**SnoN regulates mammary gland alveologenesis and onset of lactation by promoting prolactin/Stat5 signaling**

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**SUMMARY**

Mammary epithelial cells undergo structural and functional differentiation at late pregnancy and parturition to produce and secrete milk. Both TGF-β and prolactin pathways are crucial regulators of this process. However, how the activities of these two antagonistic pathways are orchestrated to initiate lactation has not been well defined. Here, we show that SnoN, a negative regulator of TGF-β signaling, coordinates TGF-β and prolactin signaling to control alveologenesis and lactogenesis. SnoN expression is induced at late pregnancy by the coordinated actions of TGF-β and prolactin. The elevated SnoN promotes Stat5 signaling by enhancing its stability, thereby sharply increasing the activity of prolactin signaling at the onset of lactation. SnoN−/− mice display severe defects in alveologenesis and lactogenesis, and mammary epithelial cells from these mice fail to undergo proper morphogenesis. These defects can be rescued by an active Stat5. Thus, our study has identified a new player in the regulation of milk production and revealed a novel function of SnoN in mammary alveologenesis and lactogenesis in vivo through promotion of Stat5 signaling.

**KEY WORDS:** SnoN, TGF-β, Mammary gland, Lactation, IrECM, Stat5, Mouse

**INTRODUCTION**

SnoN (Ski novel protein; Skil – Mouse Genome Informatics) is a member of the Ski family of proteins that contains both pro-oncogenic and anti-onscogenic activities in human cancer (Deheuninck and Luo, 2009; Jahchan and Luo, 2010; Jahchan et al., 2010; Pan et al., 2009). It is a critical regulator of transforming growth factor-β (TGF-β) signaling (Deheuninck and Luo, 2009; Jahchan and Luo, 2010; Luo, 2004). TGF-β, signaling through the Smad proteins, is a potent inhibitor of epithelial cell proliferation and acts to suppress tumor development at the early stages of carcinogenesis (Bierie and Moses, 2006; Chen and Wang, 2009; Heldin et al., 2009; Tian and Schiemann, 2009). SnoN interacts with Smad2, Smad3 and Smad4 in both the cytoplasm and nucleus to repress their ability to activate TGF-β target genes, thereby blocking the cytostatic response of TGF-β (Deheuninck and Luo, 2009; Jahchan and Luo, 2010; Krakowski et al., 2005). This ability of SnoN to antagonize the tumor suppressor activity of TGF-β is likely to be responsible for its pro-oncogenic activity. In addition, SnoN is also a potent activator of p53 (Trp53 – Mouse Genome Informatics). SnoN expression is significantly upregulated in response to cellular stress, and this high level of SnoN can induce stabilization and activation of p53, leading to increased senescence and apoptosis (Pan et al., 2009). This ability might account for the anti-tumorigenic activity of SnoN (Deheuninck and Luo, 2009; Jahchan and Luo, 2010; Pan et al., 2009).

Most previous studies have focused on the function of SnoN in cancer cells, but little is known about its physiological function in normal adult epithelial cells. SnoN is found to be ubiquitously expressed in all adult tissues at a low level (Nomura et al., 1999; Pearson-White and Crittenden, 1997). SnoN expression can be induced by TGF-β, upon tissue injury and in response to a variety of cellular stress signals (Deheuninck and Luo, 2009; Jahchan and Luo, 2010), suggesting that it might play a role in coordinating various cellular processes in response to environmental cues. In our studies, we employed the mammary gland as a model system to determine SnoN regulation and function during tissue morphogenesis. Mammary gland development takes place in distinct steps during puberty, pregnancy and lactation, and is a complex process that involves lineage-specific differentiation of mammary progenitor populations into ductal and alveolar progenitors (Bruno and Smith, 2011). We have shown that SnoN is detected in the luminal epithelial cells lining the ducts and epithelial cells of lobuli and terminal ducts, and its expression is low in the virgin gland, but is sharply upregulated at late pregnancy, peaking by around day 18.5 of pregnancy and lasting until day 1-2 of lactation, before dropping dramatically afterwards (Jahchan et al., 2010). Incidentally, this peak SnoN expression correlates with the profound structural and functional changes in the mammary alveoli (Neville et al., 2002; Oakes et al., 2006) that involve significant expansion of the alveolar luminal space and secretory activation, allowing milk production to take place (Anderson et al., 2007; Richter et al., 2000). A successful secretory activation also requires the closure of tight junctions (González-Mariscal et al., 2008; Itoh and Bissell, 2003; Nguyen and Neville, 1998; Nguyen et al., 2001), which is necessary to establish epithelial cell contacts and maintain cell polarity (Feigin and Muthuswamy, 2009; Inman and Bissell, 2010; Knust, 2000; Vasioukhin and Fuchs, 2001), permitting vectorial secretion. The expression pattern of SnoN suggests that it might play an important role in the differentiation of the secretory alveolar epithelial cells, and that pregnancy hormones could regulate its expression.

