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Mouse Mammary Epithelial Cells Express the Na-K-Cl Cotransporter, NKCC1: Characterization, Localization, and Involvement in Ductal Development and Morphogenesis

JONATHAN M. SHILLINGFORD, KEIKO MIYOSHI, MICHAEL FLAGELLA, GARY E. SHULL, AND LOTHAR HENNIGHAUSEN

Laboratory of Genetics and Physiology (J.M.S., K.M., L.H.), National Institutes of Health, Bethesda, Maryland 20892; Department of Biochemistry (K.M.), School of Dentistry, University of Tokushima, Tokushima 770-8504, Japan; and Department of Molecular Genetics, Biochemistry, and Microbiology (M.F., G.S.), University of Cincinnati, Cincinnati, Ohio 45221

Despite the fact that physiological evidence points to the existence of a functional Na-K-Cl cotransporter in the mammary gland, the molecular identity of this transport process remains unknown. We now show that the Na-K-Cl cotransporter isoform, NKCC1, is expressed in mammary tissue. Developmental profiling revealed that the level of NKCC1 protein was significantly influenced by the stage of mammary gland development, and immunolocalization studies demonstrated that NKCC1 was present on the basolateral membrane of mammary epithelial cells. To examine whether functional NKCC1 is required for mammary epithelial cell development, we used NKCC1 −/− mice. We demonstrate that NKCC1 −/− mammary epithelium exhibited a significant delay in ductal outgrowth and an increase in branching morphogenesis during virgin development. These effects were autonomous to the epithelium as assessed by mammary gland transplantation. Although the absence of NKCC1 had no apparent effect on gross mammary epithelial cell morphology during lactation, pups born to NKCC1 −/− mice failed to thrive. Finally, analysis of NKCC1 protein in mouse models that exhibit defects in mammary gland development demonstrate that high levels of NKCC1 protein are indicative of ductal epithelial cells, and the presence of NKCC1 protein is characteristic of mammary epithelial cell identity. (*Molecular Endocrinology* 16: 1309–1321, 2002)

THE MAMMARY GLAND is a highly metabolic tissue responsible for the process of milk synthesis and secretion to nurture the young. To fulfill this role, this tissue expresses a wide variety of membrane transporters (1). These are thought to be necessary to support both the metabolic activity of the cell and the active process of milk synthesis and secretion. Based on radioactive tracer flux studies, it has been suggested that the lactating rat mammary gland expresses a functional Na-K-Cl cotransporter (2, 3). However, the molecular identity of this transporter has not yet been elucidated.

The cation-chloride cotransporter family is composed of the Na-K-Cl cotransporters (NKCC1, NKCC2) and the K-Cl cotransporters (KCC1–4) (4). Although NKCC1 is expressed in a wide variety of tissues (5, 6), NKCC2 is expressed exclusively in the kidney (7, 8). NKCC1 has been shown to be present at the basolateral membrane of a number of secretory epithelial tissues where it is responsible for chloride secretion (9–11). In contrast, NKCC2 is located on the apical membrane of the cells of the thick ascending limb where it mediates absorption of chloride (8). In addition to its proposed role in chloride secretion (reviewed in Ref. 12), NKCC1 has also been shown to be involved in cell-volume regulation (13, 14) and cell proliferation (15–17).

Analysis of 2.44×10^6 expressed sequence tag (EST) clones in the mouse database revealed that NKCC1 is highly expressed in normal and neoplastic mammary tissue, suggesting that it may play a role in mammary gland development. Indeed this hypothesis is supported by a recent study, which demonstrated that the transport activity of NKCC1 is increased in mammary tissue culture cells upon NKCC1 phosphorylation stimulated by the PRL receptor (PRLR)-Jak2-signal transducer and activator of transcription 5 (Stat5) pathway (18). The generation of NKCC1 −/− mice (19) allowed us to study its *in vivo* function in the mammary gland.

In this paper we demonstrate that mammary epithelial cells exhibit developmental regulation of NKCC1 mRNA and protein. Utilizing a mammary transplantation approach and NKCC1 −/− mice, we further show that the absence of NKCC1 in mammary epithelial

Abbreviations: EST, Expressed sequence tag; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; PRLR, PRL receptor; SMA, smooth muscle actin; Stat5, signal transducer and activator of transcription 5; TEB, terminal end bud; TRITC, tetramethylrhodamine isothiocyanate; WAP, whey acidic protein.

cells results in a significant increase in ductal side branching and reduced outgrowth of the mammary epithelium. Finally, we provide evidence that high levels of NKCC1 protein are characteristic of ductal epithelial cells, and *trans*-differentiation of mammary epithelium results in the loss of detectable NKCC1 protein.

RESULTS

Analysis of NKCC1 Expression During Mammary Gland Development

Screening of the GenBank mouse EST database with a full-length cDNA clone of mouse NKCC1 revealed a number of matching EST clones in several mammary gland libraries (Table 1). A total of 2.44×10^6 sequences are deposited in the EST database; of the top 60 matching clones, 37 were of mammary tumor and mammary gland origin, which represents an approximate 10-fold enrichment of clones matching NKCC1 in the mammary gland libraries compared with the other libraries present in the database.

To assess the expression pattern of NKCC1 mRNA during mammary gland development, primers specific for NKCC1 were used in RT-PCR experiments. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is included as a control for equivalence of the cDNAs (Fig. 1, panel 5). As can be seen in Fig. 1, panel 1, NKCC1 mRNA was detected in the virgin mammary gland (4-wk and 10-wk), during pregnancy (d 5, 9, 12, and 16) and during lactation (d 1, 5, and 10), but less so at involution (d 1, 6, and 10). β-Casein and whey acidic protein (WAP) mRNA, markers of functionally differentiating epithelium, were detected at early pregnancy (Fig. 1, panel 4) and at late pregnancy/early lactation (Fig. 1, panel 5), respectively. The facilitative glucose transporter GLUT1 (glucose transporter 1) is expressed in mammary epithelial cells at all stages of development (30–31). RT-PCR revealed that GLUT1 mRNA increased steadily over the course of mammary gland development, declining only during the later stages of involution (Fig. 1, panel 2). Interestingly, although there is a significant increase in epithelial cell content during mammary development, as assessed by expression of CK18 mRNA (Fig. 1, panel 3), we observed a decrease in NKCC1 mRNA during pregnancy relative to the 10-wk virgin. This observation suggests that the level of NKCC1 mRNA is regulated in a developmental-specific manner.

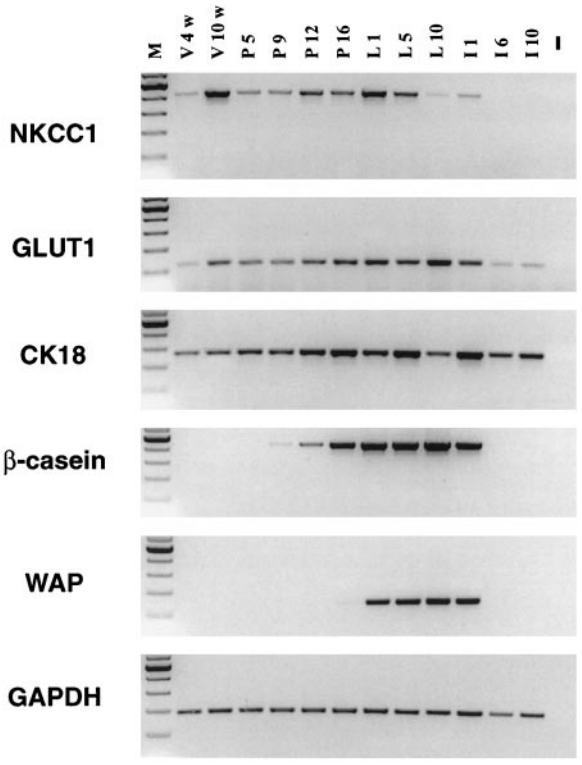

Fig. 1. Expression of NKCC1 mRNA During Mammary Gland Development

Total RNA (1 μg) was reverse transcribed into cDNA using reverse transcriptase SuperScriptII (Life Technologies, Inc.). An aliquot (1 μl) of the cDNA synthesis reaction was used for PCR analysis using gene-specific primers for NKCC1, GLUT1, β-casein, WAP, or GAPDH. PCR products (20 μl of a 50 μl reaction) were separated by electrophoresis on a 2% agarose gel containing 0.5 μg/ml ethidium bromide. The amplification products were viewed and photographed under UV light using a gel imaging system. M, Markers; v4, 4-wk-old virgin; v10, 10-wk-old virgin; p5, pregnancy d 5; p9, pregnancy d 9; p12, pregnancy d 12; p16, pregnancy d 16; L1, lactation d 1; L5, lactation d 3; L10, lactation d 7; I1, involution day 1; I6, involution d 6; I10, involution d 10; −, no cDNA control.

