Fatty acid synthase is required for mammary gland development and milk production during lactation

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Suburu J, Shi L, Wu J, Wang S, Samuel M, Thomas MJ, Kock ND, Yang G, Kridel S, Chen YQ. Fatty acid synthase is required for mammary gland development and milk production during lactation. Am J Physiol Endocrinol Metab 306: E1132–E1143, 2014. First published March 25, 2014; doi:10.1152/ajpendo.00514.2013.—The mammary gland is one of the few adult tissues that strongly induce de novo fatty acid synthesis upon physiological stimulation, suggesting that fatty acid is important for milk production during lactation. The committed enzyme to perform this function is fatty acid synthase (FASN). To determine whether de novo fatty acid synthesis is obligatory or dietary fat is sufficient for mammary gland development and function during lactation, Fasn was specifically knocked out in mouse mammary epithelial cells. We found that deletion of Fasn hindered the development and induced the premature involution of the lactating mammary gland and significantly decreased medium- and long-chain fatty acids and total fatty acid contents in the milk. Consequently, pups nursing from Fasn knockout mothers experienced growth retardation and preweanling death, which was rescued by cross-fostering pups to a lactating wild-type mother. These results demonstrate that FASN is essential for the development, functional competence, and maintenance of the lactating mammary gland.

In most tissues, fatty acid synthesis is relatively quiescent, and the reason for its quiescence remains unclear. It is possible that nonproliferating tissues do not demand fatty acid synthesis or that dietary fatty acids are sufficient to fulfill the physiological fatty acid requirement of these tissues. Nonetheless, expression of FASN is observed in a variety of adult tissues, with the strongest expression in the liver, adipose, lung, and brain, where it is a critical enzyme for metabolic homeostasis (10, 27). Whole body knockout of FASN in mice is embryonically lethal due to impaired blastocyst implantation in the uterus. Even heterozygous FASN knockout mice exhibit impaired growth and survival, suggesting that fatty acid synthesis is critical for normal development (7). Tissue-specific knockout of FASN has provided valuable insight to the role of de novo fatty acid synthesis in the liver, pancreas, brain, macrophage, heart, intestine, and adipose. In these cell types, FASN has been shown to be a critical regulator of systemic energy homeostasis, atherosclerotic lesions, diet-induced obesity, diabetes, inflammation, and cardiac stress (5, 6, 16, 21, 26, 39).

Overexpression of FASN has also been strongly associated with many cancer types and is under extensive study as a potential cancer drug target (19). Interestingly, although fatty acids may be produced de novo or taken up from the diet, no study to date has described adequate compensation by dietary fatty acids following FASN knockout in vivo.

The mammary gland is a unique lipid-metabolizing tissue. Although the mature, resting mammary gland does not demand fatty acid synthesis, expression of lipogenic genes, and specifically FASN, is strongly induced upon the morphological changes associated with pregnancy and lactation (22, 23). During that time, the mammary gland undergoes four phases of functional differentiation: early proliferation (early pregnancy), secretory differentiation (midpregnancy), secretory activation (parturition), and lactation (25). During lactation, mice are estimated to produce a total of ~5 ml of milk per day, of which ~30% is triacylglycerides (1).

Fatty acid synthase in the mammary gland is quite unique relative to other tissues. Comparative analysis of fatty acids in the liver and mammary gland shows a shorter average fatty acid chain length in the mammary gland (30). Supporting this finding was the identification and purification of a mammary gland-specific thioesterase II enzyme, which is responsible for the early cleavage of de novo fatty acids from FASN and, thus, generation of short- and medium-chain fatty acids (<16 carbons) (15). In accordance with its unique lipid synthesizing function, the mammary gland has been shown to have the highest expression of FASN of the tissues analyzed. Deletion of Fasn in these tissues results in the death of homozygous mice. The results we have described support the notion that FASN is the key committing enzyme for de novo fatty acid synthesis during lactation.

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capabilities, the mammary gland has been hypothesized to have a distinct program that regulates lipid metabolism for the preparation and execution of lactation by the concerted efforts of epithelial cells and adipocytes (2, 12, 22, 25). Gene expression studies revealed that activation of fatty acid synthesis in the mammary epithelium occurs in response to parturition and the onset of lactation. This increased expression of lipogenic genes is significantly larger than their relative expression in the liver, supporting the hypothesis that the mammary gland is undergoing changes to fulfill a very specific function (23, 33).

