Seipin deficiency leads to increased endoplasmic reticulum stress and apoptosis in mammary gland alveolar epithelial cells during lactation†

Ahmed E. El Zowalaty¹.², Rong Li¹.², Weiqin Chen³ and Xiaoqin Ye¹.².∗

¹Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Georgia, Athens, Georgia, USA; ²Interdisciplinary Toxicology Program, University of Georgia, Athens, Georgia, USA and ³Department of Physiology, Augusta University, Augusta, Georgia, USA

∗Correspondence: Department of Physiology and Pharmacology, College of Veterinary Medicine and Interdisciplinary Toxicology Program, University of Georgia, Athens, GA 30602, USA. Tel: +1-706-542-6745; Fax: +1-706-542-3015; E-mail: ye@uga.edu

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Abstract

Seipin is an integral endoplasmic reticulum (ER) membrane protein encoded by Berardinelli–Seip congenital lipodystrophy type 2 (BSCL2/B scl2 ) gene. Most litters (59%) from B scl2 −/− dams mated with wild type (WT) ( B scl2 +/+ ) males did not survive postnatal day 5 (PND5) and pups ( B scl2 −/− ) lacked milk in their stomachs. The survived litters had reduced pup survival rate at PND21. It was hypothesized that seipin was critical for lactation. B scl2 was upregulated and highly detected in the lactation day 1 (LD1) WT mammary gland alveolar epithelial cells. LD1 B scl2 −/− mammary glands lacked adipocytes and alveolar clusters and had varied alveolar morphology: from interconnected mammary gland alveoli with dilated lumen and sloughed epithelial cells to undifferentiated mammary gland alveoli with unexpanded lumen. Comparable levels of whey acidic protein (WAP, a major component in rodent milk) staining and Nile Red lipid droplet staining between WT and B scl2 −/− LD1 alveolar epithelial cells indicated normal milk protein synthesis and lipid syntheses in LD1 B scl2 −/− mammary glands. Significantly reduced percentage of larger lipid droplets was detected in LD1 B scl2 −/− alveoli with unexpanded lumen. There was no obviously impaired proliferation detected by PCNA staining but increased apoptosis detected by cleaved caspase-3 staining in LD1 B scl2 −/− alveolar epithelial cells. Increased expression of protein disulfide isomerase and binding immunoglobulin protein in the LD1 B scl2 −/− mammary gland alveolar epithelial cells indicated increased ER stress. This study demonstrates increased ER stress and apoptosis in LD1 B scl2 −/− mammary gland alveolar epithelial cells and reveals a novel in vivo function of seipin in lactation.
Summary Sentence

Our findings that pups from Bsc12−/− dams lacked milk and had reduced survival rate as well as that LD1 Bsc12−/− mammary gland alveolar epithelial cells had increased ER stress and apoptosis reveal a novel in vivo function of seipin in lactation.

Key words: seipin, mammary gland alveolar epithelial cells, lactation, ER stress, apoptosis.

Introduction

The mammary gland undergoes tremendous side branching and alveogenesis to prepare for lactation [1]. A lactating mouse can secrete ~30 g of lipids in milk during the 20 days of lactation [2], and 98% of milk lipids are triglycerides [3]. A lactating mouse mammary gland can rapidly take up injected radiolabeled fatty acids and convert them into lipid droplets [4]. Microlipid droplets formed in the endoplasmic reticulum (ER) of a lactating mouse mammary gland consist of a triacylglycerol-rich core coated with a layer of proteins and polar lipids. This coating enhances aggregation of lipids into droplets. The lipid droplets are released from the ER in the mammary gland epithelium and fused with cytoplasmic lipid droplets, which are precursors of milk lipids [3] and are surrounded by the bilayer milk lipid globule membrane [5]. Endoplasmic reticulum is also the site for synthesis of milk proteins [6]. Interestingly, seipin is identified as a protein in the milk lipid globule membrane [7].