Mammary alveologenesis and lactogenesis are controlled by the coordinated actions of several cytokines, in particular TGF-β and prolactin. Prolactin expression increases during pregnancy and remains elevated during lactation (Freeman et al., 2000; Oakes et al., 2008), when it plays a key role in promoting lactogenesis and milk secretion (Brisken et al., 1999; Goffin et al., 1999; Kelly et

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al., 2002; Oakes et al., 2008). Upon activation by prolactin, Stat5a and the highly homologous Stat5b translocate into the nucleus to regulate the expression of various target genes, including Rankl (Tnfrsf11), cyclin D, caseins, Elf5, Wap, connexin 26 (Gjb2 – Mouse Genome Informatics), connexin 32 (Gjb1 – Mouse Genome Informatics), Socs1, Socs2, Socs3 and Igf2, all of which are known to be crucial for alveolar proliferation, milk protein production, secretory activation and the establishment of tight junctions (Hennighausen and Robinson, 2008; Kirch et al., 1997; Miyoshi et al., 2001; Oakes et al., 2008). Furthermore, Stat5 deficiency in the mammary gland leads to loss of lobulo-alveolar development and lactation failure due to decreased expression and secretion of milk proteins (Cui et al., 2004; Liu et al., 1997; Teglund et al., 1998; Udy et al., 1997). The actions of prolactin can be antagonized by TGF-β (Bailey et al., 2004; Jhappan et al., 1993; Pierce et al., 1993), of which two specific isoforms, TGF-β2 and TGF-β3, are expressed at high levels during mid-pregnancy and start to decrease in late pregnancy (Robinson et al., 1991). As TGF-β signaling inhibits proliferation and functional differentiation of mammary epithelial cells (Moses and Barcellos-Hoff, 2011), repressing TGF-β signaling is crucial for the initiation of lactation.

We used SnoN−/− mice to dissect the functions of SnoN during late pregnancy and early lactation in vivo (Pearson-White and McDuffie, 2003). The phenotype analysis in the SnoN−/− mice is complemented by the in vitro differentiation of the non-transformed MCF-10A human mammary epithelial cells in a three-dimensional (3D) culture system, which recapitulates aspects of tissue architecture, gene expression and differentiation (Alcaraz et al., 2008; Debnath et al., 2003; Muschler et al., 1999; Muschler and Streuli, 2010; Streuli et al., 1991; Streuli and Bissell, 1990; Xu et al., 2009a). We show here that SnoN plays a crucial role in alveologenesis and lactogenesis by coordinating TGF-β and prolactin signaling.

MATERIALS AND METHODS

Mouse strains, cells and DNA constructs

The SnoN−/− mice were obtained from Dr Pearson-White (Pearson-White and McDuffie, 2003). Primary mammary epithelial cells were isolated from the virgin glands as described previously (Fata et al., 2007; Novaro et al., 2003) and maintained in 1:1 DMEM:F12, 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone and 100 ng/ml of cholera toxin, and 100× penicillin/streptomycin (Debnath et al., 2003). Small hairpin RNA (shRNA) targeting human SnoN (Krakowski et al., 2005) and anti-Ki67 (Vector Laboratories) were used as the peroxidase substrate (SK-4105, Vector Laboratories). E-cadherin immunostaining was performed with anti-E-cadherin (BD Biosciences; 1:50), and ZO-1 staining with anti-ZO-1 (Invitrogen; 1:50). TUNEL assay on paraffin-embedded sections was performed using the DeadEnd Fluorometric TUNEL system kit (Promega).

Transfection and retroviral infection

Transfections of cells were performed using the Lipofectamine Plus (Invitrogen), and establishment of stable pools and clones by retroviral infection was performed as described (Debnath et al., 2003; Pan et al., 2009).

RT-PCR and western blotting

Frozen thoracic second and third mammary glands were homogenized and lysed in RLT buffer (Jahchan et al., 2010) for total RNA extraction and in lysis buffer (Jahchan et al., 2010) for protein assays. qPCR was performed using following primers: mouse WAP: F: 5'-TGCCCTCTG-CGCCTTGGAAATCTACT-3'; R: 5'-GACACAGTCAGGTTGCGAGCATTT-3'. Human and mouse SnoN: F: 5'-AGAGACTCTTGTTCCCAAGT-3'; R: 5'-CATGCTAAACTTCTCTTATTTCCT-3'. For western blotting, 30 μg tissue or cell extracts were analyzed.