De novo fatty acid synthesis in the mammary gland is responsible for producing short- and medium-chain fatty acids for milk production (29) and provides ~15–40% of the total fatty acid content of milk (30). Sterol regulatory element-binding protein-1 is a well-known transcriptional activator of lipogenic genes (14), and its activation in the mammary gland is important for the induction of fatty acid synthesis during lactation (23). In vivo deletion of SCAP, the activator of SREBP proteins, in the mammary gland significantly decreases fatty acid synthesis and the growth rate of nursing pups, and feeding mothers a high-fat diet only partially rescues this effect (24). Gene expression analysis suggests that de novo lipogenesis in the mammary gland can be modulated by a high-fat diet, whereby increased dietary fat consumption results in a marked decrease in enzymes of the fatty acid synthesis pathway and fewer short-chain fatty acids in milk (23, 24, 30).

Lipid metabolism is clearly an integral component of mammary gland physiology; however, no study has investigated the absolute requirement of fatty acid synthesis during mammary gland development and lactation. We sought to determine the role of FASN in the mammary gland by generating a conditional mammary gland-specific Fasn knockout mouse (KO) from Fasn floxed mice (5), in which deletion of Fasn is made specific to the mammary epithelial cells through the activation of a Cre recombinase gene regulated by the whey acid protein (WAP) promoter (37). We report that loss of FASN in mammary epithelial cells decreases the growth and survival of nursing pups, alters the fatty acid profile of milk produced by lactating mothers, and hinders the development and maintenance of a functional lactating mammary gland.

**EXPERIMENTAL PROCEDURES**

*Transgenic mice.* Mammary epithelial cell-specific Fasn knockout mice were generated by crossing floxed Fasn mice (Fasn<sup>fl/fl</sup>) kindly provided by the laboratory of Dr. Clay Semenkovich, Washington University) (5) with whey acidic protein-Cre recombinase transgenic mice (WAP<sup>Cre</sup>) (The Jackson Laboratory). At 6 wk, females of either Fasn<sup>fl/fl</sup>WAP-Cre<sup>(WT)</sup> or Fasn<sup>fl/fl</sup>WAP-Cre<sup>(KO)</sup> genotype were bred with Fasn<sup>fl/fl</sup>H11002 (WT) mouse was euthanized at the same lactation day and pregnancy number. In cross-fostering experiments, only mothers that one, two, or three pregnancies. Following each pregnancy and lactation day 25 (L25) or the day at which all pups had deceased. For Fasn<sup>fl/fl</sup>WAP-Cre<sup>(KO)</sup> mothers whose mammary glands were harvested prior to L25, an age-matched Fasn<sup>fl/fl</sup>WAP-Cre<sup>(WT)</sup> mouse was euthanized at the same lactation day and pregnancy number. In cross-fostering experiments, only mothers that gave birth within 2 days of each other were eligible for growth monitoring after cross-fostering. Histopathological evaluation of all tissues was performed by board-certified veterinary pathologists.

All animals were maintained in an isolated environment in barrier cages and fed a standard rodent diet. Animal care was conducted in compliance with the state and federal animal welfare acts and the standards and policies of the Department of Health and Human Services. The protocol was approved by the Wake Forest University Institutional Animal Care and Use Committee.

**Measurement of pup growth.** The date of parturition was considered lactation day 1 (L1). Pups from each pregnancy were weighed daily between 22 and 26 h from previous weigh-in from L2 to L25. Litters of more than eight pups were culled to eight pups on L2 to prevent competition among pups for the mother’s milk supply. To minimize error, up to six pups were weighed at once, followed by sequential measurements of one additional pup, up to eight pups. For litters of more than six pups, the seventh and eighth pups were distinguished by a tail marking on L8 and were consistently weighed as the seventh and eighth pups for daily measurements. Each measurement was divided by the number of pups weighed to determine the average pup weight of each litter. The average pup weight for all litters monitored was then determined.

