Scribble promotes alveologenesis in the pregnant mammary gland for milk production

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Abstract

Mammary glands are comprised of ducts and terminal lobules that form tree-like structures. Luminal epithelial cells in these lobules undergo differentiation into alveolar cells in pregnancy to support milk production. This study reveals that Scribble (SCRIB), a scaffold protein expressed in progesterone receptor (PGR)-positive cells, plays a critical role in mammary gland alveologenesis in mice. We conditionally deleted *Scrib* using a *Pgr-Cre* driver. PGR is heterogeneously expressed throughout the luminal epithelium. *Scrib* loss in mammary glands by *Pgr-Cre* (*Scrib^{f/f}Pgr^{Cre/+}*) shows inefficient alveologenesis and terminal end bud (TEB)-like morphology during pregnancy, resulting in poor milk production and subsequent death of pups after delivery. The differentiation of PGR-positive epithelial cells into *Elf5*-expressing alveolar cells is defective in *Scrib^{f/f}Pgr^{Cre/+}* mice. These changes are reflected in reduced activation of JAK2 and PAK1, resulting in downregulation of pSTAT5, a critical transcriptional factor for alveologenesis. These results provide evidence that SCRIB impacts PGR-positive cell lineage during alveologenesis, which impacts milk production and the health of offspring.

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Introduction

Functional mammary glands contribute to the health of offspring by providing nutrients and immunoglobulins via milk production (Palmer *et al.* 2006). Lobules and ducts inside the mammary glands form tree-like structures, with lobules developing alveoli, blooming flower-like structures that support milk production in pregnancy (Palmer *et al.* 2006, Paine & Lewis 2017). Branching ducts work as pipes to transport milk from alveoli to a nipple (Palmer *et al.* 2006). At puberty, terminal end buds (TEBs) with a spoon-like structure are observed at the tips of each duct and contribute to ductal elongation and branching (Sternlicht 2006, Briskin & Ataca 2015). TEBs regress once they reach the end of fat pads, and further branching occurs in response to estrous cycles to form lobular structures (Sternlicht 2006). During every pregnancy cycle, mammary epithelia undergo extensive proliferation and differentiation to establish milk producing epithelia (alveologenesis) that undergo involution at the cessation of nursing of pups and associated with changes in expressions of female hormone receptors for progesterone (P₄), estrogen (E₂) and prolactin (Prl) (Saji *et al.* 2000, Ismail *et al.* 2002, Palmer *et al.* 2006, Briskin & Ataca 2015). Mammary glands are primarily comprised of luminal epithelial cells, basal epithelial (myoepithelial) cells and stromal fat cells (Briskin & Ataca 2015). Luminal epithelial

cells are major players in mammary gland development and differentiation, further classified by expressions of hormone receptors or transcriptional factors (Briskin & Ataca 2015). Differentiation and function of luminal epithelial cells are highly dependent on P₄, E₂ and Prl as well as expression patterns of their receptors throughout puberty to adult stages (Lydon *et al.* 1995, Saji *et al.* 2000, Grimm *et al.* 2002, Mallepell *et al.* 2006). The progesterone (P₄) receptor (PGR) is one of the core molecules to determine cell type and is also critical to the regulation of mammary development and function (Briskin & Ataca 2015). In mice, newly formed corpora lutea produce higher levels of P₄ from day 3 of pregnancy and onward; P₄ level drops upon parturition (Cha *et al.* 2012). PGR is non-uniformly expressed in luminal epithelia, with high expression in TEBs and ductal tips followed by decreased expression as pregnancy progresses (Hovey *et al.* 2001, Ismail *et al.* 2002). Reports using mouse genetic models have shown that PGR-expressing epithelial cells contribute to cell proliferation and alveologenesis (Beleut *et al.* 2010, Lee *et al.* 2013, Briskin & Ataca 2015).

To explore functions of specific genes in mammary epithelial cells, *MMTV-Cre* and *Wap-Cre* are primarily used (Wagner *et al.* 1997). Since *MMTV-Cre* is induced not only in luminal but also in basal epithelial cells, it poses difficulties in investigating the function of target genes in certain epithelial lineages (Wagner *et al.* 1997).

Wap-Cre is induced specifically in alveolar cells, but efficient gene recombination occurs when females experience lactation (Wagner *et al.* 1997). Previously, we and others established mouse lines with Cre drivers expressed in specific epithelial cells: *Pgr-Cre* and *Ltf-Cre* (Soyal *et al.* 2005, Daikoku *et al.* 2014). *Pgr-Cre* is expressed in PGR-positive cells and highly induced particularly in TEBs and the lobular tips of each duct (Soyal *et al.* 2005), whereas *Ltf-Cre* is induced in ductal epithelia (Daikoku *et al.* 2014). These Cre drivers are useful to investigate functions of specific genes of interest in a cell lineage-specific manner.

