Src kinase pathway is involved in NFAT5-mediated S100A4 induction by hyperosmotic stress in colon cancer cells

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Chen M, Sastry SK, O'Connor KL. Src kinase pathway is involved in NFAT5-mediated S100A4 induction by hyperosmotic stress in colon cancer cells. *Am J Physiol Cell Physiol* 300: C1155–C1163, 2011. First published February 2, 2011; doi:10.1152/ajpcell.00407.2010.—S100A4 is associated with the progression of many types of cancers as well as several nonmalignant conditions. However, how it is regulated by intracellular signaling and/or at the transcriptional level has not been extensively studied. We recently demonstrated that S100A4 is partially regulated by nuclear factor in activated T cell 5 (NFAT5) downstream of integrin $\alpha 6\beta 4$. NFAT5 is a mammalian osmotic regulator. To study the regulation of S100A4 by NFAT5 in a more readily inducible model, colon cancer cells were subjected to hyperosmotic stress. We found that S100A4 is induced in a subset of colon cancer cell lines, and the ability to induce S100A4 depends on the methylation status of S100A4. The osmotic stress response elements were identified in the first intron region of S100A4 by S100A4 luciferase reporter assays. Depletion of NFAT5 by small interfering RNA abolished S100A4 induction. Furthermore, chromatin immunoprecipitation assays showed that NFAT5 is induced to bind to the first intron region. Inhibition of Src kinase pathways reduced S100A4 induction by affecting NFAT5 transactivation and protein levels. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to study the function of S100A4 induction in colon cancer cells under the condition of hyperosmotic stress; the results suggest that S100A4 induction contributes to cell survival. In conclusion, this study demonstrates that hyperosmotic stress induces S100A4 through NFAT5, and Src and chromatin remodeling are involved. In addition, the induction of S100A4 contributes to cell survival. Given that the gastrointestinal tract is periodically exposed to hyperosmotic stress, this study may uncover a novel signaling pathway that could contribute to GI cancer progression.

metastasin; TonEBP; osmotic stress; osmotic stress response element

S100A4, also known as metastasin-1 (mts1), CAPL, and fibroblast-specific protein, is a calcium binding EF [helix(E)-loop-helix(F)]-hand protein belonging to the S100 superfamily. S100A4 is expressed in highly motile cell types, including T-lymphocytes, neutrophils, macrophages, platelets, endothelial cells, fibroblast, and tumor cells. Notably, cell motility has been implicated as a major function controlled by S100A4 (15). Increasing evidence demonstrates that S100A4 is associated with the progression of a variety of cancers, including breast, prostate, pancreatic, gallbladder, colon, gastric, and thyroid (7, 15, 31, 32, 34, 37, 38, 44). The documented functions of S100A4 fit into several hallmarks of cancer such as anti-apoptosis (survival), metastasis (motility and invasion), proliferation, angiogenesis, and inflammation (42). However,

S100A4 also contributes to nonmalignant conditions, such as rheumatoid arthritis and disorders of cardiovascular, nervous, and pulmonary systems (14).

Despite the instrumental role of S100A4 in a variety of normal and pathological conditions, investigations on the regulation of this molecule at the transcriptional level are limited. One important mechanism of regulation of S100A4 is through the methylation status of its promoter. Specifically, studies show that CpG sites in the first intron region of S100A4 are hypomethylated in several cancers but are hypermethylated in normal or nonmetastatic cancer cells (33, 46). Currently, there is no mechanistic evidence to show how S100A4 promoter is demethylated, although our recent study showed that integrin $\alpha 6\beta 4$ contributes to this regulation (6). Several other signaling pathways are involved in regulating S100A4, including ErbB2 (16) and β-catenin/T-cell factor (TCF) (43). However, in a recent study using a breast cancer model, the ubiquitously expressed nuclear factor in activated T cell 5 (NFAT5) was shown to play an important role in the regulation of S100A4 (6).

Hyperosmotic stress can also activate NFAT5 (2); therefore, NFAT5 is also known as tonicity responsive enhancer binding protein (TonEBP). In response to hyperosmotic stress, NFAT5 is activated at multiple levels, such as protein abundance, nuclear translocation, and transactivation. Activated NFAT5 then binds to its cognate response DNA element and leads to transcriptional regulation of the osmocompensatory genes, such as aldose reductase (AR), sodium/*myo*-inositol cotransporter (SMIT), urea transporter (UT-A), and heat shock protein 70, as well as several proinflammatory cytokine genes, such tumor necrosis factor-α (TNF-α), which is unrelated to osmoregulation (4, 29).

The role of NFAT5 in tissues has not been studied extensively except in the immune system and kidney; however, there is evidence that its role as an osmolarity sensor in other systems may warrant further investigation. Ho et al. (13) showed that both lymphoid and liver microenvironments have higher osmolarity compared with serum. Gastrointestinal epithelia are exposed to elevated postprandial osmolarity (17, 19, 25). Finally, several studies have shown that osmotic stress can induce proinflammatory cytokine production, Cox-2, and drug metabolizing enzymes, such as cytochrome *P*-450 3As expression in gastrointestinal (GI) cancer cells, thus suggesting that osmotic stress may have physiological relevance to GI function (3, 29).

Based on the regulation of S100A4 by NFAT5 (6), and given that the colon is periodically exposed to osmotic stress (28), we propose that hyperosmotic stress induces NFAT5-mediated S100A4 expression in colon cancer cells. Here, we provide the evidence that hyperosmotic stress induces S100A4 expression in a subset of colon cancer cells with hypermethy-

lated first intron region. We further identify that the osmotic response elements (ORE) of S100A4 are located in the first intron region, and one NFAT5 binding site is essential for S100A4 transcriptional regulation by hyperosmotic stress. Furthermore, we find that the Src kinase pathway is involved in this NFAT5-mediated regulation of S100A4. We also demonstrate that the induction of S100A4 plays an important role in cell survival under the condition of osmotic stress.

MATERIALS AND METHODS

Colon cancer cell lines and osmotic stress. Clone A, LS174T, HCT-8, and DLD-1 cells were maintained in RPMI 1640 (osmolarity = 279 mosM); SW480 and SW620 in high-glucose DMEM (osmolarity = 309 mosM); HT-29 and HCT-116 in McCoy medium (osmolarity = 298 mosM); KM12C and KM20 in Eagle's minimum essential medium (osmolarity = 304 mosM) supplemented with 1 mM sodium pyruvate, nonessential amino acids, and MEM essential vitamin mixture; and Caco2 cells in MEM plus 1% nonessential amino acids. Media for all cells were supplemented with 10% FCS and 1% penicillin and streptomycin. For experiments, 70% confluent cells were subjected to hyperosmotic shock by adding excess 100 mM NaCl at indicated times. Hypoosmotic stress is induced by adding 1:1 dilution of medium with water. For cells returned to isotonic conditions, cells were treated with excess 100 mM NaCl for 24 h, cells were then rinsed three times with media, and then fresh isotonic media were added and cultured for the times noted. The expression of S100A4 was determined by Western blot analysis and Q-PCR.

Bisulfate sequencing and cell treatment. For DNA methylation studies, cells were treated with 1 μM 5-aza-2′-deoxycytidine (DAC) in fresh medium daily for 3 or 5 days before genomic DNA was extracted. Identification of methylated CpG residues within the DNA was determined by bisulfate conversion and pyrosequencing of the first intron region of the S100A4 promoter ($+203$ to $+662$; accession number Z33457) and was performed by EpigenDx (Worcester, MA).

Western blot analysis. Total cell lysates (80 μg protein) prepared in RIPA buffer (150 mm NaCl, 0.5 mm EGTA, 0.5% sodium deoxycholate, 1% SDS, 1% Triton X-100, 50 mm Tris·HCl, pH 7.4, 15 μg/ml protease inhibitor cocktail, and 1 mM PMSF) were separated by either 15% SDS-PAGE (for S100A4) or 7.5% SDS-PAGE (NFAT5), transferred to PVDF membrane, and probed with antibodies for S100A4 (gift from Dr. Anne Bresnick, Albert Einstein College of Medicine) and NFAT5 (Applied Bio Reagent). The blots were stripped and reprobed with β-actin (Sigma) as the loading control.

