Cell Confluence-induced Activation of Signal Transducer and Activator of Transcription-3 (Stat3) Triggers Epithelial Dome Formation via Augmentation of Sodium Hydrogen Exchanger-3 (NHE3) Expression*

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Hsiao-Wen Su†, Hsuan-Heng Yeh†, Shainn-Wei Wang†§, Meng-Ru Shen‡, Tsu-Ling Chen‡, Pawel R. Kielia**, Fayez K. Ghishan**, and Ming-Jer Tang‡‡

From the †Institute of Basic Medical Sciences, ‡Institute of Molecular Medicine, ‡Department of Pharmacology, ‡‡Department of Physiology, and ‡§Center for Gene Regulation and Signal Transduction Research, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan and the **Departments of Pediatrics and Physiology, Steele Children’s Research Center, University of Arizona Health Sciences Center, Tucson, Arizona 85724

Cell confluence induces the activation of signal transducer and activator of transcription-3 (Stat3) in various cancer and epithelial cells, yet the biological implications and the associated regulatory mechanisms remain unclear. Because confluent polarized epithelia demonstrate dome formation and sodium influx that mimic the onset of differentiation, we sought to elucidate the role of Stat3 in association with the regulation of selective epithelial transporters in this biological phenomenon. This study established the correlation between Stat3 activation and cell confluence-induced dome formation in Madin-Darby canine kidney cells (MDCK) by following Stat3 activation events in dome-forming cells. Epifluorescent and confocal microscopy provided evidence showing specific localization of phosphorylated Stat3 Tyr705 in the nuclei of dome-forming cells at initial stages. The relationship was further elucidated by the establishment of tetracycline-inducible expression of constitutive Stat3 mutant (Stat3-C) in MDCK cells or expression of dominant negative Stat3 (Stat3-D) stable cell lines (MDCK and NMuMG). Dome formation was promoted by the expression of Stat3-C but inhibited by Stat3-D. Two trans-epithelial transporters, NHE3 and ENaC α-subunit, were found to be increased during cell confluence. Interestingly, NHE3 expression could be specifically up-regulated by Stat3-C but inhibited by Stat3-D through promoter regulation, whereas NHE1 and ENaC α-subunit were not affected by Stat3 expression. Application of NHE3 shRNA, NHE3 inhibitors (EIPA and S3226) suppressed confluence-induced dome formation in MDCK or NMuMG cells. These results demonstrate a cell confluence-induced Stat3 signaling pathway in epithelial cells in triggering dome formation through NHE3 augmentation.

Domes are multicellular hemicyst structures unique to polarized epithelia in culture (1) and are functionally equivalent to differentiated epithelium with trans-epithelial solute transport (2–4). They occur sporadically in small areas during cell confluence and mark the initial differentiation process of a functional epithelial monolayer. While turning into an impermeable substratum with preceded expression of tight junction proteins (3), the dome structure sets off with diminishing cellular adherence ability as a result of liquid accumulation between the cell layer and the underlying support (2–4). This feature is conceived by the coordinated development of trans-epithelial transport systems in morphologically polarized cells. In renal, intestinal, and other epithelia, this phenomenon involves primarily a differential localization and activation of apical sodium transporters (sodium channels and Na+/H+ exchangers) and ubiquitously expressed basolateral Na+,K+-ATPase to maintain Na+ and fluid homeostasis (2, 4–8). Most importantly, the net unidirectional transport of Na+ coincided with intracellular alkalization has been implicated in dome formation (4, 9, 10).

The epithelial cell line, Madin-Darby canine kidney (MDCK)2 (11), is a model system widely used to study dome formation-associated renal trans-epithelial transport during cell confluence (2). At least three possible mechanisms exist to coordinate the vectorial sodium transport across MDCK monolayer from apical to basolateral sites. One is the ouabain-sensitive Na+/K+-ATPase mechanism, the second is the furosemide-sensitive Na+/Cl−/K+/Cl− co-transport mechanism, and the third is the amiloride-sensitive ENaC (12–14). Because MDCK cells used to be considered a model cell line of distal nephron, the role of NHE3, a marker for proximal tubule epithelium in dome formation, has not been implicated in MDCK cells before. Because abundant studies indicated the involvement of Na+/H+ exchangers (NHEs) and epithelial sodium channel (ENaC) during renal development or epithelial cell dif-

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† To whom correspondence should be addressed: Dept. of Physiology, College of Medicine, National Cheng Kung University, 1 Da-Hsueh Road, Tainan 701, Taiwan. Tel.: 886-6-235-3535, ext. 5425; Fax: 886-6-236-2780; E-mail: mtjhang1@mail.ncku.edu.tw.

‡ The abbreviations used are: MDCK, Madin-Darby canine kidney cells; Stat, signal transducer and activator of transcription-3; PBS, phosphate-buffered saline; LCD, low cell density; HCD, high cell density; HA, hemagglutinin; NHE, Na+/H+ exchangers; DAPA, DNA affinity precipitation assay; Me2SO, dimethyl sulfoxide.

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f erentiation (15–19), we hypothesized that these sodium transporters, coordinately with Na⁺,K⁺-ATPase, participate in the initiation of dome formation in MDCK cells. The physiological roles of NHE are to maintain intracellular and systemic pH, transcellular absorption of NaCl and NaHCO₃, and intracellular volume and body fluid balance (20–22). Nine NHE isoforms have been indentified to date (6). Among them, NHE1 is ubiquitously expressed and localized at the basolateral membrane of polarized epithelial cells, whereas NHE2, NHE3, and more recently NHE8 have been localized to the apical membrane of small intestine, colon, and renal tubular cells (6, 23). In mammalian kidney, NHE3 is the key factor acting to reabsorb sodium and water across the epithelial cells of renal proximal tubule (21).

