The iFat-1 Transgene Permits Conditional Endogenous n-3 Polyunsaturated Fatty Acid Enrichment both in vitro and in vivo

By

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Based on their highly bioactive properties in membrane phospholipids, there is growing recognition that dietary n-3 polyunsaturated fatty acids (PUFA) may be of significant benefit in the prevention and treatment of many lifestyle related pathologies, however direct evidence is lacking. The fat-1 transgenic mouse, a genetic model of n-3 PUFA enrichment, is a useful tool in nutritional research which has provided enhanced insight into the health effects of lifelong n-3 PUFA exposure. However, the influence of timing of n-3 PUFA exposure on health related outcomes remains unclear. This thesis describes the functional characterization of the novel Cre recombinase dependent inducible fat-1 (iFat-1) transgene. In the presence of Cre, the iFat-1 transgene was found to reduce phospholipid n-6/n-3 PUFA ratios both in vitro (100%) and in vivo (upwards of 70%), suggesting that the iFat-1 transgene has potential application to address temporal effects of n-3 PUFA in health and disease.
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LIST OF ABBREVIATIONS

AA: Arachidonic acid
ALA: Alpha-linolenic acid
C. elegans: Caenorhabditis elegans
COX: Cyclooxygenase
DHA: Docosahexaenoic acid
EPA: Eicosapentaenoic acid
FAME: Fatty acid methyl ester
Hprt: Hypoxanthine-guanine phosphoribosyltransferase
IP: Intraperitoneal
LA: Linoleic acid
LOX: Lipoxygenase
LT: Leukotriene
MUFA: Monounsaturated fatty acids
PC: Phosphatidylcholine
PCR: Polymerase chain reaction
PE: Phosphatidylethanolamine
PG: Prostaglandin
PI: Phosphatidylinositol
PS: Phosphatidylserine
PUFA: Polyunsaturated fatty acids
RT-PCR: Reverse transcriptase polymerase chain reaction
SFA: Saturated fatty acids
TLC: Thin layer chromatography
TX: Thromboxane
WT: Wildtype
4-OHT: 4-hydroxytamoxifen
Chapter One

INTRODUCTION
1.0 INTRODUCTION

The etiology and pathogenesis of prevalent non-communicable diseases such as cancers, cardiovascular disease and other inflammatory related pathologies involve lifestyle related components, of which sub-optimal dietary habits and physical inactivity have been identified as major disease predisposing factors (1). Based on their inflammatory modulating capacities and other bioactive distinctions, n-3 PUFA are proposed to have health promoting properties in the prevention and amelioration of a broad spectrum of lifestyle related pathologies, however at present their relative therapeutic efficacy remains poorly defined (2-4).

Although in vitro and epidemiological analysis represent a useful foundation for exploring the biological effects of n-3 PUFA, respective issues of system simplicity and recall bias greatly limit extrapolation of results derived from such investigations. As such, direct nutritional manipulation of fatty acid composition in animal models represents a very important avenue for pre-clinical research and discovery in PUFA research. However, while experimental diets can be carefully matched for many components, including % energy derived from major macronutrient classes, small undesirable variability inevitably persist.

Although mammals possess the enzymes necessary to convert α-linolenic acid (ALA) to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the major bioactive n-3 PUFA species, they lack the capacity for direct biosynthesis of n-3 PUFA from n-6 PUFA. However through expression of the fat-1 n-3 desaturase gene derived from the Caenorhabditis elegans (C.elegans) worm a mouse model endowed this unique
capability has been developed (5). Within the context of identical nutritional settings fat-1 transgenic mice and their wildtype (WT) controls develop distinctly opposing fatty acid profiles, in which the tissues of fat-1 transgenic mice are markedly enriched in n-3 PUFA (5). However, while the fat-1 transgenic mouse is a highly efficacious tool in PUFA related research, the constitutive nature of transgene expression presents a less dynamic approach to tissue PUFA modification than that achievable through standard diet-based manipulation, thus restricting the use of this mouse model to investigations concerning the effects of lifelong n-3 PUFA enrichment. As such, the ability to control temporal aspects of fat-1 transgene activation may significantly extend the application of this genetic based model for n-3 PUFA research.

The overall objective of this project was to characterize the functionality of a newly developed transgenic mouse model containing a version of the fat-1 n-3 desaturase gene which has been rendered conditionally reliant on Cre-recombinase for its activation, termed the inducible fat-1 (iFat-1) transgenic mouse. This work was carried out using complementary in vitro and in vivo validation methods and has served to provide several promising advancements towards establishing the iFat-1 transgenic mouse as a beneficial tool in PUFA related research.
Chapter Two

LITERATURE REVIEW

UNDERSTANDING THE ROLE OF N-3 POLYUNSATURATED FATTY ACIDS IN HEALTH AND DISEASE: EVIDENCE GAINED FROM RESEARCH EMPLOYING THE FAT-1 TRANSGENIC MOUSE
2.1 Introduction

Polyunsaturated fatty acids (PUFA) are integral constituents of membrane phospholipids with key roles in the coordination of cell function (6). PUFA contain two or more double bonds in their molecular structure and can be further differentiated by the relative position of these double bonds. There are two major families of PUFA, n-3 polyunsaturated fatty acids (n-3 PUFA) and n-6 polyunsaturated fatty acids (n-6 PUFA), a designation which signifies the position of the first double bond when counting from the methyl terminal end of the hydrocarbon chain. The n-3 and n-6 PUFA family are each composed of an 18-carbon precursor molecule, α-linolenic acid (ALA; 18:2n-6) and linoleic acid (LA; 18:2n-6) respectively. However, it is through the major long-chain derivatives of these species, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), in the n-3 PUFA family, and arachidonic acid (AA; 20:4n-6), in the n-6 PUFA family, that PUFA elicit the bulk of their biological activities within cells (6).

Dietary n-3 PUFA, particularly the fish-oil derived EPA and DHA species, are increasingly credited with possessing health promoting properties in the prevention and treatment of prevalent chronic human pathologies. However, with the exception of a few limited parameters related to cardiovascular health (7), the ability to make firm recommendations pertaining to the therapeutic benefits of n-3 PUFA on disease specific outcomes is largely restricted by the existence of a complex and often contradictory landscape of *in vitro*, human and animal based evidence (8-10). Therefore, well designed experimental systems are essential to clarifying the fundamental mechanisms by which membrane n-3 PUFA status impacts cellular physiology and pathology.
The fat-1 transgenic mouse model offers an elegant approach to assist in delineating the specific effects of tissue n-3 PUFA composition on the modulation of various health related outcomes (5). This review will explore how evidence gained from research employing the fat-1 transgenic mouse model has contributed to fundamental knowledge concerning the biological implications of tissue n-3 PUFA status in health and disease. Specific reference will be given to studies exploring the potential protective effects of n-3 PUFA in conditions of excess inflammation, cancer and neurological health. The relative strengths, caveats and relevance of this genetic approach to tissue n-3 PUFA enrichment will also be considered.

2.2 PUFA and Health: Contextual Background

2.2.1 Sources and Metabolism

With the exception of ALA and LA, the mammalian genome encodes the complete complement of enzymatic machinery necessary for the biosynthesis of all fatty acid (FA) species involved in human health and development. Therefore, LA and ALA are the only fatty acid species historically classified as being essential from diet (11). Plant sources such as canola oil, soybean oil, flaxseed oil, walnuts and green leafy vegetables are rich in ALA (11). Conversely, principle sources of LA include corn oil, safflower oil, sunflower oil and cereal grains (11). In addition to metabolic origins, long-chain PUFA can be directly obtained through diet. AA is a component of animal fats and eggs whereas fatty fish and fish-oils are known to contain particularly high levels of EPA and DHA, but are subject to variation based on fish-type and aquaculture practices (12,
13). Marine microalgae, stearidonic acid enriched oils and fortified foods, also represent promising and emerging adjuvant and/or alternative means of increasing long-chain n-3 PUFA status (14).

In mammals, endogenous long-chain PUFA synthesis proceeds through a series of alternating desaturation and elongation steps catalyzed by a common enzymatic cascade involving acyl-coenzymeA desaturases and elongases. Within this pathway, the Δ5- and Δ6-desaturase enzymes facilitate the introduction of cis-double bond at carbon-5 and -6 respectively, whereas specific elongases (Elovl2 and Elovl5) catalyze the two carbon extension of fatty acyl-CoA molecule (15). Both sets of enzymes generate their corresponding modifications to the fatty acyl chain in relation to its carboxylic acid moiety (15). The first rate limiting step within the n-3 branch of the desaturation and elongation pathway involves Δ6-desaturase facilitated metabolism of ALA to stearidonic acid (18:4n-3). Through a successive 2-carbon elongation and Δ5-desaturation step stearidonic acid is converted to EPA, the major 20-carbon metabolite of the n-3 PUFA family. Downstream elongation of EPA results in the production of docosapentaenoic acid (22:5n-3) followed by tetracosapentaenoic acid (24:5n-3). In a final Δ6-desaturation step, tetracosapentaenoic acid is reduced to tetracosahexaenoic acid (24:6n-3), which can then be translocated to the peroxisome for subsequent retro-conversion to DHA via a one-step β-oxidation reaction (16, 17). In an analogous series of reactions, LA can be converted to its major 20-carbon and 22-carbon metabolites, AA and docosapentaenoic acid (DPA; 22:5n-6) respectively. The 24-carbon intermediates of each of the n-3 and n-6 PUFA pathway can also serve as substrates for further elongation to very-long chain PUFA species ranging from 24-34+ carbons in length. Although present in relatively
small amounts throughout the body, these very-long chain PUFA species are selectively enriched in tissues such as the retina and testes where they perform specialized roles in maintenance of tissue integrity and function (18, 19).

The standard Western-style diet is typified by a high intake of n-6 PUFA and low intake of n-3 PUFA, reportedly in the excess of 10-20:1, of which, the plant derived essential fatty acid species comprise the vast majority of all PUFA consumed (20). Despite possessing the requisite enzymatic machinery necessary for long-chain PUFA synthesis, the overall bioconversion efficiency through the endogenous PUFA cascade is very limited in humans. For example, general estimates of conversion efficiency, based on the recovery of labelled metabolites in tracer studies, suggest that human adults are capable of metabolising ~0.2%-5% and ~0.1% or less of ALA to EPA and DHA respectively, with β-oxidation for fuel and carbon recycling accounting for the major metabolic fate of ALA (21). Similarly, it has been estimated that only 0.2%-2% of LA is converted to AA (22-24). Moreover, owing to shared nature of the PUFA biosynthetic cascade substrate competition exists between n-3 PUFA and n-6 PUFA for metabolic elongation and desaturation (25). Therefore, the relative dietary excesses of LA inundate the pathway towards favorable long-chain n-6 PUFA production, further restricting tissue levels of long-chain n-3 PUFA synthesis. This imbalance in n-6/n-3 PUFA ratio is proposed to significantly contribute to the risk and progression of numerous chronic human diseases, particularly those with inflammatory undertones (1, 26, 27).
2.2.2 General Biological Properties of n-3 PUFA

Long-chain PUFA are selectively enriched within membrane phospholipids where they regulate a wide variety of physiological processes thus influencing cell function and response to environmental stimuli. Despite structural similarities and shared metabolic pathways n-6 and n-3 PUFA do not appear to be functionally interchangeable (28). The biological intricacies of n-6 and n-3 PUFA are multifold, encompassing functional differences in inflammatory mediator synthesis, gene expression and membrane architecture.

In addition to sequential elongation and desaturation within the PUFA biosynthetic pathway, EPA and AA can also serve as substrates for eicosanoid synthesis. Eicosanoids, which include prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT), amongst other oxidized 20-carbon PUFA derivatives, are a highly bioactive class of signalling molecules which play critical roles in shaping the nature of the inflammatory response (26). EPA can undergo cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) mediated metabolism to 3-series prostanoids (PG and TX) and 5-series LT respectively. The corresponding products of AA metabolism are prostanoids of the 2-series and LT of the 4-series. When directly compared with their AA-derived counterparts, eicosanoids produced through EPA metabolism are known to possess potentially favourable inflammatory modulating properties, of which, a reduced efficiency in both COX-2 expression and interleukin-6 (IL-6) secretion by PGE\textsubscript{3} (29) as well as less potent stimulation of neutrophil chemotaxis by LTB\textsubscript{5} (30) are amongst the most noted differences. On the basis of such functional distinctions, AA is frequently assigned potent pro-inflammatory effects whereas EPA is described as a far less aggressive
inflammatory stimulus. While such crude classifications cannot fully recapitulate the complexity of molecular interactions whereby eicosanoids regulate inflammatory signalling, it is generally accepted that eicosanoids produced through metabolism of AA have a higher net pro-inflammatory potential than those derived from EPA and that n-6/n-3 PUFA reductions achieved through long-chain n-3 PUFA supplementation might prevent and/or ameliorate a variety of chronic human diseases through inflammatory modulating mechanisms.

While the effects of n-3 PUFA on the synthesis of eicosanoids are most classically recognized, they by no means encompass the full myriad, or potentially even the most efficacious, of mechanisms whereby n-3 PUFA are capable of influencing cell function. In recent years two novel classes of long-chain n-3 PUFA derivatives the resolvins and protectins, have been uncovered. Evidence from cell culture and animal models indicates that, alongside AA derived lipoxin A₄, resolvins and protectins reduce neutrophil infiltration, decrease expression of pro-inflammatory cytokines and actively assist in returning inflamed tissues to their respective homeostatic set-points (31). EPA and DHA have been demonstrated to down-regulate the expression of pro-inflammatory cytokines in cell culture through mechanisms which appear to involve suppression of nuclear factor-κB (NF-κB) activity (32, 33). Moreover, at the most superficial level, DHA and EPA also compete with AA for incorporation into membrane phospholipids and the relative degree of this incorporation therefore influences membrane fluidity and function of membrane proteins. Evidence indicates that DHA, is capable of modifying the structure of specialized membrane microdomains termed lipid rafts and caveolae, which are highly ordered membrane regions that appear to serve as platforms for colocalization
of transmembrane- and membrane associated proteins/receptors involved in various signal transduction cascades (34). Although the influence of n-3 PUFA on the structure, function and behaviour of these microdomains remains an area of considerable debate; increases of raft size and modification of actin remodelling in raft regions are among the proposed mechanisms by which n-3 PUFA modulate the interface between extracellular cues and nuclear responses to control immunomodulation (35, 36).

As knowledge regarding the molecular mechanism by which n-3 PUFA regulate cellular physiology continues to expand there is growing awareness that current patterns of dietary n-3 PUFA are likely unsuitable to support optimal cell function. Numerous organizations throughout the world have released dietary recommendations in support of a combined intake of EPA and DHA of ~500mg/day (37), however epidemiological evidence from numerous geographical locales, including Canada, underline the fact that many population demographics appear to be falling short of this recommendation (38-40). To date, no dietary reference intakes (DRI) values have yet to be established for long-chain PUFA. The ability to conclusively define dietary n-3 PUFA guidelines tailored towards chronic disease risk reduction and/or treatment requires clarification of the molecular and cellular mechanisms whereby PUFA regulate health and disease related outcomes.

Significant progress towards understanding the role of n-3 PUFA has been made in recent years due to the development of the fat-1 transgenic mouse. Following its initial description in 2004 by Kang et al., the fat-1 transgenic mouse model has gained far reaching application as a tool in PUFA research (5). Not only has this mouse model assisted in corroborating previous observed associations between tissue PUFA status and
disease phenotype, but it has also offered potentially novel mechanistic insights into the impact of tissue n-3 PUFA enrichment on cellular physiology and pathology.

