglycerol (DAG) (reviewed in (29,30)). Adipocytes, but not preadipocytes, express high levels of GPR120 in the rank order of BAT>> iWAT ~ eWAT > mWAT (31). In this section, we focus on the role of GPR120 activation by fish oil in governing brown/beige fat activation.

1.2.1.1 Activation of brown fat-specific transcriptional program by n-3 PUFA

Humans contain a measurable amount of classical brown fat that expresses constitutively active uncoupling protein 1 (UCP1) compared to beige fat that has temporal and reversible UCP1 activation dependent on environmental stimuli such as cold temperature and excess nutrition (32). Provided the continuous nature of the thermogenic function, a small increase of BAT mass and/or activity are thought to exert a profound impact on overall energy expenditure. Classical brown fat is found in certain anatomical depots such as the cervical, supraclavicular, perirenal, axillary, and paravertebral areas (33).

Several research groups including our laboratory have tested the possibility that dietary fish oil or GPR120 agonists augment the mass and/or activity of classical brown fat (26,31,34,35). As an *in vitro* model of brown adipocytes, Kim et al. isolated brown fat precursor cells from the interscapular BAT (iBAT), and then n-3 PUFA (i.e., EPA and DHA) were added during differentiation and compared with other fatty acids (oleic acid and palmitic acid) (26). Treatment with 100 μM of EPA and DHA, but not oleic and palmitic acid, significantly promoted brown fat-specific transcriptional programming, resulting in an increase of uncoupled respiration. Supporting these results, Pahlavani et al. showed that EPA (50-100 μM) treatment increased thermogenesis in HIB1B cells, brown preadipocytes derived from a murine brown fat tumor (35). In addition, Kim el al. demonstrated that EPA-induced transcriptional activation of brown adipogenesis relied on microRNA (miRNA)-mediated epigenetic mechanisms in primary murine brown adipocytes. Functional clusters of brown fat-specific miRNAs including miR193b/365, miR30b, and miR378 were induced by EPA treatment (26). More importantly, transcriptional activation of brown adipogenesis as well as miRNA production were dependent on GPR120 signaling. Stimulation with GW9508, a GPR120 agonist, recapitulated the effects of EPA. Conversely, depleting GPR120 expression by siRNA (26) or genetic deletion (31), attenuated EPA-mediated activation of brown adipogenesis. These data suggest that GPR120 signaling precedes the transcriptional activation of brown adipogenesis, in part through miRNA-mediated epigenetic mechanisms.

The effects of EPA on BAT thermogenesis were validated in HF diet-fed C57BL/6 mice. EPA supplementation, either provided as pure EPA (35,36) or fish oil formulation (24,26,31,34), reduced HF-diet induced obesity and metabolic dysfunction by increasing

thermogenic energy expenditure. In addition, these *in vivo* metabolic benefits were concurrent with GPR120 activation and brown-specific miRNA production (26). A further metabolic insight into the role of GPR120 on BAT thermogenesis was found in GPR120 null mice. These mice failed to upregulate cold-induced BAT genes and demonstrated loss of brown fat-specific gene profiles and morphology, and ineffective maintenance of body temperature with cold exposure (37). Taken together, these results suggest a novel epigenetic signaling axis of EPA/GPR120/miRNAs is involved in regulating brown fat function (26). In addition, GPR120-dependent FGF21 (fibroblast growth factor 21) secretion was proposed to propagate the BAT-driven thermogenic responses to other tissues, e.g., liver and muscle, via autocrine and paracrine signaling of FGF21 (31).

Rosell et al report an intriguing intrinsic feature of GPR120 in BAT (38). Cold treatment itself promotes ~2-fold induction of GPR120 in BAT, but not in WAT (38), suggesting that the intrinsic ligand for GPR120 may be synthesized in brown fat as a part of cold adaptation. From this aspect, it is worthwhile to dissect BAT lipid metabolism during cold adaptation. In fact, n-3 PUFA is endogenously synthesized in brown fat, but not in white fat, in mice and rats (39,40). In addition, Inuits, indigenous people of the Canadian Arctic, possess selected alleles of fatty acids desaturases, thereby facilitating endogenous n-3 PUFA synthesis (41). Moreover, an induction of GPR120 by fish oil intake synergistically promotes thermogenesis compared to cold exposure alone (26,38). The elevated levels of n-3 PUFA in BAT via endogenous biosynthesis as well as high fish consumption would synergistically contribute to keeping Inuits warm against cold temperature by promoting brown thermogenesis. Further research is warranted to understand the exact nature of cold-induced GPR120 induction in BAT in the context of

lipid profile changes, n-6/n-3 ratio, membrane fluidity, and thermogenic heat release.

In conclusion, GPR120 activation, either by endogenous synthesis of n-3 PUFA in cold or by dietary n-3 supplementation, plays an essential role in boosting brown fat-specific transcriptional programming including miRNA-mediated epigenetic mechanisms and FGF21 signaling (Figure 1A).

1.2.1.2 Role of GPR120 activation in beige fat differentiation by n-3 PUFA

Oh et al. first demonstrated that white adipocytes express high levels of GPR120, but not GPR40 (42). The stimulation of GPR120 by n-3 PUFA, or its chemical agonist, promotes glucose uptake by sensitizing insulin signaling (29). Supporting this notion, genetic ablation of GPR120 in mice results in hepatic steatosis and insulin resistance (43). In humans, lack of GPR120 signaling activity due to a genetic mutation in the GPR120 gene (p.R2700H) is correlated with increased risk of obesity (43). Despite several controversies surrounding the anti-obesogenic role of GPR120 in humans, evidence derived from experimental animals indicates that GPR120 activation increases lipid combustion and reduces adiposity.

Several groups of scientists provided direct evidence that fish oil promotes beige fat development from adipogenic precursor cells (24,28,37,44,45). Zhao et al first reported that 200 μM EPA treatment of stromal vascular (SV) cells isolated from mouse inguinal fat effectively induced beige-specific signature gene profiles, and increased mitochondrial energy expenditure (28). Laigelesia et al investigated the metabolic effects of EPA (100-200μM) on human adipogenic stem cells isolated from subcutaneous fat. Incubation with EPA (100-200μM) during adipogenic differentiation promoted mitochondrial biogenesis and induced thermogenic gene expression and specific beige markers such as CD137 (37).

Similarly, Fleckenstein-Elsen et al demonstrated that EPA, but not arachidonic acid (ARA, C20:4n-6), promoted beige adipocyte formation from primary hADS (45). The conversion of uncommitted SV cells into beige fat by fish oil is GPR120 dependent. Treatment with GW9508, a chemical agonist of GPR120, recapitulated the white-to-beige conversion by fish oil (31). Conversely, beige conversion by EPA was abolished in SV cells prepared from GPR120 null mice or in the presence of AH7614, a chemical antagonist of GPR120 (31). However, GPR120 activation by EPA in fully-mature white adipocytes does not cause beige conversion, suggesting that EPA acts on beige adipocyte recruitment rather than by promoting trans-differentiation (28).

The involvement of GPR120 in beige adipocyte differentiation was further confirmed in animal studies. Quesada-Lopez et al. demonstrated that feeding adult mice with GW9508 for 7 days, significantly upregulated thermogenic genes (i.e., UCP1, PGC1α, FGF21, SIRT3), and caused browning of inguinal WAT (iWAT) (31). The simultaneous administration of CL316243, a β3-specific adrenergic receptor agonist, with GW9508 showed a synergistic increase in oxygen consumption rate and browning of WAT. In contrast, genetic ablation of GPR120 completely abolished cold- and β3 agonist-induced WAT browning. This particular study demonstrated that elevated levels of FGF21 are linked with GPR120 activation in both WAT and BAT, suggesting a novel signaling axis in which adipocyte GPR120 links with FGF21, a key endocrine hormone for fatty acid β−oxidation (31). Consistent with this study, TUG-891, another GPR120-specific agonist, significantly promotes fat oxidation and adipocyte browning through Gq/α-mediated calcium release, mitochondrial depolarization, and mitochondrial fission (46). In summary, multiple pathways work in concert to facilitate beige fat induction through GPR120

activation via either dietary n-3 PUFA supplementation or by chemical agonism (Figure 1B).

1.2.1.3 Regulation of innate immunity by n-3 PUFA in beige fat differentiation

White adipose tissue is an important endocrine organ releasing numerous adipokines that can alter inflammatory status and insulin sensitivity. Adipose tissue inflammation is mediated by the inflammatory responses of adipose tissue macrophages (ATMs). Oh et al. demonstrated the anti-inflammatory function of GPR120 in adipose tissue (42). Activation of GPR120 by n-3 PUFA or chemical agonists preferentially promotes anti-inflammatory M2 macrophage polarization and protects from HF diet-induced metabolic dysfunction (42). In the context of WAT browning, the physiological relevance of type 2 innate immune responses has been recently highlighted (47). ADRB3 pathway activation by cold stress initiates type 2 innate immune responses and alternative M2 macrophage polarization, which mediates UCP1-positive beige fat development. M2 macrophages have been shown to provide local catecholamine within WAT (21). In addition, groups of type 2 innate lymphoid cells (ILC2s) and eosinophils are the major source of type 2 cytokines IL-33, IL-4, and IL-13 that are necessary for proliferation and commitment of beige precursor cells into beige adipocytes (48,49). Despite numerous indications, a missing link remains as to whether fish oil supplementation (or GPR120 activation itself) amends the immunological makeup of WAT into a favorable state for proliferation of beige progenitor cells.

New evidence suggests FGF21 is an important mediator for type 2 innate immune responses in WAT. Huang et al. reported that FGF21 acts on adipocytes in an autocrine manner to promote the production of CCL11, which subsequently promotes eosinophil

recruitment, IL-4 release, M2 macrophage polarization, and proliferation and differentiation of beige progenitor cells into thermogenic beige fat cells (50). Consistently, Quesada-Lopez et al. have demonstrated that GPR120 activation failed to induce beige fat induction in FGF21 null mice and vice versa (31), suggesting that a GPR120/FGF21 axis is essential for WAT browning. Given the indispensable function of GPR120 on FGF21 production, it is likely that fish oil-induced beige fat may trigger type 2 innate immune responses and eosinophil recruitment in WAT. To validate this notion, it is necessary to evaluate the signaling relay of FGF21/CCL11/type 2 lymphoid cells proposed by Huang et al (50) in GPR120 null mice.

In summary, the activation of GPR120 in inguinal adipose tissue mediates the production of FGF21, resulting in immunological remodeling of adipose tissue such as recruitment of type 2 innate immune responses, recruitment of eosinophils, ILC2s and M2 macrophages, and proliferation of beige precursor cells. (Figure 1B).

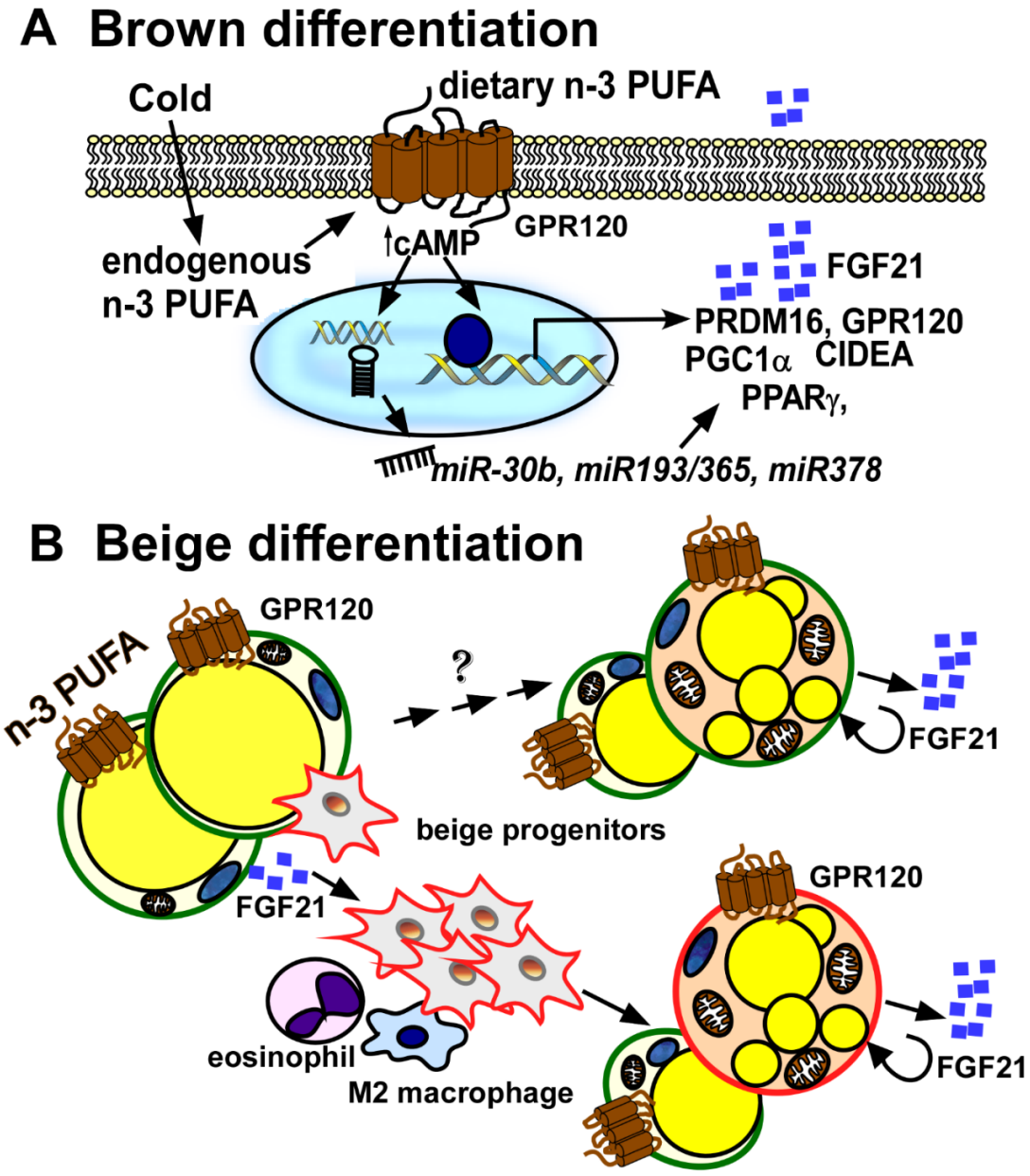

Figure 1 Activation of brown and beige thermogenesis by n-3 PUFA by GPR120-dependent mechanisms

1.2.2 Thermogenic activation by n-3 PUFA through GPR120-independent mechanisms

1.2.2.1 Thermogenic activation by n-3 PUFA through TRPV1

N-3 PUFA supplementation triggers multiple signaling pathways for thermogenic reprogramming, which GPR120 activation alone cannot explain. Activation of the sympathetic nervous system (SNS) plays a critical role in non-shivering thermogenesis by innervating BAT and, to a lesser extent, WAT. Detected in the skin, cold is a strong afferent signal to stimulate the SNS in the hypothalamus. Stimulation of the SNS induces efferent signaling acting upon BAT and WAT to produce catecholamines, which activate β3-adrenergic receptors (ADRB3), produce cAMP, and thereby triggers the thermogenic program (51). Though the sympathetic outflow signaling to BAT/WAT could be identical, the afferent signal to SNS by food-borne molecules originates from temperature-sensing mechanism in the gut (52,53). An example of a dietary component is capsaicin, the major pungent component in hot red peppers. Dr. Saito's group has extensively investigated the ability of capsaicin (or capsinoids) to bind to transient receptor potential vanilloid 1 (TRPV1), a nonselective calcium channel located on peripheral sensory neurons in the gut, sending out a thermogenic stimulus to the SNS (10,54,55).