In general, STAT signaling events are triggered by membrane receptors in response to wide varieties of cytokines and growth factors to regulate physiological responses (24–26). Receptors for cytokines are devoid of intrinsic kinase activity and require the mediation of Janus kinases (JAKs) for STAT phosphorylation (24, 25). However, recently, a ligand-independent activation of Stat3 (signal transducer and activator of transcription-3) by cell confluence has been demonstrated in both cancer and normal epithelial cell lines (27, 28). These studies revealed cdk2- (cyclin-dependent kinase 2)-regulated activation of Stat3 by cell growth arrest and envisioned a novel role of JAK-dependent Stat3 signaling in modulating the survival-related physiological functions during cell confluence. This raises the possibility that signaling via Stat3 (or their cognate family members involved in the growth control, such as Stat1 and 5 (25)), as a result of cell confluence, modulates trans-epithelial sodium transport and lead to dome formation. STAT proteins, which include 7 latent cytoplasmic transcription factors, are normally activated through phosphorylation of their tyrosine residues by several tyrosine kinases. The key tyrosine residue phosphorylated in Stat1 is Tyr701, in Stat3 is Tyr705, and in Stat5 is Tyr694 (24). Phosphorylated STATs are dislodged from the receptor or non-receptor associated kinase complex and may cross-talk between each other to form immediate hetero- (e.g. Stat1-Stat3) or homodimers (e.g. Stat1-Stat1 or Stat3-Stat3) (24). They are then translocated into the nucleus where they work coordinately with different co-activators to induce transcription of distinct genes via binding to their cognate cis-elements within their promoter regions (26, 29).

We report here that cell confluence-induced activation of Stat3 regulates dome formation in MDCK and NMuMG (normal mouse mammary gland) cells by augmentation of NHE3 transporter. By coupling dome formation as a biological trait to chase Stat3 signaling events in MDCK or NMuMG cells during cell confluence, a causal effect of Stat3 resulting from cell-cell contact on dome formation was revealed. We found that the morphological characteristics of dome formation in terms of their number and size as a result of the transcriptional regulation by constitutively active or dominant negative forms of Stat3 correlated with Stat3-mediated expression of NHE3. Lastly, by functional inhibition of NHE3 activities, we demonstrated a novel Stat3-mediated NHE3 augmentation signaling pathway in regulating differentiation of a functional epithelial monolayer during cell-cell contact.

EXPERIMENTAL PROCEDURES

Cell Culture and Stable Transfections—MDCK cells and NMuMG cells were maintained in Dulbecco’s modified minimal essential medium supplemented with 10% fetal calf serum under 5% CO₂ at 37 °C. The constitutively active form Stat3 (Stat3-C) plasmid was kindly provided by Dr. James Darnell, Jr. (30). To generate the tetracycline-inducible Stat3-C system, we subcloned the Stat3-C gene from pRC/CMV plasmid to rtTA-responsive pTRE2-hyg plasmid (Clontech Laboratories, Inc.). Stable transfections of inducible FLAG-tagged Stat3-C (30) to MDCK cells were conducted by using 20 μl of Lipofectamine to establish clonal lines constitutively expressing rtTA encoded by the pTet-ON regulator plasmid. The pTet-ON plasmid contains a neomycin resistance gene to permit G418 selection (0.5 mg/ml; Clontech Laboratories, Inc.). One of these rtTA clones was selected for secondary stable transfection with rtTA-responsive pTRE2-hyg hygromycin plasmid containing Stat3-C, and colonies were selected with 0.2 mg/ml hygromycin B (Invitrogen; Carlsbad, CA). Stable clones were screened after 24 h of induction with 10 μg/ml doxycycline (Sigma) for Stat3-C expression by Western blot using anti-FLAG antibody. For the dome formation assay, MDCK (2.5 × 10⁵) cells or NMuMG (3 × 10⁵) cells were plated on 6-cm culture dish in medium containing 10% fetal calf serum, and the medium was changed every 2 days until dome formation. To generate cells stably expressing a dominant negative Tyr705 mutant form of Stat3 (Stat3-F), subconfluent MDCK cells grown on 6-cm dish were co-transfected with 1 μg of pRC/CMV neomycin resistance empty vector and 4 μg of pMS1 plasmid encoding Stat3-F (31) using 20 μl of Lipofectamine (Invitrogen). One day after transfection, cells were re-seeded on a 10-cm dish at an appropriate density in the medium containing 0.5 mg/ml G418. Neomycin-resistant cell clones were selected and screened for exogenous Stat3-F expression by immunoblotting with anti-Stat3 and anti-Stat3 Tyr705(P) polyclonal antibodies. Two positive clones (clones 11 and 12) expressing Stat3-F were used for dome formation assay. To generate cells stably expressing HA epitope-tagged Stat3-D, a DNA binding domain mutant kindly provided by Dr. T. Hirano (32), subconfluent MDCK (and NMuMG) cells grown on a 6-cm dish were transfected with 4 μg of pCAGGSneo plasmid encoding Stat3-D using 20 μl of Lipofectamine. Neomycin-resistant cell clones were selected following the aforementioned procedures and were screened for exogenous Stat3-D expression by immunoblotting with anti-HA monoclonal antibody. Multiple positive clones expressing Stat3-D were obtained for dome formation assay. The dome number and diameter were quantified by counting and measuring domes from at least 20 independent fields at high power magnification (×100). EIPA (NHE inhibitor, Sigma) and S3226 (NHE3 inhibitor) were used for inhibition of dome formation. Confluent cells were incubated with each inhibitor at different concentrations until the dome appeared in control cells. S3226 was kindly provided by Dr. Robert J. Wilkins (33). Oncostatin M was purchased from PeproTech EC Ltd (London, UK).

Preparation of Nuclear Extracts—Nuclear extracts were collected according to the method of Wang et al. (34) with minor
modifications. Cells grown to various degrees of confluence on 6-cm plastic dishes were washed three times with phosphate-buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM dibasic sodium phosphate, and 2 mM monobasic potassium phosphate) and scraped off the plate after incubation with 400 μl of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, and 10 mM KCl) on ice for 10 min. Cells were precipitated by centrifugation at 7,500 × g for 20–30 s, and the pellet was resuspended in 100 μl of buffer C (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.2 mM EDTA, 420 mM NaCl). The resulting suspension was centrifuged at 7,500 × g for 2 min. The supernatants were collected and stored at −70 °C. Buffers A and C contained 0.5 mM dithiothreitol, 2 μg/ml leupeptin, 1 mM orthovanadate, 2 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride.

DNA Affinity Precipitation Assay (DAPA)—This assay was performed according to the method of Wang et al. (34) with minor modifications. The binding assay was performed by mixing 200 μg of nuclear extract proteins, 2 μg of biotinylated m67 oligonucleotides specific to Stat3 binding (14), and 20 μg of streptavidin-agarose beads in TE buffer (pH 7.9). The mixture was incubated at room temperature for 1 h with rotation followed by precipitation at low speed in a microcentrifuge and washed with cold PBS three times. The bound proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE), followed by Western blot analysis probed with Stat1 or Stat3 antibody (Cell Signaling Technology; Danvers, MA).

