S100A4 REGULATION AND FUNCTION

by

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DEDICATION

I have dedicated this dissertation to the following persons:

My parents,

who have given me endless love and support,

who have been my role models, teaching me the importance of hard work,

and who have shown me how to be a better person each day.

My parents-in-law,

who have given their support and patience.

My husband,

who has been working so hard to take care of our family

and who has shared the happiness, sufferings, and challenges.

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S100A4 is a calcium binding and tumor associated protein. Despite its critical roles in multiple aspects of cancer progression and non-malignant disease development, the investigations on the regulation of S100A4 are limited. This dissertation focuses on exploring the mechanisms of S100A4 regulation by integrin α6β4 in breast cancer cells, and hyperosmotic stress response in colon cancer cells, as well as the functional aspects of S100A4 in these two models.

Using breast cancer cell lines, I found that S100A4 is upregulated by integrin α6β4 and its expression correlates well with integrin α6β4 expression. Using siRNA, promoter analysis and chromatin immunoprecipitation (ChIP), I demonstrate that S100A4 is regulated by NFAT5 in breast cancer cells. To study the regulation of S100A4 by NFAT5 in a more readily inducible model, I used colon cancer cells under hyperosmotic stress as the model. I found that the osmotic stress response elements are located in the first intron region of S100A4 by luciferase reporter assays. Inhibition of Src kinase pathways reduced S100A4 induction by affecting NFAT5

transactivation and protein levels. Inhibition of DNA methyltransferases stimulated expression of S100A4 in cells lacking the α6β4 integrin, whereas demethylation inhibitors suppressed expression in α6β4 integrin expressing cells. Alterations in DNA methylation were confirmed by bisulfate sequencing, thus suggesting that integrin α6β4 signaling can lead to the demethylation of select promoters. Suppression of S100A4 by siRNA leads to decreased chemoinvasion of breast cancer cells and cell viability of colon cancer cells under hyperosmotic stress. The data suggest that NFAT5 in conjunction with DNA demethylation are important components of S100A4 regulation.

The interaction of S100A4 with cytoskeleton proteins suggests that S100A4 could be a potential regulator of actin cytoskeleton reorganization. Using GST pull-down and immunoprecipitation assays, I demonstrate that Rhotekin is a direct and specific partner of S100A4. Confocal microscopy showed that S100A4 co-localizes with Rhotekin. Using RNAi, I found that suppression of both S100A4 and Rhotekin leads to the loss of Rho-dependent membrane ruffling and a reduction in invasive growth in three-dimensional culture. My data suggest that interaction of S100A4 and Rhotekin alters the functional output of Rho signaling to confer an invasive phenotype in breast cancer cells.

In summary, this dissertation highlights the novel regulation and signaling of S100A4. Further elucidating the mechanism of the underlying function will help us to develop a potential therapeutic target for cancer metastasis.

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Chapter 5

CHAPER 1: INTRODUCTION[1]

1.1. S100A4 in cancer progression and non-malignant condition

S100A4, a calcium binding EF-hand protein, belongs to the S100 superfamily that contains at least 21 family members. It was cloned independently from various cell types under different names including metastasin-1 (mts1), CAPL, and fibroblast specific protein (FSP1), 18A2, pEL98, p9Ka, 42A, and calvasculin (*1, 2*). Increasing evidence demonstrates that S100A4 is associated with the progression of a variety of cancers, including breast, prostate, pancreatic, gallbladder, colon, gastric, and thyroid (*3-10*) and has been considered as a valuable prognostic marker for several tumors including breast and colon (*9, 11*). The role of S100A4 on tumor progression, and specifically on tumor metastasis, was also documented in several types of cancer by experimental metastasis models and transgenic mouse models (*1, 2*). In addition, mounting evidence also shows that S100A4 contributes to non-malignant conditions, such as rheumatoid arthritis and disorders of cardio-vascular, nervous and pulmonary systems (*12*). For example, S100A4 is over-expressed in rheumatoid arthritis synovial tissue and presents as

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[1] Part of this was originally published in Journal of Biological Chemistry. 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, 2009. 284(3): p. 1484-1494. © the American Society for Biochemistry and Molecular Biology and Chen M, Sastry SK, and 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, 2011. 300(5): p. C1155-116 The Am Physiol Soc, used with permission.

bioactive multimeric form, suggesting the inflammation function of S100A4 (*13, 14*). One recent study showed that in liver injury, fibrosis and cancer, S100A4 could be considered a marker for a specific subset of inflammatory macrophages (*15*). As a tumor metastasis associated protein, the documented functions of S100A4 fit into several hallmarks of cancer such as anti-apoptosis (survival), metastasis (motility and invasion), proliferation, angiogenesis, and inflammation (*16*).

1.2. S100A4 tissue distributions, subcellular localization, and signaling transduction

S100A4 was initially identified as a fibroblast marker (*17*). Later on, investigation on S100A4 expression demonstrated that it is expressed in highly motile cell types, including T-lymphocytes, neutrophils, macrophages, platelets, endothelial cells, fibroblast, and tumor cells (*18-21*). Notably, cell motility has been implicated as a major function controlled by S100A4 (*3*). One recent study showed that the recruitment of macrophages to inflammation sites *in vivo* is impaired by loss of S100A4, similarly, the primary bone marrow macrophages (BMMs) derived from S100A4-deficient mice have defects in chemotactic motility *in vitro*, therefore, indicating that S100A4 acts as a regulator of physiological macrophage motility (*22*).

S100A4 has been shown to localize in the nucleus, cytoplasm, and the extracellular space (*2*) which suggests that S100A4 functions through intracellular and extracellular action. Intracellularly, S100A4 interacts with target proteins such as p53 (*23*), heavy chain of non-muscle myosin IIA (MHC-IIA) (22), tropomyosin (*24*), liprin β1 (*25*) and several unknown proteins. Studies showed that S100A4 interacts with p53, inhibits p53 phosphorylation by PKC, and facilitates p53 degradation, thereby represses p53-mediated transcriptional activity (*1, 23*) .

This interaction with p53 implicates the function of S100A4 in apoptosis and cell cycle progression. Similarly, interaction of S100A4 with MHC-IIA inhibits MHC-IIA phosphorylation and promotes myosin disassembly and this has been well defined as one mechanism of S100A4 to mediate cell motility (*1, 26-28*).

S100A4 is secreted by different types of cancer cells and stromal cells; therefore, it participates in paracrine and autocrine signaling. For example, extracellular S100A4 can act as an angiogenesis factor by stimulating endothelial cell motility (*29*), MMP13 activity (*30*), and interacting with endothelial plasminogen co-receptor annexin II to stimulate plasmin formation (*28*). Extracellular S100A4 can also activate NF-κB in several cancer cell lines through induction of phosphorylation and subsequently degradation of IκBα (*31*) as well as binding to amphiregulin, an EGFR ligand, and enhancing EGFR/ErbB2 receptor signaling and cell proliferation (*32*)

1.3. S100A4 gene structure and regulation

The S100A4 genes in human, mouse, and rat are located on chromosome 1q21, 3f3, and 2q34 respectively (*33*). The mouse S100A4 gene has three exons and two introns (*34*). The first exon is non-coding and located in the 5'-UTR of the gene. The two EF-hands both in N-terminal and C-terminal are encoded in the second and the third exon respectively (*34*). The human S100A4 gene has four exons and the first two exons are non-coding. Two spliced variants of S100A4 are found in human (*35*); however, their significance in terms of function is unknown (*1*). Previous studies based on mouse and rat mammary adenocarcinoma cell lines (*36-39*), NIH 3T3 fibroblasts (*40*), and human lymphoma cell lines (*41*) demonstrated that the first intron

region serves partially as a cell type specific enhancer (*42*) and appears to be essential to regulate S100A4 expression. By comparison between the S100A4-positive highly metastatic mouse adenocarcinoma cell line CSML 100 and the S100A4-negative non-metastatic counterpart CSML0, at least six elements were identified in the intron region and contribute to the enhancer activity of S100A4, including Sp1, activating protein-1 (AP-1), nuclear factor NF-κB, κB recognition component (KRC), core binding factor (CBF), and minisatellite DNA binding protein (Msbp), (*42*).

Despite the instrumental roles of S100A4 in a variety of normal and pathological conditions, understanding of the regulation of this molecule at the transcriptional level, especially in human cells, are limited. Several signaling pathways have been shown to regulate S100A4 in different types of cancer. For example, ERBB2 regulates S100A4 in medulloblastoma (*43*), while, in colon cancer, β-catenin/TCF directly regulates S100A4 which induces migration and invasion *in vitro* and metastasis *in vivo* (*44*). Another important mechanism of S100A4 regulation is through epigenetic control. Specifically, studies show that CpG sites in the first intron region of S100A4 are hypomethylated in several cancers but are hypermethylated in normal or non-metastatic cancer cells (*45, 46*). Currently, there is no mechanistic evidence to show how S100A4 promoter is demethylated. Furthermore, treatment of these cells with DNA methyltransferase inhibitor re-expresses S100A4 (*46, 47*). In addition, the cooperation of signaling pathways involved in S100A4 regulation and the epigenetic alteration is unknown. In Chapter 3, by using a breast cancer model, I provide evidence to demonstrate that the ubiquitously expressed Nuclear Factor in Activated T cell 5 (NFAT5) in conjunction with DNA methylation play important role in the regulation of S100A4 downstream of integrin α6β4 (*48*).

1.4. Integrin α6β4 and cancer progression

Integrins are receptors for the extracellular matrix (ECM), which are composed of a non-covalently linked α-subunit and β-subunit. The combination of α-subunits and β-subunits defines the specificity of extracellular ligand for integrin. In general, integrins have two major functions. The first is an adhesive function that secures cells to the surrounding ECM or, in the case of cell motility, provides traction for locomotion. Their second function is to transduce signals that are essential for cells to sense and integrate cues from the extracellular matrix, which include signals for directed cell motility, anchorage-dependent survival, and growth (*49, 50*). As a result, integrin signaling and function are critical for most biological events in higher eukaryotes, both under normal and pathological conditions. In recent years, one integrin species, the α6β4 integrin, which was originally identified as tumor-specific antigen-180 (*51*) has garnered much attention for its ability to promote an invasive and metastatic phenotype in different types of carcinomas including breast, colon, and pancreatic carcinomas (*52-54*).

Integrin α6β4 is unique among integrins due to its extremely large cytoplasmic domain (*55*) and serves as the receptor for laminin (*56*). It is mostly expressed in epithelial tissue, endothelial and Schwann cells (*57*). In cells of epithelial origin, the integrin α6β4 nucleates the formation of hemidesmosomes that link the cytokeratin cytoskeleton to the laminins found in the basement membrane, which are essential for epithelial integrity (*58*). During wound healing or the epithelial to mesenchymal transition (EMT), the α6β4 integrin is phosphorylated, released from hemidesmosomes and then binds the actin cytoskeleton (*59*). Under these conditions, the α6β4 integrin promotes cell motility (*60*). Increased expression of the α6β4 integrin is a poor

prognostic factor for breast cancer (*61, 62*), as well as various solid tumors (*63, 64*), and is associated with an invasive (*65, 66*) and metastatic phenotype (*67*). Exogenous expression of the α6β4 integrin in MDA-MB-435 cells substantially increased the ability of these cells to form lamellae, polarize, migrate (*68*) and invade a reconstituted basement membrane (Matrigel) (*69*). Importantly, these observations have been extended to the MDA-MB-231 (*70*) and Sum159 (*71*) cell lines and have been validated *in vivo* in the ErbB2 breast cancer mouse model where targeted deletion of the β4 subunit reduces tumor invasion and progression (*72*).

Integrin α6β4 signaling also activates the PI3K/Akt pathway (*66, 73*), cooperates with several receptor tyrosine kinases (RTK) pathways such as EGFR signaling (*74*) and contributes to cancer cell survival and angiogenesis. However, studies on the mechanisms of how α6β4 integrin contributes to tumor invasive phenotype are limited. The capabilities that the α6β4 integrin can activate several transcription factors such as NF-κB (*75, 76*) and AP-1 (*72*) and NFATs (*77*) suggest that integrin α6β4 can regulate some pro-invasive and pro-survival genes expression. In Chapter 3, I present that a tumor metastasis associated gene, S100A4 is regulated by integrin α6β4 through NFAT5 and plays an important role in breast cancer chemo-invasion.

1.5. NFAT and cancer progression

NFAT family is closely related to Rel/NF-κB transcription factor and consists of five members: NFAT1 (also known as NFATc2 and NFATp), NFAT2 (also known as NFATc1 and NFATc), NFAT3 (also known as NFATc4), NFAT4 (also known as NFATc3 and NFATx), and NFAT5 (also known as tonicity enhancer binding protein, TonEBP) (*78, 79*). Among them, NFAT1-4 is regulated by the calcium-calcineurin signaling pathway. NFAT5 is not regulated by

calcium-calcineurin signaling due to lack of a calcineurin binding domain. While they were initially identified as the DNA-binding factors in T cell activation, NFATs have been found ubiquitously expressed in all tissues and cells and play fundamental roles in immune system and developmental program (*79*).

The role of NFATs in cancer progression is emerging (*78*). NFATs have been implicated in different types of cancers such as B cell lymphomas, chronic myeloid leukemia, T cell acute lymphoblastic leukemia (*79*) and several solid tumors including pancreatic (*80, 81*), breast, and colon cancers (*82-85*). An important study implicating that NFATs, particularly NFAT1 and NFAT5, are pro-migratory and pro-invasive transcription factors is through their link with integrin α6β4 signaling in breast and colon cancers (*77*). Downstream of integrin α6β4, both protein level and activity of NFAT1 and NFAT5 are increased, interestingly; NFAT1 and NFAT5 expression are correlated with α6β4 integrin expression in tumor samples from patients with invasive ductal breast carcinoma (*77*). Importantly, expression of active NFAT1 and NFAT5 promotes distinct migratory and invasive phenotypes in breast cancer cell lines (*77*). This study suggested that a different NFAT isoform may regulate a non-overlapping subset of gene expression (*77, 78*). Our lab and others demonstrated that NFAT1 regulates autotaxin in breast cancer cells (*85*) and Cox-2 in colon (*84*) and breast cancer cells (*83*). These observations support the hypothesis that integrin α6β4 promotes tumor migration and an invasive phenotype through activation of select transcription factors, which in turn regulates pro-invasive gene expression. In Chapter 3, I demonstrate that integrin α6β4 affects S100A4, a tumor metastasis related protein, gene expression through chromatin remodeling. In addition, I identified that

downstream of integrin α6β4, NFAT5 plays important roles in regulation of S100A4 in breast cancer cells.

1.6. NFAT5 and osmotic stress

NFAT5 mRNA expression is ubiquitous; however, detection at the protein level is more restricted. NFAT5 can be activated by T-cell activation (*86*), integrin α6β4 clustering (*48*), and hyperosmotic stress (*87*). Among them, hyperosmotic stress-mediated activation of NFAT5 is well-characterized and is of particular interested because it is the only documented osmotic stress responsive transcription factor in mammalian cells; therefore, NFAT5 is also called tonicity responsive enhancer binding protein (TonEBP). NFAT5 is a hybrid between NFATc and NF-κB family (Rel family) according to its structure and DNA homology. Like the other transcription factors, NFAT5 contains an N-terminal nuclear export signal (NES), a transactivation domain (AD1), a putative nuclear localization signal (NLS), Rel-homology domain (RHD), two modulation domains (MD1 and MD2) and two other activation domains in the C-terminal (AD2 and AD3) (*88, 89*). However, several special properties make it stand out from NFATc and NF-κB. Unlike the other NFATc, NFAT5 forms constitutive dimer in order to bind DNA with its dimerization surface in the C-terminal half of the RHD. NFAT5 binds a stricter DNA sequence such as TGGAAC/A/T than NFATc (*87, 88, 90*). Compared to NF-κB, which forms a heterodimer, there is no evidence to show NFAT5 dimerizes with other NFATc. Furthermore, no cytoplasm protein which has the similar function with IκB has been found for NFAT5 (*87*).

In response to hyperosmotic stress, NFAT5 is controlled at multiple levels, such as protein abundance, nuclear translocation, and transactivation; but the relative contribution at

each level in different system has not been fully studied. Like other transcription factors, NFAT5 is bidirectionally trafficked between the nucleus and cytoplasm due to its NLS and NES. 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 as TNF-α which is unrelated to osmoregulation (*91, 92*). In addition, it has been suggested that several kinases such as p38, ATM, Fyn, and PKA are involved in activation of NFAT5 under hyperosmotic stress, however, whether these kinases can activate NFAT5 through direct or indirect phosphorylation remains controversial (*93-97*).

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*., showed that both lymphoid and liver microenvironments have higher osmolarity as compared with serum (*98*). Gastrointestinal epithelia are exposed to elevated postprandial osmolarity (*99-101*). Finally, several studies have shown that osmotic stress can induce proinflammatory cytokine production, Cox-2, and drug metabolizing enzymes, such as cytochrome P450 3As expression in GI cancer cells, thus suggesting that osmotic stress may have physiological relevance to GI function (*92, 102*).

Based on the regulation of S100A4 by NFAT5 (*48*), and given that the colon is periodically exposed to osmotic stress (*103*), I proposed that hyperosmotic stress induces NFAT5-mediated S100A4 expression in colon cancer cells. In Chapter 4, I provide evidence that hyperosmotic stress induces S100A4 expression in a subset of colon cancer cells with a

hypermethylated first intron region. I further identified 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, I found that the Src kinase pathway is involved in this NFAT5-mediated regulation of S100A4. I also demonstrated that the induction of S100A4 plays an important role in cell survival under the condition of hyperosmotic stress.

1.7. Rho signaling and tumor cell migration, invasion and metastasis

Rho small GTPases belong to the Ras superfamily and consist of at least 20 members of 20-30 KDa GTP-binding proteins in mammalian cells (*104*). Like Ras, most Rho GTPases act as the molecular switch in many cellular processes and cycle from GTP bound active state to GDP bound inactive state. The cycling between these two states is controlled by the positive regulators, guanine nucleotide-exchange factors (GEFs), and the negative regulators, GTPase activating proteins (GAPs) and guanine nucleotide-dissociation inhibitors (GDIs) (*104*). The major function of Rho small GTPases is to regulate actin cytoskeleton reorganization in response to receptor activation which in turn regulates GEF (*105*). The three best characterized Rho GTPases including Rho (A, B, C isoforms), Cdc42, and Rac, have been documented to play distinct roles in actin reorganization mainly through its various effectors. For example, RhoA is involved in stress fiber formation and focal adhesion, Cdc42 is engaged in filopodial formation, and Rac regulates lamellipodia and ruffle formation. Studies also showed that these three Rho small GTPases coordinate signaling during cell migration (*106, 107*). In addition, through their

effectors, small Rho GTPases are involved in a variety of signaling pathways such as gene regulation, vesicle trafficking, cell cycle progression, and transformation (*108*).

The effectors of small Rho GTPases such as Rho, Cdc42 and Rac comprise of a variety of proteins including lipid kinases, scaffold proteins, and serine/threonine kinases (*109*). Specifically, Rho effectors were classified into three categories: class I includes Rhotekin, Rhophilin and protein kinase N (PKN); class II includes ROCK I and II; and class III includes Citron. The mammalian homolog of Drosophila diaphanous (mDia) was also considered as another class of Rho effector (*110*). Among them, ROCK and mDia have been extensively studied. The role of RhoA in cell migration at one time was considered dispensable. Constitutive activation of RhoA negatively regulates cell migration due to excess stress fiber formation and adhesion forces (*111, 112*), which is mediated by Rho/ROCK signaling. ROCK inactivates myosin phosphatases by phosphorylation of its myosin-binding subunit as well as directly activates myosin light chain and as a consequence, enhances actomyosin contractility (*110*). However, in cells with epithelial origin, RhoA is active in the leading edge, promotes membrane ruffling and facilitates cell motility (*113-116*). One study also showed that Rho is required for cell migration in mouse embryonic fibroblast through mDia (*117*). In addition, ROCK and mDia also cooperates or antagonizes each other in Rho-induced actin reorganization (*118, 119*). Interestingly, these studies were done in epithelial cells and fibroblasts respectively. How RhoA regulates different aspects of actin reorganization in fibroblast and in epithelial cells is unknown.

The Rho subgroup of Rho GTPases has three members: RhoA, RhoB, and RhoC which share about 85% amino acid sequence identity (*120*). Given that Rho plays important roles in cell migration, actin cytoskeleton reorganization, and focal adhesion, it is well accepted that Rho

signaling should play roles in tumor invasion and metastasis. Indeed, RhoA and RhoC have been shown to be involved in different stages of tumor progression such as loss of cell polarity and cell junctions, intravasation and vascularization (*121*). There is a substantial amount of evidence to support the involvement of aberrant expression of Rho, especially RhoC in the metastasis capacity of different types of cancers such as breast, colon, prostate, lung, head and neck, and pancreatic cancers (*121, 122*). In contrast, most studies suggest that RhoB acts as a tumor suppressor (*122*). Although RhoA, B, and C share overlapping effectors, whether the preference of each isoform to different effectors contributes to distinct effect on cell behavior is not well understood.

Rhotekin was initially identified as a putative target for Rho and interacts with RhoA and RhoC equally well (*123*). Rhotekin is also a scaffold protein. The search for Rhotekin interacting proteins had been focused on the C-terminal domain since it contains a consensus binding motif for class I PDZ proteins. Recent studies showed that Rhotekin interacts with vinexin, Lin7B, PIST, and septin which are considered to play some roles in cell polarity, focal adhesion, and septin organization (*124-126*). Rhotekin was also found to be overexpressed in metastatic colon cancer cells (*127*) and gastric adenocarcinoma cells and confers resistance to apoptosis through activation of NF-κB (*128*). In addition, interaction of Rhotekin and TIP-1 together with active Rho strongly activate SRE (serum response element) (*129*). Although being classified as a class I Rho effector, the role of Rhotekin involved in Rho-mediated downstream signal transduction such as actin cytoskeleton reorganization remains largely unknown.