Similar to capsaicin, fish oil induces brown/beige fat through activation of the SNS. Kim et al. demonstrated that fish oil supplementation for 10 weeks increased oxygen consumption and core body temperature in mice (24), which was abolished by propranolol, a potent β-blocker. Consistently, elevated levels of cAMP in serum and peripheral tissue after fish oil consumption was reported (24,26). The gut-brain-adipose tissue axis seems to be essential, as evidenced by browning effects were abolished in mice with the removal of

the vagal nerve, vagotomy, or genetic deletion of TRPV1 (24). In agreement with this notion, n-3 PUFAs are well-known ligands for TRPV1 (56). Ohyama et al. proposed new evidence that capsaicin-mediated TRPV1/SNS axis activation may stimulate β2-adrenoceptors (ADRB2) in white fat rather than the β3-adrenoceptor. Furthermore, this study demonstrated that synergistic SNS activation by sub-ambient temperature (17°C) and TRPV1 activation in the gut by capsaicin strongly enhanced WAT browning, resulting in > 2-fold weight loss relative to that caused by cold or capsaicin alone (57).

Collectively, these studies suggest that n-3 PUFA, independent of GPR120-mediated signaling, serve as a stimulator for TRPV1 in the gut, activating the SNS to trigger ADRB3-mediated signaling cascades in both BAT and WAT (Figure 2A). It is worth testing whether the combined strategy of chronic sub-ambient temperature and dietary regimen with fish oil will constitute a practical approach to promote brown/beige thermogenesis in humans.

1.2.2.2 Impact of n-3 PUFA-driven oxylipins on thermogenic activation of beige adipocytes

The Western diet is deficient in n-3 fatty acids (n-6/n-3=15~17), and a high ratio of dietary n-6/n-3 is associated with increased risk of various metabolic diseases including obesity (58). Pisani et al first addressed the impact of n-6/n-3 ratio on beige fat conversion (59). By using *h*ADS, it has been shown that n-6 PUFA, *i.e.*, linoleic acid (LA, C18:2n-6) and arachidonic acid (ARA, C20:4n-6), strongly inhibit adipocyte browning. This inhibitory effect was associated with ARA-derived eicosanoids of PGE2 and PGF2 via cyclooxygenase (COX) activity (59). Ghandour et al have compared the effective thermogenic potential of a n-6 enriched diet (n-6/n-3=30) composed of linoleic acid (LA,

C18:2n-6) and oleic acid (OA, C18:1n-6) with an n-3 supplemented diet (n-6/n-3=3.7) mostly composed of alpha-linolenic acid (ALA, C18:n-3) (60). This study revealed that n-2 prostaglandin series derived from n-6 PUFA, *i.e.*, PGF2 and PGE2, were inhibitors of adaptive thermogenesis. More importantly, it was proposed that a high intake of n-3 PUFA and its oxygenated lipid mediator prostacyclin (PGI2) promotes beige fat formation by suppressing PGE2 and PGF2 production in adipocytes, implicating the competition between n-3 vs. n-6 FA for COX activities (60). In accord with this idea, PGI2 or a stable analog of prostacyclin promotes browning of *h*ADS through mechanisms associated with cognate receptor IP-R/cAMP signaling and upregulation of PPARγ (61). Taken together, these studies suggest that the n-6/n-3 ratio *per se* is an important modulator for white-to-beige thermogenic conversion through oxygenated lipid mediators (oxylipins) independent of GPR120-mediated signaling cascades (Figure 2B).

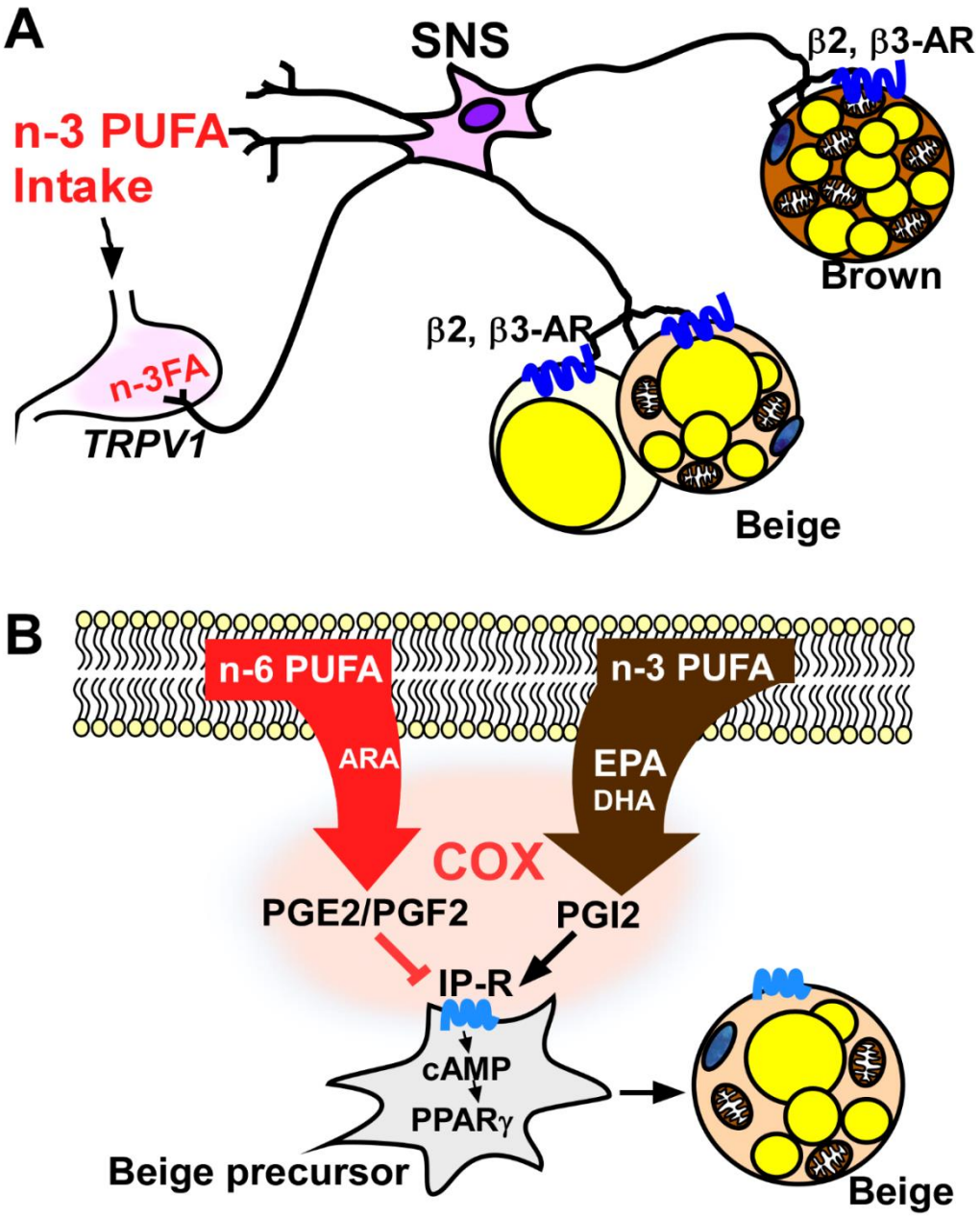

Figure 2 Activation of brown and beige thermogenesis by n-3 PUFA by GPR120-independent mechanisms.

1.3 Current knowledge, controversies, and directions for future research

We summarized recent studies that investigated the role of n-3 PUFA in influencing thermogenic function in animals and relevant cell models of brown and beige adipocytes. n-3 PUFA possess unique properties to promote brown/beige thermogenesis compared to other long-chain saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), or n-6 PUFA (26,45). This agrees with literature suggesting that n-3 PUFA are better ligands for GPR120 than n-6 PUFA (29). Moreover, these data are consistent with recent results that metabolites of n-3 PUFA are much more potent agonists for the TRPV1 receptor than metabolites of ARA (62). n-3 PUFA seemingly exhibit distinctive mechanisms on brown vs. beige precursor cells, although GPR120 is involved in both brown and beige fat development. Based on literature, we suggest a molecular framework that n-3 PUFA act on brown precursor cells at the transcriptional level during brown adipogenesis (Figure 1A), whereas n-3 PUFA alter the microenvironment of WAT at the level of recruitment and proliferation of beige precursors (Figure 1B). In agreement with this current hypothesis, trans-differentiation of white to beige adipocytes was not triggered by n-3 PUFA (28). Herein we categorized β3-specific adrenergic receptor activation by n-3 PUFA as a GPR120-independent pathway. Given that adaptive thermogenesis is an integrative response linking the central nervous system to peripheral adipose tissues, the activation of sympathetic neurons by n-3 PUFA can be regarded as a concurrent and synergistic signal parallel to the GPR120-dependent peripheral responses rather than exclusive to each other.

One important but unanswered question is the thermogenic effectiveness among the different dietary n-3 PUFA, i.e., DHA, EPA, ALA or docosapentaenoic acid (DPA,

C22:5n-3). To date, most studies tested the thermogenic function of n-3 PUFA by using fish oil that contains mixtures of EPA and DHA, and no study was conducted to evaluate the thermogenic function of ALA or DPA alone. Despite some controversies, EPA seems to be a stronger thermogenic stimulus than DHA in general, based on studies on murine brown adipocytes or hADS (26). One reasonable answer is found in studies conducted by Dr. Pisani's group. A fascinating hypothesis is proposed that oxylipins derived from different PUFA are downstream effectors to modulate thermogenic remodeling of beige fat. In particular, Pisani et al claimed that PGI2 is a pro-thermogenic eicosanoid wherein EPA is a better substrate of COX for PGI2 production rather than DHA (63). Conversely, it is suggested that DHA is insufficient to compete with ARA and less effective in suppressing anti-adipogneic oxylipin production of PGF2 and PGE2, although DHA features a stronger effect on inhibiting COX2 activity. This notion seems to align with the several cohort studies demonstrating EPA, but not DHA, is effective in reducing triglyceride levels in humans (64,65). However, the likelihood of the impact of n-6/n-3 ratio on beige fat development warrants additional support from other researchers, as all studies were published from a single group (63-65). Also, it is uncertain how competition between n-3 vs. n-6 PUFA toward COX activity affects recruitment of beige precursor or the immunological setting of WAT. Further research is required to test these hypotheses in a relevant system. For example, use of the transgenic fat-1 mouse that converts n-6 PUFA into n-3 endogenously would be a useful model system to investigate the function of a low n-6/n-3 ratio in regulating adaptive thermogenesis probably without the influence of GPR120 signaling. Moreover, future studies to distinguish the thermogenic function of plant-based n-3 PUFA, e.g., flaxseed oil or nut oil (rich in ALA), from marine-based fish

oil (rich in DHA and EPA) would be essential to establish a solid role of a low n-6/n-3 ratio in regulating adaptive thermogenesis.

There are some caveats since most cell-based studies used approximately 100-200 μM of EPA, which may not be attainable by oral intake of a fish oil-containing diet or pills. Nonetheless, these studies suggest a new research avenue that n-3 PUFA-enriched diets possess the potential to alter the fate of adipogenic stem cells into mitochondria loaded, fat-burning beige adipocytes rather than fat-depositing white adipocytes.

1.4 Human clinical studies supporting the thermogenic function of n-3 PUFA

The physiological relevance of brown/beige fat in obesity has been well established in humans (66). It is estimated that healthy adults contain upward of ~60 g of brown/beige fat (<0.1% of body weight), which could be responsible for >20 % of daily energy expenditure (67). Unfortunately, induction of thermogenic fat in response to cold exposure is severely compromised in obese individuals (68,69). Conversely, loss of thermogenic activity, whether classical brown or beige fat, contributes to obesity. There are numerous studies supporting that n-3 PUFA decreased inflammation and improved insulin sensitivity in metabolically unhealthy humans (70-76). However, the implication of thermogenic function of n-3 PUFA is less evident in human clinical trials, despite the clear indication in the aforementioned animal studies. This is likely due to technical difficulties in measuring thermogenic energy expenditure in humans; thus, few studies were designed to assess thermogenic heat release or energy expenditure. In this section, we revisit some of the previous human clinical trials showing that n-3 PUFA affect metabolic rates and adiposity, which may help us provide insight into the thermogenic function of n-3 PUFA in humans.