**Milk collection.** Mothers were separated from pups for 45 min to 2 h on L2, L10, and L18. Mothers were anesthetized by intraperitoneal (ip) injection of 6.25 mg/30 g body wt Avertin (Sigma T48402). A 40X solution of Avertin was made by dissolving 500 mg/ml in tert-amyl alcohol and stored at 4°C. A 1X solution was made with PBS from the 40X solution. Following anesthesia, mice were injected with 200 μl of 10 IU/ml ip (2 IU per mouse) oxytocin (Sigma O4375) to induce milk release from the mammary gland. Nipples were gently massaged, and secreted milk was collected in a capillary tube. Approximately 10 μl of milk was extracted from each gland (~100 μl total), pooled together into one microcentrifuge tube, snap-frozen in liquid nitrogen, and stored at −80°C until analyzed. All drug solutions were filtered through a 0.22-μm filter and stored at 4°C.

**Histochemistry.** The right inguinal mammary gland was excised and spread between two microscope slides, fixed overnight in 10% buffered formalin, and processed for embedding in paraffin. Routine processing and staining was done with hematoxylin and eosin (H&E). Immunohistochemical (IHC) staining was performed with mouse anti-FASN primary antibody (BD Biosciences 610962) and guinea pig anti- adipophilin primary antibody (20R-AP002, Fitzgerald Industries, Flask, MA). Primary antibody was incubated at 4°C overnight in a humidity chamber followed by HRP-conjugated goat anti-mouse (GE Healthcare NA931V) or donkey anti-guinea pig (706-165-148; Jackson Immunoresearch, West Grove, PA) secondary antibody. DAB chromogen was applied for 3–8 min according to the package insert (K3467; Dako, Carpinteria, CA). TUNEL staining was performed using a DeadEnd Colorimetric Assay kit (Promega G7360) according to the manufacturer’s instructions. A positive TUNEL control included a DNase I digestion that was processed separately from all other samples. A negative TUNEL control was processed in the absence of any rTdT enzyme.

**Immunofluorescent staining** was performed with mouse anti-FASN primary antibody (BD Biosciences 610962) and guinea pig anti-adipophilin primary antibody (20R-AP002, Fitzgerald Industries) followed by Alexa fluor 488 goat anti-mouse (Life Technologies A-11001) and Alexa fluor 594 goat anti-guinea pig (Life Technologies A-11076) secondary antibody. Following this step, DAPI (4',6-diamidino-2-phenylindole; Sigma D9542) nuclear stain was incubated for 5 min followed by mounting with ProLong Gold Antifade Reagent (Life Technologies P10144).

**Mammary gland whole mount.** The left inguinal mammary gland was excised and spread between two microscope slides and fixed overnight in Carnoy’s fixative (60% 200 proof ethanol, 30% chloroform, 10% glacial acetic acid). Following fixation, mammary glands were subjected to a series of ethanol washes to rehydrate: twice with 70% ethanol for 15 min, twice with 50% ethanol for 10 min, twice with 30% ethanol for 10 min, twice with 10% ethanol for 10 min, and twice with H<sub>2</sub>O for 5 min. After rehydration, mammary glands were
stained overnight in carmine aluminum staining solution followed by a series of ethanol washes to dehydrate: twice in 70% ethanol for 15 min, twice in 95% ethanol for 15 min, and twice in 100% ethanol for 15 min. Mammary glands were placed in xylene until all fat was cleared and mounted onto a microscope slide using Cytoseal-60 mounting medium. Carmine aluminum staining solution was made by placing 1 g of carmine (Sigma C1022) and 2.5 g of aluminum potassium sulfate (Sigma A7167) in 500 ml of $^2$H$_2$O and boiling for 20 min. Final volume was adjusted to 500 ml with H$_2$O. A thymol crystal was added as a preservative. Carmine staining solution was stored at 4°C.

**Microscopy.** All images were captured with either a Hamamatsu Nanozoomer 2.0HT with Olympus objectives and NDP image software or an Olympus VS110 microscope equipped with Olympus objectives and VS-ASW FL image software. Image frames were created using Olympus Olyvira software. Average lumen size was quantified by measuring the largest diameter of 8–10 alveolar lumens at ×40 magnification in four to five glands of one to two mice for each genotype and lactation day. TUNEL stains were quantified by averaging the number of TUNEL-positive cells per 15 random fields of view at ×20 magnification for each gland and lactation day.

**Fatty acid methyl ester analysis.** Fatty acid methyl ester (FAME) analysis was performed on milk collected from lactating mothers as previously described (3). Results from each milk collection were averaged for each pregnancy.

**Statistics.** Statistics were performed using Microsoft Excel, Daniel’s XL Toolbox Plugin 5.05, and MedCalc software v. 12.7. A two-tailed Student’s t-test was performed to identify statistical significance between two groups at a given point.

