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S100P/RAGE signaling regulates microRNA-155 expression via AP-1 activation in colon cancer

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Abstract

Accumulating evidence indicates that elevated S100P promotes the pathogenesis of cancers, including colon cancer. S100P exerts its effects by binding to and activating the Receptor for Advance Glycation End-products (RAGE). The effects of up-regulated S100P/RAGE signaling on cell functions are well documented. Despite these observations, little is known about the downstream targets of S100P/RAGE signaling. In the present study, we demonstrated for the first time that activation of RAGE by S100P regulates oncogenic microRNA-155 (miR-155) expression through Activator Protein-1 (AP-1) stimulation in colon cancer cells. Ectopic S100P up-regulated miR-155 levels in human colon cancer cells. Conversely, knockdown of S100P resulted in a decrease in miR-155 levels. Exogenous S100P induced miR-155 expression, but blockage of the RAGE with anti-RAGE antibody suppressed the induction of miR-155 by exogenous S100P. Attenuation of AP-1 activation through pharmacological inhibition of MEK activation or genetic inhibition of c-Jun activation using dominant negative c-Jun (TAM67) suppressed miR-155 induction by exogenous S100P. Also, S100P treatment stimulated the enrichment of c-Fos, an AP-1 family member, at the miR-155 host gene promoter site. Finally, a functional study demonstrated that miR-155 knockdown decreases colon cancer cell growth, motility, and invasion. Altogether, these data demonstrate that the expression of miR-155 is regulated by S100P and is dependent on RAGE activation and stimulation of AP-1.

Keywords

colon cancer; S100P; RAGE; miR-155; AP-1; miR-155 sponge

Introduction

Despite the advancements in the diagnosis and treatment of colon cancer, colon cancer remains the 2nd leading cause of death due to cancer in the United States [1]. Approximately

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half of the patients diagnosed with colon cancer will develop liver metastasis [2]. Metastasis is the major cause of death in cancer patients and is largely considered incurable due to a lack of effective therapy other than hepatic resection [3, 4]. Metastasis is a complex multi-factorial and multi-step process which promotes the detachment, migration, and proliferation of malignant lesions from the primary tumor site to distant site [5, 6]. Defining the gene targets underlying the metastatic process is essential for the development of an effective targeted therapy [7]. To identify the novel genes that play key roles in colon cancer metastasis, our group focused on determining downstream target genes involved in the S100P/RAGE signaling pathway.

S100P, a 95 amino acid protein which was first purified from human placenta with a restricted cellular distribution, is a member of the S100 family of calcium-binding proteins of the EF-hand type [8]. Marked expression levels of S100P have been reported in both primary and metastatic lesions [9–14]. A number of studies have strongly linked S100P to cell proliferation, invasion, and migration in cancers, including colon cancer [12, 15–18]. S100P is among three signature genes that were shown to promote liver metastasis in an orthotopic mouse model of colorectal cancer [19]. Over expression of S100P is associated to poor prognosis and survival in patients with breast and lung cancer [12, 20]. On the contrary, blockage of S100P inhibits colon cancer growth and metastasis, while also improving mice survival [11, 21, 22]. Our group has previously shown that S100P expression is regulated by the PGE₂/EP4 signaling through cAMP response element-binding protein (CREB) activation in colon cancer cells [10]. S100P is known to bind to the receptor for Advanced Glycation End-products (RAGE), a member of the immunoglobulin superfamily of cells surface molecules. RAGE can also be activated by other ligands, including other S100 family members, to activate the MAP-kinase and NF-kappa B pathways [15, 17, 23]. S100P activation of RAGE stimulates growth, invasion and migration in colon cancer cells. However, the downstream signaling events in the S100P/RAGE signaling axis remain to be identified.

MicroRNAs are a class of small noncoding RNA of about 22 base pairs that have emerged as a key player in various cellular and pathogenic processes that includes cellular development, immunological response, tumorigenesis, invasion and metastasis. MiR-155 has been implicated in the pathogenesis of colon cancer as well as other malignancies [24]. Having both oncogenic and inflammatory properties, miR-155 is a prime example of a microRNA that links inflammation and cancer [25]. With respect to colon cancer, elevated levels of miR-155 have been observed in primary colon cancers and metastatic lesions [26]. Although miR-155 has been shown to target transcripts involved in DNA repair [25], the targets important for the metastatic phenotype associated with colon cancer are not known. Furthermore, the upstream signaling events that regulate the expression of miR-155 in colon cancers cells remain to be elucidated.