In Chapter 3, I demonstrate that S100A4 is essential for breast cancer chemoinvasion. The pro-migratory effects of S100A4 are partially due to the interaction between S100A4 and cytoskeleton proteins such as non-muscle myosin IIA. S100A4 colocalizes with myosin IIA at the leading edge of migrating cells (*130*) and affects myosin IIA assembly which, in turn, promotes directional cell migration (*131, 132*). These observations imply that S100A4 could serve as a regulator of actin cytoskeleton reorganization. In Chapter 5, I provide evidence to show that Rhotekin is a binding partner for S100A4, thus suggesting the connection between S100A4 with Rho signaling. I also demonstrate that the cooperative signaling between S100A4 and Rhotekin promotes membrane ruffling in EGF-stimulated MDA-MB-231 cells while suppressing stress fiber formation. These observations indicate a switch in Rho signaling to facilitate lamellar formation and invasive growth in 3D.

CHAPTER 2: MATERIALS AND METHODS

2.1. Cell lines and treatments:

Breast carcinoma cells MDA-MB-435 were stably transfected with vector only (MDA/mock, clones 6D2 and 6D7) or the integrin β4 subunit cDNA (MDA/ β4, clones 5B3 and 3A7) were obtained from Arthur M. Mercurio (University of Massachusetts Medical School, Worchester (*69*)); MDA-MB-468 and BT-20 cells from Janet Price (UT MD Anderson, Houston, Texas); and all other breast cancer cell lines including MDA-MB-231 cells from ATCC. Cells were cultured as described previously (*68, 133*). For all studies, cells were given fresh growth medium the day prior to harvest and harvested at 70% confluence.

Colon cancer cell lines: Clone A, LS174T, HCT-8, and DLD-1 cells were maintained in RPMI1640 (osmolarity = 279 mOsml); SW480 and SW620 in high glucose DMEM (osmolarity = 309 mOsml); HT-29 and HCT-116 in McCoy medium (osmolarity = 298 mOsml); KM12C and KM20 in Eagle's Minimum Essential Medium (osmolarity = 304 mOsml) supplemented with 1 mM sodium pyruvate, non-essential amino acids and MEM Essential Vitamin mixture; and Caco2 cells in MEM plus 1% non-essential 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 hrs,

then cells were rinsed 3 times with media; then, fresh isotonic media were added and cultured for the times noted. The expression of S100A4 was determined by western blot and Q-PCR.

2.2. Antibodies and Reagents:

Integrin β4 clone 439-9B (Chemicon-Millipore), integrin α6 clone GoH3 (Chemicon-Millipore), NFAT1 (Santa Cruz; cat. #sc-7296), NFAT5 (Affinity Bio-Reagents; cat. #PA1-023), and S100A4 for Western blot (gift from Dr. Anne Bresnick, Albert Einstein College of Medicine) (*131*). S100A4 for Inmmunostaining (Dako), anti-c-myc coupled agarose beads (clone 9E10, Santa Cruz Biotechnology), monoclonal anti-Flag (clone M2, Sigma), HA-probe (F-7, 1:100, Santa Cruz) Alexa Fluor 488 goat anti-rabbit IgG (molecular Probes), Cy3-conjugated Affinitpure Donkey anti-mouse IgG (Jackson immune Research), Phalloidin-TRITC (Sigma) Actin (Sigma, cat #A5441). Stripping solution (Pierce), Matrigel (BD Bioscience)

2.3. DNA methylation analysis:

For DNA methylation status analysis, cells were treated with 0.1 or 1 μM 5-aza-2′-deoxycytidine (DAC) or 80 μM S-adenosyl-methionine (SAM) in fresh medium daily for 3 or 5 days or 100 mM excess NaCl for 24 hrs as note. Tricostatin A (TSA; 1μM) treatment was given 24 hrs before harvest. The 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 (+147 to +600; accession number Z33457) and was performed by EpigenDx (Worcester, MA).

2.4. Plasmids and transfection:

NFAT5 luciferase reporter was obtained from Addgene and contained three consensus NFAT5 sites from promoter region of human aldose reductase gene that was cloned into a pGL3 luciferase reporter with minimal SV40 promoter (*92*). 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 promoter. Genomic DNA was purified by GenElute^{TM} 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' GGGGTACCACAAGGTCCTCTGTGTTGCTC 3', anti-sense 5' GAAGATCTGACAGCAGTCAGGATCTGGGA 3', and anti-sense 5' GAAGATCTAGTCAGGCCAACACAACTCACC 3'. Primers used to generate pGl4.10 S100A4 (+58/+1010) were: sense 5'GGGGTACCGTGAGTTGTGTTGGCCTGACT and anti-sense 5' GAAGATCTGACAGCAGTCAGGATCTGGGA 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'-GCGGCTGTGCCTGGTTTCTCCTACTGCAGGCCCC-3' and those for NFAT5 site 5 were based on: 5'- GTGGATAGACTGAGTGAGGGGCGAACAAAATGGTGTTGTTGAGCAAG-3'. All of the constructs and their mutants were confirmed by sequencing.

For luciferase reporter assays, cells grown in 24-well plate were co-transfected 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 hrs. Then, cells were induced by 100 mM excess NaCl or left in isotonic medium for 24 hrs. 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 co-transfection of NFAT5 transactivation reporter, GalDBDNFAT5AD (Dr. Maurice B. Burg, NIH) and Gal4 luciferase reporter pGL4.31 (Promega) together with pRL-TK Renilla control reporter. After Twenty four hours, cells were pretreated with 10 µM PP2 or PP3 for 30 mins, and then cells were exposed to hyperosmotic stress for 24 hrs prior to luciferase activity assay.

Rhotekin constructs: Flag-RTKN-FL, Flag-RTKN-ΔRBD, Flag-RTKN-Cent, Flag-RTKN-C, Myc-RTKN-FL, Myc-RTKN-ΔRBD, Myc-RTKN-RBD, Myc-RTKN-Cent, and Myc-RTKN-C were obtained from Dr. Kohichi Nagata (Institute for Developmental Research, Alchi human Service Center, Alchi, Japan) and had been described (*134*). Plasmids pGEX-4T-1-mouse mDia RBD (aa −2 to 304), pGEX-4T-3-mouse ROCK-II RBD (aa 800–1137) and pGEX-mouse citron RBD (aa 1124–1286) were obtained from Dr. Shuh Narumiya (Kyoto University Faculty of Medicine, Japan) and describe previously (*135*).

For construction of plasmid of HA-RTKN-FL, mouse cDNA was used as the template to amplified the full-length RTKN and inserted into EcoR V and Xba I site of pcDNA3.1-HA vector by using the following primers: mRTK-HA-FL-Sense 5' GCGATATC

ACAGATTGCGCATCCTGGA 3', mRTK-HA-FL-Antisense 5'GCTCTAGA TGACTTCATCACAACAGTGCCT 3'.

GST-class I Rho effector RBD constructs: for construction of plasmids GST-Rhophilin2 RBD and GST-PKN1-RBD, cDNA from MDA-MB-435 cells was used as the template. The following primers are used to amplify RBD domain of Rhophilin2 and PKN1. GST Rhophilin-2RBD: sense 5' CGGGATCCCAGCCGCTGGAGAAGGAGAA 3', antisense: 5' CGGCTCGAG GCATCTGCAGGTCTGAGTTGACG 3'. GST-PRK1RBD: sense 5' CGGGATCCCAGAGTGAGCCTCGCAGCTGGTCC 3', anti-sense 5' CCG CTCGAG GGGAAGCACCACGTGGGCGT 3'. The PCR products were inserted into BamH I and Xho I site of pGEX-6P-3 vector. All of the plasmids were confirmed by sequencing.

2.5. Western blot:

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, 1 mM PMSF) were separated by appropriate percentage of acrylamide (SDS-PAGE, reducing conditions), transferred to PVDF membrane and probed with the indicated antibodies.

2.6. Q-PCR:

Total RNA (1µg, extracted from cells using Trizol reagent) was used to prepare cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Expression of target genes was then assessed by Comparative Ct ($\Delta\Delta$Ct) using commercially available probes

and master mix reagent and performed on a StepOnePlus[TM] 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.

2.7. Chromatin immunoprecipitation (ChIP):

ChIP assays were performed as modified from the protocol published previously (*48*). 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 1μg 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 TE buffer, and used as templates for PCR amplification by using the following primers: sense 5' ATGGCCTCTGCAGCTTCTCTT 3' and anti-sense 5' TGCGCAAGTCTTGGAGATTCG 3'.

2.8. Cell fractionation:

Cells were pretreated with 10 μM PP2, PP3 (Calbiochem), or DMSO for 30 mins 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).

2.9. Invasion assays:

Matrigel (5 µg BD Biosciences) was dried onto the upper side of Transwell chambers (6.5 mm diameter, 8 µm pore size; Corning). One hour before the assay, Matrigel was rehydrated with DMEM and the bottom surface was coated with 10 µg/ml laminin-1. 100 nM LPA or 50 ng/ml HGF in DMEM/BSA or DMEM/BSA was added to lower chamber. Cells ($5x10^4$) were placed in the top chamber and allowed to invade for 4 hrs. Non-invading cells in the top chamber were removed using a cotton swab and cells in the bottom chambers were fixed with methanol, stained with 1% crystal violet and quantified visually. Values for triplicate membranes are reported as a mean value ± the standard deviation as described previously (*68*).

2.10. shRNA and siRNA treatment:

For stable knockdown of β4 integrin expression, cells were stably transfected with pLKO.1-puro lentiviral constructs (Sigma) containing one of two shRNAs targeting β4 (#4; CCGGGAGGGTGTCATCACCATTGAACTCGAGTTCAATGGTGATGACACCCTCTTTTT G and #5, CCGGCGAGGTCACATGGTGGGCTTTCTCG AGAAAGCCCACCATGTGACCTCGTTTTTG) or a control sequence (#2, CCGGCCCATGAAGAAAGTGCTGGTTCTCGAGAACCAGCACTTTCTTCATGGGTTTTT G)

For stable reduction S100A4 expression in MDA-MB-231 cells, Lentivirus-mediated shRNA construct pLKO.1-puro targeting human S100A4 sequence or non-targeting sequence were obtained from Sigma-Aldrich. The targeting sequence for human S100A4 is 5' CGCCATGATGTGTAACGAATT 3'. Virus packaging was done as described previously (*136*).

In brief, the control or S100A4-specific shRNA construct was co-transfected with Mission lentiviral packaging mix (Sigma-Aldrich) into 293T cells using polyethylenimine (PEI) based on 1:3 ratio of DNA (1μg): PEI (3μg). The viral supernatant was collected 48 hrs after transfection. Then MDA-MB-231 cells were infected with virus containing media and stable transfectants were selected with puromycin (2μg/ml).

For siRNA electroporation: Cells from 70% confluent cultures were suspended by trypsinization and rinsed three times with DMEM. Cells (3×10^6) were electroporated with 200 nM siRNAs specific for an individual target or a control (non-targeting) sequence (Dharmacon, Inc.) as reported previously (*85*). Individual sequences for NFAT5 are CAACAUGCCUGGAAUUCAAUU (#3) and CAGAGUCAGUCCACAGUUUUU (#5). Dharmacon SMARTPool siRNAs were used for all other targets. Cells were then kept in normal growth medium for 24-96 hrs and then assessed for target gene expression using Q-PCR and immunoblotting analysis as indicated.

2.11. MTT assay:

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

2.12. RhoA activity, GST-fusion protein affinity binding, and immunoprecipitation assays:

RhoA activity was assessed with the Rhotekin binding assay as described previously (*115*). GST-fusion protein affinity binding and immunoprecipitation assays were carried out in lysis buffer (50 mM Tris, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 10 µg/ml protease inhibitor cocktail, and 1 mM PMSF). GST-fusion protein coupled beads (35µl) were incubated with cell lysates or purified S100A4 for 30 min at 4°C. For immunoprecipitation, 10 µl of anti-c-myc coupled agarose beads (clone 9E10, Santa Cruz Biotechnology) and 1µg monoclonal anti-Flag (clone M2, Sigma) were incubated with precleared cell lysates at 4°C overnight. Then the beads were rinsed 3 times with washing buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 10 mM $MgCl_2$, 10 µg/ml protease inhibitor cocktail [Sigma], and 1 mM PMSF). The GST-beads coupled protein and immunoprecipitated proteins complexes were resuspended in 2× Laemmli SDS sample buffer, separated by SDS-PAGE and immunoblotting as indicated.

2.12. Immunocytochemistry staining:

Cells ($2.5x10^4$) from different treatments as noted were seeded on glass coverslips coated with 50 µg/ml collagen I (BD Bioscience) for 2 hrs, then cells were treated with 5 ng/ml EGF (Pepro Tech) for 5 mins. Cells were fixed, permeabilized, and immunostained as done previously (*115*). Primary antibodies anti-S100A4 (1:400, Dako) and/or HA-probe (F-7, 1:100, Santa Cruz) and secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (molecular Probes) or Cy3-conjugated Affinitpure Donkey anti-mouse IgG (Jackson immune Research) were used.

Phalloidin-TRITC (Sigma) was used to stain F-actin. The slides were mounted in VECTASHIELD mounting medium for fluorescence (Vector Laboratories, Inc) and analyzed with confocal microscopy or total internal reflection fluorescence microscopy (TIRF).

2.13. Three-dimensional culture:

MDA-MB-231 cells were grown in three-dimensional culture as done previously to assess invasive growth (*137*). Briefly, 100 μl of growth factor reduced Matrigel (BD Bioscience) were added into each wells of 8-well chamber slide. Cells (5000) in 200 μl DMEM/F12 plus 2% FBS were seeded into each well after Matrigel was solidified. Then 10% of Matrigel in medium were loaded on top of the cells. The medium were changed every other day. After 3 days, the samples were observed under microscope. Eight representative fields for each condition were chosen to assess the percentage of invasive growth. Then 20 μl of Matrigel containing colonies were smeared onto slides, fixed with 4% PFA, permeabilized and immunostained as described above.

2.13. Statistical analysis:

Data were analyzed by t-test and presented as mean value ± SD. The significant level was set up at 95% confidence. $P < 0.05$ was considered as statistically significant.

CHAPTER 3: INTEGRIN α6β4 CONTROLS EXPRESSION OF S100A4/METASTASIN-1 IN BREAST CARCINOMA CELLS[2]

3.1. Abstract

The integrin α6β4 is associated with carcinoma progression by contributing to apoptosis resistance, invasion and metastasis, due in part to the activation of select transcription factors. To identify genes regulated by the α6β4 integrin, gene expression profiles of MDA-MB-435 cells that stably express integrin α6β4 (MDA/β4) and vector-only transfected cells (MDA/mock) were compared and analyzed using Affymetrix GeneChip® analysis. The results show that there are 36 genes implicated in cell motility and metastasis including S100A4/metastasin are altered by integrin α6β4 expression. S100A4 expression correlated well with integrin α6β4 expression in established cell lines. Suppression of S100A4 by siRNA resulted in a reduced capacity of α6β4 expressing cells to invade a reconstituted basement membrane in response to LPA. Using siRNA, promoter analysis and chromatin immunoprecipitation (ChIP), I demonstrate that S100A4 is regulated by NFAT5, thus identifying the first target of NFAT5 in cancer. In addition, inhibition

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of DNA methyltransferases stimulated expression of S100A4 in cells lacking the α6β4 integrin, whereas demethylase inhibitors suppressed expression in α6β4 integrin expressing cells. Alterations in DNA methylation were confirmed by bisulfate sequencing, thus suggesting that integrin α6β4 signaling can lead to the demethylation of select promoters. In summary, the data suggest integrin α6β4 confers a motile and invasive phenotype to breast carcinoma cells by regulating pro-invasive and pro-metastatic gene expression.

3.2. Results

3.2.1. S100A4 is upregulated by overexpression integrin β4 in MDA-MB-435 cells

NFAT and AP-1 are transcription factors that can promote tumor invasion that are known to signal downstream from the α6β4 integrin (*72, 77*). However, the extent of the changes in gene transcription and what genes are altered as a result of integrin α6β4 signaling has not been established. To test the hypothesis that the α6β4 integrin can regulate the expression of genes that can promote a motile and invasive phenotype, Affymetrix GeneChip® analysis on MDA-MB-435 clones that were mock transfected (MDA/mock, clones 6D2 and 6D7) or stably transfected with the α6β4 integrin (MDA/β4, clones 3A7 and 5B3) was performed and analyzed as published previously (*138*). The results showed that there are 36 genes reported to affect cell motility were altered by integrin α6β4 expression (*48*). I chose S100A4 that was associated with cell motility, invasion and metastasis for further investigation.

S100A4, also known as fibroblast specific protein or metastasin, is a metastasis-associated protein documented to promote the metastatic process in several types of cancer including breast, gastric, pancreatic and thyroid cancers (*1*) . As shown in Figure 3.1A, S100A4

expression in the β4-expressing cells is over 140-fold higher than non-expressers as determined by Q-PCR. This overexpression extends to both increased intracellular and extracellular protein levels (Fig. 3.1B).
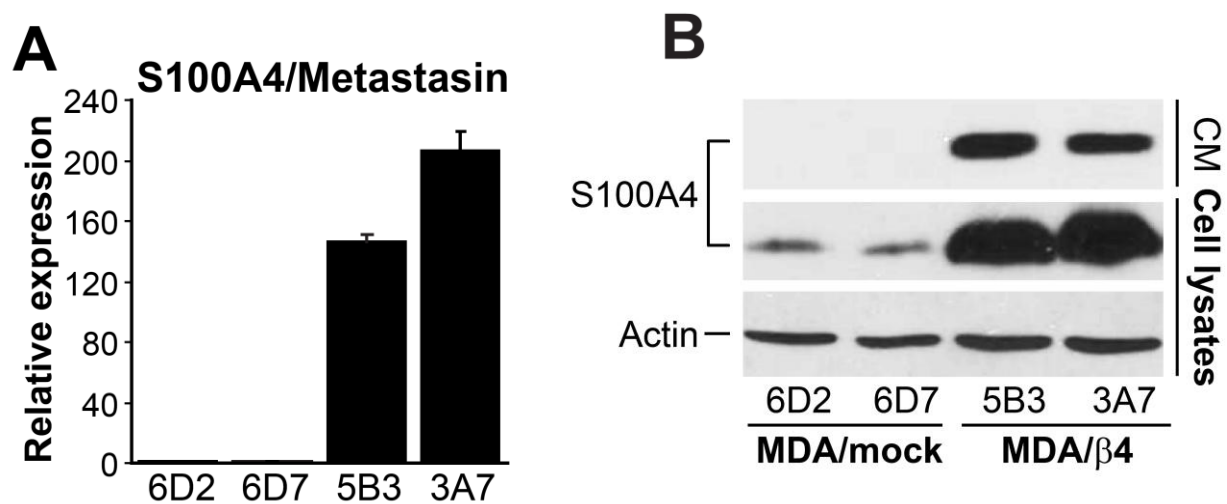
Figure 3. 1. <u>S100A4 is upregulated by α6β4 integrin expression.</u>

Figure 3.1. Total RNA (**A**) or protein (**B**) was isolated from the MDA-MB-435 clones 6D2 and 6D7 (MDA/mock; null for the β4 subunit) and 5B3 and 3A7 (MDA/β4; express the α6β4 integrin) and assessed for S100A4 expression by Q-PCR (A) or immunoblotting (B). For extracellular S100A4 (B), conditioned media (CM) represents 50 μl of serum free medium removed from cultures just prior to harvesting the cells for protein. For Q-PCR, expression was normalized to 18S rRNA levels and reported as a value relative to the clone 6D2. Values represent the mean ± the standard deviation.

3.2.2. S100A4 expression is correlated with integrin β4 expression in breast cancer cell lines

To determine if S100A4 expression correlated with integrin α6β4, I assessed various breast carcinoma cell lines for S100A4 by immunoblotting (Fig. 3.2A). The cell surface expression of the β4 integrin was performed by FACS (Fig. 3.2B). Notably, expression of S100A4 is found in all cell lines that express the α6β4 integrin with the exception of MCF7 cells. Of these cells, MCF7 is the only cell line that does not display a mesenchymal phenotype. Interestingly, some cell lines showed higher molecular weight bands that run at a molecular mass equivalent to a trimer (≈ 35kD) or tetramer (≈ 47kD) of S100A4. To confirm conclusively that these bands are specific for S100A4, I electroporated BT-20 cells with siRNA specific for S100A4 or a non-targeting control (NT) prior to immunoblotting for S100A4. As shown in Figure 3.2C, the S100A4 siRNA effectively reduced the expression of the higher molecular weight bands, thus confirming that these bands represent S100A4. Therefore, these data demonstrate that expression of S100A4 correlates well with expression of integrin α6β4.

3.2.3. S100A4 is important for chemo-invasion in breast cancer cells

To determine whether S100A4 contributes to the invasive phenotype mediated by the α6β4 integrin, I utilized MDA-MB-231, the highly invasive breast carcinoma cell line that has been previously shown to utilize the α6β4 integrin for chemoinvasion (*70*). S100A4 expression was suppressed using specific siRNAs. Chemoinvasion assays were performed on S100A4 siRNA, non-targeting siRNA treated cells, or untreated cells and compared. LPA was used as the chemoattractants since LPA signaling cooperates with the α6β4 integrin (*68*). The loss of S100A4 expression (Fig. 3.3A) reduced the invasion of MDA-MB-231 cells toward LPA by

approximately 70% compared to untreated or non-targeting siRNA transfected cells (Fig. 3.3B). These data indicate that S100A4 is important for tumor cell invasion, an activity that can predispose cells for metastasis.

3.2.4. NFAT5, but not NFAT1, in part regulates S100A4 expression

While the biochemical mechanisms governing how S100A4 contributes to an invasive and metastatic phenotype are becoming clear (*131, 139*), how S100A4 expression is regulated on the transcriptional level is poorly understood. Using bioinformatic analysis of the region, I find that the S100A4 promoter contains multiple NFAT consensus binding sites. As NFAT is known to function downstream of the α6β4 integrin, I tested the potential role of NFAT1 and NFAT5 in the regulation of S100A4 expression using specific siRNAs to target their downregulation in the MDA/β4 transfectants. As shown in Figures 3.4A and B, effective silencing of NFAT5, but not NFAT1, by specific siRNAs reduced S100A4 expression in the MDA/β4 cells. The reduction in S100A4 expression due to loss of NFAT5 was confirmed using two individual siRNAs to target NFAT5 (Fig. 3.4C, D). Of note, reduction of NFAT1 expression with the siRNA used here was shown previously to reduce autotaxin expression (*85*).