Reverse Transcription-PCR—Total cellular RNA was extracted with the RNeasy Mini kit (Qiagen, Valencia, CA). For RT-PCR, first-strand cDNA was synthesized from 0.2–1 μg of total RNA with an oligo-dT primer and the Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI). The sequences of PCR primers were as follows: forward primer (5′-CTC ATG GTC TTC CAT GAA CTC AAC-3′) and reverse primer (5′-ACT GTA CAG GTG CTT GTA CTC TG-3′) were designed from Canis familiaris NHE3 (NCBI accession XM_545197), and the resulting PCR product was 318 bp. Forward primer (5′-GTC TCT TTC ATC ATT GTC TT-3′) and reverse primer (5′-GAT GTG GCC CTC CAC GTA CT-3′) were designed from Mus musculus NHE3 (NCBI accession XM_993032), and the resulting PCR product was 360 bp. Forward primer (5′-CGA AAG TCA GGT TGC TGG TC-3′) and reverse primer (5′-AAG GAG TGG TGC TCT AGG TC-3′) were designed from M. musculus Stat3 (NCBI accession NM_011486), and the resulting PCR product was 337 bp. Forward primer (5′-GGT GCT GGT GCT GAG TA-3′) and reverse primer (5′-GAC CAC CTG GTC CTC AGT GT-3′) were designed from C. familiaris GAPDH (NCBI accession NM_00103012), and the resulting PCR product was 585 bp. Forward primer (5′-GGT GAG GCC GGT GCT GAG TA-3′) and reverse primer (5′-GAC AAC CTC GTC CTC AGT GT-3′) were designed from M. musculus GAPDH (NCBI accession no. BC083149), and the resulting PCR product was 585 bp. The PCR protocol performed with the canine NHE3 was: 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s (35 cycles), followed by 72 °C for 7 min. The PCR protocol performed with the mouse NHE3 was: 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 45 s (32 cycles), followed by 72 °C for 7 min. The PCR protocol performed with the Stat3 and GAPDH primers was: 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 1 min (28 cycles), followed by 72 °C for 10 min.

Transfection and Dual Luciferase Assay—The Stat3 reporter pm67 and its vector pTATA TK-luc were kindly provided by Dr. James Darnell, Jr. (35). The Renilla luciferase reporter pRL-TK was purchased from Promega. For the Stat3 reporter gene assay, cells (2 × 10⁵ cells) were plated in 6-cm dishes and co-transfected with 4 μg of pTATA TK-luc or m67 pTATA TK-luc with 0.5 μg pRL-TK by using the Arrest-In™ transfection reagent (Open Biosystems, Huntsville, AL) according to the manufacturer’s instructions. After 12 h of transfection, the transfectants were reseeded into 6-well plates at high cell density (5 × 10⁵ cells/well) or at low cell density (5 × 10⁴ cells/well). After 24 h of incubation, cells were subjected to the dual luciferase assay. NHE3 promoter activity assay was conducted in mock-transfected MDCK cells or in cells stably transfected with inducible Stat3-C expression vector (St5C-3). Cells (2 × 10⁵ cells) were plated in 6-cm dishes and transiently transfected with a Renilla luciferase reporter gene containing plasmid alone (either phRG-b vector, pNHE3 – 450/+58) or in combination with a pCMV-Luc vector (firefly luciferase). Transient transfection was carried out by the Arrest-In™ reagent (Open Biosystems, Huntsville, AL) according to the manufacturer’s instructions. phRG-b vector is a promoterless vector harboring the Renilla luciferase gene (Promega), and pNHE3 – 450/+58 is the phRG-b vector fused with a rat NHE3 promoter (–450 to +58 region) to drive Renilla luciferase expression (36), as canine NHE3 promoter sequence has not been described. The pCMV-Luc contained the firefly luciferase gene driven by cytomegalovirus (CMV) promoter (a gift kindly provided by Dr. Wen-Tsan Chang, College of Medicine, National Cheng-Kung University). After 12 h of transfection, the transfectants were reseeded in 6-well plates and cultured in the medium with or without doxycycline (10 μg/ml) for 20 h. The cells were washed three times in PBS, and the lysates were prepared by scraping the cells from plates in the presence of 1 × passive lysis buffer (Promega). Dual luciferase assays for Renilla luciferase and firefly luciferase activities were then performed on cell lysates using the Dual Luciferase Assay System (Promega) and a Sirius luminometer (Berthold Detection System, Pforzheim, Germany).

RNA Interference—To knock-down NHE3 expression in NMuMG epithelial cells, 19-mer short hairpin RNA (shRNA) against mouse NHE3 was synthesized by Open Biosystems. Sequences for shNHE3 are: 5′-GCG TCT GTC TCA TAT TTC T-3′ and 5′-AGA AAT ATG AGA CAG CAC G-3′. For stable transfection, shNHE3 was cloned in pm2 expression vector (Open Biosystems) and then transfected into NMuMG cells using Arrest-In™ transfection reagent (Open Biosystems) according to the manufacturer’s instructions (Open Biosystems). After 24 h of transfection, cells were subjected to puromycin (1 μg/ml) selection for at least 1 week. Subsequently, multiple puromycin-resistant cell clones were selected and screened for NHE3 expression by immunoblotting with NHE3 polyclonal antibody. Two positive clones (clones 4 and 10) with minimal NHE3 expression were picked for dome formation assay as described above.
**Immunofluorescence and Confocal Study—**MDCK cells were cultured at different time intervals followed by washing three times with PBS and fixed with 4% buffered paraformaldehyde for 20 min at room temperature. After cells were washed three times in PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature, they were incubated with Stat3 Tyr705(P) or Stat3 (Cell Signaling) polyclonal antibody for 1 h. Cells were then washed and incubated with Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen) and Hoechst 33258 for 1 h. The immunofluorescent images were taken by confocal microscopy (Olympus, FV-1000).

