Published in final edited form as:

Cancer Prev Res (Phila). 2008 December ; 1(7): 532–545. doi:10.1158/1940-6207.CAPR-08-0030.

Gene signaling pathways mediating the opposite effects of prepubertal low and high fat n-3 polyunsaturated fatty acid diets on mammary cancer risk

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Abstract

Background—In rats, prepubertal exposure to low fat diet containing n-3 polyunsaturated fatty acids (PUFAs) reduces mammary cell proliferation, increases apoptosis, and lowers risk of mammary tumors in adulthood, while prepubertal high fat n-3 PUFA exposure has opposite effects. To identify signaling pathways mediating these effects, we performed gene microarray analyses and determined protein levels of genes related to mammary epithelial cell proliferation.

Methods—Nursing female rats and rat pups were fed low (16% energy from fat) or high fat (39% energy from fat) n-3 or n-6 PUFA diets between postnatal days 5 and 24. cDNA gene expression microarrays were used to identify global changes in the mammary glands of 50-day-old rats. Differences in gene expression were confirmed using real time quantitative PCR, and immunohistochemistry was used to assess changes in the PPAR γ and cyclin D1 levels. DNA damage was determined using 8-hydroxy-2' deoxyguanosine assay.

Results—Expression of the antioxidant genes thioredoxin, heme oxygenase, NADP-dependent isocitrate dehydrogenase and metallothionein III as well as PPAR γ protein were increased in the mammary glands of 50-day-old rats prepubertally fed the low fat n-3 PUFA diet. Prepubertal exposure to the high fat n-3 PUFA diet increased DNA damage and cyclin D1 protein, and reduced expression of BRCA1 and cardiostrophin-1.

Conclusions—Reduction in mammary tumorigenesis among rats prepubertally fed a low fat n-3 PUFA diet was associated with an up-regulation of antioxidant genes, whilst the increase in mammary tumorigenesis in the high fat n-3 PUFA fed rats was linked to up-regulation of genes which induce cell proliferation and down-regulation of genes which repair DNA damage and induce apoptosis.

Keywords

8-hydroxy-2'-deoxyguanosine; 8-OhdG; Brca1; breast cancer 1; polymerase chain reaction; PCR; polyunsaturated fatty acid; PUFA

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Introduction

A high dietary intake of n-3 polyunsaturated fatty acids (PUFAs), as present in fish and some vegetable oils, (canola and linseed), can reduce the risk of developing breast cancer (1,2,3) and inhibit metastasis (4) in human studies. However, some studies report either no change or a significant increase in breast cancer risk among women who consumed high levels of n-3 PUFAs (5,6,7,8). Data from animal studies also have generated conflicting data: some studies show that a high dietary intake of n-3 PUFAs inhibits mammary tumorigenesis (9,10), while this effect is not seen in other studies (11,12). Perhaps reflecting these inconsistencies, we found that prepubertal exposures to a low or a high fat n-3 PUFA diet had opposing effects on later breast cancer risk (13). A low fat n-3 PUFA diet reduced later susceptibility to develop carcinogen-induced mammary tumors, but a high fat n-3 PUFA diet increased cancer risk (13).

We have extended our previous study to identify changes in gene signaling pathways in the mammary glands that could explain the opposing effect of prepubertal low and high fat n-3 PUFA exposures on mammary tumorigenesis. Our earlier study showed that these diets differentially affected cell proliferation and apoptosis in the mammary gland and, therefore, we were particularly interested in studying genes involved in these pathways (13). Since prepubertal exposure to both low and high fat n-3 PUFA diet induced lipid peroxidation (13), genes regulating oxidative damage were also of interest. Therefore, we performed a gene microarray analysis, focusing on antioxidant genes and genes associated with proliferation and apoptosis. The analysis, done utilizing tissues from our previous study, used novel analytic approaches to find functionally relevant gene expression pathways in the context of nutrigenomic animal studies. We also determined protein levels of two key genes linked to cell proliferation: cyclin D1 which is downstream of multiple pathways leading to increased cell proliferation (14,15), and peroxisome proliferation-activated receptor gamma (PPAR γ) which inhibits cell proliferation and induces differentiation (16). The latter gene was of particular interest, since n-3 PUFAs serve as ligands for PPAR γ .

Materials and Methods

Diet administration

Rats were exposed to low and high fat n-3 and n-6 PUFA diets during prepuberty as previously described (13). Briefly, timed pregnant Sprague-Dawley rats were obtained from Charles River (Wilmington, MA) on gestation day 10 and fed AIN93G diet. After delivery, ten female pups from 3–4 litters were housed per a nursing dam. Nursing dams were either kept on a semi-purified AIN93G diet or switched to one of the three experimental diets when the offspring were 5 days old. The dietary groups were: 1) low fat n-6 PUFA reference diet (AIN93G diet); 2) high fat n-6 PUFA diet; 3) low fat n-3 PUFA diet; and 4) high fat n-3 PUFA diet. The low fat diets contained 16% energy from fat and the high fat diets contained 39% energy from fat. Corn oil was the source of n-6 PUFAs (contains 60% n-6 PUFAs and 1% n-3 PUFAs) and menhaden oil of n-3 PUFAs (contains 25% of n-3 PUFAs and 2% n-6 PUFAs). The low-fat n-3 PUFA diet consisted of 35 grams/kg of menhaden oil and 35 grams/kg of corn oil, while the high fat n-3 PUFA diet consisted of 70 grams/kg of menhaden oil and 120 grams/kg of corn oil. The low fat n-6 PUFA diet consisted of 5 grams/kg menhaden oil and 65 grams/kg of corn oil, and the high fat n-6 PUFA diet consisted of 15 grams/kg of menhaden oil and 175 grams/kg of corn oil. Although the absolute levels of n-3 and n-6 PUFAs varied, the n-6 PUFA: n-3 PUFA ratio was kept similar in the n-6 PUFA diets (13:1 in the low fat n-6 PUFA diet and 12:1 in the high fat n-6 PUFA diet). Similarly, the n-3 PUFA: n-6 PUFA ratio was also kept similar in the n-3 PUFA diets (1:1 for the low fat n-3 PUFA diet and 1:2 in the high fat n-3 PUFA diet). This eliminated the possibility of differences between the low and high fat n-3 PUFA diet being caused by a change in the n-3: n-6 PUFA ratio.

Pups initially consumed the diets through the dam's milk from postnatal day 5 through postnatal day 15. It has been shown that the dam's milk closely reflects what is taken in via the diet in terms of fatty acids (17). Although still nursing, pups begin to consume food pellets at about on postnatal day 15, and therefore at that age onwards, they obtained n-3 and n-6 PUFAs both through milk and through consuming food pellets. At day 26, all pups were weaned and switched to the reference AIN93G diet.