1.4.1 Clinical evidence on the thermogenic function of n-3 PUFA

One of the most noticeable effects of n-3 PUFA supplementation is reduced fat mass. Based on the meta-analysis involving 11 randomized clinical trials, Zhang et al revealed that n-3 PUFA intake significantly reduced serum levels of triglyceride and waist circumference without affecting body mass index (BMI) (77). Results reported from this study confirm the potential function of n-3 PUFA on reducing visceral fat. However, conclusions from this report are uncertain due to limitations, such as small-scale randomization and poor quality control. The fat loss effect by fish oil was also noted in a study of insulin-resistant adults conducted by Ramel et al (78). A total of 324 participants aged 20-40 years with BMI 27.5-32.5 were randomly assigned 0-2.1g/day of n-3 PUFA for 8 weeks. This study revealed that 2.1g/day n-3 PUFA was linked to a significant decrease in body weight, plasma levels of fasting insulin, glucose and triglyceride, and improved insulin sensitivity (78). However, these studies were not designed to understand the underlying mechanisms, and thus it is difficult to infer a thermogenic function of fish oil solely due to reduced adiposity.

The very first crossover-study to determine the effects of n-3 PUFA on resting metabolic rate (RMR), basal energy expenditure, and body composition was conducted by Couet el al. Supplementation of 6g/day of fish oil for three weeks resulted in reduced body fat mass. It also decreased respiratory exchange ratio (RER), indicating increased fuel usage from fat, and increased basal lipid oxidation without altering resting metabolic rate (79). This study posed several limitations such as small size (only six participants), gender imbalance (5 males and one female), and seasonal differences between two cohorts. Noreen et al. conducted another study to determine the oxidative potential after fish oil intake (80).

In this study, 6 weeks of fish oil supplementation (4g/day) significantly increased lean body mass and reduced fat mass in healthy adults (total of 44 men and 34 women), although significant differences in basal metabolic rate (BMR) or RER were not observed (80). In parallel with these results, the inclusion of fish or fish oil in randomized trials of weight-loss-diets offered increased weight loss in healthy humans (81,82). Most recently, Jannas-Vela et al. reported an interesting study (83) that determined the BMR and substrate oxidation in young healthy males subjects after 12 weeks of fish oil supplementation (2g/d EPA, 1g/d DHA) in comparison to olive oil intake. The authors identified that fish oil intake increased fatty acid and carbohydrate oxidation in the winter season, but not in summer, regardless of RMR. These results imply that fish oil-mediated thermogenic activation may require additional environmental stimuli, such as cold temperature (83). In the same context, the effects of n-3 PUFA were augmented with exercise (84), another signaling factor to promote adaptive thermogenesis (85). Another important aspect to consider is gender differences in WAT browning. In contrast to the study performed by Jannas-Vela et al. (83), Logan et al. reported that 12 weeks of fish oil supplementation (3g/day) significantly increased resting BMR as well as exercise-induced fat oxidation in females (86). This result is also consistent with the finding that women contain a detectably higher amount of brown fat mass than males (6).

1.4.2 Limitations of studies and directions for future research

The thermogenic effects of n-3 PUFA in human clinical trials, mostly fish oil supplementation, remain inconclusive, despite a strong correlation between fish oil intake and reduced visceral adiposity. The inconsistency between animal studies and human clinical trials seems to originate from the confounding factors of study design and technical

difficulties in brown fat identification in humans. Unlike experimental design for rodents, direct stimulation of ADRB3 via pharmacological agonists or chronic exposure to suboptimal low temperature is not appropriate for human clinical trials. Hence, better study designs are required to address the thermogenic function of n-3 PUFA intake in adaptive thermogenesis in humans. In agreement with this notion, the reduced adiposity by fish oil supplementation seems to become increasingly evident with additional signaling cues for thermogenesis such as calorie restriction, cold temperature, and exercise (81-83). Therefore, fish oil supplementation in combination with other lifestyle modifications could be a better strategy to promote adaptive thermogenesis. This hypothesis needs further research with well-controlled and large-scale human trials in both genders. In addition, direct evidence such as ^{18}F-2-deoxy-glucose (FDG) uptake using positron emission tomography (PET)-scans should be provided to establish stronger links with adaptive thermogenesis in humans (87).

1.5 Prenatal exposure of n-3 PUFA, BAT development, and childhood obesity

Pregnancy, especially in the late gestation period, is the critical window of time for fetal growth including classical BAT development. Augmentation of classical BAT development at the time of birth and increased retention in childhood could be a promising intervention strategy to counteract obesity and metabolic syndrome. Until now, several non-nutritional factors have been shown to increase the development of BAT formation in the fetus. These include thyroid hormone, catecholamines (88,89) or maternal cold exposure (90), suggesting that activation of maternal sympathetic innervation promotes ADRB3 signaling in the fetus, thereby enhancing the development of BAT. Currently, there is growing information that maternal or infant nutritional status alters BAT

mass/activity in newborns and its maintenance in adulthood. Malnutrition at late gestational phases, such as low protein intake (91-93) or HF diet (94), results in reduced BAT development in animal models.

Maternal supplementation of n-3 PUFA increases the n-3 PUFA concentration in the placenta and uterus cord blood, suggesting that n-3 PUFA are effectively transferred to the fetus in utero (95). Pregnant women are recommended to take 600~800 mg of n-3 PUFA daily during pregnancy, and no adverse effects were found with up to 2.8 g/day of n-3 PUFA at the late gestation period until delivery (96). Several human studies report a negative correlation between maternal n-3 PUFA intake and prevalence of childhood obesity (97-100). In a large population-based cohort study with 4830 mothers, Vidakovic et al. showed that higher maternal n-6/n-3 ratio was correlated with higher risk of childhood obesity (97). In another prospective cohort study, Moon et al. identified that maternal n-3 FA intake was associated with offspring lean body mass in 12,583 participants (99). More interestingly, a long-term follow-up study showed that fish oil supplementation during pregnancy in obese mothers had long-lasting effects on reduced adiposity in their offspring (100). Intriguingly, a recent study by Rudolph et al. supports this model demonstrating that low perinatal n-6/n-3 ratio serves as a metabolic cue to attenuate the susceptibility against diet-induced obesity in adult offspring (101).

Based on these results, it is conceivable that maternal fish oil intake may promote prenatal BAT reprogramming via the aforementioned mechanisms or may delay the postnatal degeneration of BAT in early life (Figure 3A). Our group is currently undertaking a pilot study to address the metabolic benefit of maternal n-3 PUFA nutrition on fetal BAT development in animals. While our results are promising, this possibility is still to be

reported in the primary literature. In addition, the broader implications of maternal n-3 PUFA supplementation on gestational diabetes, obesity outcomes, and BAT activity in mothers in addition to infants remains to be determined in the context of thermogenic activation.

1.6 N-3 PUFA supplementation and aging-mediated reduction in thermogenesis

The probability to detect brown/beige adipose tissue is inversely correlated with age, implying that thermogenic potential declines with senescence (6). Understanding the exact cause and identifying an intervention strategy to revert this thermogenic reduction have strong clinical implications for improving metabolic health. Berry et al. have found that old mice (1-year-old) failed to activate cold-induced beige thermogenesis compared to young mice (2-month-old) (102). They identified that 1) cellular aging of beige progenitors is driven by cell cycle inhibitors (i.e., p21cip and p16Ink4a, and p19Arf) and stress-activated kinases p38, and 2) senescent beige precursors are unable to proliferate and differentiate upon cold stimulus. Blockade of cellular senescence by genetic deletion of Ink4a/Arf or a small molecule inhibitor of p38 reversed the aging-mediated decrease in thermogenesis (102).

Based on the proposed mechanism above, dietary molecules that possess the ability to suppress cellular senescence of beige precursor cells could be effective in rejuvenating WAT-browning with aging. Compared to other health benefits, the anti-aging function of n-3 PUFA is less clear and poorly understood (103). Although the exact mechanism was not presented, several clinical trials have reported anti-aging effects of n-3 PUFA; marine n-3 PUFA intake (DHA+EPA) was associated with significant attenuation of telomere shortening in patients with coronary artery disease (104) and elderly individuals (105).

Regarding this mechanism, Chen et al. revealed that fish oil supplementation suppressed aging-mediated reduced-telomere activity by maintaining redox homeostasis; DHA intake was effective in suppressing overexpression of p16 and p53, which are metabolic culprits to promote cellular senescence (106). Supporting this concept, fish oil supplementation improved energy expenditure and promoted RMR in elderly females (86). It remains to be determined whether the proposed axis activation of 'redox-telomere-cell cycle inhibition' will be relevant to beige precursor cells (Figure 3B).

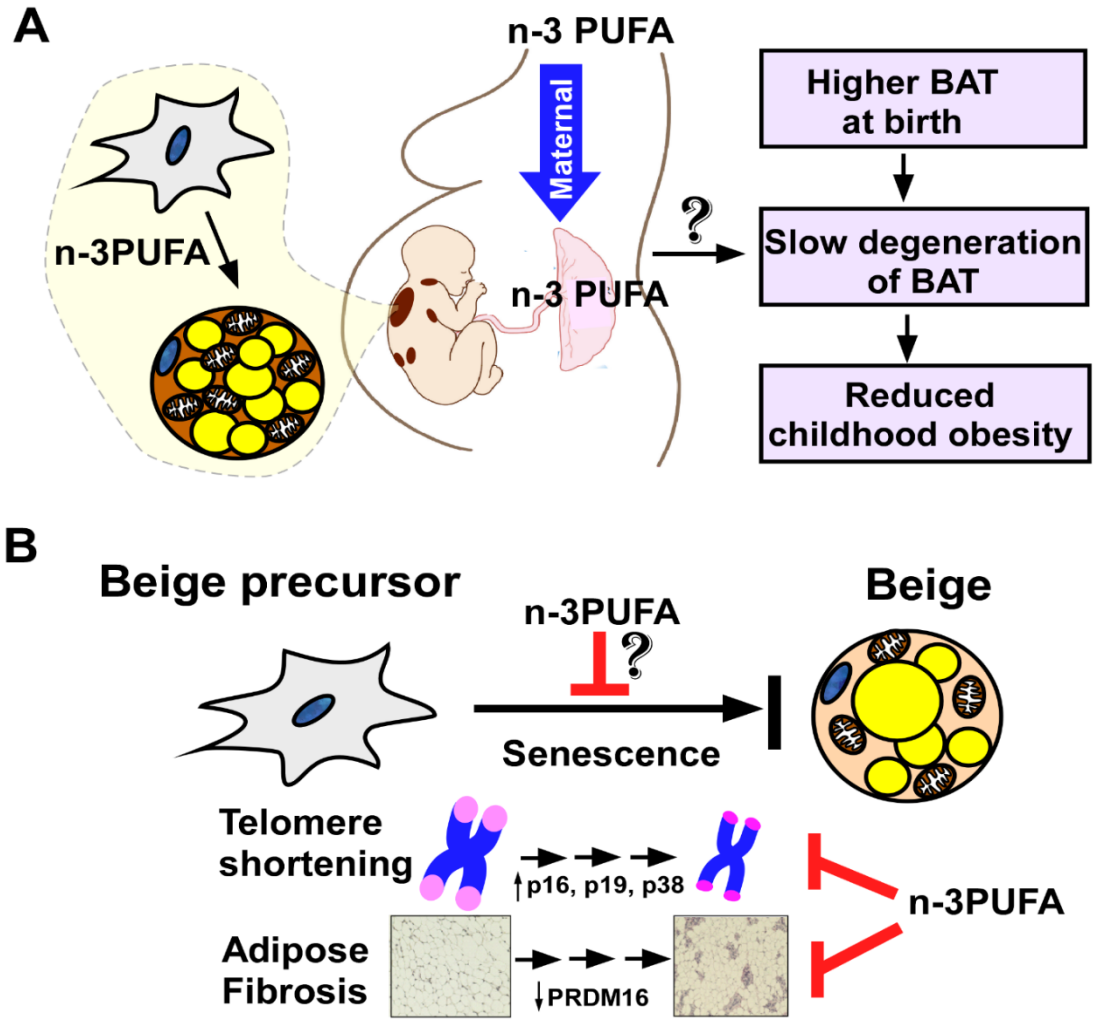

Figure 3 Nutritional perspectives regarding n-3 PUFA intake on thermogenic activity during pregnancy and senescence.

CHAPTER 2. EXPERIMENTS AND RESULTS

2.1 Introduction

Brown adipose tissue (BAT) is a specialized fat that dissipates excess energy into heat (non-shivering thermogenesis) through mitochondrial uncoupling protein 1 (UCP1) (107). Current research renews the metabolic function of BAT by revealing BAT as a crucial regulator in maintaining energy balance by increasing thermogenic energy expenditure. A significant amount of BAT is found in healthy adults as well as most children and adolescents (6,108,109), but not in the obese adults, suggesting that loss of active BAT depots is a contributing factor to obesity. Childhood obesity predisposes adults with metabolic susceptibility to obesity and type 2 diabetes (T2D) (1,110,111). Therefore, identifying early regulatory factors to prevent childhood obesity is critical to combat the current obesity epidemic. The fetal and neonatal stages are critical for fetal BAT development, which are expected to have long-term impacts on offspring BAT function (112). However, limited amount of studies has been conducted regarding the effect maternal nutrition on prenatal development of BAT. The status of n-3 PUFA is important to fetus as determining factors of health and disease in both infancy and later life (113). During the pregnancy stage, mother's metabolism needs to be adapted in order to satisfy the continuous draining of substrates and the proper growth and development of the fetus (114). Despite the well-established physiological relevance of BAT in obesity outcome, it is unclear whether the amount of BAT at birth or the rate of BAT loss (either by degeneration or by transdifferentiation into WAT) are associated with susceptibility to obesity in later life. In particular, it is poorly understood whether prenatal BAT development through dietary intervention could be a manageable target to attenuate the

risk for childhood obesity.

Human BAT depots are found in the deeper neck (cervical), supraclavicular, paravertebral, perirenal, and axillary areas (115-117) and possesses 50-times greater respiratory activities than white adipose tissue (WAT) (2). These BAT depots are comprised of 1.5% of total body mass (roughly 5% of total fat mass), and up to 90% depots could be activated BAT (118). An image-guided mapping of rodent adipose depots reveals the topological analogy of BAT between rodents and humans (119), which provides the feasibility to use rodent in studying human BAT. Also, the comparable functional analysis demonstrated that human supraclavicular BAT features functional similarity with rodent interscapular BAT regarding mitochondrial activity and thermogenic potential (2). Regarding the timeline for BAT development, late pregnancy (at the last trimester) is the critical time for human BAT formation (4,120). The human brown adipocytes in the interscapular are rapidly lost after birth via either degeneration and replacement with white adipocyte, or transdifferentiation into white adipocytes. However, brown adipocytes reside in the deeper neck and supraclavicular regions, remain active to adolescence and adulthood until they lost thermogenic potential with the progression of obesity, type 2 diabetes, or aging (110). Given these developmental similarities, the regulation of interscapular BAT in rodents during pregnancy seems to be translatable to BAT around the neck and supraclavicular in humans.

