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Reproductive abnormalities in mice expressing omega-3 fatty acid desaturase in their mammary glands

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Abstract The *Caenorhabditis elegans* n-3 fatty acid desaturase (Fat-1) acts on a range of 18- and 20-carbon n-6 fatty acid substrates. Transgenic female mice expressing the *Fat-1* gene under transcriptional control of the goat *β*-casein promoter produce milk phospholipids having elevated levels of n-3 polyunsaturated fatty acids (PUFA). However, females from this line were also observed to have impaired reproductive performance characterized by a smaller litter size $(2.7 \pm 0.6$ vs. 7.2 ± 0.7; $P < 0.05)$ than wildtype controls. While there is a close association between PUFA metabolism, prostaglandin biosynthesis, and fertility; reproductive problems in these mice were unanticipated given that the *Fat-1* transgene is primarily expressed in the lactating mammary gland. Using multiple approaches it was found that *Fat-1* mice have normal ovulation and fertilization rates; however fewer embryos were present in the uterus prior to implantation. Small litter size was also found to be partly attributable to a high incidence of post-implantation fetal resorptions. Embryo transfer experiments revealed that embryos developing from oocytes derived from transgenic ovaries had an increased rate of post-implantation resorption, regardless of the uterine genotype. Ovary transplantation between *Fat-1* and C57BL/6 wildtype females revealed that non-ovarian factors also contributed to the smaller litter size phenotype. Finally, surgical removal of the mammary glands from juvenile *Fat-1* mice increased the subsequent number of implantation sites per female, but did not lessen the high rate of post-implantation resorptions. In conclusion, we herein report on a system where an exogenous transgene expressed predominately in the mammary gland detrimentally affects female reproduction, suggesting that in certain circumstances the mammary gland may function as an endocrine regulator of reproductive performance.

Abbreviations

PUFA	Polyunsaturated fatty acid
PG	Prostaglandin
LA	Linoleic acid
ALA	α-Linoleic acid
COX	Cyclooxygenase
AA	Arachidonic acid
DGLA	Dihomo-gamma-linolenic acid
EPA	Eicosapentaenoic acid

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Introduction

A successful pregnancy outcome reflects the ovulation of a competent oocyte, fertilization, pre-implantation development, implantation, and post-

implantation growth of the fetus. Deficiencies at any of these stages can lead to decreased reproductive performance or reproductive failure. All of these events are potentially influenced by dietary polyunsaturated fatty acids (PUFA) where the relative proportions of n-6 and n-3 PUFA in the diet can affect fertility (Wathes et al. 2007). Oocytes and sperm contain high levels of PUFA, and the PUFA composition of cell membranes is critical during fertilization. Further, dietary PUFA content has been reported to affect follicle size (Bilby et al. 2006) and number (Robinson et al. 2002) in dairy cattle. Supplementing the diets of mice with n-3 PUFA decreased fertilization in vivo and subsequent development of the pre-implantation embryo (Wakefield et al. 2008). Exposing oocytes to an environment high in n-3 PUFA adversely affected embryo morphology and their ability to develop to the blastocyst stage.

As PUFA are the substrates for prostaglandin (PG) biosynthesis (reviewed by Wathes et al. 2007), their intake can also influence PG availability. Prostaglandins have multiple roles in female reproduction including ovarian, uterine, placental, and pituitary function. Dietary PUFA become incorporated into cell membrane phospholipids and are the substrates for PG biosynthesis. The more biologically active 2-series PGs are synthesized from n-6 PUFA. The less active 1- and 3-series PGs are derived from dihomo-gamma-linolenic acid (DGLA; 20:3) and eicosapentaenoic acid (EPA: 20:5 n-3), respectively. It has been found that uterine expression of cyclooxygenase (COX)-2, the rate-limiting enzyme in the conversion of AA to prostacyclin, is essential for normal implantation and decidualization (Lim et al. 1999). Prostaglandins are known to influence oviduct contractility which facilitates sperm and embryo transport through the oviduct (Wijayagunawardane et al. 2001). Arachidonic acid (AA: 20:4 n-6) is the preferred substrate for COX-2.