Mammary epithelial cell differentiation in 3D culture

Cultrex basement membrane extract (60 μl; Trevigen) was placed in a well in an 8-well chamber, and 2000 MCF-10A cells were seeded and allowed to differentiate for 6 days. Staining of MCF-10A cells was carried out as described before (Debnath et al., 2003). Antibodies used for immunostaining were anti-α6-integrin (Chemicon), anti-GM130, anti-E-cadherin and anti-β-catenin (all from BD Biosciences), anti-cleaved-casparse 3 (Cell Signaling), anti-SnoN (Krawkowksi et al., 2005) and anti-Ki67 (Vector Laboratories). All images were analyzed using laser scanning confocal microscopy (Zeiss 510 UV/Vis Meta). Data from two or three independent experiments were plotted as mean ± s.e.m. Two-tailed unpaired Student’s t-tests were employed to evaluate the statistical significance.

RESULTS

SnoN affects mammary alveologenesis, functional differentiation and milk secretion

We examined the morphological and functional differentiation of SnoN−/− mammary glands at day 18.5 of pregnancy and day 0.5-2 of lactation. In contrast to wild-type (WT) SnoN+/+ glands, SnoN−/− glands exhibited markedly fewer alveoli and a greatly reduced epithelial content as shown by the whole-mammary staining at both day 18.5 of pregnancy and day 0.5 of lactation (Fig. 1A,B). Histological analysis revealed that the SnoN−/− alveoli were small and poorly differentiated, with small lumens containing very few milk droplets at day 18.5 of pregnancy (Fig. 1C). In conjunction with the significantly reduced epithelial content, SnoN−/− alveolar cells exhibited a much lower rate of proliferation than that observed in SnoN+/+ cells (Fig. 1D). Interestingly, there was no apparent difference in apoptosis, as shown by TUNEL staining, or in cadherin junction structure, as shown by the normal level and basal localization of E-cadherin (Fig. 1C).

The impaired structural differentiation observed at late pregnancy and early lactation suggested that SnoN−/− females might display defects in lactation and may have difficulty nursing pups. We therefore examined the process of secretory activation and tight junction formation in the mutant mice. During secretory activation, the large cytoplasmic lipid droplets (CLDs) in the luminal alveolar cells during late pregnancy are replaced by small lipid droplets located in the luminal surface of WT epithelial cells (Fig. 2A). By contrast, in the SnoN−/− mammary glands, large CLDs remained in the luminal alveolar cells after parturition at day 0.5 and day 1 of lactation (Fig. 2A, arrow), suggesting that secretory activation failed to occur. This phenomenon is typically observed in female mice that fail to lactate and nurse their pups (Palmer et al., 2003; Palmer et al., 2006; Schwertfeger et al., 2003; Wagner et al., 1997; Watkin et al., 2008). As tight junctions are required to establish epithelial cell contacts and maintain cell polarity and because SnoN−/− secretory alveolar cells lacked organized cell-cell contacts, we investigated whether zonula occludens 1 (ZO-1; Tjp1 – Mouse Genome Informatics) was properly expressed and localized. We examined the morphological and functional differentiation of SnoN−/− females might display defects in lactation and may have difficulty nursing pups. We therefore examined the process of secretory activation and tight junction formation in the mutant mice. During secretory activation, the large cytoplasmic lipid droplets (CLDs) in the luminal alveolar cells during late pregnancy are replaced by small lipid droplets located in the luminal surface of WT epithelial cells (Fig. 2A). By contrast, in the SnoN−/− mammary glands, large CLDs remained in the luminal alveolar cells after parturition at day 0.5 and day 1 of lactation (Fig. 2A, arrow), suggesting that secretory activation failed to occur. This phenomenon is typically observed in female mice that fail to lactate and nurse their pups (Palmer et al., 2003; Palmer et al., 2006; Schwertfeger et al., 2003; Wagner et al., 1997; Watkin et al., 2008). As tight junctions are required to establish epithelial cell contacts and maintain cell polarity and because SnoN−/− secretory alveolar cells lacked organized cell-cell contacts, we investigated whether zonula occludens 1 (ZO-1; Tjp1 – Mouse Genome Informatics) was properly expressed and localized. Immunostaining revealed that ZO-1 was absent or mislocalized in all SnoN−/− secretory alveolar cells compared with the WT structures, which displayed intense ZO-1 apical staining (Fig. 2B).
SnoN promotes differentiation

**SnoN regulates mammary epithelial cell proliferation, polarity and differentiation**

To characterize further the mechanism by which SnoN regulates morphogenesis and differentiation of the alveoli, primary mammary epithelial cells (MECs) were isolated from SnoN−/− mammary glands or SnoN+/− littersmates and cultured on a 3D laminin-rich extracellular matrix (IrECM). Whereas SnoN+/− MECs underwent morphological differentiation in the 3D culture to form organized acinus-like structures, SnoN−/− cells failed to differentiate properly. SnoN−/− acini were much smaller and displayed impaired morphology and a disrupted basal polarity (Fig. 2D). Moreover, the number of SnoN−/− cells in each acini was significantly lower than that in the SnoN+/− acini at days 3 and 5 of culture (Fig. 2D). This impaired differentiation of the SnoN−/− cells in 3D correlates well with the defective development of the SnoN−/− mammary glands during late pregnancy and early lactation.