**RESULTS**

**Generation of mammary gland-specific fasn KO mice.** Mice with mammary epithelial cell-specific Fasn KO were developed by crossing mice homozygous for the floxed Fasn alleles (5) with transgenic mice bearing a Cre recombinase transgene controlled by the WAP promoter (Jackson Laboratory). Knockout of Fasn in this model occurs only in the mammary epithelial cells, not in mammary adipose tissue, and only upon pregnancy and lactation. To determine whether multiple pregnancies increased the effect of Fasn KO, female mice were subjected to one, two, or three pregnancies. By lactation day L15 of the first pregnancy (L15P1), FASN staining was observed in a large number of epithelial cells during lactation, suggesting incomplete knockout. However, staining was largely absent by L17 of the second pregnancy (L17P2) and decreased further by L16 of the third pregnancy (L16P3). In contrast, very strong FASN staining was observed in WT mice during all three pregnancies. Virgin mice also showed positive FASN staining (Fig. 1A).

Despite an obviously gradual deletion of Fasn over the course of multiple pregnancies, time-dependent deletion was also observed over the course of the lactation period. On L2 of pregnancy 3 (L2P3) approximately one-half of the epithelial cells stained strongly positive for FASN. This number decreased to less than 25% by L10P3, and FASN was nearly completely absent in all epithelial cells by L16P3 (Fig. 1B).

**Fasn KO in lactating mammary gland decreases pup growth and survival.** Because FASN is responsible for producing short- and medium-chain fatty acids in milk (30) and fatty acids are important nutritional components of milk for the mother’s growing pups, we sought to determine how FASN KO affects the growth of pups nursed by KO mothers for each pregnancy. No significant differences in the average litter size or the mother’s age at parturition were observed between WT and KO mothers for all three pregnancies (Table 1). In the first pregnancy, all pups showed similar growth rates regardless of the mother’s genotype (Fig. 2A). Interestingly, although the growth curves were similar, we observed a significantly greater preweanling death rate for pups from KO mothers than for pups nursed by WT mothers. While 75% of pups from WT mothers survived to weaning age (L25), only 61% of pups from KO mothers survived to weaning age during the first pregnancy ($P = 0.01$; Fig. 2B).

In the second pregnancy, there was a significant decrease in the growth rate of pups from KO mothers compared with pups from WT mothers (Fig. 2C). No pups from KO mothers survived beyond L19, whereas 75% of pups from WT mothers survived to weaning age ($P < 0.0001$; Fig. 2D). In the third pregnancy, pups from KO mothers displayed a similar trend in the growth of pups compared with the second pregnancy (Fig. 2E). Also, no pups from the third pregnancy of KO mothers survived beyond L9, whereas all pups from WT mothers survived to weaning age ($P < 0.0001$; Fig. 2F). In all, the growth and survival trends of pups from KO mothers over the course of three pregnancies correlates with the progressive deletion of FASN.

It has been previously reported that both homozygous and heterozygous KO of FASN leads to developmental complications (7). To further demonstrate the specificity of the FASN KO phenotype, pups from the second pregnancy of KO mothers were genotyped to determine whether growth complications might have possibly been due to unexpected expression of the WAP-Cre recombinase and premature KO of FASN. As expected, pups demonstrated 50% Mendelian inheritance of the Cre recombinase transgene (data not shown), providing evidence that the premature death of KO pups was not a result of erroneous expression of WAP-Cre in pups.

**Deletion of FASN induces premature involution and cell death.** Litters from KO mothers typically perished all together in a time frame of 2–3 days (Fig. 3), and mammary glands of KO mothers were immediately harvested following the deaths of all their pups. We harvested mammary glands from lactating, age-matched, WT mothers from the same pregnancy and on the same lactation day as KO mothers to analyze any changes that had occurred in KO mammary glands during the lactation period. Although the difference in pregnancy 1 appeared less dramatic, whole mount mammary gland analysis showed a consistent and marked difference in the density of glands in KO mothers compared with WT mothers for all three pregnancies. All age-matched virgin mice showed normal tree-like branching (Fig. 4A). Closer examination with H&E staining further demonstrated a marked difference in the alveolar density between KO and WT mammary glands. Adequate formation of actively secreting alveoli was evident in KO mice on L15P1. However, compared with WT mice, KO mice had fewer alveoli and showed a larger proportion of adipocytes.