To determine definitively whether NFAT5 binds the S100A4 promoter, I performed ChIP analysis on the MDA/mock and MDA/β4cells. The second intron region 3' to the transcriptional start site (equivalent to the first intron in mice) contains a transcriptional enhancer that is critical for the regulation of S100A4. This region contains key CpG residues that suppress S100A4 expression when methylated (*140*). Notably, this region also contains two NFAT consensus binding sites. Here, I immunoprecipitated NFAT5 and assessed whether it was associated with

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this region of the S100A4 promoter. As shown in Figure 3.4E, immunoprecipitation of NFAT5, but not an IgG control, brought down this regulatory region of the S100A4 promoter in both the MDA/mock and the MDA/β4 cells. These data show that NFAT5 is definitively associated with the S100A4 promoter and suggest that negative regulators suppress NFAT5 action in the MDA/mock cells.

3.2.5. S100A4 is regulated by NFAT5 downstream of integrin α6β4

To verify the results with the MDA/β4 cells, I treated MDA-MB-231 cells, which endogenously express the α6β4 integrin, with siRNA targeting NFAT1, NFAT5 or a non-targeting siRNA. Previous studies demonstrated that MDA-MB-231 cells exhibit α6β4 integrin-dependent migration and invasion (*70*), a process facilitated by NFAT molecules (*77*). I found that S100A4 expression is reduced by siRNA targeting of NFAT5 in the MDA-MB-231 cells but not by targeting NFAT1 using both Q-PCR (Fig. 3.5A) and immunoblotting analysis (Fig. 3.5B). These data were confirmed using single duplexes targeting NFAT5 as performed with the MDA/β4 cells (Fig. 3.5C).

To confirm the role of integrin α6β4 in mediating S100A4 expression, I stably transfected MDA-MB-231 cells with commercially available lentiviral shRNA constructs targeting the β4 integrin subunit. Constructs 4 and 5, which reduce the cell surface expression of β4 integrin by 3 and 2-fold, respectively (data not shown), significantly decreased S100A4 expression (Fig. 3.5D). This is in contrast with construct 2, which was unable to reduce β4 integrin expression. Collectively, these data indicate that integrin α6β4 expression leads to the NFAT5-dependent transcriptional upregulation of S100A4.

A

S100A4

Oligomer

Monomer —

Actin —

		97
		66
		45
		31
		21
		14

Integrin α6β4 -   −   −   +   +   +   +   +   +   −   +

Cell Line -   Hs578T   BT549   MDA-175   MCF7   T47D   MDA-468   MDA-231   BT20   **Mock β4**

MDA-435

B
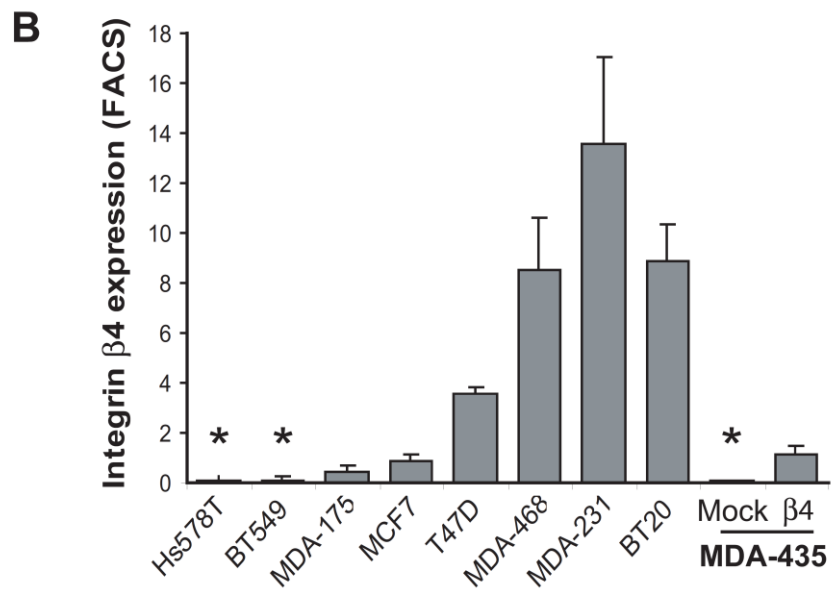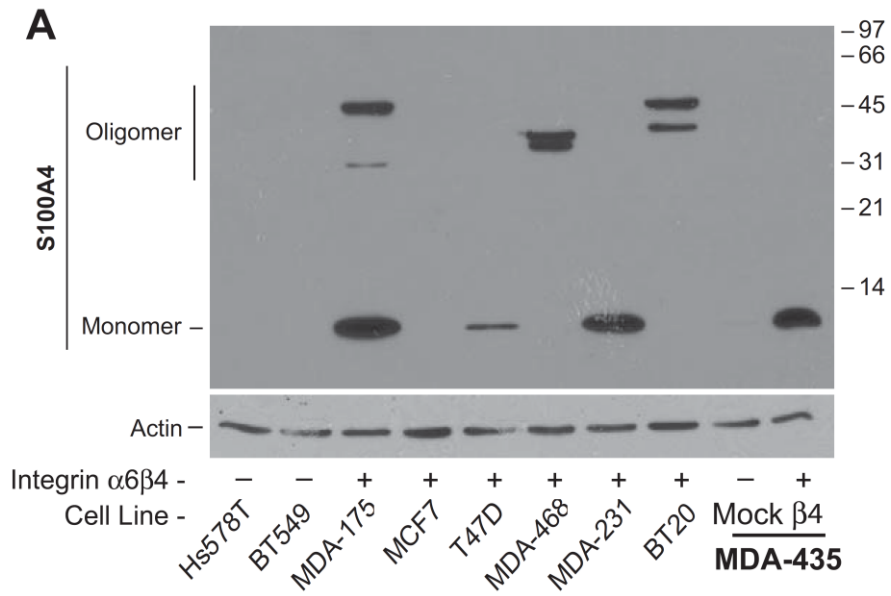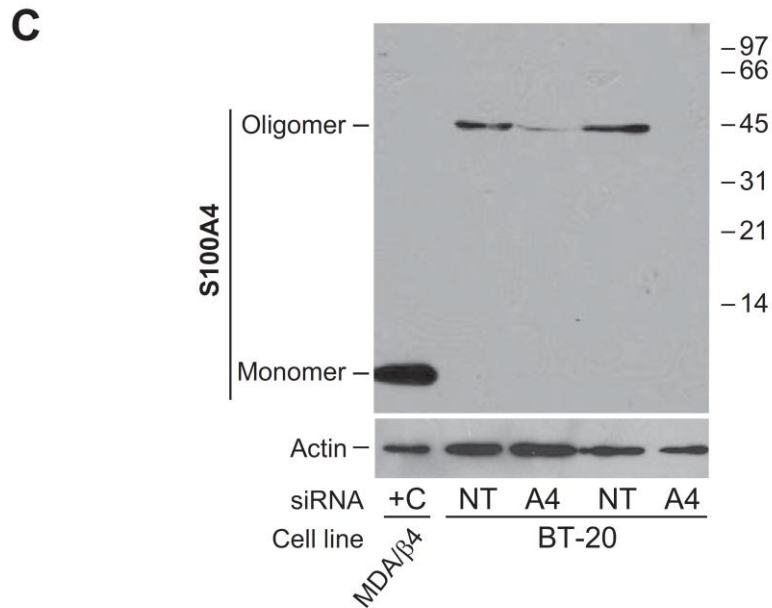
Figure 3.2. <u>S100A4 expression correlates with integrin α6β4 expression.</u>

Figure 3.2. (A) The indicated breast carcinoma cell lines and MDA-MB-435 clones were harvested at 70% confluence under normal culturing conditions. Cleared whole-cell lysates were submitted to SDS-PAGE and immunoblotted for S100A4 (upper panel) or actin (lower panel). **(B)** Cells were assessed for β4 integrin content by FACS analysis performed by L. Nicole Towers. Data are reported as the average fold difference in mean fluorescence as compared to secondary antibody only control ± standard deviation from three separate experiments. Asterisk (*) denotes cell lines also determined to be negative for β4 integrin expression by immunoblotting analysis (data not shown). **(C)** BT-20 cells were electroporated with 200 nM siRNA specific for S100A4 (A4) or non-targeting siRNA (NT), as noted, and then cultured for 48 hrs. Cells were then harvested and cell lysates immunoblotted for S100A4. Cell extract from a MDA/β4 transfectant serves as a positive control for the monomeric form (+C).
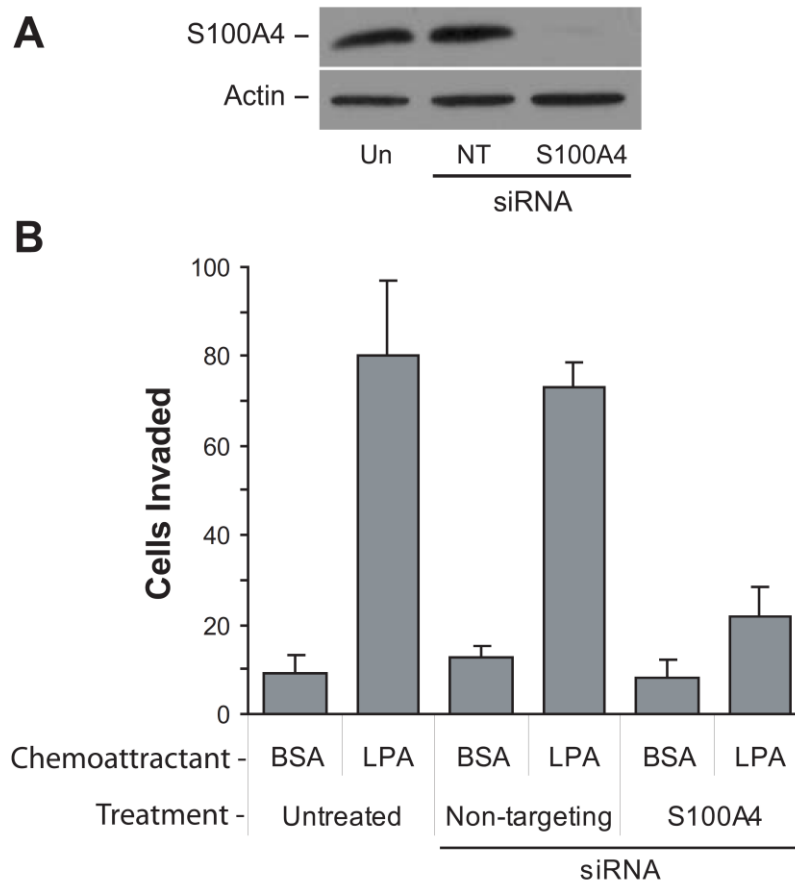
Figure 3. 3. <u>S100A4 is important for chemoinvasion of breast carcinoma</u>

Figure 3. 3. MDA-MB-231 cells were electroporated with nothing (Un), non-targeting siRNA (NT) or siRNA targeting S100A4. After 48hrs, cells were assessed for S100A4 expression by immunoblotting (A) or chemoinvasion toward 100 nM LPA (B) as described in the Experimental Procedures. Asterisk (*) denotes $p < 0.002$ for treated compared to untreated control and $p < 0.0001$ for treated compared to non-target control.
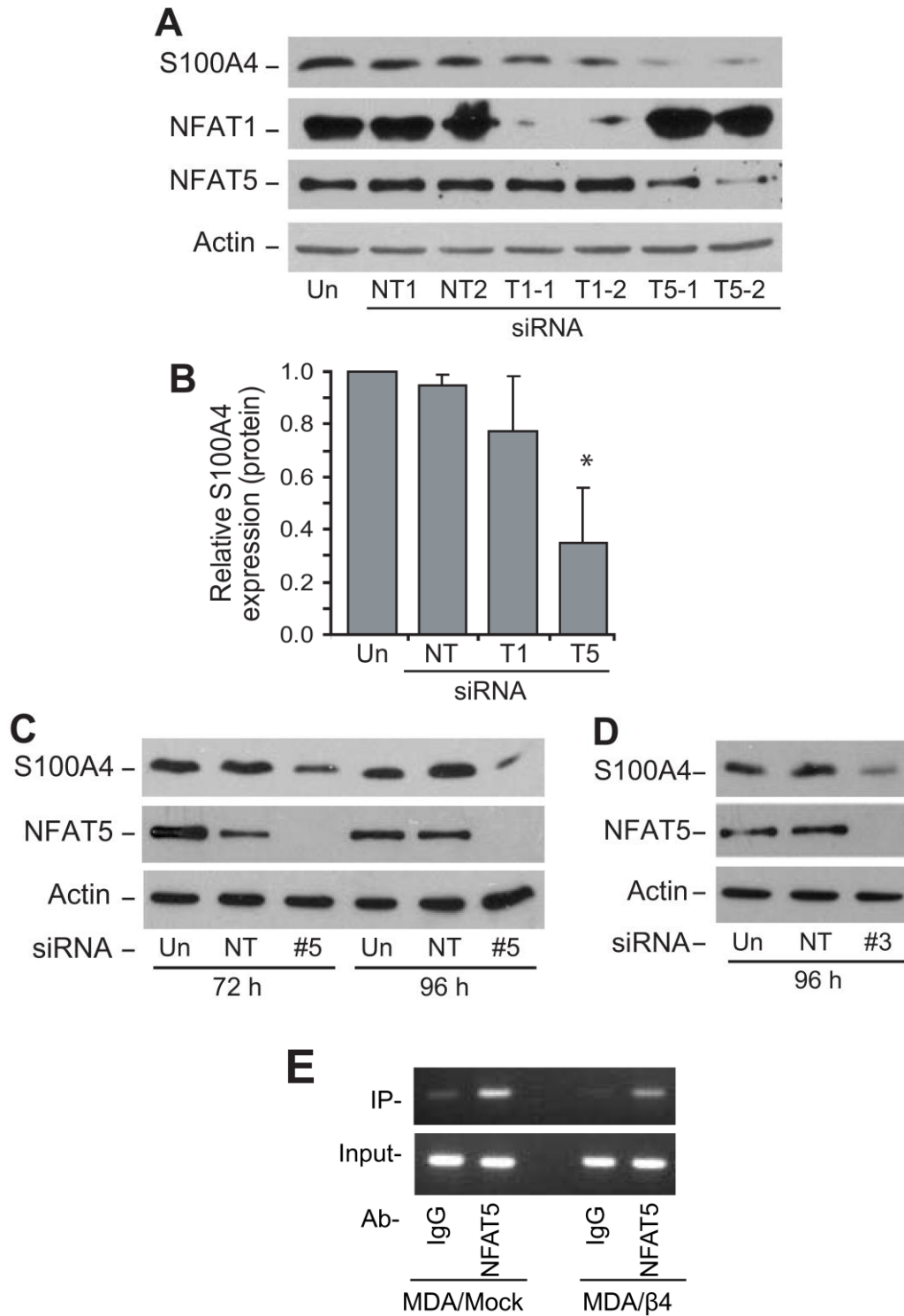
Figure 3. 4. NFAT5, but not NFAT1, controls the transcriptional regulation of S100A4 in MDA/β4 cells.

Figure 3.4. (Previous page) (A, B) MDA/β4 clone 5B3 cells were left untreated (Un) or transfected with either 200 nM (1) or 400 nM (2) of Dharmacon siRNA SMARTPools that are non-targeting (NT) or directed against either NFAT1 (T1) or NFAT5 (T5). After 48 hrs, cell lysates were harvested and immunoblotted for S100A4, NFAT1, NFAT5 and actin, as indicated (A). Blots from two separate experiments were quantified by densitometry and averaged (B). Bars in (B) represent the mean expression ± standard deviation. The asterisk (*) indicates a p-value < 0.05 compared to either untreated or NT controls. (C, D) MDA/β4 cells were treated with individual siRNAs targeting NFAT5 for 72 or 96 hrs and then cell lysates were immunoblotted for S100A4, NFAT5 and actin. (E) MDA/mock and MDA/β4 cells under normal culturing conditions were crosslinked with formaldehyde. Nuclei were then isolated, DNA fragmented and NFAT5-containing chromatin immunoprecipitated. The S100A4 promoter associated with NFAT5 was then amplified as described in the Experimental Procedures section and compared to an IgG control.
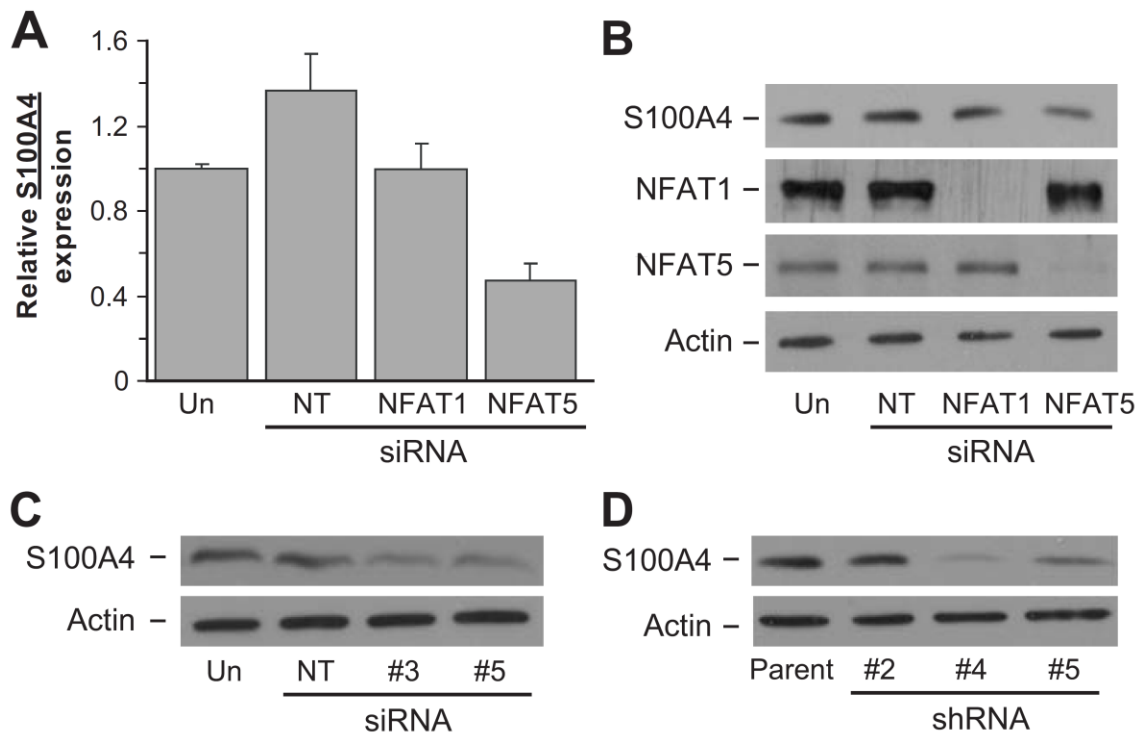
Figure 3. 5. S100A4 expression is controlled by NFAT5 and integrin α6β4 in MDA-MB-231 cells

Figure 3.5. (**A**, **B**) MDA-MB-231 cells were left untreated (Un) or transfected with 200 nM of siRNA SMARTPools that are non-targeting (NT) or directed against either NFAT1 or NFAT5. Duplicate cells cultures were then harvested 48 hrs later and analyzed by Q-PCR for S100A4 mRNA expression (**A**) or protein expression by immunoblot analysis (**B**). Blot was stripped and reprobed for NFAT1, NFAT5 and actin. For Q-PCR, p-values for NFAT5 samples compared to Un or NT controls was < 0.001. (**C**) MDA-MB-231 cells were treated with individual siRNAs targeting NFAT5 for 72 hrs and then cell lysates were immunoblotted for S100A4, NFAT5 and actin. (**D**) MDA-MB-231 cells were stably transfected with lentiviral shRNA constructs that target the integrin β4 subunit (#4 and #5) or that were ineffective in reducing β4 expression (#2). S100A4 expression of these cell populations was compared to the parental cell line by immunoblot analysis. Reduction in integrin β4 expression by shRNA #4 and 5 was confirmed by FACS analysis (data not shown).

3.2.5. S100A4 is regulated by DNA demethylation downstream of integrin α6β4

S100A4 message levels are upregulated by the α6β4 integrin over 140-fold in the MDA-MB-435 cells (Fig. 3.1) (*48*). However, the observations that siRNA-mediated knockdown of NFAT5 reduces S100A4 message levels by only 40% and NFAT5 is present on the S100A4 promoter in the absence of α6β4 expression suggests that additional mechanisms regulate S100A4 expression. Previous studies suggest that the methylation status of the S100A4 promoter regulates S100A4 expression (*46, 141*). Given the high degree of S100A4 upregulation by the α6β4 integrin, I tested the hypothesis that the α6β4 integrin also modulates S100A4 expression by affecting DNA demethylation. For these experiments, I treated MDA-MB-435 clones with inhibitor of DNA methyltransferases (DAC). Inhibition of DNA methyltransferases, but not inhibition of histone deacetylation with TSA, elevated S100A4 protein and mRNA in the MDA/mock cells, but did not affect MDA/β4 cells (Fig. 3.6A and B). These data are consistent with the concept that the S100A4 promoter in the MDA/β4 transfectants is demethylated and, therefore, unaffected by DAC treatment. In MDA/mock transfectants the S100A4 promoter would normally be methylated, DAC treatment results in the removal of repressive methyl groups from the S100A4 promoter and a dramatic upregulation of S100A4 expression. To confirm that active demethylation functions in S100A4 regulation downstream of the α6β4 integrin, MDA/β4 transfectants or MDA-MB-231 cells were treated with an inhibitor of DNA demethylases (SAM). As shown in Figure 3.6C, inhibition of DNA demethylation by SAM treatment led to a decrease in S100A4 expression in both the MDA/β4 transfectants and in MDA-MB-231 cells.