Table 1. Full-Length Murine NKCC1, Accession No. NM_009194, Was Screened Against the Mouse EST Database Using the BLAST 2.1 Basic Search (http://www.ncbi.nlm.nih.gov)

Library Description	Total Clones	No. of Clones
Tumors of mammary origin (10 libraries)	216,565	31
Embryo (pooled)	15,247	7
Salivary gland	19,301	4
Mammary gland (nonlactating)	39,637	3
Mammary gland (lactating)	42,324	3
Kidney tumor	19,484	2
Two-cell embryo (fertilized)	3,314	2
Brain (newborn)	3,443	2
Retina	21,141	2
Myotubes	3,403	2
Optic cup (embryo d 11.5)	307	1
One-cell embryo	7,577	1

The table represents descending numbers of EST clones present in each cDNA library with a significant homology (>90%, >250 bp) to murine NKCC1. The mouse EST database contained 2.44×10^6 sequences at the time of running the database search. Of the top 60 EST matches, 37 (62%) were of mammary tumor and nontumor origin.

Immunlocalization of NKCC1 Protein in Mammary Tissue

The mammary gland is unique in that the ratio of the major cell types (adipocytes and ductal and secretory epithelial cells) changes dramatically during development. Although adipocytes predominate in the virgin tissue, secretory epithelial cells constitute the major cell type present during lactation. This complicates the interpretation of data obtained by RT-PCR and Northern and Western blot analyses because they represent a heterogeneous population of cells. Therefore, the localization of NKCC1 was examined by immunohistochemistry using an N-terminal antibody (α-NT) raised against rat NKCC1. The characteristics of this antibody have been described previously (27), and we confirmed the specificity of the antibody in sections of salivary gland and kidney (data not shown).

Immunolocalization of NKCC1 in sections prepared from 6-wk-old virgin mammary tissue revealed distinct NKCC1 staining at the plasma membrane of ductal epithelial cells (Fig. 2A, *arrow*) but not in the cytoplasm. Longitudinal sections (Fig. 2B) and cross-sections (Fig. 2C) of individual mammary ducts further established that NKCC1 was absent from the apical membrane (Fig. 2, B and C, *arrowheads*) and was present exclusively on the basolateral membrane of the ductal epithelial cells. NKCC1 protein was also detected in the plasma membrane of cells present within the terminal end bud (TEB) structures of the mammary gland (Fig. 2D, *arrowhead*) although its membranous localization could not be accurately determined. Detectable expression of NKCC1 protein was not identified in any other cell type of the mammary gland, including fibroblasts, adipoctytes, endothelial cells, or the cap cells of the TEB (data not shown). Furthermore, in support of the complete absence of detectable NKCC1 protein in NKCC1 $-/-$ mice (19), NKCC1 was not detected in mammary ductal epithelial cells in these mice (data not shown).

To examine whether changes in systemic hormone levels may influence the expression pattern and/or levels of NKCC1 protein, we determined the localization of NKCC1 in mammary tissue isolated from virgin mice (5–7 wk) at different stages of the estrous cycle. These experiments revealed no apparent difference in the pattern of overall protein expression or cell localization throughout the estrous cycle (data not shown). Thus, expression was homogeneous in the ductal epithelium, and NKCC1 was localized to the basolateral membrane at all stages.

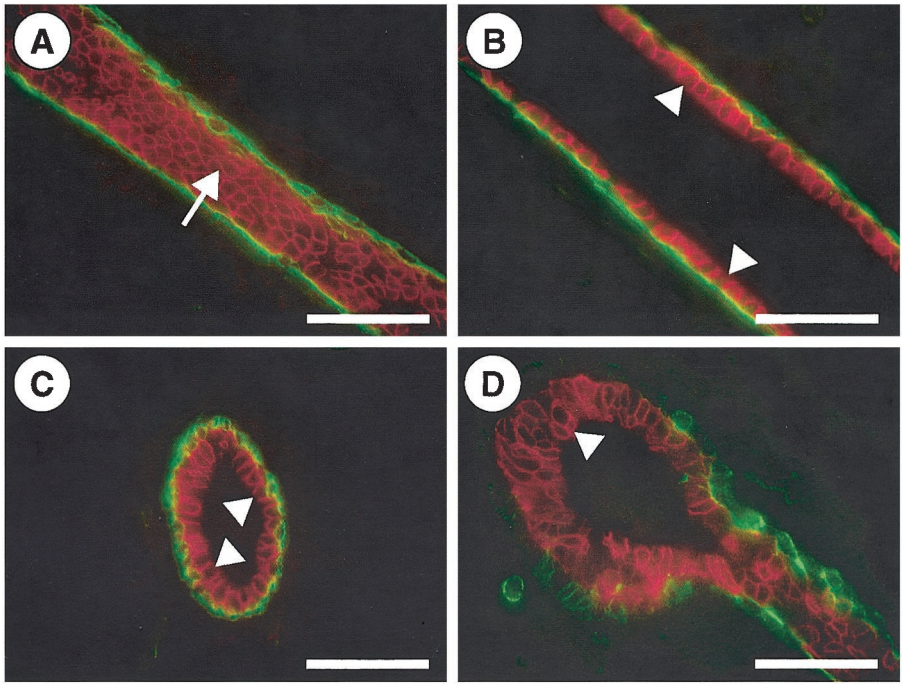

Fig. 2. Immunolocalization of NKCC1 Protein in Ductal Epithelial Cells

Paraffin-embedded mammary gland sections (5 μm) from a 6-wk-old virgin mouse were incubated with a rabbit polyclonal anti-NKCC1 (1:1000) and a mouse monoclonal anti-SMA (1:1000) antibody. Bound antibody was visualized using appropriate fluorescent-conjugated secondary antibodies (NKCC1, *red*; smooth muscle actin, *green*). A, Longitudinal section of a duct demonstrating membranous localization of NKCC1 (*red*) in ductal epithelial cells (*arrow*). SMA (*green*) highlights the myoepithelial cells surrounding the ductal epithelial cells. B, Longitudinal section of a duct demonstrating localization of NKCC1 (*red*) on the basolateral membrane of ductal epithelial cells, but not the apical membrane (*arrowheads*). C, Cross-section of a duct demonstrating basolateral but not apical (*arrowheads*) localization of NKCC1 (*red*) in ductal epithelial cells. D, Longitudinal section of a TEB structure exhibiting membranous staining of NKCC1 (*red, arrowhead*). *Bar,* 100 μm.

Developmental Profile of NKCC1 During Mammary Gland Development

Because it was evident from the RT-PCR data that NKCC1 mRNA in mammary tissue was developmentally regulated, we investigated whether the cellular localization and/or level of NKCC1 protein was influenced by developmental stage. Therefore we examined the localization of NKCC1 protein at various time points using immunohistochemistry. NKCC1 protein was detectable on the basolateral membrane in all ductal epithelial cells during virgin gland development (Fig. 3, A–C). Further studies established that NKCC1 protein was first detected in the mammary epithelium at 3 wk of age and up to 35 wk of age in virgin animals (data not shown). During pregnancy, NKCC1 protein was evident in only a few ductal epithelial cells (Fig. 3, D and E, *arrowheads*) and was much reduced in rudimentary alveoli (Fig. 3, D and E, *arrows*). At lactation, considerably lower levels of NKCC1 protein were observed in mature alveoli (Fig. 3F) compared with those observed during virgin development (Fig. 3A). The apparent differences observed between the high levels of NKCC1 mRNA (Fig. 1, panel 1) and the low levels of NKCC1 protein (Fig. 3F) at lactation d 1 (L1) may simply reflect the epithelial content of the gland at this