In contrast to the first pregnancy, mammary glands from KO mice in the second and third pregnancies appeared to be undergoing involution at day L16–17, whereas age-matched, WT mice of the same lactation day and pregnancy displayed large and actively secreting alveoli (Fig. 4B).
To verify that KO glands were experiencing early involution, we compared WT and KO mammary glands at various time points during each lactation period and performed a TUNEL assay to analyze cell death. As expected, glands from both WT and KO mice had experienced involution by L25P1. KO glands, in accordance with having fewer alveoli at L15, also showed fewer epithelial cells at L25 compared with WT mice (Fig. 5A). In the second pregnancy, WT glands had developed mature alveoli and were abundantly secreting at L12 and L17, whereas KO glands showed a decreased number of alveoli at L12 and showed histological signs of involution at L17 (Fig. 5B). In the third pregnancy, we analyzed glands at L2, L10, L16, and L19 to gain a more comprehensive understanding of the histological progression during the lactation phase in KO mice. At L2, both WT and KO mice showed comparable development of actively secreting alveoli. However, by L10, a discrepancy in the number and size of alveoli was apparent, and by L16 and L19, glands were clearly undergoing involution (Fig. 5C), as they appeared very similar to glands of L25P1. TUNEL staining showed no difference in cell death at L15P1 (Fig. 5D), nor L2 and L10 of the third pregnancy (Fig. 5F). However, in accord with their marked histological changes, there was a statistically significant increase in TUNEL-positive cells at L17P2 and L6P3, validating cell death and involution at these time points (Fig. 5, D–G). A positive and negative TUNEL control using DNase I digestion.
and no TdT enzyme, respectively, confirmed the results of the TUNEL staining (Fig. 5H).

**FASN KO hinders mammary gland maturation but not secretory activation during lactation.** As we previously mentioned, the deletion of FASN gradually increased over the course of lactation and multiple pregnancies. Therefore, we questioned whether KO glands were strictly involuting early, as a consequence of increased FASN deletion, or if they also experienced developmental deficits during lactation. Glands experiencing involution, whether WT or KO, had a much smaller lumen size than actively lactating glands. However, analysis of lumen measurements at earlier time points during lactation demonstrated a statistically significant difference (P < 0.001) between WT and KO glands at days L12 and L10, but not at L2 (Fig. 6A). Interestingly, a comparison of FASN-positive vs. FASN-negative lumens showed no difference in lumen size at either L2 or L10 in KO glands with mosaic deletion of FASN (Fig. 6B). Despite the differences observed in lumen size, KO glands still showed adequate formation of lipid droplets on the luminal side of epithelial cells at all stages of lactation (Fig. 6, C–E). This was true even in lumens where FASN had been deleted, as shown by immunofluorescent double staining for adipophilin and FASN on L10P3 (Fig. 6F).

**Fasn KO in the lactating mammary gland alters the lipid profile in milk.** FASN is responsible for the production of short- and medium-chain fatty acids (<16 carbons), as well as a substantial portion of long-chain fatty acids (16–20 carbons), whereas dietary sources are the major supply for very long-chain fatty acids (>20 carbons) (28). Because we identified a deficit in the growth and survival of pups nursed by KO mothers, we hypothesized that FASN KO in the mother’s mammary gland was affecting the fatty acid profile of the milk, and thereby, pups were succumbing to premature death, in part, by malnutrition. To test this hypothesis, we performed FAME analysis on milk collected from lactating mothers on days L2,

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<tr>
<th>Total no. of litters</th>
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<th>KO</th>
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<td>6.3 ± 0.27</td>
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<td>7.1 ± 0.21</td>
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<td>7.6 ± 0.45</td>
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<td>7.0 ± 0.95</td>
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<td>8.5 ± 0.29</td>
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<td>34</td>
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<td>10.0 ± 0.30</td>
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<tr>
<td>10.0 ± 0.26</td>
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<td>19.0 ± 0.71</td>
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<td>19.2 ± 0.99</td>
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<td>31.5 ± 1.41</td>
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<td>30.0 ± 2.18</td>
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Values represent means ± SE. WT, wild type; KO, Fasn (fatty acid synthase) knockout.