To demonstrate that the α6β4 integrin alters DNA methylation, I assessed the first intron region of the S100A4 promoter, which is known to contain a transcriptional enhancer that is regulated by DNA methylation. Using bisulfate pyrosequencing, I analyzed the +147 to +600 region of the S100A4 promoter for methylated CpG residues. As shown in Figure 3.6D, MDA-MB-435 clones that express the α6β4 integrin reduced CpG methylation content in the 7 CpG residues present in the enhancer. Four of the CpG residues in this region, specifically at positions 1, 3, 4 and 5, show a high level of methylation in the MDA/mock cells that is collectively 8-fold higher than the MDA/β4 transfectants (Fig. 3.6E). Together, these observations demonstrate that demethylation of the S100A4 promoter is an active process and a key regulator of S100A4 expression that is stimulated by the α6β4 integrin.

Several other genes are highly upregulated by integrin α6β4 expression in addition to S100A4, including FST, Nkx2.2, PDLIM4, CAPG and autotaxin (*142*). I find that FST, Nkx2.2, PDLIM4 and CAPG, but not autotoxin, are substantially upregulated by DAC treatment in the MDA/mock transfectants, but not in the MDA/β4 transfectants (*142*). Collectively, these data indicate that DNA demethylation of select promoters is an important component of α6β4 integrin-mediated gene regulation. Notably, these observations are not based on clonal variation since the observation extends to multiple promoters. Importantly, this is the first evidence that an integrin can affect the methylation status of a promoter.
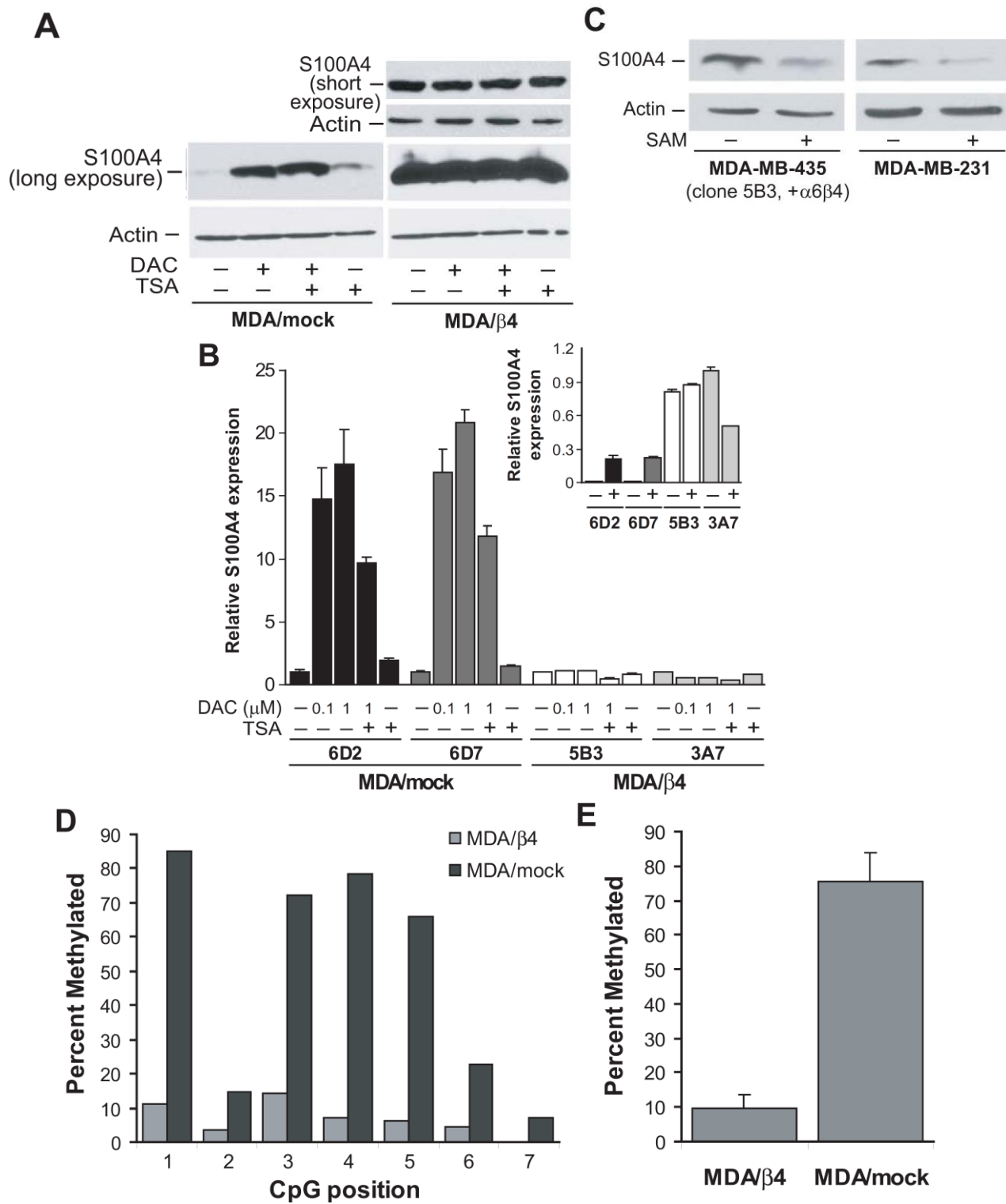
Figure 3. 6. Effect of DNA methyltransferase inhibitor, DAC, and demethylation inhibitor, SAM, on S100A4 expression and DNA methylation status analysis in S100A4 first intron region in MDA/mock and MDA/ β4 cells.

Figure 3.6. (Previous page) MDA/mock and MDA/ β4 transfectants were cultured in the presence or absence of 0.1 or 1 μM DAC for three days, as noted. Where indicated, 1 μM TSA was added for the final 24hrs of culture. Duplicate cultures of each clone were then harvested to assess the level of S100A4 by immunoblot **(A)** or Q-PCR **(B)** analysis. Immunoblots in (A) are from the same gel with the same exposure time. A shorter exposure of the S100A4 blot from a smaller amount of the same samples (1 sec exposure) showed that the loading between the MDA/ β4 samples was similar. Q-PCR values are reported as fold change relative to control for each clone. Inset in B represents relative S100A4 level between clones using 3A7 (MDA/β4) control cells as a value of 1. **(C)** MDA-MB-435 clone 5B3 and MDA-MB-231 cells were treated with SAM (80 μM), a methyl donor known to inhibit demethylases, for three days under normal culturing conditions prior to harvest and immunoblotting cell lysates for S100A4 and actin. **(D)** Genomic DNA from MDA/mock and MDA/β4 transfectants containing the first intron region of the S100A4 promoter (+203 to +662) was assessed for CpG residue methylation by bisulfate conversion and PCR pyrosequencing. The levels of methylation of each of the 7 CpG residues in this region are reported. (**E**) The percent methylation of CpGs at positions 1, 3, 4 and 5 were averaged and reported as mean ± standard deviation.

3.3. Discussion

The ability of the integrin α6β4 to promote an invasive phenotype is well-documented. Several pathways have been implicated in this phenomenon including cooperation with receptors for growth factors such as EGF (*143*), HGF (*144, 145*) and LPA (*68*), and the subsequent activation of PI3K, Akt, Rac, Rho and phosphodiesterases. Despite the mechanistic delineation of immediate downstream signaling events, how transcriptional events downstream of the integrin α6β4 affect these signaling events and subsequent tumor cell invasion has received little attention. Previous studies have shown that the α6β4 integrin can signal to multiple transcription

factors. Here, I expand on these observations and define to what extent signaling through the α6β4 integrin can affect gene transcription. I further show that the α6β4 integrin regulates a coordinated program of genes that predispose the cell to a migratory and invasive phenotype, including genes such as metastasis-associated S100A4. Importantly, I demonstrate for the first time that an integrin can affect the DNA methylation pattern of the promoters of select genes, including S100A4.

S100A4 is a member of the S100 family of calcium binding proteins and has been given several names including metastasin, fibroblast-specific protein and CAPL. Analysis of S100A4 expression has revealed that it is associated with a metastatic phenotype in multiple types of carcinoma, including breast, prostate, pancreatic, gastric and thyroid (*139*). Interestingly, the α6β4 integrin is associated with an invasive phenotype in each of these types of carcinomas (*60, 146*). In breast cancer, S100A4 can promote hormone-independent growth and metastasis of MCF-7 breast carcinoma cells in nude mice, which are normally non-metastatic (*147*). Conversely, crossing mice that overexpress S100A4 in the mammary epithelium with mouse models of metastasis (e.g. MMTV-neu or GRS/A) dramatically increases the incidence of metastasis (*148, 149*); however, by itself S100A4 is non-tumorigenic (*149*). Intracellularly, S100A4 can induce cell motility (*150*), in part through its ability to interact with myosin-IIA (*130, 151*). In addition, extracellular S100A4 can stimulate MMP-13 activity, possibly contributing to tumor invasion (*139*). In some breast tumor cell lines, I observe a form of S100A4 that runs at a higher molecular weight than expected for the 11.7 kD monomer, which may be oligomers of S100A4. Attempts to reduce these bands to a monomer using strong reducing agents, urea or excessive heat were unsuccessful (unpublished observations). While the

nature of these oligomers are undefined, interestingly, other members of the S100 family can be crosslinked by transglutaminases (*152*). Therefore, one possibility is that transglutaminases crosslink S100A4 to form oligomers; it is unclear how S100A4 oligomers may contribute to tumor biology. Typically, S100A4 forms a non-covalent symmetric homodimer, and it is this dimeric form that is expressed by metastatic and invasive cell lines (e.g. MDA-MB-231) (*139*). The other possibility could be due to post-translational modification. Recent study reported that S100A4 is sumoylated in human articular chondrocytes and sumoylation of S100A4 is required for S100A4 nuclear localization and IL-1β-induced MMP-13 production (*153*).

Despite the strong data supporting a role for S100A4 in tumor metastasis, little is known about the regulation of S100A4 other than it can be upregulated during EMT (*154*), by ErbB2 signaling (*43*) and through promoter demethylation (*140, 141*). This study demonstrates that the α6β4 integrin can stimulate the dramatic upregulation of S100A4 expression. Importantly, I determined that S100A4 expression correlates well with the expression of the α6β4 integrin in breast carcinoma cell lines, with the exception of MCF7. Of all the cell lines examined, MCF7 is the only cell line that does not display a mesenchymal phenotype and has been used previously to model EMT downstream of exogenously expressed Snail (*155*). S100A4 is a well accepted marker for EMT. The ability of integrin α6β4 signaling to activate the S100A4 promoter suggests that the integrin α6β4 may control the expression of a subset of genes during EMT and thus be an integral part of the process. This is an intriguing concept considering that the α6β4 integrin, and thus its oncogenic potential and ability to regulate pro-invasive genes, is released from hemidesmosomes during EMT. However, more work is needed to determine how much the α6β4 integrin contributes to EMT.

Downstream of the α6β4 integrin, S100A4 expression is stimulated through two distinct modes: through NFAT5 and by altering the DNA methylation status of the S100A4 promoter. The removal of methyl groups from CpG residues initially opens the promoter for activation, but itself does not activate the promoter. Transcription factors are needed for activation to occur. Here, I implicate NFAT5 in the activation of the S100A4 promoter. NFAT was first identified in T-cells where, upon T-cell activation and nuclear transport, NFAT promotes specific transcription to promote mobilization of T-cells and elicit an immune response (*156*). Jauliac et al. (*77*) were the first to identify the importance of NFAT1 and NFAT5 in the invasion and motility of carcinoma cells. Importantly, this role for NFAT was identified in the MDA-MB-435 cell model where integrin α6β4 promotes the transcriptional upregulation of NFAT1 and NFAT5 and the subsequent activation of these factors (*77*). Few targets of NFAT transcription factors have been identified in carcinoma cells, which includes autotaxin as defined by our group (*142*) and Cox-2 (*83, 84*), both of which are NFAT1 targets. Here, I extend these studies by identifying S100A4 as a target of NFAT5. Interestingly, the data show that NFAT5 is present on the S100A4 promoter in the absence of signaling from the α6β4 integrin, thus suggesting that other conditions controlled by integrin α6β4 determine whether NFAT5 present on the promoter can drive promoter activity.

Notably, besides S100A4, several other genes such as FST, PDLIM4, CAPG and Nkx2.2 are also dramatically enhanced by integrin α6β4 expression (*48*). Of these genes, FST (*157*) and PDLIM4 (*158*) are known to be regulated by DNA methylation. Treatment of cells with methyltransferase inhibitors, such as DAC, in the absence of this integrin can recapitulate the effect of α6β4 integrin expression (*85*). Finally, I uncovered evidence that the S100A4 promoter

is hypomethylated in MDA-MB-435 cells expressing integrin α6β4 but hypermethylated in the absence of this integrin. Methylation of CpG sites within a promoter is controlled by the balance of DNA methyltransferases and demethylases; however, the exact mechanisms governing the selectiveness toward specific promoters are unknown. Once methylated, promoters are generally silenced either by disruption of transcription factor binding sites or binding of methyl binding proteins such as MBDs and MeCPs, which recruit histone modifying agents to the promoter for effective chromatin silencing (*159*). Certainly the results demonstrate that the α6β4 integrin can affect the expression of genes normally silenced by promoter methylation and that the S100A4 promoter specifically is hypomethylated in MDA-MB-435 cells expressing the α6β4 integrin. Whether the α6β4 integrin stimulates DNA demethylases directly by altering specific signaling pathways or indirectly through the upregulation or inhibition of key genes is not clear. However, these results indicate that this cell model is ideal to study how selective promoter demethylation is achieved and will be the focus of future studies.

In a recent publication from the Mercurio's lab (*160*), several of the published gene array databases from breast cancer patients were mined for correlations with β4 integrin subunit mRNA expression. They confirmed the prevalence of α6β4 integrin overexpression in basal intrinsic subtype of breast cancer and defined what they refer to as a "β4 signature". Notably, few of these genes identified in the analysis are found in the β4 integrin signature. There are several likely reasons for this observation. Their analysis was made using whole tissue homogenates, which includes gene expression profiles from the cells of the tumor microenvironment such as immune infiltrates and stromal cells. Therefore, this β4 signature incorporates the genes expressed by cancer cells and cells from the microenvironment, as well as

genes altered due to the interactions between the two cell populations. The analysis takes into account only those genes expressed by the cancer cells under controlled *in vitro* conditions. Secondly, many of the genes identified in the study are genes whose expression levels are increased during EMT, such as S100A4. Stromal cells including fibroblasts and macrophages are known to express S100A4 (*19*) . In the presence of a desmoplastic stroma and immune infiltrate, genes in this class are likely to be masked by expression in the stroma and appear not to be significantly altered. Finally, the α6β4 integrin is well documented to cooperate with growth factor signaling to mediate its effects (*115, 143-145*). Therefore, it is likely that gene expression augmented by the α6β4 integrin will depend on cellular context, which growth factor receptors are stimulated and cell origin.

Investigations using the MDA-MB-435 cell line cannot escape the controversy surrounding this cell line stemming from reports suggesting that it is may be derived from a melanoma (*161, 162*). Extensive work from the MD Anderson Cancer Center shows that these cells express breast-specific (non-melanocyte) markers and can be induced to secrete milk proteins and lipids (*163*). Furthermore, these cells preferentially grow when implanted into mammary fat pads compared to subcutaneous injection (*164*), similar to other breast cancers but unlike melanoma cell lines (JE Price, personal communications). Many of the markers that MDA-MB-435 cells share with melanomas are typically found in neuroendocrine cells. Therefore, MDA-MB-435 cells may actually be derived from a tumor of neuroendocrine origin, a tumor type not well-recognized in the breast cancer literature, rather than melanocyte origin. However, should the MDA-MB-435 cells conclusively be shown to be of melanoma origin through more reliable methods such as DNA footprinting, the studies presented here would have

important implications for melanoma metastasis. The α6β4 integrin has been shown to be expressed in more aggressive melanomas (*165*) where, like in breast and other carcinomas, it promotes an invasive and metastatic phenotype.

In summary, using siRNA and promoter analysis, I found that S100A4 is the first target of NFAT5 reported in cancer. I also determined that S100A4 which is known to be regulated by DNA methylation is dramatically upregulated by integrin α6β4 expression and that the α6β4 integrin promotes the demethylation of the S100A4 promoter. Together with previous results (*48, 85*), my studies suggest integrin α6β4 confers a motile and invasive phenotype in breast carcinoma cells, in part, by regulating transcription factors including NFAT and chromatin remodeling such as promoter demethylation to modulate the expression of pro-invasive genes. Importantly, this is the first report that an integrin can affect gene transcription through chromatin remodeling.

CHAPTER 4: SRC KINASE PATHWAY IS INVOLVED IN NFAT5-MEDIATED S100A4 INDUCTION BY HYPEROSMOTIC STRESS IN COLON CANCER CELLS[3]

4.1. Abstract

S100A4 is associated with the progression of many types of cancers as well as some non-malignant conditions. However, how it is regulated by intracellular signaling and/or at the transcriptional level has not been extensively studied. I recently demonstrated that S100A4 is partially regulated by NFAT5 downstream of integrin α6β4. NFAT5 is a mammalian osmotic regulator. In order to study the regulation of S100A4 by NFAT5 in a more readily inducible model, colon cancer cells were subjected to hyperosmotic stress. I 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 siRNA abolished S100A4 induction. Furthermore, chromatin immunoprecipitation (ChIP) 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

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 (GI) tract is periodically exposed to hyperosmotic stress, this study may uncover a novel signaling pathway that could contribute to GI cancer progression.

4.2. Results

4.2.1. Hyperosmotic stress induces S100A4 expression in colon cancer cell lines

NFAT5 is an osmoregulator in mammalian cells (*87*) and regulates S100A4 expression in breast cells (*48*). Here, I 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 hrs or were treated with 100 mM NaCl at various times before harvest for analysis of S100A4 expression. As shown in Figure 4.1, 25 mM hypertonic NaCl treatment for 24 hrs induced S100A4 expression with higher induction at 100 mM excess NaCl (A). Time course analysis by immunoblotting and Q-PCR showed that S100A4 induction appeared at 16 hrs after treatment and is near maximal after treatment for 24 hrs (Fig. 4.1B and C). In contrast, the more permeable solute urea did not induce S100A4 expression (Fig. 4.1D). 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.

In order to test how long S100A4 induction is sustained, Clone A cells were exposed to hyperosmotic stress for 24 hrs before returning cells to isotonic medium. As shown in Figure 4.2, S100A4 induction was sustained up to 96 hrs 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.
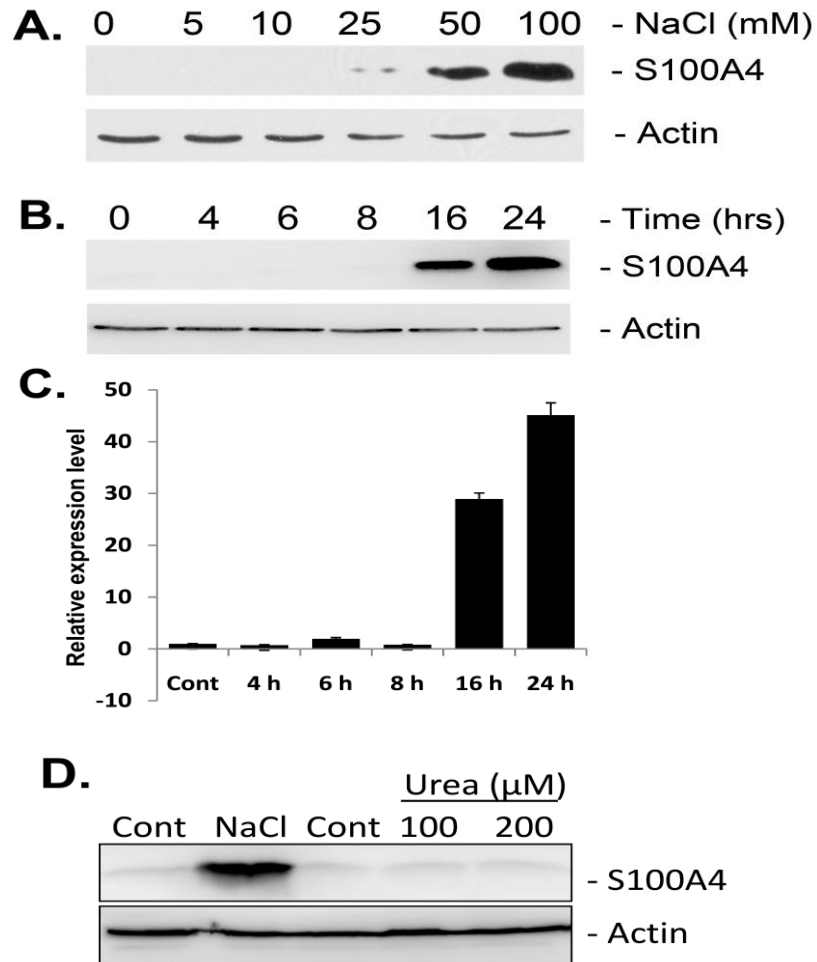
Figure 4. 1. S100A4 is induced in Clone A colon cancer cells by hyperosmotic stress.

Figure 4. 1. (Previous page) (**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**). (**D**) Clone A cells were treated with 100 mM excess NaCl, 100 mM and 200 mM Urea or left in regular medium (Control) for 24 hrs, then cells were harvested for S100A4 immunoblotting. Blots were stripped and reprobed for actin as a loading control. All data are representative of at least three separate experiments.
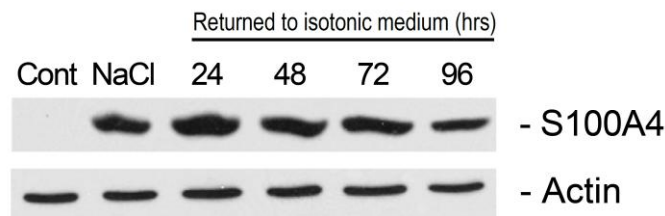
Figure 4. 2. <u>The induction of S100A4 is sustained.</u>

Figure 4.2. Clone A cells were placed under hyperosmotic shock with 100 mM excess NaCl for 24 hrs, then plates were rinsed with PBS for 3 times before returning 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.