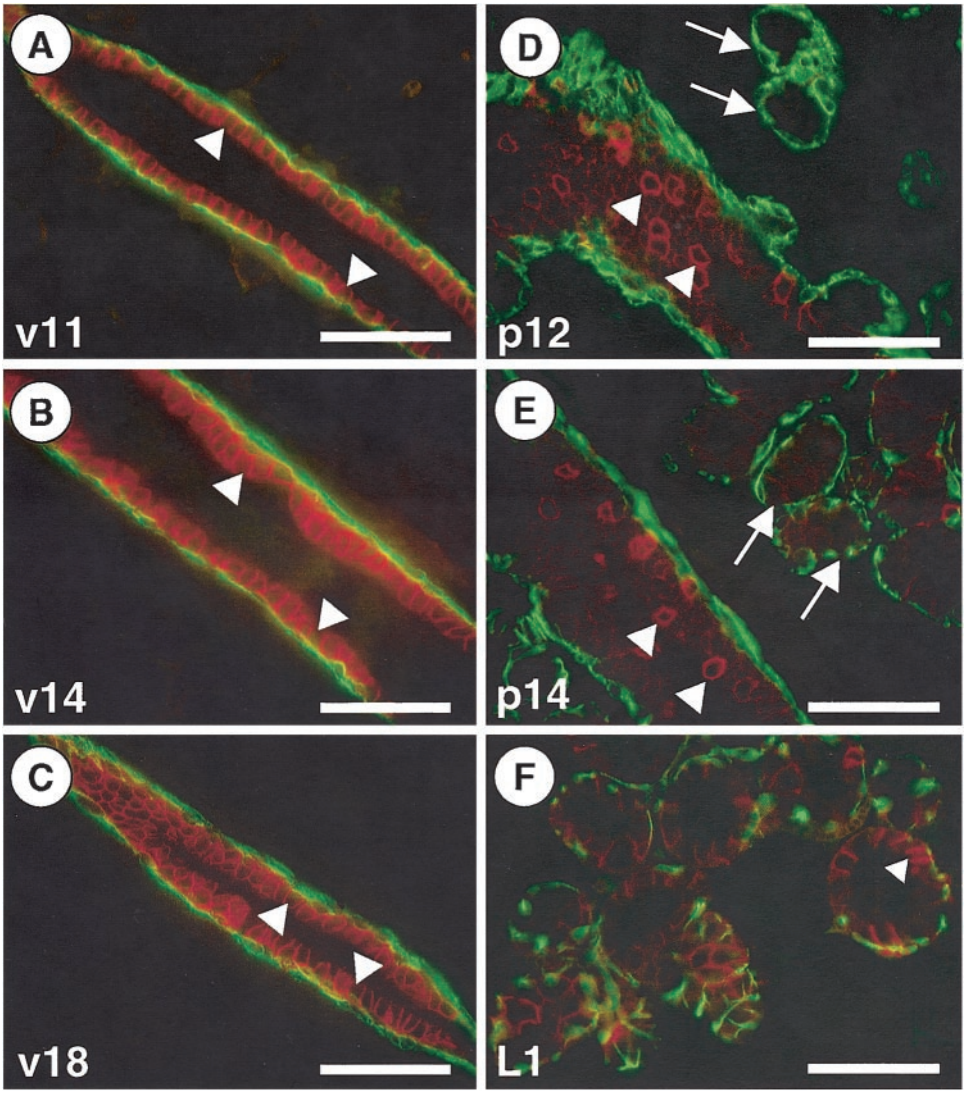

Fig. 3. Immunolocalization of NKCC1 Protein During Mammary Gland Development

Sections (5 μm) derived from mammary gland at different stages of development were incubated with anti-NKCC1 and anti-SMA primary antibodies and subsequently visualized with fluorescent-conjugated secondary antibodies (NKCC1, *red*; SMA, *green*). A, 11-wk-old virgin; B, 14-wk-old virgin; C, 18-wk-old virgin; D, pregnancy d 12; E, pregnancy d 14; and F, lactation d 1. NKCC1 protein is evident on the basolateral membrane of ductal epithelial cells during virgin development (A–C), but not the apical membrane (*arrowheads*, A–C). During pregnancy (D and E), high levels of NKCC1 protein are evident in a few cells within the ducts (*arrowheads*, D and E) but are much reduced in developing alveoli (*arrows*, D and E). At lactation, NKCC1 protein is apparent at low levels in mature alveoli (*arrowhead*, F). *Bar,* 100 μm.

stage of development. Taken together, these results suggest that NKCC1 protein is regulated in a developmental-specific manner. In particular, NKCC1 protein is present at high levels in the ductal epithelial cells during virgin development but appears to be greatly down-regulated in developing alveoli during pregnancy. These data support the results obtained by RT-PCR and suggest that NKCC1 may play a more prominent role in ductal epithelial cell development as opposed to alveolar development.

Analysis of Ductal Outgrowth in Transplanted NKCC1 −/− Mammary Epithelium

To further analyze and understand the role that NKCC1 may play in mammary epithelial cell development, we performed a series of transplantation experiments using NKCC1 −/− mammary epithelium. These experiments were performed for several specific reasons. First, although the ovary, oviduct, and uterus were reported to be histologically normal in NKCC1 −/− females (32), it does not rule out the possibility that the secretion of hormones is normal. Indeed the smaller size of the NKCC1 −/− mice compared with +/− and wild-type littermates might be due to alterations in circulating hormone levels, which would in turn affect mammary gland development. Thus, although we observed reduced outgrowth of mammary epithelium in 8-wk-old NKCC1 −/− mice (data not shown), it is not possible to distinguish between direct and indirect effects of NKCC1 inactivation on mammary gland development in the native mouse. Further, NKCC1 −/− mice exhibit a high incidence (30%) of death at weaning age (19). Taken together, these factors preclude a thorough study of *in vivo* mammary gland development in NKCC1 −/− mice.

For the transplantation procedure, small pieces of mammary gland isolated from female NKCC1 −/− and +/− littermates were transplanted into the cleared fat pads of athymic nude mice, and the development of the mammary gland was assessed at different time points. Although athymic nude mice possess lower levels of systemic estrogen and progesterone (33), contralateral transplantation of NKCC1 −/− and +/− mammary tissue in the same mouse allowed valid comparisons to be made. Analyses of transplanted mammary epithelium 3 wk after transplantation (n = 3) demonstrated that although the NKCC1 −/− transplants (Fig. 4B) filled the fat pad to the same extent as +/− epithelium (Fig. 4A), there was a considerable increase in secondary ductal branching in NKCC1 −/− epithelium (Fig. 4D) compared with the +/− transplants (Fig. 4C). At 8 wk posttransplantation, the increased ductal branching of NKCC1 −/− epithelium (Fig. 4H) remained evident, and there was a persistence of TEB-like structures (Fig. 4H) compared with +/− epithelium (Fig. 4G). In addition, NKCC1 −/− epithelium failed to fill the entire fat pad (Fig. 4F) compared with the +/− transplants (Fig. 4E). It was estimated in three separate transplants that NKCC1 −/− transplanted epithelium occupied approximately 70% of the fat pad compared with 100% for NKCC1 +/− epithelium. By 12 wk posttransplantation, NKCC1 −/− epithelium (Fig. 4J) had fully penetrated the fat pad similar to +/− epithelium (Fig. 4I), but the defects in ductal side branching were still apparent (Fig. 4L) when compared with +/− epithelium (Fig. 4K).

To quantitate the observed increase in ductal branching, we counted secondary branch points emanating from five primary ducts within each transplant (Table 2). This revealed a significant 2-fold increase in side branches in NKCC1 −/− epithelium compared with +/− epithelium, pointing to a role for NKCC1 in branching morphogenesis in the mammary gland. Despite a significant increase in ductal branching, we did not observe a concomitant increase in cell proliferation at any of these time points as assessed by immunofluoresence staining using proliferating cell nuclear antigen (PCNA) (data not shown).

High Levels of NKCC1 Protein Are Associated with PCNA-Negative Cells During Pregnancy

We demonstrate that high levels of NKCC1 protein are only maintained in a few ductal cells during pregnancy (Fig. 3, D and E, *arrowheads*). Because it has been proposed that NKCC1 is involved in proliferation, we examined the localization of NKCC1 and PCNA using immunofluorescence (Fig. 5). No PCNA-positive cells were observed in ductal epithelial cells during virgin development (Fig. 5A) except for some cells within the TEB structures, which showed evidence of NKCC1-strong positive, PCNA-negative (Fig. 5B, *arrow*) and NKCC1-strong positive, PCNA-positive (Fig. 5B, *arrowhead*) cells. At pregnancy, three distinct populations of cells were observed; NKCC1-weak positive, PCNA-negative cells (Fig. 5, C–F, *yellow arrowhead*); NKCC1-weak positive, PCNA-positive cells (Fig. 5, C–F, *white arrowhead*); and NKCC1-strong positive, PCNA-negative cells (Fig. 5, C–F, *white arrow*). Interestingly, no NKCC1-strong positive, PCNA-positive cells were observed in either ductal (Fig. 5, C and D) or alveolar structures (Fig. 5, E and F) during pregnancy. These results suggest that actively dividing and recently divided cells down-regulate NKCC1 protein, which may be a prerequisite for cell division. Alternatively, because high levels of NKCC1 protein are apparent in ductal epithelial cells, the NKCC1-strong positive, PCNA-negative cells may represent the persistence of nondividing virgin ductal epithelial cells that have maintained a less differentiated state.