Q-PCR. cDNA was prepared from total RNA (1 μg, extracted from cells using TRIzol reagent), using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Expression of target genes was then assessed by comparative C_t ($\Delta\Delta C_t$) using commercially available probes and master mix reagent and performed on a StepOnePlus 96-well instrument as described by the manufacturer (Applied Biosystems). The expression level of each gene was normalized by 18S RNA and reported as a relative level to a specified control.

Luciferase reporter constructs and luciferase assay. NFAT5 luciferase reporter was obtained from Addgene and contained three consensus NFAT5 sites from a promoter region of human aldose reductase gene that was cloned into a pGL3 luciferase reporter with minimal SV40 promoter (29). S100A4 luciferase reporters were generated by amplifying fragments of the S100A4 regulation region by PCR and then cloning them into the *Kpn*I and *Bgl*II site of the pGL4.10[luc2] basic vector (Promega), which lacks a basic promoter. Genomic DNA was purified by GenElute Mammalian Genomic DNA Miniprep kit (Sigma). From the S100A4 genomic DNA sequence (accession number Z33457), the following primers were used to generate S100A4 reporter pGl4.10 S100A4 ($-632/+1010$) and pGl4.10 S100A4 ($-632/+77$): sense 5′-GGGG-TACCACAAGGTCCTCTGTGTTGCTC-3′, anti-sense 5′-GAA-GATCTGACAGCAGTCAGGATCTGGGA-3′, and anti-sense 5′-

GAAGATCTAGTCAGGCCAACACAACTCACC-3′. Primers used to generate pGl4.10 S100A4 ($+58/+1010$) were sense 5′-GGGGTAC-CGTGAGTTGTGTTGGCCTGACT-3′ and anti-sense 5′-GAA-GATCTGACAGCAGTCAGGATCTGGGA-3′. Mutants for putative NFAT5 binding site 4 and site 5 were generated using the pGl4.10 S100A4 ($+58/+1010$) construct using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Primers for mutating the NFAT5 site 4 were based on the sense strand sequence 5′-GCGGCTGTGCCTGGTTTCTC-CTACTGCAGGCCCC-3′ and those for NFAT5 site 5 were based on 5′-GTGGATAGACTGAGTGAGGGGCGAACAAAATGGTGTTGT-TGAGCAAG-3′. All of the constructs and their mutants were confirmed by sequencing.

For luciferase reporter assays, cells grown in 24-well plates were cotransfected with either 0.25 μg of the NFAT5 or a S100A4 reporter along with pRL-TK Renilla control reporter as 50:1 ratio for 24 h. Then cells were induced by 100 mM excess NaCl or left in isotonic medium for 24 h. Cells were collected, and luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega). The data are presented as a relative value to internal control or fold induction as individual control.

NFAT5 transactivation was performed by cotransfection of NFAT5 transactivation reporter GalDBDNFAT5AD (Dr. Maurice B. Burg, National Insititutes of Health) and Gal4 luciferase reporter pGL4.31 (Promega) together with pRL-TK Renilla control reporter. After 24 h, cells were pretreated with 10 μM 4-amino-5-(4-chlorophenyl)-7-
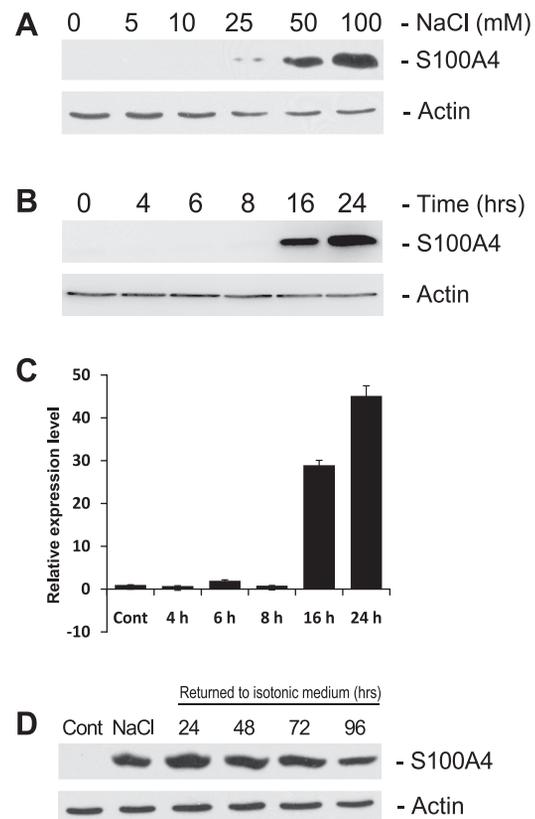

Fig. 1. S100A4 is induced in Clone A colon cancer cells by hyperosmotic stress. Clone A cells were treated with indicated amount of excess NaCl for 24 h (*A*) or 100 mM excess NaCl for different times (*B*), and then cells were harvested for S100A4 immunoblotting (*A*, *B*) or Q-PCR analyses (*C*). Clone A cells were placed under hyperosmotic shock with 100 mM excess NaCl for 24 h (*D*), and then plates were rinsed with PBS for 3 times before being returned to isotonic conditions for the indicated times. Samples were then analyzed for S100A4 protein expression. Blots were stripped and reprobed for actin as a loading control. All data are representative of at least three separate experiments.

(*t*-butyl)pyrazolo[3,4-d]pyramidine (PP2, a specific src family kinase inhibitor) or 4-amino-7-phenylpyrazolo[3,4-d]pyramidine (PP3, a PP2 negative control) for 30 min, and then cells were exposed to hyperosmotic stress for 24 h before luciferase activity assay.

Cell fractionation. Cells were pretreated with 10 μM PP2, PP3 (Calbiochem), or DMSO for 30 min followed by treatment with 100 mM excess NaCl at the indicated time points. Cell fractionation was performed by using NE-PER nuclear and cytoplasmic extraction reagents, as recommended by the manufacturer (Pierce).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were performed as modified from the protocol published previously (6). In brief, cells were cross-linked with 1% formaldehyde and terminated with 0.125 M glycine. Nuclei were isolated, sonicated to fragment DNA (average length of 500–700 bp), and centrifuged to pellet debris. Equal amounts of DNA from each sample were incubated with 1 μg anti-rabbit NFAT5 antibody or normal rabbit IgG and protein A/G-Sepharose beads (Amersham Biosciences) at 4°C overnight. Washed immunoprecipitates were digested with proteinase K, followed by a 65°C incubation to reverse the cross-linking. DNA was then precipitated with 0.5 μg/μl of glycogen overnight. The precipitated pellets were collected by microcentrifugation at top speed, resuspended in 20 μl Tris-EDTA (TE) buffer, and used as templates for PCR amplification by using the following primers: sense 5′-ATGGCCTCTGCAGCTTCTCTT-3′ and anti-sense 5′-TGCGCAAGTCTTGGAGATTCG-3′.

siRNA treatment. Small interfering RNA (siRNA) was electroporated into cells as described previously (6). In brief, suspended cells (3 × 10⁶) were electroporated (350 V with 500 μF capacity) with either SmartPool siRNA targeting S100A4 or nontargeting siRNA control or were left as electroporated only control. After siRNA treatment for 48 h, cells were subjected to different experiments. Cell lysates were used to detect the efficiency of siRNA treatment by immunoblotting.

MTT assay. Clone A cells (2 × 10³) electroporated with siRNA targeting S100A4 or nontargeting control were seeded in each well of a 96-well plate. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed in triplicate by adding 20 μl (MTT) (5 mg/ml) to each well and incubated at 37°C for 3 h. To dissolve the formazan precipitate, 100 μl of stop-mix solution containing 90% isopropanol and 10% DMSO were added. OD 570 was read and recorded every day for a 6-day period.