Carcinogen-induced mammary tumorigenesis

When the rats were 50 days of age, some were administered 10 mg of the mammary carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) (Sigma Chemical Co., St. Louis, MO) by oral gavage. The low fat n-6 PUFA control group was composed of 23 rats, the high fat n-6 PUFA group had 25 rats, the low fat n-3 PUFA group had 25 rats, and the high fat n-3 PUFA group had 24 rats. Carcinogen was dissolved in peanut oil and given in a volume of 1 ml. Animals were checked weekly for mammary tumors by palpation. Tumor growth was measured using a caliper and the length, width, and height of each tumor were recorded. Animals were sacrificed when the tumor burden was approximately 10% of total body weight. All remaining animals, including those that did not develop tumors, were sacrificed 18 weeks after DMBA administration. All animal procedures were approved by the Georgetown University Animal Care and Use Committee, and the experiments were performed following the National Institutes of Health guidelines for the proper and humane use of rats in biomedical research.

Tumor incidence was calculated by the methods developed by Kaplan and Meier, followed by the log rank test. Differences in final tumor incidence among groups were compared using Chi-square test. The differences were considered significant if the p-value was less than 0.05. All probabilities were two-tailed.

Mechanisms mediating the effects of prepubertal PUFA diets on mammary tumorigenesis

To identify changes induced by prepubertal dietary PUFA exposures that might have mediated the effects of these diets on mammary tumorigenesis, we obtained serum and removed the 3rd thoracic and 4th abdominal mammary glands from 26- and 50-day old rats that were fed different PUFA diets during prepuberty (but not exposed to DMBA). The 3rd thoracic and 4th abdominal mammary glands from each side were removed because these are the easiest to locate and remove, anatomically. The glands were snap frozen in liquid nitrogen and stored at -80°C, except for one of the 4th glands which was processed for immunohistochemistry. Total RNA from the other 4th mammary gland was used in the microarray analysis, while total RNA from the 3rd mammary gland was used for real time PCR studies. Therefore, there was total RNA from each animal used in both the microarray studies and the real time PCR studies. RNA from the animals was not pooled. An additional set of rats was used to measure BRCA1 expression, cyclin D1 protein, PPAR γ protein, and DNA damage levels.

RNA Extraction

Total RNA was extracted from the mammary glands of rats exposed to low fat or high fat n-3 PUFA and n-6 PUFA diets. Frozen tissue samples were placed in 1" $\times$ 1" plastic bags, pulverized on dry ice, transferred to 35 mL conical Oakridge tubes (Nalgene, Rochester, NY), and weighed. Tissues were homogenized in 1 ml of TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA) per 50 mg of tissue using a PowerGen 35 hand held homogenizer (Fisher Scientific, Pittsburgh, PA) with RNase free disposable OMNI-Tips (Fisher Scientific) for 30 seconds. From this point, procedures were followed according to the manufacturer's instructions for use of the TRIzol Reagent. The quantity and quality of RNA was measured by comparing the optical density ratios (OD₂₆₀/OD₂₈₀) obtained using a Beckman DU640 Spectrophotometer (Beckman, Fullerton, CA). There were a total of 6 rats fed the low fat n-3

PUFA diet, 6 fed the low fat n-6 PUFA diet, 5 fed the high fat n-6 PUFA diet, and 5 fed the high fat n-3 PUFA diet.

Microarray Hybridization

We used GF300DS rat filters (Research Genetics Inc., Huntsville, AL) that contain 5531 known genes, 192 “housekeeping” genes, and 192 control genes on each filter. To synthesize the labeled cDNA probe, 2 µg total RNA was incubated at 70°C for 10 min with 2 mg of oligo dT and then chilled on ice for 2 min. The primed DNA was incubated at 37°C for 90 min in a solution containing 1× first strand, 3 mM DTT, 1 mM dGTP/dTTP, 300 Units of reverse transcriptase, 50 mCi of [³³P] dCTP and 50 mCi of [³³P] dATP. The second strand was synthesized by adding 1× reaction buffer, 100 units DNA polymerase I, 500 ng of random primers, 1 mM dGTP/dTTP, 50 mCi of [³³P] dCTP and 50 mCi of [³³P] dATP. The reaction was incubated for 2 hrs at 16°C. Radiolabeled probe was purified using a BioSpin-6 chromatography column (Bio-Rad, Hercules, CA) and denatured by boiling for 3 min.

Filters were prehybridized in Microhyb solution (ResGen) for 2 hrs at 42°C. Purified probe was added to the hybridization roller tube containing the prehybridized GeneFilter, and incubated for 12–18 hrs at 42°C in a Robin Scientific Roller Oven. Each hybridized GeneFilter was washed twice in 2× SSC, 1% SDS at 50°C for 20 min and once at 55°C in 0.5× SSC, 1% SDS for 15 min. Hybridization signals were detected by phosphorimage analysis, using a Molecular Dynamics Storm phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Microarray Data Analysis

All data processing and analysis was implemented in the Mathworks MATLAB™ programming software environment (Fig 1a).

Data Preprocessing—The raw intensity of each spot on a filter was imported into the Pathways™ 4.0 software (Research Genetics, Inc.), which was used to correct the local nonspecific binding of the probe to filter for each spot (background correction). Raw intensity data were normalized such that every raw intensity value from a filter was divided by the mean intensity values of that array. For some genes, the use of radiolabeled probes can produce signal bleeding into adjacent spots. We used an algorithm that iteratively identifies signal bleed effects and includes genes found to be free of bleeding effects in at least 70% of all arrays (in preparation). All signals identified as being compromised were excluded from further analysis.

Dimensionality Reduction/Gene Filtering—Following preprocessing, dimensionality in the remaining data was reduced by eliminating those genes least likely to contain relevant discriminant and/or biological information. We first applied, in a supervised manner, a series of simple univariate statistical filters: Student’s t-test, t-test for unequal variances (assumes normal distribution of the data), and the Wilcoxon test (nonparametric distribution-free) were applied without correction for multiple comparisons. Since the distribution of the data among and within replicate experiments and for individual genes cannot be determined accurately in such high dimensions (18), both logarithm transformed and nontransformed data also were compared. The level of significance was set at $p < 0.05$, using the inflated type I error (many false positives) and reduced type II error (few false negatives) to exclude those genes least likely to be truly differentially expressed. A further filter of relative fold changes ≤ 0.56 for down-regulation and ≥ 1.8 -fold for up-regulation was applied to identify those genes most likely to have biologically relevant changes in expression among groups. Following this dimensionality reduction, all spots in all arrays that remained within the reduced dimensional data set, and were determined to be free of signal bleeding, were visually inspected to assess further whether or not these signals should be included for subsequent data analysis. This

approach generated a final reduced dimensional data set of 282 genes (dimensions) that was used for data analysis.

Gene Selection, Data Visualization, and Classifier Construction—While dimensionality reduction applied univariate criteria, for gene selection we used an approach designed to preserve the joint discriminant power of genes within the reduced dimensional data set. Thus, the individual gene selection algorithm excludes genes based on weak joint discriminant power, while the multilayer perceptron (MLP) identifies genes where the joint discriminant power is high (classification accuracy of >70%). The profile selection algorithm first eliminates genes by their lack of contribution to the Fisher's discriminant components of the data set, and further eliminates those genes that least change the trace of a weighted Fisher's scatter matrix (19). Comparisons were made between the low n-3 versus high n-3 PUFA array filters using the mean intensity normalized results obtained from each filter for the genes in the 282-dimensional data set.