**Western Blotting—**Cells were grown to various degrees of confluence, and proteins were extracted using lysis buffer (20 mM HEPES, pH 7.9, 0.5% Nonidet P-40, 7.5% glycerol, 300 mM NaCl, 1 mM EDTA) containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.2 units/ml aprotinin, 1 mM diethiothreitol, 10 mM NaF, 1 mM Na3VO4 and 20 μg/ml leupeptin). For each blotting analysis, 50 μg of clarified cell extract was resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-ECL). The membranes were blocked with 5% nonfat milk for at least 1 h followed by an overnight incubation in primary antibody. Immunodetection was performed using antibodies against the Stat3 Tyr705(P), total Stat3, Stat3 Ser727(P), Stat1 Tyr701(P), total Stat1, Stat5 Tyr694(P) (all from Cell Signaling Technology), FLAG (Sigma), HA (Roche Applied Science; Indianapolis, IN), Ki-67 (Santa Cruz Biotechnology; Santa Cruz, CA), PCNA (Zymed Laboratories Inc.), Lamin A/C (Santa Cruz Biotechnology), β-actin (Sigma), NHE1 (Chemicon), ENaC-subunit (Santa Cruz Biotechnology), Na+,K+-ATPase α1 (Santa Cruz Biotechnology), or Na+,K+-ATPases β1 (Upstate; Charlestown, VA) followed by horseradish peroxidase-conjugated goat secondary antibodies (Invitrogen). The bands were visualized using enhanced chemiluminescence (ECL) according to the manufacturer’s instructions (PerkinElmer; Boston, MA).
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**FIGURE 2.** High cell density triggers Stat3 transcriptional activation in MDCK cells. A, MDCK cells seeded at LCD (1 × 10^6 cells/10-cm dish) and HCD (6 × 10^5 cells/10-cm dish) were monitored by phase-contrast microscopy after 1 day in culture. B, equal amounts of whole cell lysates from LCD or HCD cultures were subjected to Western blot analysis with the indicated antibodies to correlate Stat3 Tyr705 phosphorylation with cell density. C, equal amounts of nuclear extracts from LCD or HCD cultures were subjected to DAPA (upper panel) and Western blot analysis by using indicated antibodies (lower panel) to correlate cell density with Stat3-DNA binding and the level of Tyr705 phosphorylation of nuclear Stat3, respectively. Nuclear extract from NIH 3T3 cells stimulated with IL-6 was used as the positive control (PC) for DAPA. Bead only is a negative control without the m67 probe. The Ki-67 and PCNA are cell proliferation markers. A nuclear matrix protein Lamin A was used as an internal control. D, pm67 (Stat3 reporter, firefly luciferase) or pTATA TK-luc plasmid was cotransfected with pRL-TK into MDCK cells. After 12 h of transfection, cells were cultured at LCD (5 × 10^4 cells per well of 6-well plate) or HCD (5 × 10^5 cells per well of 6-well plate) for another one day. The firefly luciferase activity was normalized to Renilla luciferase activity as a transfection control. The Stat3 reporter pm67 contains 4 repeats of the Stat3 binding site (TTACGGGAA). The resulting values were then normalized to those of pTATA TK-luc vector without any insert. Each bar represents the mean ± S.E. from three independent experiments in triplicate. ***, p < 0.001; Student’s t test. E, MDCK cells were cultured under low or high density for 24 h and treated with conditioned media (CM) collected at low density (L) or high density (H) for an additional 8 h. Equal amounts of cell lysates harvested under each condition were subjected to Western blot analysis using Stat3 Tyr705(P) or Stat3 antibody. β-Actin was used as an internal control.

**Statistics**—All data were expressed as means ± S.E. Differences between groups were determined by Student’s t test. Multigroup comparisons were determined by one-way analysis of variance. Differences in the comparison were considered to be significant when p values were less than 0.05.

**RESULTS**

Cell Confluence Induces Stat3 Tyr705 Phosphorylation and DNA Binding, Which Is Correlated with Dome Formation in MDCK Cells—MDCK cells were monitored daily to establish the sequence of events from seeding to reaching confluence and to dome formation (Fig. 1A). They formed initial cell islets and proliferated to reach confluence with few highly cell-condensed clusters occasionally present at day 4. These cells organized into small blister-like structures, which represented early stage of dome formation at day 5 and progressed to reach relatively larger dimensions at day 6. These domes continued to bulge with decreased adherence to plate and kept developing for an additional week associated with floating cells and apoptotic bodies (data not shown). Cell growth reached a plateau on day 4, and the cell number was sustained as 100% confluent on day 5–6 based on a standard growth curve (data not shown). These results indicated that the initial stages of dome formation with inhibited cell proliferation were captured within the selected time frame.

Possible phosphorylation events of Stat1, Stat3, or Stat5 during cell culture at different stages were investigated (Fig. 1B). The level of Stat3 Tyr705(P) increased to 3-fold of the control at day 3 and continued to rise until day 5. This correlated with formation of large patches of cells in confluence at day 3, highly condensed cell clusters at day 4, and early dome formation at day 5. A 33% drop of Stat3 Tyr705(P) was observed at day 6. The Stat3 Ser727(P) was maintained at low levels and increased slightly from day 5 to day 6. Because Stat3 expression was constitutively maintained from day 0 to day 6 (Stat1 and Stat5 not shown), phosphorylation of Stat3 at Tyr705 rather than at Ser727 showed a better time correlation with confluence-induced dome formation. There was no apparent correlation of Stat1 Tyr705(P) or Stat5 Tyr693(P) with the described sequence of events, except for increased phosphorylation of Stat1 at Tyr705 prior to reaching confluence (Fig. 1B).