Owing to the short life span of the mouse primary MEC and the early growth arrest of the SnoN−/− primary MEC, we were unable to manipulate these cells genetically and perform subsequent rescue experiments. We therefore employed the non-transformed human mammary epithelial cell line MCF-10A and inhibited SnoN expression by stable expression of small hairpin RNA targeting SnoN (shSnoN). Three clones with the highest efficiency of SnoN knockdown were characterized (Fig. 3A). In the conventional 2D culture, these shSnoN clones displayed a morphology identical to that of the control cells, albeit with a moderately decreased growth rate and an increased sensitivity to the cytostatic response of TGF-β, as expected (Fig. 3B,C).

When cultured in the 3D IrECM, the parental or control MCF-10A cells proliferated and underwent morphological differentiation to form polarized and highly organized multicellular acinar-like structures (Fig. 3D, top), similar to those displayed by WT primary MEC. By contrast, the shSnoN colonies displayed abnormal morphologies, ranging from colonies with smaller sizes to colonies containing dead cells (Fig. 3D, bottom). Because MCF-10A cells cultured in 3D IrECM were heterogeneous, we categorized the acinar-like colonies based on the size and morphology as ‘normal’ (defined as bigger than 60 μm in diameter, and number of cells/acinus, organization of the luminal epithelial layer and basal integrin staining all within the normal range), ‘intermediate’ (less than 60 μm) and ‘dead’. As quantified in Fig. 3D, whereas 75% of the control acini were of normal size and morphology by day 7, the majority of the shSnoN acini were in the intermediate and dead categories with only 25% of the acini being of normal size. Immunofluorescence staining combined with confocal microscopy showed that both the diameter of the shSnoN acini and the number of cells in each shSnoN acinus lagged that of the control starting at day 3 of culture and persisting until the end of culture (Fig. 3E,F). Notably, the shSnoN acini never caught up with the WT acini in size even after 10 days on IrECM (data not shown). The smaller sizes of the shSnoN acini were due to reduced proliferation as no difference in the rate of apoptosis between control and shSnoN cells was observed (data not shown). By contrast, whereas 55% of the cells in the control acini were BrdU positive, only 28% of the cells in the shSnoN acini were positive for BrdU (Fig. 3G), suggesting that the reduction in SnoN expression led to reduced proliferation in 3D cultures.

In addition to reduced proliferation, the shSnoN acini also displayed defects in apical-basal polarity. Whereas epithelial cells in the control acini established proper basal α6-integrin staining and apical localization of the cis-Golgi matrix protein GM130 (Golga2 – Mouse Genome Informatics), loss of SnoN disrupted

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Fig. 1. SnoN affects mammary alveolar development in mouse. (A, B) Alveolar atrophy is observed in the SnoN−/− mammary glands at day 18.5 of pregnancy (A) and day 0.5 of lactation (B). Left: whole-mount images from SnoN+/+ and SnoN−/− glands. Middle and right: Hematoxylin and Eosin (H&E) staining of mammary tissue sections at low (middle) and high (right) magnifications. The graphs to the right show the quantification of the epithelial content in three pairs of mice for day 18.5 pregnancy (P<0.0001) and in seven SnoN+/+ and six SnoN−/− mice at day 0.5 of lactation (P=0.0004). (C) H&E, TUNEL and E-cadherin staining at day 18.5 of pregnancy. (D) Pcna staining on alveolar structures at day 18.5 of pregnancy. Quantification of Pcna-positive nuclei in three pairs of glands (P=0.0140) is shown to the right. At least 1000 nuclei from each gland were counted. Error bars indicate the mean ± s.e.m. of three pairs of glands from each genotype.

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In addition, expression of a milk protein, β-casein (Rosen et al., 1999), was reduced in the SnoN−/− glands at late pregnancy, and the levels of whey acidic protein (Wap) mRNA showed at least a 90% reduction at early lactation (Fig. 2C).