4.2.2 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 (*46*). To test whether methylation of CpG sites in the first intron region are involved in hyperosmotic stress induced S100A4 expression, I focused on the first several CpG sites playing important roles in S100A4 regulation. These specific CpG sites are illustrated in Figure 4.3A. 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 were subjected to DNA methylation analysis. As shown in Figure 4.3B, 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 immunoblotting analysis (Fig. 4.3C). 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 my observation in Clone A cells, I submitted a panel of established colon carcinoma cells to a 24-hr osmotic stress treatment. As shown in Figure 4.3D, 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. 4.3D), S100A4 was not induced. To determine if 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 (4, 5, and 6) in Clone A cells were most affected by DNA methyltransferase treatment, I focused on these sites in the other colon cancer cell lines. As illustrated in Figures 4.3D 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, I 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. However, I did not observe any changes of the methylation status on CpG site 4, 5 and 6 after osmotic stress in Clone A cells (Fig. 4.4), which suggest that other chromatin remodeling events other than DNA methylation are involved.
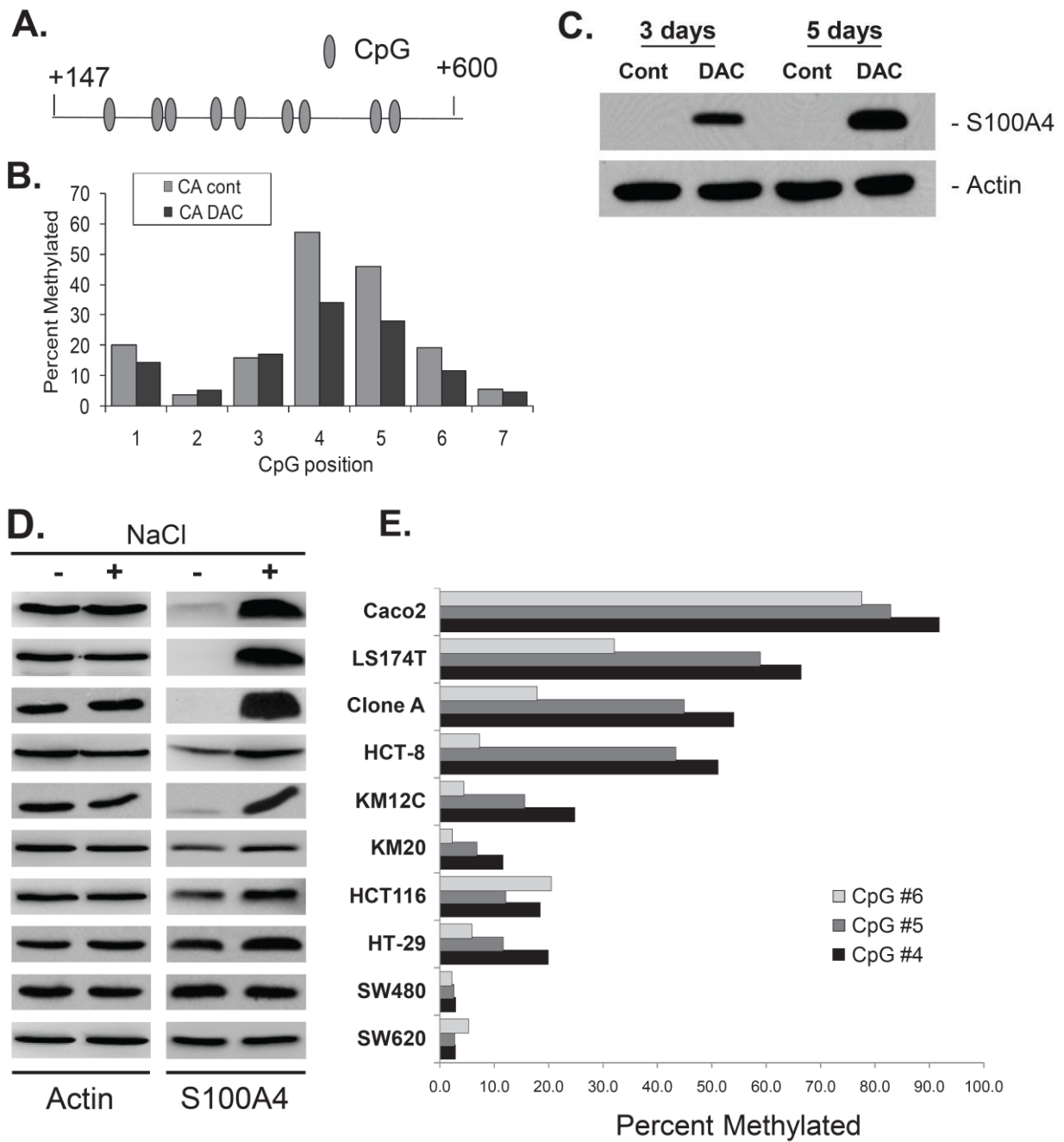
Figure 4. 3. S100A4 is regulated by DNA methylation in Clone A colon cancer cells and S100A4 induction in colon cancer cells correlates with DNA methylation status

Figure 4.3. (Previous page) (**A**) Depiction of CpG sites in the first intron region targeted for methylation status analysis. (**B, C**) Clone A cells were treated with 1μM of the DNA methyltransferase inhibitor DAC or DMSO for 3 or 5 days, then assessed for S100A4 promoter methylation status (**B**; 5 day; 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 hrs and then collecting cell lysates for S100A4 expression. (**E**) Genomic DNA isolated from colon cells was subjected to methylation status analysis.
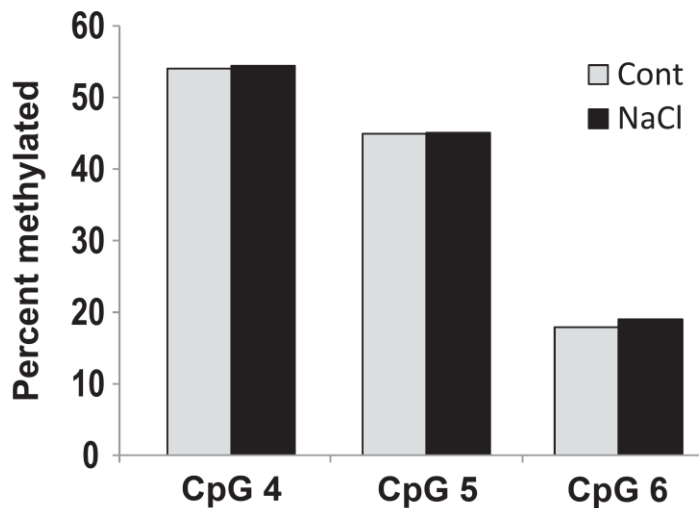
Figure 4. 4. Hyperosmotic stress does not change the methylation status of the first intron region of the S100A4 promoter.

Figure 4.4. Clone A cells were treated with 100 mM excess NaCl for 24 hrs, and then genomic DNA isolated from cells was subjected to methylation status analysis.

4.2.3 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, I treated Clone A cells with hyperosmotic NaCl for 24 hrs and then detected the NFAT5 protein level by immunoblotting analysis. As shown in Figure 4.5A, NFAT5 was dramatically upregulated in response to hyperosmotic stress in colon cancer cells. In addition, cells that were transfected with a NFAT5 reporter construct prior to hyperosmotic stress showed a 2.5-fold activation of NFAT5 compared to control (Fig. 4.5B). As NFAT5 is translocated into the nucleus following osmotic stress, I used a cell fractionation assay to determine if NFAT5 was translocated to the nucleus upon hyperosmotic shock. Consistent with previous findings (*87*), I found that although NFAT5 resides in the nucleus, hyperosmotic stress induced a rapid translocation of NFAT5 into the nucleus within 30-60 mins (Fig. 4.5C). To further ascertain whether NFAT5 is required for the induction of S100A4, Clone A cells were electroporated with siRNA targeting NFAT5 or non-targeting siRNA and were treated with 100 mM excess NaCl overnight. As seen in Figure 4. 5D, the induction of S100A4 was completely abolished by NFAT5 siRNA treatment compared to untreated and non-targeting siRNA controls. Given that NFAT5 is a bidirectional osmotic regulator, I hypothesize that hypoosmotic stress may have the opposite effect on NFAT5-mediated S100A4 expression in colon cancer cells. For this experiment, I submitted SW480 cells to hypoosmotic shock by 1:1 dilution of media and water for 24 hrs. The cells were collected for

immunoblotting and probed for S100A4 and NFAT5. The results showed that hypoosmotic stress decreased NFAT5 and S100A4 expression (Fig. 4. 5E). 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.

4.2.4 Hyperosmotic stress-responsive elements are located in the first intron region of the S100A4 promoter

My 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, I generated a series of S100A4 promoter luciferase reporter constructs. As illustrated in Figure 4.6A, different fragments of the regulatory regions including the proximal promoter region and the first intron region of S100A4 were cloned into pGL4.10 basic vector. Cells were then co-transfected with one of these constructs and with TK-Renilla prior to hyperosmotic shock treatment for 24 hrs. The luciferase activity of cell lysates was then measured. As shown in Figure 4. 6B, luciferase activity from the S100A4 reporters containing the first intron region, pGl4.10-632/+1010 and pGl4.10 +58/+1010, was stimulated about 5-fold 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.
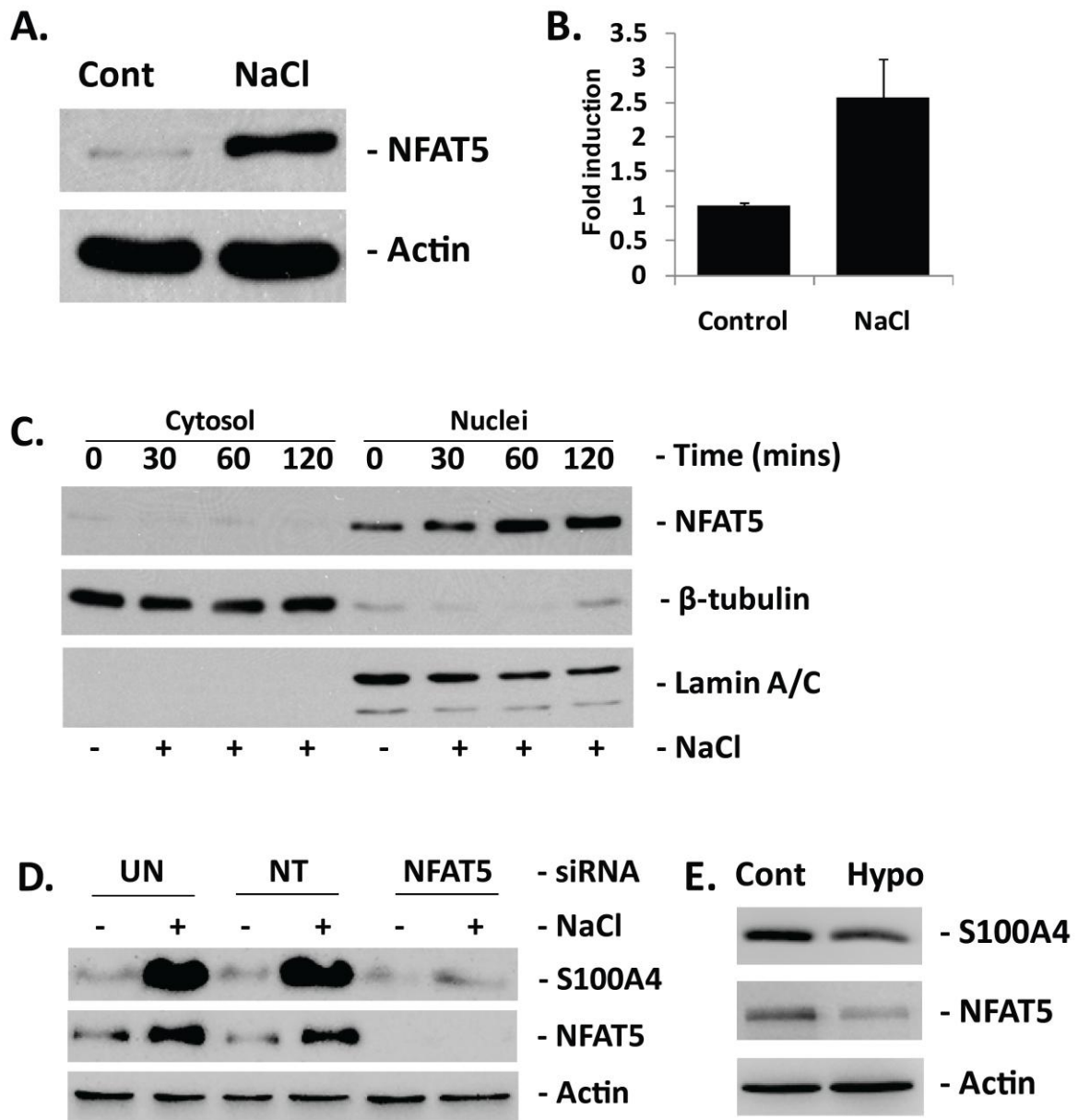
Figure 4. 5. <u>NFAT5 is activated and required for S100A4 induction in response to hyperosmotic stress in Clone A colon cancer cells</u>

Figure 4.5. (Previous page) (**A**) Clone A cells were induced with hyperosmotic stress in 100 mM excess NaCl for 24 hrs, then cell lysates were analyzed for NFAT5 expression by immunoblotting. (**B**) Clone A cells were co-transfected with pRL-TK Renilla control reporter and NFAT5 luciferase reporter containing the NFAT5 binding sites from the human aldose reductase gene promoter. After 24 hrs, cells were exposed to 100 mM excess NaCl for additional 24 hrs prior to luciferase activity analysis. Experiments were performed in triplicate, and data were presented as fold induction compared to control. (**C**) NFAT5 localization in response to hyperosmotic shock. Clone A cells were exposed to 100 mM excess NaCl for the indicated times, 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 siRNA targeting NFAT5, non-targeting control (NT), or electroporated only control (UN) were treated with 100 mM excess NaCl for 24 hrs 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 hrs, and then cells were assessed for S100A4 and NFAT5 expression.
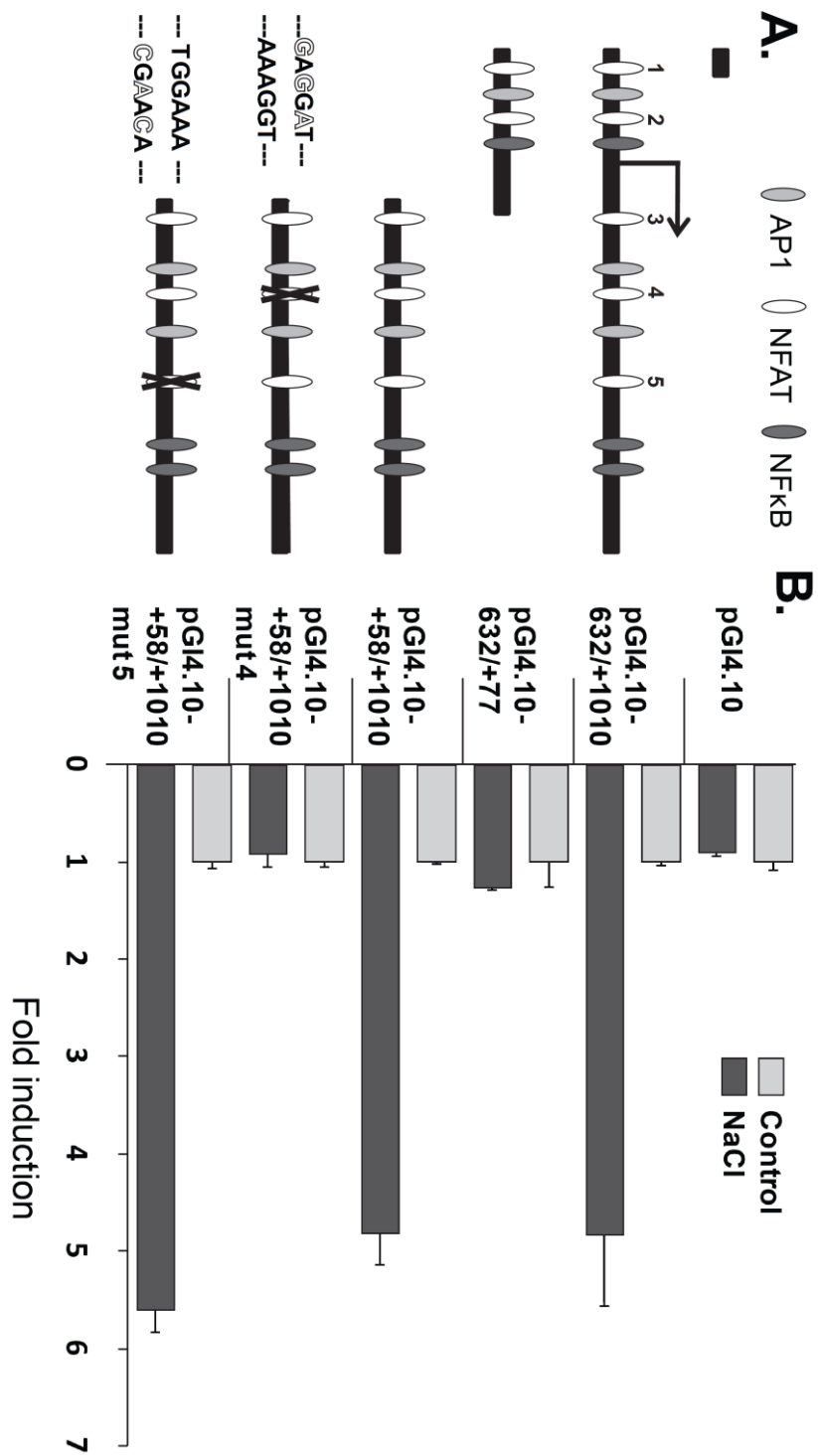
Figure 4. 6. Hyperosmotic response elements are located in the first intron region of S100A4.

Figure 4.6. (Previous page) (**A**) A schematic of transcription factor binding motifs in the regulatory region of S100A4. (**B**) Cells were co-transfected with the indicated S100A4 luciferase reporter along with pRL-TK Renilla control reporter for 24 hrs. Cells were then induced with 100 mM excess NaCl for an additional 24 hrs 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.

By scanning the NFAT5 transcription factor binding sites, I found five putative NFAT binding sites in the regulatory region (Fig. 4.6A). 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 11bp sequence TGGAAANNYNY (N represents as any nucleotide and Y represents as pyrimidine) (*166*) To determine if 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. I changed the putative binding site T<u>GGA</u>AA in reverse direction (site 4) to GA<u>GGA</u>T and putative binding site T<u>GGA</u>AA in sense direction (site 5) to <u>CGAAC</u>A. As shown in Figure 4. 6B, 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 hrs, then the ChIP assay was performed using NFAT5 antibody or normal rabbit IgG as control. As shown in Figure 4.7, 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.
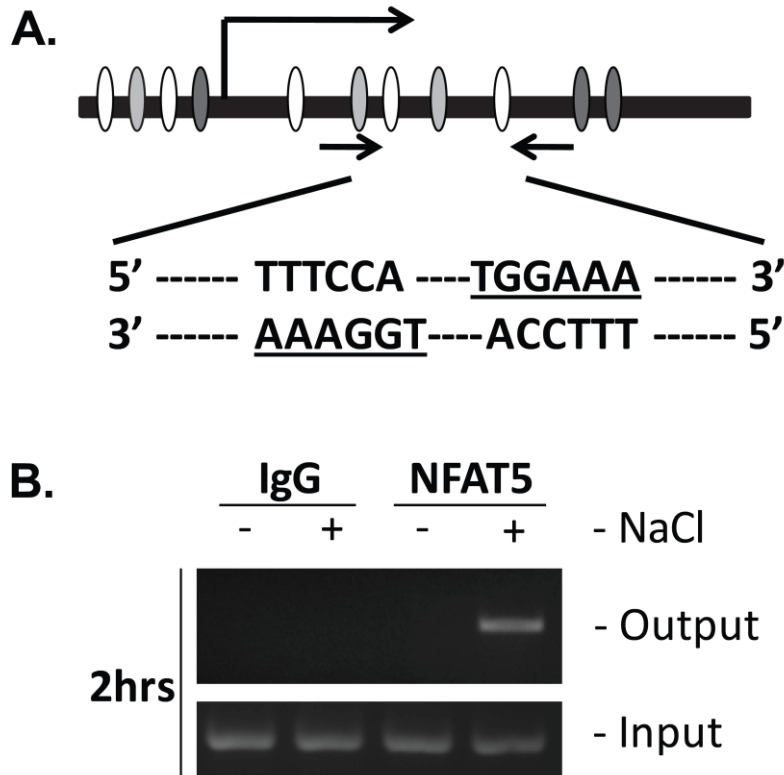
Figure 4. 7. NFAT5 binds to the first intron region where the putative NFAT5 binding sites are located.

Figure 4.7. (**A**) A schematic of the S100A4 regulation region with consensus NFAT5 binding sites and the primers for ChIP assay. (**B**) Cells were treated with 100 mM excess NaCl for 2 hrs, then cells were harvested and subjected to ChIP assay using antibody against NFAT5 or normal rabbit IgG control, and the expected 281 bps product amplified.