Seipin is an integral ER membrane protein encoded by Berardinelli–Seip congenital lipodystrophy type 2 (BSCL2/Bsc12) gene [8]. It has been shown that seipin physically interacts with the sarco/ER Ca2+-ATPase in adipocytes [9]. Seipin is required for adipocyte differentiation and lipid droplet accumulation [8, 10–13]. Seipin deficiency results in lipodystrophy and muscle hypertrophy in human and mouse [10, 14–16]. Knockout of seipin in mice or in the fibroblasts of a human patient with seipin-based lipodystrophy results in small lipid droplets and an increase in the number of lipid droplets [17]. A recent study demonstrates that seipin is required for converting nascent to mature lipid droplets, possibly through seipin at ER-lipid droplet contact sites [18]. Milk production from lactation also involves lipid droplet formation [5]. Since seipin is involved in converting nascent to mature lipid droplets [18] and is a protein in the milk lipid globule membrane [7], it suggests that seipin might play a role in lactation.

We have been investigating the roles of seipin in pubertal development and reproduction using Bsc12−/− mice [15, 16]. Previously, we reported abnormalities in 5 weeks old Bsc12−/− mouse mammary glands, such as enlarged lymph nodes, longer and wider mammary gland ducts, and more terminal end buds [16], suggesting altered pubertal mammary gland development in Bsc12−/− mice and in vivo role of seipin in mammary gland development. We also observed that many neonatal pups (Bsc12+/−) from Bsc12−/− females mated with Bsc12+/− males died within a few days of birth. This observation prompted us to investigate Bsc12−/− mammary glands in lactation. Here, we reported a novel role of seipin in lactation.

Materials and methods

Animals

Bsc12−/− mice in C57BL/6J background were derived from an original colony at Baylor College of Medicine with backcrosses to C57BL/6J background for five generations [10]. Genotyping was done as previously described [15, 16]. Bsc12+/− (wild type, WT) and Bsc12+/− females were used as the genotype control for Bsc12−/− females. They were housed in polypropylene cages with free access to food and water on a 12 h light/dark cycle (0600–1800) at 23 ± 1°C with 30%–50% relative humidity at the College of Veterinary Medicine animal facility at the University of Georgia. The animals were sacrificed by CO2 inhalation followed with cervical dislocation. At least three mice in each group were analyzed for each parameter. All methods used in this study were approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC) Committee and conform to National Institutes of Health guidelines and federal law.

Litter size and survival

Young virgin control (Bsc12+/+ and Bsc12+/−) and Bsc12−/− females were mated with WT stud males. Pregnancy was determined by the increase of body weight (>30%) and the continuous changes of the belly shapes. Delivery was monitored and litter size at birth (postnatal day 1 [PND1]) from each dam was recorded. The litters were observed and the number of pups in each litter was recorded at weaning (PND 21). There were 53 litters from control dams and 22 litters from Bsc12−/− dams.

Lipid droplet analysis

Frozen sections (10 μm) of fourth inguinal mammary glands were stained with Nile Red (N3013, Sigma) in the dark for 20 min at room temperature and counterstained with 4’,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI). The sizes of all visible lipid droplets in representative areas (images with 80 × magnification) on lactation day 1 (LD1, the day new pups were found) Bsc12+/− mammary glands and Bsc12−/− mammary glands (areas with expanded alveolar lumen and areas with less expanded/unexpanded alveolar lumen, indicated by denser nuclei in the DAPI staining) were quantified using ImageJ. The percentages of lipid droplets with readings >2000 and >1000 from each image were calculated for comparisons (N = 3–4 mice/group).

Histology and whole mount of mammary gland

The LD1 females were euthanized, and the mammary glands were dissected. The left side mammary glands were snap-frozen. The right fourth inguinal mammary gland was fixed in formalin, dehydrated in a series of 50%, 70%, 80%, 90%, 100% ethanol, and two changes of xylene, embedded in paraffin for histology. The right fourth inguinal mammary glands from another set of LD1 mice were used for whole mount as previously described [16, 19, 20]. The lumen area and epithelial area of all alveoli in a representative 40× image in each WT mammary gland, as well as a representative 40× image with expanded lumen and a representative 40× with less expanded/unexpanded lumen in each Bsc12−/− mammary gland were quantified using ImageJ. The ratio of lumen area to epithelial area of each alveolar was obtained. The average ratio of all alveoli in one 40× image represented one data point of each mouse (N = 3).
In situ hybridization
Sense and antisense cRNA probes were synthesized as previously described [15, 21]. The template for mouse Bscl2 cRNA probe synthesis was amplified from mBscl2 cDNA using primers mBscl2e3F: 5′-GTGCCATCTCAGGAC-3′ and mBscl2e6R: 5′-CTGCAGTGTGGCAGTAC-3′. In situ hybridization was carried out as previously described [15, 21] on sections from gestation day 13.5 (D13.5, WT) and LD1 (WT and control females when they were mated with WT (Bscl2+/−)).