Thus, the absence of SnoN impairs structural differentiation, secretory activation, tight junction formation and milk production at the onset of lactation. Consistent with this, no pups derived from SnoN−/− females survived beyond 3 days after birth following the first pregnancy, whereas 93% of pups from WT mothers and 95% of pups born from heterozygous SnoN+/− mothers survived, and the stomach contents of the newborn pups contained very little milk (data not shown). Similar to many mouse strains with lactation defects, SnoN−/− females were able to overcome these lactation defects during their second pregnancies and nurse their pups (data not shown) (Gallego et al., 2001; Ormandy et al., 1997). Taken together, these data suggest that SnoN plays a crucial role in the morphological and functional differentiation of mammary epithelial cells during late pregnancy and early lactation.
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this polarity with α6-integrin detected everywhere and GM130 relocated to the basal or lateral surfaces or in some cases entirely absent (Fig. 3H). It is important to note that this defective apical-basal polarity might not be the direct result of an early growth defect induced by the loss of SnoN. Even after 10 days on lrECM, defects in polarity persisted (data not shown), suggesting that the defects in shSnoN acini are not simply due to a delay in morphogenesis.

Taken together, our analysis indicates that loss of SnoN impairs MEC proliferation, morphogenesis and differentiation. At day 6 of culture, whereas 75% of the control acini were polarized, differentiated and reached a proper size, only 13% of the shSnoN acini did so (Fig. 3H).

To confirm that the impaired differentiation of the shSnoN acini is caused by the absence of SnoN and not due to an off-target effect of shRNA, we performed ‘rescue’ experiments by re-introducing a WT SnoN (WTsnoN) that is resistant to shSnoN inhibition back into shSnoN cells. Stable shSnoN clones expressing WTsnoN to a level comparable to the WT and SnoN+/- glands and cultured on lrECM for 5 days. Blue, DAPI; green, α6-integrin. Right: quantification of the number of cells per acinus in the SnoN+/- and SnoN-/- 3D cultures at day 3 (P<0.0001) and day 5 (P<0.0001). At least 100 acini were quantified for each sample. Error bars represent the mean ± s.e.m. of the acini counted for each sample.

SnoN regulates alveologenesis and Stat5 stability independently of the Smads

We determined next whether SnoN regulates MEC differentiation through a Smad-dependent or -independent pathway. As MCF-10A cells expressing shSnoN showed elevated Smad activity, we investigated whether reducing Smad expression in shSnoN cells would rescue the defective differentiation. Introduction of an siRNA against Smad2, Smad3 or both (data not shown) effectively decreased Smad expression (Fig. 4E), but did not rescue the growth and differentiation defects (Fig. 4F,G). Furthermore, overexpression of Smad2 (Fig. 4H) or Smad3 (data not shown) in MCF-10A cells (to mimic the elevated Smad activity in shSnoN cells) had no effect on acinar differentiation and polarity (Fig. 4I,K), although these cells showed enhanced TGF-β-dependent growth inhibition (Fig. 4I). The phenotypes of Smad overexpression were therefore very different from that of reducing SnoN. These data suggest that the effect of SnoN on MEC differentiation in 3D is likely to be Smad independent.

SnoN regulates prolactin-mediated Stat5 signaling

Several cytokines and signaling pathways have been shown to regulate MEC differentiation in 3D culture and during late pregnancy and early lactation in vivo. These include the PI3K-AKT pathway, the Rac1 GTPase, the laminins-integrins-focal adhesion kinase (FAK) pathway and the prolactin pathway, in addition to others (Katz and Streuli, 2007; Muschler and Streuli, 2010; Streuli and Akhtar, 2009; Xu et al., 2009a). To determine which pathway(s) might be affected by SnoN to regulate mammary epithelial differentiation, we compared the expression and activities of various molecules in the WT and SnoN-/- mammary glands. No difference was observed in the activity, expression levels and localization of MAPK, AKT, FAK, β1-integrin, E-cadherin or β-catenin (data not shown). Furthermore, staining with anti-laminin showed an intact basement membrane around the shSnoN acini (data not shown), suggesting that SnoN did not directly affect the extracellular matrix-epithelial cell interaction.

Interestingly, tyrosine phosphorylation of Stat5 was markedly diminished in SnoN-/- glands (Fig. 5A). This attenuated phosphorylation was largely due to a decrease in the levels of Stat5 proteins in the SnoN-/- glands (Fig. 5A). Immunohistochemistry staining for Stat5 in mammary tissue sections at day 0.5 of lactation further confirmed that SnoN-/- secretory alveolar cells expressed less Stat5 in both the cytoplasm and the nucleus (Fig. 5B). Consistent with this, expression of cyclin D1 (Cnd1 - Mouse Genome Informatics) protein and connexin 32 mRNA of the gap junction complex, two important Stat5 targets (Miyoshi et al.,
...was also reduced in the SnoN−/− glands at day 0.5 of lactation (Fig. 5A,D). As Stat5 directly induces the transcription of multiple milk protein genes (Kelly et al., 2002; Oakes et al., 2008), this decrease in Stat5 signaling is likely to be responsible for the significant reduction in milk protein expression in the SnoN−/− mice. Moreover, prolactin signaling has been shown to enhance tight junction formation and to maintain the impermeability of the mammary epithelium and closure of tight junctions during lactation. Thus, the decreased Stat5 signaling in the SnoN−/− mice might also be responsible for the disruption of these apical tight junctions (Itoh and Bissell, 2003; Nguyen and Neville, 1998; Stelwagen et al., 1999).