4.2.5 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 (*93, 167*). Importantly, Src acts as a regulator for cell volume and transactivates NFAT5 under the condition of osmotic stress (*167*). To test whether Src is involved in NFAT5-mediated S100A4 induction by hyperosmotic stress in colon cancer cells, I 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, -- prior to exposing cells to hyperosmotic stress. I found that inhibition of the SFK pathway by PP2 dramatically abrogated the induction of S100A4 at both the protein and RNA levels (Fig. 4.8A and B, respectively). In addition, NFAT5 expression was also decreased by PP2 treatment. Further assays utilizing transfection of the NFAT5 reporter (Fig. 4.8C) and S100A4 reporter constructs into Clone A cells (Fig. 4.8D) 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 mins, cells were exposed to hyperosmotic shock for 24 hrs, and a cell fractionation assay was performed. As shown in Figure 4.8E, 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, I used the NFAT5 transactivation reporter Gal4dbd-TonEBP/OREBP, which contains the NFAT5 transactivation domain (548-1531) fused to the Gal4dbd (*90, 168*). Clone A cells were co-transfected with the NFAT5 transactivation reporter together with a Gal4 luciferase reporter.
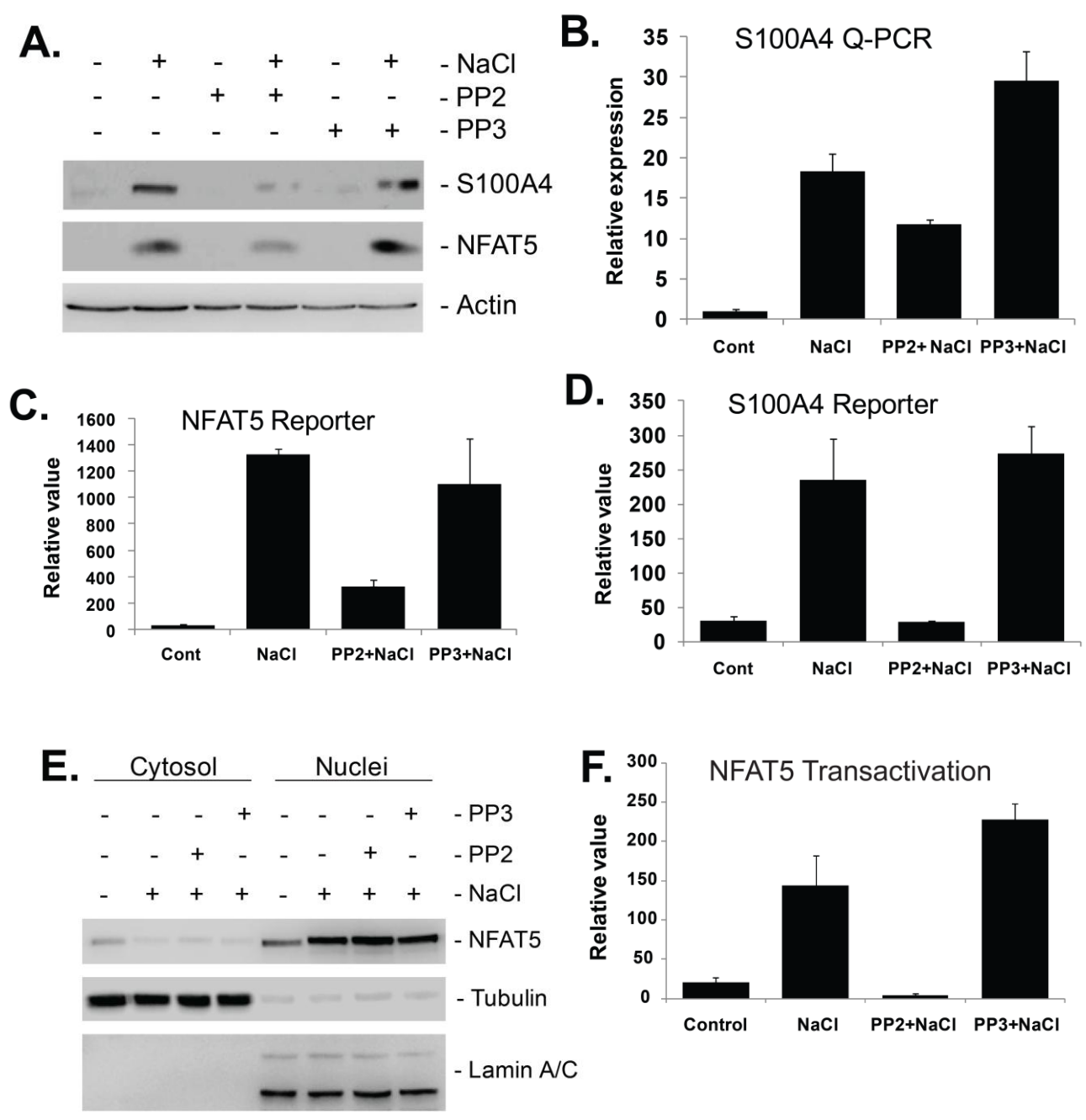
Figure 4. 8. <u>Inhibition of Src kinase pathway hinders hyperosmotic stress-induced S100A4 expression through inhibition of NFAT5 expression and NFAT5 transactivation but not nuclear translocation in Clone A cells.</u>

Figure 4.8. (Previous page) Clone A cells were pretreated with 10 μM PP2 or PP3, the PP2 negative control, for 30 mins before inducing hyperosmotic stress by 100 mM excess NaCl for 24 hrs; 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 co-transfected with NFAT5 reporter (**C**) or pGl4.10 S100A4 (+58/+1010) reporter (**D**) with pRL-TK-Renilla, 24hrs before treating cells as done in (**A**). After 24-hr treatment, cell lysates were assessed for luciferase activity. (**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 co-transfected 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 pGL4.31[luc2P/GAL4UAS/Hygro] (Promega) together with 5 ng pRL-TK Renilla control reporter. After 24 hrs, cells were pretreated with 10 μM PP2 or PP3 for 30 mins, and then cells were induced by hyperosmotic shock for 24 hrs prior to luciferase activity assay.

As shown in Figure 4.8F, I 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.

4.2. 6 Effect of S100A4 knocking down on cell survival

Because osmotic shock induces cell death, I hypothesized that S100A4 induction may contribute to cell survival. To test this concept, I 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 Figure 4.9A, the reduction of S100A4 resulted in decreased cell viability under the hyperosmotic condition. Notably, S100A4 reduction has little impact on cell viability under normal culturing conditions (Fig. 4.9B). Figure 4.9C, shows the successful reduction of S100A4 expression at representative time points. 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.

4.3. Discussion

In this study, I demonstrated that hyperosmotic stress induces S100A4 expression in colon cancer cells. Although this phenomenon had been shown in the kidney (*169*), my study expands this observation in colon cells to demonstrate that the Src kinase pathway and NFAT5 are required in this induction. In addition, I further identify that the osmotic stress response elements are located in the first intron region of S100A4. Importantly, I observed that the ability of hyperosmotic stress to induce S100A4 depends on the methylation status of S100A4. I 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.
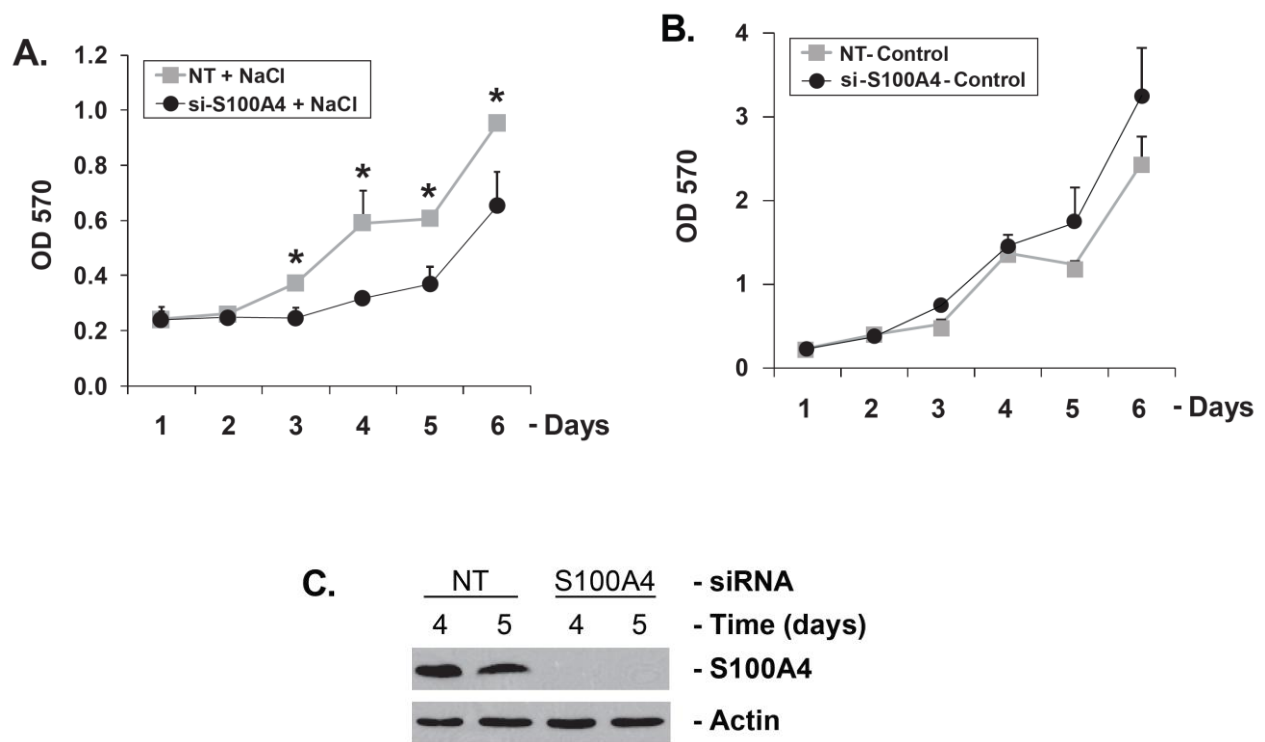
Figure 4. 9. S100A4 facilitates cell viability under hyperosmotic

Figure 4.9. (**A, B**) Clone A cells were electroporated with 200 nM siRNA targeting S100A4 or non-targeting (NT) control, then seeded into a 96-well plate. Cells were then exposed to hyperosmotic (100 mM excess NaCl) or normal culturing conditions 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 panels A and B represent standard deviation from triplicate determinations. Asterisks in (**A**) represent a p-value of less than 0.04. Notably in (**B**), S100A4 reduction leads to a consistent increase in cell viability, but it does not reach statistical significance.

In contrast, I did not observe changes in DNA methylation status when cells were induced with hyperosmotic shock. 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 (*170*). Nucleosomes, which are wrapped with DNA and packed into the fundamental organization unit of chromatin, are often depleted at active promoters (*171*). It has been shown that hyperosmotic stress can induce a rapid and reversible loss of nucleosomes around OREs (*172*). The depletion of nucleosomes may bypass DNA methylation; relax chromatin, thus allowing ORE access for transcription factors such as NFAT5. I 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 hrs. 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 P450 3As expression (*173, 174*). In this study, consistent with NFAT5 activation in response to hyperosmotic stress (*87*), I 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. I 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 my 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, I found that the inhibition of SFKs abrogates S100A4 induction by hyperosmotic stress in a dose-dependent manner. SFKs are the non-receptor protein tyrosine kinases, which consist of nine members and have many critical cell functions, including cell adhesion, invasion, proliferation, survival, and angiogenesis during tumor development (*175*). In response to hyperosmotic stress, several SFK members, such as FYN, HCK, FGR, SRC, and YES, are shown to be activated (*167*); this activation may be through the proposed osmosensing receptors for mammalian cells such as EGFR, integrin, and G-protein coupled receptors (*167*). 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 (*90, 93*). In agreement with these findings, my data demonstrated

that the specific SFK inhibitor, PP2, dramatically blocked NFAT5 transactivation but did not affect NFAT5 nuclear translocation. In addition, I 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 (*176*). 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 (*177*), studies on the effects of this hyperosmolarity in the gut have been focused on NF-κB-mediated cytokine production (*174, 178*), as osmotic stress has been suggested to be related to inflammation (*179, 180*). In most tissues, the osmolarity is thought to be around 300 mOsmol and osmolarity in the inflammation fluid could be increased to approximately 425-450 mOsmol (*180*). Under physiological conditions, the osmolarity of intraluminal contents after meal intake is increased in the colon (*181*). From detailed studies in pigs, the osmolarity in the lumen of the GI tract can increase to as much as 430 mOsmol/kg water after meal intake (*100*). Here, I used 25-100 mM excess NaCl to induce hyperosmotic stress, suggesting that the range of osmolarity used here has physiological and pathophysiological relevance.

My 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 (*182, 183*). For example, S100A4 is upregulated in the hypertrophic rat and human heart. Recombinant S100A4 promotes growth and survival of cardiac myocytes (*183*). In pancreatic cancer cells, knocking down S100A4 leads to increased sensitivity of cancer cells to gemcitabine

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treatment as well as increase in apoptosis and cell cycle arrest (*182*). This observation supports the contribution of S100A4 to pancreatic cancer chemoresistance (*182*). S100A4 confers these functions possibly through either intracellular or extracellular modes. Intracellularly, S100A4 interacts with target proteins such as p53, and extracellularly, S100A4 activates NF-κB through induction and subsequent degradation of the NF-κB inhibitor IκBα (*31*). 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 (*184*), which could lead to accumulation of DNA mutations by exposure to a hyperosmolar microenvironment (*185*). 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, my 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, I 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, my data suggest that hyperosmotic stress may affect GI physiology and potentially contribute to GI cancer progression.

CHAPTER 5: S100A4 BINDS RHOTEKIN AND THEIR COOPERATIVE SIGNALING CONFERS AN INVASIVE PHENOTYPE IN BREAST CANCER CELLS

5.1. Abstract

S100A4 is a calcium binding protein and tumor metastasis associated factor that has been suggested to promote motility and invasiveness of different types of cancer. This migratory promoting effect, in part, is due to the interaction of S100A4 with actin and actin binding proteins. Rho signaling through its effectors plays critical roles in actin cytoskeleton reorganization. I found that S100A4 interacts with Rho binding domain of Rhotekin (TRBD), one of the Rho effectors, thus suggesting a connection between the two pathways. To test whether this interaction is specific, I performed GST pull-down assays using Rho binding domain from different Rho effectors such as Rhotekin, Rhophilin, PKN, ROCK II, and Citron. These results showed that S100A4 specifically interacts with Rho binding domain (RBD) of Rhotekin but not the RBD of other Rho effectors. To determine whether this interaction is direct and calcium-dependent, I incubated purified S100A4 with GST-TRBD in the presence and absence of calcium. I found that the interaction is direct and calcium-dependent. The *in vivo* interaction of these two proteins was further confirmed by immunoprecipitation of exogenous mutated forms of Rhotekin with endogenous S100A4 from MDA-MB-231 cells. Interestingly, S100A4 did not bind TRBD using the same residues as Rho, as determined by using a triple-A mutant of Rhotekin that is unable to bind Rho. Immunocytochemistry staining followed by TIRF

microscopy showed that Rhotekin and S100A4 are co-localized at the leading edge of the migrating cells. To examine the function of this interaction, RNAi was used to suppress Rhotekin and/or S100A4 in MDA-MB-231. F-actin staining showed that the cooperation of S100A4 and Rhotekin enhanced membrane ruffling and the suppression of actin contractile fibers in the body of the cells. Taken together, I showed that S100A4 is a partner of Rhotekin and the interaction is specific, direct, through the Rhotekin Rho binding domain and calcium-dependent. Based on the function of this interaction, I propose that interaction of S100A4 and Rhotekin may change the functional output of Rho signaling through spatial regulation of Rho signaling, therefore, conferring an invasive phenotype in breast cancer cells.

5.2. Results

5.2.1. S100A4 specifically and directly interact with Rho effector Rhotekin in calcium dependent manner

I initially observed that S100A4 was precipitated with the RBD of Rhotekin while probing cell lysates for RhoA activity. As S100A4 is a tumor metastasis associated protein and Rho signaling through Rho effectors plays essential roles in cancer progression, I decided to investigate this interaction further.

Rho effectors have been classified into three groups depending on the regions of Rho to which they bind. class I includes Rhotekin, PKN/PRK, and Rhophilin; class II includes ROCKI and ROCKII; and class III contains citron (*186*). To test the specificity of S100A4 binds to Rhotekin, a representative effector from each class plus mDia1 and Pak Rac/Cdc42 binding domain (PBD) were chosen; the ability of S100A4 to bind the GTPase binding domain of each

effector was tested. As shown in Figure 5.1A, S100A4 specifically interacted with RBD of Rhotekin, a class I Rho effector, but not the other Rho effectors, GST alone or PBD.

The binding of calcium to S100A4 causes a conformational change which facilitates the interaction of S100A4 with its targets (*1*). To determine whether the interaction between S100A4 and the RBD of Rhotekin is direct and calcium regulated, bacterially-expressed Rhotekin RBD (TRBD) was incubated with 10 ng-1 μg of purified S100A4 in the presence or absence of EGTA. As shown in Figure 5.1B, a significant amount of purified S100A4 was pulled down with GST-Rhotekin RBD beads. In addition, the presence of EGTA abolished this binding. These data suggest that the interaction between S100A4 and Rhotekin RBD is direct and requires calcium.

Rhotekin belongs to class I Rho effectors that have the similar characteristics of Rho binding domain at the N-terminal. The amino acid sequence at Rhotekin RBD share about 30% identity with Rhophilin and the serine/threonine kinase PKN (*123*). I next tested whether S100A4 interacts with other class I Rho effectors. For this experiment, GST-fusion proteins containing Rho binding domain of PKN1 and Rhophilin2 were incubated with 100 ng purified S100A4 protein. As showed in Figure 5.1C, only RBD from Rhotekin bound S100A4.

To confirm that the interaction of Rhotekin and S100A4 can also occur *in vivo*, MDA-MB-231 cells, which express high levels of endogenous S100A4 were transfected with myc-tagged Rhotekin constructs including full-length (FL), ΔRBD, RBD, central domain (Cent) and C-terminal as depicted in Figure 5.1D. Fusion proteins were immunoprecipitated with anti-myc IgG agarose beads and analyzed for S100A4 association by immunoblotting analysis. These results showed that endogenous S100A4 was immunoprecipitated with full-length or RBD

Rhotekin fusion proteins (Fig. 5.1E, top panel: lane 3 and 5) but not the C-terminal, ΔRBD, or central domain fusion proteins (Fig. 5.1E top panel). Middle and bottom panels in Figure 5.1E showed that the comparable input of cell lysates as probed for S100A4 and the expression levels of Rhotekin construct immunoblotted with anti-myc antibody. Similarly, transfection of Flag-tagged Rhotekin full length, C-terminal and ΔRBD mutants into MDA-MB-231 cells, S100A4 immunoprecipitated with only the Rhotekin full-length fusion protein. The C-terminal and ΔRBD mutants did not show any interaction with S100A4 (Fig.5.1F, top panel). Collectively, the data showed that S100A4 directly and specifically interacts with Rhotekin through its Rho binding domain and this interaction is calcium-dependent.

5.5.2. S100A4 and active Rho bind to different residues of Rhotekin

The ability of S100A4 to bind directly to the RBD of Rhotekin raises the question whether active Rho and S100A4 might bind to the same motif. To test this possibility, I utilized a Rhotekin RBD triple A mutant construct, which contains alanine substitution at Arg37, Arg 39 and Asp40 respectively and has been shown to bind to GTP-bound Rho with reduced affinity (*187*). The bacterial expressed GST-fusion Rhotekin RBD or RBD triple A mutants were incubated with 100 ng purified S100A4 protein. The results showed that TRBD triple A mutant also binds S100A4 to the same degree as the wild-type RBD (Fig.5.2A). To test the difference of these two proteins bind to GTP-bound Rho, MDA-MB-231 cells were plated on collagen I-coated plates and treated with 100 nM LPA for 5 mins. Figure 5.2B shows the GTP bound active RhoA has less binding to TRBD triple A mutant compared to TRBD. These data demonstrated that S100A4 binds to different residues of Rhotekin than does active Rho.