RESULTS

Hyperosmotic stress induces S100A4 expression in colon cancer cell lines. NFAT5 is an osmoregulator in mammalian cells (2) and regulates S100A4 expression in breast cells (6). Here, we sought to test whether S100A4 can be induced by hyperosmotic stress. For these experiments, Clone A colon carcinoma cells were treated with excess NaCl at differing dosages to stimulate hypertonic shock for 24 h or were treated with 100 mM NaCl at various times before harvest for analysis of S100A4 expression. As shown in Fig. 1, 25 mM hypertonic NaCl treatment for 24 h induced S100A4 expression with higher induction at 100 mM excess NaCl (Fig. 1A). Time-course analysis by immunoblotting and Q-PCR showed that S100A4 induction appeared at 16 h after treatment and is near maximal after treatment for 24 h (Fig. 1, *B* and *C*, and see online supplemental Fig. S1 at the *AJP-Cell Physiol* website). In contrast, the more permeable solute urea, which causes hyperosmolality without hypertonicity, did not induce S100A4 expression (data not shown). In addition, sorbitol-mediated hyperosmotic stress similarly stimulated S100A4 expression in Clone A cells (data not shown). These results demonstrate that S100A4 is an osmotic stress-responsive protein in Clone A colon cancer cells.

To test how long S100A4 induction is sustained, Clone A cells were exposed to hyperosmotic stress for 24 h before returning cells to isotonic medium. As shown in Fig. 1D, S100A4 induction was sustained up to 96 h when cells were returned to isotonic condition. These data indicate that S100A4 induction may involve epigenetic mechanisms or, alternatively, extension of S100A4 protein half-life.
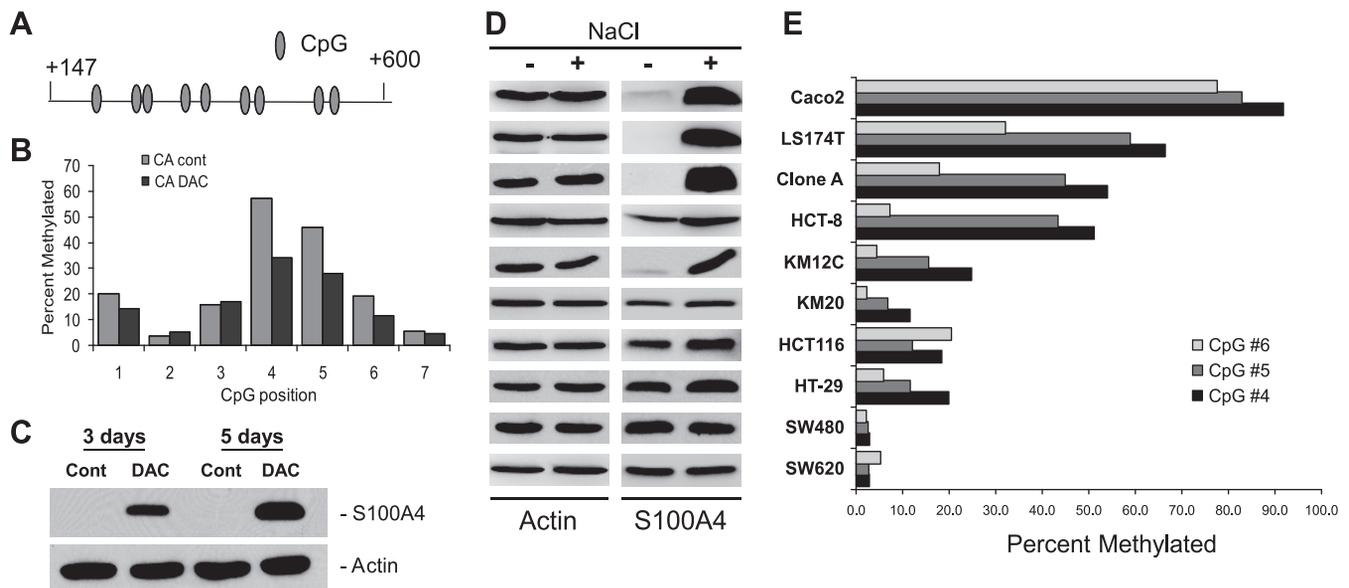

Fig. 2. S100A4 induction in colon cancer cells correlates with DNA methylation status of the first intron region of S100A4. *A*: depiction of CpG sites in the first intron region targeted for methylation status analysis. *B* and *C*: clone A cells were treated with 1 μM of the DNA methyltransferase inhibitor DAC or DMSO for 3 or 5 days and then assessed for S100A4 promoter methylation status (*B*; 5 days; EpigenDX) or S100A4 protein expression (*C*). *D*: several other Colon cancer cell lines were assessed for hyperosmotic shock-mediated S100A4 induction by treating cells with 100 mM excess NaCl for 24 h and then collecting cell lysates for S100A4 expression. *E*: genomic DNA isolated from colon cells was subjected to methylation status analysis.

S100A4 induction in response to hyperosmotic stress is correlated with methylation status of first intron region of S100A4. DNA hypomethylation of the S100A4 gene is responsible for gene activation in human colon carcinoma cells (33). To test whether methylation of CpG sites in the first intron region are involved in hyperosmotic stress-induced S100A4 expression, we focused on the first several CpG sites playing important roles in S100A4 regulation. These specific CpG sites are illustrated in Fig. 2*A*. Clone A cells were treated with 1 μM of the DNA methyltransferase inhibitor DAC for 3 or 5 days, and then the genomic DNA from the 5-day treatment was subjected to DNA methylation analysis. As shown in Fig. 2*B*, inhibition of methyltransferase decreases methylation percentage specifically at CpG sites 1, 4, 5, and 6. Furthermore, S100A4 expression levels were confirmed at both 3- and 5-day treatment by immunoblot analysis (Fig. 2*C*). These data suggest that DNA methylation in the first intron region of the S100A4 promoter can regulate S100A4 expression in Clone A colon cancer cells.

To determine the universality of our observation in Clone A cells, we submitted a panel of established colon carcinoma cells to a 24-h osmotic stress treatment. As shown in Fig. 2*D*, a dramatic induction of S100A4 was also observed in Caco2, LS174T, HCT-8, and KM12C colon carcinoma cells. For those cells with low S100A4 expression, a slight stimulation was noted in KM20, HCT116, and HT29. However, in cells with high expression levels of S100A4, such as SW620 and SW480 (Fig. 2*D*), S100A4 was not induced. To determine whether the response to osmotic stress in different colon cancer cell lines correlated with the methylation status of the first intron region of S100A4, the genomic DNA of these cell lines were submitted for bisulfate conversion and pyrosequencing of the first intron region of the S100A4 promoter. Because three CpG sites (sites 4, 5, and 6) in Clone A cells were most affected by DNA methyltransferase treatment, we focused on these sites in the other colon cancer cell lines. As illustrated in Fig. 2, *D* and *E*, two cell lines SW620 and SW480, with no change in S100A4 expression in response to osmotic stress, had very low levels of DNA methylation in these three sites where methylation is 2.9% (position 4), 2.7% (position 5), and 5.3% (position 6) in SW620 cells and less than 3% in these three sites in SW480 cells. Cell lines with greater S100A4 induction, including Caco2, LS174T, and Clone A had the highest levels of DNA methylation. For example, the methylation percentage in Caco2 cells on sites 4, 5, and 6 were 91.8%, 82.9%, and 77.6%, respectively. In the cell lines with marginal upregulation of S100A4 expression, the level of methylation in this region of the promoter was intermediate. Taken together, we conclude that the methylation status of the first intron region positively correlates with the responsiveness of cells for hyperosmotic induction of S100A4 in colon cancer cells.