Immunohistochemistry and immunofluorescence
Frozen sections (10 μm) of fourth inguinal mammary glands were used for immunohistochemistry and immunofluorescence as previously described [15, 22] using the following antibodies: anti-protein disulfide isomerase (PDIs) antibody (1:200, ab2792, Abcam), anti-cleaved caspase-3 antibody (1:300, Asp175, Cell Signaling), anti-E-Cadherin (E-Cad) antibody (1:300, Cell Signaling), anti-whey acidic protein (WAP) antibody (D3H8P, Cell Signaling), anti-BiP antibody (Ab21685, Abcam) (Table 1).

Table 1. A list of antibodies used in the study.

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<th>Antibody name</th>
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<th>Dilution</th>
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<td>Binding immunoglobulin protein (BiP)</td>
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Statistical analysis
Data are presented as mean ± SD wherever applicable. Two-tailed Fisher exact test was used for litter size and pup survival rate. Two-tailed unequal variance Student t-test was used for litter size, percentage of larger lipid droplets, and cleaved caspase-3 quantification. Ranking test coupled with two-tailed unequal variance Student t-test was used for ratios of alveolar lumen area/epithelial area. Significance level is set at P < 0.05.

Results

Bscl2−/− females have impaired lactation
Bscl2−/− females had comparable mating activity with age-matched control females when they were mated with WT (Bscl2+/+) stud males. The average numbers and sizes of implantation sites examined on gestation day 13.5 were comparable between control and Bscl2−/− females [23], indicating no obvious defects in early pregnancy events, such as ovulation, fertilization, embryo development, embryo implantation, and placental development in Bscl2−/− dams. However, among the 22 litters delivered by Bscl2−/− females (2–4 months old, mated with WT stud males), 13 of them had 1–10 pups per litter at birth (PND1) but all live pups at birth in these 13 litters were dead by PND5 due to lack of milk in their stomachs (Figure 1A). The remaining 9 litters from Bscl2+/− females survived PND5 and were weaned at PND21. Therefore, the litter survival rate from Bscl2−/− dams (9/22 = 40.9%, P < 0.0001) was significantly decreased compared to the control (Bscl2+/+ and Bscl2−/+; 53/53 = 100%) (Figure 1B). The nine survived litters from Bscl2−/− dams had comparable litter size at PND1 to that from the control dams but had significantly decreased litter size at PND21 compared to that from the control dams (Figure 1C), indicating reduced pup (Bscl2−/−) survival rate in the survived litters from Bscl2−/− dams (Figure 1D). The litter sizes at PND21 in both groups were significantly lower than their respective litter sizes at PND1 (Figure 1C).