BAT development is modulated by epigenetic modifications that are heritable and reversible changes in gene expression occur without altering DNA sequences through DNA methylation, chromatin histone remodeling, and noncoding RNAs such as miRNAs (121-123). Nearly a dozen miRNAs have been identified in promoting the transcriptional

program of brown adipogenesis (124). On the other hand, the site-specific acetylation (Ac) and methylation (Me) status on the lysine (K) residues of histone tails, especially at H3K9 and H3K27, play essential roles in adipogenesis by controlling gene activation or repression(125,126). Maternal nutrition is a key epigenetic modulator for fetus development. However, it is largely unknown whether the BAT epigenome is a viable target for obesity control through maternal nutrition.

Accumulating evidence has supported that n-3 PUFA promotes brown adipogenesis and adaptive thermogenesis (7,25,27,28,127). We have previously demonstrated that eicosapentaenoic acids (EPA) promote the brown adipogenic program through a miRNA-dependent epigenetic mechanism in the murine primary brown adipocytes (26), suggesting that maternal n-3 PUFA nutrition may be effective in reinforcing embryonic BAT developmental program during pregnancy. This study aimed to investigate the impact of early n-3 PUFA exposure through maternal nutrition on the fetal BAT development. Here, we demonstrate that maternal n-3 PUFA intake during pregnancy and lactation enhanced brown transcriptional programming through miRNA and histone modification-mediated epigenetic regulations. These results open a new research avenue emphasizing that ‘boosting prenatal BAT development’ could be a novel therapeutic target for attenuating childhood obesity through thermogenic energy expenditure.

2.2 Central Hypothesis, Purpose, and Specific Aims

A. Purpose of the Study

The purpose of the study is to investigate the effect and the underlying mechanisms of maternal n-3 PUFA supplementation on fetal brown adipose tissue development and risks for obesity and long-lasting metabolic benefits.

B. Central Hypothesis

Maternal supplementation of n-3 PUFA during pregnancy and lactation promotes the embryonic BAT development of neonates, thereby increasing the postnatal BAT retainment and decreasing the risk of obesity and T2D in later life.

C. Specific Aims

Specific Aim 1: Investigate the effect and underlying epigenetic mechanism of maternal n-3 PUFA supplementation during gestation and lactation on offspring's brown adipose tissue development and thermogenic activity in C57BL/6 mice.

Specific Aim 2: Identify the metabolic significance of maternal n-3 PUFA supplementation in postnatal BAT retainment and long-term metabolic benefits.

Specific Aim 3: Evaluate the metabolic benefits of maternal n-3 PUFA intake during pregnancy and lactation in the mother mice.

2.3 Materials and Methods

Animals

All animal experiments were conducted according to the protocols approved by the

Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska-Lincoln. C57BL/6 male and female mice were purchased at 8-10 weeks of age from the Jackson Laboratory. After 3 days of acclimatization period, mice were put into the breeding colony. Female mice were randomly assigned to two diet groups receiving either a diet containing 3% of n-3 PUFA from the fish oil (FO) or a diet devoid of n-3 PUFA (Cont). The AIN-93G rodent formulation was modified for the fat composition containing 15% of total calorie from fat either 10% palm oil (Cont) or fish oil (FO), the other 5% of total calorie from soybean oil as a source of essential FA. Gas Chromatography (GC) was used to analyze fatty acid profiles of each diet and dietary composition in **Table 1.** The same diets were maintained throughout gestation and lactation. The pregnancy of the female mice last for 19-21 days, and the pups (n=18 each group) were weaned (3 weeks postpartum). Necropsy was conducted at weaning, to collect blood, liver, interscapular BAT (iBAT), and inguinal (iWAT) and epididymal WAT (eWAT). Tissue samples were snap-frozen in liquid nitrogen and kept at -80 °C for further analysis.

Energy expenditure by metabolic cage

To measure the effect of maternal n-3 PUFA intake on energy expenditure, pups (n=5 each group) were individually placed into the metabolic cage (TSE systems) for six days (two days of adaptation and four days of measurement). Indirect oxygen consumption (VO2) and carbon dioxide production (VCO2) were used to calculate metabolic parameters. Energy expenditure (EE) and respiratory exchange rate (RER=VCO2/VO2) were calculated and obtained from the TSE systems software and plotted into a figure with hourly time point.

Cold exposure, measurement of rectal temperature and heat release

To measure the long-term effect of maternal n-3 PUFA supplementation on the offspring's thermogenic potential, male pups from both maternal fish oil or control diet (n=6 per group) were switched to a standard AIN-93G diet (no additional n-3 PUFA). At week 11, mice were exposed to cold temperature (6°C) acutely (1-3 hour) or for 24 hours. To measure the core body temperature, a rectal thermometer (Kent Scientific Corp) was used. The probe was positioned into the anal ducts of the mice and three readings of each time point were recorded. Infrared (IR) camera (A655sc, FLIR Systems) was used to detect thermal release and to capture images of the surface body temperature. FLIR Research IR program software was used to display surface heat release via color palette representing temperatures between 22 and 34 °C.

Gas Chromatography (GC) for fatty acid analysis

To determine FA profiles in the red blood cells, whole blood was collected, and red blood cells were precipitated by centrifugation (6000×g for 15 minutes). 200µl of the packed volume of red blood cells were transferred to a fresh glass vial and total lipids were extracted. They were then subjected to FA methylation by 14 % boron trifluoride (BF3)-methanol reagent (Sigma, USA) at 100 °C for 1 hour to form fatty acid methyl ester (FAME). Agilent Technologies HP-88 column (100m x 0.25mm x 0.2 µm film thickness) was used. The individual FA peak was identified by comparing its relative retention times with the commercial mixed-FA standard (NuCheck PreP), and the area percentages for all resolved peaks were analyzed using the GC Chemstation software.

Blood Chemistry

To measure plasma glucose, insulin, and cAMP levels, immunoassays were conducted by using mouse glucose assay (Crystal Chem), ultra-sensitive mouse insulin

ELISA kit (Crystal Chem), and mouse cyclic adenosine monophosphate (cAMP) parameter assay kit (R&D Systems), respectively, in accordance to the manufacturer's protocol.

qPCR of mRNA and microRNA analysis

Total RNA was extracted using Trizol® reagent (Invitrogen) from homogenized tissues. RNA was purified using DNase treatment & removal kit (Invitrogen), and 2 μg of RNA was converted into cDNA (iScript, BioRad) via reverse transcription. Relative gene expressions were determined based on the $2^{-\Delta\Delta CT}$ method with normalization of the raw Ct value to 18s. For miRNA analysis, miRNA was converted to cDNA using the miScript reverse transcription kit (Qiagen) according to the manufacturer's instructions. MiRNA was measured by using the commercial miScript Universal Primer, with the miScript primer assay kit (Qiagen). Primers of miR-30b, -193b, -365, and RNU6-2 were purchased from Qiagen. The Ct values were normalized to RNU6-2 (U6 small nuclear).

Western blot analysis

Total protein was extracted from tissue by homogenizer using RIPA buffer along with a protease inhibitor and phosphatase inhibitor. Proteins were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane (0.45 μm, Thermogenic Scientific) using a wet-transfer method. Antibody targeting UCP1 (14670), PPARγ (2444) and β-actin (4967) were purchased from Cell Signaling Technology. PRDM16 (sc-55697) was purchased from Santa Cruz Biotechnology. Blots were visualized with a FluorChemTME imaging system (Protein Simple).

Chromatin immunoprecipitation assay (ChIP)

The murine brown adipocyte cell-line (HIB1B) was a generous gift from Dr.

Johannes Klein (University of Lubeck, Lubeck, Germany), who has generated the cell line from brown fat of newborn or 6-8 week old of C57BL/6 or FVB mice (128). HIB1B cells were differentiated and treated with EPA (100 μM), as we described previously (26). Chromatin immunoprecipitation assay was performed using CHIP-IT® Express kit according to the manufacturer's instructions (Active Motif, USA). Cells were fixed with 1% formaldehyde to cross-link DNA and proteins. Chromatins were isolated and DNA was sheared into 200-1000 bp length. Fifty μl of each sample was removed as the input control. The samples were incubated with magnetic beads-AcH3K27 antibody complex (Cell Signaling Technology, USA) overnight. Next, magnetic beads were washed, and chromatins were eluted and reversely cross-linked. After treated with Proteinase K, DNA was ready to be used immediately for real-time qPCR analysis using SYBR Green (QuantStudio 6, life technology). The Ucp1 and Pgc1α promoter regions were assessed in acetylation histone 3 at lysine 27 (H3K27) ChIP samples. Fold of enrichment was calculated and presented according to the methods provided by the manufacturer.

Primer sequence: Ucp1 proximal promoter, 5'-CCCACTAGCAGCTCTTTGGA-3' and 5'-CTGTGGAGCAGCTCAAAGGT-3'; Pgc1α CRE region, 5'-CAAAGCTGGCTTCAGTCACA-3' and 5'-AAAAGTAGGCTGGGCTGTCA-3' (129).

Liver triglycerides content

Total liver lipids were extracted from 100mg liver tissue (n=4 each group) using Folch method by adding chloroform/methanol with the ratio 2:1. After extracted at 60℃ for 2 hours and filtered through 70mm (Whatman) in glass funnels, 1% Triton X-100 was added (Fisher BioReagents). Subsequently, samples were dried under nitrogen at 60℃, and were resuspended in nucleus free water for further analysis. Triglyceride content was

measured by the commercial TG quantification colorimetric kit (BioVision).

Statistical Analysis

All data are presented as mean ± SEM. Independent samples from control group and treatment group were analyzed and compared using two-tailed Student's t-test, with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All statistical analyses were conducted by Graph Pad Prism 7 (Version 7.03).

2.4 Results

2.4.1 Maternal n-3 PUFA supplementation decreased WAT mass and n-6/n-3 ratio

To investigate the effects of n-3 PUFA on BAT development on offspring, C57BL/6 female were fed either a diet containing with 3% of n-3 PUFA from fish oil (FO) or an isocaloric diet absent of additional n-3 PUFA (Cont) during pregnancy and lactation. Both male and female pups were weaned at 3 weeks postpartum. There was a significant decrease in body weight in FO compared to Cont in both genders. Although there was no difference in liver weight, WAT was significantly reduced in both genders, indicating that maternal n-3 PUFA intake decreased white fat accumulation. A similar trend was observed in iBAT mass, although the differences did not reach a statistical significance (Fig 4A-C).

To investigate the impact of maternal n-3 PUFA intake in the pups, FA profile was determined in the red blood cells of the weaned pups. There was a significant decrease in n-6/n-3 PUFA ratio in the maternal n-3 PUFA received group (FO) compared to the control, indicating that maternal n-3 PUFA was effectively delivered from mothers to the pups (Fig 4D). Concomitantly, the content of arachidonic acid (ARA), a precursor for pro-inflammatory eicosanoids, was significantly decreased in the maternal n-3 PUFA group (Fig 4E). Also, there was a trend towards a decrease in plasma glucose ($p=0.09$) and insulin

concentrations (p=0.1) in the maternal n-3 PUFA group (Fig 4F, G), indicating that maternal n-3 PUFA intake may have an impact on the glucose tolerance and insulin sensitivity in pups at weaning. Adrenaline, released via the action of the sympathetic nervous system, plays a vital role in brown and beige adipose tissue thermogenic program (130). Our results also indicated that there was a significant increase in cAMP levels ($p<0.01$) in the plasma of FO group (Fig. 1H), which is a downstream target upon β3-adrenergic receptor (ADRB3) activation, thereby regulating lipolysis and UCP1-mediated thermogenic responses (131).

2.4.2 Maternal n-3 PUFA supplementation promoted the fetal BAT development

Despite there were no differences in iBAT mass, maternal n-3 PUFA intake was linked with a darker in color in the picture of BAT taken upon sacrifice (Fig 5A), and lower lipid accumulation in the H&E staining image (Fig 5B). Similar to the results from red blood cells, maternal FO intake significantly altered the lipid profile in the iBAT with a 5-fold reduction in n-6/n-3 ratio and a 2-fold decrease in ARA content compared to control (Fig 5C). To identify the effect of maternal n-3 PUFA supplementation on BAT activities, we measured the brown-specific gene expression levels in the iBAT. There was a significant increase in Ucp1, Cidea, Prdm16, and Pgc1α, along with the Gpr120, a membrane receptor of long chain fatty acid receptor in both gender pups (Fig. 5D-F). In accordance with the increased brown-specific transcripts, protein expressions of UCP1, PRDM16, and GRP120 were higher in the maternal FO-fed group (Fig 5G). The mitochondrial DNA content showed a trendency towards an increase in the maternal n-3 PUFA group, but it was not statistically significant (p=0.08) (Fig 5H).

2.4.3 Maternal n-3 PUFA supplementation modulated epigenetic factors for BAT developments.

We have previously demonstrated that n-3 PUFA potentiate brown adipogenesis in the primary murine brown precursor cells via miRNA-mediated epigenetic mechanisms (26). After confirming that maternal n-3 PUFA upregulated BAT transcriptional programs of the offspring (Fig 5), we sought to determine whether the similar epigenetic mechanisms are involved in this process. The functional cluster of miRNAs of miR-30b, miR-193b, and miR-365 was significantly increased in maternal FO received pups (Fig 6A), recapitulating our in vitro results (26). Intriguingly, the BAT with maternal n-3 PUFA exposure showed a substantial increase of Drosha, an RNA double-strand ribonuclease and critical component of the microprocessor for initiating the cleavage of pri-miRNA into stem-loop pre-miRNA (Fig 6B), in both gene and protein expression (Fig 6C, D). These results suggest that nuclear processing of pri-miRNA might be upregulated by early exposure of FO during fetal BAT development.