NKCC1 as a Marker of Ductal Epithelial Cells

The presence of a high level of NKCC1 protein in cells during virgin mammary gland development (Fig. 6A), relative to pregnancy (Fig. 6B) and lactation (Fig. 6C), suggested that NKCC1 might serve as a useful marker of ductal epithelial cells. To further establish this hy-

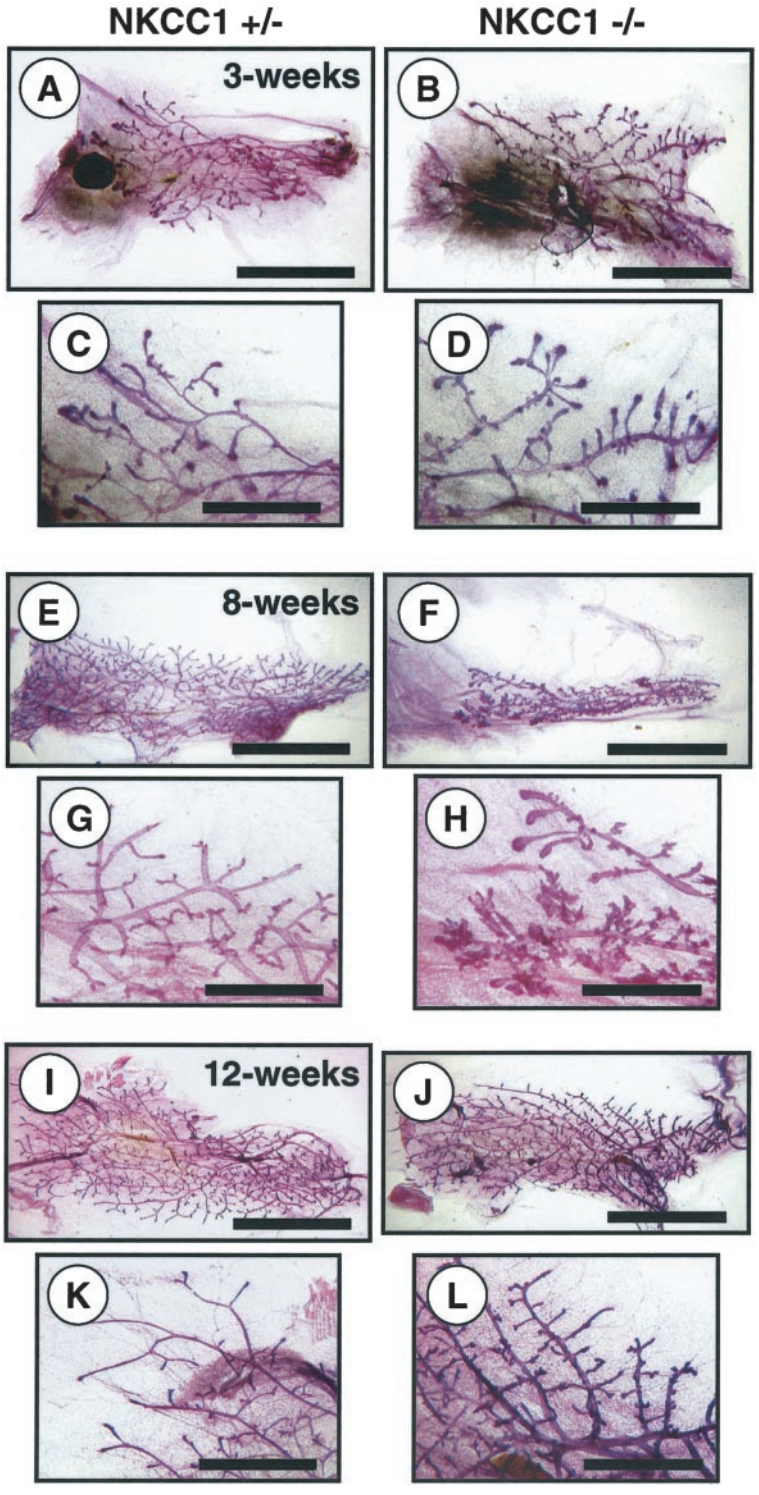

Fig. 4. Whole-Mount Analysis of NKCC1 −/− Transplants Reveals Reduced Epithelial Outgrowth and Increased Ductal Branching During Virgin Development

Mammary glands were isolated from NKCC1 −/− and NKCC1 +/− littermates. Small pieces were cut from the gland and transplanted into 3-wk-old athymic nude mice whose endogenous mammary epithelium had been removed. Mice were left for 3–12 wk, after which time they were euthanized and the glands excised and processed for whole-mount staining. Analyses of 3-wk-old mice (A–D) reveal normal ductal outgrowth in NKCC1 −/− transplants (B) compared with NKCC1 +/− transplants (A) but an increase in ductal branching (*cf*. D and C). By 8 wk (E–H), NKCC1 −/− epithelium failed to fill the entire fat pad (F) compared with NKCC1 +/− epithelium (E), and the increase in ductal branching remained evident (*cf*. H and G). At 12 wk posttransplantation (I–L), NKCC1 −/− epithelium filled the entire fat pad (J) similar to NKCC1 +/− epithelium (I) but showed persistence of ductal branching (*cf*. L and K). *Bar* in A, B, E, F, I, and J, 1 mm. *Bar* in C, D, G, H, K, and L, 0.5 mm.

Table 2. Quantitation of Secondary Ductal Branching in NKCC1 −/− Transplanted Mammary Epithelium

	NKCC1 +/−	NKCC1 −/−
3 wk	25 ± 1.7	54 ± 3.2[a]
8 wk	56 ± 3.1	114 ± 4.7[a]
12 wk	54 ± 6.2	108 ± 5.1[a]

Secondary ductal branch points emanating from five primary ducts were counted at each stage of development in NKCC1 −/− and NKCC1 +/− epithelial transplants. The results presented are the mean ± SEM of three separate transplants.
[a] $P < 0.001$.

pothesis, we used mice deficient in genes that are necessary for the development of the mammary gland epithelium at pregnancy. Thus mice with deficiencies in the PRLR (20, 34), the tyrosine kinase Jak2 (21), and the transcription factors signal transducer and activator of transcription 5a and 5b (referred to as Stat5 −/− mice) (22) were used. Each of these models fails to undergo pregnancy-mediated proliferation, and the epithelium maintains ductal-like features at parturition (23, 24). We reasoned that if a high level of NKCC1 protein is a marker of ductal epithelial cells, each of these models should exhibit maintenance of NKCC1 similar to that observed in virgin ductal epithelium. Indeed immunofluoresence analyses established that PRLR −/−, Jak2 −/−, and Stat5 −/− epithelium failed to down-regulate NKCC1 protein at parturition and maintained high levels of NKCC1 (Fig. 6, D–F), as reported previously (23). Furthermore, the rudimentary alveoli that branch off of the main ducts, which we have termed ductoli (23), fail to down-regulate NKCC1 protein levels consistent with the maintenance of ductal-like characteristics (Fig. 6, D–F). These results support the hypothesis that high levels of NKCC1 protein are indicative of ductal epithelial cells and suggest that NKCC1 may prove useful in characterizing other mouse models that exhibit defects in mammary gland development.

NKCC1 as a Marker of Mammary Epithelial Cell Identity

Although NKCC1 is developmentally regulated, as demonstrated by RT-PCR and immunofluorescence analyses, it is expressed throughout mammary gland development. To establish whether NKCC1 expression is a hallmark of normal mammary epithelial cells, we used a previously described mouse model (25) in which the stabilization of the cell-adhesion molecule/transcription factor β-catenin could be specifically induced in mammary epithelial cells. Examination of these mice revealed profound changes in mammary epithelial cell development, the formation of cyst-like structures, and the deposition of keratin indicative of *trans*-differentiation into epidermis (26). Because NKCC1 expression is observed in normal alveolar epithelial cells at lactation (Fig. 7, A and B), we examined whether a loss of NKCC1 expression might be associated with this *trans*-differentiation process. These results established that areas within the mammary gland that had undergone initial stages of *trans*-differentiation, as assessed by cytoplasmic accumulation of β-catenin, had lost detectable membranous expression of NKCC1 protein (Fig. 7, C–F, *arrowheads*), as described previously (26). In contrast, morphologically normal areas of mammary epithelial cells demonstrated detectable NKCC1 protein on the basolateral membrane and normal membranous expression of β-catenin (Fig. 7, C–F, *arrows*). Taken together, these results suggest that detectable NKCC1 expression is a general feature of mammary epithelium, and its loss is associated with a loss of normal mammary epithelial cell identity.

DISCUSSION

Shennan (2) in 1989 first described physiological evidence that suggested the existence of a Na-K-Cl cotransport system in mammary tissue. Thus, using radioactive tracer experiments, it was demonstrated that the inward movement of Cl^- occurred via a transport mechanism dependent on the presence of both external Na^+ and K^+. Furthermore, this transport process was inhibited by furosemide, a known inhibitor of the NKCCs. Based on these data, and the data presented herein, it is clear that the mammary gland expresses a functional Na-K-Cl cotransporter (NKCC1).