Recent studies provide insights into the regulation of miR-155 and indicate a context dependent regulation of the promoter of the miR-155 host gene (MIR155HG), also known as B cell integration cluster (BIC) [27, 28]. In breast cancers, miR-155 is induced by transforming growth factor β /SMAD signaling which involves a SMAD response element within the MIR155HG promoter (–454) [29]. Also BRCA1 has been shown to epigenetically regulate miR-155 [30]. In lymphoma cells, both AP-1 (–40 nt from TSS) and NF-kappa B sites (–1150 and –16797 nt) have been demonstrated to control the expression of the MIR155HG promoter [28, 31]. In human retinal epithelial cells, miR-155 can be regulated by cytokines via JAK/STAT pathway [32]. Taken together these studies implicate important regulatory elements within the MIR155HG promoter that may be targets of activation by the S100P/RAGE signaling pathway in colon cancers.

Clearly, miR-155 promotes the tumorigenic phenotype in colon cancer cells [33]. Furthermore miR-155 is up-regulated by AP-1 and NF-kappa B, two transcription factors implicated in RAGE signaling [34–36]. Taken together, this information suggests that S100P/RAGE signaling might function through regulating the expression of miR-155. Our study here investigates the effects of S100P/RAGE activation on miR-155 and the biological consequences of knockdown of miR-155 in colon cancer cells.

Materials and Methods

Cell culture and maintenance of cell lines

DLD-1, HEK-293T, LS174T and SW480 cell lines were obtained from the American Type Culture collection (ATCC Manassas, VA). All the cell lines were maintained as previous described [10, 37]. HEK-293T, LS174T, and SW480 cells were cultured in 1X high glucose Dulbecco's modified Eagle's medium (DMEM) with sodium pyruvate (Invitrogen) supplemented with 10% fetal Bovine serum (FBS), and 1% penicillin-streptomycin (P/S). DLD-1 cells were maintained in Roswell Park Memorial Institute medium (RPMI) 1640, also supplemented with 10% FBS and 1% P/S. SW480 cells stably transfected with S100P (SW480/S100P) or empty vector (SW480/pcDNA) were maintained in 1X DMEM supplemented with 10% FBS, 1% P/S and 250 µg/mL G418 selections. Also, LS174T cells with stable S100P knockdown (LS174T/ShS100P) or empty vector (LS174T/PLKO1) were maintained in 1X DMEM supplemented with 10% FBS, 1% P/S and 2 µg/mL puromycin. The empty vector or miR-155 sponge transduced SW480/S100P cells were maintained in 250 µg/mL G418 and 2 µg/mL puromycin supplemented medium. DLD-1 and LS174T cells transduced with miR-155 sponge or empty vector were maintained in RPMI 1640 and 1X DMEM (respectively) that were supplemented 10% FBS, 1% P/S and 2 µg/mL puromycin.

RNA isolation and qRT-PCR analysis

Small RNAs were isolated using the mirVana kit and protocol (Ambion, Austin Texas). The isolated RNAs were subjected to reverse transcriptase reaction using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Pittsburgh PA) reverse transcriptase kit and protocol. Gene specific stem loop primers for miR-155, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCCCT and U6, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATATG (IDT, Chicago, IL) were added to the reverse transcriptase reaction mix. The reaction mixtures were incubated for 10 min at 25°C followed by 50°C for 30 min, and 85°C for 5 min. The cDNA (50 ng) was added to 20 µL reaction mix containing 0.5 µM of forward miR-155 primer, GCGGTTAATGCTAATCGTGAT or U6 primer, GCGCGTCGTGAAGCGTTC (IDT) in 1X Roche Master mix for realtime PCR reaction. The reaction mix were incubated at 95 °C for 3 minutes, 95 °C for 10 seconds, 60 °C for 20 seconds and 72 °C for 30 seconds. Matured miR-155 transcript levels were determined by comparative Ct method ($2^{-\Delta\Delta C_t}$). The $\Delta\Delta C_t = \Delta C_t \text{ miR-155}_{\text{target}} - \Delta C_t \text{ U6}_{\text{reference}}$. The results were normalized using the empty vector or vehicle control.