Next, we examined the role of maternal n-3 PUFA intake on histone modifications. There was a remarkable decrease in histone deacetylase 1 (HDAC1). The reduced HADC1 levels were linked with an increase of acetylation (Ac), but a decrease of tri-methylation (me3), at histone H3 lysine 27 (H3K27) site (Fig 7A), a critical post-translational modifications (PTM) mark for brown adipogenesis(126). The decrease H3K27me3 was consistent with decreased gene expression of JmjC domain-containing protein 3 (Jmjd3), an H3K27-specific demethylase (132), in FO group compared to control (Fig 7B). Intriguingly, no specific increase in acetylation levels at H3K9, another critical regulatory site for brown adipogenesis. However, there was a distinct increase in methylation status

(H3K9me2) with maternal n-3 PFUA intake (Fig 7A). Consistently, the expression of euchromatic histone N-lysine methyltransferase 1 (Ehmt1), a methyltransferase to H3K9(113), was significantly increased without altering expression levels of Jumonji Domain Containing 1A (Jmjd1a, same with Kdm3a or Jmdm2a), an H3K9-specific demethylase (Fig 7B). These site-specific epigenetic modulations by maternal n-3 PUFA were linked with increased brown adipocyte differentiation evidenced by increased protein expression of PPARγ, fatty acid synthase (FAS) and fatty acid binding protein aP2 in the iBAT compared to control (Fig 7C).

To further ascertain whether the modulation of H3K27Ac occurs at the promoter/enhancer region of Ucp1 and Pgc1α, we conducted chromatin immunoprecipitation (ChIP) assay by pulling down the DNA with the antibody targeting to H3K27Ac in the murine HIB1B was more than a 2-fold enrichment at H3K27Ac in the n-3 PUFA treated group compared with the control both in the Ucp1 enhancer region and Pgc1α-cAMP response element (CRE) binding region (Fig 7D). Taken together, these results suggest that maternal FO exposure alters the epigenetic signature of histone acetylation and methylation and miRNA abundance in the fetal BAT, thereby enhancing brown-specific transcriptional program.

2.4.4 Maternal n-3 PUFA supplementation conferred long-lasting thermogenic benefits to offspring

To gain an insight as to whether augmented BAT activity by maternal FO intake exerts extended metabolic benefits, we conducted additional experiments. The weaned mice with or without maternal n-3 PUFA intake were kept in the same diet without additional n-3 PUFA until they were used for measurement of energy expenditure (at 5-

week of age) and cold resistance (at 11-week of age) (Fig 8A). The pups that received maternal n-3 PUFA showed significantly higher ($p=0.04$) energy expenditure especially at night (Fig 8B). There were no significant differences in RER ($p=0.3$), implicating no difference in the substrate use between groups (Fig 8C). Subsequently, upon mice reaching 11 weeks of age (8 weeks after maternal nutrition), mice were subjected to acute cold treatment (6°C), and rectal temperature was recorded. At the 3-hour time point, core body temperature was significantly higher in the maternal n-3 PUFA fed group compared to control group (Fig 8D). Additionally, under the IR camera, pups from maternal FO group presented a higher heat release on the surface than control mice (Fig 8E). After 24-hour cold exposure, adipocyte morphology was visualized by H&E staining. In iBAT, fat accumulation was lower in maternal FO-received mice than Cont, indicating the higher BAT activity (Fig 8F). Similarly, cold exposure induced the massive brown-like adipocyte morphology in the inguinal WAT (iWAT) in the FO group, but not in the Cont group (Fig 8F). Consistently, UCP1 protein expression was higher in both iBAT (2-fold) and iWAT (3.5-fold) of the FO group than control (Fig 8G). The brown-specific gene expression levels, i.e., UCP1, PGC1α and PRDM16, were maintained higher in the iBAT of the FO group than control (Fig 8H). In addition, the browning related gene expressions of Ucp1, Pparγ, and Pgc1α were significantly higher in the iWAT of FO group than the control (Fig 8I). However, the transcript levels of sarcoplasmic/endoplasmic reticulum calcium-ATPase (SERCA2b), a responsive gene for calcium-dependent thermogenesis(133), was not different between groups (Fig 8I). In addition, the two gene expressions that are responsible for another UCP1-independent thermogenic program via creatine-driven substrate cycle, i.e., CKMT1, CKMT2, were not detectable in both groups. These results

suggest that maternal FO supplementation not only maintains stronger thermogenic potential in the iBAT but also links with enhanced adaptive thermogenesis (UCP1-dependent) in the iWAT. Taken together, augmented BAT formation by maternal FO may also mediate extended metabolic benefits in later life.

2.4.5 Health beneficial effects of n-3 PUFA supplementation during pregnancy and lactation from maternal perspective

10-week-old C57BL/6 female mice (n=4 each group) was put into breeding colony, supplemented with either 15% total calorie from fat Control diet (5 % from soybean oil, and 10% total calorie from palm oil,) or FO diet (5 % from soybean oil, and 10% total calorie from fish oil, in which 3% is from n-3 PUFA), and same diets were maintained throughout gestation and lactation. Each maternal mouse has an average of three pregnancies before the final sacrifice period. To assess the effect of FO intake on body weight regulation during pregnancy, body weight was measured before breeding and after breeding. Results showed that FO intake significantly decreased the postpartum obesity (Figure 9A). To confirm the effect of n-3 PUFA on blood lipid profile modification, same assays were performed as in the pups. GC results demonstrated a significant decrease in n-6/n-3 ratio (Figure 9B. $p<0.001$) in the FO supplementation mother, and a significant decrease in arachidonic acid content in the red blood cell lipid profile (Figure 9B. $p<0.001$), both of which followed the same trend in pups presented in figure 4. Additionally, there was a significant decrease in fasting blood glucose in the FO-group mother (Figure 9C. $p<0.001$), and a trend towards decrease in plasma insulin (Figure 9E).

Maternal n-3 PUFA intake also exerted health benefits to mothers in a tissue-specific manner. In liver, there was a significant decrease in both liver weight and liver

triglycerides content, confirmed by liver H&E staining showing a dramatic decrease in liver TG (Figure 9F, 9G, 9H). In brown adipose tissue, there was a trend towards decrease in the BAT weight, accompanied by a decrease in lipid droplet size and content in the H&E staining (Figure 9I, 9J). Also, there was an increase of UCP1 gene expressions in brown adipose tissue (Figure 9K). In the epididymal white adipose tissue, there was a trend towards lower eWAT weight and smaller lipid droplets, and no difference in UCP1 expression (Figure 9L, 9M, 9N). In subcutaneous white adipose tissue, there was a significant decrease in sWAT weight, followed by a significant increase in sWAT UCP1 mRNA expression. Also, H&E staining showed smaller lipid droplets and a more brown-like feature in the mother subjected to FO supplementation (Figure 9O, 9P, 9Q).

2.5 Discussion

As a major nutrient, long chain fatty acid modulates energy metabolism via multiple mechanisms, e.g. as ligands for PPARs and FFARs (134). Recently, emerging evidence has gradually associated n-3 PUFA with increased brown adipogenesis and beige fat conversion (28,31,37). Since brown adipose tissue was formed prenatally, and whether or not maternal n-3 PUFA promotes fetal BAT development is unknown, this study focused on the maternal nutrition and aimed to identify the role of maternal n-3 PUFA intake on offspring's BAT development and activity. This study is significant to identify the early strategy of promoting BAT development and activity, which can be an effective approach to retain more BAT activity to later life in pups, also combatting the risks of obesity and metabolic syndrome. Furthermore, accumulating studies identified the thermogenic potentials of n-3 PUFA and brown/beige adipogenic properties of a low n-6/n-3 ratio in the diet (59,135). Here, we demonstrated that n-6/n-3 ratio was significantly decreased both in

the pups and mother, along with a dramatic decrease in the pro-inflammatory eicosanoids precursor, arachidonic acid (*Fig.1, Fig.5*), suggesting that n-3 PUFA was effectively delivered from the mom to the pups and significantly increased the thermogenic potential and attenuated the inflammatory eicosanoids production.

The differentiation into adipocyte has two major steps: multipotent stem cells became committed pre-adipocyte, followed by an induction of terminal differentiation into mature adipocytes, which are modulated by lineage-specific transcription factors, and more importantly, some epigenetic modifications (136). Specifically, microRNA regulations (124,137-139) and histone modifications (140-142) were suggested to play critical roles during the brown adipocyte differentiation. MicroRNAs (miRNAs) are short-chain and none-coding RNAs associated with post-transcriptional regulation of gene expression, which is also closely related with various tissue development (143).The miR-193b-365 cluster was demonstrated to maintain brown adipocyte differentiation by suppressing preadipocytes myogenic potentials and targeting at prdm16 and PPARγ, which are the brown adipogenesis positive regulators (144). Another noticeable miRNA, microRNA 30b, was reported to induce UCP1 and CIDEA, brown-specific gene expressions, and increased mitochondrial respiration, possibly through the mechanism of repressing Rip140, which is a corepressor of genes associated with glucose uptake, fatty acid oxidation, as well as mitochondrial biogenesis (26,145). Previously, our laboratory also showed that n-3 PUFA promoted brown adipogenesis via miRNA networks (26). Here, we demonstrated that maternal n-3 PUFA intake upregulated brown-adipogenesis functional clusters microRNA 30b, microRNA 193b, and microRNA 365 expressions, indicating that n-3 PUFA potentiates brown adipogenesis via microRNA-mediated mechanism. Histones are a

family of positively-charged proteins that are wrapped around by DNA to form the compact nucleosomes, which consist of histone octamers of H2A, H2B, H3, and H4. Histone post-translational modifications (PTMs) have been suggested to be associated with the replication, transcription, and the repair of DNA. Also, Histone acetylation, methylation, and phosphorylation have effects on the structure of chromatin (146). Mechanistically, histone modifications are related with regulations of gene expressions and multiple cellular activities, including cell differentiation and cell cycle control (147), and various diseases such as diabetes and cancer (148,149). Recently, emerging evidence supports histone deacetylase 1 (HDAC1) as a negative regulator of brown thermogenic program (129,150). Here, our results indicated that maternal n-3 PUFA supplementation enhanced offspring brown adipose tissue development by decreased HDAC1 expression and increased Histone acetylation at lysine 27 in the brown fat, which may serve as the underlying mechanisms by which n-3 PUFA potentiates BAT development.

The health benefits of maternal n-3 PUFA fatty acids intake on offspring have long been received attention by different researchers (20,21,151-153). It has been demonstrated that adequate n-3 PUFA during pregnancy and early childhood is of vital importance (154). There was a maternal feeding study performed in C57BL/6 mice suggested that maternal n-3 PUFA intake reduced pups body fat at weaning. Additionally, there was a significant increase in the insulin sensitivity, and a significant decrease in the plasma cholesterol and triglycerides levels in pups at 15 weeks of age (155). However, the health beneficial effects of n-3 PUFA of maternal intake on offspring BAT development remain elusive. Here, we demonstrated in mice model that maternal n-3 PUFA supplementation was effectively delivered to pups, reduced fat accumulation, promoted brown adipogenesis-related gene

UCP1, PGC1-α, CIDEA, PRDM16, and GPR120 expressions, and promoted brown adipose tissue-specific protein expression (Fig.1-4).

Although accumulating evidences regarding n-3 PUFA promotes brown/beige adipose tissue thermogenesis have been well-elucidated in mouse and in vitro cell models, the implication of this effect in human remain to be clarified. There are some studies supporting that n-3 PUFA administration in human could reduce body weight and plasma triglycerides, decrease inflammation, and increase insulin sensitivity (78,156-162). However, the amount of human studies directly associating n-3 PUFA with energy expenditure/thermogenesis is limited. Interestingly, there are some human studies focusing on the health beneficial effect of maternal n-3 PUFA intake during pregnancy and lactation on offspring. A human study done by Foster et al. investigated the effect of DHA 800 mg/day on women at 25-29 weeks gestation until the last day of the pregnancy. Results indicated that arachidonic acid, as the precursor for pro-inflammatory eicosanoids, was decreased and DHA was increased at 36 weeks of pregnancy red blood cell lipid profile analysis. There was no significant increase in the direct developmental indicators (body weight, BMI) at 2 years or 4 years age. However, there was a significant correlation between BMI z-score and the length of breastfeeding in months at both two and four years (100). These findings indicated that DHA can be effectively delivered to the offspring, and that DHA has anti-inflammatory effect. Also, in another feeding study in pregnant women by Bergmann et al. (163), normal maternal diet supplemented with 300mg n-3 PUFA lowered body weight and body mass index at offspring in 21 months of age. In another maternal feeding study, mothers supplemented 1.83g DHA and 0.80g EPA from gestation to the end of lactation, the results showed no difference in the body mass index at age 7

(164). Taken together, many researches have been done regarding the health-beneficial effect of n-3 PUFA in human, influences of maternal n-3 PUFA intake on offspring seem to be promising but remain to be investigated. Despite some conflicting results in the human feeding studies, the underlying reasons may due to the complexity of human study design or the races, gender, or health conditions, individual difference of the human object, and the length of the study and the time of measurements might contribute to the conflicting results. Future human studies are needed to confirm the effect of n-3 PUFA on thermogenesis and energy expenditure at the clinical study level, and also the effect of maternal n-3 PUFA on offspring's brown adipose tissue development and from the energy expenditure perspective.

In summary, maternal n-3 PUFA supplementation was effectively delivered to pups and increased n-3 PUFA concentration in the pup's blood, at the same time, decreasing the pro-inflammatory eicosanoids precursor arachidonic acid. Additionally, maternal n-3 PUFA intake significantly decreased fat accumulation and increased BAT activity, confirmed by brown specific markers in both gene and protein expressions. Interestingly, microRNA 30b, 193b, and 365 were upregulated in the maternal n-3 PUFA supplementation. Concomitantly, Histone modifications at acetylation at K27 and di-methylation at K9 were increased, which may serve as mechanisms by which maternal FO intake potentiates offspring's brown adipose tissue development. This study offers an insight for the role of maternal n-3 PUFA supplementation on fetal brown adipose tissue development, non-shivering thermogenesis, and the long-lasting metabolic benefits extended to later life of the offspring. This study also provides scientific foundation for further clinical trials regarding the health benefits of n-3 PUFA.