Figure 1. Litter sizes and litter survival rates. (A) PND1 pups from Bscl2−/− (+/−) and Bscl2−/+ (−/+) dams. White arrows indicated milk spots in the stomachs. (B) Litter survival rates from control (Con: +/+ and +/−, N = 53) and Bscl2−/+ (−/−, N = 22) nursing mothers (*P < 0.05). (C) Litter sizes at PND1 and PND21. N = 53 survived litters from control nursing mothers, nine survived litters from −/+ nursing mothers, and 13 deceased litters from −/− nursing mothers. *P < 0.05, compared to respective PND1; $P < 0.05, compared to PND21 control; †P < 0.05, compared to the other two PND1 litter sizes; error bar, standard deviation. (D) Pup survival rate at PND21 in the survived litters. N = 431 pups in 53 survived litters from control nursing mothers, and 69 pups in nine survived litters from −/+ nursing mothers; *P < 0.05.
There was interlobular connective tissue but gen-
erally absent of adipocytes in the LD1 mammary glands. Histology confirmed the clusters of secretory mammary gland alveoli that were surrounded by adipocytes, indicated by perilipin 1 (Plin1), a lipid droplet associated protein [24], in the LD1 mammary gland alveoli that were surrounded by adipocytes, (G–H1) Plin1 immunofluorescence. (G1–H1) Enlarged from the rectangle areas in G and H, respectively. Green, Plin1 staining of adipocytes (red star); blue, counterstaining with DAPI; scale bars, 1 cm (A), 400 μm (B), 200 μm (C–H), or 25 μm (C1–H1). (I) Ratios of lumen area/epithelial area. *P < 0.05, compared to +/+; †P < 0.05, compared to −/− with enlarged lumen; N = 3; error bar, standard deviation.

**Expression of whey acidic protein in lactation day 1 mammary glands**

The observations of insufficient milk in pups’ stomachs (Figure 1A) and reduced postnatal pup survival rate (Figure 1D) suggested insufficient milk production in the Bssl2−/− mothers. It appeared that Bssl2 was also detectable in the WT adipocytes, but at a much lower level than in the LD1 WT alveolar epithelial cells (Figure 3B and D). The same Bssl2 antisense probe also detected some signals in the LD1 Bssl2−/− mammary gland (Figure 3C), but the signals were much weaker than that in the LD1 WT mammary gland (Figure 3B). No specific signal was detected in an LD1 WT mammary gland using a sense Bssl2 probe (Figure 3E). The high expression of Bssl2 in the LD1 WT mammary gland alveolar epithelial cells suggested a local function of Bssl2 in the mammary gland epithelium.

**Expression of Bssl2 in lactation day 1 mammary glands**

In situ hybridization indicated that Bssl2 had a relatively low level of expression in the D13.5 WT mammary gland (Figure 3A). Bssl2 was upregulated and highly detected in the LD1 WT mammary gland alveolar epithelial cells (Figure 3B and D). The lack of adipocytes in the mammary gland was consistent with lipodystrophy in the Bssl2−/− females [16].

Expression of whey acidic protein in lactation day 1 mammary gland

The observations of insufficient milk in pups’ stomachs (Figure 1A) and reduced postnatal pup survival rate (Figure 1D) suggested insufficient milk production in the Bssl2−/− mammary glands. To determine milk protein production in the Bssl2−/− LD1 mammary gland alveolar epithelial cells, WAP, a major component in rodent milk [25], was examined in LD1 mammary gland frozen sections using immunofluorescence. WAP was detected in all mammary gland epithelial cells, with more intense labeling on the apical side of epithelial cells (Figure 3B and D). It appeared that Bssl2 was also detectable in the WT adipocytes, but at a much lower level than in the LD1 WT alveolar epithelial cells (Figure 3A and B). The same Bssl2 antisense probe also detected some signals in the LD1 Bssl2−/− mammary gland (Figure 3C), but the signals were much weaker than that in the LD1 WT mammary gland (Figure 3B). No specific signal was detected in an LD1 WT mammary gland using a sense Bssl2 probe (Figure 3E). The high expression of Bssl2 in the LD1 WT mammary gland alveolar epithelial cells suggested a local function of Bssl2 in the mammary gland epithelium.
level of WAP in the mammary gland epithelial cells between WT and \( \text{B}sc\text{l2}^{-/-} \) LD1 mammary glands (Figure 4). No specific signal was detected in the negative control (data not shown). These data demonstrated no defective synthesis of WAP in the \( \text{B}sc\text{l2}^{-/-} \) LD1 mammary gland alveolar epithelial cells.