We next evaluated whether increased phosphorylation of Stat3 at Tyr705 during dome formation resulted in augmented Stat3 DNA binding by DAPA. Nuclear fractions of cells at different stages were reacted with Stat3-specific binding oligomer m67, and precipitated proteins were profiled by Western blotting. Stat3 but not Stat1 was detected in the precipitate (Fig. 1C, upper panel). The specificity and validity of the DAPA were reflected by the detection of Stat1 and Stat3 in a heterologous positive control. Importantly, the same time course pattern was observed for Stat3 DNA binding and Tyr705 phosphorylation of nuclear Stat3 (Fig. 1C, upper and lower panel, respectively), corresponding with decreased cell proliferation markers, such as Ki-67 and PCNA (Fig. 1C, lower panel), and the initiation of dome formation at days 4 ̼ 5. Cell confluence enhanced Stat3-DNA binding to 3.5-fold and the nuclear Stat3 Tyr705(P) levels to 5-fold on day 5 compared with those on day 1. From day 5 to day 6, there was 60% reduction of Stat3-DNA binding and 70% reduction of the nuclear Stat3 Tyr705(P) levels. These results suggest that Stat3 Tyr705(P) contributes to the active nuclear pool of Stat3 and may have possible downstream signaling consequences reflected in a distinctive differentiation features during cell confluence and early stages of dome formation.
Cell Density Triggers Stat3 Transcriptional Activation in MDCK Cells—Whether cell density is a factor independent of cell proliferation and capable of activating Stat3 for DNA binding was investigated by seeding cells at low cell density (LCD) or high cell density (HCD). After culture for 1 day, cells at LCD formed islets, while cells at HCD were more confluent (Fig. 2A). Stat3 Tyr705(P), but not Stat3 Ser727(P) or total Stat3, in cells cultured at HCD was markedly higher than that in LCD (Fig. 2B), indicating a density effect on Stat3 Tyr705(P) phosphorylation. Increased Tyr705 phosphorylation correlated also with the higher nuclear Stat3 DNA binding in HCD, as determined by DNA affinity precipitation assay (Fig. 2C, upper panel). Because levels of Ki-67 and PCNA proteins were similar in both HCD and LCD cultures, we concluded that the elevation of nuclear Stat3 Tyr705(P) was independent of cell proliferation (Fig. 2C, lower panel). We also found that Stat3 transcriptional activity in HCD was about 10-fold higher than that in LCD as assessed by Stat3 reporter gene assay (Fig. 2D). The induction level of Stat3 reporter activity was markedly higher than the Stat3-DNA binding effect in HCD, because the Stat3 reporter, pm67, contains 4-time repeats of Stat3 binding site, whereas m67 used in DAPA assay contains no repeat of Stat3 binding site. These results strongly suggest that cell density triggers phosphorylation of Stat3 at Tyr705, which corresponds with the nuclear translocation of Stat3 to increase DNA binding affinity and transcriptional activity. We considered that high cell density-triggered Stat3 activation might be mediated by stimulatory soluble factors derived from dense culture. Therefore, conditioned medium collected from high density cells were applied to low density cells and vice versa for 8 h. As shown in Fig. 2E, the level of Stat3 Tyr705(P) in low density cells was not affected by conditioned medium, ruling out the possibility that cell density induced Stat3 Tyr705(P) is mediated by soluble factors.

Stat3 Tyr705(P) Is Localized in the Nuclei of MDCK Cells at the Initial Stages of Dome Formation—We examined the physical localization of Stat3 Tyr705(P) in spares, confluent, and post-confluent MDCK cells by confocal microscopy (Fig. 3). On days 3 and 4, Stat3 Tyr705(P) was co-localized with Hoechst-positive nuclei in cell clusters at relatively condensed area of confluent patches. Once the dome was formed on day 5, most of the dome-forming cells exhibited Stat3 Tyr705(P) in the nuclei, whereas the surrounding adherent cells exhibited mostly cytosolic Stat3 Tyr705(P) pattern. On day 6, most of the Stat3 Tyr705(P) was predominantly localized at the cytoplasm, not overlapping with Hoechst-stained nuclei in dome forming and surrounding adherent cells. This observation confirmed that the reduction of Stat3 activity on day 6 correlated with depletion of nuclear Stat3 Tyr705(P). These results suggest the correlation of Stat3 activation with cell density at the initial stages of dome formation.

Constitutive Expression of Dominant Active Stat3 (Stat3-C) Promotes Dome Formation—To examine whether Stat3 indeed promotes dome formation in MDCK cells, we established stably transfected MDCK cells with tetracycline inducible (Tet-On) expression of FLAG-tagged Stat3-C, a constitutively active form of Stat3 (30). Upon Stat3-C induction by a tetracycline analogue doxycycline for 5 days, no apparent changes with respect to the morphology or growth characteristics were found in any of the selected clones (data not shown). Doxycycline induction of Stat3-C expression in the selected stable transfectants (St3C-3 and St3C-4) was dose-dependent in contrast to the control mock-transfected cells expressing rTAT
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A.

B.

C.

D.

E.

F.

FIGURE 4. Stat3 activation regulates dome formation in MDCK cells. A, doxycycline induced dose-dependent Stat3-C expression in two tetracycline inducible MDCK clones (St3C-3 and St3C-4 cells). MDCK transfectants expressing either rtTA alone (mock) or the rtTA-controlled FLAG-tagged Stat3-C (St3C-3 and St3C-4) was treated with doxycycline (0, 1, 10 μg/ml) for 5 days. Cell lysates were subjected to Western blot analysis with antibodies against FLAG, Stat3, or β-actin. B, dome formation in mock, St3C-3, and St3C-4 cells in response to doxycycline treatment for 5 days visualized by phase-contrast microscopy. The number (C) and the diameter (D) of domes on day 5 were quantified by averaging from at least 20 independent fields (>100) in three independent experiments. E, selected MDCK clones expressing dominant negative Stat3 mutants (Stat3-F: Stat3-Y705F mutant; Stat3-D: DNA binding domain mutant) were cultured for 5 days, and their levels of Stat3 expression and dome formation were characterized for Stat3 expression and Tyr705 phosphorylation (Fig. 4E). Compared with the control cell line, the 5th day cultures of stable Stat3-F transfectants (clones 11 and 12) exhibited elevated cellular Stat3 expression but reduced Stat3 Tyr705(P) (Fig. 4E, left panel), while the stable Stat3-D transfectants (clones 2, 3, 5, and 10), exhibited elevated levels of Stat3 and Stat3 Tyr705(P) (Fig. 4E, right panel). These data suggest that overexpression of Stat3-F partially reduced the overall Stat3 Tyr705 phosphorylation, whereas overexpression of Stat3-D did not. However, overexpression of Stat3-D inhibited Stat3 transcriptional activity in MDCK cells as assessed by Stat3 reporter gene assay (data not shown). Quantitative estimation of dome formation in the corresponding 5th day cell cultures reflected the negative effects of Stat3-F and Stat3-D expression on the number of domes (Fig. 4F). Both Stat3F-11 and Stat3F-12 clones exhibited fewer domes (~18/field and ~11/field respectively) than Stat3-C expression in St3C-4 cells. This indicated that constitutively active Stat3 promoted dome formation in terms of their number and size in an expression level-dependent fashion (Fig. 4, A, C, and D). Interestingly, expression of Stat3-C did not cause accelerated dome formation before day 5. Therefore, it is unlikely that Stat3-C promoted dome formation through increasing cell proliferation and cell density. In support of this hypothesis, equivalent numbers of live cells were counted during cell confluence and dome formation in mock-transfected and Stat3-C-expressing MDCK cells (data not shown). This observation reinforces the notion that Stat3-C-mediated augmentation of dome formation (both their number and size) is cell confluence-dependent and that it reflects a distinct Stat3-mediated mechanism involved in promoting the extent of initial dome formation.