CHAPTER 3. DISCUSSION, LIMITATION, AND FUTURE STUDIES

3.1 Discussion

Emerging evidence suggests that n-3 PUFA potentiate thermogenic programs in brown and beige adipocytes (28,31,37). Although pregnancy is a critical time frame for BAT development (112), it is largely unknown whether the availability of n-3 PUFA to the fetus could modulate embryonic BAT development. This study is designed to identify the effects of maternal n-3 PUFA intake on fetal BAT development, potential mechanisms, and long-term metabolic consequences. Here, we demonstrated that maternal n-3 PUFA intake altered the neonates' FA profiles by reducing the n-6/n-3 PUFA ratio in the neonates, suggesting that n-3 PUFA is effectively delivered from mothers to the pups (Fig 4). The maternal n-3 PUFA intake (3%) was sufficient to potentiate transcriptional programming of the brown adipogenesis (Fig 5) via mechanisms involved in miRNA production (Fig 6) and histone modification (Fig 7). Furthermore, the augmented thermogenic activities endowed offspring with long-lasting metabolic benefits (Fig 8). Based on these results, we proposed that maternal n-3 PUFA intake is a molecular driver in promoting epigenetic liability of brown differentiation via a two-pronged mechanism through brown-specific miRNA biogenesis and histone modulation (Fig 9). It is intriguing to evaluate the potential of prenatal BAT as a new and feasible therapeutic target to promote thermogenic energy expenditure via maternal dietary intervention.

3.1.1 Metabolic relevance of BAT in energy metabolism and use the rodent as a feasible model

Human BAT depots are found in the deeper neck (cervical), supraclavicular, perirenal, axillary, and paravertebral areas (115-117). Human supraclavicular BAT

possesses 50-times greater respiratory activities than white adipose tissue (WAT) (2). These BAT depots are comprised of 1.5% of total body mass (roughly 5% of total fat mass), and up to 90% depots could be activated BAT (118). By using the ^{18}F-labeled fluorodeoxyglucose positron emission tomography/computerized tomography (PET/CT), substantial brown fat activities are found in ~96% of healthy lean adults, but in only ~20% of obese subjects (67), emphasizing the metabolic significance of BAT in adiposity control. By using stable isotope methods, it has been revealed that human BAT directly participates in whole-body lipolysis and thermal regulation in response to both prolonged (165) and mild cold exposure (166). An image-guided mapping of rodent adipose depots reveals the topological analogy of BAT between rodents and humans (119), which provides the feasibility to use rodent in studying human BAT. Supporting this notion, comparable functional analysis demonstrated that human supraclavicular BAT features the functional similarity with rodent interscapular BAT regarding mitochondrial activity and thermogenic potential. It is also shown that brown adipose tissue in both human and rodents exerted comparable UCP1 activity per mitochondrion (2).

In terms of timeline for BAT development, mouse BAT is formed at the last week of pregnancy out of approximately 21-day of total gestation period. Despite humans have longer gestation period than rodents, late pregnancy is the critical time for human BAT formation (4,120). In humans, BAT appears at the mid-gestation period and its BAT development mostly happens at the last trimester of pregnancy (110). BAT expands its maximum size at the early postnatal period, and stays fairly stable in size until adolescence, and degenerates into white adipose tissue with the progression of age (110). Given the developmental similarities of BAT between rodents and humans, the regulation of

interscapular BAT in rodents during pregnancy seems to be translatable, at least offering an important insight for human BAT regulation.

3.1.2 Regulation of fetal BAT development by maternal n-3 PUFA intake

The health benefits of maternal n-3 PUFA intake on offspring have received extensive attention (21,151,153). Since the synthesis of n-3 PUFA in the fetus side is limited, the transfer of n-3 PUFA cross the placenta from the mother to the fetus is vital and necessary when it comes to maternal nutrition (167). The maternal supplementation of n-3 PUFA increases the n-3 PUFA concentration in placenta and uterus cord blood, suggesting that maternal n-3 PUFA is effectively transferred to fetus prenatally (168). Despite the multiple metabolic benefits of maternal n-3 PUFA intake to the offspring (156,157,159,162), the effects of maternal n-3 PUFA intake on modulating the fetal BAT development and thermogenic activities remain elusive. Indeed, per our knowledge, our study is the first to demonstrate maternal n-3 PUFA supplementation decreases n-6/n-3 ratio in the neonatal BAT (Fig 2C). Our results also provide scientific evidence that maternal n-3 PUFA supplementation promoted brown adipogenesis-related gene expressions including UCP1, PGC1α, CIDEA, and PRDM16 (Fig 2). It is notable that activation of brown-specific genes in the iBAT relies on GPR120, a membrane sensor for n-3 PUFA. There was a robust increase in GPR120 mRNA expression in iBAT in response to the maternal supply of n-3 PUFA in both genders. These results align with our previous finding that EPA-induced brown adipogenesis is dependent on GPR120 (26). It is also compatible with the literature showing that knockout of GPR120 or treatment of GPR120 antagonist dampened the thermogenic activities (31). Consistent with this notion, genetic ablation of GPR120 in mice results in hepatic steatosis and insulin resistance (43). In

humans, lack of GPR120 signaling activity due to a genetic mutation in the GPR120 gene (p.R2700H) is correlated with increased risk of obesity (43).

Intriguingly, augmented BAT activity by maternal n-3 PUFA was sustained for at least 8 weeks post-weaning (Fig5); the pups received the maternal n-3 PUFA supplementation not only maintained the higher BAT activities in the iBAT but also exerted higher adaptive thermogenesis in the iWAT (Fig5). Our results are consistent with the recent report that low perinatal n-6/n-3 ratio is important to attenuate susceptibility against diet-induced obesity in adult offspring (101). Human studies also support an inverse correlation between maternal n-3 PUFA intake and prevalence of childhood obesity. Specifically, a higher risk of childhood obesity was correlated with a higher maternal n-6/n-3 fatty acid diet ratio, while maternal n-3 PUFA intake was linked with a lean body fat mass of children (97-100). In contrast, there exist some inconsistent human studies that fail to observe the negative correlation between maternal n-3 PUFA and adiposity of the offspring (169-171). These studies might be compounded by the variables such as supplementation periods, dose, and the ratio of DHA to EPA. Future human clinical trials with the long-term follow-up research design are warranted to establish the effect of maternal n-3 PUFA on thermogenesis and energy expenditure at the time of birth, childhood and adolescence.

3.1.3 Epigenetic regulation of BAT by early exposure to maternal n-3 PUFA

In terms of the underlying mechanism, we propose that maternal n-3 PUFA intake promotes brown transcriptional program of neonatal BAT via the synergistic action of miRNAs and histone modifications.

MiRNAs are short non-coding RNAs associated with post-transcriptional

regulation of gene expression, which play a critical role in BAT differentiation (124,137-139). The blockage of miRNA biogenesis by adipocyte-specific deletion of dicer significantly impairs BAT development (172). The miR-193b/365 was first identified as a brown-specific miRNA cluster to promote brown adipogenesis by suppressing the myogeneic-lineage differentiation in the Myf5+ precursor cells(173). Also, miR-30b was reported to induce brown-specific gene expressions and increased mitochondrial respiration through degradation of the transcriptional corepressor receptor-interacting protein 140 (RIP140) (26,145). Previously, we demonstrated the cell-autonomous function of n-3 PUFA in promoting brown adipogenesis in the primary brown precursor cells, leading to an increase of clusters of brown-specific miRNAs including miR-30b and miR-193b/365 (26). In agreement with our in vitro study, n-3 PUFA exposure during pregnancy recapitulated the involvement of the same profile of miRNAs including miR-30b, and miR-193/365 cluster (Fig 3A). Interestingly, augmented BAT thermogenesis is correlated with an increase of the Drosha gene and protein expression (Fig 3C, D). Drosha is a ribonuclease and a critical component of the microprocessor for initiating the cleavage of pri-miRNA into stem-loop pre-miRNA (174). It is conceivable that n-3 PUFA availability may alter microprocessor activity resulting in pre-miRNA biogenesis in the iBAT, thereby augmenting the microbiome of iBAT. In supporting this idea, deletion of fat-specific DGCR8, a subunit of the microprocessor complex that recognizes and mediates the processing of pri-miRNA into pre-miRNA, resulted in defective BAT formation and severe cold intolerance (175). We are currently investigating whether maternal n-3 PUFA intake alters the miRNA processor activities, thereby increasing the pri-miRNAs processing rate in the nucleus.

Histone modifications represent another important mode of epigenetic regulation of transcription in directing cell-lineage specification and tissue-specific gene expression. Accumulating evidence suggests that BAT development is governed by dynamic changes in post-translation modification via acetylation and methylation on the N-terminal tail region of histones(126). In general, histone acetylation of lysine (K) side chains decreases positive changes and reduces chromatin compactness, thereby contributing to transcriptional activation. Histone acetylation is regulated by the balance between histone acetyltransferase (HAT) and histone deacetylases (HDACs). Jin et al. showed that Gcn5/PCAF, a HAT enzyme, facilitates brown adipogenesis (176). The inhibition of HDAC1 (129) or HDAC9 is linked with transcriptional activation of brown adipogenesis (131,153). In contrast, deacetylase activity of HDAC3 is required for thermogenic adipose program (177). Histone methylation occurs on lysine (K) or arginine (R) residue, thereby altering the DNA accessibility to the binding of chromatin modifiers and transcription factors or epigenetic readers. It is important to note that site-specific H3K9 methylation by G9a represses brown differentiation (151), while methyl transfer by EHMT1 promotes BAT-selective thermogenic program (113). Controversially, deletion of demethylase JMJD1a, demethylase at H3K9, is linked with obesity (152,178). Another critical epigenetic site for histone methylation is H3K27. The reduction of H3K27me3 by demethylase enzymes of UTX (129) or JMJD3 (132) is essential for brown adipogenesis. Recently, the dynamic interplay between histone acetylation and methylation at H3K27was revealed during brown adipogenesis. The β3−adrenergic stimulation induced dissociation of HDAC1, resulting in increased H3K27 acetylation followed by H3K27me3 demethylation thus allowing transcription in the isolated brown precursor cells (129).

Most aforementioned epigenetic histone modifications during brown differentiation were derived from the animals or cells with specific deletions of epigenetic writers (enzymes that catalyze the addition of epigenetic marks) or erasers (enzymes that catalyze the removal of epigenetic marks). To our surprise, little information is available about how environmental factors such as diet modify epigenetic markers for brown adipogenesis. The transcriptional activation of the fetal brown thermogenic program by n-3 PUFA were linked with altered PTMs, i.e., enhanced H3K27Ac and diminished H3K27me3, through the coordinated modulation of epigenetic erasers between HDAC1 and JMJD3. In addition, the enhanced H3K9me2 status by n-3 PUFA intake was associated with an increase of epigenetic writer EHMT1 without altering the demethylase JMJD1 (Fig 4A, B). These results unanimously suggest that prenatal n-3 PUFA exposure serves as an epigenetic modulator, thereby potentiating transcriptional brown thermogenic program.

3.2 Limitation and future studies

One caveat of our study design is that we cannot distinguish the effects of prenatal n-3 PUFA exposure from the n-3 PUFA supply through lactation. Aware of this limitation, we plan to conduct new experiments to address the separate role of prenatal BAT development and lactation by collecting BAT at birth or swapping the pups during the lactation period. To ascertain the long-term metabolic benefits, we are currently investigating whether maternal n-3 PUFA intake confers the resistance against high fat diet-induced obesity and insulin resistance in adulthood. Despite numerous indications in experimental animals, our current understanding regarding the thermogenic function of n-3 PUFA in humans is inconsistent (97-101,169-171). In particular, the role of maternal n-3 PUFA supplementation on thermogenic activities in offspring, presumably through

epigenetic modifications, has yet to be established. As pregnant women are recommended to take approximately 600~800 mg of n-3 PUFA daily during pregnancy (3,5), the exact role of n-3 PUFA on fetal BAT development and its long-term metabolic benefits through enhanced brown thermogenic potential should be addressed in humans. Here, we presented initial evidence that fetal BAT development could be a feasible therapeutic target to attenuate childhood obesity via maternal n-3 PUFA supplementation.

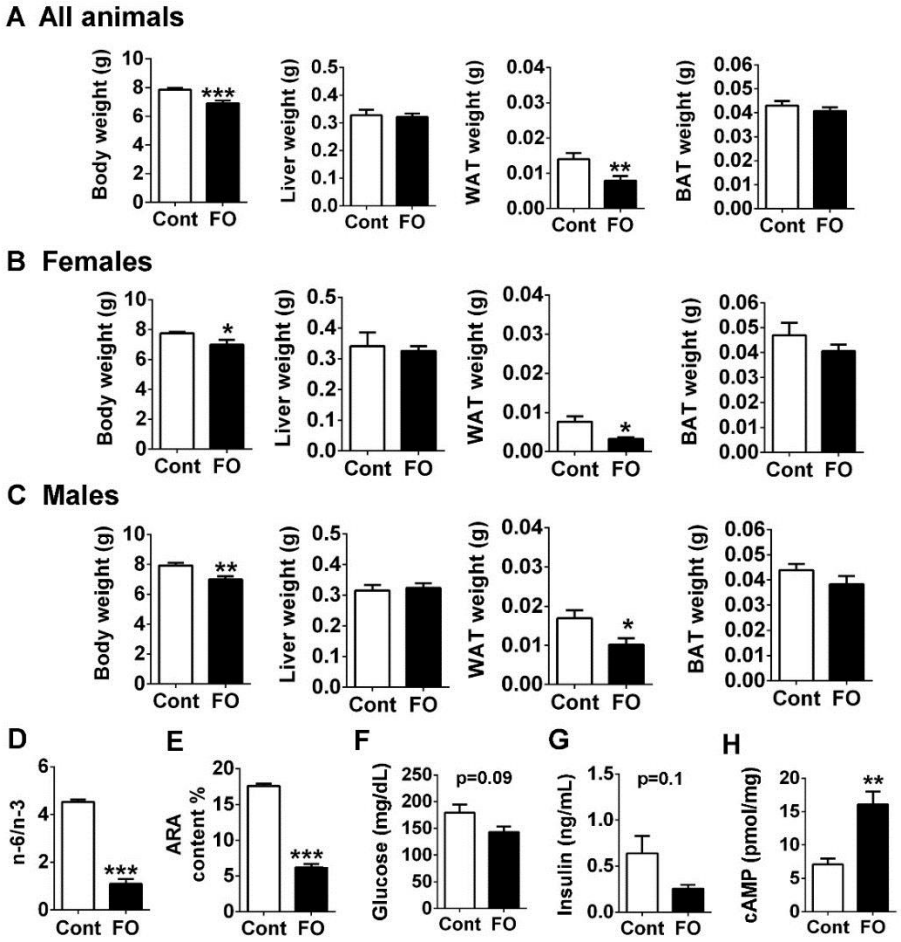

Figure 4 Maternal n-3 PUFA supplementation decreased offspring's WAT accumulation and altered blood lipid profile at the time of weaning. A-C. Effects of maternal n-3 PUFA supplementation on body weight, liver weight, epididymal WAT, and interscapular BAT weight from all pups (A, n=18 each group), female pups (B, n=8) and male pups (C, n=10). D. n-6/n-3 PUFA ratio by GC analysis. E. Arachidonic acid content (%). F. Plasma glucose level. G. Insulin concentration by ELISA. H. cAMP concentration. All data was expressed as mean ± SEM.* $p<0.05$, **$p< 0.01$, and ***$P < 0.001$ by Student's t-test.