We first ran the gene selection algorithm on all samples to select a 20-gene data set for visualization, to assess the likelihood that we would retain sample separability. We obtained 3-dimensional projections by both Principal Component Analyses (PCA) and Discriminant Component Analyses (DCA) (19,20). PCA is an unsupervised method that maximizes the capture of variance within the data set. DCA is a supervised method based on a Fisher separability matrix that uses class information to maximize separation among treated groups while minimizing variability within groups (19,20). For each projection, the reconstruction error was calculated by dividing the sum of the variances captured by the three-top principal or discriminant components by the total variance spanned by all gene dimensions.

We then used a leave-three out method and ran the algorithm through 100 iterations to select 100 gene profiles, each profile containing 20 genes (Fig 1b). At each gene selection iteration, (i) three random samples from each group are excluded, (ii) a 20 gene profile is obtained, (iii) this profile is used as the input data to train an MLP classifier, (iv) each classifier is trained using a leave-two-out method (100 training iterations), and (v) validated against the four samples left out prior to gene selection at the first step in the process. Twenty-gene profiles that produced MLPs with a classification accuracy of >70% for the independent data set were collated into a final gene list. For the classifier step, MLP prediction models were built using three-layers, one output node, and three hidden nodes. Weighted inputs were fed into the hidden layer and then transferred to the outer layer, with both layers using a tan-sigmoid transfer function. The MLP was trained using a Quasi-Newton numerical optimization technique ("trainbfg" Matlab™ routine; a back-propagation method).

Real Time PCR

To ensure that validation was independent of the microarrays, we used RNA from mammary glands not used in the microarray studies, as described above. RNA was extracted from each individual mammary gland and then cDNA reverse-transcribed from 100 µg/ml of total input RNA using Taqman Reverse Transcription Reagents as described by the manufacturer (Applied Biosystems, Foster City, CA). Next, PCR products were generated from the cDNA samples using the Taqman Universal PCR Master Mix (Applied Biosystems) and Assays-on-Demand (Applied Biosystems) for the appropriate target gene. The 18S Assay-on-Demand (Applied Biosystems) was used as an internal control. All assays were run on 384 well plates so that the cDNA sample from each mammary gland was run in triplicate for the target gene and the endogenous control. Real time PCR was performed on an ABI Prism 7900 Sequence Detection System and the results assessed by relative quantitation of gene expression using the $\Delta\Delta C_T$ method.

Immunohistochemistry to determine changes in protein levels

Formalin-fixed tissue sections (5µm) obtained from the 3rd thoracic mammary glands of five 50-day-old rats per group were deparaffinized in xylene, hydrated through graded alcohol and incubated with 3% H₂O₂ for 10 min to block endogenous peroxidases. Non-specific binding was blocked with normal rabbit serum from the Vectastain Elite ABC Kit (Vector Laboratories, Inc.) for 20 min., blocked, incubated with cyclin D1 antibody (1:700) (#RB-212, Lab Vision Corporation, CA) or PPAR γ antibody (1:100) (#H-100, Santa Cruz Biotechnology, CA), washed, treated with biotinylated goat anti-serum to mouse IgG followed by incubation with Streptavidin-peroxidase conjugate (Dako Cytomation The Ark Kit, Carpinteria, CA). Antigen-antibody complexes were visualized by 3' 3-diaminobezidine and counterstained with hematoxylin stain, dehydrated and mounted. Control slide was incubated with normal mouse serum. For cyclin D1, the percentage of positive cells was determined by calculating the number of cells that had positive staining (only darkly stained cells were counted) per 1000 cells per mammary gland structure (terminal end buds, lobules and ducts). PPAR γ expression, in turn, was assessed in terminal end buds, lobules and ducts using a scale of 0–5 for percentage staining and 0–3 for staining intensity. The combined values were used for statistical analysis. Slides were blindly evaluated with help of the Metamorph software.

8-hydroxy-2'-deoxyguanosine (8-OHdG) Enzyme Immunoassay

DNA damage was measured in the serum using an 8-OHdG enzyme immunoassay (Oxis Health Products, Inc., Portland, OR). Serum was centrifuged at 2000 $\times$ g for 90 min and the supernatant collected. The level of 8-OHdG was measured in the supernatant as described by the manufacturer. Each sample was run in triplicate and the mean value was calculated.

Statistical analysis for real time PCR and 8-OHdG

Results for the data obtained on 8-OHdG enzyme, and real time PCR were analyzed with SigmaStat software using one-way analysis of variance (ANOVA), separately at 26- and 50-days of age. Where appropriate, between-group comparisons were done using Tukey's multiple comparisons test. The differences were considered significant if the p-value was less than 0.05. All probabilities were two-tailed.

Results

Mammary tumorigenesis

As previously reported (13), rats fed a low fat n-3 PUFA diet during prepuberty developed significantly fewer mammary tumors than rats fed a high fat n-3 PUFA diet (Fig. 2). Additionally, mammary tumor incidence was significantly lower in the rats fed a prepubertal low fat n-3 PUFA diet compared with the reference diet (p=0.0327). Conversely, rats fed a prepubertal high fat n-3 PUFA diet exhibited significantly increased mammary tumor incidence, compared with the reference diet (p=0.0198). No changes in mammary tumor latency or the number of tumors per rat (tumor multiplicity) were noted between the two groups.

Microarray results

n-3 and n-6 PUFA diets induce different patterns of gene expression—Our first approach to data analysis was to determine whether n-3 and n-6 PUFA diets produce different patterns of gene expression. This comparison would establish our ability to find genetic changes associated with exposure to two different types of PUFAs. Since n-3 and n-6 PUFAs induce significantly different changes in gland morphology (13), we expected this comparison to also produce marked changes in mammary gene expression. Indeed, if we could not separate these two groups, it would be unlikely that we could separate any other groups. Therefore, the initial

goal was to identify a subset of genes differentially expressed between all n-3 PUFA exposed (low + high) and all n-6 PUFA exposed (low + high) mammary glands (Table 1).

To determine if these genes are truly differentially regulated in a meaningful pattern, subgroups of samples were used for gene selection, neural network training and evaluation, as described in the Materials and Methods section. The remaining samples (those not used for gene selection or neural network training/evaluation) were used as an independent data set for testing the neural network classifier.

Fig. 3b shows DCA visualization of the top 20 potentially discriminant genes from the n-3 and n-6 PUFA-exposed glands, capturing 97% of the cumulative variance in the data. To confirm the discriminant power of this data set, a multilayer perceptron (MLP; non-linear neural network classifier) was built to predict in an independent sample set whether they are from an n-3 or n-6 PUFA-exposed mammary gland. We achieved 100% accuracy (no misclassifications) during training, $79 \pm 3\%$ accuracy with the evaluation data set, and $73 \pm 2\%$ accuracy for predicting the dietary exposure in the independent data set.