Scribble (SCRIB) is a scaffold protein involved in a variety of cellular functions by binding to other proteins responsible for specific signaling pathways, including epithelial cell polarity (Bonello & Peifer 2019). There are reports on the importance of SCRIB in mammary glands (Zhan *et al.* 2008, Godde *et al.* 2014, Baker *et al.* 2016). In MCF10A, a mammary gland cell line with no PGR or estrogen receptor expression, *Scrib* knockdown suppresses myc-induced apoptosis, and transplantations of those cells resulted in tumorigenesis (Zhan *et al.* 2008). Mammary glands with *Scrib* knockout (KO) using *MMTV-Cre* also showed a tumorigenic phenotype due to compromised cell polarity and excessive expansion of ductal epithelia (Godde *et al.* 2014). Another study systemically knocked down *Scrib* in females before pregnancy; these mice showed decreased cell proliferation and alveologenesis because of reduced prolactin receptor (PRLR) expression on cell surfaces, while milk production was not affected (Baker *et al.* 2016). These studies with different phenotypes suggest that SCRIB has complex functions in mammary glands depending on cell types or physiological states, such as pregnancy.

Here, we show that SCRIB expressed in the PGR-positive cell lineage is critical for alveologenesis in pregnancy. *Scrib^{f/f}Pgr^{Cre/+}* females show poor milk production, resulting in the death of their offspring promptly after delivery. This defect is not observed in *Scrib^{f/f}Ltf^{Cre/+}* females. The phenotype in *Scrib^{f/f}Pgr^{Cre/+}* females is not due to dysregulated cell polarity or abnormal cell death during early pregnancy, as previously reported in *Scrib* deletion in whole mammary glands. *Scrib* deletion in PGR-positive cell lineages results in the failure of luminal cells to differentiate into mature alveolar cells, depicted by reduced levels of *Elf5* expression and a large accumulation of PGR-positive cells in lobular epithelia. In addition, pJAK2, pPAK1 and pSTAT5, which are indispensable factors for alveologenesis, are reduced in *Scrib^{f/f}Pgr^{Cre/+}* tissues. Tridimensional (3D) imaging of mammary glands on days 4 and 12 of pregnancy shows that *Scrib^{f/f}Pgr^{Cre/+}* tissues have TEB-like structures with higher levels of cell proliferation, suggesting abnormal differentiation of PGR-positive cell lineages. Our model using PGR-positive cell-specific gene deletion illuminates new insights into context-dependent SCRIB functions.

Materials and methods

Mice

Pgr^{Cre/+}, *Ltf^{Cre/+}* and *Scrib^{f/f}* mouse lines were generated as previously described (Soyal *et al.* 2005, Hartleben *et al.* 2012, Daikoku *et al.* 2014). Conditional knockout mice of Scribble were generated by mating floxed females with either *Pgr^{Cre/+}* or *Ltf^{Cre/+}* males. To confirm induction of *Ltf-Cre*, *Rosa26^{TdTomato}Ltf^{Cre/+}* females were generated by mating *Rosa26^{TdTomato}* females (Jackson Lab) with *Ltf^{Cre/+}* males. All mice used in this study were housed under a constant 12-h/12-h light/dark cycle in the Cincinnati Children's Animal Care Facility according to NIH and institutional guidelines for the use of laboratory animals. All protocols were approved by the Cincinnati Children's Animal Care and Use Committee. Mice were provided *ad libitum* with autoclaved Laboratory Rodent Diet 5010 (Purina) and UV light-sterilized reverse osmosis/deionized constant-circulation water *ad libitum*.

Preparation of pregnant females and collection of mammary glands

Three adult females were randomly chosen and housed with a WT fertile male overnight in separate cages; the morning of finding the presence of a vaginal plug was considered successful mating (day 1 of pregnancy). Plug-positive females were then housed separately from males. The morning of finding delivered pups was defined as lactation day 1. Litter size and number of live/dead pups were counted until lactation day 2. To confirm pregnancy, plug-positive females were examined as follows: In plug-positive day 4 mice, one uterine horn was flushed with saline to confirm the presence of blastocysts. On day 5 of pregnancy, mice were intravenously injected with a blue dye solution (1% Chicago Blue in Saline, 100 µL/mouse) 4 min before killing to confirm embryo implantation (Psychoyos 1973). On day 12 of pregnancy, the implantation sites are blatant. The fourth mammary glands which are located in the distal to the sternum were collected from each female as previously described (Plante *et al.* 2011).