2.3 Key Insights from fat-1 Transgenic Mice

Fat-1 transgenic mice have been specifically engineered to ubiquitously express a humanized version of the fat-1 gene, an n-3 desaturase derived from the roundworm Caenorhabditis elegans (C. elegans), which has been optimized for efficient expression within mammalian cells and placed under the control of a chicken β-actin promoter (41). Expression of the fat-1 transgene thereby affords this mouse model the unique capacity for endogenous synthesis of n-3 PUFA through the metabolism of n-6 PUFA, which is otherwise not possible in mammals. As a result, fat-1 transgenic mice exhibit a characteristic enrichment in major n-3 PUFA species, at the expense of n-6 PUFA, resulting in a relative balancing of the n-6/n-3 PUFA ratio in all major tissues and organs (42-46). Furthermore, several comparative evaluations have suggested that these genetically driven changes in tissue n-3 PUFA composition are similar to those achievable through n-3 PUFA diet feeding trials (45, 47, 48).

Research employing the fat-1 mouse is uniquely advantageous because it evades major diet-derived complicating factors (43). That is, subtle within-study variability between experimental diets, including differences in oil source and purity, palatability and oxidative susceptibility, may significantly impact an observed phenotypic response to a given dietary PUFA manipulation (43). Since fat-1 transgenic mice can be maintained on the exact same diet as their wild-type counterparts, yet develop opposing PUFA compositional profiles, they are well-guarded from such diet-based confounding
influences and therefore represent a well-controlled model for the study of n-3 PUFA and
the n-6/n-3 PUFA ratio in vivo (43).

Figure 2.1: PUFA metabolism in fat-1 transgenic mice (based on substrate preference of
the C. elegans fat-1 gene product (49)). Asterisk denotes site of unsaturation by the FAT-1 enzyme.

2.3.1 Inflammation

Inflammation is an innate component of the body’s defense system that functions
as a first-line defender against tissue insults which otherwise threaten host health, such as
those arising from infection, chemical irritants and trauma. However when this
protective response becomes dysregulated a pathological inflammatory state can ensue.
The inflammatory dampening capacities of tissue n-3 PUFA enrichment in conditions of
excess acute and chronic inflammation have been well supported through applications
involving the fat-1 transgenic mouse and have been summarized in Table 2.1
In particular, the protective effects of lifelong endogenous n-3 PUFA enrichment on DSS-induced colitis, a widely used model of inflammatory bowel disease, have been the focus of several investigations to date (42, 52, 54, 56).

Inflammatory bowel disease is a classification used to describe the spectrum of chronic inflammatory conditions afflicting the colon which include ulcerative colitis and Crohn’s disease. Fat-1 transgenic mice have been consistently demonstrated to have improved histopathological outcomes in DSS-induced colitis relative to their WT controls (42, 52, 54, 56). Hudert et al. reported that in addition to possessing a lowered long-chain n-6/n-3 PUFA ratio (1.7 versus 30.1), the colons of fat-1 transgenic mice were characterized by a significant increase in n-3 PUFA derived lipid mediators (PGE₃, LTB₅, resolving E₁, resolvin D₃, neuroprotectin D₁), reductions in both NF-kB activity and pro-inflammatory cytokine expression (TNFα, iNOS, IL-1β) and up-regulated expression of mucoprotective factors (42). In another study, conducted by Gravaghi et al., it was shown that, compared to controls, fat-1 transgenic mice with acute and chronic DSS-induced colitis experience significant reductions in a breadth of pro-inflammatory cytokines, decreased cox-2 expression and increased PGE₂ production (52). It is interesting to note that this latter finding is in opposition to that of Hudert et al., who reported no evident differences in major AA-derived eicosanoid species between fat-1 mice and their WT controls, but in good agreement with observations from nutrition based n-3 PUFA studies in experimental models of colitis (10, 57). Both Gravaghi et al. and Hudert et al. employed comparable DSS-induction strategies and therefore the reason for this discrepancy is not immediately clear, however the reduced sample size of Hudert et al. (n=3 vs n=40) may offer a plausible explanation. Recently, it was also
demonstrated that the lessened severity of inflammatory damage in fat-1 transgenic mice with chronic experimental colitis is associated with significant reductions in splenic and colonic Th17 cells, a T-cell subset involved in the persistence of chronic colon inflammation (54). Collectively, research employing the fat-1 transgenic mouse has helped to shed light on the seemingly complex network of mechanism through which tissue n-3 PUFA enrichment may dampen inflammation and emphasizes the need for further research in this area. Interestingly it has also been noted that while the inflammatory dampening properties exerted through fat-1 mediated endogenous n-3 PUFA enrichment confer beneficial effects on inflammatory related pathology, a coincident impairment in resistance to bacterial infection may also occur and should be considered in future research (58).
<table>
<thead>
<tr>
<th>Inflammatory Model</th>
<th>fat-1 Phenotype&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Ref</th>
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</table>
| Colitis            | ↓ Clinical and histopathological severity  
|                    | ↑ PGE<sub>3</sub>, LTB<sub>5</sub>, Resolin E<sub>1</sub>, Resolvin D<sub>3</sub> Neuroprotectin D<sub>1</sub>  
|                    | ↓ NF-κB activity  
|                    | ↓ pro-inflammatory gene expression (TNF-α, IL-1β, iNOS)  
|                    | ↑ mucoprotective factors  
|                    | ↓ histopathological severity (chronic colitis only)  
|                    | ↓ histopathological severity  
|                    | ↓ Th17 cells in spleen (systemic) and colon (local)  
|                    | ↓ expression of Th17 cytokines (IL17F, IL-21)  
|                    | ↑ expression of IL-27 (Th17 cell suppressive)  
|                    | ↓ histopathological severity  
|                    | ↓ Th17 cells in spleen (systemic) and colon (local)  
|                    | ↓ expression of Th17 cytokines (IL17F, IL-21)  
|                    | ↑ expression of IL-27 (Th17 cell suppressive)  
|                   | (52)                                                                                      |     |
| Hepatitis          | ↓ inflammatory liver injury  
|                    | ↑ plasma TNF-α (systemic inflammation)  
|                    | ↓ hepatic pro-inflammatory gene expression (TNF-α, IL-1β, IL-6)  
|                    | ↓ apoptosis  
| Pancreatitis       | Acute: ↓ plasma IL-6, ↑ pulmonary neutrophil infiltration  
|                    | Chronic: ↓ pancreatic fibrosis  
| Acute Lung Injury  | ↓ histopathological severity  
|                    | ↑ ventilator compliance  
|                    | ↓ leukocyte invasion and protein extravasation  
|                    | ↓ TXB<sub>4</sub>  
| Allergic Airway    | ↓ airway resistance  
| Response           | ↑ leukocyte invasion and pro-inflammatory cytokines (several)  
|                    | ↑ resolvin E<sub>1</sub> and neuroprotectin D<sub>1</sub> lung tissue  
|                    | (51)                                                                                      |     |

Table 2.1: Applications of fat-1 transgenic mice in models of inflammation. <sup>1</sup>in relation to WT control mice
2.3.2 Cancers

To date, the fat-1 transgenic mouse has been combined with various cancer models and in doing so has facilitated enhanced insight into the tumour suppressive effects of lifelong n-3 PUFA enrichment in models of melanoma (59), colitis associated colon cancer (56, 60), prostate cancer (61), hepatocellular carcinoma (62-64) and breast cancer (65) (Table 2.2). In the earliest of such reports it was demonstrated that relative to WT controls, fat-1 transgenic mice maintained on a 10% safflower oil diet experienced significant reductions in tumour incidence and growth rate following implantation of mouse melanoma B16 cells (59). The differential tumour phenotype of fat-1 transgenic mice was associated with stark reductions in total lipid n-6/n-3 PUFA ratio, decreased PGE₂ and increased PGE₃ production in both tumour and tumour adjacent tissue (59).

Reductions in tumour incidence and average tumour volume in response to azoxymethane/DSS-induced colitis associated colon tumourigensis have also been confirmed through application of the fat-1 transgenic mouse model (56, 60). Modified lipid mediator profiles and decreased NF-κB level/activity are amongst some of the associations by which lowering the n-6/n-3 PUFA ratio by fat-1 are linked to tumour suppressive effects in this and other cancer models (56, 60, 62, 64).

Research conducted in association with the fat-1 transgenic mouse has also served to emphasize the involvement of up-regulated apoptotic signalling networks in n-3 PUFA mediated chemoprevention. PTEN is a tumour suppressor which functions to down-regulate activation of the PI3K/Akt survival pathway, ultimately reducing the Akt mediated post-transcriptional phosphorylation and inactivation of Bad, a pro-apoptotic
factor (66). Indeed, expression of the PTEN was demonstrated to be significantly up-regulated in melanoma bearing fat-1 transgenic mice, a finding which, coincident with in vitro results, is suggested to involve a PGE3 dependent mechanism (59). Evidence from fat-1 transgenic mice also supports a role of tissue n-3 PUFA enrichment in enhancing tumour cell apoptosis through PTEN independent mechanisms. When fat-1 transgenic mice were crossed with prostate specific PTEN knockout mice, a spontaneous prostate cancer model, fat-1 expressing mice experienced a ~4.5 fold reduction in prostate n-6/n-3 PUFA ratio and a significant attenuation in tumour burden (61). Moreover, this fat-1 associated blunting of prostate tumour growth was comparable to the tumour differential observed when prostate specific PTEN knock-out mice, not expressing fat-1, were maintained on either n-6 PUFA (n-6/n-3=40) or n-3 PUFA (n-6/n-3=1) enriched diets (61). In this dietary arm it was also noted that the n-3 PUFA diet group experienced significant reductions in the level of phosphorylated Bad and increased apoptosis in prostate tissue, however such apoptotic outcomes were unfortunately not reported in combination with the fat-1 transgenic mouse model (61).

Syndecan-1 is a transmembrane heparan sulfate proteoglycan, which has been shown to be up-regulated, through peroxisome proliferator-activated receptor-γ dependent mechanisms, following DHA treatment in cultured human prostate and breast cancer cells where it has been demonstrated to promote apoptosis and therefore may represent a novel mechanism whereby tissue n-3 PUFA status can modulate tumour growth (67-69). Syndecan-1 is up-regulated within the prostate tissue of PTEN knockout mice fed a high n-3 PUFA diet (68). In parallel, relative to their WT controls the mammary glands of fat-1 transgenic mice are also enriched in syndecan-1 (70). While
many aspects of the n-3 PUFA-syndecan-1 association remain yet to be elucidated *in vivo*, particularly as it pertains to tumour related outcomes, syndecan-1 dependent down regulation of phosphorylated MEK/Erk and Bad have been identified as candidate pathway(s) through which n-3 PUFA may promote apoptosis in the fat-1 transgenic mouse model (71).

Recently, evidence from our lab has demonstrated that lifelong enrichment of n-3 PUFA within the mammary gland, either through endogenous synthesis (fat-1 mediated) or diet (3% fish-oil feeding), is associated with reductions in tumour volume and multiplicity in MMTV-neu(ndl)-YD5 mice, a model for the study of human epidermal growth factor receptor 2-positive breast cancers (65). Although the mechanisms underlying these chemoprotective effects remain yet to be elucidated it is possible that modified syndecan-1 expression could be involved. Additionally, or perhaps alternatively, n-3 PUFA mediated modulation of mammary gland development may also underlie these effects (72). The mammary gland develops through a dynamic process which extends throughout life, with critical windows occurring *in utero*, pre-puberty/puberty and pregnancy, and involves a similar set of morphological and functional aspects between rodents and humans (73, 74). Pubertal onset is associated with the development of highly proliferative structures, referred to as terminal end buds, which function to rapidly drive ductal elongation and branching throughout the mammary fat pad (73, 74). Given their highly proliferative nature terminal end buds have been identified as primary sites of neoplastic transformation within the rodent mammary gland (75). By contrast, pregnancy/lactation induced mammary gland differentiation is protective against lifetime risk of breast cancer (76). Evidence indicates that early life
exposure to n-6 PUFA and n-3 PUFA can differentially modulate breast cancer susceptibility through mechanisms which may involve alteration of the structural and functional organization within the developing mammary gland in favour of a less highly proliferative state (77, 78). In support of this contention, Lui et al. have recently shown that in addition to a significant enrichment in n-3 PUFA content, particularly 22:5n-3 and DHA, the mammary gland of virgin fat-1 transgenic mice developed a lobulo-alveolar like branching morphology similar to that induced by pregnancy (79).
<table>
<thead>
<tr>
<th>Cancer</th>
<th>Model</th>
<th>fat-1 Phenotype</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
<td>Injected</td>
<td>↓ tumour incidence and growth rate, ↑ PGE₃, ↓ PGE₂, ↑ PTEN expression (tumour suppressor gene)</td>
<td>(59)</td>
</tr>
<tr>
<td>Colitis-associated colon cancer</td>
<td>Chemical</td>
<td>↓ tumour incidence, number and volume, ↓ NF-κB activity and target gene expression (iNOS), ↑ anti-proliferative gene expression (TGF-β)</td>
<td>(60)</td>
</tr>
<tr>
<td></td>
<td>Chemical</td>
<td>↓ tumour incidence and number, ↑ colonic apoptosis, ↓ n-6 PUFA derived eicosanoids, ↑ PGE₃</td>
<td>(56)</td>
</tr>
<tr>
<td>Prostate</td>
<td>Genetic</td>
<td>↓ total average tumour volume</td>
<td>(61)</td>
</tr>
<tr>
<td></td>
<td>Injected</td>
<td>↓ tumour size and volume 4 days post-inoculation, ~100% palpable tumour regression by 12 days</td>
<td>(63)</td>
</tr>
<tr>
<td></td>
<td>Genetic</td>
<td>Blocked hepatic neoplasia in 92% of mice, ↓ hepatic NF-κB levels, Modified expression of inflammatory and cell-cycle regulatory gene networks (microarray)</td>
<td>(62)</td>
</tr>
<tr>
<td></td>
<td>Chemical</td>
<td>↓ tumour number and volume, ↓ liver injury, ↓ plasma TNF-α (systemic inflammation), ↓ hepatic cox-2 expression, ↑ resolvin and protectin precursors</td>
<td>(64)</td>
</tr>
<tr>
<td>Mammary</td>
<td>Genetic</td>
<td>↓ tumour volume and multiplicity, Results comparable dietary arm with 3% fish-oil</td>
<td>(65)</td>
</tr>
</tbody>
</table>

Table 2.2: Applications of fat-1 transgenic mice in models of cancer. ¹in relation to WT control mice
2.3.3 Neurological Health

The fat-1 transgenic mouse model has also been privy to several investigations concerned with the role of n-3 PUFA enrichment, namely DHA, in aspects of neurological function and disease. Gene and protein microarray analysis of brain tissue from fat-1 transgenic mice and their WT controls has revealed that signalling networks implicated in inflammation, apoptosis, neurotransmission, neuronal growth and synapse formation are differentially modified in the presence of the fat-1 transgene (80). In support of such associations it was found that, relative to their WT n-6 PUFA rich controls, 18-week old fat-1 transgenic mice exhibit elevated levels of hippocampal DHA and 22:5n-3, enhanced neurogenesis and neuritogenesis and improved spatial learning performance in the Morris Water Maze test (81). Additionally, another study showed that following 12 weeks of 10% safflower oil feeding fat-1 transgenic mice had a markedly different PUFA composition within cortical phospholipids, which included a 220% increase in DHA, relative to controls (82). Moreover, cortical COX-2 protein levels were significantly reduced in the presence of the fat-1 transgene (82). Collectively these findings reiterate that under “healthy” conditions, n-3 PUFA enrichment differentially impacts numerous aspects of neurophysiology which might have profound functional implications on the balance between health and disease, particularly as it pertains to aspects of age-associated cognitive decline and neuro-inflammation.