Arachidonic acid is also the preferred substrate for the *Caenorhabditis elegans* n-3 fatty acid desaturase (*Fat-1*) that acts on a range of 18- and 20-carbon n-6 fatty acids (Spychalla et al. 1997). This integral membrane protein targets n-6 fatty acids at the 2-position of phospholipids in cellular membranes. Transgenic mice expressing the omega-3 fatty acid desaturase (*Fat-1*) gene from *Caenorhabditis elegans* under the control of the goat beta-casein promoter produce milk with elevated levels of omega-3 polyunsaturated fatty acids (Kao et al. 2006). Herein, it is

reported that *Fat-1* transgenic female mice also display impaired fertility and multiple reproductive defects. We further dissect the influence of transgene presence in different tissues on reproductive performance, and postulate that transgene overexpression in the mammary gland could be implicated with the impaired fertility phenotype.

Materials and methods

Animals

Animal experimentation was conducted in accordance with the regulations of the American Association for Accreditation of Laboratory Animal Care in fully-accredited facilities at the University of California, Davis. Transgenic mice (C57BL/6J × DBA) expressing the *Caenorhabditis elegans* n-3 fatty acid desaturase gene (GenBank accession number L41807) under the control of the goat β-casein promoter (Roberts et al. 1992) from the pBC1 mammary expression vector (Invitrogen, Carlsbad, CA) were generated by pronuclear microinjection as described (Kao et al. 2006). All transgenic *Fat-1* mice used in these experiments were heterozygous females derived from backcrossing the only founder male found to have a milk phenotype to C57BL/6 females, while controls were C57BL/6 wildtype (WT) female mice. Mice were fed a standard rodent diet (Formulab Chow 5008, Ralston Purina Co., St. Louis, MO). Southern banding patterns from transgenic mice revealed that two copies of the transgene integrated at a single insertion site in a tail to tail orientation (Kao et al. 2006). Transgene transmission rates from heterozygous individuals in this line were never seen to differ from 1:1, further supporting a single transgene insertion site.

Baseline reproductive parameters

To assess fetal parameters at mid-gestation, *Fat-1* ($n = 11$) and WT ($n = 5$) female mice were bred to fertile males and sacrificed at 12 days post coitus (DPC). The day of seminal plug detection was defined as 0.5 DPC. Following sacrifice, the total number of normally-developed and resorbing fetuses was determined. Females were determined to be pregnant if there was at least 1 viable pup present within the uterus at 12 DPC.

Ovulation rate

Transgenic *Fat-1* ($n = 9$) and WT ($n = 7$) female mice were housed with fertile WT males. Females were checked daily for the presence of a vaginal plug and were sacrificed at 0.5 DPC. Oviducts were isolated and placed into a drop of M2 media (Sigma-Aldrich; St. Louis, MO) supplemented with 10 mg/ml hyaluronidase (Sigma) to aid in dissociation of the cumulus mass. The oviduct was then flushed, the cumulus cells dissociated, and the total number of ovulated oocytes counted using a dissecting microscope.

Blastocyst recovery/embryonic development

Embryonic development was determined by flushing uterine tracts from *Fat-1* ($n = 12$) and WT ($n = 3$) females at 3.5 DPC. The uterine tract was dissected and placed into a drop of M2 media and the utero-tubal junctions cut to allow for the flow of media out of the horns upon flushing. A 27 gauge needle was navigated through the cervix and into the lumen of one uterine horn where M2 media was gently released from the syringe to flush embryos into a Petri dish. The process was then repeated on the opposite horn. The number of unfertilized oocytes and developing embryos was quantified using a dissecting microscope.

Embryo collection, in vitro culture and transfer

Six-week old *Fat-1* ($n = 12$) and WT ($n = 12$) female mice were superovulated by injection of 5 IU pregnant mare serum gonadotropin (Sigma) followed by 5 IU of human chorionic gonadotropin (Sigma) 48 h later. Females were housed individually with a fertile male following human chorionic gonadotropin injection, and were sacrificed the following morning (0.5 DPC) upon detection of a vaginal plug. Oviducts were isolated and placed into a drop of M2 media containing 10 mg/ml hyaluronidase to dissociate the cumulus mass. The oviduct was ruptured and the resulting 1-cell embryos were collected and washed through three additional drops of M2 media to remove excess hyaluronidase. Embryos were then placed into drops of potassium simplex optimized medium (Specialty Media, Phillipsburg, NJ) under mineral oil and cultured at 37°C, 5% CO_2 in a humidified atmosphere until 3.5 DPC at which point they were transferred into pseudopregnant recipients. Pseudopregnant transgenic

and WT females ($n = 5$–6 recipients/group) served as recipients for either WT or *Fat-1* embryos at 3.5 DPC. Psuedopregnancy was induced by mating recipients with vasectomized males. The uterus was then exposed through a paralumbar incision before cultured embryos (6.4 ± 0.3 embryos/recipient) were transferred into the uterus of each recipient. Females were allowed to recover and were sacrificed at 12 DPC for determination of the total number of viable and resorbing fetuses.