Table 1. Litter statistics for growth and survival curves

**Fig. 2.** Deletion of FASN hinders growth and survival of nursing pups. Average weight of pups and their survival were monitored from L2 through L25 following the first (A), second (B), and third (C) pregnancies. Error bars represent SE. *P < 0.05, **P < 0.01, ***P < 0.001. P values for survival curves are indicated in the panel.

**Fig. 3.** Preweanling death occurs as a whole litter. Survival of pups was monitored for each litter following the second (A) and third (B) pregnancies of KO mothers. Each line represents the survival of pups in a single litter from a KO mother. Typically, litters perished over the course of 2–3 days.
L10, and L18 during each pregnancy and quantified the presence of each fatty acid.

Milk analysis from the first pregnancy showed significant decreases in 14:0 (P < 0.001), 14:1 (P = 0.03), 16:0 (P < 0.01), 18:0 (P < 0.05), 18:2 (P < 0.01), and total fatty acid (P < 0.01) in milk from KO mothers compared with WT (Fig. 7A).

Milk analysis from the second pregnancy showed significant decreases in 14:0 (P < 0.001), 14:1 (P < 0.001), 16:0 (P < 0.001), and 22:0 (P = 0.03), and a similarly decreasing trend in the total fatty acid (P = 0.09) (Fig. 7B). Finally, milk analysis from the third pregnancy showed significant decreases in 14:0 (P < 0.001), 16:0 (P < 0.001), 18:0 (P < 0.001), 20:0 (P = 0.02), and total fatty acid (P = 0.03) (Fig. 7C).

Growth and survival deficiencies of pups are rescued by nursing from a WT mother. To clearly demonstrate that the functional differences in lactation from impaired mammary gland development and the consequential growth and survival trends in pups were in fact due to the Fasn KO in the mammary gland of lactating mothers, we swapped the litters of age-matched KO and WT mothers between day L1 and day L3 and monitored the growth of pups being nursed by the surrogate mother. Again, there were no significant differences in litter size or the age of all mothers at parturition between KO and WT mothers for all three pregnancies (Table 2). Similarly to our previous results (Fig. 2), pups born to a WT mother but nursed by a KO mother showed a significant decrease in
growth and survival. When mothers were swapped after their first pregnancy, we observed a significant decrease in the growth and survival \( (P = 0.0003) \) of pups born to a WT mother and nursed by a KO mother (Fig. 8A). During the second and third pregnancies, WT pups nursed by a KO mother showed significantly decreased growth and survival \( (P < 0.0001; \text{Fig. 8, B and C}) \). Importantly, pups born to a KO mother and nursed by a WT mother were phenotypically rescued, showing significantly greater growth and survival (Fig. 8).

**DISCUSSION**

Fatty acid synthesis is an important aspect of mammary gland function during lactation. Previous reports have de-
scribed the importance of lipogenesis for mammary gland milk secretion and its modulation by dietary fat (25, 30). However, no study has investigated the requirement of FASN in the mammary gland. Our study demonstrates that FASN is essential for functional development and maintenance of the lactating mammary gland. We show that FASN KO mice develop fewer alveoli, have impaired alveolar development during lactation, undergo premature mammary gland involution, and have an altered milk fatty acid profile. These perturbations ultimately manifest in significant growth retardation and premature death of nursing offspring.

Deletion of FASN in our model was spatially restricted to mammary epithelial cells and temporally restricted to late pregnancy and lactation (37). The mammary gland undergoes significant apoptosis during involution (17); however, cells that survive the involution phase may act as alveolar progenitors that repopulate the epithelium in subsequent pregnancies (35). Our data from the second and third pregnancies are very similar, suggesting that two pregnancies were likely sufficient for a phenotypic outcome in our KO mice. Other studies using the WAP-Cre mouse model to knock out a gene of interest have also reported using multiple pregnancies to achieve complete knockout and a phenotype (8, 34, 36). The fact that expression of WAP-Cre begins in late pregnancy, increases sharply following parturition, and is maintained during lactation (37) likely explains the progressive loss of FASN. While most, but not all, L10 KO mammary gland samples during the third pregnancy showed a large decrease in FASN staining,
there was even more variability in the extent of FASN staining among L2 KO samples from the third pregnancy. This suggests that activation of Cre was not uniform in all mammary glands between different KO mice and possibly within the glands of each KO mouse, and instead was likely dependent on the progression of lactation. Such variation explains the steady separation between the growth curves of KO and WT mice as glands progressively lost FASN. Moreover, the tendency for litters to perish as a whole suggests that only after the majority of glands were devoid of FASN was the lactating mother unable to support her nursing pups. The fact that survival of KO pups steadily decreased from day L11 to L17, a rather large, 6-day window when the majority of pups perished, further demonstrates the varying rates of Fasn deletion between lactating mice. Concordantly, some mammary glands of KO mothers did not produce sufficient quantities of milk for fatty acid analysis on day L18, which was most likely a result of premature involution of that gland from Fasn deletion. Finally, because deletion of Fasn occurs as early as late pregnancy, pups born to WT mothers could possibly have had an early growth advantage over pups born to KO mothers. This could explain the significant difference in average pup weight as early as day L2 in pups born to and nursed by a KO mother. It may also explain the time delay to observe the same growth and survival effects during the third pregnancy of the cross-fostering experiments as those observed in the second and third pregnancies of pups born to and nursed by KO mothers. It is important to note that the mothers monitored for the growth