Carmine alum staining of whole mount mammary gland

Carmine alum staining was performed as previously described (Plante *et al.* 2011). Briefly, the fourth mammary glands were attached to glass slides and fixed in Carnoy's fixative. Tissues were stained in Carmine Alum solution overnight (O/N) and dehydrated in ethanol and xylene. Pictures were taken using Nikon Eclipse 90i upright microscope.

Immunofluorescence (IF)

IF was performed as previously described (Yuan *et al.* 2018). IF using frozen sections (12 µm) was performed using the following antibodies: Scribble (1:500, sc-28737, Santa Cruz), PGR (1:300, 8757; Cell Signaling technology), E-Cadherin (1:300, 3195s, Cell Signaling Technology), CK8 (1:1000, TROMA-1, Hybridoma Bank), Ki67 (1:300, RM-9106-S, Thermo Fisher Scientific), Cleaved Caspase-3 (1:300, 9661s, Cell Signaling technology),

ZO-1 (1:300, 14-9776-82 Thermo Fisher Scientific), Radixin (1:300, sc-6408, Santa Cruz), pPAK1(Ser144)/pPAK2(Ser141) (1:300, 2606s, Cell Signaling Technology). For signal detection, secondary antibodies conjugated with Cy-2, Cy-5, Alexa 488, Alexa 594 or Alexa 647 (1:300, Jackson Immuno Research) were used. Nuclear staining was performed using Hoechst 33342 (5 µg/mL, H1399, Thermo Fisher Scientific). Tissue sections from control and experimental groups were processed on the same slide. Pictures were taken using Nikon Eclipse TE2000-E. Numbers of Ki67-positive cells and nuclei were quantified using Image J (NIH).

Immunohistochemistry (IHC)

IHC using paraffin sections (6 µm) was performed with the following antibodies: β-casein (1:300, sc-166530, Santa Cruz), pJAK2 (1:300, sc-294, Santa Cruz) and pSTAT5 (1:300, 9314s, Cell Signaling Technology). Signals were detected with DAB (Sigma) by following the manufacturer's protocol and counterstained with either hematoxylin (for β-casein and pJAK2) or eosin (pSTAT5). Pictures were taken using Nikon Eclipse E800.

In situ hybridization

Frozen sections from mice with each genotype were processed on the same slide. *In situ* hybridization with ³⁵S-labeled cRNA probes was performed as described (Briskin et al. 2002) using probes for *Prlr* (Briskin et al. 2002) and *Tnfrsf11*. Signals were visualized under a Nikon Eclipse E800 with dark-field. Fluorescence *in situ* hybridization was adopted based on previously established DIG *in situ* hybridization (Cha et al. 2014). In brief, following proteinase K (5 mg/mL) digestion and acetylation, slides were hybridized at 55°C with the DIG-labeled *Elf5* probe. Anti-Dig-peroxidase was applied onto hybridized slides following washing and peroxide quenching. Color was developed by TSA (Tyramide signal amplification) Fluorescein according to the manufacturer's instructions (PerkinElmer). Signals were visualized under a Nikon A1 Confocal Microscope. Following primers were used for the probes: 5'-GCATCGCTCTGTTCTG-3' and 5'-TTTTCGTGC TCCCTCCTTC-3' for *Rankl*; 5'-AGCCAGTGTTATGGG TGCTG-3' and 5'-GGGGAAGTTAGGTTTCAGGG-3' for *Elf5*.

Tridimensional visualization of mammary glands

Whole mount staining, tissue clearing and 3D visualization of mammary glands were performed as previously described (Yuan et al. 2018) with some modification in the procedure of fixation. The fourth mammary glands were attached to glass slides and fixed in acetone at -20°C, O/N. Anti-CK8 antibody (1:300, TROMA-1, Hybridoma Bank), anti-PGR antibody (1:300, 8757; Cell Signaling technology), anti-Ki67 antibody (1:300, RM-9106-S, Thermo Scientific) and anti-Scrib antibody (1:300, sc-28737, Santa Cruz) were used as primary antibodies. Anti-rabbit antibody conjugated with Alexa 594 (1:300, Jackson ImmunoResearch) and anti-rat antibody conjugated with Cy2 (1:300, Jackson ImmunoResearch) were used as secondary antibodies. Dehydration in methanol was

followed by tissue clearing by BABB (Benzyl alcohol: Benzyl benzoate (1:2); each reagent from Sigma-Aldrich) for 1 h at room temperature. The samples were then stored in the dark until 3D imaging acquisition. 3D images were acquired by a Nikon multiphoton upright confocal microscope (Nikon A1R) using LWD 10X objective with 3 µm Z-stack.