Ovary transplants

Ovaries were transplanted between 6 week old *Fat-1* ($n = 10$) and WT ($n = 10$) females as described by Cargill et al. (1999) to compare the reproductive potential of WT females harboring *Fat-1* ovaries, and *Fat-1* females harboring WT ovaries. In addition, C57BL/6 WT ($n = 10$) mice underwent a sham surgery where both ovaries were swapped between two individual WT females. Following surgery, females were recovered for 2 weeks, at which point they were mated and post-partum litter sizes recorded. All dams were rebred and sacrificed at 12 DPC of their second gestation for the assessment of fetal development parameters, as described above.

Surgical mastectomy

To determine whether transgene expression in the mammary glands was affecting reproductive function *Fat-1* ($n = 11$) and WT ($n = 10$) female mice were mastectomized at 21 days of age. At this age the mammary epithelium is positioned about the nipple and can be surgically-resected to leave a "cleared mammary fat pad" (Faulkin and Deome 1960). Briefly, a Y-shaped incision was made along the ventral midline to the rear legs under ketamine/xylazine anesthesia. After reflecting back the skin, the epithelium-containing region of each of all ten mammary glands was removed by cautery before the incision was closed with sutures. After recovery, females were mated to fertile males at 42 days of age, where the day of mating was determined by the presence of a vaginal plug. Females were sacrificed at 12 DPC, at which point the number of viable and resorbing fetuses was determined. As a further control, *Fat-1* females ($n = 19$) that did not undergo surgical mastectomy were mated and the total number of viable and

resorbing fetuses was determined following sacrifice at 12 DPC.

Transgene mRNA expression analysis

Expression of mRNA from the *Fat-1* transgene in the uterus, ovary and mammary glands was assessed by RT-PCR. Extraction of RNA from tissues was performed by the single-step extraction method (Chomczynski and Sacchi 1987). First-strand cDNA synthesis was performed using 1 to 3 μg of RNA, 0.5 μg oligo(dT)$_{18-20}$, 10 mM dNTP mix, and sterile water to a total volume of 14 μL. The mixture was heated to 65°C for 5 min before adding 4 μL of 5× first-strand buffer, 0.1 M dithiothreitol, 200U Super-Script III reverse transcriptase (Invitrogen, Carlsbad, CA). The mixture was then heated to 50°C for 60 min, followed by heat inactivation at 70°C for 15 min. cDNA samples were subjected to PCR using a goat β-casein exon 1 forward primer (5′TCCATT CAGCTTCTCCTTCA3′) and a *Fat-1* internal reverse primer (5′TTCCATGATGGCATTGCTT′). These primers were designed to span the transgenic goat β-casein intron 1 such that spliced mRNA would yield a 634-bp fragment whereas genomic DNA would produce a 2,655-bp fragment. β-actin forward (5′GACGGCCAGGTCATCACTAT3′) and reverse (5′AGTCCGCCTA-GAAGCACTTG3′) primers that generate a 406-bp amplicon from the β-actin gene served as a positive control.

Milk phenotype

To confirm the milk phenotype of transgenic mice, milk samples were collected from lactating (day 10–12 of lactation) transgenic β-casein *Fat-1* mice ($n = 6$), a line of mice expressing the *Fat-1* gene under the control of the CMV (enhancer)/β-actin promoter (Kang et al. 2004) ($n = 8$), and WT ($n = 9$) females that had all been raised on a standard rodent diet (Formulab Chow 5008, Ralston Purina). Milk phospholipid fatty acid composition was analyzed by gas chromatography according to Kao et al. (2006).

Statistical analysis

Data were analyzed with the JMP 8.0 statistical analysis program (SAS Institute, Cary, NC) using one-way ANOVA. Significant differences were determined using Student's t test at $P < 0.05$.