Expression and isolation of human recombinant S100P protein

The expression and purification of human recombinant S100P protein were performed as previously described [15]. Briefly, full-length human S100P cDNA was cloned into pTrcHis2 vector and chemically transformed into one shot TOP10 competent Escherichia coli (E. coli) cells (Invitrogen). Transformed E. coli cells were cultured on a 270 RPM shaker and at 37°C to $A_{600} = 0.6$, at that point 1 mM isopropyl-1-thio-β-D-galactopyranoside was added and cells were cultured for another 4 hours at 37 °C and 270 RPM. The cells were harvested by centrifuging at 3000 g at 4°C and stored at –80°C for

later use. Next, the bacterial cells were sonicated for 13 minutes with cooling to release the proteins. His-S100P and other proteins were then separated from cellular debris using centrifugation. The His-S100P protein isolation was performed under Native condition using Probond resin kit, according to the manufacturer protocol (Invitrogen). In order to avoid purification of unspecific protein, the probond resins were washed 4 times with 50 mM Imidazole and another 4 times with 100 mM Imidazole. The His-S100P protein was then eluted with 250 mM Imidazole in native buffer and concentrated using Amicon Ultra-15 (Millipore, Billerica MA), estimated and then analyzed using SDS-PAGE and Western blot. Purified S100P proteins were stored at -20°C in 5% glycerol. The proteins from the empty vector-transformed TOP10 cells were used as a control to monitor for protein purity.

Western blotting

SW480 colon cancer cells were seeded at 2 million cells per well in a six-well plate and cultured for 24 hours. The cells were then starved overnight in 2 mL 1X OptiMEM medium (Invitrogen). Purified human recombinant S100P protein (200 nM) or vehicle were added to the cells and incubated for 0 min, 15 min, 30 min, and 60 min. Later the cells were harvested and nuclear proteins were isolated using the nuclear extraction kit and protocol (Pierce, Rockford IL). The proteins concentrations were estimated using the Bradford assay (Bio-rad, Hercules CA) and 50 μg of nuclear protein were denatured in 1X loading buffer at 98°C for 5 min, loaded and run in 10% acrylamide gel. Western blotting analysis was conducted with primary anti-human antibodies recognizing phospho c-Fos (Cell signaling, catalog number - 5348), total c-Fos (Cell signaling, catalog number - 2250), phospho c-jun (Cell signaling, catalog number - 2361), total c-jun (Cell signaling, catalog number - 9165), phospho NF-kappa B p65 (Cell signaling, catalog number - 3033), total NF-kappa B p65 (Cell signaling, catalog number - 8242) and lamina A (Cell signaling, catalog number - 4777). Secondary antibodies conjugated with horseradish peroxidase were incubated with the membrane for 1 hour at room temperature. Membranes were washed with phosphate-buffered saline with 0.05% Tween 20 (Sigma-Aldrich). Signals were detected using the Western Lightening Plus – ECL (Perkin Elmer, Inc, Waltham, MA).

Pharmacological study

SW480 colon cancer cells were plated at 2×10^6 cells per well in a six-well plate and incubated overnight. The culture medium was then aspirated and replaced with fresh 1X optiMEM medium (starving medium) and again incubated overnight. After that the cells were pre-treated with 10 μM U0126 (Sigma Aldrich, St. Louis MO) for 30 minutes and 0.5 μM CAY10512 (Sigma Aldrich) for 1 hour. Following this, 200 nM of purified human recombinant S100P proteins were added and cells incubated for 1 hour at 37°C . Next, treated cells were harvested and small RNAs isolated using the mirVana kit (Ambion). The miR-155 levels were analyzed using the real time qRT-PCR.

Luciferase assays

LS174T and SW480 cells were transfected using TransIT (Mirus Bio LLC, Madison WI) according to the manufacturer's specification. A total of 1×10^6 cells were seeded in each well in six-well plate and incubated overnight. The cells were then co-transfected overnight with MIR155HG_{promoter}/pGL3 and renilla or pGL3_{basic} and renilla. The transfected medium was replaced with fresh optiMEM medium and incubated for 24 hours. Next, the cells were pre-treated with pharmacological inhibitor for MEK (10 μM U0126) or NF-kappa B (0.5 μM CAY10512) for 4 hours. The cells were then subjected to 200 nM human recombinant S100P treatments for 1 hour and later harvested with luciferase 1X passive lysis buffer (Promega, Madison WI). The experimental results were analyzed with luminator (Berthold detection system, Sirius). All transient transfection were carried out overnight using TransIT.