To more specifically elucidate the role of n-3 PUFA in the prevention of various forms of neuropathology the fat-1 transgenic mouse has been combined with select disease models. Neuro-protective effects of fat-1 mediated n-3 PUFA enrichment have recently been described in combination with models of epilepsy (83), Alzheimer’s...
disease (84) and Parkinson’s disease (85). However, despite marked increases in retinal DHA content, presence of the fat-1 transgene was found to be neither of detriment nor benefit to retinal function, photoreceptor cell loss or rhodopsin levels in two separate transgenic models of heritable retinal degeneration (86, 87). Similarly these studies showed that fat-1 mediated n-3 PUFA enrichment was also of no consequence to biomarkers of retinal function, under non-pathological conditions (86, 87). Still others have reported that fat-1 mediated elevation in retinal DHA is linked to exacerbated susceptibility to light induced stress (88) and enhanced peroxidation induced retinal protein modifications (88, 89), however the functional consequences of these findings remain to be determined.

It should be noted that some degree of discrepancy in the ability of the fat-1 transgenic mouse to facilitate conversion of n-3 to n-6 PUFA within brain tissue under different nutritional settings has been noted (48, 80, 85). Therefore, future applications of the fat-1 transgenic mouse model for the study of neurological health would likely benefit from detailed characterization of brain PUFA composition using dietary dose response experiments.

2.3.4 Other applications of the fat-1 transgenic mouse

Amongst other applications, the fat-1 transgenic mouse has recently offered insights into protective effects of lifelong endogenous n-3 PUFA enrichment on parameters of skeletal, cardiovascular and metabolic health. Fat-1 transgenic mice displayed significant reductions in ovariectomy induced bone loss within the femur, tibia and vertebrae relative to their WT controls (90). Additionally, work focused on
characterization of fat-1 induced changes in fatty acid composition within the vertebrae and femur has revealed a significant positive correlation between major long-chain n-3 PUFA species and bone mineral density and biomechanical strength in healthy young adult mice (44, 91, 92). However, the relative contribution of these early life associations between n-3 PUFA and bone mineralization in the prevention of later life bone loss have yet to be fully established. Presence of the fat-1 transgene has also been associated with reduced inflammation and atherogenesis in apoE knockout mice (93), attenuated severity of high fat diet induced inflammation, insulin resistance and non-alcoholic fatty liver disease (94, 95), improved glucose tolerance (96) as well as protection against STZ-induced diabetes (97).

2.4 Fat-1 Transgenic Mice: Strengths and Limitations

The fat-1 transgenic mouse model was developed by Kang et al. using a classical pronuclear injection strategy (41). For this, a mammalian optimized version of the fat-1 n-3 desaturase gene coupled to a chicken β-actin promoter was introduced into fertilized mouse oocytes from which a transgenic line showing a widespread, heritable, pattern of endogenous tissue n-3 PUFA enrichment was identified and so coined the “fat-1 transgenic mouse” (41). Expression of the fat-1 transgene within this mouse line is reported to begin as early as the embryo stage of development and proceeds throughout life (43). The fat-1 transgenic mouse model therefore has valuable application in PUFA related research because it is conducive to investigating the protective effects of tissue n-3 PUFA enrichment without a requirement for dietary manipulation between experimental groups, thus streamlining experimental design while simultaneously
controlling for diet based variability (43). However, since the timing of transgene activation is fixed, results obtained through this transgenic approach to tissue n-3 PUFA enrichment are ultimately restricted to interpretation within the context of lifelong n-3 PUFA exposure.

It is becoming increasingly acknowledged that early life nutritional exposures may significantly influence later life disease risk and severity (98, 99). For example, it has been demonstrated that discrete in utero exposure to a nutritionally high n-6 PUFA environment increases terminal end bud number within the mammary gland as well as the incidence of carcinogen induced mammary tumourigenesis in female rodents (77, 100). Conversely, in utero and early post-natal exposures to n-3 PUFA have demonstrated some benefits in reducing the incidence of later life mammary tumourigenesis (78, 100, 101). Similarly the in utero and early-post natal period has been suggested to represent sensitive windows through which PUFA compositional exposures may influence later life risk of allergic disease (102) as well as parameters of bone strength/development (103). As such it is possible that n-3 PUFA exposure during these early periods of development may greatly underscore any given phenotype observed using the fat-1 transgenic mouse. Therefore while the fat-1 transgenic mouse represents a highly useful model for studying the prophylactic effects of lifelong n-3 PUFA enrichment, hypotheses concerned with the more dynamic intricacies of n-3 PUFA enrichment and the n-6/n-3 PUFA ratio on health and disease related outcomes remain best addressed through nutritional manipulation approaches.
Chapter Three

LITERATURE REVIEW

STRATEGIES FOR INDUCIBLE TRANSGENE ACTIVATION
USING Cre-loxP TECHNOLOGY
3.1 Introduction

In the early 1980’s several preliminary reports documenting the production of transgenic mouse lines harbouring, expressing and heritably transmitting heterologous DNA sequences were published (104-108). The functional significance of these early milestones in mammalian genomic engineering was immediately clear; the capacity to selectively modify the mouse genome would provide a gateway to explore and integrate previously unattainable facets of genetics, molecular biology, nutrition and medicine. Over the past three decades the tools and techniques in animal transgenesis have rapidly evolved (109). One such advancement that has proven particularly instrumental is Cre-loxP technology.

Cre is a 38 kDa recombinase enzyme from bacteriophage P1, which when introduced into mammalian cells retains its capacity for catalyzing site-specific recombination between 34 base pair loxP (locus for X-over; P1) consensus sequences, thus presenting a powerful tool for in vivo somatic mutagenesis (110). Through the selective positioning of loxP sites it is possible to produce a variety of Cre-mediated genomic manipulations in vivo, however most frequently investigators choose to orient the consensus sequences in such a manner that the DNA segment between them is permanently deleted from the genome (111). In addition to the more intuitive application in loss-of-function studies (conditional knock-out), the excisive properties of Cre-loxP have also been exploited to attain conditional control over the activation of transgenes (112). This can be achieved by positioning of a loxP flanked (floxed) STOP cassette between cDNA, coding for the protein of interest, and its upstream promoter (112). Under basal conditions the floxed STOP cassette presents a transcriptional/translation
barrier to cDNA expression. But, in the presence of Cre the floxed STOP cassette is physically removed from the genome through excisive recombination resulting in irreversible activation of cDNA expression. Moreover, the advent of systems which superimpose ligand inducible constraints over the timing of Cre-mediated recombination has provided a means to exogenously control the timing of Cre-mediated transgene activation.

Presently two major strategies for ligand regulated control over Cre-mediated recombination exist: (1) tamoxifen inducible Cre and (2) tetracycline inducible Cre. This short review will consider the basic technology underlying each of these strategies as well as the inherent strengths and limitations governing the use of ligand regulated recombinases.

### 3.2 Tamoxifen Inducible Cre

To date, numerous variants of the tamoxifen inducible Cre-loxP system have been developed (113-116). However they all take advantage of the same basic strategy, generation of a fusion peptide in which Cre is linked to a mutated version of the ligand binding domain of the estrogen receptor (117). The resulting Cre-ER fusion peptide is rendered insensitive to endogenous estrogen and therefore in the absence of ligand remains sequestered in the cytoplasm bound to heat shock protein 90 (Hsp90) (118). When tamoxifen, or its metabolic product 4-hydroxytamoxifen (4-OHT), is administered, ligand binding liberates Cre-ER thereby allowing nuclear translocation and subsequent Cre mediated recombination at floxed genomic regions. Following cessation of
tamoxifen treatment Cre-ER is exported from the nucleus and once again sequestered in
the cytoplasm.

3.2.2 Tetracycline Inducible Cre

The tetracycline inducible reversible switch system described by Gossen and
Bujard takes advantage of critical components of the transposon Tn10 Tet-resistance
operon of *Escherichia coli*, the tetracycline repressor (TetR) and its specific binding
affinity for tetO operator sequences (119, 120). When adapted for Cre-loxP technology
three key transgenic components are required; (a) a tetracycline-controlled transcriptional
activator protein (adapted from TetR) under the control of a ubiquitous or tissue specific
promoter; (b) Cre linked to a tetracycline response element (TRE) promoter which is
typically composed of 7 identical tetO operator sequences in tandem array upstream of a
constitutive CMV minimal promoter and (c) a floxed transgene of interest (121). Using
this system, the timing of Cre expression, and associated floxed transgene activation, can
be controlled at the transcriptional level through administration of tetracycline or, more
commonly, its derivative doxycycline and cessation of drug administration causes the
reversal of this effect.

Both Tet-ON and Tet-OFF versions of the tetracycline inducible system have
been described. In the Tet-OFF version, transgene expression is regulated by the
tetracycline responsive transactivator protein (tTA) which consists of tetR fused to the C-
terminal domain of herpes simplex virus transcriptional activator protein VP16 (119).
This modification converts tetR from a repressor to a transcriptional activator (119).
Therefore, in the Tet-OFF system Dox represses Cre expression by binding to tTA and preventing it from interacting with the TRE. Conversely, in the Tet-ON system a reverse tetracycline transactivator protein (rtTA), or a variant thereof, activates expression from TRE promoters only in the presence of tetracycline/doxycycline (120, 122). Although both Tet-OFF and Tet-ON systems have been used for the generation of tetracycline/doxycycline inducible Cre-loxP mice, the reversed pharmacology of the Tet-ON approach is generally deemed preferential since only an acute bout of drug treatment is required.

3.4. General Strengths and Limitations of Inducible Cre Systems

Ligand inducible Cre recombinases offer the obvious advantage of temporal freedom over the timing of floxed transgene activation. Although relatively rare, toxic effects of Cre arising from illegitimate recombination at “cryptic” loxP sites within the mammalian genome have been reported (123-125). Therefore, ligand-inducible Cre recombinases are also advantageous because they provide a means of conditionally regulating genomic exposure to Cre, thus reducing the probability of Cre-toxicity, though not completely eliminating it (126).

Inducible Cre systems can be subject to complexity arising from incomplete efficiency of recombination and/or ligand-independent expression of Cre resulting in respective issues of tissue mosaicism and background “leaky” excision of floxed alleles. To this end, the application of well-characterized Cre-reporter lines is useful in validating the spatiotemporal specificity and tight ligand-dependency of inducible Cre
expression systems (127). However data on recombination efficiency gleaned from crossing a given Cre-expression line with a Cre-reporter strain must be cautiously interpreted as it is often not directly translatable to that achievable with an alternative floxed transgene (128). Amongst other factors, the genomic environment encompassing a floxed locus can significantly impede the accessibility of Cre to loxP sites (128). As such, independent Cre-loxP crosses require individual authentication of recombination characteristics and often ligand treatment tailoring (129).

Currently the use of tamoxifen-inducible systems far exceeds the tetracycline-inducible systems for site specific recombination. One key factor underpinning this difference is the complexity of the breeding strategy involved in the development of each type of ligand-inducible system. Since ligand sensitivity and recombinase activity are contained within a single fusion protein, one less round of breeding is required in the generation of tamoxifen inducible Cre-loxP mice than their tetracycline inducible counter parts, which typically necessitate a triple transgenic approach. In an attempt to streamline this breeding issue, bi-cistronic vectors possessing both rtTA as well as a TRE promoter driving Cre expression within a single core transgenic construct have been developed, however reports of leaky tetracycline/doxycycline independent Cre expression in vivo have limited their success (130, 131). Moreover, since Cre-ER only requires a shift in intracellular localization to facilitate Cre-mediated recombination the kinetics of excisive recombination events may be faster with tamoxifen based systems. However, many details regarding the time required for maximal recombination efficiency still remain unclear (132).
Ligand appropriateness is also an important consideration. Although Cre-ER fusion proteins are modified such that they do not bind endogenous estrogen, the reciprocal is not true of tamoxifen and the endogenous estrogen receptor. Tamoxifen is a competitive antagonist of the estrogen receptor with application in breast cancer therapy (133). Therefore, even though tamoxifen administration is acute, tamoxifen-inducible Cre-loxP experiments interested in estrogen sensitive outcomes such as mammary gland development/tumourigenesis and reproduction should be interpreted with extreme caution (118). Additionally, issues of transient infertility and impaired gastrointestinal function in tamoxifen inducible Cre mice have also been reported (134). By comparison, within the dose range required for induction in tetracycline/doxycycline systems, ligand toxicity does not generally appear to be an issue (121).

3.4 Conclusion

Ligand inducible Cre-recombinases are powerful tools which can be used to achieve temporal control over the activation of a desired transgene. Although the development and use of these model systems can be very demanding, careful consideration of the strengths and caveats associated with such systems prior to study onset can help ensure appropriate model selection, accurate interpretation of results and optimization of time and resources.
Chapter Four

RATIONALE, OBJECTIVES, HYPOTHESIS and
EXPERIMENTAL DESIGN
4.1 Rationale

Dietary n-3 PUFA, particularly fish-oil derived EPA and DHA, are increasingly acknowledged as potentially powerful modifiers of human health and disease. Despite this, research efforts aimed at elucidating the role of dietary n-3 PUFA for the prevention and treatment of chronic human pathology have rendered largely inconsistent results. Based on their unique method of endogenous tissue n-3 PUFA accretion, fat-1 transgenic mice have emerged as a well-controlled model with utility in clarifying the biological effects of tissue n-3 PUFA status and mechanisms by which numerous facets of health including inflammation, cancer and neuropathology are affected (43). In this model, expression of the fat-1 transgene appears to be constitutive, encompassing all stages of the life-cycle and windows of development (41). However, timing of exposure may be a critical determinant of the relative impact of n-3 PUFA on disease risk and treatment efficacy. As such, the identification of sensitive biological windows and/or tissue specific mechanisms by which n-3 PUFA elicit health related benefits may be of enhanced therapeutic relevance. To this end, the conditional iFat-1 transgenic mouse model has been developed. The iFat-1 transgene has been engineered to contain a loxP flanked STOP cassette between the fat-1 coding DNA sequence and its ubiquitous CAG promoter. By virtue of transgene design, the iFat-1 transgenic mouse is anticipated to provide fundamental insight into the temporal-specific mechanisms by which tissue n-3 PUFA status regulates a diverse array of health and disease related outcomes.
4.2 Objectives

The objectives of this thesis work were to:

1. Employ an \textit{in vitro} approach to demonstrate that the iFat-1 transgene is capable of stringent Cre-inducible activation of \textit{fat-1} expression and associated changes in membrane fatty acid composition.

2. Use a comparable \textit{in vivo} approach to characterize the functional utility of iFat-1 transgenic mouse as a model of Cre-inducible n-3 PUFA enrichment.

4.3 Hypothesis

The overall hypothesis of this thesis work is that, the presence of Cre will lead to specific activation of iFat-1 expression and associated changes in membrane phospholipid fatty acid profile. Additionally, it is anticipated that Cre-mediated changes in the ratio of n-6/n-3 PUFA will be consistent with those achievable with the constitutive fat-1 transgene \textit{in vivo}. 
4.4 Experimental Design

*In vitro* validation of the iFat-1 transgene was carried out using the human embryonic kidney (HEK) 293T cell line. Experimental plasmids for co-transfection experiments consisted of (a) an expression vector containing the iFat-1 transgenic construct (DMA1-express), (b) a plasmid capable of constitutive expression of Cre (pCAG-Cre) and (c) the backbone vector of pCAG-Cre as negative control (Figure 3.1). Inducibility of the iFat-1 transgene was assessed based on transcriptional expression profile and changes in membrane fatty acid composition in the presence and absence of Cre. Fatty acid composition was determined by gas liquid chromatography.

To investigate *in vivo* inducible expression of the iFat-1 transgene, R26-Cre-ER\textsuperscript{T2} (Tam-Cre) male mice were crossed with iFat-1 female mice. Resultant experimental progeny were one of four potential genotypes: WT, iFat-1, Tam-Cre and TamCre/iFat-1. Mice possessing the Tam-Cre transgene carry a modified version of Cre which renders its activation dependent on administration of the drug tamoxifen (135). Therefore, double hybrid TamCre/iFat-1 progeny derived from experimental breeding should be capable of, body-wide, tamoxifen inducible iFat-1 activation and associated changes in membrane PUFA composition. Commencing at 6-7 weeks of age, TamCre/iFat-1 transgenic mice were treated with a five day course of tamoxifen or vehicle control. In order to investigate potential background “leaky” expression characteristics of the iFat-1 transgene, untreated WT and iFat-1 mice were used. All mice were euthanized at 9-10 weeks of age and liver, kidney, muscle and brain tissue collected for subsequent transcriptional (liver) and fatty acid compositional analysis by RT-PCR and gas liquid chromatography respectively (Figure 3.1). Tissues from age matched C57BL/6 mice
expressing the constitutive fat-1 transgene were included in analysis as reference positive controls for fat-1 expression where appropriate.