Results

Baseline reproductive parameters

Pregnant *Fat-1* and WT females were sacrificed at 12 DPC and the uterine tracts removed and inspected (Fig. 1). A number of differences were noted in *Fat-1* females, including a greater incidence of resorbing fetuses (1.9 ± 0.8 vs. 0.0 ± 0.0; $P < 0.05$) and significantly fewer implantation sites (4.6 ± 0.9 vs. 7.2 ± 0.7) than in WT dams. Furthermore, *Fat-1* dams carried an average of only 2.7 ± 0.6 viable pups per gestation compared with WT dams which carried an average of 7.2 ± 0.7 viable pups per gestation ($P < 0.05$). The ratio of surviving *Fat-1* and WT pups in litters from *Fat-1* females did not differ from those expected based on Mendelian segregation laws (data not shown).

Ovulation and pre-implantation development

The ovulation rate of *Fat-1* females (6.9 ± 0.7 ovulated oocytes) was not different from that for WT females (6.6 ± 0.8), indicating that decreased ovulation did not account for the small litter size in *Fat-1* females. Pre-implantation embryo development was similarly assessed by flushing uteri collected from females at 3.5 DPC. *Fat-1* females had significantly fewer developing embryos (3.3 ± 0.8) within their uterus than WT females (8.3 ± 0.3).

In vitro embryo culture and transfer

The number of 1- and 2-cell embryos within the oviduct at 0.5 DPC was not different between *Fat-1* and WT females, indicating that fertilization rate was unaffected by the transgene. Furthermore, there was no significant difference in the number of developed morula (5.0 ± 0.8 vs. 5.5 ± 0.5) or blastocysts (4.9 ± 0.7 vs. 5.5 ± 0.5). To test whether the genotype of recipients affected reproductive competency, WT and *Fat-1* blastocysts were transferred to either WT or *Fat-1* pseudopregnant recipients. Females were allowed to carry their pregnancies until 12 DPC, at which time baseline reproductive parameters were
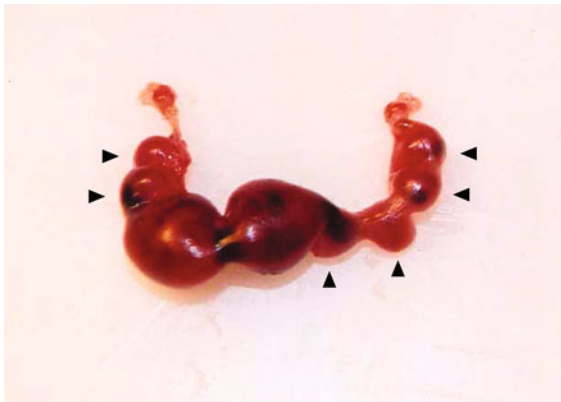
Fig. 1 Photo of a representative reproductive tract from a pregnant β-casein *Fat-1* female. Two normally-developing pups are accompanied by six implantation sites undergoing resorption. Sites undergoing resorption are denoted by *arrow heads* (▶)

measured. The total rate of implantation (implantation sites/embryos transferred) did not differ significantly between groups (Fig. 2). However, implanted embryos derived from *Fat-1* dams underwent resorption at a higher rate by 12 DPC, regardless of the recipient genotype.

Ovary transplants

We next transplanted ovaries across genotypes to determine if the reproductive effect was ovary-specific. Wildtype females harboring *Fat-1* ovaries had significantly fewer live pups (1.3 ± 0.7 vs. 5.3 ± 0.6) per gestation than sham control females, indicating that the presence of a *Fat-1* ovary had a deleterious effect on fertility (Fig. 3). *Fat-1* females carrying WT ovaries also had smaller litter sizes (3.0 ± 0.6 vs. 5.3 ± 0.6) compared to sham controls, suggesting that the presence of the transgene in non-ovarian tissues also contributed to the impaired reproductive phenotype seen in control *Fat-1* females. It should be noted that sham control females had significantly fewer live pups (5.3 ± 0.6) in the first gestation following surgery than WT females that had not undergone ovarian transplantation (8.7 ± 1.0).