**Lipid droplets in lactation day 1 \( \text{B}sc\text{l2}^{-/-} \) mammary gland**

In addition to milk protein, another important component of milk is fat. Nile Red lipid droplet staining of frozen LD1 mammary gland sections showed some very large fluorescent irregular smears in the WT that were absent from LD1 \( \text{B}sc\text{l2}^{-/-} \) mammary gland sections (Figure 5). These smears most likely indicated adipocytes that were absent from the LD1 \( \text{B}sc\text{l2}^{-/-} \) mammary gland (Figure 2). Although the LD1 mammary gland structures differed greatly (Figure 2), the density of lipid droplets appeared comparable between LD1 WT and \( \text{B}sc\text{l2}^{-/-} \) mammary glands in areas with alveoli (Figure 5). The sizes of the lipid droplets varied greatly in both WT and \( \text{B}sc\text{l2}^{-/-} \) LD1 mammary glands (Figure 5). Quantitative data (Figure 5J) revealed that the percentage of larger lipid droplets (with ImageJ reading > 1000) in the areas of \( \text{B}sc\text{l2}^{-/-} \) LD1 mammary glands with expanded alveolar lumen (Figure 5D-F) was comparable with that of \( \text{B}sc\text{l2}^{+/+} \) LD1 mammary glands (Figure 5A-C), while significantly higher than that in the areas of \( \text{B}sc\text{l2}^{-/-} \) LD1 mammary glands with less expanded/unexpanded alveolar lumen, indicated by denser nuclei in the DAPI staining (Figure 5G-I). These data indicated that lipid synthesis was not impaired but lipid droplet aggregation might be impaired in the \( \text{B}sc\text{l2}^{-/-} \) mammary gland with undifferentiated alveoli.

**Alveolar epithelial cell proliferation and apoptosis in lactation day 1 \( \text{B}sc\text{l2}^{-/-} \) mammary gland**

Since milk protein WAP synthesis and lipid synthesis in the \( \text{B}sc\text{l2}^{-/-} \) mammary glands were not obviously impaired, to find out the causes for reduced milk production, cell proliferations and apoptosis in the LD1 mammary glands were detected by PCNA staining and cleaved caspase-3 staining, respectively. Almost all alveolar epithelial cells (with round nuclei) were PCNA positive in both WT and \( \text{B}sc\text{l2}^{-/-} \) LD1 mammary glands (Figure 6A, B1 and B2). However, some \( \text{B}sc\text{l2}^{-/-} \) LD1 alveoli had less PCNA positive epithelial cells (Figure 6B2) because these alveoli lacked epithelial cells (Figure 6B2, D3 and H), most likely due to increased apoptosis (Figure 6D3). Cleaved caspase-3 positive cells were rarely detected in the WT LD1 mammary gland alveoli (Figure 6C) but more frequently detected in the \( \text{B}sc\text{l2}^{-/-} \) mammary gland alveoli (Figure 6D), which could be in the epithelial cells on the alveolar epithelium (Figure 6D1), or detached epithelial cells in the alveolar lumen (Figure 6D2 and D3). All the cleaved caspase-3 positive cells were alveolar epithelial cells in both WT and \( \text{B}sc\text{l2}^{-/-} \) LD1 mammary glands. Quantification of total cleaved caspase-3 positive cells per 10× image (Figure 6E) or per 10^4 nuclei (counted by ImageJ) (Figure 6F) showed over 10-fold increase of cleaved caspase-3 positive cells in the \( \text{B}sc\text{l2}^{-/-} \) alveoli. Some LD1 \( \text{B}sc\text{l2}^{-/-} \) alveoli had a few or were depleted of epithelial cells (Figure 6D3), which could explain reduced number of cells with round nuclei in the PCNA staining (Figure 6B2). E-Cad staining revealed organized alveolar epithelial cells in the LD1 WT mammary gland (Figure 6G), but disorganized and often “flattened” or absent, especially in the alveoli with dilated lumen and sloughed epithelial cells, in the LD1 \( \text{B}sc\text{l2}^{-/-} \) mammary gland (Figure 6H). The \( \text{B}sc\text{l2}^{-/-} \) alveoli with scattered epithelial cells or depletion of epithelial cells most likely resulted from epithelial cell apoptosis as seen in Figure 6D3. These data indicated normal alveolar epithelial cell proliferation but increased alveolar epithelial cell apoptosis in the \( \text{B}sc\text{l2}^{-/-} \) LD1 mammary glands.