We show that NKCC1 is expressed at its highest levels during virgin mammary tissue development, where it is restricted to the basolateral membrane of the ductal epithelial cells. The down-regulation of NKCC1 protein during pregnancy and lactation provides evidence that, at least at the level of overall protein, NKCC1 is regulated in a developmental-specific manner. The physiological reason for this observation is unclear at present but may be related to the phosphorylation of the NKCC1 protein during pregnancy (18). Thus the activity of the transporter increases 3-fold upon phosphorylation, meaning that a concomitant 3-fold reduction in the level of protein will result in an equivalent rate of transport. Interestingly, the population of cells that maintains a high level of NKCC1 protein during pregnancy is nonproliferating as assessed by PCNA immunohistochemistry. Therefore, it is tempting to speculate that the down-regulation of NKCC1 is a prerequisite for and/or consequence of cell division. An alternative explanation is that these cells represent the maintenance of a nondividing, less differentiated virgin ductal epithelial cell population. Analysis of other proteins, such as the PR (36, 37), may provide further information as to the nature of this epithelial cell population.

To ascertain whether the high level of NKCC1 protein observed during virgin mammary gland development was characteristic of ductal epithelial cells, we

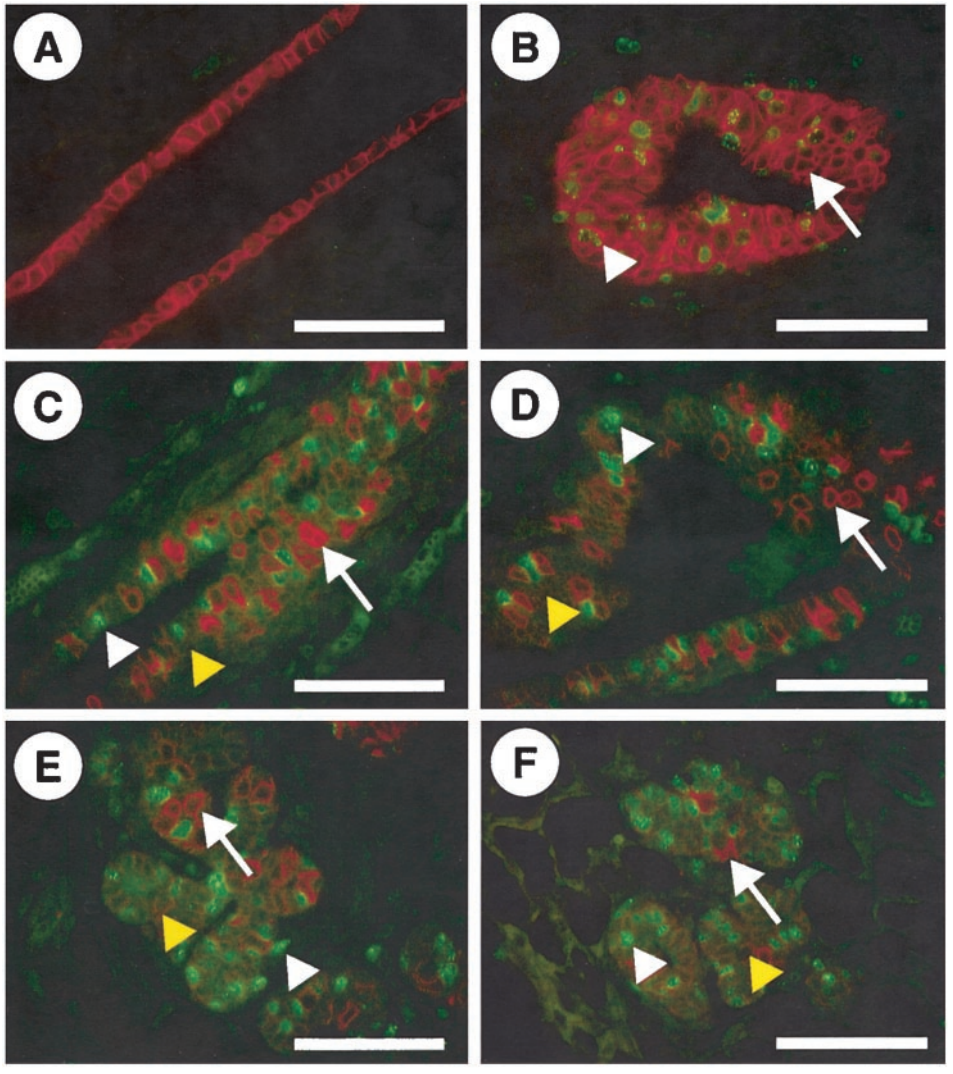

Fig. 5. High Levels of NKCC1 Protein During Pregnancy Are Associated with PCNA-Negative Cells

Sections (5 μm) derived from 6-wk-old virgin (A and B) and pregnancy d 12 (C–F) mammary tissue were incubated with anti-NKCC1 and anti-PCNA primary antibodies and subsequently visualized with fluorescent-conjugated secondary antibodies (NKCC1, *red*; PCNA, *green*). Ductal epithelial cells were NKCC1-strong positive, PCNA-negative (A). In contrast, there was evidence of NKCC1-strong positive, PCNA-negative (B, *arrow*) and NKCC1-strong positive, PCNA-positive cells (B, *arrowhead*) in the TEB structures. During pregnancy, ductal structures (C and D) and alveolar structures (E and F) exhibited three distinct populations of cells: NKCC1-weak positive, PCNA-negative (C–F, *yellow arrow*); NKCC1-strong positive, PCNA-negative (C–F, *white arrow*); and NKCC1-weak positive, PCNA-positive (C–F, *arrow*). *Bar,* 100 μm.

examined the levels of NKCC1 protein in mammary epithelium of mice deficient in PRLR, Jak2, or Stat5. These models were used because they all exhibit a failure of mammary epithelium to develop during pregnancy, as we reported previously (23, 24, 38, 39). This phenotype is manifested at parturition in a significant decrease in epithelial content and, based on histological sections, the persistence of ductal-like epithelial cells (ductoli). The ductal nature of these cells was supported by the observation that high NKCC1 protein levels were maintained in all three mouse models at parturition (23, 24), providing molecular evidence that these cells retained ductal features. These results suggests that examination of NKCC1 protein levels in other mouse models that exhibit similar mammary gland defects may provide useful insights into the nature of the mammary epithelial cells present.

The Role of NKCC1 in Mammary Epithelial Cell Development

Based on the available physiological data and the known roles of this Na-K-Cl cotransporter, our original hypothesis was that functional deletion of NKCC1 would affect the process of milk secretion. However, analysis of mammary tissue obtained from NKCC1 $-/-$ mice at parturition revealed no significant differences on a histological level (data not shown). Thus