Chromatin immunoprecipitation

For chromatin immunoprecipitation, 2×10^6 of LS174T or SW480 colon cancer cells were seeded in each well in a six-well plate and cultured for 24 hours. Next the cells were starved with 2 mL 1X OptiMEM medium for 24 hours and then treated with 200 nM of purified human recombinant S100P or vehicle for 1 hour. Cells were then fixed with 1% formaldehyde for 10 min and 0.125 M glycine was added to stop fixing and avoid over fixing of the cells. Next, the cells were washed twice with cold 1X PBS, harvested by spinning down at 1000 Xg for 1 min, then suspended in cold-RIPA buffer and stored at -80°C . To fragment the DNA, the cells were sonicated to obtain fragmented DNA of about 600 bp, repeatedly for 10 seconds with 50 seconds cooling for 8 times at 90 amplitude. The lysates were subjected to immunoprecipitation with anti-human antibodies for c-Fos (Cell signaling, catalog number-5348) using the EpiSeeker ChIP kit and protocol (ABCAM). Realtime PCR was performed to assess enrichment of AP-1 at MIR155HG promoter using AP-1 forward primer - TGTAGGTTCCAAGAACAGGCAGGA and reverse primer - ACTTGTGACTCATAACCGACCAGG; GAPDH forward primer - GCTA CTAGCGGTTTTACGGG and reverse primer - TGACTGTGGAACAGGAGGAG.

Generation of stable miR-155 knockdown (sponge) cell lines

Transient transfection of retroviral particles and transduction were performed as previously described [38]. Briefly, retrovirus particles were generated through transient cotransfection of HEK-293T cells with retroviral expression vectors plus packaging vectors using modified calcium phosphate precipitation procedure. HEK-293T cells were seeded at 2.5 million cells onto 100 mm tissue culture dish that was pre-coated with poly-D glycine (Sigma-Aldrich) for 30 minutes and cultured overnight at 37°C with 10 mL of freshly supplemented high glucose DMEM medium. Later, plasmid DNA was precipitated by mixing 10 μg of retroviral vector, 10 μg of vesicular stomatitis virus G protein expression vector, and 10 μg of pVPack dGI packaging vector with ethanol and 3 M of sodium acetate and then stored at 4°C overnight. The following day, 30 μL of 2.5 M CaCl_2 was added to re-suspend the DNA. Another round of DNA precipitated was carried out by the addition of 0.5 mL of 2X HEPES-buffered saline (0.5% HEPES, 0.6% NaCl, 0.1% dextrose, 0.01% anhydrous Na_2HPO_4 , 0.37% KCl [pH 7.10]) and mixing. The precipitates were incubated at room temperature for 20 minutes and then added in dropwise fashion cells. The cells were cultured at 37°C with 5% CO_2 for 20 h before the medium was replaced with 12 mL of freshly supplemented DMEM medium. At 48 hour the viral supernatant were collected, spun for 1.5 minutes at 1000 Xg and then filtered with 0.45- μm -pore-size surfactant-free cellulose acetate filter. The filtered supernatants were used to infect DLD-1, LS174T and SW480/S100P cells. A day prior to infection, 500,000 cells were seeded on six-well plates and incubated overnight. The medium was changed with fresh supplemented DMEM (10% FBS and 1% P/S). Polybrene was added to the viral supernatant, as well as, the transfection medium to the final concentration of 4 $\mu\text{g}/\text{mL}$. At this point, the viral supernatant was then added to the cells. After 8 hours, 3 mL of fresh completed medium were added to the cells and cultured for additional 48 hours. The infected cells were selected with 2 $\mu\text{g}/\text{mL}$ puromycin.

Colony formation assay

Transduced miR-155 sponge or empty vector cells were seeded in 100 mm dish plates in the amount of 1000 cells per dish. The cells were then incubated in 20 mL 1X DMEM supplemented with 10% FBS, 1% P/S, 2 $\mu\text{g}/\text{mL}$ and incubated at 37°C and 5% CO_2 for 3 weeks. At the end of the incubation, the culture medium was aspirated and colonies were stained in methylene blue dye (0.5% methylene blue and 50% methanol) at room temperature for 10 minute. The plates were gently rinsed with water and visible colonies counted.

Motility and invasion assay

Cell motility and invasion were performed as previously described [10, 37]. In a 24 well plate, 600 μ L cultured medium was added to each well prior to the placement of 8 μ m pore Falcon transwell. The cells bearing stable miR-155 knockdown or empty vector were resuspended with culture medium to make a final volume of 100 μ L and then seeded into the transwell and cultured for 24 hours or 48 hours at 37 °C, 5% CO₂. Next, the incubation medium at the upper and bottom chambers were aspirated and the insert placed upside down. The surfaces of the inserts were quickly stained with crystal violet stain (0.5% crystal violet in 20% methanol) for 1 minute at room temperature. The crystal violet solutions on the inserts were removed by gently dipping and rinsing them in distilled water three times. Non-motile cells inside the inserts were removed with wet cotton swap, repeatedly. The inserts were then allowed to air dry overnight. Experiment was repeated one more time. The number of motile cells (cells penetrating through the membrane) and invaded cells (cells that had passed through the membrane) were counted under a light microscope.