NFAT5 is activated and required for S100A4 induction in the condition of hyperosmotic stress. NFAT5 is an osmotic stress regulator and has been shown to regulate S100A4 expression in breast cancer cells downstream of integrin α6β4 signaling. To determine the effect of hyperosmotic stress on NFAT5 in colon cancer cells, we treated Clone A cells with hyperosmotic NaCl for 24 h and then detected the NFAT5 protein level by immunoblot analysis. As shown in Fig. 3*A*, NFAT5 was dramatically upregulated in response to hyperosmotic stress in colon cancer cells. In addition, cells that were
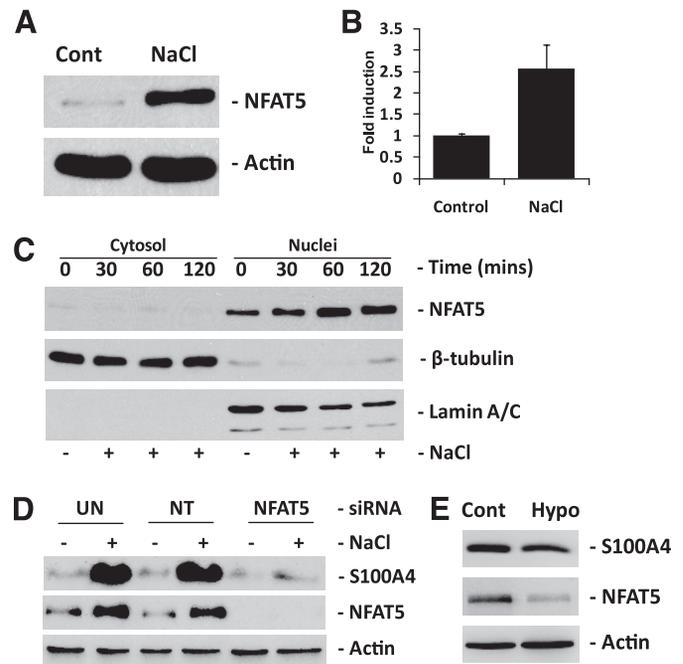

Fig. 3. Nuclear factor-inactivated T cell 5 (NFAT5) is activated and required for S100A4 induction in response to osmotic shock in colon cancer cells. *A*: Clone A cells were induced with hyperosmotic stress in 100 mM excess NaCl for 24 h, and then cell lysates were analyzed for NFAT5 expression by immunoblotting. *B*: Clone A cells were cotransfected with pRL-TK Renilla control reporter and NFAT5 luciferase reporter containing the NFAT5 binding sites from the human aldose reductase gene promoter. After 24 h, cells were exposed to 100 mM excess NaCl for additional 24 h before luciferase activity analysis. Experiments were performed in triplicate, and data were presented as fold induction compared with control. *C*: NFAT5 localization in response to hyperosmotic shock. Clone A cells were exposed to 100 mM excess NaCl for the indicated times, and then cell fractionation was performed by using NE-PER nuclear and cytoplasmic extraction reagents as recommended by manufacturer (Pierce). NFAT5 nuclear localization was assessed by immunoblotting. β-Tubulin and lamin A/C were used as cytoplasmic and nuclear protein markers as well as loading controls, respectively. *D*: Clone A cells were electroporated with SmartPool small interfering RNA (siRNA) targeting NFAT5, nontargeting control (NT), or electroporated-only control (UN) were treated with 100 mM excess NaCl for 24 h and then assessed for S100A4 and NFAT5 expression. *E*: SW480 cells were induced with hypoosmotic shock by adding medium diluted 1:1 with water for 24 h, and then cells were assessed for S100A4 and NFAT5 expression.

transfected with a NFAT5 reporter construct before hyperosmotic stress showed a 2.5-fold activation of NFAT5 compared with control (Fig. 3*B*). As NFAT5 is translocated into the nucleus following osmotic stress, we used a cell fractionation assay to determine whether NFAT5 was translocated to the nucleus upon hyperosmotic shock. Consistent with previous findings (2), we found that although NFAT5 resides in the nucleus, hyperosmotic stress induced a rapid translocation of NFAT5 into the nucleus within 30–60 min (Fig. 3*C*). To further ascertain whether NFAT5 is required for the induction of S100A4, Clone A cells were electroporated with siRNA targeting NFAT5 or nontargeting siRNA and were treated with 100 mM excess NaCl overnight. As seen in Fig. 3*D*, the induction of S100A4 was completely abolished by NFAT5 siRNA treatment compared with untreated and nontargeting siRNA controls. Given that NFAT5 is a bidirectional osmotic regulator, we hypothesize that hypoosmotic stress may have the opposite effect on NFAT5-mediated S100A4 expression in

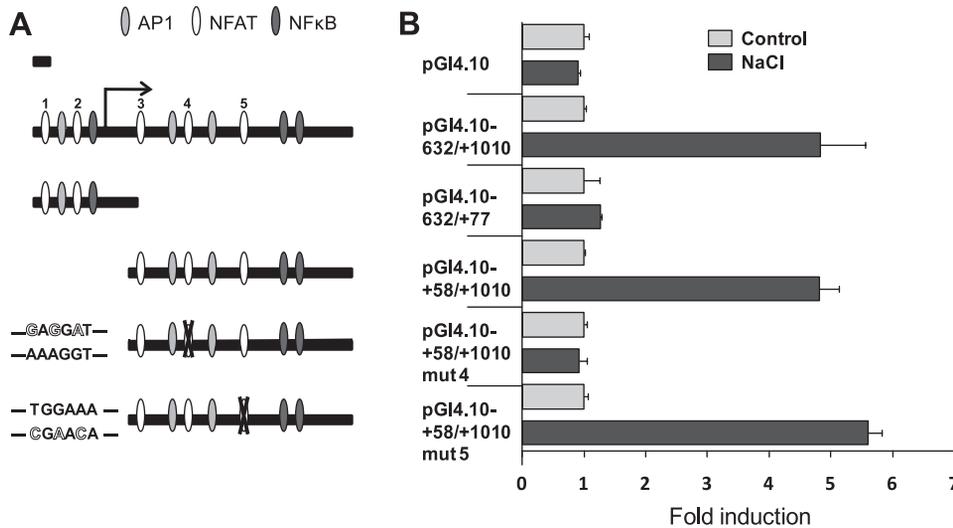Fig. 4. Hyperosmotic response elements are located in the first intron region of S100A4. *A*: schematic of transcription factor binding motifs in the regulatory region of S100A4. *B*: cells were cotransfected with the indicated S100A4 luciferase reporter along with pRL-TK Renilla control reporter for 24 h. Cells were then induced with 100 mM excess NaCl for an additional 24 h before cells were assessed for luciferase activity using a dual-luciferase reporter assay system. The data are presented as fold induction from individual controls and are representative of three different experiments.

colon cancer cells. For this experiment, we submitted SW480 cells to hypoosmotic shock by 1:1 dilution of media and water for 24 h. The cells were collected for immunoblotting and probed for S100A4 and NFAT5. The results showed that hypoosmotic stress decreased NFAT5 and S100A4 expression (Fig. 3*E*). These data demonstrate that, in response to hyperosmotic stress, NFAT5 is activated at multiple levels, and the activation of NFAT5 is required for S100A4 induction in colon cancer cells.

Hyperosmotic stress-responsive elements are located in the first intron region of the S100A4 promoter. Our data suggest that S100A4 induction by hyperosmotic shock is regulated on the transcriptional level by NFAT5. To determine what regions of the S100A4 are responsible for this regulation, we generated a series of S100A4 promoter luciferase reporter constructs. As illustrated in Fig. 4*A*, different fragments of the regulatory regions of the proximal promoter region and the first intron region of S100A4 were cloned into pGL4.10 basic vector. Cells were then cotransfected with one of these constructs and with TK-Renilla before hyperosmotic shock treatment for 24 h. The luciferase activity of cell lysates was then measured. As shown in Fig. 4*B*, luciferase activity from the S100A4 reporters containing the first intron region pGl4.10−632/+1010 and pGl4.10 +58/+1010 was stimulated about fivefold by osmotic shock. In contrast, the reporter construct based on the proximal promoter region pGl4.10−632/+77 did not show any induction. These results suggest that S100A4 is transcriptionally regulated by osmotic shock, and the osmotic-response elements are located in the first intron region.