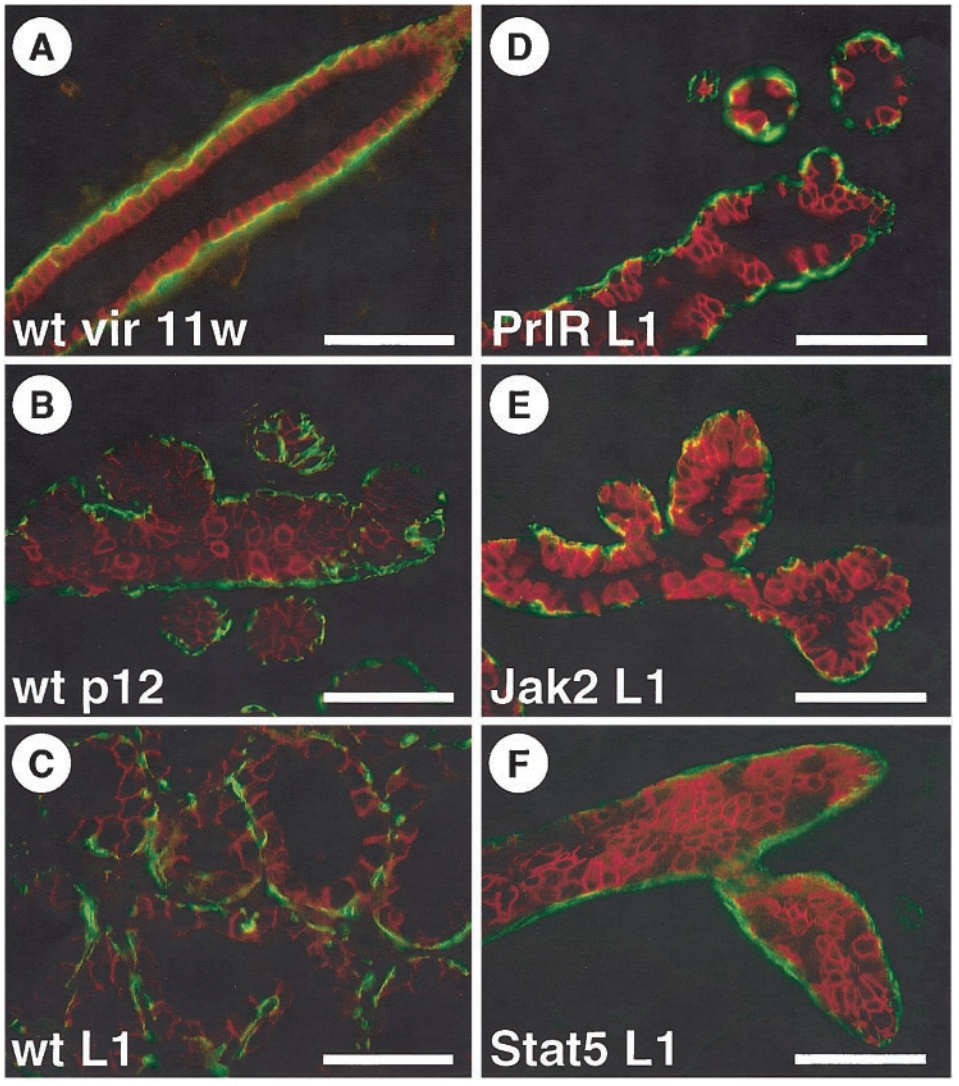

Fig. 6. NKCC1 Protein Expression as a Marker of Ductal Epithelial Cells

Sections (5 μm) derived from wild-type (A–C), PRLR −/− (D), Jak2 −/− (E), and Stat5 −/− (F) mammary tissue were incubated with anti-NKCC1 and anti-SMA primary antibodies and subsequently visualized with fluorescent-conjugated secondary antibodies (NKCC1, *red*; SMA, *green*). Wild-type ductal epithelial cells show evidence of a high level of NKCC1 protein (A), which is apparent in only a few ductal epithelial cells at pregnancy (B) and greatly reduced at lactation d 1 (C). Despite hormonal stimulation during pregnancy, a high level of NKCC1 protein is maintained in PRLR −/− (D), Jak2 −/− (E), and Stat5 −/− (F) epithelial cells at parturition (lactation d 1) similar to that observed in wild-type epithelium during virgin development (A). *Bar,* 100 μm.

there was evidence of lipid droplets, expanded alveoli, and milk secretion within the lumen. Interestingly, despite the normal appearance of the mammary epithelium in native NKCC1 −/− mice at lactation, pups born to these mice failed to thrive, suggesting that secondary defects (5, 19, 32), independent of mammary gland development, may be responsible for the decreased pup survival. Indeed NKCC1 −/− mice exhibit severe motor defects and, as a consequence, may not nurse their pups effectively. Unfortunately, the possible effect of NKCC1 deletion on lactational competence cannot be accurately assessed using the mammary transplantation approach because the nipple is separated from the mammary epithelium as a result of the transplantation procedure. Thus the role of NKCC1 during lactation would be better addressed utilizing a mouse model in which the temporal- and spatial-specific deletion of the *NKCC1* gene can be achieved. Although there was no significant difference in the length of time it took for NKCC1 −/− mice to become pregnant, two of six NKCC1 −/− females failed to successfully deliver their pups. This deficiency may have been due to systemic circulatory abnormalities (19) and possible consequential placental defects.

The high levels of NKCC1 protein observed in ductal epithelial cells during virgin development prompted an investigation into the possible role of this transporter in ductal epithelial cell development. Analyses of NKCC1

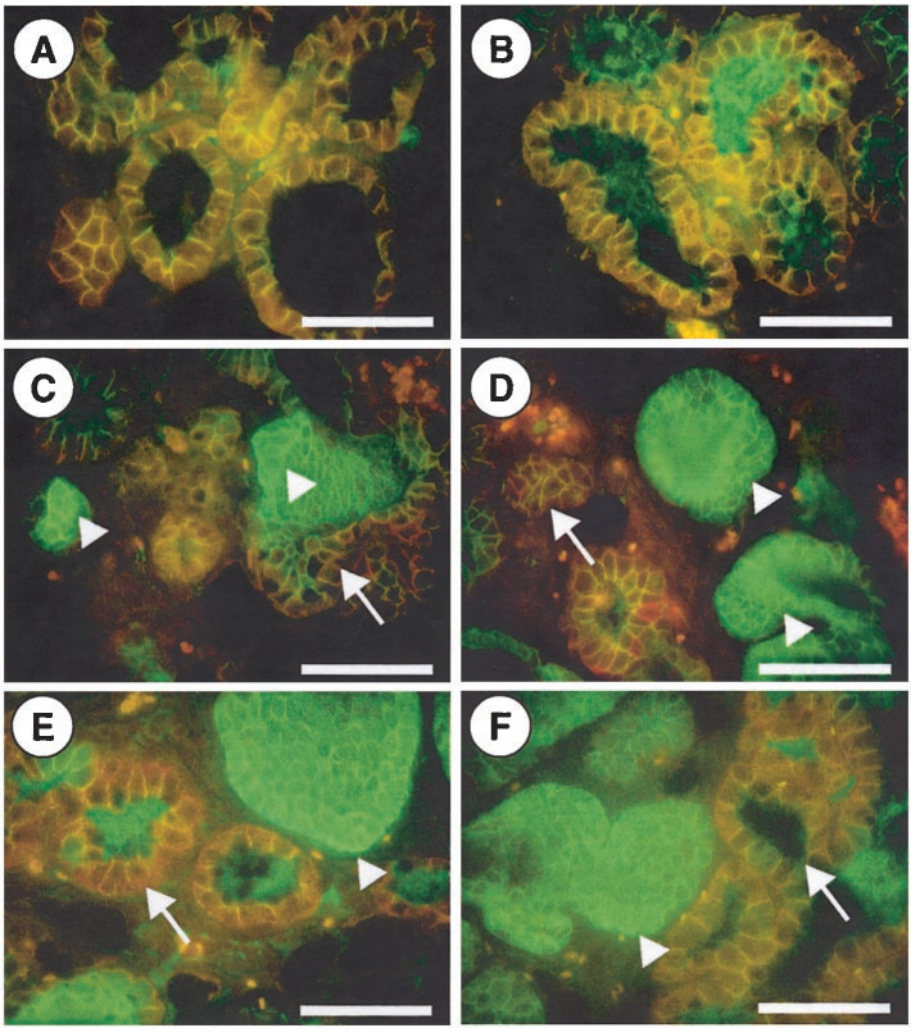

Fig. 7. Loss of Mammary Epithelial Cell Characteristics Is Associated with the Loss of NKCC1 Protein

Sections (5 μm) derived from wild-type mammary tissue at lactation d 1 (A and B) and mammary tissue expressing a dominant stable form of β-catenin (lactation d 1, C and D; lactation d 2, E and F) were incubated with anti-NKCC1 and anti-β-catenin primary antibodies and subsequently visualized with fluorescent-conjugated secondary antibodies (NKCC1, *red*; β-catenin, *green*). Wild-type alveolar cells possess detectable membranous staining of NKCC1 and β-catenin (A and B). In the mutant mouse model, NKCC1 protein is lost in areas that appear morphologically abnormal and exhibit cytoplasmic accumulation of β-catenin (C–F, *arrowhead*). In contrast, membranous staining of NKCC1 and β-catenin are maintained in areas that appear normal (C–F, *arrow*). *Bar,* 100 μm.

$-/-$ mammary transplants revealed a significant increase in secondary branching at all stages of virgin mammary gland development studied. Furthermore, in contrast to transplanted mammary epithelium derived from NKCC1 $+/-$ mice, NKCC1 $-/-$ transplanted epithelium failed to fill the fat pad at 8 wk posttransplantation. This observation suggests a novel and as yet undescribed role for this transporter in the determination of cell growth and development. Furthermore, these results demonstrate that NKCC1 expression is an important determinant of branching morphogenesis in early virgin mammary tissue. Because branching of epithelial tissues is a complex process that undoubtedly involves the cooperation of many diverse gene products, further studies will be needed to address the precise role of NKCC1 in ductal cell development. The homogeneous expression of NKCC1 in all mammary ductal epithelial cells, including cells at branch points, suggests that NKCC1 performs a housekeeping function. Although the ductal cells of virgin mammary tissue are largely nonsecretory, the individual ducts maintain a luminal space. Therefore it might be possible that NKCC1 expression, in concert with other membrane transport systems, may contribute to luminal expansion of the ducts. However, if NKCC1 is involved in the process of luminal expansion, its role is somewhat redundant because the virgin mammary ducts present in NKCC1 $-/-$ mice maintain a luminal space.