Low fat and high fat diets induce different patterns of gene expression—We then determined whether some genes are differentially expressed in mammary glands exposed to a high fat vs. a low fat diet irrespective of whether the fat is n-3 or n-6 PUFA (Table 2). We used the same data and data analysis approaches, but combined the gene expression profiles for the analysis such that we compared all low fat (n-3 + n-6 PUFA) with all high fat (all n-3 + all n-6 PUFA) exposed rats. The data in Fig. 3b represent the multidimensional scaling from 20-dimensions to 3-dimensions and capture 80% of the cumulative variance in the data. We used three samples from each group that were not used for either gene selection or network training/evaluation, as the independent data test set. We achieved 100% accuracy (no misclassifications) during training, $78 \pm 3\%$ accuracy with the evaluation data set, and $80 \pm 4\%$ accuracy for predicting the dietary exposure in the independent data sets. The data in Fig. 3b provide compelling evidence that low and high fat diets differentially affect mammary gland gene expression.

Molecular profiles can predict reduced mammary tumor risk conferred by exposure to a low n-3 PUFA diet—Having established the ability of gene expression profiles to discriminate between dietary exposures to n-3 and n-6 PUFAs and between exposures to low and high fat diets, we asked whether we could identify a gene set that discriminates between the effects of diet on susceptibility to mammary carcinogenesis. Thus, we wanted to determine whether we could identify a gene subset that would discriminate between mammary glands with a low cancer susceptibility (low n-3 PUFA diet) and those with a “normal/high” susceptibility (high n-3, low n-6 and high n-6 PUFA diets) (Table 3).

The data in Fig 3c represent the multidimensional scaling from 20-dimensions to 3-dimensions and capture 96% of the cumulative variance in the data. The projection shows that the samples from the two cancer susceptibilities are linearly separable in gene expression data space. Since we have a limited number of replicates in the low n-3 PUFA exposed group, we built and trained a neural network classifier using genes selected from all 6/6 low n-3 PUFA-exposed samples and 8/11 n-6 PUFA-exposed samples. This approach left all high n-3 PUFA and 3/11 n-6 PUFA samples as independent data for classifier testing. We achieved 100% accuracy (no misclassifications) during training and evaluation. While the high accuracy of predicting the low cancer susceptibility (during evaluation) in part reflects the use of all these data for gene selection, the evaluation data is a “leave-two-out” method (2 samples are randomly excluded from each iteration during evaluation of the training set). The independent test data are exclusively from high susceptibility profiles but we achieve a robust $92 \pm 1\%$ accuracy for predicting this phenotype.

Genes differentially expressed in the mammary glands of rats prepubertally fed a low fat n-3 PUFA diet versus a high fat n-3 PUFA diet—In the final analysis, a total of 91 genes (excluding ESTs) were identified as differentially expressed in the mammary glands of rats prepubertally fed a low fat n-3 PUFA diet, when compared to a high fat n-3 PUFA group (Table 4), using the criteria established above. Four separate statistical tests were used to identify these genes. Since PUFAs induce lipid peroxidation that can potentially increase oxidative stress (21,22,23), and we previously demonstrated an increase in lipid peroxidation in these animals (13), we looked at the list of 91 genes specifically for genes with these implied or known functions in the gene subset. Four genes that were found to be up-regulated in the mammary glands of low fat n-3 PUFA-fed rats were directly and/or indirectly associated with protection from oxidative stress: thioredoxin (24), heme oxygenase (25), NADP-dependent isocitrate dehydrogenase (26), and metallothionein III (27). Differential expression of all four genes was confirmed by real time PCR (Fig. 4). All four genes were expressed at a significantly higher level in the 50-day-old prepubertally low fat n-3 PUFA fed rats than in the control rats fed low fat n-6 PUFA diet. Only heme oxygenase was expressed at a lower level in the high fat n-3 PUFA group than in the controls. These changes were not seen in the mammary glands of 26-old day rats. Further, at 50 days of age, none of these genes were differentially expressed between the low and high fat n-6 PUFA groups, but in the microarray analysis they were significantly down-regulated in the mammary glands of high fat n-3 PUFA groups, when compared to all the other three groups (data not shown). To ensure validation, the glands used were obtained from different rats than those from which the RNA was extracted for the microarray analysis.

We also expected the gene expression microarray experiments to implicate other key signaling pathways involved in the effect of dietary exposures on tumorigenicity, such as apoptosis. From within this dataset, our analyses identified cardiotrophin-1 as a candidate gene for further evaluation. Cardiotrophin-1 phosphorylates, and thereby activates, the Akt protein (28), a signaling molecule implicated in several key functions in the mammary gland, including promoting cell survival (29,30). In our earlier study, we found that prepubertal exposure to a high fat n-3 PUFA diet elevates Akt phosphorylation (13). We confirmed differential expression of cardiotrophin-1 mRNA by real time PCR in the high fat n-3 PUFA group ($p < 0.001$) (Fig. 5). This increased expression of cardiotrophin-1 in the 50-day-old mammary glands of the high fat n-3 PUFA group was not observed in the 26-day-old mammary glands of the high fat n-3 PUFA group.

Cyclin D1 and PPAR γ protein levels

Some of the genes in Table 1 are those which have been linked to cell proliferation and differentiation. Instead of confirming their expression using RT-PCR, we decided to assess protein levels of two genes closely linked to cell proliferation: cyclin D1 which increases proliferation and PPAR γ which induces differentiation and reduces cell proliferation. The data indicated that the levels of cyclin D1 were elevated in the mammary glands of rats exposed to a high fat n-3 PUFA diet during prepuberty ($p < 0.001$) (Fig. 6a), consistent with increased cell proliferation reported in these rats (13). PPAR γ protein levels were significantly elevated in the mammary glands of prepubertally low fat n-3 PUFA diet fed rats ($p = 0.035$) (Fig. 6b), and this is in agreement with reduced cell proliferation noted in their mammary glands (13).

BRCA1 Expression

The changes in genes repairing oxidative damage led us to hypothesize that DNA damage repair pathways may be altered. BRCA1 is a tumor suppressor gene that affects DNA damage repair and is strongly implicated in a high proportion of inherited breast cancers (31,32). We measured BRCA1 mRNA expression in the mammary glands of 26 and 50-day-old rats fed low or high n-3 PUFA or n-6 PUFA diets during prepuberty by real time PCR.

26-day-old rats fed the high fat n-3 PUFA diet exhibited a significant decrease in Brca1 expression compared to the reference diet and the low fat n-3 PUFA diet ($p<0.001$) (Fig. 7a). At 50 days of age, there was a significant 30% reduction in Brca1 expression in the rats fed the high fat n-3 PUFA diet compared to the rats fed the low fat n-3 PUFA diet ($p=0.026$), but no significant differences in Brca1 expression were observed between the rats fed either of the n-3 PUFA diets and the reference diet (Fig. 7b).