Statistical analysis

Data are displayed as mean ± S.E.M. The data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. *P* < 0.05 was considered statistically significant.

Results

Scrib deletion in PGR-expressing cells results in poor milk production

To examine the role of *Scrib* across cell lineages, we generated *Scrib^{fl/fl}Pgr^{Cre/+}* and *Scrib^{fl/fl}Ltf^{Cre/+}* females by mating mice harboring a specific Cre-driver with floxed *Scrib* mice (Hartleben et al. 2012). We found that almost 30% of pups from *Scrib^{fl/fl}Pgr^{Cre/+}* dams die within 2 days of delivery (Fig. 1A). The major cause of death appears to be malnourishment, as the pups from *Scrib^{fl/fl}Pgr^{Cre/+}* dams have poor milk accumulation in their stomachs (Fig. 1B). These Cre drivers are expressed in mammary glands (Soyal et al. 2005, Daikoku et al. 2014). We do observe the loss of *Scrib* in a group of mammary epithelial cells by IF in *Scrib^{fl/fl}Pgr^{Cre/+}* females (Fig. 2 and Supplementary Fig. 1A, B, see section on supplementary materials given at the end of this article).

We then asked whether milk production is compromised in *Scrib^{fl/fl}Pgr^{Cre/+}* females. Two markers for milk production, Oil-red-O (Fig. 3A) and β-casein staining (Fig. 3B), indicate poor milk secretions in these mutant mice. Normally, alveolar epithelial cells undergo extensive proliferation during lactogenesis in response to increasing demands of milk (Jahchan et al. 2012). In agreement with the reduced milk production, *Scrib*-deletion in PGR-positive cells compromises proliferation depicted by Ki67 staining at the lactation stage (Fig. 3C and Supplementary Fig. 2).

Surprisingly, *Scrib* deletion using *Ltf*-Cre driver, which is also induced in mammary epithelial cells (Supplementary Fig. 1B) (Daikoku et al. 2014), does not result in obvious problems with the mothers in nursing pups. The different phenotypes between *Scrib^{fl/fl}Pgr^{Cre/+}* and *Scrib^{fl/fl}Ltf^{Cre/+}* mice are perhaps due to differential *Pgr* and *Ltf* expression: PGR is highly expressed in TEBs (Ismail et al. 2002), while *Ltf* is expressed in the ductal epithelia (Supplementary Fig. 1B) (Daikoku et al. 2014). In addition, *Ltf* expression levels are higher during involution rather than pregnancy stages (Neville & Zhang 2000). There are reports showing that *Ltf* is poorly expressed in PGR- and PRLR-positive cells (Scheele et al. 2017, Casey et al. 2018) which are critical for alveologenesis (Briskin & Ataca 2015).

Figure 1 *Scrib^{fl/fl}Pgr^{Cre/+}* females fail to nourish new born pups. (A) The rate of dead pups among total pups from females with each genotype. *n*: the number of total dams. The numbers of total dead pups/total delivered pups are noted within brackets above each bar. Data are means $\pm$ S.E.M., **** $P < 0.0001$ and n.s.: not significant by one-way ANOVA followed by Bonferroni *post hoc* test. (B) Representative pictures of pups from dams with each genotype on the next day of delivery (lactation day 2). Arrowheads indicate milk spots. Scale bar: 10 mm.

Taken together, these observations suggest that *Scrib* is critical to the preparation of mammary glands for milk production, particularly related to PGR-expressing cells.

***Scrib* deletion in PGR-expressing cells results in abnormal alveogenesis and associated with reduced *Prl* signaling**

We sought to explore the mechanism by which *Scrib* deletion in PGR-positive cells affects milk production during pregnancy. There have been reports that PGR signaling is important for mammary gland side branching, epithelial expansion and alveogenesis

during development and pregnancy (Beleut *et al.* 2010, Lee *et al.* 2013, Briskin & Ataca 2015). To examine when mammary development is affected by *Scrib*-deletion in PGR-positive mammary epithelial cells, we performed whole-mount staining of mammary glands by Carmine Alum solution (Plante *et al.* 2011). Whole mount observations captured bigger terminal tips of each duct in *Scrib^{fl/fl}Pgr^{Cre/+}* mammary glands (Fig. 4A, top). This morphology is further accentuated in the mid-stage of pregnancy (day 12); *Scrib^{fl/fl}Pgr^{Cre/+}* tissues show TEB-like structures, compared to fine branching observed in ductal tips of floxed or *Scrib^{fl/fl}Ltf^{Cre/+}* females (Fig. 4A, middle). Eventually, *Scrib^{fl/fl}Pgr^{Cre/+}* mammary glands develop flattened lobules in contrast to the blossoming alveoli of floxed and *Scrib^{fl/fl}Ltf^{Cre/+}* females (Fig. 4A, bottom). The enlarged TEB-like lobules in *Scrib^{fl/fl}Pgr^{Cre/+}* mammary glands are more evident during the early stages of a second pregnancy (Fig. 4B).