By scanning the NFAT5 transcription factor binding sites, we found five putative NFAT binding sites in the regulatory region (Fig. 4*A*). Three of these sites are in the intronic region; two of these three sites have the consensus sequence for NFAT5 binding, which is characterized by an 11 bp sequence TGGAAANNYNY (N represents as any nucleotide and Y represents as pyrimidine) (24) To determine whether the binding of NFAT5 to these two sites plays an important role in S100A4 regulation under the condition of hyperosmotic stress, site-directed mutagenesis was performed on the pGl4.10+58/+1010 construct. We changed the putative binding site TGGAAA in reverse direction (site 4) to GAGGAT and putative binding site

TGGAAA in sense direction (site 5) to CGAACA. As shown in Fig. 4*B*, mutation in site 4 not only decreased the basal level (data not shown) but also completely blocked the induction of luciferase activity by osmotic shock. However, mutation of site 5 had no effect. These results implicate the site 4 NFAT5 binding site as essential for S100A4 transcriptional regulation.

To test whether NFAT5 binds to this regulatory region, Clone A cells were treated with 100 mM excess NaCl for 2 h and then the ChIP assay was performed using NFAT5 antibody or normal rabbit IgG as control. As shown in Fig. 5, NFAT5 bound to this intron region only when cells were treated with hyperosmotic NaCl. These results, together with the luciferase reporter assays, demonstrate that NFAT5, when bound to site 4 of the intronic region of the S100A4 promoter, is an important component in the regulation of S100A4 in colon cancer cells under the condition of hyperosmotic stress.

Effect of Src pathway on NFAT5-mediated S100A4 induction in colon cancer cells. Hyperosmotic stress can activate Src family kinases (SFK) such as Src and Fyn (8, 22).
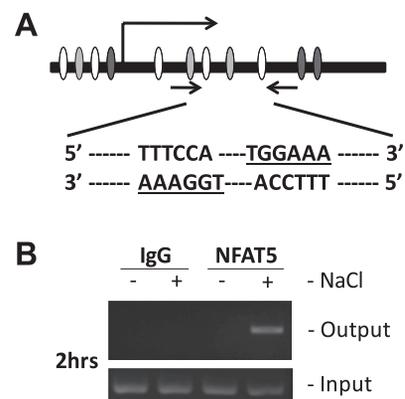

Fig. 5. NFAT5 binds to the first intron region where the putative NFAT5 binding sites are located. *A*: schematic of the S100A4 regulation region with consensus NFAT5 binding sites and the primers for chromatin immunoprecipitation (ChIP) assay. *B*: cells were treated with 100 mM excess NaCl for 2 h, then cells were harvested and subjected to ChIP assay using antibody against NFAT5 or normal rabbit IgG control, and the expected 281 bps product were amplified.

Importantly, Src acts as a regulator for cell volume and transactivates NFAT5 under the condition of osmotic stress (8). To test whether Src is involved in NFAT5-mediated S100A4 induction by hyperosmotic stress in colon cancer cells, we pretreated Clone A cells with 10 μM PP2, a specific SFK inhibitor, or the same concentration of PP3 (a structurally related but inactive analogue of PP2) before exposing cells to hyperosmotic stress. We found that inhibition of the SFK pathway by PP2 dramatically abrogated the induction of S100A4 at both the protein and RNA levels (Fig. 6, A and B, respectively). In addition, NFAT5 expression was also decreased by PP2 treatment. Further assays utilizing transfection of the NFAT5 reporter (Fig. 6C) and S100A4 reporter constructs into Clone A cells (Fig. 6D) confirmed that inhibition of the Src pathway decreases the induction of NFAT5 and S100A4 reporter activity. To determine whether inhibition of Src kinase pathway affects NFAT5 activity, Clone A cells were pretreated with either 10 μM PP2 or PP3 for 30 min, cells were exposed to hyperosmotic shock for 24 h, and a cell fractionation assay was performed. As shown in Fig. 6E, hyperosmotic shock induced the translocation of NFAT5 into the nucleus; however, inhibition of Src did not affect this nuclear translocation.

To analyze whether the inhibition of Src kinase affects NFAT5 transactivation, we used the NFAT5 transactivation reporter Gal4dbd-TonEBP/OREBP, which contains the NFAT5 transactivation domain (548–1531) fused to the Gal4dbd (11, 18). Clone A cells were cotransfected with the NFAT5 transactivation reporter together with a Gal4 luciferase reporter. As shown in Fig. 6F, we found that hyperosmotic shock dramatically induced NFAT5 transactivation, which was completely inhibited by the Src inhibitor PP2 but not the negative control PP3. In summary, these data demonstrated that the Src kinase pathway is involved in S100A4 induction by hyperosmotic shock by affecting NFAT5 protein level as well as its transactivation but not NFAT5 nuclear translocation.

Effect of S100A4 knock down on cell survival. Because osmotic shock induces cell death, we hypothesized that S100A4 induction may contribute to cell survival. To test this concept, we knocked down S100A4 expression in Clone A cells by siRNA and then performed an MTT assay under the condition of hyperosmotic stress. As shown in Fig. 7A, the reduction of S100A4 resulted in decreased cell viability under the hyperosmotic condition. Figure 7C shows the successful reduction of S100A4 expression at representative time points. Notably, S100A4 reduction has little impact on cell viability under normal culturing conditions (Fig. 7B). In conclusion, these data suggest that S100A4 induction acts as the survival factor, either through increased proliferation or cell survival under the condition of osmotic stress.