In contrast to Stat5, the expression of other key components in the prolactin signaling pathway, including prolactin receptor (Prlr), Jak2, Socs1 (data not shown) and Cbl, was not altered significantly in the SnoN−/− glands (Fig. 5C), suggesting that SnoN targets mainly Stat5.

Expression of Stat5 proteins in SnoN mutant cells rescues acinar morphogenesis and differentiation
If reduction of Stat5 signaling is responsible for the defects in MEC differentiation in the absence of SnoN, restoration of Stat5 signaling in shSnoN cells should rescue the disrupted acinar morphogenesis and differentiation. To test this, we infected shSnoN mammary cells with retroviruses expressing STAT5A 1*6 or STAT5B 1*6, both of which contain two amino acid substitutions, H299R and S711F. These amino acid changes cause prolonged tyrosine phosphorylation and nuclear translocation of Stat5 following prolactin stimulation (Onishi et al., 1998). As confirmed in Fig. 5E, elevated tyrosine phosphorylation of Stat5 was observed in shSnoN cells expressing these Stat5 proteins. When cultured on lrECM, both STAT5A 1*6 and STAT5B 1*6 were able to rescue the acinar size, polarity and morphogenesis to a level similar to that found in control MCF-10A cells (Fig. 5F). Staining with various markers indicated that proliferation and polarity were re-established (Fig. 5G,H). As quantified in Fig. 5G,H, the percentage of STAT5A 1*6 and STAT5B 1*6 colonies that displayed a normal morphology was ~55% and ~48% respectively, compared with only 17% of the shSnoN acini (Fig. 5H). These data suggest that SnoN might regulate MEC proliferation and differentiation by modulating the expression and/or activity of Stat5 proteins.

SnoN enhances Stat5 stability
SnoN could potentially regulate Stat5 expression through several possible mechanisms, including induction of Stat5 transcription or enhancement of Stat5 protein stability. To test these possibilities, we first performed real-time quantitative PCR to compare Stat5a and Stat5b mRNA levels in SnoN+/+ and SnoN−/− mammary glands.
No significant difference was observed between the two groups (Fig. 6A; data not shown). To assess whether SnoN affects Stat5 protein stability, we performed a pulse-chase assay to measure Stat5 half-life in cells transfected with Stat5a with or without SnoN. Cells were treated with cycloheximide (CHX) to inhibit new protein synthesis, and the cellular level of Stat5a was measured at different time points by western blotting to follow its natural decay (Fig. 6B, left). As shown in Fig. 6B, SnoN extended the half-life of Stat5a from 4 hours to >6 hours (right).

We further examined Stat5 stability in MCF-10A cells upon prolactin (Prl) stimulation and compared it with that in cells either lacking SnoN (shSnoN cells) or overexpressing SnoN. In all three cell lines, one hour of prolactin treatment induced a rapid and efficient nuclear translocation of Stat5, as expected (Fig. 6C, 0 hours). We then removed prolactin from the culture and monitored the expression levels of Stat5 at 3 and 5 hours after prolactin removal. As shown in Fig. 6C, control MCF-10A cells showed a moderate decrease in Stat5 expression (top) at 5 hours after prolactin removal. Interestingly, shSnoN cells exhibited a more prominent reduction in Stat5 expression at both 3 and 5 hours following prolactin withdrawal (middle), whereas cells overexpressing SnoN displayed persistent strong nuclear Stat5 expression beyond 5 hours of prolactin removal (bottom). Thus, in the absence of SnoN, Stat5 is degraded more rapidly, whereas in the presence of high SnoN levels, Stat5 is stabilized. Taken together, these findings suggest that SnoN promotes Stat5 stability, leading to elevated prolactin signaling.

If SnoN enhances Stat5 stability by antagonizing the Smad proteins, overexpression of Smads is expected to block the stabilization of Stat5 by SnoN. To test this, we co-transfected Smad2 together with Stat5a and/or SnoN and assessed whether Smad2 affected stabilization of Stat5 by SnoN. As shown in Fig. 6D, not only did the expression of Smad2 alone have no effect on Stat5 expression, it did not decrease the enhancement of Stat5 expression by SnoN. In addition, mSnoN defective in binding to the Smad proteins was as efficient as WT SnoN in enhancing Stat5 expression (Fig. 6E), again supporting the idea that SnoN stabilization of Stat5 is Smad independent.