Constitutive Expression of Dominant Negative Stat3 Mutants Suppresses Dome Formation—We examined whether the expression of dominant negative Stat3 could offset the dome formation in MDCK cells. Clonal lines of MDCK cells stably transfected with Stat3-F (a tyrosine 705 mutant) or HA-tagged Stat3-D (a DNA binding domain mutant) were selected and characterized for Stat3 expression and Tyr705 phosphorylation (Fig. 4E). Compared with the control cell line, the 5th day cultures of stable Stat3-F transfectants (clones 11 and 12) exhibited elevated cellular Stat3 expression but reduced Stat3 Tyr705(P) (Fig. 4E, left panel), while the stable Stat3-D transfectants (clones 2, 3, 5, and 10), exhibited elevated levels of Stat3 and Stat3 Tyr705(P) (Fig. 4E, right panel). These data suggest that overexpression of Stat3-F partially reduced the overall Stat3 Tyr705 phosphorylation, whereas overexpression of Stat3-D did not. However, overexpression of Stat3-D inhibited Stat3 transcriptional activity in MDCK cells as assessed by Stat3 reporter gene assay (data not shown). Quantitative estimation of dome formation in the corresponding 5th day cell cultures reflected the negative effects of Stat3-F and Stat3-D expression on the number of domes (Fig. 4F). Both Stat3F-11 and Stat3F-12 clones exhibited fewer domes (~18/field and ~11/field respectively) than
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the Neo control (~28/field), whereas all of the Stat3-D stably transfectant cells showed dramatically suppressed dome formation in comparison with the control MDCK cells (Fig. 4F). Although Stat3D 2 and 5 cells exhibited residual domes, there was no difference in dome formation among these four Stat3-D transfectants. While the reason for the smaller effect of Stat3-F on dome formation is not immediately clear, it appears that inhibition of Stat3 DNA binding by overexpression of Stat3-D is more potent in masking the effects of endogenous Stat3. Regardless, these results show clearly that inhibition of Stat3 by overexpression of either Stat3-F or Stat3-D negatively affected dome formation, implicating the pivotal role of activated Stat3 in this process.

Cell Confluence Augments Expression of NHE3 through Stat3 Activation in MDCK Cells—One of the requirements for successful dome formation in epithelium is the activation of sodium transport from apical to basolateral site (2, 4, 8, 37). Our results point to the sodium transporters, NHE3 and ENaC α-subunit, induced by cell confluence, as the most likely candidates involved in dome formation. As shown in Fig. 5, the protein expression levels of NHE3 and ENaC α-subunit increased from day 1 to day 5 following confluence and then decreased after day 6, which bears similarity to the Stat3 Tyr705 phosphorylation pattern, as shown in Fig. 1B. Of other sodium transporters, Na+,K+-ATPase α1 was slightly increased from day 0 to day 2 but decreased afterward, while Na+,K+-ATPase β1 and NHE1 were stably maintained during the 6-day period (Fig. 5A).

Although the above data suggest that both the NHE3 and ENaC α-subunit are the major candidates augmented in parallel with Stat3 activation, only NHE3 expression was specifically promoted by Stat3-C. As shown in Fig. 5B, NHE3 expression, in contrast to that of NHE1 or ENaC α-subunit, was augmented by doxycycline in a dose-dependent manner in St3C-3 cells but not in the mock-transfected control cells. Comparison of the NHE3 expression levels between Stat3-D transfectants (clones 2, 3, 5, and 10) and the control MDCK cells by Western blot showed substantial negative effect of Stat3-D in reducing the level of NHE3 but not NHE1 (Fig. 5C). Because Stat3 mutants affected the expression of NHE3 in dense culture, we considered whether a density factor was required for Stat3-mediated NHE3 expression. MDCK cells under low or high density were treated with Stat3-activating cytokine, oncostatin M (OSM), for 1 day. As shown in Fig. 5D, the expression level of NHE3 in dense culture was markedly higher than in sparse culture with the similar induction level of Stat3 Tyr705(P). However, OSM only mildly enhanced NHE3 expression under high cell density conditions. These results indicate that activation of Stat3-triggered NHE3 expression depends on cell density.

To test whether Stat3 augmented NHE3 through up-regulation of NHE3 mRNA level, semi-quantitative RT-PCR was performed to detect the mRNA level of NHE3 in St3C-3 (Fig. 5E) and Stat3-D cells (Fig. 5F). Induction of Stat3-C mRNA by doxycycline was associated with increased NHE3 mRNA level in St3C-3 cells but not in the control mock-transfected cells (Fig. 5E). Conversely, the expression of exogenous Stat3-D mRNA was associated with reduced NHE3 mRNA level in St3D-3 cells in comparison with MDCK control cells (Fig. 5F). Collectively, these results strongly suggest the effect of Stat3 in promoting NHE3 protein and mRNA expression, likely mediated through a transcriptional mechanism.

Stat3 Regulates NHE3 Promoter Activity in MDCK Cells—To address the potential transcriptional mechanism of Stat3-induced NHE3 expression, we utilized Stat3-C inducible (St3C-3) and non-inducible mock cells transiently co-transfected with a firefly luciferase reporter plasmid (pCMV-Luc; transfection efficiency control) in combination with Renilla luciferase plasmid.
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These results indicate that Stat3-C up-regulates NHE3 mRNA expression through a transcriptional mechanism.