DNA Damage in PUFA-fed Rats

DNA damage has been associated with increased breast cancer risk (33,34). We measured DNA damage caused by oxidative stress by determining 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in the serum of low and high fat n-3 PUFA fed rats and rats fed the reference diet. Rats fed the high fat n-3 PUFA diet had the highest level of DNA damage at both 26 ($p<0.001$) and 50 days of age ($p<0.001$) (Fig. 8). Furthermore, rats fed the low fat n-3 PUFA diet had the lowest amount of DNA damage at these two ages, when compared with the reference diet-fed rats and the rats fed the high fat n-3 PUFA diet ($p<0.05$).

Discussion

In our earlier study, prepubertal dietary exposure to a low fat n-3 PUFA diet reduced, while a high fat n-3 PUFA diet increased, susceptibility to develop carcinogen-induced mammary tumors in rats (13). These observations were confirmed in the present study. The increase in mammary tumorigenesis in rats fed the high fat n-3 PUFA diet during prepuberty was unexpected, mostly because prepubertal exposure to a high fat n-6 PUFA had no effect on mammary tumorigenesis and n-3 PUFAs are generally considered protective against breast cancer. For example, findings in athymic nude mice show that n-3 PUFAs inhibit the growth of human breast cancer cells (35,36,37). However, a diet containing menhaden oil, when given after carcinogen administration, did not modify the growth of N-nitrosomethylurea-induced mammary tumors in rats (11). Further, when the amount of n-3 PUFAs relative to n-6 PUFAs in the diet was increased, DMBA-induced mammary tumor incidence and tumor weights were increased in rats (12).

To identify molecular pathways that may have mediated the opposing effects of the low and high prepubertal n-3 PUFA exposures on mammary cancer risk, we performed gene microarray analyses. The ability of our microarray analysis to accurately identify independent samples as those obtained from rats fed n-3 PUFA diets *versus* n-6 PUFA diets, and those obtained from rats fed low versus high fat diets, regardless of the source of fat, shows that the genes selected are expressed/repressed in both patterns and at levels consistent with the model. This is an appropriate and rigorous test of the approach, since the initial goal was simply to determine if the molecular profiles are consistently different. Building a multigene predictor is a more efficient and less resource intensive test of the selected genes than would be obtained by confirming expression of multiple genes via real-time PCR or Western assays.

Several genes involved in protecting the mammary gland from oxidative DNA damage were up-regulated in the low fat n-3 PUFA fed rats, supporting the findings that this diet reduced DNA damage and mammary tumorigenesis. The altered genes included thioredoxin, heme oxygenase, metallothionein III, and NADP-dependent isocitrate dehydrogenase. Each of these genes has an antioxidant function, and can protect cells against DNA damage due to oxidative stress and reactive oxygen species (38,39,27,40,41,42). Changes in gene expression were seen only in 50-day-old rats but not in younger rats; i.e., at the time when they were still on the special diets. These results suggest that the changes in antioxidant genes and genes regulating cell survival may reflect functional, long-term changes in the cell membrane fatty acid composition. It also is possible that the changes in gene expression are manifested in the context of mature mammary gland, but not during pubertal development.

Consistent with the increase in expression of antioxidant genes, rats exposed prepubertally to the low fat n-3 PUFA diet incurred lower levels of DNA damage, as indicated by the reduced levels of 8-OHdG. This observation could also reflect several other differentially altered functions, besides a change in the antioxidant genes. For example, the high fat n-3 PUFA diet might have induced high levels of DNA damage as a consequence of the increasing lipid peroxidation (43,44) and a decrease in apoptosis (13). The failure of cells to undergo apoptosis in the prepubertally high fat n-3 PUFA diet fed rats may be related to the observation that cardiotrophin-1 was up-regulated in their mammary glands. Cardiotrophin-1 phosphorylates Akt, leading to promotion of cell survival (28). We confirmed the increase in cardiotrophin-1 expression by real time PCR. Previously, we have shown that phosphorylated Akt is increased in the mammary glands of rats fed the high fat n-3 PUFA diet during prepuberty (13). Increased activation of Akt in the prepubertally high fat n-3 PUFA-fed rats implies that signaling through this pathway could be inhibiting apoptosis in cells with damaged DNA, with a consequent increase in tumorigenesis.

The effect of prepubertal exposure to a high fat n-3 PUFA diets on DNA damage repair pathways was also assessed by measuring the level of BRCA1 mRNA expression. Germline BRCA1 mutations are linked to familial breast cancers (45,46), reflecting its function in DNA damage repair and recombination, processes related to maintenance of genomic integrity, control of cell proliferation, and regulation of gene transcription (47,31). A previous study has reported up-regulation of BRCA1 expression by n-3 PUFAs in breast cancer cell lines, but a reduced expression in normal mammary cells (48). We observed a decrease in *Brca1* mRNA expression in the mammary glands of rats exposed prepubertally to the high fat n-3 PUFA diet, and this could have further contributed to the increased levels of DNA damage in these rats. Thus, both the down-regulation of antioxidant genes, and impairment in the DNA repair mechanisms in the prepubertally high fat n-3 PUFA diet fed rats might have contributed to the increase in their mammary tumorigenesis. Up-regulation of cyclin D1 expression in these rats might also be involved in increasing their mammary tumorigenesis, and perhaps explains the increase in cell proliferation noted in these rats (13).

The results generated in this study also shed light as to why low fat n-3 PUFA diet, when consumed prior to puberty onset, reduces mammary cancer risk, even though it increases lipid peroxidation (13). Cells have multiple ways to defend themselves from the toxic effects of free radicals, including increased activation of heme oxygenase (HO) which in turn generates antioxidant products. We found that during adulthood, rats fed a low fat n-3 PUFA diet during prepuberty, exhibited increased expression of HO and several antioxidants, suggesting that this dietary exposure can cause a permanent up-regulation of antioxidant genes. Further, low fat n-3 PUFA groups exhibited reduced expression of cardiotrophin-1, promoting apoptosis, and perhaps explaining why rats exposed to a low fat n-3 PUFA diet during prepuberty exhibited reduced levels of DNA adducts. Our results also implicated up-regulation of PPAR γ expression in the mammary glands of these rats. n-3 PUFAs act as ligands for this receptor (16) which is known to inhibit cell proliferation and induce differentiation (16). Thus, elevated PPAR γ levels in the mammary glands of rats fed a low fat n-3 PUFA diet during pregnancy may be linked to their reduced mammary cancer risk.

In conclusion, we studied plausible mechanisms explaining why rats fed prepubertally a low fat n-3 PUFA diet are at reduced mammary cancer risk and why those fed a high fat n-3 PUFA diet are at an increased risk. Based on the findings, increased antioxidant gene expression is strongly implicated in the reduction in mammary cancer risk. The increase in risk, in turn, may be caused by increased cell proliferation and survival, and DNA damage. We further show that the levels of *Brca1* expression are reduced in these rats. Together these changes could explain why rats prepubertally fed the high fat n-3 PUFA diet subsequently are at an increased risk of developing mammary cancer.