**SnoN is induced by both TGF-β and prolactin signaling**

We have shown previously that SnoN expression is elevated dramatically in late pregnancy and early lactation (Jahchan et al., 2010). A key question is what factors or pathways are responsible...
for the sharp induction of SnoN expression in late pregnancy. Because SnoN induction coincides with the presence of TGF-β and prolactin and as SnoN is already a known TGF-β target, we investigated how the combined action of prolactin and TGF-β affects SnoN expression. To do this, primary MECs or the EpH4 mouse mammary epithelial cells were treated with TGF-β, prolactin, or both, and SnoN expression was monitored by semi-quantitative RT-PCR. As reported before, treatment of cells with TGF-β alone induced SnoN expression, whereas prolactin alone had little effect on SnoN expression (Fig. 7 A,B). By contrast, stimulation with both TGF-β and prolactin simultaneously resulted in an enhanced increase in SnoN expression, suggesting that they induce SnoN expression in a coordinated manner.

The coordinated induction of SnoN by TGF-β and prolactin may explain the sharp increase in SnoN expression during late pregnancy, a period in which both TGF-β and prolactin are expressed at considerable levels. This increased SnoN level might repress the inhibitory activity of TGF-β on lactogenesis, while simultaneously enhancing the pro-lactogenic activity of prolactin and Stat5a at the onset of lactation. Thus, SnoN functions as the key point of crosstalk to coordinate the actions of TGF-β and prolactin pathways to enable alveologenesis and lactogenesis (Fig. 7E).

DISCUSSION

We show here that SnoN plays a key regulatory role in mammary gland alveologenesis and lactogenesis by coordinating the activities of TGF-β and prolactin signaling. At late pregnancy, SnoN expression is sharply induced by the combined action of TGF-β and prolactin. This heightened SnoN expression can repress TGF-β/Smad signaling, relieving its inhibition of the prolactin pathway, and at the same time directly promoting prolactin signaling by stabilizing Stat5. Deletion of SnoN results in dramatically reduced Stat5 expression and activity, leading to failure in alveolar morphogenesis, milk production, secretory activation and tight junction formation. Consistent with the regulation of Stat5 by SnoN, expression of an active Stat5 in cells lacking SnoN rescues the defective epithelial cell differentiation. Thus, our study has
identified a new player in the regulation of milk production and has revealed a novel function of SnoN as a crucial positive regulator of Stat5/prolactin signaling in the mammary gland at late pregnancy. This is also the first demonstration of a crucial function of SnoN in normal adult tissue development and epithelial differentiation.

Defective lactation has been observed in mouse models with either elevated TGF-β expression, defective tight junctions or aberrant Stat5 signaling. Mammary-specific expression of TGF-β1 under the control of the WAP promoter resulted in reduced milk protein expression in vivo (Jhappan et al., 1993), and mice expressing a constitutively active TβRII receptor (MMTV-TβRIIAAD) did not lactate properly as a result of increased apoptosis and decreased proliferation (Siegel et al., 2003). Although the SnoN−/− females exhibited defects in lactation, we did not observe any significant difference in apoptosis at day 18.5 of gestation between WT and SnoN−/− littermates. Thus, the phenotype of the SnoN−/− mammary gland is consistent with, but cannot be fully explained by, the elevated TGF-β/Smad signaling activity.

Prolactin/Stat5 signaling has been shown to regulate tight junction formation, and mice lacking Stat5 show a loss of lobulo-alveolar

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**Fig. 6. SnoN enhances Stat5 stability.**
(A) stat5a mRNA levels in SnoN+/+ (n=5) and SnoN−/− (n=6) littersmates from late pregnancy/early lactation showed no difference by qRT-PCR. (B) Measurement of SnoN half-life. 293T cells transfected with Stat5 with or without Flag-SnoN were treated with 50 µg/ml of cycloheximide for the indicated times. The expression levels of Stat5 and SnoN were measured by western blotting with anti-Stat5 and anti-Flag, respectively. Anti-tubulin was used as a loading control. Quantification of the blot is shown to the right. (C) MCF-10A cells expressing vector control, shSnoN or Flag-SnoN were transfected with Stat5 and Prlr, and treated with 5 µg/ml of ovine prolactin for one hour. At 0, 3 or 5 hours after removal of prolactin, immunofluorescence staining of Stat5a (green) was performed. (D) 293T cells were transfected with Stat5a with or without Flag-SnoN, Flag-Smad2, or both. Stat5a levels were measured by western blotting. (E) 293T cells were transfected with Stat5a with or without WT-SnoN and mSnoN. Stat5a level was measured by western blotting.