**Figure 3.1** - Experimental design/groups used for *in vitro* (upper panel) and *in vivo* (lower panel) validation of the iFat-1 transgene. ¹ Included control for fatty acid compositional analysis only.
Chapter Five

THE iFat-1 TRANSGENE PERMITS CONDITIONAL ENDOGENOUS n-3 PUFA ENRICHMENT BOTH IN VITRO AND IN VIVO
5.1 Abstract

Fat-1 transgenic mice, which convert n-6 polyunsaturated (PUFA) to n-3 PUFA, are an extensively used tool in nutritional research. However, due to the constitutive nature of transgene expression, this model cannot be used to directly investigate the impact of timing of n-3 PUFA exposure on health related outcomes. To circumvent this issue the Cre-inducible fat-1 (iFat-1) transgenic mouse has been developed. The aim of this study was to characterize the functional utility of the iFat-1 transgene as a model of Cre-inducible endogenous n-3 PUFA enrichment. Cre-independent expression of fat-1 associated mRNA transcripts was detected both in vitro and in vivo. However, changes in membrane PUFA composition, a functional measure of FAT-1 enzyme activity, remained reliant upon Cre as an activating factor signifying that background transgene expression is non-functional. Co-transfection of human embryonic kidney (HEK) 293T cells with iFat-1 and a Cre expression plasmid permitted a near balancing of the n-6/n-3 PUFA ratio within major phospholipid fractions (p<0.01). Similarly, 9-10 week old Tam-Cre/iFat-1 double hybrid mice transiently treated with tamoxifen at 6-7 weeks of age experienced an approximate 2-fold, or more, reduction (p<0.05) in the n-6/n-3 PUFA ratio of liver, kidney and muscle phospholipids relative to vehicle treated controls. However, these in vivo effects were considerably blunted in relation to age-matched constitutive fat-1 reference controls, a finding which may be partially attributed to the difference in duration of fat-1 gene expression. Collectively these findings suggest that the iFat-1 transgene/transgenic mouse may be a promising tool to better understand the temporal effects through which n-3 PUFA impact health related outcomes, however further model characterization is first necessary.
5.2 Introduction

In recent years, dietary n-3 PUFA, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have garnered strong research and public interest based on their potentially pleiotropic health related benefits (8, 136). However, while various lines of evidence support the involvement of dietary n-3 PUFA in the prevention and treatment of prevalent lifestyle related pathologies such as cancer, cardiovascular disease and insulin resistance, still others have reported no, or even inverse, associations (8). As such, clarification of the role of n-3 PUFA in health and disease is greatly needed.

In contrast to unmodified mammalian systems, fat-1 transgenic mice are able to directly convert n-6 PUFA to corresponding n-3 PUFA through constitutive expression of the fat-1 transgene, an n-3 desaturase native to the roundworm Caenorhabditis elegans (C. elegans) (5). To date, this transgenic mouse model has assisted in providing enhanced insight into the protective effects of lifelong endogenous tissue n-3 PUFA enrichment on numerous health related outcomes including; cancer chemoprevention (56, 59-65), inflammatory/immune pathology (42, 46, 51-55), neurological disease (83, 84), bone strength and development (44, 90-92), cardiovascular disease and metabolic health (93-96). However, while this well-controlled model is able to efficiently evade the diet-based confounding variability inherent to traditional PUFA feeding approaches (43), it is not able to control the timing of tissue n-3 PUFA enrichment; a factor increasingly proposed to have potential bearing on the relative therapeutic efficacy ascribed to dietary n-3 PUFA (99, 102, 103). As such, the ability to more clearly define the full temporal spectrum through which n-3 PUFA can elicit health related benefits may assist in better refining the potential therapeutic applicability of this highly bioactive class of fatty acids.
The bacteriophage P1 derived Cre-loxP recombinase system is a commonly employed approach to achieve conditional mutagenesis within mammalian cells. This technology is based on the specific affinity of Cre for site specific recombination between loxP consensus sequences producing irreversible genomic excision of intervening DNA sequence (111). Through selective inclusion of a loxP flanked (floxed) regulatory STOP element between a transgene and its driving promoter, Cre-inducible control over transgene activation can be successfully achieved (137). Moreover, since the Cre-loxP recombination system require, at minimum, two independent genetic components to produce conditionality, floxed transgenic mice harbour a more versatile research potential than that which is achievable with more classical transgenic approaches. While alternative murine models with both broad (138) and tissue specific (139, 140) patterns of heterologous C. elegans fat-1 gene expression have been reported, a model capable of Cre-inducible fat-1 expression has yet to be described. To this end, the Cre-inducible fat-1 (iFat-1) mouse has been developed.

This study was designed to evaluate the utility the iFat-1 transgene as a model of Cre-inducible fat-1 expression. Function of the iFat-1 transgene was screened in vitro using co-transfection experiments in the HEK 293T cell line. For in vivo characterization of the R26-Cre-ERT² (Tam-Cre) mouse line was selected (135). Tam-Cre mice ubiquitously express a human estrogen receptor-Cre fusion protein which is reliant on administration of the drug tamoxifen for nuclear translocation and subsequent Cre-mediated recombination (135). Using these complementary in vitro and in vivo approaches we describe, for the first time, a novel transgenic approach for Cre-inducible endogenous n-3 PUFA enrichment.
5.3 Materials and Methods

5.3.1 Transgenic Construct and iFat-1 Model Development

The iFat-1 transgenic construct and mouse model were commercially developed (GenOway, Lyon, France). The fat-1 cDNA, codon optimized for efficient mammalian expression, required for this work was generously donated by Dr. Jing Kang (Massachusetts General Hospital/Harvard Medical School). The iFat-1 transgene was designed to consist of a loxP flanked STOP element positioned between the fat-1 coding DNA sequence and its upstream CAG promoter, a chicken β-actin/CMV immediate early enhancer fusion promoter which has been demonstrated to drive a strong ubiquitous pattern of transgene expression (141) (Figure 5.1). The loxP flanked regulatory STOP sequence contains a C-terminal portion of the yeast HIS3 gene and an SV40 polyadenylation signal, which is intended to effectively prevent CAG driven transcription from extending beyond the STOP cassette (112). Moreover, a downstream translation block, consisting of a synthetic nucleotide with false translational initiation codon (ATG) and 5’ splice donor site has also been included within the STOP cassette as a second-line of defense against uncontrolled transgene activation (112). Therefore, the iFat-1 transgenic construct should permit stringent Cre-inducible control over transgene activation and associated endogenous n-3 PUFA biosynthesis.

A validated Quick Knock-in™ approach was used to introduce a single copy of the iFat-1 transgenic cassette into the hydroxanthine phosphoribosyltransferase (Hprt) locus of the X-chromosome through homologous recombination in E14Tg2a embryonic stem cells, a derivative of the 129P2/OlaHsd E14 embryonic stem cell line. The Hprt
gene codes for a housekeeping protein integral to the Salvage Pathway of nucleotide synthesis, an enzymatic cascade which is reliant on the recycled degradation products of nucleotide metabolism as substrate in the synthesis of purine nucleotides. In E14Tg2a embryonic stem cells a 35 kb portion of the $Hprt$ locus encompassing the promoter and first 2 exons has been deleted rendering this cell line solely dependent on the de-novo pathway for nucleotide synthesis for survival (142). The iFat-1 transgenic cassette was designed to simultaneously restore $Hprt$ gene function, through introduction of human equivalents of the missing gene region, and by inserting the iFat-1 transgene immediately upstream of this locus (Figure 5.2). Correctly targeted E14Tg2a clones were therefore positively selected based on resistance to Hypoxanthine, Aminopterin and Thymidine (HAT) medium, which effectively blocks the de-novo pathway of nucleotide synthesis. Targeted transgenesis through restoration of Hprt function in E14Tg2a and E14Tg2a-derivatives is a commonly used approach for the generation of transgenic mouse lines (143-150).

The presence of successful recombination events in HAT resistant E14Tg2a clones was validated by southern blot analysis prior to generation of male chimeras through C57BL/6J blastocyst injection. Highly chimeric males were bred with C57BL/6J females to generate F1 progeny. Germ-line transmission of the iFat-1 transgene to F1 progeny was confirmed by polymerase chain reaction and southern blot analysis. The resultant iFat-1 F1 female heterozygous progeny were subsequently transferred from GenOway to the University of Guelph, at which point a breeding colony of heterozygous females was established. Since the iFat-1 transgene is X-linked, males cannot inherit this transgene from paternal origins. The iFat-1 breeding colony was established and is
maintained through backcrossing of iFat-1 heterozygous females with WT C57BL/6N males (Charles River). This strategy ensures that a subset of females and males within every generation inherits the iFat-1 transgene.

**Figure 5.1:** Schematic representation of the iFat-1 transgene. The iFat-1 transgenic construct consists of a loxP flanked STOP cassette positioned between the codon optimized *fat-1* coding cDNA and upstream ubiquitous CAG promoter. The STOP cassette contains a C-terminal portion of the yeast *His3* gene, an SV40 polyadenylation signal, a false ATG and a 5’ splice donor site (112). This transcriptional regulatory mechanism, with back-up translational block, has been demonstrated to prevent functional activation of downstream transgenes with 100% efficiency in the absence of Cre recombinase (112).
Figure 5.2: Construct map of DMA1-HR the iFat-1 targeting vector (as provided by manufacturer, GenOway). Major structural features of the iFat-1 transgene including floxed STOP cassette and fat-1 coding DNA sequences (cds) have been highlighted. A single copy of the iFat-1 transgene was specifically targeted to the Hprt locus of the X-chromosome using GenOway’s validated Quick Knock-in™ approach.
5.3.2 Plasmid Constructs

The DMA1-express plasmid (9771bp), a conditional expression vector containing the iFat-1 transgenic construct, was obtained from GenOway (Lyon, France) (Figure 5.3). All other plasmids were obtained through the Addgene plasmid repository, deposited by Dr. Connie Cepko (Harvard Medical School). pCAG-Cre (5871bp) is a plasmid in which the Cre expression is under direct control of the constitutively expressed CAG promoter (Addgene plasmid 13775) (151). The backbone vector of pCAG-Cre, pCAGEN (4798bp), was used as a negative control (Addgene plasmid 11160) (152). pCALNL-GFP (6844bp), a green florescence Cre-reporter plasmid was also utilized for initial protocol work-up (Addgene plasmid 13770) (151).

Upon receipt, all experimental plasmids were immediately isolated, amplified and purified for later use. For bacterial transformation with the DMA1-express plasmid, 1 µL of plasmid DNA was added to 100 µL E. coli DH5α. The mixture was incubated on ice for 30 minutes, transferred to a 42°C water bath for 1 minute, then immediately returned to ice and serial diluted to a concentration of 1:1000 in LB broth (Sigma L2542). pCAG-Cre, pCAGEN and pCALNL-GFP plasmids were received as pre-transformed DH5α bacterial stab cultures (Addgene). For each plasmid, bacterial colonies were isolated by streaking on LB agar plates coated with 100 µg/mL ampicillin and incubating overnight at 37°C. A single transformed colony was isolated and subsequently amplified by gently agitation overnight at 37°C in 500 µL LB broth containing 100 µg/mL ampicillin. All plasmids were purified using Wizard® Plus Maxipreps DNA Purification System (Promega, A7270). Purified plasmid DNA was eluted in 1.5 mL DEPC treated water and
quantified via spectrophotometry (ThermoScientific NanoDrop 2000). Purity of experimental plasmids was also assessed via restriction enzyme digest.

Figure 5.3: Construct map of DMA1-express plasmid (as provided by manufacturer, GenOway). Major structural features of the iFat-1 transgene including floxed STOP cassette and fat-1 coding DNA sequences (cds) have been highlighted. The DMA1-express plasmid, containing the iFat-1 transgene, was transfected into HEK 293T cells in the presence and absence of Cre.
5.3.3 Cell Culture, Co-transfection and Fatty Acid Treatment

Transcriptional and functional expression characteristics of the iFat-1 transgene were investigated \textit{in vitro} via co-transfection experiments employing HEK 293T cells and the transfecting agent polyethyleneimine (PEI) (Sigma Aldrich 408727), both of which were generously provided by Dr. Nina Jones (Department of Molecular and Cellular Biology, University of Guelph). For routine maintenance, HEK 293T cells were grown in a standard culture medium composed of high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) (ThermoScientific SH30022.01), supplemented with 10% fetal bovine serum (Sigma F1051) and 1% penicillin/streptomycin (Sigma A5955). Cells were sub-cultivated at approximately 80% confluence (~twice weekly) using 0.25% trypsin/EDTA (Sigma T4049) to disrupt adherent interactions. For each co-transfection reaction, 750 µL of OptiMEM serum free medium (Invitrogen Gibco® 31985) and 25 µL of 2 mg/mL PEI were added to a total of 12 µg, of equally balanced, plasmid DNA. The transfection solution was briefly vortexed then incubated at room temperature for 5 minutes prior to edge-wise application to cells plated in 10 cm culture dishes (Falcon 353003) containing 10 mL of culture medium.

In order to characterize the transcriptional profile of the iFat-1 transgene in the presence and absence of Cre cells were seeded at an approximate density of 1x10^6 cells per plate in standard culture medium and allowed to adhere overnight. The next day plated cells were co-transfected with either (a) DMA1-express and pCAG-Cre (iFat-1/Cre), for Cre-mediated iFat-1 expression or (b) DMA1-express and pCAGEN (iFat-1/Emp), as a control for Cre-independent iFat-1 expression. Twenty-four hours after
cotransfection cells were lysed and total RNA was harvested for the purpose of reverse transcriptase polymerase chain reaction (RT-PCR), as described in section 5.3.7.

Functional enzyme expression parameters of the iFat-1 transgene were indirectly assessed based on relative fatty acid compositional changes. For this, cells were seeded at a density of approximately 0.5x10⁶ cells per plate. The next day standard culture medium was aspirated and replaced with freshly prepared 100 μM LA supplemented medium, as substrate, for a period of 72 hours. In order to prepare 10 mL of 100 μM LA supplemented medium, 0.1 g of bovine serum albumin (Roche 10735078001) was added to 9.5 mL of DMEM containing 1% penicillin/streptomycin, gently mixed and pre-equilibrated to 37°C in a water-bath. LA (NuCheck Prep, cat# U-59-A), solubilized in anhydrous ethanol, was dried under a gently stream of nitrogen gas. The dried fatty acid was re-suspended in 500 μL of fetal bovine serum and heated at 37°C, with gentle vortexing every 5 minutes to aid in solubilisation. The DMEM and FBS solution were combined, thoroughly mixed by pipetting and directly applied to a 10 cm culture dish containing adherent HEK 293T cells. Twenty four hours following initiation of fatty acid treatment, cells were transfected as previously outlined. Cells were harvested 48 hours post-transfection at which point total cellular lipids were extracted and membrane phospholipid fatty acid composition determined via thin layer chromatography (TLC), as detailed in section 5.3.8.
5.3.4 Experimental Animals and Diet

Male heterozygous Tam-Cre mice were bred with female heterozygous iFat-1 mice, which had been backcrossed onto a C57BL/6 background for 3 generations. Since dosage compensation is achieved in female cells through a process of random X chromosome inactivation early in development, female iFat-1 heterozygous progeny would be expected to display variegated patterns of iFat-1 expression following Cre-mediated activation (153). To avoid this confounding factor, female iFat-1 mice would be required to be bred to homozygosity and therefore only male, hemizygous, progeny were used for the purpose of this study.