Acknowledgments

The work was supported by a predoctoral fellowship grant from Department of Defense to S.O.M. and grants to L.H.C. from NCI (5 RO1 CA89950 and 1 U54 CA100970), the Susan G. Komen Breast Cancer Research Foundation and American Institute for Cancer Research. S.O.M. is currently a recipient of a National Cancer Institute Cancer Prevention Fellowship.

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Figure 1.

A) Microarray data analysis procedure. Data was preprocessed such that raw intensity values were divided by the mean intensity values for that array for normalization. Dimensionality was reduced by eliminating ESTs and genes least likely to contain relevant biological information. Gene filtering was accomplished by applying a series of univariate statistical filters, setting the level of significance at $p < 0.05$ with additional filtering of fold changes ≤ 0.56 down-regulation and ≥ 1.8 fold up-regulation. **B)** Multilayer Perceptron (MLP) classification for separation of prepubertal dietary treatment groups. A gene selection algorithm was run on all samples to select a 20-gene data set for visualization that was then used to train an MLP classifier. The classifier was trained using the leave-two-out method (100 training iterations) and then validated against the 4 samples left out prior to gene selection at the first step. Finally, the 20 top potentially discriminant genes were used in a DCA-based multidimensional scaling algorithm to visualize the data set for separability.

Figure 2. DMBA-induced mammary tumor incidence in the rats exposed to a low fat or high fat n-3 or n-6 PUFA diets between postnatal days 5 and 25. Tumor incidence was analyzed using Kaplan-Meier's survival analysis, and was significantly increased in the high fat n-3 PUFA group ($\chi^2=10.5$, $p=0.0148$). The low fat n-6 PUFA diet is the reference diet.

Figure 3. Discriminant component analysis (DCA) of the molecular profiles in the mammary gland of 50-day-old rats exposed prepubertally to low or high-fat n-3 or n-6 PUFA diets. **A)** The molecular profile of n-3 PUFA versus n-6 PUFA-exposed mammary glands; **B)** the molecular profile of low versus high fat mammary glands; and **C)** the molecular profile of glands of rats exhibiting low (low fat n-3 PUFA) versus normal/high susceptibility (all the other groups) to develop mammary cancer. The top 20 potentially discriminant genes were selected and visualized via DCA to evaluate whether these profiles project into separable data space.

Figure 4.

Expression of genes involved in oxidative damage repair; i.e., thioredoxin (**A**); heme oxygenase (**B**); NADP-dependent isocitrate dehydrogenase (**C**); and metallothionein III (**D**), in the mammary glands of 50-day-old rats exposed prepubertally to low or high fat n-3 PUFA diet or the reference low fat n-6 PUFA diet, studied using real time PCR. Data were normalized to 18S and expressed as a fold difference compared to the reference diet using the $\Delta\Delta C_T$ method. Values represent the mean of twelve rats per group $\pm$ SEM. One-way ANOVA: **A**, **B**, **C**, **D**, $p < 0.001$. **a, b, c** represent dietary treatments that are significantly different from one another ($p < 0.05$).

Figure 5.

Cardiotrophin-1 expression in the mammary glands of 26- and 50-day-old rats exposed prepubertally to a low or high fat n-3 PUFA diet or the reference low fat n-6 PUFA diet. Cardiotrophin-1 expression was examined through real time PCR. Data were normalized to 18S and expressed as a fold difference compared to the reference diet using the $\Delta\Delta C_T$ method. Values represent the mean of twelve rats per group $\pm$ SEM; One-way ANOVA: cardiotrophin-1, $p < 0.001$. **a** and **b** represents dietary treatments that are significantly different from one another ($p < 0.05$) as determined via a Tukey test.

Figure 6.

A) Cyclin D1 and **B)** PPAR γ expression in the mammary glands of 50-day-old rats. Protein levels were determined using immunohistochemistry and as the percentage of cells staining positive for cyclin D1/PPAR γ , or using a combined visual scale to determine percentage of positive cells and staining intensity for PPAR γ . **a**, **b**, and **c** represent dietary treatments that are significantly different from one another ($p < 0.05$) as determined by a Tukey test.

Figure 7.

Brca1 expression in the mammary glands of (A) 26- and (B) 50 day-old rats exposed prepubertally to a low or high fat n-3 PUFA diet or the reference low fat n-6 PUFA diet. Brca1 expression was examined through real time PCR. Data were normalized to 18S and expressed as a fold difference compared to the reference diet using the $\Delta\Delta C_T$ method. Values represent the mean of twelve rats per group $\pm$ SEM. **A)** One-way ANOVA: $p < 0.001$; **B)** one-way ANOVA: $p < 0.026$. **a** and **b** represent dietary treatments that are significantly different from one another ($p < 0.05$) as determined by a Tukey test.

Figure 8. 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in the serum of (A) 26- and (B) 50-day-old rats exposed prepubertally to a low-fat or high-fat n-3 PUFA diet or the reference low fat n-6 PUFA diet. Values represent the mean of twelve rats per group $\pm$ SEM. One-way ANOVA: $p < 0.001$ at both ages. **a, b, c** are dietary treatments that are significantly different from one another ($p < 0.05$) as determined by a Tukey test.

Top 20 gene list from gene profiles generated by Wang Gene Selection-MLP iterations separating n-3 versus n-6 PUFAs

Table 1

Probe_ID	Acc	UGCluster	Name	Symbol
5275	AA899822	Rn.153980	Tripartite motif-containing 35	Trim35
5032	AA923885	Rn.164851	Transcribed locus	
3490	AA818634	Rn.1408	Enoyl Coenzyme A hydratase domain containing 2	Echdc2
3128	AA818572	Rn.1078	Transcribed locus	
3123	AA818548	Rn.106849	Interleukin 33	Il33
2793	AA818743	Rn.79807	Dystonin	Dst
2788	AA818736	Rn.38987	Pinin	Pnn
2787	AA818727	Rn.162119	Transcribed locus	
2705	AA819828	Rn.22432	Transcribed locus	
2681	AA859035	Rn.8398	ATP-binding cassette, sub-family G (WHITE), member 1	Abcg1
2535	AA958011	Rn.88085	Mitogen activated protein kinase 14	Mapk14
2456	AA858736	Rn.3504	Response gene to complement 32	Rgc32
2389	AA818475	Rn.1889	DC2 protein	Dc2
2225	AA997865	Rn.10627	Thymosin beta-like protein 1	Tmsbl1
2070	AA818893	Rn.33877	EST	
1540	AA998890	Rn.6589	Annexin A3	Anxa3
395	AA818398	Rn.6036	Glutathione S-transferase, mu type 3	Gstm3
271	AA817875	Rn.98782	Transcribed locus	
168	AA998372	Rn.10877	Dual specificity phosphatase 5	Dusp5
29	AA819165	Rn.1659	Histone cluster 1, H4b	Hist1h4b