Cell Confluence-induced Stat3 Activation Regulates NHE3 Expression and Dome Formation in NMuMG Cells—To examine whether cell confluence induced Stat3 Tyr705 phosphorylation and NHE3 expression applies to other dome-forming epithelial cells, we employed normal mouse mammary epithelial NMuMG cells. As shown in Fig. 7A, cell confluence-induced activation of Stat3 and augmented expression of NHE3 in NMuMG cells were similar to those observed in MDCK cells although the time frame from seeding to dome formation extended up to 8 days. Cell confluence induced an early onset of Stat5 Tyr694(P) with no further increase at day 6. Stat1 phosphorylation at Tyr701 was induced late (day 6) and did not appear to correlate with maximal induction of NHE3 expression observed already on day 4 (Fig. 7A). We also observed that Stat3 Tyr705(P) level was decreased on the 8th day. These results suggest similar pattern of Stat3 Tyr705(P) activation and NHE3 augmentation during dome formation in NMuMG and MDCK cells. Furthermore, cells seeded at HCD displayed higher level of Stat3 Tyr705(P) (1.5–2-fold) and Stat3 transcriptional activity (6-fold) than cells seeded at LCD (Fig. 7B and C). In addition, NHE3 expression during dome formation in NMuMG cells was also regulated by Stat3, as evidenced by lowered NHE3 protein (clones 2, 7, and 18; Fig. 7D) as well as mRNA levels (clones 7 and 18, Fig. 7E) in NMuMG cells stably transfected with dominant negative Stat3-D. Moreover, dome formation was significantly reduced in all Stat3-D transfectants compared with control NMuMG cells (Fig. 7F). These results again support the notion that cell density-dependent transcriptional activation of Stat3 and NHE3 expression are the key factors driving dome formation.

NHE3 Is Required for Epithelial Dome Formation—To further examine whether NHE3 augmentation was indeed required for dome formation and did not simply coincide with the process, RNA interference technique was employed to specifically knock-down NHE3 expression. Individual colonies of NMuMG cells stably transfected with shRNA of NHE3 (shNHE3) were selected and characterized for NHE3 expression. Compared to control cell lines (NMuMG with or without pSM2 vector), the 6th day cultures of stable shNHE3 transfectants (clones 4 and 10) exhibited lower levels of NHE3 and reduced dome formation (Fig. 8A). Quantitative estimation of dome formation in the corresponding 6th day cell cultures revealed the dramatic effects of NHE3 knockdown. The number of domes observed per microscopic field (×100) was decreased from ~48/field in NMuMG control cells or ~46/field in pSM2-transfected control cells to ~5/field and ~1/field in shNHE3–4 and shNHE3–10 clones, respectively (Fig. 8A). These results strongly suggest the role of NHE3 in regulating the initiation stage of dome formation.

The role of NHE3 in dome formation was further examined using NHE3 inhibitors, EIPA (specific NHE family inhibitor), and S3226 (specific NHE3 inhibitor). Dome formation was assessed in confluent MDCK or NMuMG cells treated with Me2SO (vehicle control) or with a respective inhibitor. As shown in Fig. 8B, after 24 h of treatment with increasing concentrations of EIPA or S3226, the number of domes in both
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**FIGURE 7.** Cell confluence-induced Stat3 activation regulates NHE3 expression and dome formation in NMuMG cells. A, whole cell lysates of NMuMG collected every 2 days were subjected to Western blot analysis with the indicated antibodies. β-Actin was used as an internal control. B, cell lysates collected from LCD (seeding at 1 × 10⁶ cells/10-cm dish) or HCD (seeding at 6 × 10⁶ cells/10-cm dish) of NMuMG cells cultured for 24 h were subjected to Western blot analysis with the indicated antibodies. C, pm67 (Stat3 reporter, firefly luciferase) or pTATA TK-luc plasmid was cotransfected with pRL-TK (Renilla luciferase) into NMuMG cells. After 12 h of transfection, cells were cultured at LCD (5 × 10⁵ cells per well of the 6-well plate) or HCD (5 × 10⁵ cells per well of the 6-well plate) for another one day. The firefly luciferase activity was normalized to Renilla luciferase activity as a transfection control. The resulting values were then normalized to those of pTATA TK-luc vector without any insert. Each bar represents the mean ± S.E. from three independent experiments in triplicate. D, cell lysates from NMuMG cells expressing Stat3-D with HA tag were collected on day 6 and were analyzed by Western blot with the indicated antibodies. E shows NHE3, Stat3, and GAPDH mRNA levels in wild-type and two individual Stat3-D-transfected NMuMG cells (S3226 and S3226-18), respectively, as assessed by RT-PCR analysis using primers specific to mouse NHE3, Stat3, or GAPDH. The results indicate that dominant negative Stat3 inhibits the NHE3 mRNA level. F, dome formation was assessed in wild-type and Stat3-D-transfected NMuMG cells corresponding to D by counting domes under at least 20 non-overlapping (×100) fields in three independent experiments. Values are plotted as means ± S.E. from at least three independent studies. ***, p < 0.001; Student's t test.

MDCK (upper panel) and NMuMG (lower panel) cells was decreased substantially in a concentration-dependent manner. EIPA appeared more effective in reducing the number of formed domes than S3226. This may indicate the involvement of other members from the NHE family in the process of dome formation, but may also reflect differences in stability of EIPA and S3226, thus requiring higher concentrations of the latter inhibitor to remain effective throughout the 24-h experiment. The observed increase in dome formation after Me₂SO treatment was not unexpected, because Me₂SO is a known differentiation inducer that stimulates dome formation (38).

**DISCUSSION**

Activation of Stat3 during cell confluence has been shown to play important physiological roles in cell growth arrest and survival (27, 28, 39). This has led us to speculate that Stat3 signaling events in cell differentiation may be functionally and biologically relevant to dome formation in epithelial cells. This study provides support for a distinct Stat3/NHE3 signaling pathway during cell confluence to coordinate epithelial dome formation. The present line of evidence includes the following: 1) cell confluence induces Stat3 signaling through Tyr⁷⁰⁵ phosphorylation, nuclear translocation, and specific DNA binding; 2) translocation of Stat3 Tyr⁷⁰⁵(P) into the nucleus is associated with an increase in cell density and up-regulation of NHE3 promoter activity during the initial but not the latter stage of dome formation; 3) the extent of dome formation during the initiation stage is governed by Stat3 downstream effectors, which most likely involves NHE3; and 4) inhibition of NHE3 diminishes confluence-induced dome formation.