Litters were weaned at 3 weeks of age at which point tail biopsies were collected and immediately stored at -20°C for subsequent genotypic characterization. Experimental male progeny identified as either: Tam-Cre\(^{(-/-)}\)/iFat-1\(^{(-)}\) (WT), Tam-Cre\(^{(-/-)}\)/iFat-1\(^{(+)\ (iFat-1\)}}\) and Tam-Cre\(^{(+/+)}\)/iFat-1\(^{(+)\ (Tam-Cre/iFat-1\)}}\) were used for the purpose of this study. All breeders and their progeny were maintained on a specially formulated modified AIN-93G diet containing 10% w/w safflower oil diet (Research Diets, #D04092701), rich in LA (Table 5.1). The fatty acid composition of this experimental diet has been previously verified through in-house gas chromatography analysis (65).

Two study branches were used for in vivo validation of the iFat-1 transgene (refer to Figure 3.1). Potential inherent background expression characteristics of the iFat-1 transgene were addressed using WT and iFat-1 mice. These mice were maintained on the experimental diet throughout the study duration but received no additional treatment. Conversely, the Cre-inducible activation profile of the iFat-1 transgene was considered
using Tam-Cre/iFat-1 double hybrids. Commencing at 6-7 weeks of age, Tam-Cre/iFat-1 double hybrids were treated with either 100 µL of 10 mg/mL tamoxifen free-base (Sigma Aldrich, T-5648) suspended in corn-oil (Sigma C8267) or 100 µL of vehicle control, via one daily intraperitoneal (IP) injection administered for a course of 5 consecutive days.

Mice were housed 1-3/cage in a temperature and humidity controlled room on a diurnal 12 hour light/dark cycle. To prevent potential cross-contamination between treatment groups, mice were separately housed based on treatment strategy (154). All mice were euthanized at 9-10 weeks of age by CO₂ asphyxiation. Kidney, liver, skeletal muscle and brain tissues were immediately harvested, snap frozen in liquid nitrogen and stored at -80°C for later analysis. Where appropriate, corresponding tissues from age matched constitutive fat-1 mice (5), backcrossed on a C57BL/6 background, were included in analysis as positive reference controls of fat-1 expression. The animal utilization protocol for this study was approved by, and conducted in accordance with, the University of Guelph’s Animal Care Committee (protocol 12G002).
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<tr>
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<table>
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<tr>
<th>Ingredients</th>
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<tr>
<td>t-Butylhydroquinone</td>
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</tr>
<tr>
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<table>
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</tr>
<tr>
<td>15:0</td>
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</tr>
<tr>
<td>16:0</td>
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<tr>
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<tr>
<td>16:2</td>
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<tr>
<td>16:3</td>
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</tr>
<tr>
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<tr>
<td>18:0</td>
<td>2.3</td>
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</tr>
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</tr>
<tr>
<td>20:1</td>
<td>0.0</td>
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<tr>
<td>20:2</td>
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</tr>
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<tr>
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</tr>
<tr>
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</tr>
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</tr>
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<tr>
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<td>Total</td>
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<tr>
<td>Monounsaturated (%)</td>
<td>12.1</td>
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<td>Polyunsaturated (%)</td>
<td>79.1</td>
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</table>

**Table 5.1:** Composition of 10% w/w safflower oil modified AIN-93G diet (as provided by manufacturer, Research Diets Inc.)
5.3.5 Tamoxifen Preparation

A stock solution of 10 mg/mL tamoxifen free-base solubilized in ethanol/corn-oil was freshly prepared immediately before initiation of tamoxifen treatment and stored, protected from light, at 4°C for the duration of the 5 day induction period. Briefly, 100 µL of anhydrous ethanol was added to a 10 mg aliquot of tamoxifen free-base and heated at 55°C on dry heat-block with frequent intermittent vortexing to facilitate tamoxifen dissolution. 900 µL of sterile corn-oil was added and the solution was sonicated to homogeneity (30 seconds). Vehicle control was prepared in an analogous fashion, through omission of tamoxifen from the protocol.

5.3.6 Genotyping by PCR

Tail biopsies were incubated overnight, at 55°C on a dry-heat block, with 35 µL of 10 mg/mL proteinase K (Invitrogen 25530-015) and 500 µL tail lysis buffer consisting of 50 mM Tris, 100 mM NaCl, 1% sodium dodecyl sulphate and 25 mM EDTA. DNA was extracted from digested tail samples by adding 500 µL of buffer saturated phenol (Invitrogen Ultrapure™ 15513-039), vortexing and spinning at 18,800 x g for 10 minutes at 4°C (ThermoScientific Legend Micro 21R Centrifuge). The upper, DNA containing, phase was transferred to a new microcentrifuge tube containing 1 mL of ice-cold 95% ethanol. Samples were gently mixed by inversion and incubated at -20°C for a minimum of 20 minutes before centrifugation at 18,800 x g for 10 minutes at 4°C to precipitate DNA. The supernatant was discarded and DNA was concentrated through sequential washing in ice-cold 70% and 100% ethanol with respective centrifugation for 10 and 4
minutes at 18,800 x g at 4°C. The resulting DNA pellet was thoroughly re-suspended in 30 μL of Tris-EDTA (TE) buffer.

Ambion® DNAZap™ solutions (Invitrogen, AM9890) were used to free all polymerase chain reaction (PCR) preparation equipment and surfaces of contaminating nucleic acid. For each reaction, 0.5 μL of genomic DNA was combined with 24.5 μL of PCR master-mix solution and amplified using an Applied Biosystems Veriti 96 Well Thermal Cycler. To screen samples for the presence of the Tam-Cre transgene, PCR master mix was prepared to a final concentration of 0.2 mM dNTP (ThermoScientific Fermentas R0192), 0.5 μM of each forward and reverse primer and 1X PCR buffer, 2 mM MgCl₂ and 0.625 units Platinum® Taq DNA polymerase (Invitrogen 10966-034). For iFat-1 reactions, PCR master mix was composed of 0.3 mM dNTP, 0.4 μM of each forward and reverse primer and 1X PCR buffer, 1.5 mM MgCl₂ and 1.5 units of Platinum® Taq. All master-mix solutions were made up in UltraPure™ nuclease-free sterile water (Invitrogen 10977-015).

For each sample, 10 μL of final PCR product was mixed with 2 μL of 6X loading dye (ThermoScientific Fermentas R0611), loaded onto a 2% agarose gel containing ethidium bromide (Fisher Scientific BP1302-10) and separated via gel electrophoresis at 110V for 50 minutes. Gels were imaged under UV light using the FluorChem HD2 imaging system and amplicon size estimated relative to a 100-basepair ladder (ThermoScientific Fermentas SM0241). Primer sequences, thermal cycling parameters and expected product sizes are provided in Table 5.2.
5.3.7 RNA Isolation and RT-PCR

All RNA preparation surfaces and equipment were treated with Ambion® RNaseZap® (Invitrogen AM9780) to eliminate potential sample degrading nuclease.

Total RNA was isolated from one 10 cm plate containing co-transfected HEK 293T cells (for in vitro assays) or approximately 30 mg samples of frozen liver tissue (for in vivo assays), as per manufacturer’s instructions, using the RNAqueous®-4PCR kit (Invitrogen AM1914). To remove potential contaminating genomic DNA, RNA was treated at 37°C for 30-60 minutes with 1-3 µL of DNaseI per sample. RNA yield was assessed using a NanoDrop spectrophotometer.

First strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using oligo(dT)$_{12-18}$ and moloney murine leukemia virus reverse transcriptase (Invitrogen 28025-013) according to manufacturer’s instructions. PCR reactions for the amplification of transcripts derived from the expression of iFat-1 or rig/S15 (a housekeeping gene) were prepared by combining 1 µL of cDNA with 24 µL of PCR master mix solution consisting of: 0.3 mM dNTP, 0.4 µM of each forward and reverse primers, 1X PCR buffer, 1.5 mM MgCl$_2$ and 1.5 units of Platinum® Taq. Detailed amplification protocols are provided in Table 5.2. RT-PCR products were separated and visualized by gel electrophoresis as outlined in section 5.3.6.
<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Cycling Protocol</th>
<th>Amplicon Description</th>
</tr>
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<tbody>
<tr>
<td><strong>Genotyping (Section 5.3.6)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-CCTGATCCTGGCAATTTCG-3'(F)</td>
<td>1. 94°C - 180s; 2. 35 cycles: 94°C - 30s, 58°C - 90s, 72°C-60s; 3. 72°C - 300s</td>
<td>Tam-Cre (825bp)</td>
</tr>
<tr>
<td>5'-GGAGCGGGAGAAATGGATATG-3'(WT -R)</td>
<td></td>
<td>WT Rosa26 (650bp)</td>
</tr>
<tr>
<td>5'-AAAGTCGCTCTGAGTTGTTAT-3'(TG-R)</td>
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<tr>
<td>5'-ACGTCAGTAGTCATAGGAACTCGGTGTCG-3'(F)</td>
<td>1. 94°C - 120s; 2. 35 cycles: 94°C - 30s, 55°C - 30s, 68°C - 60s; 3. 68°C - 420s</td>
<td>iFat-1 (325bp)</td>
</tr>
<tr>
<td>5'-CCAACCCGGTGAGTTGTTG-3'(R)</td>
<td></td>
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<td><strong>RT-PCR (Section 5.3.7)</strong></td>
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<tr>
<td>5'-CTGCACCACGCCTTCAACCAACC-3'(F)</td>
<td>1. 95°C - 900s; 2. 35 cycles: 94°C - 20s, 62°C - 40s, 72°C - 60s; 3. 72°C - 600s</td>
<td>iFat-1 mRNA (250bp)</td>
</tr>
<tr>
<td>5'-ACACAGCAGCAGATTCCAGAGATT-3'(R)</td>
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<tr>
<td>5'-TTCCGCAAGTTCACCTACC-3'(F)</td>
<td>1. 95°C-60s; 2. 35 cycles: 94°C-30s, 55°C-30s, 72°C-30s; 3. 72°C-300s</td>
<td>rig/S15 mRNA (361bp)</td>
</tr>
<tr>
<td>5'-CGGGCGCCCATGCTTT-3'(R)</td>
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</table>

**Table 5.2:** Primer sequences, thermal cycling parameters and expected product sizes for PCR and RT-PCR.
Total lipids were extracted from both cell culture and tissues using a modified method of Folch et al. (155). To prepare cells for lipid extraction, LA supplemented culture medium was aspirated and each plate was treated with 1 mL of 0.25% trypsin-EDTA to facilitate lifting. After 2 minutes, at 37°C, trypsinization was halted by addition of 5 mL of DMEM, containing 5% FBS. Residual adherent cells were manually lifted by scraping. Resultant cell suspension were pelleted at 500 x g (ThermoScientific Sorvall RT-1 Centrifuge) for 5 minutes at 4°C in 17x100 mm centrifuge tubes (Falcon 352057). Supernatant was discarded and samples were subsequently rinsed twice in PBS by sequential re-suspension, centrifugation and supernatant disposal. Resulting cell pellets were directly processed for lipid extraction. Samples were suspended in 1 mL of 0.1 M potassium chloride and immediately transferred to pre-chilled acid washed tubes. A 2:1 mixture of chloroform (Fisher Scientific C298-4): methanol (Fisher Scientific A452-4) was freshly prepared and 4 mL was added to each sample. Samples were vortexed for 1 minute, flushed with a gentle stream of nitrogen gas and incubated at 4°C overnight. The following day, samples were centrifuged at 340 x g for 10 minutes at 21°C to separate phases (ThermoScientific Sorvall Legend RT+ Centrifuge). The lipid-containing chloroform layer was transferred into a disposable glass culture tube. Extracted lipids were dried under a gentle stream of nitrogen and reconstituted in 100 µL of chloroform for subsequent fatty acid analysis (Section 5.3.9).

For in vivo analysis, total lipids were extracted from 50-100 mg sections of liver, kidney, muscle and brain tissue. Briefly, tissue samples were homogenized, on ice, in 2.5 mL of 0.1 M potassium chloride (Fisher PowerGen 125) and subsequently prepared for
lipid extraction using equivalent final solvent concentrations and extraction procedures as outlined above. Following centrifugation, the chloroform layer of each sample was transferred to a corresponding pre-weighed acid washed tube, dried under a gentle stream of nitrogen and reconstituted at a concentration of 10 mg/mL in chloroform.

5.3.9 Fatty Acid Analysis

Phospholipid fractions were separated from total lipid extracts by thin layer chromatography (TLC) using 20 cm x 20 cm silica H-plates (VWR 5721-7). Plates were activated by heating at 100°C for 1 hour, scored with 2 cm lanes and spotted with 100 µL of lipid extract per sample. Plates were transferred into TLC tanks containing a freshly prepared developing solvent composed of 30 mL chloroform, 25 mL 2-propanol (Fisher A416-1), 18 mL triethylamine (Sigma CT0886), 9 mL methanol and 6 mL 0.25% (w/v) potassium chloride. When the solvent front reached a position 2-3 cm away from the top edge of the plate (~3 hours), plates were removed from their tanks and lightly sprayed with 0.1% (w/v) 8-anilino-1-naphthalene sulfonic acid (Fluka 10417). Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) bands were identified and scored, relative to spotted phospholipid standards, under UV light. Individual phospholipid fractions from each sample were scraped into corresponding labelled acid-washed pyrex tubes containing heptadecanoic acid (C17:0), as an internal fatty acid standard: PE (liver, muscle, and brain 1 µg; kidney 5 µg), PC (liver, kidney and brain 10 µg, muscle 5 µg), PS and PI (all tissues 0.5 µg). Fatty acid methyl esters (FAME) were prepared by heating phospholipid fractions in 2 mL of hexane (EMD HX0295-1) and 2 mL 14% boron triflouride-methanol solution (Sigma Aldrich B1252) at 100°C for 90 minutes. Samples were equilibrated at
room temperature for 10 minutes and 2 mL of ddH2O was added to each tube. Samples were vortexed and phases were separated via centrifugation at 21°C and 340 x g for 10 minutes. The top layer, containing hexane, was transferred to a 2 mL glass vial (Agilent 5182-0714) and dried under nitrogen gas. Samples were reconstituted in appropriate volumes of hexane, based on phospholipid fraction and source, for subsequent analysis by gas chromatography.

FAME composition of PL fractions was resolved using an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara USA) equipped with a flame ionization detector and a fused-silica polyethylene glycol capillary column (DB-FFAP; 15 m, 0.1 mm internal diameter, 0.1 µm film thickness) (Agilent Technologies 127-32H2). Samples were injected in split mode (1:200) using hydrogen as the carrier gas (30 mL/min) and eluted according to the following temperature program: 150°C for 20 seconds, ramp at 35°C/minute, hold at 170°C for 3 minutes, ramp at 9°C/minute, hold at 225°C for 30 seconds, ramp at 80°C/minute, hold at 245°C for 2.2 minutes. Chromatograms were generated using the Agilent EZChrom Elite GC Data System (version 3.3.2). Individual fatty acid peak identities were verified, by comparison with peak retention times of known fatty acid standards (Nu-Check Prep, Elysian, MN), and expressed as relative percent composition of total fatty acids.

5.3.10 Statistical Analysis

All fatty acid data is expressed as mean ± standard deviation (SD). Statistical analysis was conducted using SAS v9.1. One-way analysis of variance (ANOVA), with Tukey post-hoc test, was used to analyze fatty acid data from in vitro experiments.
Where appropriate, data was logarithmically transformed to produce normality. Fatty acid data within each in vivo study arm were analyzed by a Student’s t-tests. Significance was set at p< 0.05 for all analysis.