It is clear from the published literature that NKCC1 has many functions, including proposed roles in cell

volume regulation (13, 40), cell proliferation (15), and the cell cycle (17). Perhaps the most convincing evidence that NKCC1 may play a role in cellular proliferation is the observation that overexpression of this transporter in mouse fibroblasts leads to a transformation phenotype (17). However, if this were the case then it might be expected that functional ablation of this transporter would lead to a reduction in cellular proliferation, which is not consistent with the significant increase in secondary branching in transplanted NKCC1 −/− mammary epithelium. Indeed this notion is supported by the observation that the discrete populations of cells that possess high levels of NKCC1 protein at pregnancy were all PCNA-negative, suggesting that expression of NKCC1 is associated with nonproliferative cells. Furthermore, the maintenance of high NKCC1 protein levels in PRLR −/−, Stat5 −/−, and Jak2 −/− mammary epithelium at parturition correlates with a significant decrease in cell proliferation (23). Thus it seems, at least in the context of mammary epithelial cells, that NKCC1 may not have a prominent role in cell proliferation.

Physiological Implications of NKCC1 Activity

The mammary gland does not secrete large amounts of Cl^- or K^+ during lactation (34). On the basis of thermodynamic calculations, and the known concentrations of the major ions in milk, it has been suggested that K^+ is passively distributed across the apical membrane of the mammary secretory cell (35). In contrast, Cl^- is actively pumped back into the cell. Based on these considerations, it seems unlikely that NKCC1 is poised to secrete Cl^- and/or K^+ as it does in other secretory epithelial tissues (4). Because there are no available data on the ionic concentrations in ductal epithelial cells of the virgin gland, the physiological relevance of high levels of NKCC1 protein during virgin gland development is less clear. Although it is apparent that the transport of ions via NKCC1 is somehow influential in ductal branching, most likely in association with a cascade of cell processes, the exact nature of its role remains to be determined.

MATERIALS AND METHODS

Animals

The NKCC1 −/− mice used throughout were described previously (19). The PRLR −/− (20), Jak2 −/− (21), and Stat5 −/− (22) mice have been described previously (23–24). Athymic nude mice (nu/nu) were used in the transplantation experiments. Mice carrying a mutant β-catenin gene (25–26) have been described previously.

Antibodies

The rabbit polyclonal anti-NKCC1 antibody, which has been characterized previously (27), was a kind gift from Dr. Jim Turner, National Institute of Craniofacial and Dental Research, NIH (Bethesda, MD). Mouse monoclonal antismooth muscle actin (SMA) and anti-PCNA were obtained from Transduction Laboratories, Inc. (Lexington, KY) and DAKO Corp. (Carpinteria, CA), respectively.

Immunohistochemistry

Tissue was fixed in Tellyesniczky's fixative for 4 h before embedding in paraffin wax. Tissue sections (5 μm) were cut and mounted on polylysine-coated slides. Sections were cleared in xylene, rehydrated through an alcohol series, and treated with antigen unmasking solution (Vector Laboratories, Inc., Burlingame, CA). Sections were blocked with normal horse serum for 30 min at room temperature followed by the addition of primary antibodies (NKCC1, 1:1,000; SMA, 1:1,000; PCNA, 1:50) for 1 h at 37 C. Specifically bound antibody was visualized using fluorescein thiocyanate (FITC)- and tetramethylrhodamine isothiocyanate (TRITC)-conjugated fluorescent secondary antibodies (Molecular Probes, Inc., Eugene, OR).

Imaging Analysis

All images were captured using a DKC-5000 (Sony Ltd.) color digital camera attached to an Axioscop microscope (Carl Zeiss, Thornwood, NY) equipped with FITC and TRITC single filters and a FITC:TRITC double filter.

Transplantation of NKCC1 Mammary Tissue into the Cleared Fat Pad of Nude Mice

The fourth inguinal mammary gland was excised from littermate 8-wk-old virgin female NKCC1 +/− and NKCC1 −/− mice, and small pieces, approximately 1 mm in size, were cut from each mammary gland for transplantation. Athymic nude mice were anesthetized with avertin (20 μg/g body weight) and restrained in the supine position. A small (~2 cm) incision was made in the skin between the fourth and fifth nipple, and the skin was pulled back to expose the fourth inguinal mammary gland. The major blood vessels between the fourth and fifth mammary gland and under the lymph node were cauterized, and the endogenous mammary epithelium was carefully excised (28). Tissue isolated from a NKCC1 +/− mouse was inserted in the cleared fat pad on the right side of the animal, and tissue isolated from a NKCC1 −/− mouse was inserted in the cleared fat on the contralateral side of the animal. The incisions were closed using 9-mm wound clips, and animals were allowed to recover fully under a radiant heat source. To assess the completeness of clearing, the excised endogenous glands were processed for whole mount staining according to standard protocols.

Whole-Mount Analysis of Mammary Glands

Animals were euthanized by CO_2 administration and cervical dislocation. The mammary glands were removed, spread on glass slides, and fixed in Carnoy's fixative for 1.5 h. Whole-mount preparations were rehydrated through an alcohol series to distilled water and placed in carmine alum stain overnight. The glands were then dehydrated through an alcohol series and the fat was cleared (Histoclear). Whole-mounts were permanently mounted in mounting medium (Permount).

Isolation of Total RNA

Total RNA was isolated from frozen or fresh tissues as described previously (29). Briefly, tissue was placed in a denaturing solution containing guanidinium thiocyanate and homogenized until no solid tissue was visible. Homogenates

were placed on ice and aliquoted (600 μl) into Eppendorf (Madison, WI) tubes. Subsequently, 60 μl of 2 M sodium acetate, pH 4, 600 μl of water-saturated phenol, and 120 μl of chloroform were added to each aliquot. Samples were centrifuged at 4 C, 13,000 rpm for 30 min to separate the phases, and the aqueous phase was transferred to a fresh tube. An equal volume of chloroform was added, and the samples were centrifuged at 4 C, 13,000 rpm, for 20 min. The aqueous phase was transferred to a fresh Eppendorf tube, and an equal volume of isopropanol was added. The nucleic acid was precipitated by incubating the samples at −70 C for 30 min followed by centrifugation at 4 C, 13,000 rpm, for 30 min. The supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was allowed to air dry for 10 min at room temperature and subsequently resuspended in an appropriate volume of diethyl pyrocarbonate-treated water. The concentration of the nucleic acid solution was determined from the A_{260}/A_{280} values as measured using a spectrophotometer.

Synthesis of cDNA

Before cDNA synthesis, total RNA (5 μg) was routinely treated with ribonuclease-free deoxyribonuclease (Promega Corp., Madison, WI). Total RNA (1 μg) was reverse transcribed into cDNA using SuperScript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) following the manufacturer's protocol. Total RNA was first incubated with deoxyribonucleoside triphosphates and an oligo-$dT_{(12-18)}$ primer at 65 C for 5 min. All components were added except the reverse transcriptase, and the reaction was incubated at 42 C for 2 min. SuperScript II RT (50 U) was added to each reaction and incubated for a further 50 min. Reactions lacking reverse transcriptase were also performed. The enzyme was inactivated by heating at 85 C for 15 min, and the single-stranded RNA was degraded by treating the reaction with *Escherichia coli* ribonuclease H^- for 20 min at 37 C. The resulting cDNA was stored at −20 C.

PCR and Cycling Conditions

Primers corresponding to published mouse cDNA sequences were designed and synthesized (Lofstrand Labs Ltd., Gaithersburg, MD). A master mix PCR was made consisting of 1× Promega Corp. Buffer A, 2.5 mM $MgCl_2$, 50 μM deoxynucleoside triphosphates, 30 pmol gene-specific primer, and 2.5 U of *Taq* polymerase. The master mix was aliquoted (50 μl/reaction) into 0.2 ml strip PCR tubes to which 1 μl of cDNA was added. Reactions containing total RNA or lacking cDNA served as controls for genomic DNA contamination and reaction contamination, respectively. PCR cycling was performed using the following gene-specific primers [size of product (base pairs) *in parentheses*]: NKCC1 (500), sense 5′-ATG GTG TCA GGA TTT GCA CCC TTG-3′, antisense 5′-CTG AGG TAA GTC AGC GCT TGT GTG-3′; β-casein (534), sense 5′-ACT ACA TTT ACT GTC TCC TCT GAG-3′, antisense 5′-GTG CTA CTT GCT GCA GAA AGT ACA G-3′; GAPDH (292), sense 5′-CTC ACT GGC ATG GCC TTC CG-3′ antisense 5-ACC ACC CTG TTG CTG TAG CC-3′; WAP (248), sense 5′-CAC AGA GTG TAT CAT CTG CC-3′, antisense 5′-GTA CAT GTC ATG ACA CAG TC-3′; GLUT1 (228), sense 5′-GAT TCG CCC ATT CCT GTC TCT TC-3′, antisense 5′-CCA TCT ATA CAC AGC AGG GCA G-3′; CK18 (375), sense 5′-CGC ATC GTC TTG CAG ATC GAC A-3′, antisense 5′-GCT GAG ACC AGT ACT TGT CCA G-3′. The template was first denatured at 94 C for 2 min followed by 35 cycles (25 cycles for GAPDH, β-casein, and WAP) of denaturation (94 C, 40 sec), annealing (NKCC1 and GLUT1, 65 C, 40 sec; GAPDH, CK18, β-casein, and WAP, 58 C, 40 sec), and extension (72 C, 1 min). A final extension at 72 C for 10 min was performed. An aliquot (20 μl) of the PCR was electrophoresed on a 2% gel containing 0.5 μg/ml ethidium bromide. Amplified products were visualized under UV light and photographed. The identities of all amplified products were determined by sequencing.