Following the first gestation, females were rebred and sacrificed at 12 DPC during their second postoperative gestation. Wildtype females bearing *Fat-1* ovaries averaged only 1.1 ± 1.0 normally-developed fetuses per gestation (Fig. 4), and displayed
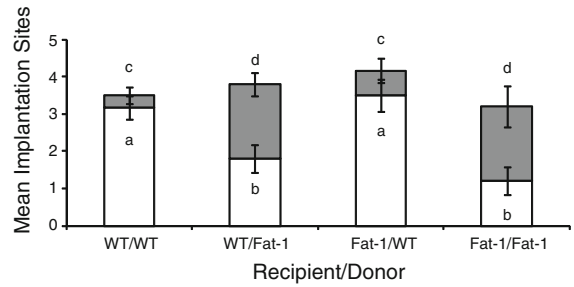

Fig. 2 Mean number of implantation sites ($\pm$SEM) that were either resorbing (*shaded*) or contained a viable fetus (*unshaded*) at 12 days post coitus following embryo transfer. The number of females in each group were WT/WT, $n = 6$; WT/*Fat-1*, $n = 5$; *Fat-1*/WT, $n = 6$; *Fat-1*/*Fat-1*, $n = 5$. Bars with different letters are significantly different ($P < 0.05$). The total number of implantation sites did not significantly differ across all groups

significantly more fetuses undergoing resorption per litter compared to *Fat-1* females harboring WT ovaries and WT sham females. However, *Fat-1* mice bearing WT ovaries again had a significantly smaller litter size compared to WT sham females. In the case of the second postoperative litter there was no difference in litter size between sham females and WT females that did not undergo the ovarian transplant procedure indicating that the litter size reduction effect of ovarian transplantation surgery itself was negligible by the second litter.

Effect of mastectomy

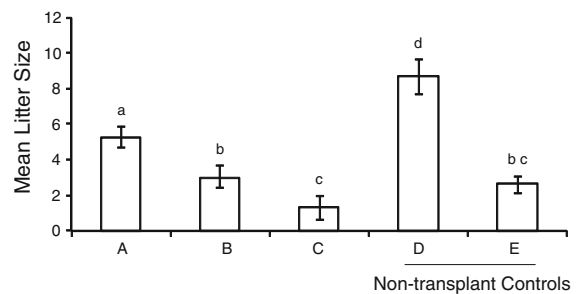The total number of implantation sites in mastectomized *Fat-1* females did not differ from that of

Fig. 3 Mean number of pups born ($\pm$SEM) to dams in various ovarian transplant treatment groups after their first gestation. (A) WT sham ($n = 7$); (B) β-casein *Fat-1* recipient of WT ovary ($n = 7$); (C) WT recipient of β-casein *Fat-1* ovary ($n = 6$); (D) WT non-surgical control ($n = 5$); (E) β-casein *Fat-1* non-surgical control ($n = 8$). Bars with different letters are significantly different ($P < 0.05$)
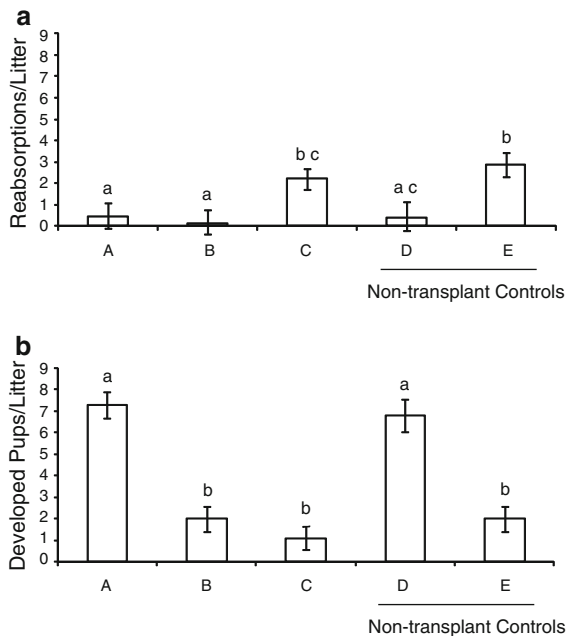
a