Table 2

Top 20 gene list from gene profiles generated by Wang Gene Selection-MLP iterations separating low fat versus high fat

Probe_ID	Acc	UGCluster	Name	Symbol
1651	AA818361	Rn.23906	Similar to WD repeat domain 36	LOC688637
249	AA866228	Rn.3005	Sushi domain containing 3	Susd3
862	AA998630	Rn.11350	Rat VL30 element mRNA	
1496	AA925096	Rn.3973	Ribosomal protein L29	Rpl29
1378	AA818949	Rn.20419	DnaI (Hsp40) homolog, subfamily B, member 12	Dnajb12
881	A1044516	Rn.144629	Mitogen-activated protein kinase 7	Mapk7
1982	AA818369	Rn.2578	Heat shock factor binding protein 1	Hsbp1
159	AA998118	Rn.6534	Myosin light chain, phosphorylatable, fast skeletal muscle	MyIpf
242	AA866277	Rn.3036	Guanine nucleotide binding protein (G protein), alpha inhibiting 2	Gnai2
259	AA859109	Rn.147231	Transcribed locus	
817	AA956549	Rn.5834	Cyclin G1	Ccng1
385	AA818858	Rn.118772	Transcribed locus, strongly similar to NP_058797.1 peptidylprolyl isomerase A [Rattus norvegicus]	
1770	AA819262	Rn.203031	Transcribed locus	
151	AA965256	Rn.84920	Myosin, light polypeptide 1	Myl1
2186	AA955550	Rn.6686	Cytochrome c oxidase subunit Vb	Cox5b
2299	A1136540	Rn.15488	Troponin T3, skeletal, fast	Tnnt3
2033	AA818986	Rn.33913	EST	
4947	AA923927	Rn.204981	Transcribed locus	
772	AA899852	Rn.5820	Granulin	Grn
2199	AA957962	Rn.98989	Secreted acidic cysteine rich glycoprotein	Sparc

Table 3

Top 20 gene list from gene profiles generated by Wang Gene Selection-MLP iterations separating low n-3 versus all others

Probe_ID	Acc	UGCluster	Name	Symbol
242	AA866277	Rn.3036	Guanine nucleotide binding protein (G protein), alpha inhibiting 2	Gnai2
1020	AA819884	Rn.2285	Transcribed locus	
231	AI136065	Rn.32973	Arrestin, beta 2	Arrb2
249	AA866228	Rn.3005	Sushi domain containing 3	Susd3
1500	AA925099	Rn.55127	Platelet derived growth factor receptor, alpha polypeptide	Pdgfra
3640	AA901378	Rn.8181	Transcribed locus	
1380	AA818954	Rn.16576	Transcribed locus	
245	AA866227	Rn.99722	Similar to RIKEN cDNA 1110005A03	RGD1306284
319	AA819862	Rn.4182	Mitochondrial carrier homolog 2 (C. elegans)	Mtch2
192	AI045437	Rn.9714	Neuropeptide Y	Npy
3120	AA818538	Rn.42527	EST, Weakly similar to T-CELL SURFACE GLYCOPROTEIN E2 PRECURSOR [H. sapiens]	
385	AA818858	Rn.118772	Transcribed locus, strongly similar to NP_058797.1 peptidylprolyl isomerase A [Rattus norvegicus]	
1200	AA998869	Rn.53971	Signal-regulatory protein alpha	Sirpa
1640	AA819591	Rn.7444	Transcribed locus, moderately similar to NP_002847.1 poliovirus receptor related 2 isoform alpha precursor [Homo sapiens]	
859	AA998607	Rn.11133	Amino adipate aminotransferase	Aadat
1370	AA818937	Rn.203008	Transcribed locus	
2200	AA963906	Rn.10529	RNA binding motif protein 16	Rbm16
691	AA818526	Rn.16849	Ring finger protein 146	Rnf146
3470	AA818571	Rn.2295	Transcribed locus	
151	AA965256	Rn.84920	Myosin, light polypeptide 1	Myl1

Table 4

List of genes that were differentially expressed between the mammary glands of rats exposed to a low versus high fat n-3 PUFA diet prepubertally, $p < 0.05$

GENE ID	ACCESSION NUMBER	UNIGENE NUMBER	GENE NAME	LN3/HN3	cell cycle proliferation	apoptosis	differentiation
Up-regulated genes							
1237	AI059137	Rn.8737	Myr3 for myosin I heavy chain (Myole)	78.1497			
2476	AA859285	Rn.2661	Macrophage migration inhibitory factor (Mif)	77.9136		x	x
922	AI071262	Rn.11349	Trans-Golgi network integral membrane protein (Tgolin1)	64.705			
1208	AA998830	Rn.11204	Brain expressed myelocytomatosis oncogene (Bmyc)	51.2979			
1539	AA998895	Rn.3539	Nucleoplasmin-related protein (Nuclear protein B23)	50.9449			
1191	AA997450	Rn.34521	G protein-coupled receptor kinase interactor 1 (GIT1)	49.9268			
2195	AA955902	Rn.9493	Myogenic differentiation 1 (Myod1)	45.0848			x
475	AA956941	Rn.10450	New England Deaconess E-box binding factor	42.5661			
575	AI070721	Rn.6281	Glial cell line-derived neurotrophic factor receptor alpha (Gfra4)	37.5564			
821	AA956736	Rn.2432	Fyn proto-oncogene (Fyn)	35.4852			x
1136	AA923854	Rn.3446	unc-50 homolog (Unc50)	31.3974			
1833	AA901147	Rn.11305	Atrophin-1 (Atn1)	28.9757			x
142	AA963308	Rn.40942	Latent transforming growth factor beta binding protein 1 (Ltbp1)	26.7251			
1809	AA874828	Rn.10962	FB1 osteosarcoma oncogene B (Fosb)	26.1847		x	x
1498	AI111925	Rn.25905	Carboxypeptidase D (Cpd)	20.7826			
1808	AA875109	Rn.6977	Pleomorphic adenoma gene-like 1 (Plagl1)	17.6601	x	x	x
2470	AA875142	Rn.761	5HT3 receptor	17.012			
731	AA817997	Rn.1214	Ribosomal protein L24	10.9046			
447	AA924772	Rn.11325	Metallothionein-III (Mt3)	9.3542			x
2455	AA858723	Rn.2366	acyl-CoA synthetase long-chain family member 1 (Acsl1)	8.5659			
402	AA900189	Rn.2140	cell division cycle 37 homolog (cdc37)	7.6244	x		
2147	AA874884	Rn.3160	Heme oxygenas (Hmox)	7.5387		x	x
1582	AI044828	Rn.967	Thioredoxin nuclear gene encoding mitochondrial protein	6.7203			
214	AI070507	Rn.8778	Fibromodulin (Fmod)	6.025			
1464	AA874962	Rn.3189	Nucleoporin 210 (Nup210)	5.3968			
1158	AA955301	Rn.2342	Mannoside acetylglucosaminyltransferase (ngat2)	5.3782			