PGR cooperatively works with Prolactin (PRL) signaling to establish milk-producing alveoli during pregnancy (Grimm *et al.* 2002, Obr *et al.* 2013, Sackmann-Sala *et al.* 2015, Bernard *et al.* 2019). With increasing PRL secretion during pregnancy, PRLR activates JAK2 (pJAK2), a downstream target, which in turn phosphorylates STAT5 (Shillingford *et al.* 2002). PAK1 is also a kinase that phosphorylates and facilitates nuclear translocation of STAT5 (Wang *et al.* 2003). There is evidence that SCRIB supports PAK1 activation by indirect binding to this protein (Nola *et al.* 2008, Zhan *et al.* 2008). Mice deficient in either of these mediators results in abnormal development and differentiation of mammary glands, indicating the importance of this signaling axis in this tissue (Briskin *et al.* 1999, Miyoshi *et al.* 2001, Shillingford *et al.* 2002, Wang *et al.* 2003). Phosphorylated nuclear STAT5 (pSTAT5) stimulates expression of paracrine factors, further expanding the signal cascades for alveogenesis (Hennighausen & Robinson 2005). STAT5 can facilitate PGR-induced transcription of RANKL (Obr *et al.* 2013), another key factor for alveogenesis. It was shown that poor cell proliferation in PGR-deficient mammary glands is rescued by RANKL expression (Beleut *et al.* 2010), indicating the importance of the PRLR-pSTAT5-RANKL axis in the PGR-positive cell lineage.

These results prompted us to ask if *Scrib*-deletion in PGR-positive cells influences these signaling pathways. First, we examined the expression of *Prlr*. *In situ* hybridization shows it is comparable between each genotype (Fig. 5A). We then investigated whether downstream processes of PRLR are affected by *Scrib* deletion. Normally, pJAK2 is present in the cytosol of alveolar cells during mid-pregnancy (Fig. 5B). However, *Scrib^{fl/fl}Pgr^{Cre/+}* cells show lower levels of pJAK2 (Fig. 5B). In the same vein, pPAK1 is also reduced (Fig. 5C). These results are associated with reduced nuclear pSTAT5 levels in *Scrib^{fl/fl}Pgr^{Cre/+}* mammary epithelia compared to those of floxed or *Scrib^{fl/fl}Ltf^{Cre/+}* females (Fig. 5D).

IF of *Scrib* (day 5 of pregnancy)

Figure 2 Deletion of *Scrib* in mammary glands by *Pgr-Cre* driver. IF of *Scrib* and CK8 in pregnancy day 5 mammary glands from females with each genotype. Arrowheads indicate the location of *Scrib*-deleted epithelial cells. Scale bar: 20 μ m. Each image is a representative from at least three independent experiments.

These results led us to examine the levels of RANKL. Our *in situ* hybridization results show that *Rankl* is decreased in epithelia from *Scrib^{f/f}Pgr^{Cre/+}* females (Fig. 5E). Taken together, these findings indicate the loss of SCRIB in PGR-positive cell lineages results in alveologenesis failure.

***Scrib*-deletion in PGR-positive cell lineages results in dysregulated differentiation and expansion of PGR-positive cell lineages**

Our observations of poor alveologenesis in *Scrib^{f/f}Pgr^{Cre/+}* mice raise the question as to how SCRIB in the PGR-positive cell lineage contributes to mammary cell differentiation into mature alveolar cells.

Abnormal epithelial cell polarity has been reported to yield abnormal functions of mammary glands (Akhtar & Streuli 2006, Jahchan *et al.* 2012). SCRIB is a cell polarity component (Bonello & Peifer 2019), and several studies featuring *Scrib* mutants show abnormal cell polarity in mammary epithelia and compromised

alveologenesis (Zhan *et al.* 2008, Godde *et al.* 2014). However, *Scrib^{f/f}Pgr^{Cre/+}* mammary glands show normal apico-basal cell polarity as depicted by apical staining of ZO-1 (Fig. 6A). We also examined cell apoptosis, which was compromised by SCRIB loss in whole mammary epithelia (Zhan *et al.* 2008, Godde *et al.* 2014). In contrast to previous reports, apoptosis defined by the staining of cleaved-caspase-3 also shows comparable levels in our mutant lines (Fig. 6B and Supplementary Fig. 3). These results indicate that SCRIB regulates PGR-positive cells specific to intracellular signaling but not cell polarity and cell death.