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Address all correspondence and requests for reprints to: Dr. Jonathan M. Shillingford, Building 8, Room 105, National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892. E-mail: jonshi@helix.nih.gov.

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REFERENCES

1. Shennan DB, Peaker M 2000 Transport of milk constituents by the mammary gland. Physiol Rev 80:925–951
2. Shennan DB 1989 Evidence for furosemide-sensitive Na+-K^+-Cl-co-transport in lactating rat mammary tissue. Q J Exp Physiol 74:927–938
3. Shennan DB, McNeillie SA 1990 Efflux of chloride from lactating rat mammary tissue slices. Comp Biochem Physiol A 95:367–371
4. Russell JM 2000 Sodium-potassium-chloride cotransport. Physiol Rev 80:211–276
5. Delpire E, Rauchman MI, Beier DR, Hebert SC, Gullans SR 1994 Molecular cloning and chromosome localization of a putative basolateral Na^+-K^+-2Cl-cotransporter from mouse inner medullary collecting duct (mIMCD-3) cells. J Biol Chem 269:25677–25683
6. Ginns SM, Knepper MA, Ecelbarger CA, Terris J, He X, Coleman RA, Wade JB 1996 Immunolocalization of the secretory isoform of Na-K-Cl cotransporter in rat renal intercalated cells. J Am Soc Nephrol 7:2533–2542
7. Igarashi P, Vanden Heuvel GB, Payne JA, Forbush III B 1995 Cloning, embryonic expression, and alternative splicing of a murine kidney-specific Na-K-Cl cotransporter. Am J Physiol 269:F405–F418
8. Kaplan MR, Plotkin MD, Lee WS, Xu ZC, Lytton J, Hebert SC 1996 Apical localization of the Na-K-Cl cotransporter, rBSC1, on rat thick ascending limbs. Kidney Int 49:40–47
9. Dharmsathaphorn K, McRoberts JA, Masui H, Mandel KG 1985 Vasoactive intestinal peptide-induced Cl secretion by a colonic epithelial cell line. Direct participation of a basolaterally localized Na,K,Cl cotransport system. J Clin Invest 75:462–471
10. Boucher RC, Hviid Larsen E 1988 Comparison of ion transport by cultured secretory and absorptive canine airway epithelia. Am J Physiol 254:C535–C547
11. Nauntofte B 1992 Regulation of electrolyte and fluid secretion in salivary acinar cells. Am J Physiol 263: G823–G837
12. Haas M, Forbush III B 2000 The Na-K-Cl cotransporter of secretory epithelia. Annu Rev Physiol 62:515–534
13. O'Neill WC, Klein JD 1992 Regulation of vascular endothelial cell volume by Na-K-2Cl cotransport. Am J Physiol 262:C436–C444
14. Klein JD, Lamitina ST, O'Neill WC 1999 JNK is a volume-sensitive kinase that phosphorylates the Na-K-2Cl cotransporter *in vitro*. Am J Physiol 277:C425–C431
15. Panet R, Atlan H 1991 Stimulation of bumetanide-sensitive Na+/K^+/Cl-cotransport by different mitogens in synchronized human skin fibroblasts is essential for cell proliferation. J Cell Biol 114:337–342

16. Panet R, Markus M, Atlan H 1994 Bumetanide and furosemide inhibited vascular endothelial cell proliferation. J Cell Physiol 158:121–127
17. Panet R, Marcus M, Atlan H 2000 Overexpression of the $Na^+/K^+/Cl^-$ cotransporter gene induces cell proliferation and phenotypic transformation in mouse fibroblasts. J Cell Physiol 182:109–118
18. Selvaraj NG, Omi E, Gibori G, Rao MC 2000 Janus kinase 2 (JAK2) regulates prolactin-mediated chloride transport in mouse mammary epithelial cells through tyrosine phosphorylation of Na+-K^+-2Cl-cotransporter. Mol Endocrinol 14:2054–2065
19. Flagella M, Clarke LL, Miller ML, Erway LC, Giannella RA, Andringa A, Gawenis LR, Kramer J, Duffy JJ, Doetschman T, Lorenz JN, Yamoah EN, Cardell EL, Shull GE 2000 Mice lacking the basolateral Na-K-2Cl cotransporter have impaired epithelial chloride secretion and are profoundly deaf. J Biol Chem 274:26946–26955
20. Brisken C, Kaur S, Chavarria TE, Binart N, Sutherland RL, Weinberg RA, Kelly PA, Ormandy CJ 1999 Prolactin controls mammary gland development via direct and indirect mechanisms. Dev Biol 210:96–106
21. Neubauer H, Cumano A, Muller M, Wu H, Huffstadt U, Pfeffer K 1998 Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoesis. Cell 93:397–409
22. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN 1998 Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841–850
23. Miyoshi K, Shillingford JM, Smith GH, Grimm SL, Wagner K-U, Oka T, Rosen JM, Robinson GW, Hennighausen L 2001 Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium. J Cell Biol 155:531–542
24. Shillingford JM, Miyoshi K, Robinson GW, Grimm SL, Rosen JM, Neubauer H, Pfeffer K, Hennighausen L 2002 Jak2 is an essential tyrosine kinase involved in pregnancy-mediated development of mammary secretory epithelium. Mol Endocrinol 16:563–570
25. Harada M, Tamai Y, Ishikawa I, Sauer B, Takaku K, Oshima M, Taketo MM 1999 Intestinal polyposis in mice with a dominant stable mutation of the β-catenin gene. EMBO J 18:5931–5942
26. Miyoshi K, Shillingford JM, Le Provost F, Gounari F, Bronson R, von Boehmer H, Taketo MM, Cardiff RD, Hennighausen L, Khazaie K 2002 Activation of β-catenin signaling in differentiated mammary secretory cells induces transdifferentiation into epidermis and squamous metaplasias. Proc Natl Acad Sci USA 99:219–224
27. Moore-Hoon ML, Turner RJ 1998 Molecular and topological characterization of the rat parotid Na+-K^+-2Cl-cotransporter1. Biochim Biophys Acta 1373:261–269
28. DeOme KB, Faulkin Jr LJ, Bern HA, Blair PE 1959 Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res 19:515–520
29. Chomczysnki P, Sacchi N 1987 Single step method of RNA extraction by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
30. Camps M, Vilaro S, Testar, X, Palacin M, Zozano, A 1994 High and polarized expression of GLUT1 glucose transporters in epithelial cells from mammary gland: acute down-regulation of GLUT1 carriers by weaning. Endocrinology 134:924–934
31. Zhao FQ, Dixon WT, Kennelly JJ 1996 Localization and gene expression of glucose transporters in bovine mammary gland. Comp Biochem Physiol B Biochem Mol Biol 115:127–134
32. Pace AJ, Lee E, Athirakul K, Coffman TM, O'Brien DA, Koller BH 2000 Failure of spermatogenesis in mouse lines deficient in the Na^+-K^+-$2Cl^-$ cotransporter. J Clin Invest 105:441–450
33. Kopf-Maier P, Mboneko VF 1990 Anomalies in the hormonal status of athymic nude mice. J Cancer Res Clin Oncol 116:229–231
34. Ormandy CJ, Binart N, Kelly P 1997 Mammary gland development in prolactin receptor knockout mice. J Mammary Gland Biol Neoplasia 2:355–364
35. Linzell JL, Peaker M 1971 Intracellular concentrations of sodium, potassium and chloride in the lactating mammary gland and their relation to the secretory mechanism. J Physiol 216:683–700
36. Atwood CS, Hovey RC, Glover JP, Chepko G, Ginsburg E, Robison Jr WG, Vonderhaar BK 2000 Progesterone induces side-branching of the ductal epithelium in the mammary glands of prepubertal mice. J Endocrinol 167: 39–52
37. Humphreys RC, Lydon J, O'Malley BW, Rosen JM 1997 Mammary gland development is mediated by both stromal and epithelial progesterone receptors. Mol Endocrinol 11:801–811
38. Hennighausen L, Robinson GW 2001 Signaling pathways in mammary gland development. Dev Cell 1:467–475
39. Shillingford JM, Hennighausen L 2001 Experimental mouse genetics—answering fundamental questions about mammary gland biology. Trends Endocrinol Metab 12:402–408
40. Hoffmann EK, Dunham PB 1995 Membrane mechanisms and intracellular signaling in cell volume regulation. Int Rev Cytol 161:173–262