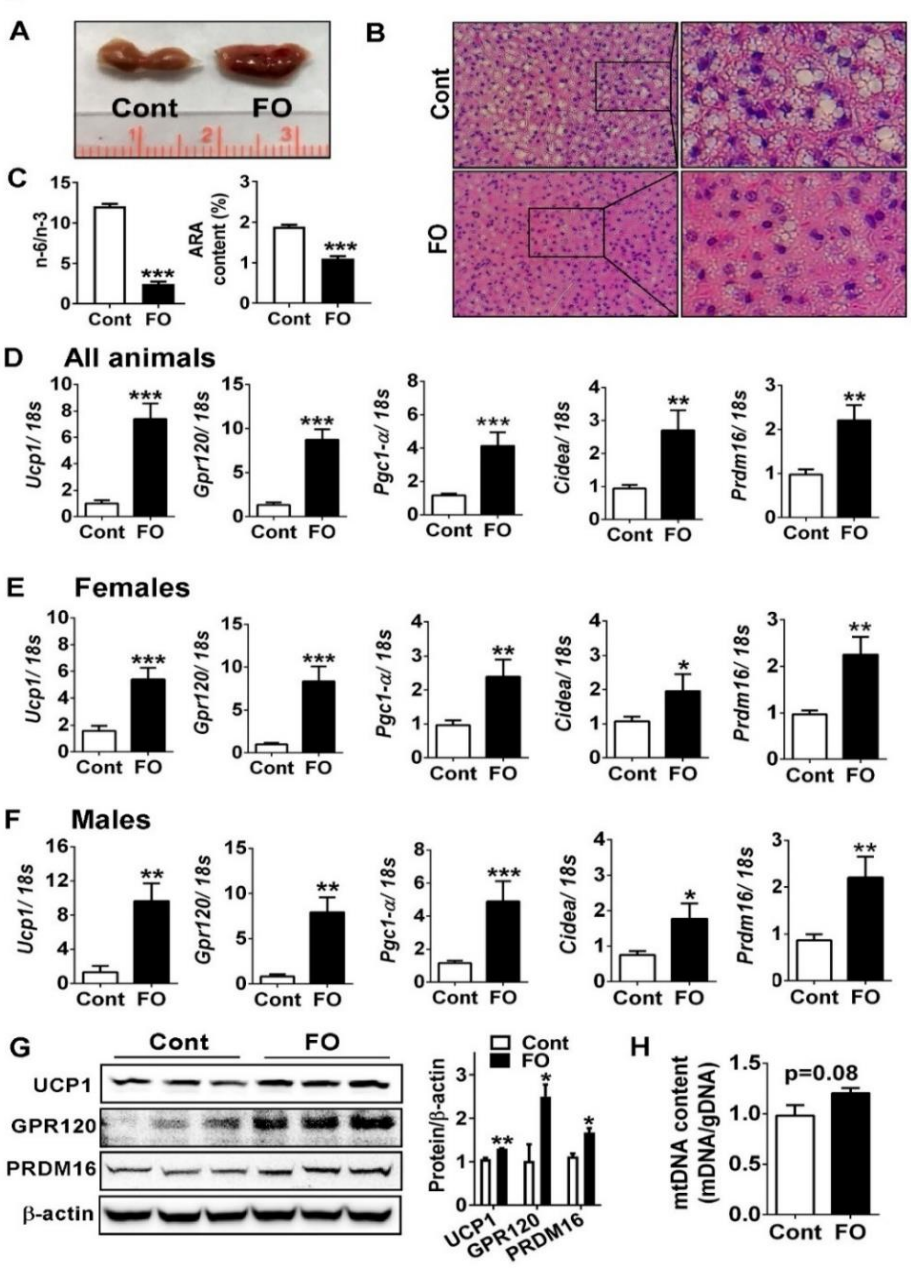

Figure 5 Maternal n-3 PUFA supplementation enhanced BAT development in offspring at the time of weaning. A. Gross image of iBAT without (Cont) or with (FO) maternal n-3 PUFA supplementation. B. Representative microscopic images with H&E staining of iBAT. C. n-6/n-3 PUFA ratio and arachidonic acid (ARA) content (%) in the iBAT (left) by GC analysis. D-F. Brown signature gene expressions of Ucp1, Gpr120, Pgc1-α, Cidea, and Prdm16 in all pups (D), female pups (E) and male pups (F) by qPCR. G. Western blot pattern of UCP1, GPR120, and PRDM16 in the iBAT (left). Relative protein intensities to β-actin quantified by Image J (right). H. Relative mitochondrial DNA to genomic DNA in the iBAT (n=6). All data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by Student's t-test.

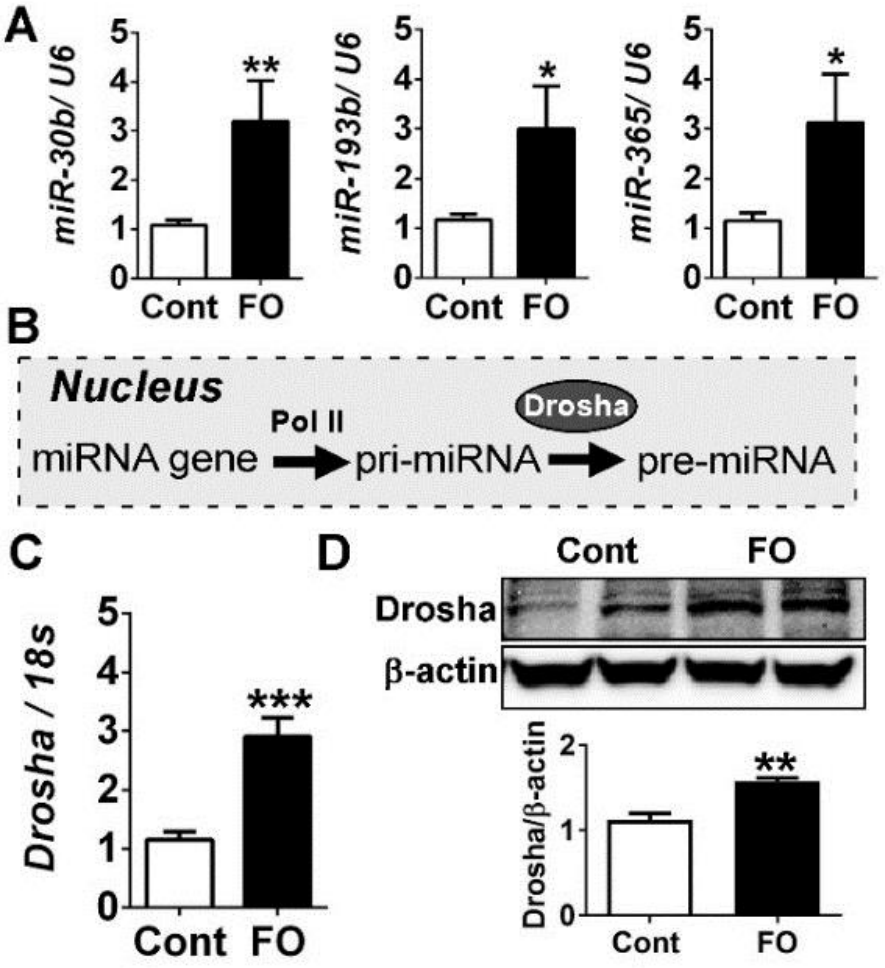

Figure 6 Maternal n-3 PUFA supplementation promoted brown adipogenesis via miRNA biogenesis at the time of weaning. A. qPCR analysis of miRNAs of miR-30b, 193b, and 365 in all pups (n=18), B. Schematic diagram of the nuclear processing of pri-miRNA by Drosha. C. Drosha mRNA expression (n=8) by qPCR, D. Protein levels of Drosha in iBAT (upper), and its relative intensity to β-actin by Image J (lower). All data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by Student's t-test.

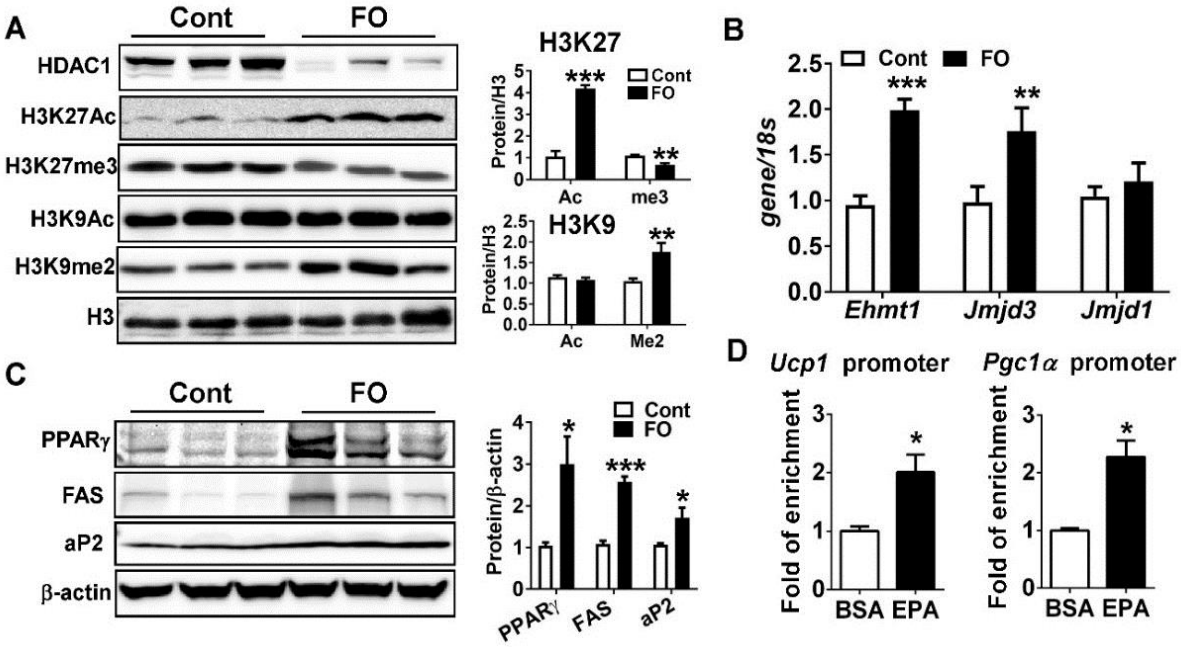

Figure 7 Maternal n-3 PUFA supplementation promoted brown adipogenesis via histone modifications at the time of weaning. A. Western blot pattern of epigenetic markers of HDAC1, H3K27Ac, H3K27Me3, H3K9Ac, H3K9me2 (left). Image J was used to determine the relative status of histone acetylation and methylation at the H3K27 and H3K9 (right). B. qPCR analysis of epigenetic enzymes of histone methyltransferase Ehmt1, and demethylases of Jmjd3 and Jmjd1. C. Western blot pattern of adipocyte proteins of PPARγ, FAS, and aP2 (left). The membrane intensity was quantified by Image J (right). D. Enrichment of H3K27Ac at the promoter region of Ucp1 and Pgc1α by ChIP assay in the HIB1B cells that were differentiated with either vehicle (BSA) or EPA (n=4). All data are expressed as mean ± SEM. *p< 0.05, **p< 0.01, ***p<0.001 by Student's t-test**.**