b

Fig. 4 Mean number of **a** resorbing fetuses and **b** normally-developing pups (±SEM) in various ovarian transplant groups at 12 days post coitus during the second post-operative gestation. (A) WT sham ($n = 7$); (B) β-casein *Fat-1* recipient of WT ovary ($n = 7$); (C) WT recipient of β-casein *Fat-1* ovary ($n = 6$); (D) WT non-surgical control ($n = 5$); (E) β-casein *Fat-1* non-surgical control ($n = 8$). Bars with different letters are significantly different from each other ($P < 0.05$)

mastectomized WT females (Fig. 5), and remained significantly higher than rates observed in non-mastectomized *Fat-1* females. However, the total number of normally-developing fetuses remained significantly lower in mastectomized *Fat-1* mice compared to mastectomized WT mice (2.0 ± 0.3 vs. 7.5 ± 0.6) as a result of an increased incidence of resorbing fetuses.

Transgene expression analysis

To determine whether there was ectopic expression of the *Fat-1* transgene in female reproductive tissues, RT-PCR was performed on ovarian and uterine tissues collected from transgenic females at daily intervals between ovulation (0 DPC) and implantation (5 DPC). At no time were these tissues found to be positive for *Fat-1* expression. As expected, *Fat-1* expression was detected in mammary gland tissue taken from both lactating and 8-week old-virgin *Fat-1* females (Fig. 6).
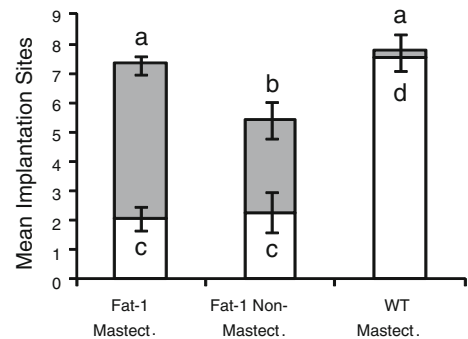

Fig. 5 Mean number of implantation sites (±SEM) at 12 days post coitus following mastectomy in β-casein *Fat-1* ($n = 11$), WT ($n = 10$), and non-mastectomized β-casein *Fat-1* females ($n = 19$). White bars represent developing pups; grey shading represents fetal resorptions. Bars with different letters are significantly different from each other ($P < 0.05$)

Milk composition phenotype

Milk phospholipid data for the substrates and products of the *Fat-1* gene are shown in Fig. 7. A significant decrease in the proportion of AA and an increase in ALA and EPA content were recorded in the milk from *Fat-1* dams relative to that in WT dams. Interestingly, the milk phospholipids from a line of mice constitutively expressing the *Fat-1* transgene under the control of the CMV (enhancer)/
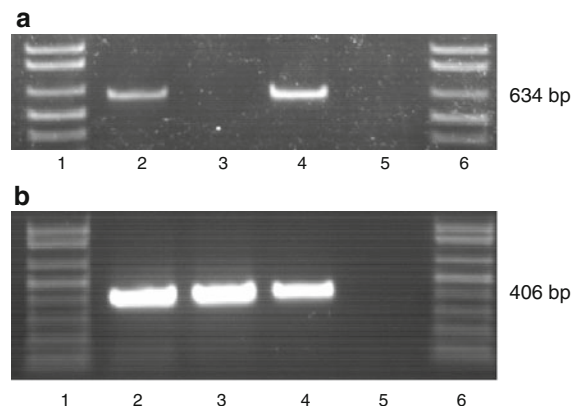
a

b

Fig. 6 RT-PCR analysis of mammary tissue from *Fat-1* and WT virgin females. The *top* gel **a** shows PCR products generated using transgene specific primers. The *bottom* gel **b** shows PCR products from primers for mouse β-actin. Lanes 1 and 6: 1 kb Plus Ladder; lane 2: β-casein *Fat-1*, mammary tissue from 8 week old-virgin female; lane 3: WT 8 week old-virgin mammary tissue; lane 4: β-casein *Fat-1* lactating mammary tissue (positive control); Lane 5: H_2O (negative control)
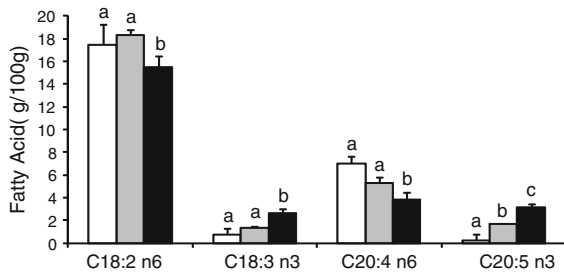
Fig. 7 Milk phospholipid profiles (±SEM) for the substrate and product of the *Fat-1* gene for samples derived from WT (white bars; $n = 9$), and transgenic females expressing the *Fat-1* gene under the control of the constitutively active β-actin promoter (Kang et al. 2004; *gray bars*; $n = 8$), or the goat β-casein promoter (*black bars*; $n = 6$). Bars with different letters within a fatty acid group are significantly different from each other ($P < 0.05$)