Fig. 7. SnoN is upregulated by both TGF-β and prolactin signaling pathways. (A,B) SnoN mRNA levels upon treatment of primary mouse MEC (A) and EpH4 cells (B) for two hours with TGF-β1 (100 pM), ovine prolactin (3 µg/ml), or both were measured by RT-PCR. β-actin was used as a loading control. (C,D) pSno2.8-LUC (C) or pSno2.8 STAT5-LUC (D) was transfected into EpH4 cells and treated for 16 hours with ovine prolactin (3 µg/ml), TGF-β1 (100 pM), or both. Error bars indicate the mean ± s.e.m. of three independent experiments performed in triplicate (C) and the mean ± s.e.m. of three replicates from one representative experiment (D). (E) Model of SnoN regulation and function in mammary epithelial cell differentiation. SnoN is induced by both TGF-β and prolactin at late pregnancy and subsequently stabilizes Stat5 to promote prolactin/Stat5 signaling while repressing TGF-β signaling to allow structural and functional differentiation of the mammary alveolar.
development and lactation failure (Cui et al., 2004; Liu et al., 1997; Teglund et al., 1998; Udy et al., 1997). Consistent with a role of SnoN in promoting Stat5 expression, SnoN<sup>−/−</sup> mammary glands show a dramatic decrease in Stat5 expression and phosphorylation, and the SnoN<sup>−/−</sup> phenotypes partially resembled that of Stat5-deficient mice with an impaired lobulo-alveolar development and defective lactation. This ability of SnoN to promote Stat5 stability and signaling is Smad independent. SnoN might stabilize Stat5 by directly interacting with Stat5 to prevent its ubiquitination and degradation or interfering with its binding to the E3 ubiquitin ligase Cbl. In addressing these possibilities, we demonstrated that there was a direct physical binding between SnoN and Stat5 in transfected cells (data not shown). However, the SnoN–Stat5 interaction could not be detected under the endogenous levels in mammary glands from pregnant females or in mammary epithelial cells in culture. Although it is still possible that this direct physical interaction could occur in vivo under specific conditions or in a transient manner to regulate Stat5 stability, other indirect mechanisms are likely to also play important roles. Notably, SnoN is not required for continuous Stat5 signaling during most of the lactation period, but is only crucial for the initial transition from pregnancy to lactation. This is the juncture at which TGF-β and prolactin signaling both exert their effects, and SnoN is in a unique position to coordinate the activity of these two antagonistic pathways to allow a sharp increase in Stat5 activity that is necessary for lactation to start. As lactation proceeds, TGF-β activity is rapidly diminished, relieving its suppressive effects on Stat5 signaling, and SnoN activity is no longer needed to repress TGF-β signaling. At this point, Stat5 activation is strong enough to be effective without the aid of the enhancing abilities of SnoN.

Although TGF-β and prolactin signaling are usually antagonistic on cell proliferation and differentiation, they function in a coordinated manner to induce SnoN expression. Consistent with this, both Smad binding sites and the consensus Stat5 binding sites were found in close proximity in the promoter region of mouse and human snoN gene (data not shown), suggesting that the Smad complex might potentially coordinate with Stat5 on DNA to activate SnoN transcription. It is possible that the dynamic interaction between Smads and Stat5 is different when both factors bind to DNA compared with when only Stat5 is bound to DNA. Thus, interactions of these proteins might produce different outcomes depending on the presence or absence of specific Smad or Stat5 binding sites in a given promoter, thus allowing the two pathways to antagonize the expression of some genes while synergistically activating that of other genes. The cooperated induction of SnoN expression then allows the simultaneous repression of TGF-β signaling and enhancement of prolactin signaling, providing an accurate switch to trigger lactation.

In addition to prolactin signaling, Stat5 proteins have been shown to mediate signaling downstream of many cytokines in a tissue- or cell type-specific manner. These cytokines include growth hormone, interleukin (II)2, II3, II5, erythropoietin, thrombopoietin and granulocyte-macrophage colony-stimulating factor (Hennighausen and Robinson, 2008). By promoting Stat5 stability, SnoN might enhance signal transduction by multiple cytokines in a tissue-specific manner. Consistent with this notion, SnoN<sup>−/−</sup> mice have been found to display defects in T-cell proliferation with reduced II2 signaling (Pearson-White and McDuflie, 2003). Thus, it is conceivable that SnoN may be required for signaling activation by many cytokines at an initial stage when cytokine concentration is still low and when the cytokine activity is still subjected to repression by TGF-β. Our study in the mammary gland thus provides the first example of a tissue-specific function of SnoN in normal adult tissues and cells and opens up new directions for future studies of the normal physiological functions of SnoN.

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Competing interests statement
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