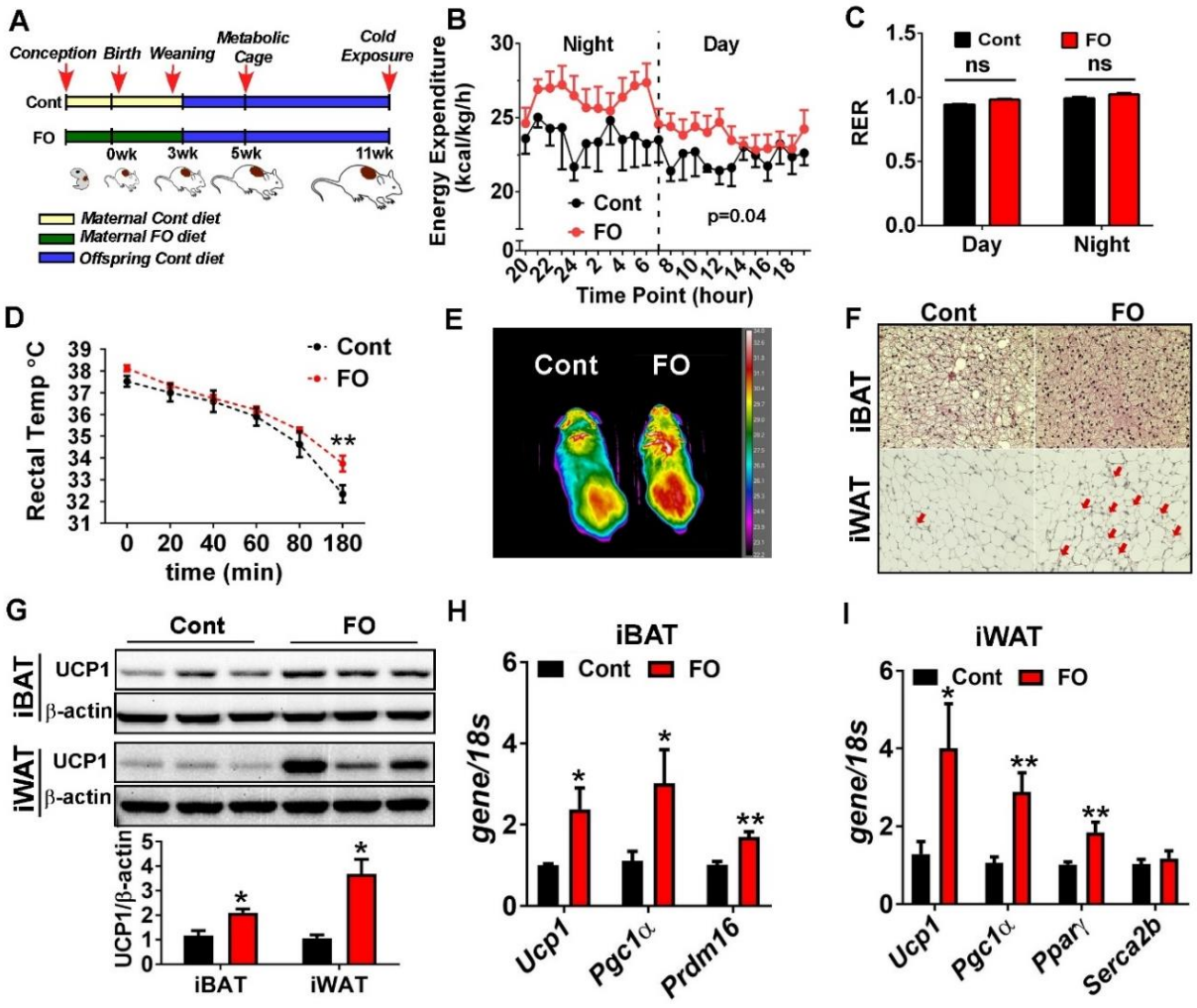

Figure 8 Maternal n-3 PUFA supplementation was associated with metabolic improvement in the later life of offspring. A. Schematic presentation of experimental design. The pups received maternal control diet (Cont)- or FO-diet (FO) were weaned at 3 weeks age, and maintained until 11 week-old with no additional dietary modification. B. Average energy expenditure for 4 days measured by metabolic cages (n=5) C. Average RER (VCO2/VO2) values for 4 days measured by using metabolic cages (n=5). D. Core body temperature measured by rectal temperature upon exposure to cold temperature (6°C) for 3 hours (n=6). E. Heat release captured by IR camera upon 3-hour cold exposure. F. Representative images with H&E staining of iBAT and iWAT after 24-hour exposure to cold temperature (6°C). Red arrows indicate the emergence of brown-like structure within iWAT. G. Western blot pattern of UCP1 in the iBAT and iWAT after 24-hour cold exposure (left). Relative UCP1 levels were quantified by Image J (right). H. Brown signature gene expressions of Ucp1, Pgc1α, and Prdm16 in the iBAT after 24-hour cold exposure (n=6), I. Gene expression levels of Ucp1, Pgc1α, PPAR□ and Scerca2b (a responsible gene for calcium-cycling dependent thermogenesis) in the iWAT after 24-hour cold exposure (n=6). All data are expressed as mean ± SEM. *p< 0.05, **p< 0.01, ***p<0.001 by Student's t-test.

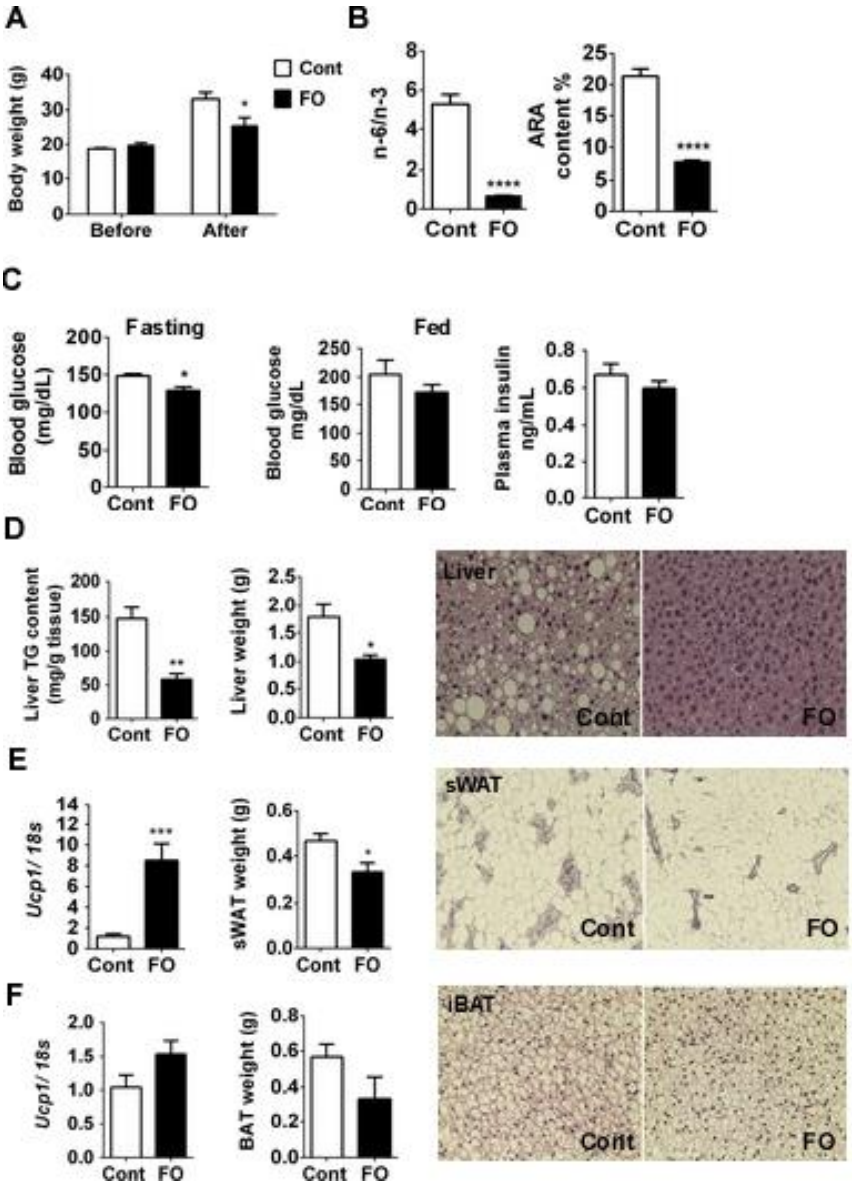

Figure 9 Health benefits of n-3 PUFA during pregnancy and lactation from maternal perspective. A. Body weight changes after an average of 3 pregnancies. B. Upon sacrifice, red blood cell lipids were extracted and detected for n-6/n-3 fatty acids ratio Band arachidonic acid content. C. Fasting blood glucose and plasma was measured for insulin concentration. D. Liver weight, triglycerides content, and H&E staining were measured. E. Subcutaneous WAT weight, Ucp1 expression, and H&E staining. F. iBAT weight, H&E staining, and Ucp1 expression were presented. All data were shown as mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Student's t -test.

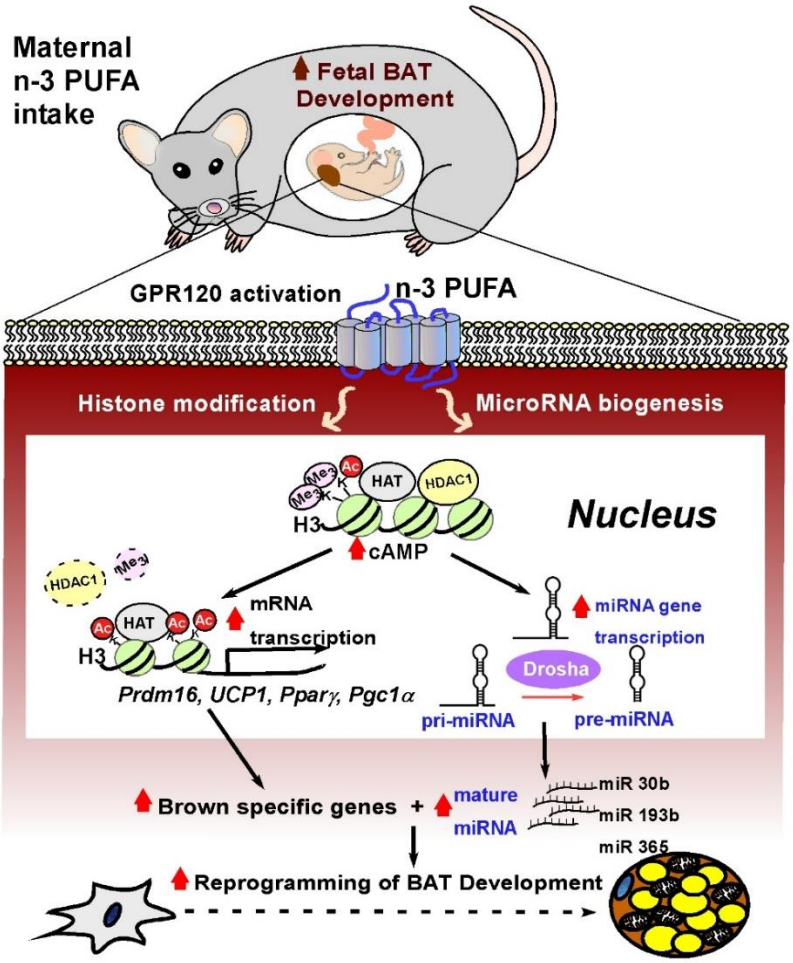

Figure 10 The proposed mechanism by which maternal n-3 PUFA supplementation potentiates fetal BAT development via epigenetic modulation. Maternal n-3 PUFA intake provides n-3 PUFA to the fetus across the placenta. In fetus, activation of GPR120, a membrane sensor for n-3 PUFA, triggers at least two epigenetic modulations, histone modifications, and miRNA biogenesis. Fetal availability of n-3 PUFA increases cAMP levels and alters PTM marks of H3K27Ac and H3K9me2, presumably with modulation of epigenetic enzymes including reduced HDAC1, resulting in enhanced transcriptional activation for brown specific genes, i.e., *Ucp1*, *Prdm16*, *Pparγ, and Pgc1α.* The augmented transcriptome by histone acetylation concurrently increases miRNA-gene transcription and Drosha expression, leading to enhanced nuclear pri-miRNA processing and mature miRNA biogenesis. With this concerted action of histone modification and miRNA regulation, early exposure to n-3 PUFA potentiates transcriptional program of the fetal BAT, which may mediate to long-lasting metabolic benefits in later life.

Table 1 Fatty acid composition of Control diet (Cont) and N-3 PUFA (FO) diet

	Cont	FO
% Calorie (in % in gram diet)		
Carbohydrate	65 (62.1)	65 (62.1)
Protein	20 (19.8)	20 (19.8)
Fat	15 (6.8)	15 (6.8)
Energy Density	3.9 kcal/g	3.9 kcal/g
Fatty acid profile (%)		
Myristic acid C14:0	0.89	7.37
Palmitic acid (PA) C16:0	35.99	18.49
Palmitoleic acid C16:1(n-7)	0.13	10.72
Stearic acid C18:0	5.91	5.44
Oleic acid C18:1 (n-9)	23.86	14.03
Linoleic acid C18:2 (n-6)	28.84	14.74
Linolenic acid C18:3 (n-3)	4.19	3.17
Eicosapentaenoic acid C20:5 (n-3)	0.03	13.36
Docosahexaenoic acid C22:6 (n-3)	0.15	12.70
Fatty acid composition		
SFA	42.79	31.30
MUFA	23.99	24.74
PUFA	33.21	43.96
Total (n-6) PUFA (%)	28.84	14.74
Total (n-3) PUFA (%)	4.37	29.22
(n-6): (n-3) ratio	6.60	0.50

APPENDIX:

Abbreviations

ADRB2—β2-adrenoceptors
ADRB3— β3-adrenergic receptor
ALA—α-linolenic acid
ARA—Arachidonic acid
BMI — Body mass index
cAMP—Cyclic adenosine monophosphate
ChIP— Chromatin immunoprecipitation
CIDEA— Cell death-inducing DNA fragmentation factor α-like effector A
COX—Cyclooxygenases
CRE— cAMP response element
DAG— Diacylglycerol
DHA—Docosahexaenoic acids
DPA — Docosapentaenoic acid
EE— Energy expenditure
EHMT1— Euchromatic histone N-lysine methyltransferase 1
EPA—Eicosapentaenoic acids
eWAT— Epididymal white adipose tissue
FA—Fatty acids
FAS— Fatty acid synthase
FDG — ^{18}F-2-deoxy-glucose
FGF21— Fibroblast growth factor 21
FO— Fish oil
GC — Gas Chromatography
GPR120— G-protein-coupled receptor 120
hADS — Human adipogenic stem cells
HAT— Histone acetyltransferase
HDAC1— Histone deacetylase 1
HF—High fat
iBAT— Interscapular brown adipose tissue
ILC2s — Type 2 innate lymphoid cells
iWAT— Inguinal white adipose tissue
JMJD3— JmjC domain-containing protein 3
MUFA— Monounsaturated fatty acids
N-3 PUFA— Omega-3 polyunsaturated fatty acids
PET— Positron emission tomography
PGC1α— Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGI2— Prostacyclin
PPARγ— Peroxisome proliferator-activated receptor gamma
RIP140— Receptor-interacting protein 140
PRDM16— PR domain containing 16
PTM— Post-translational modifications
PVDF — Polyvinylidene fluoride
RER — Respiratory exchange ratio

RMR — Resting metabolic rate
SFA— Saturated fatty acids
SNS — Sympathetic nervous system
SV— Stromal vascular
TRPV1—Transient receptor potential vanilloid receptor1
UCP1— Uncoupling protein 1
VCO2— Carbon dioxide production
VEGF— Vascular endothelial growth factor
VO2— Oxygen consumption

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