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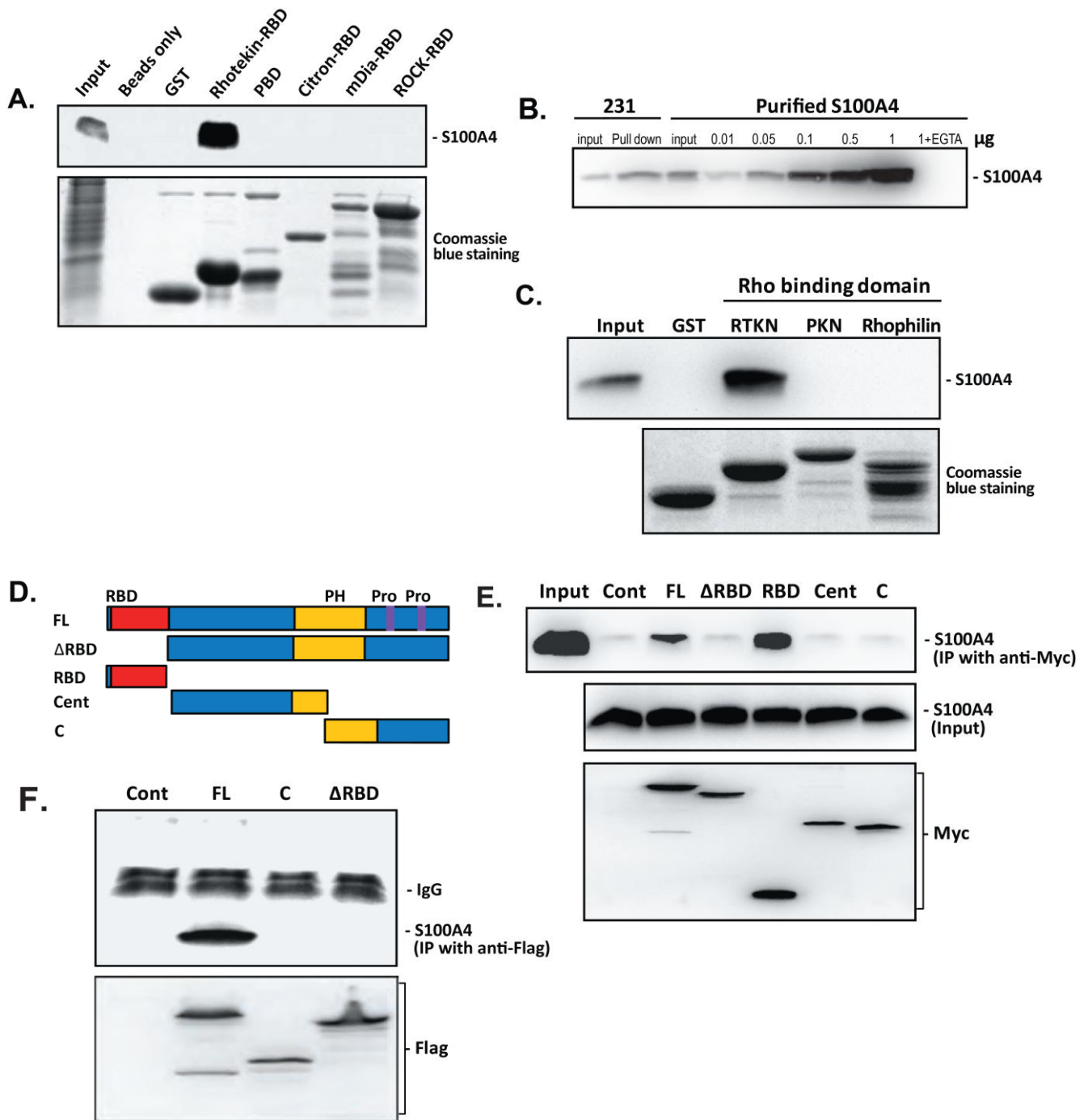
Figure 5. 1. <u>S100A4 specifically and directly binds to Rhotekin but not the other Rho effectors in a calcium-dependent manner.</u>

Figure 5.1. (Previous page) (**A**) GST-fusion proteins of different Rho binding domain of Rho effectors were purified and coupled to glutathione beads, then incubated with cell lysates from MDA-MB-231 cells. Beads were then washed and S100A4 content assessed by immunoblot analysis using 10% of the cell lysate as input control (top panel). Equal quantities of each fusion protein were separated by 10% SDS-PAGE followed by Coomassie blue staining (bottom panel). (**B**) Varying amounts of purified S100A4 were incubated with TRBD-glutathione beads in the presence or absence of 5 mM EGTA, washed and immunoblotted for S100A4. Input=10 ng S100A4. MDA-MB-231 lysate input and TRBD precipitates (pull down) represent positive controls. (**C**) GST fusion proteins of RBDs from class I Rho effectors, as indicated, or GST were purified and coupled to glutathione beads and incubated with 100 ng purified S100A4. Beads were then washed and associated proteins immunoblotted for S100A4 (top panel). Fusion protein content on beads was assessed as in (**A**) (bottom panel). 10 ng purified S100A4 was used as the input. (**D**) Domain structure of Rhotekin constructs used in (**E**) and (**F**). (**E**) MDA-MB-231 cells were transfected with myc-RTKN-FL, -ΔRBD, -RBD, -Cent, and C-terminal constructs or empty vector (Control). After 48 hrs, cells were lysed and immunoprecipitation assays were performed followed by immunoblotting for S100A4 (**E**, top panel for IP and middle panel for input) and anti-myc (middle panel). (**F**) MDA-MB-231 cells were transfected with Flag-RTKN-FL, C-terminal, and -ΔRBD constructs or empty vector (Control). After 48 hrs, cells were lysed and immunoprecipitation assays were performed followed by immunoblotting for S100A4 (top panel) and Flag (bottom panel).
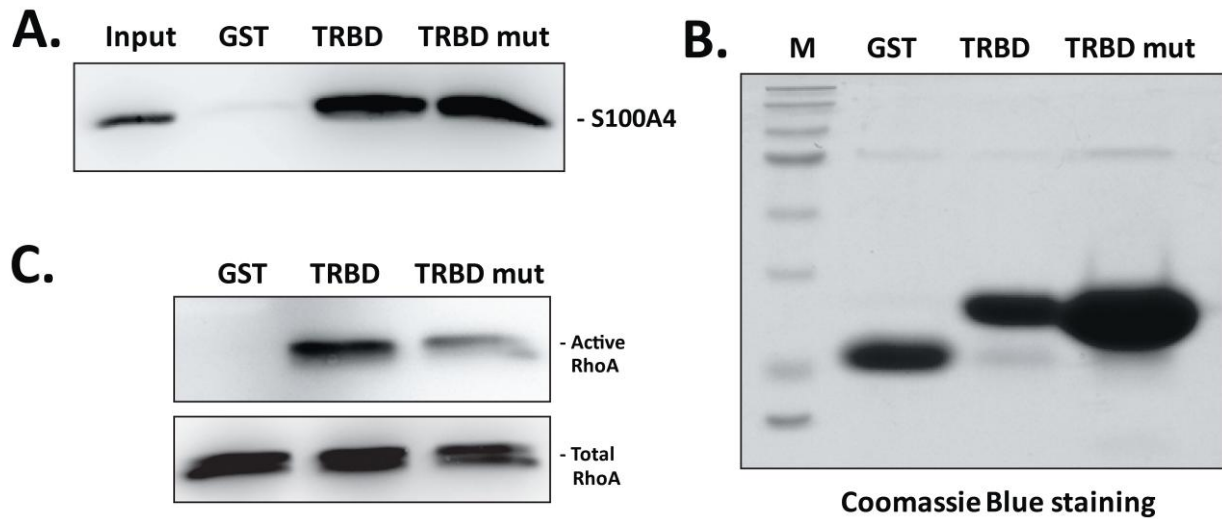
Figure 5. 2. S100A4 and RhoA bind different residues within the Rhotekin

Figure 5.2. (**A**) GST or GST fusion proteins of TRBD and TRBD triple A mutants were purified and coupled to glutathione beads and incubated with 100 ng purified S100A4. Then, beads were washed and immunoblotted for S100A4. 10 ng purified S100A4 was used as the input control. (**B**) The same amount of protein coupled beads as used in (**A**) were separated by 10% SDS-PAGE followed by Coomassie blue staining. (**C**) RhoA activity assay was performed by using GST or GST-fusion protein coupled beads with cell lysates from MDA-MB-231 cells seeded on collagen I-coated dishes and treated with 100 nM LPA for 5 min.

5.2.3. S100A4 and Rhotekin co-localize in the leading edge of migrating cells

S100A4 has been shown subcellularly localized both in the cytoplasm and nucleus (*188*). In the migrating cells, S100A4 is presented in the leading edge of the cells (*130*). In order to test whether Rhotekin and S100A4 colocalize in cells, HA-RTKN full-length construct was transfected into Hela cells, then cells were seeded on collagen I-coated coverslips and treated with 5 ng/ml EGF for 5 mins or left untreated. The immunocytochemistry staining for HA tag and S100A4 was performed and samples were analyzed by confocal microscopy. The results showed that untreated cells displayed flattened morphology with lamellipodial membrane protrusion. S100A4 and Rhotekin were colocalized in the leading edge of lamellipodia. In contrast, cells stimulated with EGF showed dramatic lamellipodial membrane ruffles. Interestingly, S100A4 and Rhotekin were concentrated and colocalized in the edge of the ruffles (Fig. 5.3). The colocalization of these two proteins was also observed in the basal surface of the cells.

5.2.4. Rho signaling is essential for membrane ruffle formation in MDA-MB-231 cells in response to EGF

Rho signaling plays a major role in actin cytoskeleton reorganization in response to growth factor stimulation. For example, RhoA has been shown to promote both actin stress fiber and membrane ruffle formation. To test whether Rho signaling is engaged in morphological change in response to EGF in MDA-MB-231 cells, bacterial expressed C3 transferase, which inactivates RhoA, B and C by ADP ribosylation, were electroporated into cells before treatment with EGF. As shown in Figure 5.4, C3 treatment impairs the lamellipodial ruffle formation.
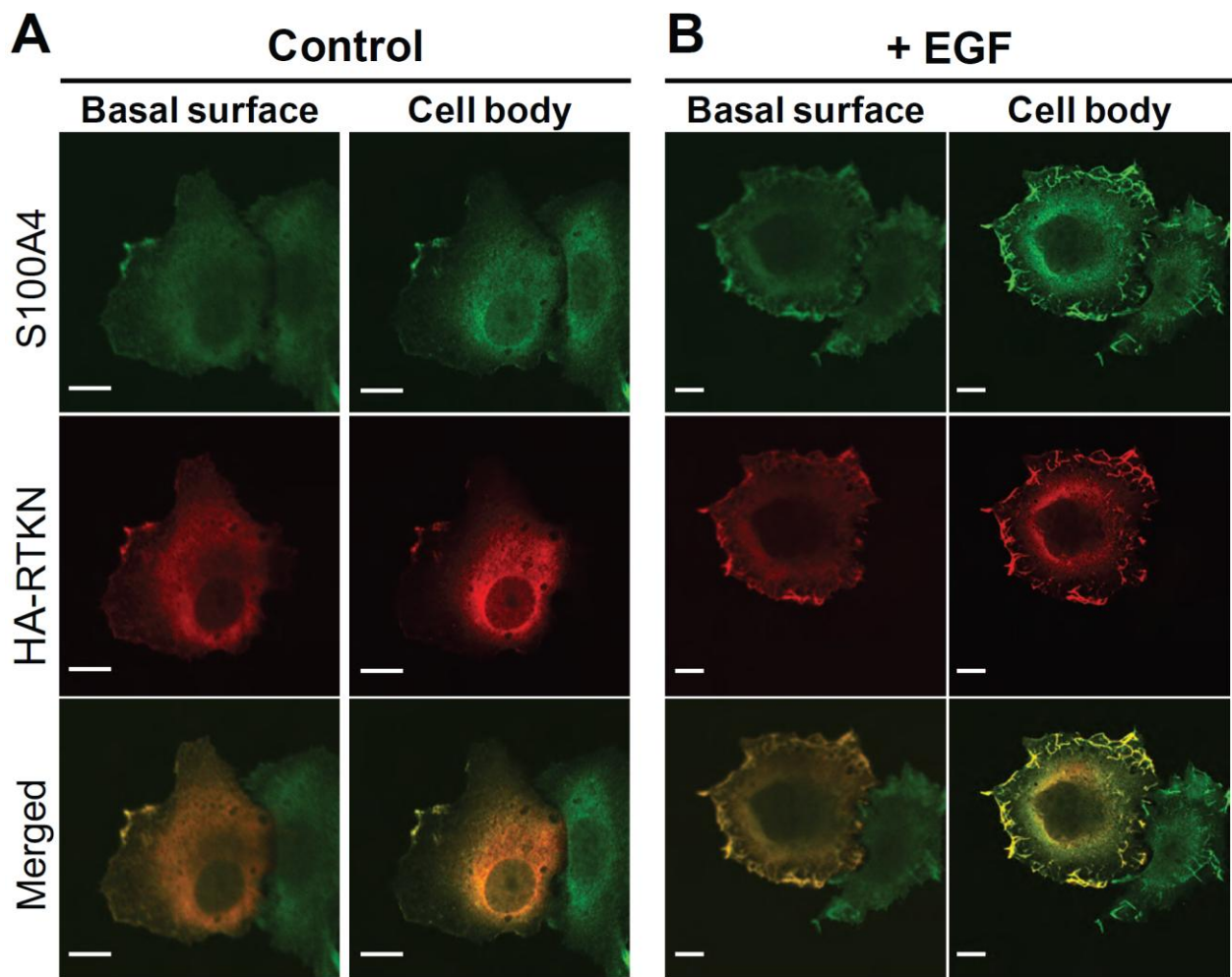
Figure 5. 3. <u>S100A4 and Rhotekin colocalize at the leading edge of migrating cells</u>.

Figure 5.3. Hela cells, which express endogenous S100A4, were transfected with HA-RTKN-FL. After 48 hrs, suspended cells (2.5×10^4) were seeded on glass coverslips coated with 50 µg/ml collagen I for 2 hrs before treatment with BSA (**A**) or EGF (5 ng/ml, **B**) for 5 mins. Cells were then fixed and immunostained for S100A4 (green) and HA (red). Images were taken every 0.5 µm starting from the basal surface. The representative images from one of three separate experiments are shown. For each condition, images from both basal level (basal surface) and 1.5 µm up to the basal level (cell body) are depicted.

Although RhoB has been shown to be upregulated at the transcriptional level by C3 treatment in murine fibroblasts, active Rho B was not observed (*189*). In this study, I electroporated C3 into cells, and the effect was observed in very short time, therefore, I do not consider that RhoB plays any roles in this effect. Rac is one of the best-characterized small RhoGTPases that mediates lamellipodial and ruffle formation. In contrast, the work in our lab demonstrated that inhibition of Rac1 by NSC23766 did not affect membrane ruffle formation in MDA-MB 231 cells (unpublished observation from Dr. Kathleen L. O'Connor's Lab). In addition, I did not consistently observe any changes in overall Rho activity in MDA-MB-231 cells in response to EGF treatment (unpublished observation) after reducing S100A4 and/or Rhotekin expression. These data may suggest that interaction of S100A4 and Rhotekin spatiotemporally regulates Rho signaling, therefore, affecting the functional output of Rho.

5.2.5. S100A4 cooperates with Rhotekin to suppress actin stress fiber formation and promote lamellipodial formation in MDA-MB-231 cells in response to EGF

Rho signaling plays important roles in cytoskeleton reorganization. As a Rho effector, the contributions of Rhotekin to cell motility are unknown*(190).* S100A4 interacts with different cytoskeleton proteins such as non-muscle myosin IIA, actin, and tropomyosin (*188*) which suggests this interaction may have biological relevance. To determine the contributions of S100A4 and Rhotekin to the organization of the actin cytoskeleton, I generated stable transfectants of MDA-MB-231 breast carcinoma cells which express either a non-targeting shRNA (shNT) or one targeting S100A4 (shS100A4). Once the efficiency of S100A4 shRNA was confirmed, shNT and shS100A4 cells were electroporated with either siRNA targeting

Rhotekin (siRtkn) or a non-targeting siRNA (siNT). Cells were then plated onto collagen-coated coverslips, treated with 5 ng/ml EGF for 5 mins and then stained for F-actin and S100A4.
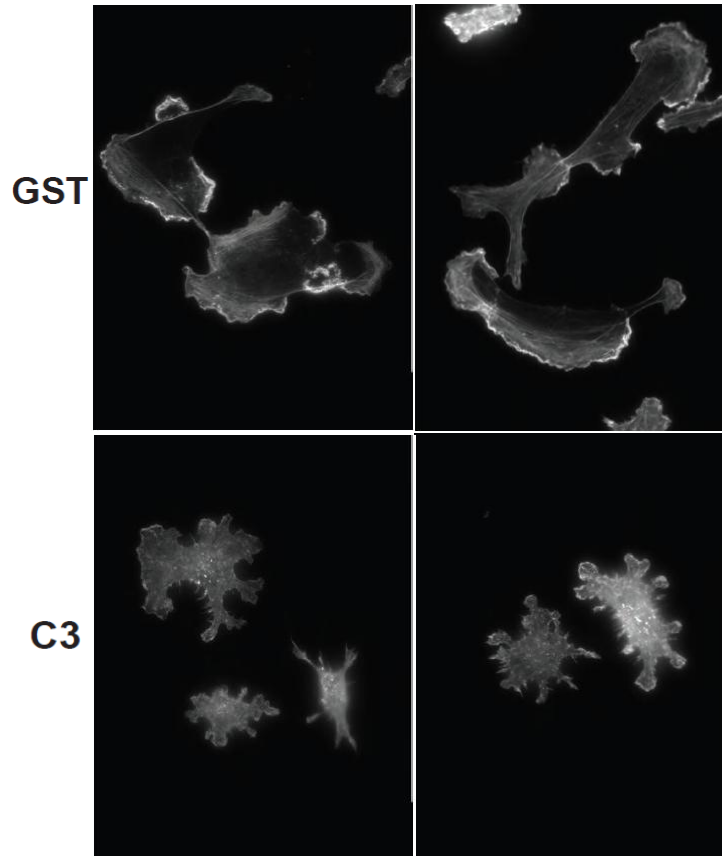
Figure 5. 4. Inhibition of Rho impairs ruffle formation in MDA-MB-231 cells in response to EGF.

Figure 5.4. MDA-MB-231 cells were electroporated (3×10^6 cell; 400V, 25µF) with 5 µg of bacterially-expressed, purified GST or C3 protein and then allowed to recover after addition of 10 µg/ml polymyxinB for 15 mins at room temperature. Cells were then plated on collagen I-coated coverslips for 2 hrs, treated with 5 ng/ml EGF for 5 mins, fixed and stained with TRITC-phalloidin. Cells were imaged by wide-field epifluorescence microscopy. Representative image are shown. Images were taken by Dr. Kathleen L. O'Connor.

As shown in Figure 5.5A, EGF-treated MDA-MB-231 cells formed large lamellae with prominent lamellipodial ruffles. Within the body of the cell, thick contractile F- actin fibers, which might be loosely referred to as stress fibers, are seen in low abundance if noted at all. However, when Rhotekin expression is reduced by siRNA, the presence of these F-actin bundles increases in number and thickness, but little effect is seen on lamellipodial ruffles (Fig. 5.5B). In contrast, when S100A4 expression is knocked down, lamellipodial ruffles become less prominent and actin polymerization at the lamellipodium is greatly stunted (Fig. 5.5C). Furthermore, as seen with Rhotekin knockdown, reduction of S100A4 expression led to an increase in contractile F-actin fibers in the cell body (Fig. 5.5D). These characteristics, quantified in panels E and F, were more dramatic when both S100A4 and Rhotekin expression were reduced by RNAi, thus suggesting cooperation between S100A4 and Rhotekin signaling.

5.2.5. S100A4 cooperates with Rhotekin to promote invasive growth of MDA-MB-231 cells in three-dimensional culture

As I have demonstrated that S100A4 and Rhotekin affects the functional output of Rho function to promote membrane ruffles in lieu of stress fiber formation in response to EGF treatment, next I focus on further demonstrating the consequences of this interaction in a more physiological relevant 3D culture condition. While RhoA can contribute to lamellae formation and migration in two-dimensions, the full importance of RhoA to tumor invasion is manifested more fully in 3D invasion, as seen specifically with the MDA-MB-231 cells (*191, 192*). For these experiments, Rhotekin and S100A4 in MDA-MB-231 cells were reduced by RNAi and then assessed for invasive growth for 4 days in Matrigel. As shown in Figure 5.6A, control cells
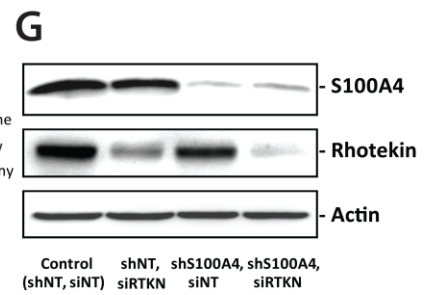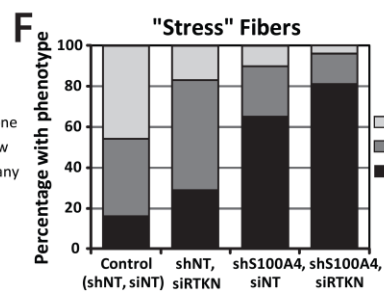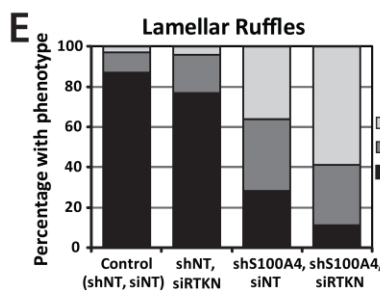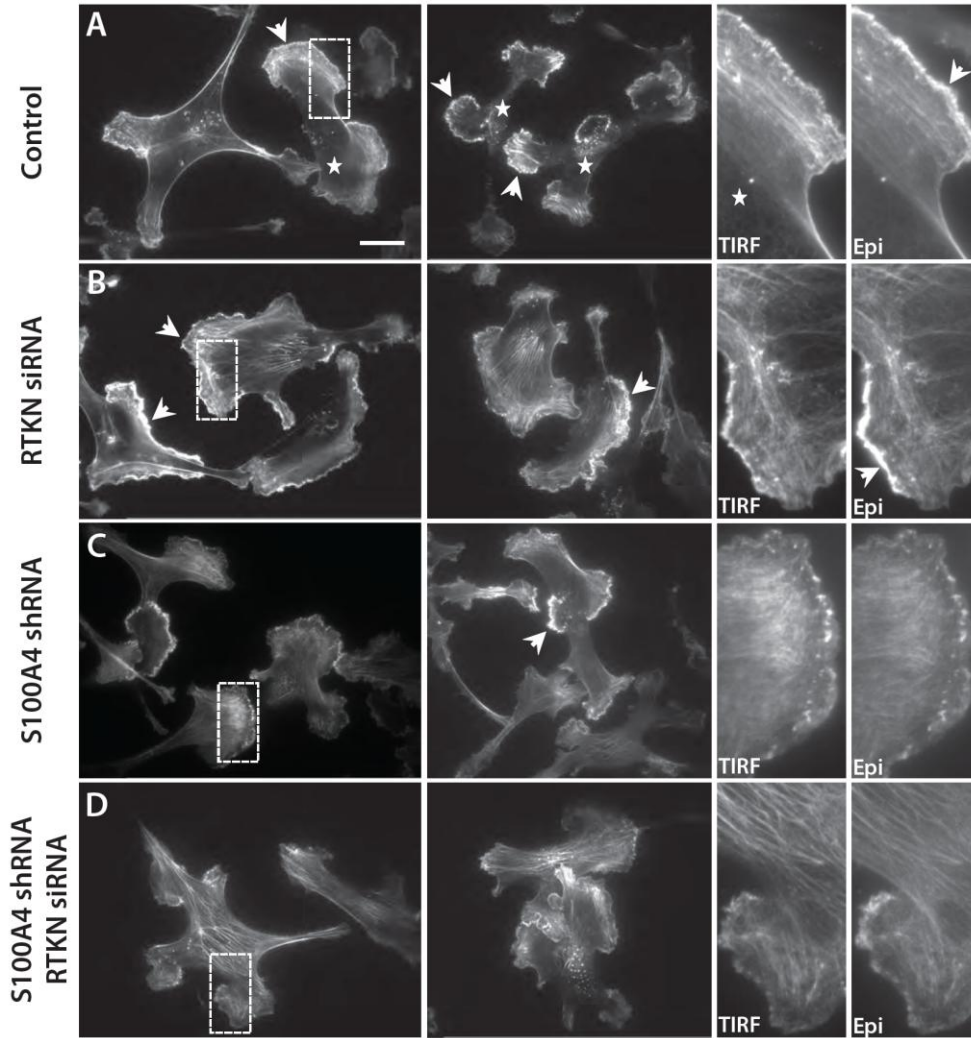
Figure 5. 5. S100A4 and Rhotekin cooperate to suppress actin stress fiber formation and promote membrane ruffle formation in response to EGF.