Fig. 6. Inhibition of Src kinase pathway hinders hyperosmotic stress-induced S100A4 expression through inhibition of NFAT5 expression and transactivation but not nuclear translocation in Clone A cells. *A* and *B*: Clone A cells were pretreated with 10 μM 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]-pyramidine (PP2, a specific src family kinase inhibitor) or 4-amino-7-phenylpyrazolo[3,4-d]pyramidine (PP3, a PP2 negative control) for 30 min before inducing hyperosmotic stress by 100 mM excess NaCl for 24 h; then cells were collected for immunoblotting to detect S100A4 and NFAT5 protein expression (*A*) or S100A4 expression by Q-PCR (*B*). *C* and *D*: Clone A cells were cotransfected with NFAT5 reporter (*C*) or pGl4.10 S100A4 (+58/+1010) reporter (*D*) with pRL-TK-Renilla, 24 h before cells were treated as done in *A*. After 24-h treatment, cell lysates were assessed for luciferase activity as performed in Fig. 4. *E*: Clone A cells were treated as in *A* and then cell fractionation was performed. NFAT5 nuclear translocation was detected by immunoblotting. *F*: Clone A cells grown in a 24-well plate were cotransfected with 0.25 μg Gal4dbd-TonEBP/OREBP, which contains NFAT5 transactivation domain (548–1531) fused to the Gal4dbd and 0.25 μg Gal4 luciferase reporter pGL-4.31[luc2P/GAL4UAS/Hygro] (Promega) together with 5 ng pRL-TK Renilla control reporter. After 24 h, cells were pretreated with 10 μM PP2 or PP3 for 30 min, and then cells were induced by hyperosmotic shock for 24 h before luciferase activity assay.
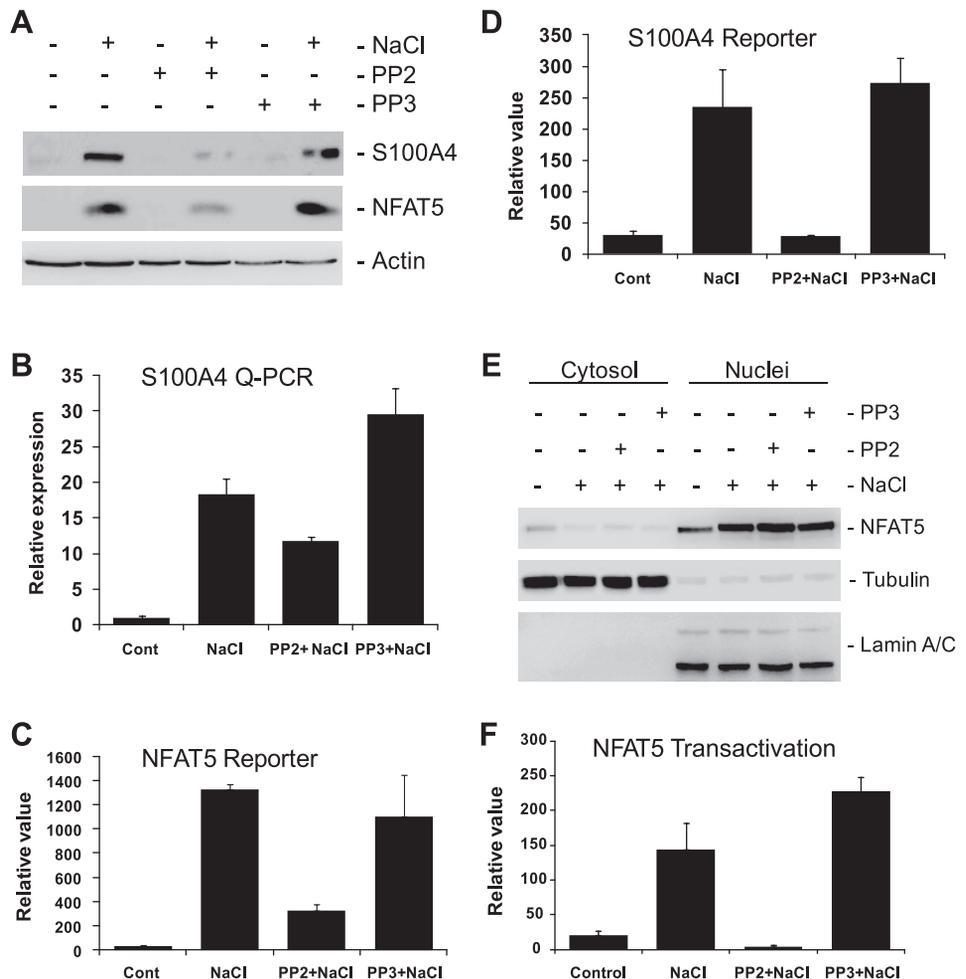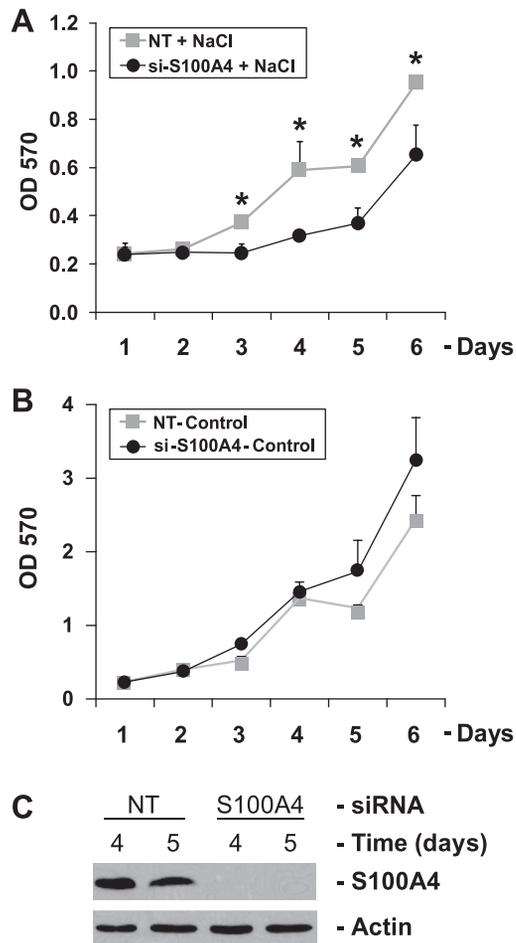
Fig. 7. S100A4 facilitates cell viability under hyperosmotic stress. *A* and *B*: Clone A cells were electroporated with 200 nM siRNA targeting S100A4 or nontargeting (NT) control and then seeded into a 96-well plate. Cells were then exposed to hyperosmotic (*A*; 100 mM excess NaCl) or normal culturing conditions (*B*), and then MTT assays were performed every day for a 6-day period. *C*: cell lysates from 4th and 5th day treatment were immunoblotted with antibody against S100A4 to test the efficiency of S100A4 siRNA. Error bars in *A* and *B* represent standard deviation from triplicate determinations. *P value of <0.04. Notably in *B*, S100A4 reduction leads to a consistent increase in cell viability, but it does not reach statistical significance.

DISCUSSION

In this study, we demonstrated that hyperosmotic stress induces S100A4 expression in colon cancer cells. Although this phenomenon had been shown in the kidney (36), our study expands this observation in colon cells to demonstrate that the Src kinase pathway and NFAT5 are required in this induction. In addition, we further identify that the osmotic stress response elements are located in the first intron region of S100A4. Importantly, we observed that the ability of hyperosmotic stress to induce S100A4 depends on the methylation status of S100A4. We found that cells with higher methylation percentages in the first intron region of S100A4 respond dramatically. Notably, DNA methyltransferase inhibitor DAC treatment could reexpress S100A4 in these cells, and the percentage of methylation of CpG sites 1, 4, 5, and 6 is decreased in response to this treatment. Furthermore, cells with lower methylation percentages displayed a constitutively high level of S100A4 and did not exhibit further increases in S100A4 expression in

response to hyperosmotic stress. These results suggest that DNA methylation of these CpG sites plays a role in suppressing S100A4 expression, and demethylation of these sites plays an active role during DAC treatment.

In contrast, we did not observe changes in DNA methylation status when cells were induced with hyperosmotic shock (supplemental data Fig. S2). This discrepancy suggests that, although DNA methylation is an important mechanism for regulation of S100A4, this regulation could be circumvented during hyperosmotic regulation through other chromatin remodeling mechanisms. Osmotic stress is known to affect the structure and function of the nucleus and to act on the genome, which leads to gene expression (12). Nucleosomes, which are wrapped with DNA and packed into the fundamental organization unit of chromatin, are often depleted at active promoters (26). It has been shown that hyperosmotic stress can induce a rapid and reversible loss of nucleosomes around OREs (45). The depletion of nucleosomes may bypass DNA methylation, relax chromatin, thus allowing ORE access for transcription factors such as NFAT5. We note that S100A4 induction in response to hyperosmotic stress has a substantial lag time both at mRNA and protein levels. However, translocation of NFAT5 demonstrated by nuclear fractionation is rapid and detected within 30–60 min. ChIP analysis further demonstrated that NFAT5 binds to the putative ORE region after hyperosmotic stress by 2 h. Notably, there are no CpG sites in the recognition site of NFAT5. These data collectively suggest that other chromatin remodeling processes, rather than DNA methylation alone, are likely involved in S100A4 induction by hyperosmotic stress and that binding of NFAT5 to the ORE might be an initiating event. However, how hyperosmotic stress affects chromatin structure and whether chromatin remodeling events are dependent on the functional ORE in the S100A4 first intron region awaits further study.

NFAT5 plays a key role in cells' response to hyperosmotic stress. The roles of NFAT5 in the kidney and immune system are well documented. However, the activation and the role of NFAT5 in the GI tract under osmotic stress have not been investigated extensively, except for the finding that NFAT5 is required for cytochrome *P*-450 3As expression (1, 23). In this study, consistent with NFAT5 activation in response to hyperosmotic stress (2), we find that NFAT5 is activated in colon cancer cells at different levels, such as transcription, translocation, and transactivation. Importantly, this activation is required for transcriptional regulation of S100A4 in response to osmotic stress. We further identified, by mutagenesis, that one of the NFAT5 sites in the intron region of the S100A4 promoter is the critical site for the osmotic response, thus defining the ORE. In agreement with our previous findings, this study demonstrates that NFAT5 is an important component for regulation of S100A4 in colon cancer cells under the condition of hyperosmotic stress. Together, these data strongly suggest that chromatin remodeling processes, in conjunction with transcription factor activation, such as that of NFAT5, are needed to fully activate the S100A4 promoter in response to hyperosmotic stress.