Our results demonstrate a distinctive role of Stat3 activation in the promotion of cell density-dependent epithelial differentiation. The specific nuclear translocation of Stat3 Tyr⁷⁰⁵(P) in the high density area and early dome structures (Fig. 3) and the effect of exogenous Stat3 downstream effectors, which most likely involve NHE3, provide solid evidence for such a unique biological consequence. Although Stat3 in most instances promotes cell proliferation or transformation (30, 40), it does not affect proliferation in MDCK cells (data not shown), ruling out the possibility that Stat3 regulates dome formation through promoting cellular proliferation. This is further supported by the documented decreased cell proliferation markers following cell confluence and by the observation of cell proliferation-independent elevation of nuclear Stat3 Tyr⁷⁰⁵(P) in HCD culture during confluence. In addition, nuclear staining of the 9th day confluent monolayer with Hoechst 33342 showed few cell divisions located only at the low cell density zone of condensation area. It has been shown that cell proliferation of normal epithelial cells is inhibited by cell contact and cell differentiation, such as dome formation, which is triggered by cell confluence. This study strongly indicates that the local nuclear accumulation of Stat3 Tyr⁷⁰⁵(P) in a confluent epithelial monolayer mediates the cell density effect to drive dome formation.

Corresponding with the cell density-induced Stat3 activation, dome formation in MDCK (or NMuMG) epithelial cells was correlated with the augmentation of NHE3 through Stat3-mediated transcriptional regulation. Early induction of NHE3 by Stat3 did not result in dome formation before cell confluence. Instead, the expression of NHE3 reached the highest level during the initial but not the later stage of dome formation and declined with decreased Stat3 activity soon after dome formation. It has been demonstrated that localization of NHE3 to the apical site in morphologically polarized epithelial cells is critical for the execution of its function (20, 22, 41). Inhibition of NHE3 expres-

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3 M. J. Tang, unpublished data.
We have found that MDCK cells exhibit markedly enhanced apoptosis at post-confluent stage on day 6. In this study, we show that Stat3 phosphorylation and activity are down-regulated at the same time, suggesting the role of Stat3 activation in cell survival. On the other hand, despite the fact that dominant negative Stat3-D reduces dome formation, it neither affects cell proliferation, nor enhances apoptosis. This may simply suggest that Stat3 activation-induced cell survival only occurs in the dome forming cells.

Stat3-mediated modulation of the cell differentiation process has also been described as a ligand-dependent phenomenon by accepting signals from specific cytokines or growth factors (24, 32, 43). For instance, Stat3 responds to oncostatin M to induce glial fibrillary acidic protein (GFAP) expression and differentiation of the astrocyte (44). However, Stat3 activation observed in our systems and as reported by others (28) are distinct from those ligand-dependent differentiation mechanisms, as disruption of cell-to-cell contact in confluent cells by calcium chelators (e.g. EDTA) reduced Stat3 activity while serum starvation could not depress cell density-induced Stat3 Tyr705 phosphorylation in MDCK cells (data not shown). In addition, CM of high density culture could not induce Stat3 Tyr705(P) in sparse cells, indicating the extracellular stimulatory factors are not the major cause for cell density-dependent Stat3 activation. However, we still cannot rule out the intracellular activating factors for induction of Stat3 Tyr705(P). These results corroborate with two previous reports which showed that neither serum starvation nor disruption of growth factor (e.g. EGF or IGF-1) or Ras-mediated signaling, influences cell density-induced Stat3 activation in fibroblast, normal breast epithelial, or breast carcinoma cell lines (27, 28). It is possible that the signaling pathway of the confluence-mediated Stat3 activation may prevalently exist in various cell types and is critical to cell contact-mediated growth inhibition or differentiation (28).

The study reported here demonstrates a novel role of Stat3 in up-regulation of NHE3 expression. To our knowledge, there has been virtually no information regarding to Stat3 regulation of NHE3. Because we did not find a classical STAT responsive sequence in the rat NHE3 promoter (~450/+58 nt) by using TFSeriorsearch (45). It is plausible that the Stat3-induced NHE3 expression during cell confluence is indirectly mediated by other Stat3-dependent transcription factors. The potential transcriptional responsive sequences such as AP-2, Sp-1, and NF-1 (46) in the rat NHE3 promoter (~450/+58 nt) as

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**FIGURE 8. NHE3 is required for epithelial dome formation.** A, NHE3 knock-down by shNHE3 inhibits dome formation. Dome formation in control NMuMG cells, pSM2- or shNHE3-transfected (4 and 10) cells was monitored by phase-contrast microscopy (×100) at day 6 in culture. Cell lysates were subjected to Western blot with the indicated antibodies. NHE3 shRNA reduced NHE3 levels in numbers 4 and 10 by 40 and 60%, respectively. Dome formation was quantitatively estimated by counting the number of domes under at least 20 independent (×100) fields. NHE3 shRNA significantly blocked dome formation in NMuMG cells in both selected clones. B, NHE3 inhibitors block dome formation in MDCK and NMuMG cells. Confluent MDCK (upper panel) or NMuMG (lower panel) cells were treated with increasing concentrations of indicated inhibitors, EIPA (1, 10, 10² nM in MeSO₄ (DMSO), specific for NHE family) and S3226 (10, 10², 10³ nM in MeSO₄ (DMSO), specific for NHE3). Cells treated with MeSO₄ (DMSO) were used as controls. After 24 h of incubation, dome formation was estimated according to the above method. Values are plotted as means ± S.E. from at least three independent experiments. A: ***p < 0.001; Student’s t test. B: p < 0.01, analysis of variance, n = 60.
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described before are important for NHE3 promoter regulation. Whether these potential responsive elements are required for Stat3-mediated NHE3 promoter regulation remains to be examined. On the other hand, the Stat3 effect on the regulation of NHE3 was observed only in HCD, suggesting Stat3 might cooperate with cell density-regulated factors on the regulation of NHE3 expression.

The molecular mechanisms of epithelial cell differentiation are of great physiological and pathophysiological importance, with cell-cell adhesion being the primary step in association with subsequent formation of tight junction, cell polarity, and microvilli (3, 47). Dome formation in confluent cells is a useful model to study epithelial differentiation. Our findings unveil a novel molecular mechanism whereby cell confluence-triggered Stat3 activation and consequent increase in NHE3-mediated transepithelial transport play critical roles in this process. We postulate that this novel Stat3/NHE3 signaling pathway induced by cell confluence, which is distinct from ligand-dependent stimulation, is accountable for modulating dome formation and epithelial differentiation. Many important questions remain to be investigated, including (1) the upstream triggering mechanisms mediating cell density-induced Stat3 activation, and (2) the detailed regulatory mechanism of NHE3 promoter by Stat3. Because dome formation mimics the terminal differentiation of renal epithelium during epithelial cell remodeling in vivo (48–50), our findings further unravel the mechanisms underlying this phenomenon to include the novel Stat3/NHE3 signaling pathways in epithelial cell differentiation.

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