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785	AA901316	Rn.7223	DCoH gene	5.0508			
2584	AI045303	Rn.13808	Coronin 7 (Coro 7)	4.2594			
2246	AI045830	Rn.35934	Aconitase 1 (Aco1)	4.2058			
231	AI136065	Rn.32973	Arrestin, beta 2 (Arbb2)	3.9432		x	
242	AA866277	Rn.3036	Guanine nucleotide binding protein (G protein), alpha inhibiting 2 (Gnai2)	3.7385		x	
2101	AA818096	Rn.241	Quinoid dehydropteridine reductase (Qdpr)	3.1367			
2466	AA875135	Rn.2803	ADP-ribosylation factor-like 5A (Arl5a)	2.9366			
813	AA926255	Rn.3520	Meprin 1 beta (Mep1b)	2.9173			
510	AA998213	Rn.10248	A kinase (PRKA) anchor protein 8 (Akap8)	2.6375	x		
859	AA998607	Rn.11133	Aminoacidpate aminotransferase (Aadat)	2.5544			
2141	AA900555	Rn.31745	N-ethylmaleimide sensitive fusion protein attachment protein alpha (Napa)	2.4051			x
1767	AA819242	Rn.1692	Glutathione synthetase (Gss)	2.3836			
208	AI070102	Rn.8653	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide (Ywhab)	2.37			
2186	AA955550	Rn.6686	Cytochrome c oxidase subunit (Cox5b)	2.2624			
5006	AA900426	Rn.2778	Ubiquitin-conjugating enzyme E2D (UBC415 homolog, yeast) (Ubc2d3)	2.1154			
4956	AA899334	Rn.2661	Macrophage migration inhibitory factor (Mif)	2.096			
146	AA965204	Rn.4037	Complement component 1, s subcomponent (C1s)	2.0794			x
1129	AA923894	Rn.6477	Telone4	2.0759			
1800	AA874973	Rn.40118	Nuclear protein E3-3	2.0512			
827	AA956889	Rn.1904	Adenylate cyclase 4 (Adcy4)	2.0485			
385	AA818858	Rn.1463	Peptidylprolyl isomerase A (Ppia)	1.96			
455	AA925731	Rn.3561	Isocitrate dehydrogenase 1 (NADPH), soluble (Idh1)	1.9171			
2207	AA957519	Rn.555	Stathmin 1 (Stmn1)	1.9014	x		x
4861	AA818749	Rn.1457	Nuclear transcription factor-Y gamma (Nfyγ)	1.8462			
837	AA964578	Rn.28	Calpactin I heavy chain	1.796			
1469	AA899597	Rn.484	Ribosomal protein L18 (Rpl18)	1.7523			
4567	AA900379	Rn.22304	HLA-B-associated transcript 3 (BAT3)	1.6102		x	x
1477	AA924274	Rn.4206	Ribosomal protein L22 (Mrpl22)	1.5434			
1218	AI043796	Rn.9686	Solute carrier family 18 A2 (vesicular monoamine), member 2 (Slc18a2)	1.5382			

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794	AA924727	Rn.2953	Collagen, type 1, alpha 1 (Col1a1)	1.4963			
216	AI070517	Rn.10421	Sensory neuron synuclein	1.486			
818	AI112794	Rn.11014	Dynein cytoplasmic 1 intermediate chain 2 (Dync1i2)	1.4444			
140	AA963258	Rn.6282	Insulin-like growth factor I (IGF-I)	1.3394		x	x
2450	AA858866	Rn.40132	5'-nucleotidase ecto (Nt5e)	1.3057			
143	AA963506	Rn.32904	Endoplasmic reticulum protein 29 (Erp29)	1.2564			
1096	AA819420	Rn.2042	Ras homolog gene family, member B (Rhob)	1.2352	x	x	
2481	AA874917	Rn.783	Biglycan (Bgn)	1.2301			
5237	AA858896	Rn.2464	Fetuin beta (Fetub)	1.1707			
832	AA964162	Rn.10838	Sphingomyelin phosphodiesterase 3 (Smpd3)	1.0946	x		
1150	AI111917	Rn.10524	Solute carrier family 16 (monocarboxylic acid transporters), member 7 (Slc16a7)	1.0614			
Down-regulated genes							
1880	AA964350	Rn.32984	Natriuretic peptide receptor 2 (Npr2)	0.0736			
5030	AA924006	Rn.40532	Tissue inhibitor of metalloproteinase 3 (Timp3)	0.1238			
1928	AI045770	Rn.11281	Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit (Cacna1a)	0.1877			
2586	AI043606	Rn.3729	Chymotrypsinogen B1 (Crb1)	0.2197			
487	AA963451	Rn.11366	Opioid binding protein/cell adhesion molecule-like (Opcml)	0.2347		x	
2579	AI029586	Rn.9781	Cholecystokinin (CCK)	0.2914		x	
2462	AA875115	Rn.2491	Endothelial differentiation, sphingolipid G-protein-coupled receptor 5 (Edg5)	0.37			
1818	AA901195	Rn.11080	Hydroxymethylbilane synthase (Hmbs)	0.3706			
2514	AA925476	Rn.32253	Coiled-coil domain containing 56 (Ccdc56)	0.3891			
217	AI070783	Rn.31889	RAB 3A interacting protein (Rab3ip)	0.3982			
526	AI030725	Rn.13361	PDZ and LIM domain 3 (Pdlim3)	0.3989			
1463	AA875174	Rn.2816	Ras-related GTP binding A (Rraga)	0.4141		x	x
2618	AI146173	Rn.2618	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit (Atp5b)	0.4163			
2607	AI059029	Rn.10379	Glycine receptor, alpha 2 subunit (Gira2)	0.4208		x	
2453	AA858888	Rn.2458	Tubulin, beta 5 (Tubb5)	0.4327			
2611	AI070064	Rn.10066	Aquaporin 5 (Aqp5)	0.4341			
2204	AA957589	Rn.11365	Erythropoietin (Epo)	0.4557			x

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1897	AI028940	Rn.11267	Vascular cell adhesion molecule 1 (Vcam1)	0.4765			
2544	AA965091	Rn.10071	Protein kinase N1 (PKN1)	0.5002			
2780	AA819300	Rn.33239	squalene epoxidase (Sqle)	0.5081			
223	AI071126	Rn.10253	Cardiotrophin-1 (Ctfl)	0.5094			x
812	AA926359	Rn.25722	Receptor-linked protein tyrosine phosphatase (PTP-P1)	0.5498			
2483	AA874919	Rn.3174	MutS homolog 2 (E. coli) (Msh2)	0.573	x	x	x
1147	AA924911	Rn.11103	Alpha cardiac myosin heavy chain	0.7165			x

Bolded genes were confirmed via real-time PCR