We performed 3D imaging of mammary glands after immunostaining of CK8 (a luminal epithelial cell marker) and PGR. PGR-expressing cells are highly populated in TEBs during puberty stages, but the number decreases in adults, especially during pregnancy (Ismail *et al.* 2002). However, *Scrib^{f/f}Pgr^{Cre/+}* mammary glands show higher levels of PGR-positive cells in lobules during mid-pregnancy (Fig. 7A). Previous studies have shown that P₄-PGR signaling regulates mammary differentiation

Figure 3 Abnormal milk production in *Scrib^{f/f}Pgr^{Cre/+}* mammary glands. (A and B) Oil red O staining (A) and IHC of β-casein (B) show poor milk production in *Scrib^{f/f}Pgr^{Cre/+}* mammary glands on lactation day 2. Scale bar: 50 μm (A) and 20 μm (B). (C) IF of Ki67 (cell proliferation marker) and CK8 (epithelial cell marker) indicates reduced proliferation of alveolar cells in *Scrib^{f/f}Pgr^{Cre/+}* mammary glands on lactation day 2. Scale bar: 50 μm. Each image is a representative from at least three independent experiments.

and proliferation via a transcriptional factor ELF5 (Oakes *et al.* 2008, Lee *et al.* 2013). ELF5 is highly induced in mature alveolar cells with a mutual exclusion with PGR (Lee *et al.* 2013) and ELF5 deletion in mammary glands results in differentiation failure of milk-producing cells (Oakes *et al.* 2008). P₄-PGR axis stimulates RANKL expression to drive the maturation of mammary epithelial cells into secretory cells by Elf5 induction (Lee *et al.* 2013). Our *in situ* hybridization shows that *Elf5* is reduced in *Scrib^{f/f}Pgr^{Cre/+}* mammary glands during mid-pregnancy (Fig. 7B). Considering the increased population of PGR-positive cells, this result indicates that cell differentiation is compromised during pregnancy.

In accord with these observations, the expression of *Rankl* is also reduced in *Scrib^{f/f}Pgr^{Cre/+}* mammary glands (Fig. 5E). We further traced back defects in the mutant alveoli. 3D Imaging during early pregnancy reveals that enlarged gland tips in *Scrib^{f/f}Pgr^{Cre/+}* show relatively high expression of PGR similar to puberty stage TEBs (Fig. 7C) (Ismail *et al.* 2002). In addition, we found increased cell proliferation in TEB-like structures of *Scrib^{f/f}Pgr^{Cre/+}* tissues, in which PGR-positive cells are highly abundant (Fig. 7D). This finding contrasts with previous observations in the systemic knockdown of *Scrib* with poor mammary cell proliferation during early pregnancy (Baker *et al.* 2016), further supporting our notion that

Figure 4 Abnormal alveologenesis in *Scrib^{f/f}Pgr^{Cre/+}* females. (A) Carmine Alum staining of whole mount mammary glands was performed using tissues from each genotype on pregnancy day 5 (top), pregnancy day 12 (middle) and lactation day 2 (bottom). Scale bar: 100 μ m. (B) Carmine alum staining of whole mount mammary glands on day 5 of 2nd pregnancy. Scale bar: 100 μ m. Each image is a representative from at least three independent experiments.

SCRIB exerts a unique function in the PGR-positive cell lineage. In summary, our results indicate SCRIB in PGR-positive cells modulates cell differentiation into mature milk-producing cells via *Elf5*-induction.

Discussion

In this study, we illuminate the critical roles played by SCRIB in PGR-positive mammary epithelial cells for epithelial differentiation during pregnancy. *Scrib*

deletion using a *Pgr-Cre* driver results in defective cell differentiation of milk-producing mature cells.