5.4 Results

5.4.1 Expression Characteristics of the iFat-1 Transgene: In vitro

5.4.1.1 Functionality of the pCAG-Cre Plasmid

In order to screen the suitability of the pCAG-Cre plasmid for conditional expression in the HEK 293T cell line, the pCALNL-GFP plasmid was employed. This Cre-reporter plasmid allows for visual detection of Cre-mediated recombination events via strict Cre-dependent expression of the green florescence protein (GFP) (151). Indeed, robust Cre-mediated GFP expression was detected in the HEK 293T cell line within 24 hours of co-transfection with pCAG-Cre and pCALNL-GFP, thus indicating that the selected Cre-expression system was a suitable candidate for in vitro validation of the iFat-1 transgene (Figure 5.4).

5.4.1.2 iFat-1 Background Expression Characteristics

The transcriptional expression characteristics of the iFat-1 transgene within the HEK 293T cell line were probed by RT-PCR (Figure 5.5). Unexpectedly, transcripts corresponding to a 250 bp region of the fat-1 coding sequence were detected both in cells co-transfected with iFat-1/Cre as well as iFat-1/Emp. All RNA samples were confirmed to be free of potential contaminating genomic DNA prior to reverse transcription to
cDNA, as outlined in Section 5.3.7 (data not shown). Although RT-PCR results demonstrated the existence of Cre-independent transcriptional expression into the \textit{fat-1} coding DNA sequence, the intensity of the observed \textit{fat-1} amplicons appeared considerably dampened in iFat-1/Emp co-transfected cells relative to iFat-1/Cre. The STOP cassette contained within the iFat-1 transgene includes an 825bp SV40 polyadenylation signal located downstream of a 550bp C-terminal portion of the yeast \textit{HIS3} gene (112). As the RT-PCR results suggest, this sequence did not appear to result in the expected efficient pre-mature transcriptional cessation from the CAG promoter of the iFat-1 transgene, consequently permitting the expression of \textit{fat-1} associated transcripts. However a precautionary translational block, composed of a false start codon immediately followed by a 5’ splice donor site, is also present within the STOP cassette as an added safeguard (112). Therefore despite the aberrant transcriptional expression downstream of the iFat-1 STOP cassette, functional FAT-1 protein expression may still remain obstructed via the translation block mechanism (communication with GenOway).

As anticipated, phospholipid fatty acid compositional changes in iFat-1/Emp co-transfected HEK 293T cells did not coincide with the observed background transcriptional effects from the iFat-1 transgene (Table 5.3-5.4). Total n-6 PUFA content (PE, PC, PS) and total n-3 PUFA content (PE, PC, PS, PI) did not significantly differ between cells co-transfected with iFat-1/Emp and control cells which had been treated with transfecting reagent alone (TF). Additionally, with the exception of a small significant difference in ALA (iFat-1/Emp; 0.2%, TF; 0.0%) within the PC fraction, iFat-1/Emp and TF treated controls were demonstrated to possess comparable relative compositions for all individual PUFA species measured. However, given that the iFat-
Emp and TF treated cells were relatively devoid of n-3 PUFA, the n-6/n-3 PUFA ratios within membrane phospholipid fractions were less consistent.

**Figure 5.4:** Screening of pCAG-Cre functionality in the HEK 293T cell line. The Cre-reporter plasmid contains a loxP flanked neomycin resistance cassette, between the CAG promoter and the GFP gene which acts as a strict functional barrier to the GFP expression in the absence of Cre recombinase (151). HEK 293T cells were transfected with pCALNL-GFP and either; pCAGEN (left panel) or pCAG-Cre (right panel). GFP expression was visually assessed 24 hours post-transfection by fluorescence microscopy. The pCAG-Cre plasmid permitted robust Cre-mediated recombination in the HEK 293T cell line and therefore represents a suitable candidate for *in vitro* validation of the iFat-1 transgene.
Figure 5.5: Transcriptional expression characteristics of the iFat-1 transgene in vitro. Cells were co-transfected with iFat-1/Emp or iFat-1/Cre and harvested for total cellular RNA 24 hours post-transfection. RT-PCR was performed to probe for the presence of transcripts corresponding to the fat-1 coding sequence. Hepatic cDNA obtained from total hepatic RNA of a constitutive fat-1 mouse was included as a reference (+) control. RT-PCR for rig/S15 cDNA, a constitutively expressed housekeeping gene encoding a small ribosomal subunit protein, was included as an indirect marker of RNA quality for each sample as per manufacturer instructions (RNAqueous®- 4PCR kit). Results demonstrate the existence of Cre-independent expression from the iFat-1 transgene.
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>PE</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2n-6</td>
<td>TF</td>
<td>iFat-1/Emp</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>29.1 ± 2.7(^a)</td>
<td>31.4 ± 2.4(^a)</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>4.8 ± 0.9(^a)</td>
<td>5.4 ± 0.4(^a)</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>3.3 ± 0.6(^a)</td>
<td>4.5 ± 0.6(^a)</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.0 ± 0.0(^b)</td>
<td>0.2 ± 0.4(^b)</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.0 ± 0.0(^b)</td>
<td>0.0 ± 0.0(^b)</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>1.8 ± 0.1(^b)</td>
<td>2.2 ± 0.3(^b)</td>
</tr>
<tr>
<td>1.9 ± 0.6</td>
<td>1.9 ± 0.3</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
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<td>48.3 ± 2.0(^a)</td>
</tr>
<tr>
<td>Total n-3 PUFA</td>
<td>3.7 ± 0.5(^b)</td>
<td>4.3 ± 0.9(^b)</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>11.8 ± 0.6(^a)</td>
<td>11.7 ± 2.9(^a)</td>
</tr>
<tr>
<td>Total SFA</td>
<td>38.7 ± 8.7</td>
<td>33.1 ± 1.3</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>13.5 ± 3.7</td>
<td>13.8 ± 0.3</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>47.8 ± 5.1</td>
<td>53.1 ± 1.2</td>
</tr>
</tbody>
</table>

**Table 5.3:** Phospholipid fatty acid composition of select n-6 and n-3 PUFA in HEK 293T cells: PE and PC. Cells were co-transfected with iFat-1/Emp or iFat-1/Cre. Cells treated with transfecting reagent in the absence of plasmid DNA (TF) were also included as controls for potential background difference in fatty acid composition attributable to Cre-independent expression of the iFat-1 transgene. Results expressed as mean ± SD from three independent experiments. Data was analyzed by one-way ANOVA with Tukey’s post-hoc test, with significance set at p<0.05. For each phospholipid fraction, means possessing different superscripts within rows denote significant differences between groups.
Table 5.4: Phospholipid composition of select n-6 and n-3 PUFA in HEK 293T cells: PS and PI. Cells were co-transfected with iFat-1/Emp or iFat-1/Cre. Cells treated with transfecting reagent in the absence of plasmid DNA (TF) were also included as controls for potential background difference in fatty acid composition attributable to Cre-independent expression of the iFat-1 transgene. Results expressed as mean ± SD from three independent experiments. N6/n-3 PUFA ratios designated non-detectable (ND) indicates the existence of sample(s) within an experimental group devoid of detectable levels of n-3 PUFA. Data was analyzed by one-way ANOVA with Tukey’s post-hoc test, with significance set at p<0.05. For each phospholipid fraction, means possessing different superscripts within rows denote significant differences between groups.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>PS TF</th>
<th>iFat-1/Emp</th>
<th>iFat-1/Cre</th>
<th>PI TF</th>
<th>iFat-1/Emp</th>
<th>iFat-1/Cre</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2n-6</td>
<td>20.3 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.5 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.5 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.5 ± 5.3</td>
<td>30.0 ± 5.8</td>
<td>22.1 ± 3.7</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.4 ± 0.8</td>
<td>1.0 ± 0.9</td>
<td>0.0 ± 0.0</td>
<td>7.2 ± 3.4</td>
<td>6.3 ± 3.4</td>
<td>1.5 ± 1.3</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>2.1 ± 2.1</td>
<td>3.3 ± 0.5</td>
<td>2.1 ± 2.2</td>
<td>0.3 ± 0.5</td>
<td>0.5 ± 0.8</td>
<td>0.0 ± 0.0</td>
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<tr>
<td>22:5n-6</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.8 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.4 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:5n-3</td>
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<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.0 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.6 ± 1.1</td>
<td>2.2 ± 0.7</td>
<td>3.9 ± 0.4</td>
<td>0.5 ± 0.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.7 ± 1.2</td>
<td>1.0 ± 1.0</td>
<td>2.6 ± 0.8</td>
<td>0.4 ± 0.6</td>
<td>0.0 ± 0.0</td>
<td>0.4 ± 0.6</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
<td>27.3 ± 4.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>32.5 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.1 ± 2.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.7 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.1 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.0 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total n-3 PUFA</td>
<td>1.3 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.7 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.8 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.1 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>n-6/n-3 ratio</td>
<td>ND</td>
<td>10.6 ± 3.1</td>
<td>1.4 ± 0.2</td>
<td>ND</td>
<td>ND</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Total SFA</td>
<td>49.3 ± 0.9</td>
<td>50.0 ± 3.7</td>
<td>50.5 ± 2.8</td>
<td>51.0 ± 7.2</td>
<td>52.7 ± 1.9</td>
<td>52.6 ± 1.3</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>22.1 ± 7.6</td>
<td>14.2 ± 2.0</td>
<td>16.7 ± 0.9</td>
<td>11.5 ± 7.0</td>
<td>6.2 ± 1.8</td>
<td>8.3 ± 2.0</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>28.6 ± 6.8</td>
<td>35.7 ± 2.4</td>
<td>32.8 ± 2.4</td>
<td>37.5 ± 2.4</td>
<td>41.1 ± 0.2</td>
<td>39.1 ± 1.0</td>
</tr>
</tbody>
</table>
5.4.1.3 iFat-1 Conditional Expression Characteristics

In comparison with controls, iFat1-/-Cre co-transfected cells experienced pronounced alterations in relative n-3 PUFA and n-6 PUFA composition with membrane phospholipids. ALA (PE, PC, PS, PI; p<0.01) and LA (PE, PC, PS; p<0.01) were the most notable species of PUFA enriched and reduced respectively. The PUFA compositional changes were most marked within the PE and PC fraction, which comprise the most abundant phospholipid populations within the membranes of HEK 293T cells. For example, significant increase in several of the downstream long-chain n-3 PUFA species, including EPA (p<0.05) and 22:5n-3 (p<0.01), with concomitant decreases in corresponding n-6 PUFA species (AA, 22:4n-6; p<0.01) were also evident with the PE fraction (Figure 5.6). Collectively, the observed change in fatty acid composition within the PE fraction was consistent with a complete balancing of the n-6/n-3 PUFA ratio in iFat-1/Cre treated cells relative to controls (1.0 p<0.01). Results from the PC fraction were in general agreement with those of the PE fraction. Surprisingly, DHA was not enriched. However, it is likely that additional incubation time was needed to allow for further elongation and desaturation from ALA given that downstream fatty acids were not enriched to the same extent as ALA, which is the direct product from the desaturation of LA by the FAT-1 protein. Total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and PUFA did not differ between experimental groups for any phospholipid fraction.
Figure 5.6: Representative gas chromatography trace of PUFA composition of PE fraction of co-transfected HEK 293T cells. Co-transfection with (A) iFat-1/Empty resulted in marked elevations in n-3 PUFA and corresponding reductions in n-6 PUFA species in relation to (B) iFat-1/Cre co-transfected cells. For detailed PUFA compositional changes within phospholipid fractions refer to Table 5.3-5.4
5.4.2 Expression Characteristics of the iFat-1 Transgene: In vivo

5.4.2.1 iFat-1 Background Expression Characteristics

Consistent with the *in vitro* results, transcripts corresponding to a 250 bp region of the *fat-1* coding sequence were detected within the hepatic tissue of mice carrying the iFat-1 transgene, irrespective of Cre-related genotype and treatment strategy (Fig 5.7). The magnitude of this aberrant iFat-1 transcriptional expression appeared comparable to that of tamoxifen treated Tam-Cre/iFat-1 transgenic mice, as PCR amplification of serial diluted cDNA could not produce marked visual differences in band intensity between experimental groups (data not shown).

In order to characterize the functional significance of the apparent intrinsic background Cre-independent transcription from the iFat-1 transgene, the phospholipid composition of non-treated iFat-1 mice was directly compared with that of WT controls. Results from major phospholipid fractions, PE and PC, in liver tissue have been presented in Table 5.5. Analogous to the *in vitro* findings, the PUFA composition of liver tissue from WT and iFat-1 transgenic mice was comparable. While an elevated total n-6 PUFA content was noted within the PC fraction of WT liver (+7.9%, p<0.05), this increase was neither associated with a significant difference in any major n-3 or n-6 PUFA species nor a significant modification of the n-6/n-3 PUFA ratio, but rather a net elevation in total PUFA content. Results from the kidney, muscle and brain tissue were in agreement with those obtained from liver tissue (data not shown).
Figure 5.7: Transcriptional expression characteristics of the iFat-1 transgene in vivo. Total RNA was isolated from liver of 9-10 week old WT and iFat-1 mice (as well as iFat-1/Tam-Cre mice treated with either vehicle or tamoxifen). RT-PCR was performed to probe for the presence of transcripts corresponding to a 250 bp portion of the fat-1 coding DNA sequence. Hepatic cDNA from a constitutive fat-1 mouse served as a reference (+) control for fat-1 transcript expression. Results demonstrate the Cre-independent expression from the iFat-1 transgene in hepatic tissue.
Table 5.5: Phospholipid composition of select n-6 and n-3 PUFA in liver tissue from WT (n=3/group) and iFat-1 (n=3/group): PE and PC. Data was analyzed by Student’s t-test, with significance set at p<0.05. For each phospholipid fraction, means possessing asterisk (*) within rows indicate significant differences between groups. Results expressed as mean ± SD. Despite apparent Cre-independent fat-1 expression from the iFat-1 transgene, WT and iFat-1 mice do not, generally, differ with respect to n-6 and n-3 PUFA composition. The phospholipid fatty acid composition of WT and iFat-1 liver tissue is also reflective of other tissue sites (data not shown).

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>PE</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>iFat-1</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>8.2 ± 0.7</td>
<td>6.6 ± 0.9</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>30.7 ± 0.8</td>
<td>30.2 ± 0.7</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>1.3 ± 0.1</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>7.5 ± 1.0</td>
<td>8.0 ± 1.3</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>2.2 ± 0.5</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Sum of n-6 PUFA</td>
<td>49.6 ± 0.7</td>
<td>47.9 ± 1.1</td>
</tr>
<tr>
<td>Sum of n-3 PUFA</td>
<td>2.4 ± 0.5</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>21.8 ± 5.7</td>
<td>24.8 ± 3.0</td>
</tr>
<tr>
<td>Total SFA</td>
<td>37.6 ± 0.8</td>
<td>38.9 ± 2.1</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>10.4 ± 0.6</td>
<td>11.3 ± 1.4</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>52.0 ± 0.5*</td>
<td>49.9 ± 1.1</td>
</tr>
</tbody>
</table>
5.4.2.2 iFat-1 Conditional Expression Characteristics

*In vivo* conditional expression characteristics of the iFat-1 transgene were assessed using tamoxifen treated and vehicle treated Tam-Cre/iFat-1 transgenic mice. The representative PUFA compositional differences from the PE fraction of liver, kidney, muscle and brain tissue have been presented (Fig 5.8-5.11). Detailed fatty acid compositional data from PE, PC, PS and PI phospholipid fractions of each tissue is provided in Appendices 1-4.