Table 2. Litter statistics for cross-fostering growth and survival curves

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<th>Pregnancy 1</th>
<th>Pregnancy 2</th>
<th>Pregnancy 3</th>
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<tr>
<td></td>
<td>WT swap</td>
<td>KO swap</td>
<td>WT swap</td>
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<tr>
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<td>Average litter size</td>
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<td>10.7 ± 0.41</td>
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Values represent means ± SE.
and survival of their pups in Fig. 2 and the mothers of the cross-fostering experiments of Fig. 8 were two different cohorts of mice. Therefore, some of the variation observed between Figs. 2 and 8 may also be a result of using different subjects.

The primary function of fatty acid synthesis in the lactating mammary gland is believed to be to produce milk fatty acids; however, FASN has also been implicated as a critical regulator of growth and survival (7). Despite a clear and often widespread depletion of FASN from mammary epithelial cells as early as day L2, KO mice still developed tree-like ducts, acini, and alveoli with grape-like clusters. Even though some KO glands developed fewer alveoli structures, these results suggest that FASN is likely not essential for the overarching structural development of the gland. Moreover, because adipophilin staining was widespread and appeared at the luminal side of epithelial cells, even when they are FASN depleted, FASN is likely not required for secretory activation. However, there was an apparent deficit in the glandular maturation of KO mice during lactation, as evidenced by their overall smaller lumen size and failure to develop alveoli comparable in size to those of WT mice. This discrepancy was observed in KO mice regardless of incomplete deletion of FASN. Hence, it is possible that the deletion of FASN affected the development of the lactating gland on a global scale or in a localized manner. The inhibition of FASN is known to decrease ErbB2 activation and its downstream signaling mediators PI3K/Akt/mTOR, which are major regulators of cellular growth, proliferation, and survival (9, 32). Indeed, transgenic expression of dominant-negative ErbB2 in mice impairs lobuloalveolar development at parturition but does not affect milk secretion (11). These findings are in agreement with our results that suggest the deletion of FASN may modulate lobuloalveolar development but does not affect secretory activation.

Despite minor developmental deficits, KO mice were capable of developing lactating glands. However, functional incompetence of FASN KO mammary glands was evidenced by the significant decrease in fatty acids and the stunted growth and premature death of pups nursed by KO mothers. Glands from KO mice during late lactation (L16–17) strongly resembled glands from WT mice undergoing involution (L25) and had a significant increase in cell death, suggesting that KO mice were experiencing premature onset of involution. Involution typically occurs in response to the loss of mechanical stimulation from cessation of breast-feeding and milk stasis (20). Thus, it is possible that the early involution observed in KO mice was a result of lost mechanical stimulation from pup weakness, malnutrition, and death. However, KO mothers showed signs of scabbing and inflammation at the nipples, suggesting that pups had not ceased to nurse, and some glands did not produce a sufficient quantity of milk for collection on L18, suggesting that glands had already involuted despite the presence of pups.