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Figure 5.5. (Previous page) MDA-MB-231 cells were stably transfected with either a non-targeting shRNA (shNT) or one targeting S100A4 (shS100A4). Cells were transiently transfected with siRNA targeting Rhotekin (siRTKN) or a non-targeting siRNA (siNT). After 48 hrs, cells were plated onto collagen coated coverslips for 3 hrs before treating with 5 ng/ml EGF for 5 mins. Cells were then stained with TRITC-Phalloidin and immunostained for S100A4. Cells were imaged using widefield epifluorescence and total interference reflection fluorescence microscopy and quantified for the presence of membrane ruffles at the lamellipodium and contractile fibers within the cell body. Representative data are shown for cells transfected with (**A**) with shNT and siNT (Control); (**B**) shNT and siRTKN; (**C**) shS100A4 and siNT; and (**D**) shS100A4 and siRTKN. Left two panels in (**A-D**) are representative images; bar in (**A**) depicts 20 μm scale for these images. The right two images are higher magnifications of the region highlighted by the rectangular box in the image to the left that were imaged either by TIRF or widefield epifluorescence (Epi). Arrows (**A-C**) represent lamellipodial ruffles; stars denote regions devoid of stress fiber-like actin filament. (**E, F**) Quantification of lamellipodial ruffles (**E**) and notable contractile f-actin fibers (**F**) present in 100 cells from each condition. (**G**) Immunoblot analysis of S100A4, Rhotekin and actin expression for cells under each experimental condition. Images were taken and processed by Dr. Kathleen L. O'Connor.

displayed a more aggressive invasive growth with "spider-like" protrusion invaded into the Matrigel. Cells with reduced Rhotekin expression did not show a significant effect on the percentage of cells with invasive growth, but comparing to control cells, the protrusions were much shorter. Cells with lowered S100A4 showed dramatic decrease of the cells with invasive growth. Interestingly, with both proteins depleted, the effects are more dramatic, most colonies tended to be smaller with few invasive protrusions. As shown in Figure 5.6B, F-actin staining after confocal microscope analysis showed that control cells, display actin rich projection, and actin was distributed to the peripheral of the cells. In contrast, when knocking down S100A4

and/or Rhotekin, the colonies are rounded with minimum projections; F-actin staining was shown in the center of the colonies. These effects were further quantified and shown in Figure 5.6C. S100A4 also acts as a paracrine and autocrine secretion factor, interestingly, a recent study showed that both intracellular and extracellular S100A4 affect TGFα-mediated branching phenotype of normal mammary gland (*193*). Based on my observation, I could not rule out the possibility that signal relay from secreted S100A4 contributes to promoting the invasive growth.

Figure 5.6. (Next page) (**A**) Representative phase-contrast images of MDA-MB-231 cells in 3D culture after reduction of S100A4 and/or RTKN expression by RNAi. Red bars represent a distance of 100 μM. (**B**) Matrigel containing colonies from cells in (**A**) were smeared onto slides, fixed, permeabilized, and stained for F-actin (TRITC-Phalloidin; red), S100A4 (anti-S100A4; green) and nuclei (DAPI; blue). The arrows indicate invasive cell protrusion into the Matrigel. Red bars represent 20 μM. (**C**) Quantification of the percentage of colonies with invasive growth from one of at least three representative experiments. Percentages are means from eight fields of 18-40 colonies in each condition and bars indicate s.d. (*p<0.01 by t-test).
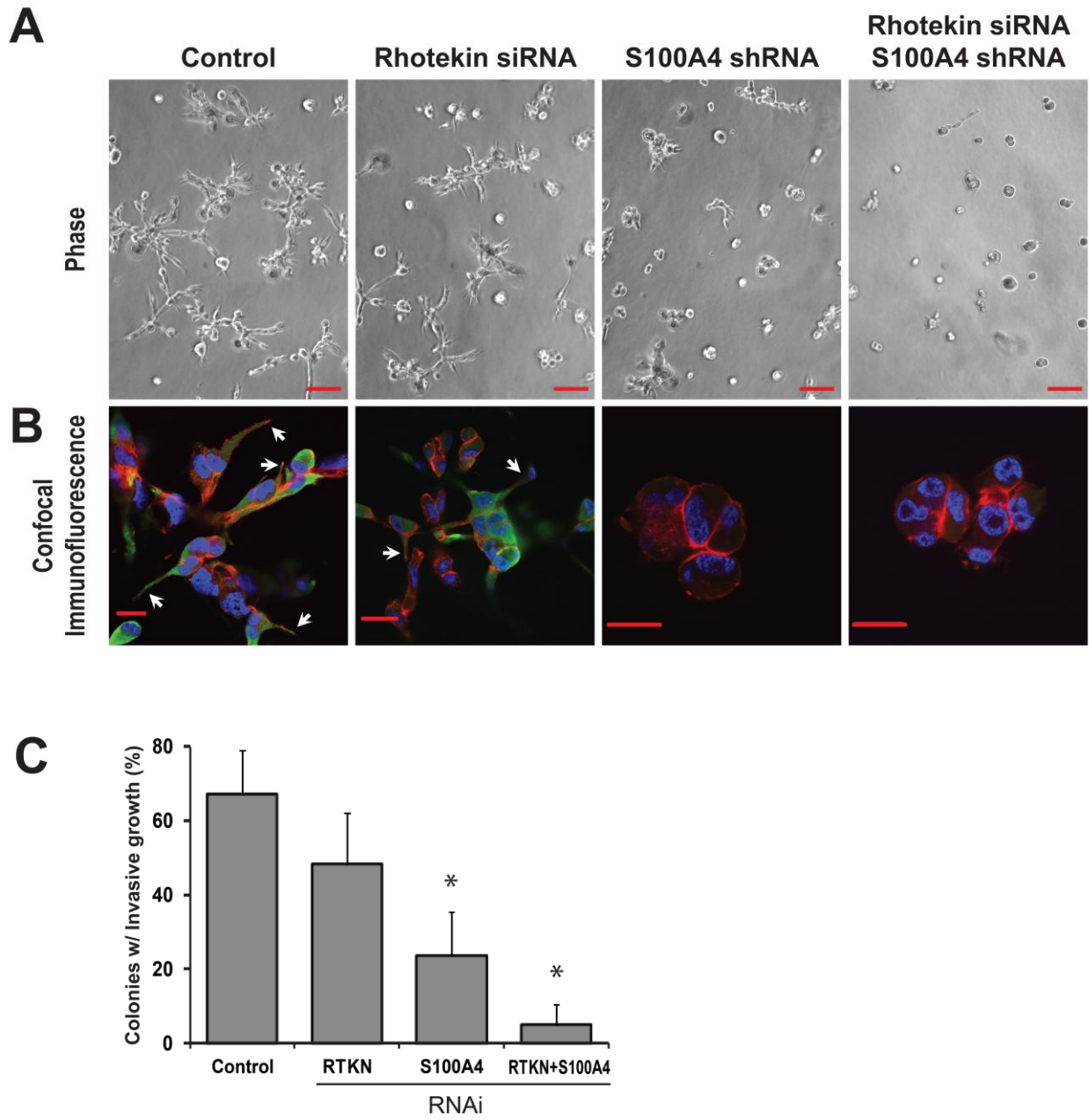
Figure 5. 6. S100A4 and Rhotekin cooperate to promote invasive growth of 3D cultures of MDA-MB-231 cells.

5.3. Discussion

S100A4 is a metastasis associated protein. Since S100A4 has no enzymatic activity, intracellularly, S100A4 interacts with its targets and changes the function of an individual target, therefore, promoting tumor migration and invasion. Elucidating the molecular mechanism of the underlying function will help us to develop a potential therapeutic target for cancer metastasis.

A variety of targets has been identified in the previous studies (*3*). For example, S100A4 interacts with non-smooth muscle myosin IIA and promotes myosin disassembly at the leading edge and this has been well defined as one mechanism of S100A4 to mediate cell motility (*1, 26-28*). Here, one of the Rho effectors, Rhotekin is demonstrated as a novel partner of S100A4. I further showed that this interaction is through Rho binding domain of Rhotekin, but use different residues from active Rho binding. This data may suggest that active Rho, Rhotekin, and S100A4 could form a complex, therefore, potentiating the signaling between Rho and S100A4. Studies showed that Rhotekin and S100A4 have the same target, such as septin (*1, 2*), which may suggest that in certain types of cells, Rhotekin and S100A4 could indirectly associate with each other. In this study, the data using purified S100A4 protein excluded this possibility, and strongly suggests that S100A4 directly interacts with Rhotekin, however, whether this interaction will affect the function of their individual partners needs to be further studied.

Rhotekin serves as a Rho effector as well as a scaffold protein and interacts with RhoA and RhoC equally well (*123*). Several proteins involved in cell polarity, focal adhesion and septin organization were found associated with Rhotekin (*124-126*). For example, Rhotekin interacts with Septin9. Interestingly, both Rhotekin and Septin9 are enriched in neurite tips of neuroblastoma Neuro2a cells (*134*), thus, suggesting that Rhotekin may regulate actin

cytoskeleton reorganization. However, how Rhotekin regulates actin cytoskeleton reorganization through Rho coupling events is not clear. In this study, I observed that S100A4 is localized in the leading edge of the migrating cells in response to growth factor. These results are consistent with a previous study (*130*). Importantly, Rhotekin is colocalized with S100A4 in the edge of the ruffles.

Membrane protrusions (lamellipodia, membrane ruffles, and filopodia) and actin stress fiber are important components of actin cytoskeleton reorganization which are regulated by three well characterized Rho GTPases including Rho, Rac and Cdc42 (*190*). Among them, RhoA is responsible for stress fiber formation in many cell types including fibroblasts (*194*). However, several studies also showed that RhoA can promote membrane ruffling and facilitate cell motility, especially in cells with epithelial origin (*113-116*). Despite the prevailing view that RhoA functions in the rear of the cells while Rac and cdc42 function at the leading edge, there is substantial mounting data that RhoA is active at the leading edge of migrating cells (*195*). Definitive evidence was finally presented with the advent of FRET-based reporter of RhoA activity which showed that RhoA activity localizes to sites of active protrusion and precedes the activation of Rac and cdc42 (*106*). Notably, this occurs not only in fibroblasts (*196*), but also in cells of epithelial origin where RhoA activation is critical for membrane ruffling (*116*).

However, a major question remains: how does RhoA promote lamellae and lamellipodia formation? It is tempting to speculate simply that the choice of one effector controls the switch between Rho's ability to promote membrane ruffles and lamellae in lieu of stress fibers; however, both membrane ruffle and stress fiber formation are mediated through the same Rho effectors, ROCK and mDia (*110*). The Rho-ROCK pathway inhibits the myosin light chain phosphatases,

resulting in an increase in myosin light chain phosphorylation. Phosphorylation of the myosin light chain enhances myosin II contractility and stress fiber formation, which is inhibitory to membrane protrusive activities (*197*) (*190*). Therefore, if Rho contributes to lamellipodial protrusions, it is necessary to temper the contractile functions of Rho to facilitate the activities of effectors such as mDia that promote actin polymerization and membrane protrusion.

In this study, I found that S100A4 is a binding partner of Rhotekin and this interaction is mediated through direct, calcium-dependant binding of S100A4 to the RBD of Rhotekin. I also demonstrate that the cooperative signaling between S100A4 and Rhotekin promotes membrane ruffling in EGF-stimulated MDA-MB-231 cells while suppressing stress fiber formation. These observations indicate a switch in Rho signaling to facilitate lamellar formation and invasive growth in 3D.

S100A4 functions intracellularly by binding to cytoskeleton proteins such as tropomyosin and non-muscle myosin IIA. Notably, myosin II is critical for the migratory process and is a convergence point for small GTPase signaling (*197*). Myosin IIA, specifically, functions predominantly at the leading edge where myosin light chains are preferentially phosphorylated downstream of RhoA signaling (*198*). S100A4 binding to myosin-IIA inhibits myosin-IIA oligomerization and thereby limits the contractile functions of this molecule (*22*). If S100A4 restricts myosin-IIA contractility, coupling Rho signaling to S100A4 through Rhotekin at the leading edge of cells would limit contractility in a spatial and temporal manner, and permit the protrusive effects of Rho signaling to dominate. While this concept is logical, the elucidation of the exact nature of this interaction and its molecular consequences will require further study.

In summary, I identified a novel interaction between the pro-metastatic protein S100A4 and the Rho effector Rhotekin. I propose that the S100A4/Rhotekin interaction changes the Rho signaling outcome by affecting how Rho assembles and modifies the actin cytoskeleton spatially. Moreover, S100A4 and Rhotekin cooperate to confer an invasive tumor phenotype in breast cancer cells through its ability to promote membrane protrusions and invasive growth.

CHAPTER 6: SUMMARY AND FUTURE DIRECTION

6.1. Summary

S100A4 is well accepted as a tumor metastasis associated protein and also an important player in several non-malignant conditions. S100A4 has been considered a valuable prognostic marker for different type of cancers as well as been suggested as a promising therapeutic target. Through the intracellular and extracellular action, the functions of S100A4 fit into several hallmarks of cancer such as anti-apoptosis (survival), metastasis (motility and invasion), proliferation, angiogenesis, and inflammation (*16*). Despite its roles in multiple aspects of cancer progression, investigations on the regulation of this molecule at the transcriptional level, especially in human cells, are limited. S100A4 is a calcium binding protein. Upon calcium binding, protein conformation of S100A4 changes, which facilitates S100A4 binding to its target protein. The interaction of S100A4 with a group of cytoskeleton proteins such as actin, tropomyosin, and myosin II implicates the role of S100A4 in cell migration. These interactions also suggest that S100A4 could be a potential regulator of actin cytoskeleton reorganization. Rho signaling is a major regulator for actin cytoskeleton reorganization. The question whether S100A4 couples to Rho signaling and in turn contributes to tumor migration and invasion needs to be addressed.

NFATs, particularly, NFAT1 and NFAT5 are activated downstream of integrin α6β4 and promote migration and invasion in colon and breast cancer cells (*77*). In Chapter 3, I hypothesized that integrin α6β4 contributes to tumor invasion and metastasis by transcriptional

regulation of promigratory and proinvasive gene expression. By using the breast cancer model, I demonstrated that S100A4 is a target gene of NFAT5 downstream of integrin α6β4; in addition, I further demonstrated that integrin α6β4 also affects S100A4 promoter demethylation. Since NFAT5 is an osmotic stress regulator in mammalian cells, in Chapter 4 I used colon cancer cells under osmotic stress as an additional model and further demonstrated this regulation mode. I found that both NFAT5 and DNA demethylation are important components of S100A4 regulation. Furthermore, I identified that Src kinases play a critical role in S100A4 expression by affecting NFAT5 expression and transactivation. The axis of Src/NFAT/S100A4 was also validated by another group (*199*). However, my studies show that integrin can affect the methylation status of a promoter and highlight a novel mechanism of how integrin α6β4 contributes to tumor progression. In Chapter 5 I confirmed that S100A4 directly and specifically interacts with Rhotekin through RBD of Rhotekin in a calcium-dependent manner, which therefore, suggests the connection between S100A4 and Rho signaling. The consequences of this link were investigated in breast cancer cells. I found that S100A4 co-localizes with Rhotekin in the leading edge of the migrating cells and cooperates with Rhotekin to promote membrane formation, suppress actin stress fiber formation in 2D, and promote invasive growth in 3D.

In summary, my studies fill the gaps of these important molecules related to tumor progression and provide the evidence that these molecules are connected and regulated at the transcriptional and epigenetic level. Specifically, I found that integrin α6β4 regulates S100A4 expression in breast cancer cells through NFAT5 in conjunction with DNA demethylation. I then validated this regulation in the colon cancer model under the condition of osmotic stress. Furthermore, I found for the first time that Rhotekin, a class I Rho effector, is a partner for

S100A4, and importantly, the functional consequences suggest that the cooperative signaling between S100A4 and Rhotekin may alter Rho signaling outcome and confer an invasive phenotype in breast cancer cells.

6.2. Future direction

S100A4 has been shown to be regulated by ErBB2, β-catenin/TCF and DNA methylation in the first intron region (*43-46*). My works suggest that S100A4 is regulated by NFAT5 in conjunction with chromatin remodeling such as DNA demethylation. Although the convergent point for the regulation of S100A4 in this study is NFAT5, NFAT5 itself is not sufficient to regulate S100A4 expression, thus suggesting that other transcriptional machineries are also involved in the regulation of S100A4 in current cell models. Specifically, binding sites for two other transcription factors such as AP1 and NF-κB that are close to NFAT5 binding sites are also found in the first intron region of S100A4. Interestingly, both hyperosmotic stress (*200-202*) and integrin α6β4 signaling (*72, 75, 76*) activate these two transcription factors. Previously, several studies found that NFAT5 did not interact with AP1 (*88, 203*), but a recent study arguably demonstrated that AP1 transcription factors c-Fos and c-Jun not only physically interact with NFAT5 but also contributes to high NaCl-mediated NFAT5 transactivation (*204*). Importantly, c-Jun and c-fos can also be activated by high NaCl (*200*). A recent study reported that NFAT5 is also associated with the NF-κB component p65, and increased NF-κB activity by hypertonicity (*205*). It will be interesting to know whether these transcription factors cooperate with NFAT5 and regulate S100A4 expression in both breast cancer and colon cancer models.

The epigenetic regulation is the combined effects of DNA methylation, chromatin-associated histone and non-histone proteins modifications, and the interplays of these modifications. It is also cell–type specific. In my study, I found that DAC can reexpress S100A4 in both breast and colon cancer cell models. However, I did not observe any changes in methylation status in colon cancer cells by hyperosmotic stress. DAC has been widely used as a potent DNA methyltransferase inhibitor; it also presents effects on histone modification such as inducing histone hyperacetylation (*206*) and decreasing histone H3-lysine 9 dimethylation, which are independent of cytosine demethylation (*207*). A recent study reported that reexpression of tumor suppressor gene RUNX3 by DNA methyltransferase inhibitor in gastric cancer cells is through histone H3-K9 methylation instead of promoter demethylation (*208*). My study found that DAC treatment can reexpress S100A4 at the comparable level as the induction of hyperosmotic stress; however, the methylation status cannot fully explain that demethylation of CpG sites is the sole mechanism responsible for S100A4 expression. Putting these into the context of S100A4 induction in response to hyperosmotic stress in Clone A cells, it could be possible that hyperosmotic stress somehow affects histone modification. In future studies, it will be important to determine the role of histone modification events coupled to transcription factors such as NFAT5.

S100A4 participates in multiple aspects of cancer progression and some non-malignant conditions. The majors function of S100A4 presented in these studies are that S100A4 cooperates with Rho effector, Rhotekin, and regulates actin cytoskeleton reorganization in 2D and invasive growth in 3D. Although these two functions confer the invasive phenotype of breast cancer cells, the mechanisms underlying these two phenomena might be different. In 2D, in

response to growth factor stimulation, membrane protrusions and actin stress fiber are regulated by Rac, Cdc42 and Rho (*190*). Specifically, RhoA is mainly engaged in actin stress fiber formation in fibroblasts. However, several studies also demonstrated that RhoA can promote membrane ruffling and facilitate cell motility, especially in cells with epithelial origin (*113-116*). Studies also showed that Rho A is localized in the leading edge of the migrating cells and coordinate with Rac and Cdc42 in the leading edge (*106*). By using FRET probes for small RhoGTPases, studies showed that RhoA, Rac, and Cdc42 are spatially and temporally regulated in the migrating cells (*209*). The cooperative signaling between S100A4 and Rhotekin promotes membrane ruffle formation; meanwhile, suppresses actin stress formation suggesting that the interaction could impact cancer cell migration and invasion through altering Rho signaling outcome. Interestingly, these two distinct actin reorganizations were observed in cells with both Rhotekin and S100A4 knocking down. Considering this interaction did not change the overall Rho activity, future research should focus on whether the interaction of S100A4 and Rhotekin spatially regulates Rho signaling in a 2D model.

Tumor invasive growth is defined as a complex, multistep program involved in the interplay of tumor cells and the microenvironment, and in turn tumor cells acquire the propensity for migration, invasion and proliferation (*210*). Rho signaling is engaged in at least two distinct types of motility in three-dimensional matrix: Rho/ROCK dependent amoeboid motility and Rac-dependent mesenchymal motility (*211*). Interestingly, these two types of migration are interchangeable. For example, MDA-MB-231 cells use a mesenchymal mode for invasion in 3D; however, when proteolysis is inhibited by protease inhibitor cocktail, MDA-MB-231 cells display a transition from mesenchymal migration to amoeboid migration (*212*). A recent study

demonstrated that RhoA activity was spatially regulated during pancreatic cancer cell invasion in live animals (*213*). Similarly, it would be interesting to investigate whether the interaction of S100A4 and Rhotekin affects Rho spatially in a 3D culture system.

A recent study suggested that both intracellular and extracellular S100A4 affect TGFα-mediated branching phenotype of normal mammary gland (*193*). In addition, both secreted S100A4 and Rhotekin have been shown to activate NF-κB(*30*) (*128*). For example, Rhotekin is overexpressed in gastric cancer and the overexpression confers resistance to apoptosis through activation of NF-κB (*128*) and extracellular S100A4 can stimulate MMP13 expression though activation of NF-κB (*30*). Whether this interaction will synergistically active NF-κB and promote MMP expression and facilitate cell survival and invasive growth needs to be studied in the future. Furthermore, I performed my studies in the cell culture system. In the future, the clinical relevance of this interaction should be further evaluated in animal models and patient samples.

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Publications

Articles:

1. Gulhati P, Bowen KA, Liu J, Stevens PD, Rychahou PG, **Chen M**, Lee EY, Weiss HL, O'Connor KL, Gao T, Evers BM, mTORC1 and mTORC2 Regulate EMT, Motility, and Metastasis of Colorectal Cancer via RhoA and Rac1 Signaling Pathways. Cancer Res, 2011. 71(9): p. 3246-3256.

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