Using pharmacological inhibitors, we found that the inhibition of SFKs abrogates S100A4 induction by hyperosmotic stress in a dose-dependent manner (supplemental data Fig. S3). SFKs are the nonreceptor protein tyrosine kinases that consist of nine members and have many critical cell functions, includ-

ing cell adhesion, invasion, proliferation, survival, and angiogenesis during tumor development (21). In response to hyperosmotic stress, several SFK members, such as FYN, HCK, FGR, SRC, and YES, are shown to be activated (8); this activation may be through the proposed osmosensing receptors for mammalian cells such as EGFR, integrin, and G protein-coupled receptors (8). Previous studies showed that inhibition of SFK or expression of the dominant-negative mutant of FYN partially blocked NFAT5-dependent transcription and transactivation but not its nuclear translocation (11, 22). In agreement with these findings, our data demonstrated that the specific SFK inhibitor PP2 dramatically blocked NFAT5 transactivation but did not affect NFAT5 nuclear translocation. In addition, we found that inhibition of SFK also slightly decreased NFAT5 protein expression. In response to hyperosmotic stress, Src activation is also required for COX-2 expression in cultured medullary epithelial cells (47). Collectively, these data suggest that Src kinase pathways are important transducers for NFAT5-mediated S100A4 gene regulation downstream of integrin signaling and hyperosmotic stress.

Although the GI tract is periodically exposed to hyperosmotic stress (27), studies on the effects of this hyperosmolarity in the gut have been focused on nuclear factor (NF)-κB-mediated cytokine production (1, 35), as osmotic stress has been suggested to be related to inflammation (40, 41). In most tissues, the osmolarity is thought to be around 300 mosM, and osmolarity in the inflammation fluid could be increased to ~425–450 mosM (40). Under physiological conditions, the osmolarity of intraluminal contents after meal intake is increased in the colon (20). From detailed studies in pigs, the osmolality in the lumen of the GI tract can increase to as much as 430 mosmol/kg water after meal intake (17). Here, we used 25–100 mM excess NaCl to induce hyperosmotic stress, suggesting that the range of osmolarity used here has physiological and pathophysiological relevance.

Our data further demonstrate that S100A4 acts as a survival factor under hyperosmotic stress in colon cancer cells. The pro-survival effect of S100A4 has been proposed previously (30, 39). For example, S100A4 is upregulated in the hypertrophic rat and human heart. Recombinant S100A4 promotes growth and survival of cardiac myocytes (39). In pancreatic cancer cells, knocking down S100A4 leads to increased sensitivity of cancer cells to gemcitabine treatment, which was coupled with an increase in apoptosis and cell cycle arrest. This observation supports the contribution of S100A4 to pancreatic cancer chemoresistance (30). S100A4 confers these functions possibly through either intracellular or extracellular modes. Intracellularly, S100A4 interacts with target proteins such as p53, and extracellulary, S100A4 activates NF-κB through induction and subsequent degradation of the NF-κB inhibitor IκBα (5). The pro-survival function of S100A4 induction might be critical for protection of epithelial cells from apoptosis and adaptation to the environment; however, chronic exposure to hyperosmotic stress may have adverse effects. Impaired DNA repair pathways and formation of DNA strand breaks appear in cells adapted to hyperosmotic stress (10), which could lead to accumulation of DNA mutations by exposure to a hyperosmolar microenvironment (9). In this respect, the sustained S100A4 induction by hyperosmotic stress further provides the survival advantage and increases the risk of genomic instability. Therefore, considering that the GI tract is

periodically exposed to osmotic shock due to fluid or food intake, the survival effect of S100A4 could facilitate development of hyperplasia in intestinal epithelia.

In summary, our study demonstrates for the first time that hyperosmotic stress induces S100A4 expression through Src-mediated NFAT5 activation in colon cancer cells, and the ability to induce S100A4 by hyperosmotic stress depends on the methylation status of S100A4. Importantly, we showed that the consequence of hyperosmotic stress in GI cells is not limited to inflammation and drug metabolism but also pro-survival mechanisms. Together with previous findings, our data suggest that hyperosmotic stress may affect GI physiology and potentially contribute to GI cancer progression.

REFERENCES

1. **Abolhassani M, Wertz X, Pooya M, Chaumet-Riffaud P, Guais A, Schwartz L.** Hyperosmolarity causes inflammation through the methylation of protein phosphatase 2A. *Inflamm Res* 57: 419–429, 2008.
2. **Aramburu J, Drews-Elger K, Estrada-Gelonch A, Minguillon J, Morancho B, Santiago V, and Lopez-Rodriguez C.** Regulation of the hypertonic stress response and other cellular functions by the Rel-like transcription factor NFAT5. *Biochem Pharmacol* 72: 1597–1604, 2006.
3. **Arbabi S, Rosengart MR, Garcia I, Jelacic S, Maier RV.** Epithelial cyclooxygenase-2 expression: a model for pathogenesis of colon cancer. *J Surg Res* 97: 60–64, 2001.
4. **Bachelder RE, Lipscomb EA, Lin X, Wendt MA, Chadborn NH, Eickholt BJ, Mercurio AM.** Competing autocrine pathways involving alternative neuropilin-1 ligands regulate chemotaxis of carcinoma cells. *Cancer Res* 63: 5230–5233, 2003.
5. **Boye K, Grotterod I, Aasheim HC, Hovig E, Maelandsmo GM.** Activation of NF-kappaB by extracellular S100A4: analysis of signal transduction mechanisms and identification of target genes. *Int J Cancer* 123: 1301–1310, 2008.
6. **Chen M, Sinha M, Luxon BA, Bresnick AR, O'Connor KL.** Integrin alpha6beta4 controls the expression of genes associated with cell motility, invasion, and metastasis, including S100A4/metastasin. *J Biol Chem* 284: 1484–1494, 2009.
7. **Cho YG, Nam SW, Kim TY, Kim YS, Kim CJ, Park JY, Lee JH, Kim HS, Lee JW, Park CH, Song YH, Lee SH, Yoo NJ, Lee JY, Park WS.** Overexpression of S100A4 is closely related to the aggressiveness of gastric cancer. *APMIS* 111: 539–545, 2003.
8. **Cohen DM.** SRC family kinases in cell volume regulation. *Am J Physiol Cell Physiol* 288: C483–C493, 2005.
9. **Dixon BP, Chu A, Henry J, Kim R, Bissler JJ.** Increased cancer risk of augmentation cystoplasty: possible role for hyperosmolal microenvironment on DNA damage recognition. *Mutat Res* 670: 88–95, 2009.
10. **Dmitrieva NI, Cai Q, Burg MB.** Cells adapted to high NaCl have many DNA breaks and impaired DNA repair both in cell culture and in vivo. *Proc Natl Acad Sci USA* 101: 2317–2322, 2004.
11. **Ferraris JD, Williams CK, Persaud P, Zhang Z, Chen Y, Burg MB.** Activity of the TonEBP/OREBP transactivation domain varies directly with extracellular NaCl concentration. *Proc Natl Acad Sci USA* 99: 739–744, 2002.
12. **Finan JD, Guilak F.** The effects of osmotic stress on the structure and function of the cell nucleus. *J Cell Biochem* 109: 460–467, 2010.
13. **Go WY, Liu X, Roti MA, Liu F, Ho SN.** NFAT5/TonEBP mutant mice define osmotic stress as a critical feature of the lymphoid microenvironment. *Proc Natl Acad Sci USA* 101: 10673–10678, 2004.

14. **Grigorian M, Ambartsumian N, Lukanidin E.** Metastasis-inducing S100A4 protein: implication in non-malignant human pathologies. *Curr Mol Med* 8: 492–496, 2008.

15. **Helfman DM, Kim EJ, Lukanidin E, Grigorian M.** The metastasis associated protein S100A4: role in tumour progression and metastasis. *Br J Cancer* 92: 1955–1958, 2005.

16. **Hernan R, Fasheh R, Calabrese C, Frank AJ, Maclean KH, Allard D, Barraclough R, Gilbertson RJ.** ERBB2 up-regulates S100A4 and several other prometastatic genes in medulloblastoma. *Cancer Res* 63: 140–148, 2003.

17. **Houpt TR.** Patterns of duodenal osmolality in young pigs fed solid food. *Am J Physiol Regul Integr Comp Physiol* 261: R569–R575, 1991.