β-actin promoter (Kang et al. 2004), and fed the same diet only differed from control milk in having a significantly elevated level of EPA.

Discussion

Transgenic female mice expressing the *Fat-1* gene under control of the mammary gland-specific goat beta-casein promoter had impaired reproductive performance. Given that the goat beta-casein promoter directs transgene expression primarily in the mammary gland, and to a lesser extent in muscle tissue (Roberts et al. 1992), this impaired reproductive phenotype was unexpected. Male *Fat-1* transgenic mice displayed normal fertility, ruling out a positional knockout of a key reproductive gene in this transgenic line. Transgenic pups were viable and segregation ratios did not differ from expected, suggesting that the presence of the transgene in the embryos themselves did not impact embryo survival.

Transplantation of ovaries from transgenic females into WT recipients revealed that transgenic ovaries were partially responsible for the diminished reproductive performance. The finding that embryos derived from *Fat-1* ovaries had an increased incidence of fetal resorption, regardless of uterine genotype, suggests that transgenic ovaries produced oocytes with inferior post-implantation survival. This finding was confirmed through embryo transfer experiments wherein significantly more embryos from *Fat-1* dams underwent resorption by 12 DPC. This phenotype is strikingly

similar to that reported for partially-fertile COX-2 deficient mice (Wang et al. 2004). In these mice, embryos implant beyond the normal "implantation window," where the uterus normally becomes receptive to implantation at 4 DPC. However, in COX-2 deficient mice the embryos implanted at 5 and 6 DPC, a delay that resulted in subsequent developmental abnormalities and frequent resorptions. In the current study high rates of resorptions were observed even when *Fat-1* embryos were transplanted into WT uteri, suggesting that embryos from oocytes produced in a *Fat-1* ovary were associated with an increased fetal resorption rate.

Fat-1 females harboring WT ovaries also produced fewer normally-developing pups than WT females, thereby suggesting an additional role for transgene expression in non-ovarian tissues. While ovulation rates and in vitro pre-implantation embryo development did not differ between *Fat-1* and WT females, the number of developed embryos in the uterus at 3.5 DPC was lower in *Fat-1* females.

To determine whether *Fat-1* transgene expression within the mammary glands was indirectly affecting reproduction, surgical mastectomy was performed to remove the primary source of known transgene expression in *Fat-1* females. This intervention increased the total number of implantation sites in *Fat-1* females compared to non-ablated *Fat-1* mice, whereas fetal resorption rates were still significantly higher in mastectomized *Fat-1* females. These findings suggest that *Fat-1* expression in the mammary glands of transgenic mice acts via an endocrine mechanism to negatively affect the number of implanted embryos. This finding is consistent with a recent report of reproductive deficiencies in *Fat-1* transgenic mice over-expressing the *Fat-1* gene under the control of the adipocyte-specific promoter, adipocyte protein-2 (aP2) (Ji et al. 2009), where heterozygous transgenic mice were unable to generate homozygous offspring. These authors concluded that "prolonged exposure to increased concentrations of n-3 fatty acids may be detrimental to reproduction". Interestingly, no reproductive problems have been reported in a line of mice that have constitutive *Fat-1* expression driven by the CMV (enhancer)/β-actin promoter (Kang et al. 2004).

Genetic mutants in fatty acid biosynthesis pathways frequently display suboptimal fertility. Of particular relevance to this study, $\Delta 6$-fatty acid desaturase (FADS2) knockout mice, which cannot

synthesize long chain PUFA from the essential linoleic (LA: 18:2 n-6) and α-linolenic (ALA: 18:3, n-3) fatty acids, are sterile (Stoffel et al. 2008). These mice do not undergo normal gametogenesis due to altered membrane properties of the gametes, a phenotype which Stoffel et al. (2008) rescued by providing a diet enriched with long chain PUFA.