Previous studies in mice have illustrated the complexities of SCRIB function in mammary glands (Table 1): *Scrib* knockdown in hormone receptor-negative mammary cells undergo tumorigenesis (Zhan *et al.* 2008). *MMTV-Cre* induced global deletion of *Scrib* in mammary epithelia resulted in ductal hyperplasia (Godde *et al.* 2014), while whole-body knockdown of Scribble in adult females showed poor alveologenesis

Figure 5 Reduced JAK2-PAK1-STAT5 signaling in *Scrib^{fl/fl}Pgr^{Cre/+}* mammary glands. (A) *In situ* hybridization of *Prlr* (brownish yellow) in pregnancy day 12 mammary epithelia from females with each genotype. Scale bar: 50 μ m. (B) IHC of pJAK2 with Hematoxylin counter staining in pregnancy day 12 mammary glands from females with each genotype. Scale bar: 20 μ m. (C) IF of pPAK1/pPAK2 in pregnancy day 12 mammary glands from females with each genotype. Scale bar: 20 μ m. (D) IHC of pSTAT5 with Eosin counter staining in pregnancy day 12 mammary glands from females with each genotype. Scale bar: 20 μ m. (E) *In situ* hybridization of *Rankl* (brownish yellow) in pregnancy day 12 mammary glands from females with each genotype. Scale bar: 50 μ m. Each image is a representative of at least three independent experiments.

Figure 6 Comparable cell polarity and apoptosis in *Scrib*^{f/f}*Pgr*^{Cre/+} mammary epithelia. (A) Cell polarity was depicted by IF of ZO-1 using pregnancy day 12 tissues. ZO-1 is localized on the apical surface of epithelia. Scale bar: 50 μm. (B) IF of cleaved caspase-3 (Cl-Cas3; apoptosis marker) and CK8 in pregnancy day 12 mammary gland tissues from each genotype. Scale bar: 20 μm. Each image is a representative from at least three independent experiments.

but normal milk production upon pregnancy (Baker *et al.* 2016). In contrast to these reports, our model using *Scrib*^{f/f}*Pgr*^{Cre/+} females demonstrates the importance of SCRIB in the PGR-positive cell lineage for alveologenesis to prepare for milk production. *Scrib*^{f/f}*Pgr*^{Cre/+} mammary glands show abnormal alveolar formation depicted by enlarged and poorly bubbled lobules akin to TEBs in mammary glands at the time of puberty. This anomaly is accompanied by impaired PRLR signaling. While PRLR levels are comparable between each genotype, phosphorylated JAK2, PAK1 and STAT5 are reduced in this mutant line, resulting in reduced levels of RANKL, which is critical for PGR-induced mammary functions

(Beleut *et al.* 2010, Obr *et al.* 2013). Lobules in *Scrib*^{f/f}*Pgr*^{Cre/+} tissues contain increased numbers of PGR-positive cells with higher cell proliferation during early pregnancy. In contrast, *Scrib*^{f/f}*Pgr*^{Cre/+} lobules fail to proliferate in response to increasing demands for milk at the lactation stage. These anomalies in cell proliferation during pregnancy or lactation indicate compromised differentiation of luminal epithelial cells. Normally, PGR is highly expressed in TEBs and lobules, which are primary drivers for mammary epithelial expansion and differentiation (Paine & Lewis 2017). The number of PGR-positive cells declines with progression of pregnancy and an increasing population of ELF5-positive cells that

Figure 7 *Scrib*^{f/f}*Pgr*^{Cre/+} mammary glands fail to differentiate into mature alveolar cells. (A) Snapshots of tridimensional (3D) imaging of mammary glands from floxed and *Scrib*^{f/f}*Pgr*^{Cre/+} females on pregnancy day 12. Tissues were stained using antibodies for CK8 (epithelial marker) and PGR. Scale bar: 50 μm. (B) Fluorescence in situ hybridization of *Elf5* in pregnancy day 12 mammary glands from females with each genotype. Scale bar: 100 μm. (C) Snapshots of 3D imaging of mammary glands from floxed and *Scrib*^{f/f}*Pgr*^{Cre/+} females on pregnancy day 4. Tissues were stained using antibodies for CK8 (epithelial marker) and PR. Scale bar: 50 μm. (D) Snapshots of 3D imaging of mammary glands from floxed and *Scrib*^{f/f}*Pgr*^{Cre/+} females on pregnancy day 4. Tissues were stained using antibodies for CK8 (epithelial marker) and Ki67. Scale bar: 50 μm. Spotty signals outside of luminal layer in A, C and D are non-specific stainings caused by blood cells. 3D images were acquired by a Nikon multiphoton upright confocal microscope (Nikon A1R) using LWD 10X objective with 3 μm Z-stack. Each image is a representative from at least three independent experiments.

Table 1 Summary of mammary phenotypes in each *Scrib*-deleted model.