18. **Irarrazabal CE, Gallazzini M, Schnetz MP, Kunin M, Simons BL, Williams CK, Burg MB, Ferraris JD.** Phospholipase C-gamma1 is involved in signaling the activation by high NaCl of the osmoprotective transcription factor TonEBP/OREBP. *Proc Natl Acad Sci USA* 107: 906–911, 2010.

19. **Kalantzi L, Goumas K, Kalioras V, Abrahamsson B, Dressman JB, Reppas C.** Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. *Pharm Res* 23: 165–176, 2006.

20. **Keusch GT, Donowitz M.** Pathophysiological mechanisms of diarrhoeal diseases: diverse aetiologies and common mechanisms. *Scand J Gastroenterol Suppl* 84: 33–43, 1983.

21. **Kim LC, Song L, Haura EB.** Src kinases as therapeutic targets for cancer. *Nat Rev Clin Oncol* 6: 587–595, 2009.

22. **Ko BC, Lam AK, Kapus A, Fan L, Chung SK, Chung SS.** Fyn and p38 signaling are both required for maximal hypertonic activation of the osmotic response element-binding protein/tonicity-responsive enhancer-binding protein (OREBP/TonEBP). *J Biol Chem* 277: 46085–46092, 2002.

23. **Kosuge K, Chuang AI, Uematsu S, Tan KP, Ohashi K, Ko BC, Ito S.** Discovery of osmosensitive transcriptional regulation of human cytochrome P450 3As by the tonicity-responsive enhancer binding protein (nuclear factor of activated T cells 5). *Mol Pharmacol* 72: 826–837, 2007.

24. **Kultz D, Csonka L.** What sets the TonE during osmotic stress? *Proc Natl Acad Sci USA* 96: 1814–1816, 1999.

25. **Ladas SD, Isaacs PE, Sladen GE.** Post-prandial changes of osmolality and electrolyte concentration in the upper jejunum of normal man. *Digestion* 26: 218–223, 1983.

26. **Lee CK, Shibata Y, Rao B, Strahl BD, Lieb JD.** Evidence for nucleosome depletion at active regulatory regions genome-wide. *Nat Genet* 36: 900–905, 2004.

27. **Lim CH, Bijvelds MJ, Nigg A, Schoonderwoerd K, Houtsmuller AB, de Jonge HR, Tilly BC.** Cholesterol depletion and genistein as tools to promote F508delCFTR retention at the plasma membrane. *Cell Physiol Biochem* 20: 473–482, 2007.

28. **Lim CH, Bot AG, de Jonge HR, Tilly BC.** Osmosignaling and volume regulation in intestinal epithelial cells. *Methods Enzymol* 428: 325–342, 2007.

29. **Lopez-Rodriguez C, Aramburu J, Jin L, Rakeman AS, Michino M, Rao A.** Bridging the NFAT and NF-kappaB families: NFAT5 dimerization regulates cytokine gene transcription in response to osmotic stress. *Immunity* 15: 47–58, 2001.

30. **Mahon PC, Baril P, Bhakta V, Chelala C, Caulee K, Harada T, Lemoine NR.** S100A4 contributes to the suppression of BNIP3 expression, chemoresistance, and inhibition of apoptosis in pancreatic cancer. *Cancer Res* 67: 6786–6795, 2007.

31. **Min HS, Choe G, Kim SW, Park YJ, Park do J, Youn YK, Park SH, Cho BY, Park SY.** S100A4 expression is associated with lymph node metastasis in papillary microcarcinoma of the thyroid. *Mod Pathol* 21: 748–755, 2008.

32. **Missiaglia E, Blaveri E, Terris B, Wang YH, Costello E, Neoptolemos JP, Crnogorac-Jurcevic T, Lemoine NR.** Analysis of gene expression in cancer cell lines identifies candidate markers for pancreatic tumorigenesis and metastasis. *Int J Cancer* 112: 100–112, 2004.

33. **Nakamura N, Takenaga K.** Hypomethylation of the metastasis-associated S100A4 gene correlates with gene activation in human colon adenocarcinoma cell lines. *Clin Exp Metast* 16: 471–479, 1998.

34. **Nakamura T, Ajiki T, Murao S, Kamigaki T, Maeda S, Ku Y, Kuroda Y.** Prognostic significance of S100A4 expression in gallbladder cancer. *Int J Oncol* 20: 937–941, 2002.

35. **Nemeth ZH, Deitch EA, Szabo C, Hasko G.** Hyperosmotic stress induces nuclear factor-kappaB activation and interleukin-8 production in human intestinal epithelial cells. *Am J Pathol* 161: 987–996, 2002.

36. **Rivard CJ, Brown LM, Almeida NE, Maunsbach AB, Pihakaski-Maunsbach K, Andres-Hernando A, Capasso JM, Berl T.** Expression of the calcium-binding protein S100A4 is markedly up-regulated by osmotic stress and is involved in the renal osmoadaptive response. *J Biol Chem* 282: 6644–6652, 2007.

37. **Rudland PS, Platt-Higgins A, Renshaw C, West CR, Winstanley JH, Robertson L, Barraclough R.** Prognostic significance of the metastasis-inducing protein S100A4 (p9Ka) in human breast cancer. *Cancer Res* 60: 1595–1603, 2000.

38. **Saleem M, Adhami VM, Ahmad N, Gupta S, Mukhtar H.** Prognostic significance of metastasis-associated protein S100A4 (Mts1) in prostate cancer progression and chemoprevention regimens in an autochthonous mouse model. *Clin Cancer Res* 11: 147–153, 2005.

39. **Schneider M, Kostin S, Strom CC, Aplin M, Lyngbaek S, Theilade J, Grigorian M, Andersen CB, Lukanidin E, Lerche Hansen J, Sheikh SP.** S100A4 is upregulated in injured myocardium and promotes growth and survival of cardiac myocytes. *Cardiovasc Res* 75: 40–50, 2007.

40. **Schwartz L, Abolhassani M, Pooya M, Steyaert JM, Wertz X, Israel M, Guais A, and Chaumet-Riffaud P.** Hyperosmotic stress contributes to mouse colonic inflammation through the methylation of protein phosphatase 2A. *Am J Physiol Gastrointest Liver Physiol* 295: G934–G941, 2008.

41. **Schwartz L, Guais A, Pooya M, Abolhassani M.** Is inflammation a consequence of extracellular hyperosmolarity? *J Inflamm* 6: 21, 2009.

42. **Sherbet GV.** Metastasis promoter S100A4 is a potentially valuable molecular target for cancer therapy. *Cancer Lett* 280: 15–30, 2009.

43. **Stein U, Arlt F, Walther W, Smith J, Waldman T, Harris ED, Mertins SD, Heizmann CW, Allard D, Birchmeier W, Schlag PM, Shoemaker RH.** The metastasis-associated gene S100A4 is a novel target of beta-catenin/T-cell factor signaling in colon cancer. *Gastroenterology* 131: 1486–1500, 2006.

44. **Takenaga K, Nakanishi H, Wada K, Suzuki M, Matsuzaki O, Matsuura A, Endo H.** Increased expression of S100A4, a metastasis-associated gene, in human colorectal adenocarcinomas. *Clin Cancer Res* 3: 2309–2316, 1997.

45. **Tong EH, Guo JJ, Xu SX, Mak K, Chung SK, Chung SS, Huang AL, Ko BC.** Inducible nucleosome depletion at OREBP-binding-sites by hypertonic stress. *PLoS One* 4: e8435, 2009.

46. **Xie R, Loose DS, Shipley GL, Xie S, Bassett RL Jr, and Broaddus RR.** Hypomethylation-induced expression of S100A4 in endometrial carcinoma. *Mod Pathol* 20: 1045–1054, 2007.

47. **Yang T, Huang Y, Heasley LE, Berl T, Schnermann JB, Briggs JP.** MAPK mediation of hypertonicity-stimulated cyclooxygenase-2 expression in renal medullary collecting duct cells. *J Biol Chem* 275: 23281–23286, 2000.