Likewise, cyclooxygenase (COX)-2 knockout mice, which are unable to synthesize PG, are also infertile (Dinchuk et al. 1995; Lim et al. 1997). Cyclooxygenase, which exists in two isoforms (COX-1 and COX-2), is the rate-limiting enzyme in the biosynthesis of prostaglandin H_2, the common substrate for various PG synthases. Targeted disruption of COX-2 in mice produces multiple reproductive deficiencies in females including effects on ovulation, fertilization, implantation, and decidualizaiton (Dinchuk et al. 1995; Lim et al. 1997). It is known that the oviduct is highly sensitive to changes in fatty acid concentrations (Scholljegerdes et al. 2007), and PG production in the bovine oviduct is known to increase contractility (Wijayagunawardane et al. 2001), which facilitates sperm and embryo transport through the oviduct.

Expression of β-casein mRNA has previously been detected in numerous non-mammary tissues of mice, including the ovary, oviduct, and uterus (Small et al. 2005; Nilsson et al. 2006; Bouma et al. 2007; Suzuki et al. 2007). It is possible that ectopic expression of the transgene in these reproductive tissues could explain our observations. However, we were unable to detect any transgene expression in the uterus or ovary from 1 DPC (ovulation) through 5 DPC (post-implantation).

The mammary glands in nulliparous female mice are characterized by the close apposition of adipocytes with mammary epithelial cells (Hovey et al. 1999) which, in the case of *Fat-1* females, abnormally express transgene-directed desaturase activity. Given the potentially-increased bioavailability of fatty acid substrates within this microenvironment (Beck et al. 1989), it is conceivable that further metabolism of these products to endocrine modulators such as fatty acids or their prostaglandin derivatives could account for the ovarian phenotype recorded herein. Alternatively, several candidate adipokines may lie behind the effects of the mammary glands on reproductive function in Fat-1 females. Prolactin is produced by adipocytes in the mammary glands (Brandebourg et al. 2007) and is crucial for luteal support and the maintenance of pregnancy (Bachelot and Binart 2007). Furthermore, both leptin and adiponectin are produced by mammary adipose tissue in nulliparous females (Lin and Li 2007; Celis et al. 2005) and have been implicated in regulating reproductive performance parameters including ovulation, implantation and embryonic survival (Mitchell et al. 2005). Regardless of the precise mechanism involved, our findings and these proposals are in keeping with previous suggestions that the mammary glands function as an ill-defined endocrine organ during events such as reproduction (Peaker 1995).

The finding that mastectomized females had a normal rate of implantation, but reduced embryonic survival, raises the question of whether the mammary glands of *Fat-1* females may have conditioned the reproductive axis prior to 21d of age. Indeed, function of the mature reproductive organs is susceptible to a range of early lifetime events (Charmandari et al. 2003). At this time it is not known whether the mammary glands expressed appreciable desaturase activity prior to mastectomy. This scenario would involve pre-pubertal activation of the β-casein promoter that is generally considered to primarily occur during pregnancy and lactation (Roberts et al. 1992). Consistent with our present findings, others have also demonstrated expression of similarly-regulated milk protein genes in the mammary epithelium of nulliparous females (McBryan et al. 2007; Robinson et al. 1995), where even the mammary epithelium from embryonic females is capable of milk protein expression (Cowie and Tindal 1971). Hence, it is plausible that early (<3 week) induction of desaturase activity may have occurred, although this hypothesis would require further testing using a regulable transgene system.

The fatty acid composition of milk phospholipids generated by our transgenic females showed that expression of the *Fat-1* transgene under control of the strong mammary-specific β-casein promoter resulted in a more pronounced shift in milk phospholipid composition and decrease in AA than in the aforementioned line expressing the *Fat-1* gene under the control of the β-actin promoter (Kang et al. 2004). This large decrease in AA is interesting given the importance of the cell membrane phospholipid composition and especially AA in PG biosynthesis, and their vital role in the establishment of a successful pregnancy.

The establishment of pregnancy is a complicated event comprising multiple levels of endocrine regulation. The data we present provides further insight into the role of PUFA in reproduction and indicates that the mammary gland has the ability to function as a regulator of the establishment and maintenance of pregnancy.

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