**Fig. 8. Phenotypic rescue of offspring by cross-fostering mothers at birth.** Litters from KO mothers were swapped with litters from WT mothers between L1 and L3 for the first (A), second (B), and third (C) pregnancies. Average pup weight and survival were monitored every day beginning on the day after swapping mothers. Error bars represent SE. *P < 0.05, **P < 0.01, ***P < 0.001. *P* values for survival curves are indicated in the panel.
Moreover, the first signs of adipocyte repopulation following forced involution began 2 days following the removal of all pups (22), but mammary glands from KO mothers were harvested immediately following the death of all their pups, and preweaning death typically occurred as a whole litter rather than as sporadic individual deaths. Therefore, it is very likely that early activation of involution in KO mice was not a mechanical response but rather occurred as a result of the loss in functionality of the lactating mammary gland. These results suggest that a lack of functional competence from the loss of FASN can trigger involution and apoptotic cell death in the lactating mammary gland. Nonetheless, it remains elusive what biological sensors may be triggered in response to the lost functionality of the gland to elicit involution and whether these sensors are similar to those during normal induction of involution.

As evidenced by the change in phenotype from cross-fostering mothers, alterations in the mammary gland milk were a major contributor to the growth and survival discrepancies observed during lactation of KO mice. It is very likely that the phenotypic outcomes were a result of insufficient milk volume due to dysfunctional lipogenesis; however, changes in the milk fatty acid profile may also have contributed to the impaired growth and survival of pups nursed by KO mothers. The literature highlights the importance of long-chain polyunsaturated fatty acids as extremely critical for visual and neuronal development (13), but the nutritional significance of FASN-derived lipids, such as 14:0, 16:0, and 18:0 fatty acids, during development remains unclear. Our data suggest that perhaps de novo synthesized fatty acids are critical for development as well. Previous reports have shown unique roles for de novo synthesized fatty acids in the liver and other tissues, including posttranslational modification, PPAR activation, and their incorporation into membrane and signaling phospholipids (4–6, 31, 39). Whether FASN-derived lipids in mammary gland milk fulfill these or similar functions remains unknown.

Few tissues in the body engage in de novo fatty acid synthesis during normal physiology (38). Nonetheless, a variety of tissue types carry the potential to initiate a lipogenic phenotype (19). The intersection between de novo and dietary fatty acids is a growing research interest, as the activation of de novo fatty acid synthesis has been implicated in various cell types linked to multiple pathophysiology, including obesity, inflammation, atherosclerosis, and cancer. The literature suggests a very distinct role for de novo fatty acids in these diseases, for which neither dietary fatty acids nor preformed fatty acids from adipose can compensate in vivo (5, 6, 19, 21, 26). Functional mammary gland development has been shown to be dependent on interactions between epithelial cells and adipocytes (2, 12), suggesting that the mammary gland is a unique model system to study the integration of local de novo fatty acid synthesis and other sources of preformed fatty acids. Large volume production of short-chain fatty acids is exclusive to the mammary gland, as only the mammary tissue expresses the unique thiosterase II enzyme that prematurely cleaves the growing fatty acyl chain from the FASN enzyme (15). Previous reports have shown that short- and medium-chain milk fatty acids are exclusively produced de novo, whereas very long-chain fatty acids are derived from preformed fatty acids, and medium- and long-chain fatty acids are derived from both (24, 29). In our model, FASN was specifically deleted in mammary epithelial cells but remained intact in mammary adipocytes. Additionally, the fatty acid profile of milk showed a significant decrease in 16:0 and 18:0 fatty acids and a near depletion of 14:0 fatty acids. These results suggest that the portion of milk fatty acids produced by de novo fatty acid synthesis in the mammary epithelial cells was not replaced following the deletion of FASN. It seems that, ultimately, other sources of fatty acids were not able to compensate for this loss, including standard dietary fatty acid consumption and de novo fatty acid synthesis in adjacent adipocytes or other tissues such as liver and adipose. Hence, our data further support the notion that de novo fatty acid synthesis is a unique source of fatty acids and that upregulation of the fatty acid synthesis pathway characterizes a specialized cellular function that cannot be substituted by increased uptake of extracellular fatty acids. Nonetheless, it would be interesting to determine whether feeding KO mothers a diet high in saturated fat could rescue the phenotypic outcome of nursing pups.

Overall, our study highlights FASN as an essential component of mammary gland physiology during lactation. However, several new questions arise from the findings of this research that may guide future studies. 1) Why do alveoli with or without FASN in KO mice seem to experience the same degree of development and secretory activation? 2) At what point following the deletion of FASN does the mammary gland begin involution, and what biological sensors are regulating this process? 3) Can a high-saturated fat diet rescue the phenotype observed in our study? An in-depth analysis focusing on the first 10–15 days of the second or third lactation period will be pertinent to answering these questions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