**Endoplasmic reticulum stress in lactation day 1 \( \text{B}sc\text{l2}^{-/-} \) mammary gland**

Since seipin is localized in the ER and mutant forms of seipin can activate the unfolded protein response (UPR) pathway and induce ER stress-mediated cell death in cultured cells [26], it was hypothesized that seipin deficiency in the \( \text{B}sc\text{l2}^{-/-} \) mammary gland caused ER stress leading to apoptosis in the alveolar epithelial cells. To test
Figure 4. Detection of WAP in LD1 mammary glands. (A–C) Wild type (+/+). (D–F) Bsc2^{+/−} (−/+−) LD1 mammary gland with enlarged alveolar lumen. (G–I) Bsc2^{−/−} (−/−) LD1 mammary gland with unexpanded alveolar lumen. Scale bar, 25 μm (A–I). (J–L) Zoom-in images of a WT (+/+) alveolus with expanded lumen showing apical WAP staining (J), two Bsc2^{−/−} (−/−) alveoli without expanded lumen showing stronger apical WAP staining (K) and relatively even cytoplasm WAP staining (L), respectively. White broken line, outline of an alveolus; scale bar, 10.6 μm.

Figure 5. Detection of lipid droplets in LD1 mammary glands using Nile Red staining. (A–C) Wild type (+/+). (D–F) Bsc2^{+/−} (−/+−) LD1 mammary gland with enlarged alveolar lumen. (G–I) Bsc2^{−/−} (−/−) LD1 mammary gland with less expanded/unexpanded alveolar lumen. Green dots, Nile Red staining of lipid droplets; blue: DAPI staining of nuclei; scale bar, 100 μm. (J) Percentage of larger lipid droplets with ImageJ readings >2000 or >1000. The three sets of data corresponded to the scenarios in B, E, and H, respectively. N = 3–4 mice/group; ∗P < 0.05; error bar, standard deviation.
in the alveoli with a few remaining epithelial cells (Figure 7G–I). Detached Bsc1l2−/− alveolar epithelial cells in the lumen appeared to have weaker PDI staining than those attached epithelial cells (Figure 7G–I). Similar pattern of BiP upregulation in LD1 Bsc1l2−/− alveolar epithelial cells was also observed (Figure 7J–L). These results indicated increased ER stress in the LD1 Bsc1l2−/− alveolar epithelial cells.

**Discussion**

This study reveals a novel in vivo role of seipin in lactation. Two main categories of components in the milk are proteins and lipids, which are originally produced in the mammary alveolar epithelial cells. Most litters (59%) from Bsc1l2−/− dams failed to survive PND5, and the survived litters had reduced postnatal survival rate, indicating insufficient milk production from the Bsc1l2−/− mothers. However, the expression level of WAP (a major component in rodent milk [25]) and the density of lipid droplets in the LD1 Bsc1l2−/− mammary alveolar epithelial cells appeared comparable to control, indicating that the Bsc1l2−/− mammary alveolar epithelial cells were functional in protein synthesis and lipid synthesis for milk production. This study demonstrates abnormal morphology in LD1 Bsc1l2−/− mammary alveoli: from undifferentiated and nonsecretory alveoli with unexpanded lumen to alveoli with dilated lumen and sloughed epithelial cells. These conditions compromise milk production in the Bsc1l2−/− mammary gland.

Seipin is an integral ER membrane protein [8] that is required for adipocyte differentiation and lipid droplet accumulation [8, 10–13]. However, heterogeneities in cell differentiation and lipid droplet accumulation were observed in the LD1 Bsc1l2−/− mammary gland epithelial cells. It was noticed that the poorly differentiated LD1 Bsc1l2−/− mammary gland alveolar epithelial cells had less polarity in WAP cellular distribution, which normally accumulates in the apical side of the differentiated alveolar epithelial cells. The poorly differentiated LD1 Bsc1l2−/− mammary gland alveoli had less percentage of larger lipid droplets, indicating impaired aggregation of lipid droplets into larger ones. These observations indicate that seipin plays an important role in mammary gland alveolar epithelial cell differentiation. The poorly differentiated LD1 Bsc1l2−/− mammary gland alveolar epithelial cells impair milk secretion, which contributes to reduced milk production in the Bsc1l2−/− lactating mice.