In relation to vehicle treated controls, tamoxifen treatment of Tam-Cre/iFat-1 mice resulted in a relative enrichment in phospholipid total n-3 PUFA content in each of the liver (PE, PC and PS; p<0.01), kidney (PE and PC; p<0.01, PS and PI; p<0.05) and muscle (PE; p<0.01, PC; p<0.05). Within all of these tissue phospholipid fractions, DHA was the predominant n-3 PUFA species enriched. However lesser contributions to total relative n-3 PUFA enrichment from 22:5n-3 within the liver (PE and PC; p<0.05, PS; p<0.01), kidney (PC; p<0.01, PE and PS; p≤0.05) and muscle (PE and PC; p<0.01) were also noted. Moreover, relative reductions within the corresponding n-6 PUFA species 22:5n-6 and 22:4n-6 were also observed within select phospholipid fractions from the liver and kidney as well as a small but significant overall decrease in total n-6 PUFA content with kidney and muscle tissue. Collectively, these PUFA compositional changes were consistent with an approximate 2 fold reduction, or more, of the n-6/n-3 PUFA ratio in each of the liver (PE: -3.6 and PS: -2.8; p<0.01, PC: -2.3; p<0.05), kidney (PE: -2.4 and PC: -2.4; p<0.01, PS: -2.5 and PI: -2.1; p<0.05) and muscle (PE: -2.0 and PC: -1.9; p<0.05) tissues. However, PUFA compositional changes within the brain tissue were less
discernible, with only a small but significant difference in the n-6/n-3 PUFA ratio evident within the PC fraction (-1.3 fold; p<0.05), which was neither associated with a marked difference in total n-6 PUFA or total n-3 PUFA between tamoxifen and vehicle treated mice. Total SFA, MUFA and PUFA did not generally differ between experimental groups.

Overall, the observed n-3 PUFA enrichment within the tissues of tamoxifen treated Tam-Cre/iFat-1 mice appeared considerably blunted in relation to age matched constitutive fat-1 reference controls. For example, the sum of the relative composition of all n-3 PUFA species of constitutive fat-1 mice exceeded that of tamoxifen treated Tam-Cre/iFat-1 mice by more than 2-fold within all phospholipid fractions of the liver, kidney and brain and by a minimum of 5-fold within the muscle tissue. The characteristic n-3 PUFA enrichment of the age-matched constitutive fat-1 mice was consistent with a near, to completely, balanced n-6/n-3 PUFA ratio in all tissue tested (Figure 5.11). Difference in PUFA composition between tamoxifen treated Tam-Cre/iFat-1 mice and constitutive fat-1 mice did not appear to be a function of oil treatment (Figure 5.11).
**Figure 5.8:** Relative fatty acid composition of total n-6 (upper panel) and total n-3 PUFA (lower panel) in tissue PE fractions of Tam-Cre/iFat-1 mice: treated with vehicle (Sham) or tamoxifen (Tam+). Results expressed as mean ± SD (n=3/group). Asterisk (*) denotes significant difference (p<0.05) between groups by Student’s t-test. Age matched constitutive fat-1 C57BL/6 males (n=2) included as reference controls for fat-1 expression.
Figure 5.9: Relative fatty acid composition of select n-6 and n-3 PUFA species in PE fraction of liver (upper panel) and kidney (lower panel) tissue of Tam-Cre/iFat-1 mice: treated with vehicle (Sham) or tamoxifen (Tam+). Results expressed as mean ± SD (n=3/group). Asterisk (*) denotes significant difference (p<0.05) between groups by Student’s t-test. Age matched constitutive fat-1 C57BL/6 males (n=2) included as reference controls for fat-1 expression.
Figure 5.10: Relative fatty acid composition of select n-6 and n-3 PUFA species in PE fraction of muscle (upper panel) and brain (lower panel) tissue of Tam-Cre/iFat-1 mice: treated with vehicle (Sham) or tamoxifen (Tam+). Asterisk (*) denotes significant difference (p<0.05) between groups by Student’s t-test. Results expressed as mean ± SD (n=3/group). Age matched constitutive fat-1 C57/BL6 males (n=2) included as reference controls for fat-1 expression.
Figure 5.11: N-6/n-3 PUFA ratios in tissue PE fractions from Tam-Cre/iFat-1 mice: treated with vehicle (Sham) or tamoxifen (Tam+). Asterisk (*) denotes significant difference (p<0.05) between groups by Student’s t-test. Results expressed as mean ± SD (n=3/group). Age matched constitutive fat-1 C57BL/6 males (const. fat-1 (+); n=2) and their WT littermates (const. fat-1 (-); n=2) were included as reference controls for fat-1 expression.
5.5 Discussion

While research employing the fat-1 transgenic mouse has lent strong support to the protective health benefits of lifelong n-3 PUFA exposure, the constitutive nature of transgene expression precludes the application of this model for investigating issues of timing of n-3 PUFA exposure. The iFat-1 transgenic mouse, which carries a Cre-inducible version of the C. elegans fat-1 gene has been developed as a potential tool to address this research gap. In contrast to the original fat-1 transgene described by Kang and colleagues (5), the iFat-1 regulatory unit has been adapted to contain both a ubiquitous CAG promoter and a floxed STOP cassette, thus introducing an overarching mechanism through which the timing and/or pattern of endogenous n-3 PUFA enrichment can be differentially controlled. This study demonstrates, for the first time, that the iFat-1 transgene is capable of facilitating Cre-inducible control over endogenous n-3 PUFA enrichment within mammalian cells and tissues.

5.5.1 iFat-1 Expression Characteristics: In vitro

The success of any given floxed transgene activation system is bound by the ability to achieve strict Cre-dependent control over transgene function. The iFat-1 transgene contains a loxP flanked STOP sequence designed to block both transcriptional and translation expression (112). In the present study, transcripts consistent with Cre-independent expression from the iFat-1 transgene were detected within the HEK 293T cell line. Nevertheless the PUFA composition of membrane phospholipids, a marker of functional FAT-1 enzyme activity, was not associated with iFat-1 status in the absence of Cre. This finding suggests that while the loxP flanked STOP cassette cannot terminate
transcriptional expression from the CAG promoter with 100% efficiency, the translation block mechanism provided an adequate barrier such that functional FAT-1 protein expression remains tightly reliant on Cre-mediated recombination as an activating factor.

As anticipated, simultaneous introduction of iFat-1 and Cre into HEK 293T cells was associated with a complete balancing of the n-6/n-3 PUFA ratio of membrane phospholipids. This balancing effect was directly attributable to an increase in the relative proportion of n-3 PUFA at the expense of corresponding n-6 PUFA species and evident within all major phospholipid fractions, though most pronounced within PE (p<0.01) and PC (p<0.01) phospholipids. The capacity of mammalian cells to endogenously convert n-6 PUFA to n-3 PUFA through heterologous expression of the humanized *C. elegans fat-1* gene was first reported by Kang and colleagues in 2001 (156). In this early report, an adenovirus mediated approach was used to specifically introduce the *fat-1* gene into cultured rat cardiac myocytes, the net result of which was a balanced n-6/n-3 PUFA ratio of total cellular lipids within a 48 hour period; an ~15 fold reduction from that of controls (156). This balancing effect has been subsequently paralleled in various other mammalian cell-lines of both murine (157, 158) and human origin (159-161). Collectively, these results indicate that the iFat-1 transgene is not only capable of producing Cre-inducible regulation over endogenous n-3 PUFA enrichment within mammalian cells, but does so in a manner that is consistent, in both magnitude and time scale, to PUFA compositional modifications achievable using constitutive approaches to *fat-1* expression *in vitro*. 
5.5.2 *iFat-1 Expression Characteristics in vivo*

Ligand dependent recombinases are advantageous because they provide an exogenous means of controlling the timing of Cre-mediated recombination events, a strategy which is well-suited to the desired research application of the iFat-1 transgenic mouse. In the present study, the Tam-Cre mouse model (135) was used to test the capacity of the iFat-1 transgene to facilitate endogenous n-3 PUFA enrichment within the tissues of young adult mice. Tam-Cre/iFat-1 mice were treated with tamoxifen or vehicle control at 6-7 weeks of age and tissues were collected for analysis 3 weeks later. Indeed, significant reductions in n-6/n-3 PUFA ratio were detected within multiple phospholipid fractions of liver, kidney and muscle tissue. In relation to vehicle treated controls, the most marked differences in PUFA composition of tamoxifen treated Tam-Cre/iFat-1 mice were evident within liver and kidney, in which significant respective increases and decreases in 22-carbon n-3 PUFA and n-6 PUFA, were detected across several phospholipid fractions. Phospholipid fatty acid analysis of muscle showed significant increases in the relative composition of 22:5n-3 and DHA within PE and PC fractions, however the n-3 PUFA composition of brain tissue remained unaltered by tamoxifen treatment. Additionally, although, aberrant Cre-independent iFat-1 transcripts were also detected *in vivo*, the phospholipid PUFA composition of WT and iFat-1 mice was, in general, similar. Therefore, analogous to *in vitro* findings, *in vivo* results support the requirement of Cre in the activation of functional FAT-1 protein expression from the iFat-1 transgene.
5.5.3 iFat-1 vs. Constitutive Fat-1 Mice

It is interesting to note that the magnitude of the PUFA compositional shift experienced by tamoxifen treated Tam-Cre/iFat-1 mice appeared considerably dampened in relation to corresponding tissues derived from age matched constitutive fat-1 reference controls. Within each of the liver, kidney and muscle of tamoxifen treated Tam-Cre/iFat-1 mice the percent composition of total n-3 PUFA was significantly increased whereas total n-6 PUFA remained relatively unaltered in relation to vehicle controls. In contrast, each of total n-3 PUFA and n-6 PUFA were visibly and correspondingly increased and decreased respectively in constitutive fat-1 mice compared with tamoxifen treated Tam-Cre/iFat-1 mice, contributing to a near to complete balancing in n-6/n-3 PUFA ratio. Several factors may account for this difference, which are detailed in the following sections.

5.5.3.1 Time for Accumulation

Expression of the constitutive fat-1 transgene begins in utero, presumably prior to implantation within the uterine wall. Opposingly, Tam-Cre/iFat-1 mice were only allotted an acute window (~3 weeks) for transgene activation and associated n-3 PUFA accumulation, which had to occur within the context of n-6 PUFA enriched mature tissues. However, unlike mammalian desaturase enzymes, which rely on fatty acyl-coenzymeA as substrate (162), the C. elegans fat-1 enzyme has been hypothesized to possess acyl-lipid desaturase activity, thus enabling direct conversion of phospholipid esterfied n-6 PUFA to corresponding n-3 PUFA products (49). Consequently, the effects of FAT-1 protein should be rapidly reflected in membrane phospholipids, and is indeed
supported by \textit{in vitro} results presented in this study. However, while time for accumulation may partially underlie the discordance between these two mouse models, given the sheer magnitude of the PUFA compositional differences, it is unlikely the sole reason.

\subsection*{5.5.3.2 Cre-Expression Model and Treatment Scheme}

Tam-Cre/iFat-1 mice were treated with tamoxifen at a dosage of 1 mg/day for 5 days IP. This dosing strategy was selected because it is not only consistent with that commonly encountered in literature \cite{163-166} but has also been successfully employed in combination with the Tam-Cre mouse model \cite{167}. However, the efficiency through which a given ligand dependent Cre-mediated recombination system can facilitate excisive recombination is multidimensional, involving influences such as the location and structure of floxed genomic sequences, genetic background, ligand treatment regime and Cre promoter, amongst various other contributing factors \cite{129}. TAM-Cre mice have been engineered to express CreER\textsuperscript{T2}, a human estrogen receptor-Cre fusion protein with enhanced sensitivity to the effects of 4-hydroxytamoxifen (4-OHT) than its CreER predecessor \cite{115}, under the control of the ubiquitous ROSA26 promoter. Nevertheless, PUFA compositional differences between tamoxifen and vehicle treated Tam-Cre/iFat-1 mice were not comparably altered throughout the body. Modifications in relative PUFA composition were principally manifested within phospholipid fractions of liver, the organ responsible for metabolism of tamoxifen to its active metabolite 4-OHT, and kidney, an organ closely associated with the peritoneal cavity. In contrast, the PUFA composition of muscle and brain tissue were less and generally unaffected by tamoxifen treatment.
respectively. Collectively, this overall distribution suggests that the tamoxifen treatment schedule and/or Cre-expression model employed in this study may have been inadequate to permit excision of the floxed STOP cassette with the efficiency necessary to produce anticipated PUFA compositional modifications. This theory is strongly supported by very recently published findings in which the Tam-Cre mouse model was crossed with a conditional Acvr1b knockout line and shown to experience only partial Cre-mediated recombination within liver and kidney and exceedingly limited recombination within brain tissue 2 weeks after administration of 4 day course of 1 mg/day 4-OHT by IP injection (168). By contrast, Acvr1b/Tam-Cre conditional knockout mice experienced almost complete Cre-mediated recombination within lung, spleen and pancreas following tamoxifen treatment (168), tissues which were not specifically analyzed within the present study.

To date, at least three independent mouse models expressing the CreER\textsuperscript{T2} fusion protein under the control of the ubiquitous ROSA26 locus have been developed (135, 166, 169). Interestingly, when the model described by Hameyer et al. was crossed with a ubiquitous lacZ reporter line, in which activation of β-galactosidase expression is controlled by Cre (127), excisive recombination from the lacZ reporter allele was detected in peripheral organs but restricted from the brain tissue of tamoxifen treated adult double hybrids. By contrast, when Tam-Cre mice were crossed with the same lacZ reporter model, β-galactosidase activity was detected in a variety of organs, including brain, at 6 weeks of age (Supplementary data (135)). This latter finding might be explained by the tamoxifen dosage used, 9mg/40g body weight/day (5mg/25g mouse/day) for 5 days by IP injection, which is several orders of magnitude higher than
that than the 1 mg/day treatment regime employed by Hameyer et al. and presented herein.

Several alternative tamoxifen routes and doses have also been used in combination with the Tam-Cre mouse model to achieve conditional knockout or conditional activation from floxed alleles (170-173). Recently it was reported that a 5-day course of subcutaneous injection of 100 μL 4-OHT in corn-oil (1mg) could lead to more efficient recombination by the Tam-Cre mouse line than that achievable by IP injection, yet data supporting this claim was unfortunately not provided (174). Future research employing the iFat-1 transgenic mouse in combination with ligand dependent, or independent, Cre expression models would clearly benefit from direct genomic assessment of Cre-mediated recombination efficiency to both aid in optimization and corroboration of PUFA related outcomes.

5.5.3.3 Inherent Expression Capacity

Although the constitutive fat-1 transgene and the iFat-1 transgene are both designed to express the same codon optimized version of the C. elegans fat-1 gene under the control of a β-actin promoter, the methods used to generate these two transgenic models are quite different. In iFat-1 transgenic mice, a single copy of the iFat-1 transgene was specifically introduced to the Hprt locus of X-chromosome, by homologous recombination in embryonic stem cells. This constitutively expressed genetic locus presents a permissive chromatin environment which has proven conducive to fostering predictable, promoter-driven, patterns of transgene expression, which are independent of the complicating factors of multi-copy insertion and position site
variegation inherent to classical pronuclear transgenesis (143). Moreover, it has been previously demonstrated that Cre can access this genomic locus and produce recombination between loxP sites (175). By comparison, the constitutive fat-1 mouse was generated using classical pronuclear transgenesis (41), a method which results in random, often multicopy, transgene integration into the genome (109). Although it has been reported that constitutive fat-1 expression is driven by a single copy of the transgene (communication with Dr. Jing Kang), the site of genomic integration remains unknown. It is well recognized that the expression of a given transgene, even one under the control of a strong ubiquitous reporter, can be markedly influenced by its surrounding genomic environment (176). If, for example, the constitutive fat-1 transgene has randomly integrated within proximity of a strong endogenous enhancer element, the inherent expression capacity of this model could potentially exceed that achievable with the iFat-1 mouse model, which may in turn lead to functional disparities in PUFA phenotype. However, clarification of the aforementioned issues regarding time for accumulation and Cre-mediated recombination efficiency should concurrently help to address this concern.