Report	Cell/Cre lines	Phenotype	Cell polarity	Proliferation	Apoptosis
Zhan <i>et al.</i> 2008	MCF10A (human, PGR-negative basal cells)	Increased tumorigenesis upon c-Myc overexpression	Compromised apico-basal polarity	Comparable	Decreased
Godde <i>et al.</i> 2014	MMTV-Cre (Whole mammary epithelia)	Ductal hyperplasia and increased tumorigenesis	Compromised apico-basal polarity	Increased in ductal luminal cells but not in TEBs at 12-weeks old	Decreased in newly-formed ductal luminal cells but not in TEBs at 6-weeks old
Baker <i>et al.</i> 2016	<i>Col1a1</i> -Dox inducible KD (Whole body)	Poor alveologenesi but normal milk production	Not affected	Reduced during early and mid-pregnancy	Not affected
This paper	<i>Pgr</i> -Cre (PGR-positive cells)	TEBs-like morphology during pregnancy with poor milk production	Not affected	Increased populations of PGR-positive cells in TEBs-like lobules during early pregnancy	Not affected
	<i>Ltf</i> -Cre (Ductal epithelia)	Normal mammary morphology and functions	Not affected	Not affected	Not affected

behave as mature secretory cells (Ismail *et al.* 2002, Lee *et al.* 2013). However, *Scrib^{f/f}Pgr^{Cre/+}* mammary glands show poor expression of *Elf5* during pregnancy. This observation is consistent with our notion that SCRIB in PGR-positive cells is critical to differentiation of mammary epithelial cells into mature alveolar cells. In contrast to the increased cell proliferation during early pregnancy, *Scrib^{f/f}Pgr^{Cre/+}* mammary glands exhibit poor proliferation at the lactational stage. This discrepancy in proliferation index can also be explained by the abnormal cell differentiation in this milieu since defects in alveologenesis consequentially cause proliferation failure during lactation (Jahchan *et al.* 2012). Although mammary gland defects may arise from decidual defects in *Scrib^{f/f}Pgr^{Cre/+}* females (Yuan *et al.* 2019), this is remote possibility because mammary defects are already present on day 4 of pregnancy as opposed to uterine anomalies which occur after initiation of decidualization on day 5 of pregnancy onward (Yuan *et al.* 2019). This observation suggests that the abnormal differentiation of mammary glands occurs independently under this milieu. This assertion is also supported by the fact that PGR is poorly expressed in mammary glands at later stages of pregnancy (Ismail *et al.* 2002) and P₄ level drops upon parturition (Cha *et al.* 2012).

Previous reports using gene knockdown in cultured cells and MMTV-Cre-induced gene deletion in tissues have showed that abnormal cell polarity in mammary epithelia results in ductal formation defects (Zhan *et al.* 2008, Godde *et al.* 2014). The role of SCRIB in regulating cell polarity is well established in other contexts (Bonello & Peifer 2019). In contrast, *Scrib* deletion by *Pgr-Cre* driver in our model does not appear to disturb apico-basal polarity. This indicates that SCRIB in the mammary gland has different functions depending on cell types. The importance of PGR for mammary gland development and functions during pregnancy and in puberty stages has previously been reported (Briskin & Ataca 2015). Our observation suggests that SCRIB exerts its function in PGR-positive cells for cell differentiation in response to specific stimuli such as P₄, E₂, Prl during early pregnancy. Considering our results, high levels of P₄ and PRL are critical factors that evoke SCRIB function in this context (Soares 2004, Cha *et al.* 2012). Since it has been reported that SCRIB binds to a variety of intracellular proteins to support their functions (Bonello & Peifer 2019), it is possible that SCRIB interacts with molecules other than PRLR downstream in different cell types. Notably, *Ltf-Cre*-induced *Scrib* deletion in mammary epithelia does not result in apparent abnormal functions of the mammary gland, although reporter proteins were visible by this Cre driver and *Scr^{ib}^{f/f}Ltf^{Cre/+}* mammary glands show reduced levels of *Elf5* compared to floxed ones. One possible reason is that *Ltf* is primarily expressed in ductal epithelia, and a study using single cell RNA-seq shows poor expression of *Ltf* in PRLR or PGR-positive cells (Casey *et al.* 2018). It is

also possible that *Elf5* expression is independent of RANKL in the LTF-positive cells. Nonetheless, this cell type seems not to be critical for SCRIB functions.

In summary, our findings reveal that SCRIB facilitates differentiation of PGR-positive cells into mature alveolar cells, contributing to nourishment of offspring. Although our studies show that SCRIB plays a role in specific cell lineages, additional studies are needed to explore the cell-specific functions of SCRIB in the mammary gland and its impact on pregnancy and lactation.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0108>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S A, J Y and A D performed experiments, S A, X S and S K D designed experiments. S A, J Y and S K D analyzed data. S A, and S K D wrote the manuscript.

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