On the other hand, some LD1 Bsc1l2−/− mammary gland alveolar epithelial cells were well differentiated and had comparable cellular distribution of WAP with control. These cells had an accumulation of larger lipid droplets comparable to the control. A recent study in Drosophila and human cells [18] demonstrates that seipin forms discrete and dynamic foci in the ER that interact with nascent lipid droplets to enable their conversion into larger, mature lipid droplets. In the absence of seipin, most nascent lipid droplets often fail to grow, which is consistent with the smaller lipid droplets seen in the poorly differentiated LD1 Bsc1l2−/− mammary gland alveoli. Interestingly, it was also reported in the above study [18] that those lipid droplets that did grow, they eventually expanded into giant lipid droplets characteristic of seipin deficiency. The two patterns of lipid droplets observed in the poorly and well-differentiated LD1 Bsc1l2−/− mammary gland alveolar epithelial cells in this study may reflect both differentiation status and the function of seipin in lipid droplet growth.

LD1 Bsc1l2−/− mammary gland alveolar epithelial cells had normal proliferation but increased apoptosis, which led to the slough
of many alveolar epithelial cells into the lumen and, subsequently, reduced alveolar epithelial cells for milk secretion. Accompanying with the increased apoptosis, there was increased ER stress in the LD1 Bsc2<sup>−/−</sup> mammary gland alveolar epithelial cells indicated by the increased expression of PDI and BiP, two ER chaperones induced during ER stress and important players in UPR pathway [26, 27]. Although it has been suggested that changes in ER stress pathway in the mammary gland during lactation are normal adaptions to the changing physiological state [28], and activation of UPR signaling pathway in responding to ER stress is initially protective [27], prolonged ER stress is proapoptotic [27]. One study reveals that mutant forms of seipin can activate the UPR pathway and induce ER stress-mediated cell death in cultured cells [26]. Another study indicates that protein phosphatase 1 (formerly 2C)-like (PP2Ce), an ER membrane targeted protein phosphatase, is highly expressed in lactating mammary gland epithelium and involved in regulating ER stress, and PP2Ce deficiency leads to loss of milk production and induction of lactating mammary gland epithelial apoptosis [29], indicating that ER stress plays an important role during lactation. The increased LD1 Bsc2<sup>−/−</sup> mammary gland alveolar epithelial cell apoptosis most likely results from increased ER stress.

ER stress was also induced in the mammary gland epithelium deficient of X-box binding protein 1 (Xbp1), a key mediator of UPR, and associated with inhibition of epithelial differentiation during lactation leading to impaired milk production [25]. It was possible that increased ER stress also contributed to the poor differentiation of some LD1 Bsc2<sup>−/−</sup> mammary gland alveolar epithelial cells.

In summary, this study reveals a novel in vivo role of seipin in lactation. Although seipin deficiency did not have obvious effects on cell proliferation of mammary gland alveolar epithelial cells or protein synthesis and lipid synthesis in the mammary gland alveolar epithelial cells, seipin deficiency led to poor differentiation and/or increased apoptosis of mammary gland alveolar epithelial cells, both of which contribute to reduced milk production and both of which could result from increased ER stress in the mammary gland alveolar epithelial cells. The molecular mechanisms of seipin in regulating ER stress during lactation remain to be elucidated.

**Figure 7.** Increased expression of PDI and BiP in Bsc2<sup>−/−</sup> LD1 mammary gland alveolar epithelial cells. (A–C and J) Wild type (+/+), PDI (A–C), and BiP (J). (D–F and K) Bsc2<sup>−/−</sup> LD1 mammary gland with most retained alveolar epithelial cells, PDI (D–F), and BiP (K). (G–I and L) Bsc2<sup>−/−</sup> LD1 mammary gland with a few remaining alveolar epithelial cells, PDI (G–I), and BiP (L). Red arrows in G–I, sloughed epithelial cells in alveolar lumen; scale bar, 25 μm.
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Conflict of Interests: The authors have declared that no conflict of interest exists.

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