5.6 Conclusion

In conclusion, this study has provided unequivocal evidence that mammalian cells and tissues can be modified through incorporation of the iFat-1 transgene to endogenously convert n-6 PUFA to n-3 PUFA in a manner which is reliant on Cre as an activating factor. Based on pronounced in vitro results and in vivo insights, the iFat-1 transgene has potential application as a versatile tool in addressing the temporal and/or
tissue specific effects of n-3 PUFA in disease prevention and treatment. However, further model characterization is first necessary.
Chapter Six

GENERAL DISCUSSION, STUDY LIMITATIONS and
FUTURE DIRECTIONS
6.1 General Discussion and Study Limitations

This thesis work describes the initial characterization of the novel iFat-1 transgenic mouse, which was developed as a tool with potential utility in addressing a unique research niche from which the constitutive fat-1 transgenic mouse is presently excluded; the categorical assessment of timing of n-3 PUFA exposure. Using a combined \textit{in vitro} and \textit{in vivo} approach the results presented herein have clearly established that the PUFA phenotype of mammalian cells and tissues modified to contain the iFat-1 transgene is Cre-inducible. While these findings are in general agreement with the hypothesized outcomes of this work, two major unexpected observations were noted: (1) Cre-independent transcription and (2) a blunted \textit{in vivo} phenotype. Overall the results of this investigation suggest that the iFat-1 transgene has powerful potential as a tool in PUFA research, however the future actualization of this potential first necessitates clarification of several currently outstanding details of transgene function, particularly \textit{in vivo}.

6.1.1 Cre-independent transcription

Cre-independent background transcriptional expression within the \textit{fat-1} coding region of the iFat-1 transgene was detected both \textit{in vitro} and \textit{in vivo}. However, whether the relative expression of these Cre-independent products occurs with low or high frequency is currently uncertain and should be better addressed in future using a quantitative approach (qRT-PCR). We have interpreted these transcriptional products to be non-functional read-through transcripts arising from inefficient transcriptional cessation by the floxed STOP cassette, however at present this remains only speculation. Additionally, while crude anti-fat-1 rabbit serum has been described (161), to the best of
our knowledge, no purified antibody preparation against the C. elegans FAT-1 protein was commercially available at the time this study was conducted. Therefore, iFat-1 expression characteristics were investigated on the basis of transcriptional profile and PUFA phenotype, which is consistent with the validation method used in other alternative in vivo applications of fat-1 n-3 desaturases (140, 177, 178). Therefore, whether Cre-independent iFat-1 transcripts result in no-product, low level FAT-1 production and/or an abnormal non-functional protein product in association with the translational block mechanism contained within the iFat-1 floxed STOP cassette is presently unknown. This latter possibility may be of particular relevance because it raises the concern for potential iFat-1 transgene associated phenotypic influences in situations where Cre mediated STOP cassette excision is not 100%, for example through potential dominant negative influences of a “non-functional” protein product.

6.1.2 Blunted in vivo Phenotype

A blunting in the magnitude of percent compositional n-3 PUFA enrichment in tamoxifen treated Tam-Cre/iFat-1 was noted in comparison to constitutive fat-1 transgenic reference controls. While several potential factors underscoring these effects were discussed in Chapter 5 they should be reiterated here as they represent significant limitations of this study. Firstly, only a single time point was used in the present study and therefore the incubation period required in the production of plateaued tissue n-3 PUFA enrichment following Cre-mediated recombination in the iFat-1 transgenic mouse remains a point of uncertainty. This consideration should be addressed in future applications of this mouse model through the implementation of pilot time-course
experiments. Additionally, we have hypothesized that poor recombination efficiency in response to the inducing strategy used in Tam-Cre/iFat-1 mice is a likely candidate factor underscoring the observed blunted phenotype. However in the present study Cre-mediated recombination efficiency was not directly assessed at the genomic level. This could be assessed in the future through the use of a PCR strategy designed to amplify alternatively sized fragments from floxed containing versus Cre-recombined iFat-1 alleles, as has been described in other Cre-loxP based validation experiments (179).

6.1.3 General Strengths and Limitations of n-3 Desaturases

In addition to the aforementioned strengths and limitations unique to the present investigation, the iFat-1 transgenic mouse is also subject to strengths and limitations central to the use of n-3 desaturase mediated approach to tissue PUFA modification in lieu of diet-based studies. That is, while this adaptation provides an efficacious means to eliminate diet-based confounding variability it does not recapitulate a natural method of tissue n-3 PUFA accretion within mammals and cannot be achieved without requisite reductions in n-6 PUFA. Therefore while FAT-1 desaturase systems provide a useful standardized tool for the investigation of n-3 PUFA in vivo, diet-based corroboration is also highly recommended.
6.2. Future Applications of the iFat-1 Mouse Model

In the present study a tamoxifen based induction approach was used, however this may not represent the most suitable induction approach for future applications of the iFat-1 transgenic mouse. There are several reasons for this, the most notable of which is that, tamoxifen is a lipid soluble molecule and therefore must be suspended in oil. While tamoxifen treatment is transient, IP injection of oil may have obvious confounding effects with respect to PUFA studies, including n-6 PUFA rich oil deposition within the peritoneal cavity and the potential of associated metabolic complications. Additionally, as discussed in Chapter 3, tamoxifen also acts as an estrogen receptor antagonist in mammary tissue and therefore CreER based expression systems are not as well situated for the study of mammary tumourigenesis, a pathological process that may be especially sensitive to timing of exposure to n-3 PUFA (99). Moreover, in addition to potential issues of tamoxifen toxicity also discussed Chapter 3, possible complicating interference with eicosanoid signalling may also arise in association with tamoxifen treatment (180). Therefore, doxycycline regulated Cre-recombinase systems may be more suitable candidates for future applications of the iFat-1 transgenic mouse. One potential approach to achieve this is by crossing the ubiquitous rtTA2S-M2 expression model recently characterized by Katsantoni and colleagues (181) with the Tg(tetO-Cre)IJaw mouse model (obtainable through the Jackson Lab #006224), in which a CMV-minimal tetO promoter drives a ubiquitous pattern of doxycycline sensitive Cre-expression (182). Double hybrid males derived from this cross could subsequently be bred with iFat-1 females with the aim of generating a novel triple transgenic mouse model capable of doxycycline inducible ubiquitous endogenous n-3 PUFA enrichment.
Overall, it is important to understand the limitations and strengths of any model system, which is critical for the appropriate interpretation of study results. Nevertheless, the iFat-1 and future hybrid models will potentially contribute significantly to enhancing our fundamental knowledge of the role of n-3 PUFA in health and disease.
Chapter Seven

REFERENCES and APPENDIX
References


89. Suh, M., Sauve, Y., Merrells, K. J., Kang, J. X. & Ma, D. W. (2009) Supranormal electroretinogram in fat-1 mice with retinas enriched in docosahexaenoic acid and...


Appendix 1 – Phospholipid Composition of Liver

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<th>Fatty Acid</th>
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<th>Tam+</th>
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<th>Tam+</th>
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<td>18.3 ± 4.8</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total SFA</td>
<td>40.0 ± 1.3</td>
<td>39.1 ± 1.3</td>
<td>38.2 ± 0.7</td>
<td>42.9 ± 0.4</td>
<td>43.7 ± 1.5</td>
<td>41.8 ± 0.7</td>
<td>51.5 ± 0.9</td>
<td>49.8 ± 1.6</td>
<td>48.8 ± 2.6</td>
<td>51.3 ± 0.9</td>
<td>49.9 ± 0.1</td>
<td>47.2 ± 2.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total MUFA</td>
<td>10.2 ± 0.7*</td>
<td>8.3 ± 0.4</td>
<td>9.1 ± 1.2</td>
<td>11.3 ± 1.7*</td>
<td>7.2 ± 0.5</td>
<td>9.1 ± 1.7</td>
<td>6.5 ± 1.2*</td>
<td>4.0 ± 0.4</td>
<td>5.5 ± 1.0</td>
<td>5.2 ± 0.4*</td>
<td>2.8 ± 0.3</td>
<td>4.0 ± 0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total PUFA</td>
<td>49.9 ± 0.8</td>
<td>52.4 ± 1.4</td>
<td>52.7 ± 1.9</td>
<td>45.9 ± 2.0</td>
<td>49.1 ± 1.7</td>
<td>49.1 ± 1.0</td>
<td>42.0 ± 1.5</td>
<td>46.2 ± 1.8*</td>
<td>46.2 ± 3.6</td>
<td>43.5 ± 0.9</td>
<td>47.3 ± 0.5*</td>
<td>48.8 ± 3.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Relative fatty acid composition of membrane phospholipids in liver tissue from Tam-Cre/iFat-1 mice treated with vehicle (Sham) or tamoxifen (Tam+). Results expressed as mean ± SD (n=3/group). Asterisk (*) denotes significant difference (p<0.05) between groups by Student’s t-test. Age matched constitutive fat-1 C57BL/6 males (n=2) included as reference controls for fat-1 expression.
Appendix 2 – Phospholipid Composition of Kidney

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>PE</th>
<th>PC</th>
<th>PS</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2n-6</td>
<td>4.9 ± 1.0</td>
<td>5.0 ± 0.5</td>
<td>5.7 ± 0.6</td>
<td>12.1 ± 2.6</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>0.0 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.6 ± 0.0</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.0</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>32.9 ± 1.2</td>
<td>34.8 ± 2.0</td>
<td>30.2 ± 0.7</td>
<td>14.9 ± 2.1</td>
</tr>
<tr>
<td>22:2n-6</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>2.2 ± 0.3</td>
<td>1.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>1.5 ± 0.1*</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>11.2 ± 1.6*</td>
<td>6.0 ± 1.2</td>
<td>0.6 ± 0.0</td>
<td>12.2 ± 2.0*</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>18:4n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.2 ± 0.0</td>
<td>0.7 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>3.5 ± 0.2</td>
<td>7.8 ± 1.5*</td>
<td>17.3 ± 0.3</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
<td>52.2 ± 0.4*</td>
<td>48.5 ± 0.8</td>
<td>38.1 ± 0.1</td>
<td>42.7 ± 1.3*</td>
</tr>
<tr>
<td>Total n-3 PUFA</td>
<td>3.7 ± 0.2</td>
<td>8.5 ± 1.4*</td>
<td>20.7 ± 0.7</td>
<td>5.0 ± 0.4</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>14.0 ± 1.0*</td>
<td>5.8 ± 0.8</td>
<td>1.8 ± 0.1</td>
<td>8.6 ± 0.6*</td>
</tr>
<tr>
<td>Total SFA</td>
<td>33.1 ± 0.2</td>
<td>32.2 ± 1.2</td>
<td>30.1 ± 1.3</td>
<td>44.0 ± 0.4</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>10.9 ± 0.2</td>
<td>10.7 ± 1.0</td>
<td>11.2 ± 0.7</td>
<td>8.3 ± 1.1</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>56.0 ± 0.2</td>
<td>57.0 ± 2.1</td>
<td>58.7 ± 0.7</td>
<td>47.7 ± 1.5</td>
</tr>
</tbody>
</table>

1Relative fatty acid composition of membrane phospholipids in kidney tissue from Tam-Cre/iFat-1 mice treated with vehicle (Sham) or tamoxifen (Tam+). Results expressed as mean ± SD (n=3/group). Asterisk (*) denotes significant difference (p<0.05) between groups by Student’s t-test. Age matched constitutive fat-1 C57BL/6 males (n=2) included as reference controls for fat-1 expression.
Appendix 3 – Phospholipid Composition of Muscle

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>PE</th>
<th>PC</th>
<th>PS</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Tam+</td>
<td>fat-1</td>
<td>Sham</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>7.5 ± 0.4</td>
<td>10.4 ± 0.5</td>
<td>17.7 ± 0.9</td>
<td>28.9 ± 2.0</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.2 ± 0.2</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.7 ± 0.0</td>
<td>0.6 ± 0.1</td>
<td>1.6 ± 0.1*</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>13.6 ± 1.5</td>
<td>13.1 ± 0.7</td>
<td>4.2 ± 0.7</td>
<td>18.8 ± 0.5</td>
</tr>
<tr>
<td>22:2n-6</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>6.3 ± 0.3</td>
<td>6.9 ± 0.2</td>
<td>2.0 ± 0.5</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>25.5 ± 2.4</td>
<td>22.1 ± 2.9</td>
<td>3.8 ± 0.0</td>
<td>4.8 ± 1.2</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.4 ± 0.2</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>18:4n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>22:3n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.6 ± 0.1</td>
<td>1.4 ± 0.2*</td>
<td>7.0 ± 0.3</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>28.3 ± 0.3</td>
<td>4.6 ± 0.5*</td>
<td>28.6 ± 1.2</td>
<td>28.6 ± 1.2</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
<td>53.9 ± 0.7*</td>
<td>50.7 ± 1.6</td>
<td>21.0 ± 2.0</td>
<td>45.5 ± 2.1</td>
</tr>
<tr>
<td>n-6:n-3 ratio</td>
<td>15.6 ± 2.0*</td>
<td>8.0 ± 1.4</td>
<td>0.6 ± 0.1</td>
<td>44.4 ± 0.8*</td>
</tr>
<tr>
<td>Total SFA</td>
<td>35.1 ± 0.9</td>
<td>35.6 ± 0.5</td>
<td>33.4 ± 0.2</td>
<td>42.9 ± 0.7</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>74.7 ± 1.0</td>
<td>72.5 ± 0.5</td>
<td>78.4 ± 0.3</td>
<td>106.1 ± 1.5</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>57.5 ± 0.6</td>
<td>57.1 ± 1.0</td>
<td>58.8 ± 0.5</td>
<td>46.5 ± 2.1</td>
</tr>
</tbody>
</table>

1 Relative fatty acid composition of membrane phospholipids in muscle tissue from Tam-Cre/iFat-1 mice treated with vehicle (Sham) or tamoxifen (Tam+). Results expressed as mean ± SD (n=3/group). Asterisk (*) denotes significant difference (p<0.05) between groups by Student’s t-test. Age matched constitutive fat-1 C57BL/6 males (n=2) included as reference controls for fat-1 expression.
Appendix 4 – Phospholipid Composition of Brain

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>PE</th>
<th>PC</th>
<th>PS</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2n-6</td>
<td>0.4 ± 0.0</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.0</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.5 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>20:5n-6</td>
<td>15 ± 0.6</td>
<td>16.7 ± 0.2*</td>
<td>13.4 ± 0.2</td>
<td>13.4 ± 0.2</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>0.0 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>6.8 ± 0.6</td>
<td>6.9 ± 0.2</td>
<td>4.8 ± 0.0</td>
<td>4.8 ± 0.0</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>17 ± 0.3</td>
<td>15.2 ± 2.3</td>
<td>0.9 ± 0.2</td>
<td>2.7 ± 0.1*</td>
</tr>
<tr>
<td>18:4n-3</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>22:3n-3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>10.0 ± 0.9</td>
<td>13.2 ± 3.6</td>
<td>28.9 ± 0.2</td>
<td>30.2 ± 0.2</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
<td>40.2 ± 0.6</td>
<td>40.0 ± 2.3</td>
<td>20.4 ± 0.1</td>
<td>20.4 ± 0.1</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>3.9 ± 0.3</td>
<td>3.1 ± 0.9</td>
<td>0.7 ± 0.2</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Total SFA</td>
<td>33.6 ± 1.4</td>
<td>32.9 ± 2.1</td>
<td>33.3 ± 1.6</td>
<td>33.3 ± 1.6</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>15.9 ± 1.8</td>
<td>13.5 ± 1.3</td>
<td>16.6 ± 1.7</td>
<td>16.6 ± 1.7</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>50.5 ± 1.3</td>
<td>53.6 ± 1.3*</td>
<td>50.1 ± 0.0</td>
<td>50.1 ± 0.0</td>
</tr>
</tbody>
</table>

1 Relative fatty acid composition of membrane phospholipids in muscle tissue from Tam-Cre/Ifat-1 mice treated with vehicle (Sham) or tamoxifen (Tam+). Results expressed as mean ± SD (n=3/group). Asterisk (*) denotes significant difference (p<0.05) between groups by Student’s t-test. Age matched constitutive fat-1 C57BL/6 males (n=2) included as reference controls for fat-1 expression.