Statistical analysis

All experiments were performed at least three times. Results are presented as means of $\pm$ S.E.M. Statistical comparisons between two groups of data were made using two-tailed unpaired Student's t test. A *p*-Value of < 0.05 was considered statistically significant. Any *p*-Value of < 0.05 is denoted as *, *p*-Value of < 0.01 as **, and *p*-Value of < 0.001 denoted ***.

Results

S100P regulates the expression of miR-155

Activation of RAGE receptor signaling by S100P is known to stimulate both ERK and NF-kappa B signaling [15, 17]. In addition, miR-155 can be up-regulated by NF-kappa B, by adrenaline in colon cancer cells [33], and by ERK signaling in human B cells [28]. Therefore, we investigated whether S100P/RAGE receptor signaling could up-regulate miR-155 expression. **Previous studies demonstrated that SW480 cells express the RAGE receptor but not S100P proteins [17, 39]. In addition, SW480 cells express a low level of miR-155 [40]. Furthermore, ectopic S100P confers a migratory phenotype onto SW480 cells [10]. To first investigate the relationship between miR-155 and S100P expressions, we evaluated whether changes in the S100P expression could alter miR-155 level in colon cancer cells. S100P or empty vector was stably transfected in SW480 colon cancer cells and S100P transcript levels in the cells were analyzed using qRT-PCR (Figure 1A). The ectopic expression of S100P resulted in significant elevation of miR-155 (Figure 1B). To validate this result, S100P expression in LS174T colon cancer cells was knocked down using a stably transfected shRNA construct which targets S100P mRNA (ShS100P). Studies by our group have previously shown that LS174T cells express S100P protein knockdown of S100P affects cell proliferation and motility [10]. We can also demonstrate that the LS174T cells also express the RAGE receptor (data not shown). S100P knockdown was once again confirmed using the qRT-PCR (Figure 1C). The knockdown of S100P led to reduced miR-155 levels in the LS174T cells (Figure 1D). The results indicate that miR-155 expression is sensitive to altered S100P levels. Altogether, these results suggest that S100P regulates miR-155 expression in human colon cancer cells.

S100P regulation of miR-155 is RAGE dependent

Since S100P is a secreted protein and ligand for the RAGE receptor, we evaluate whether S100P regulation of miR-155 expression is receptor mediated. SW480 cells were treated with purified human recombinant S100P for different time points and analyzed for changes

in miR-155 expression levels using the qRT-PCR. The miR-155 level was increased 4 fold in 20 minutes, 11 fold in 1 hour, 1.5 fold in 2 hours and returned to baseline by 24 hours (Figure 2A). The return to baseline levels by miR-155 appears to be due to S100P no longer stimulating the RAGE receptor. We observed a similar time course of miR-155 induction in LS174T cells, however the induction of miR-155 by exogenous S100P was maximal at 4 hours (Figure 2B). Next, the dependence of miR-155 induction by S100P on the RAGE receptor was evaluated. Cells were pre-treated with blocking anti-RAGE antibody and then treated with recombinant S100P for different time periods followed by qRT-PCR for miR-155 induction. Analysis of the results indicated that pre-treatment with anti-RAGE antibody suppressed S100P induction of miR-155 (Figure 2C). Overall, the results indicate that induction of miR-155 by S100P is RAGE dependent.

S100P controls miR-155 expression through AP-1 in colon cancer cells

S100P is known to stimulate AP-1 and NF-kappa B activation via RAGE engagement. First, activation of AP-1 and NF-kappa B by exogenous S100P treatment was confirmed using Western blot (Figure 3A). Subsequently, we examined whether S100P/RAGE regulation of miR-155 is via AP-1 or NF-kappa B pathways. Colon cancer cells were pre-treated with either U0126 (a MEK/MAPK kinase inhibitor) or CAY10512 (a NF-kappa B inhibitor) prior to stimulation of RAGE receptor signaling with exogenous S100P. Both U0126 and CAY10512 suppressed S100P induction of miR-155 (Figure 3). These data suggest that both AP-1 and NF-kappa B may be involved in the induction of miR-155 by S100P.

To further elucidate the involvement of AP-1 and NF-kappa B in S100P-mediated miR-155 stimulation, we monitored the effects of pharmacological blockade of AP-1 or NF-kappa B on miR-155HG promoter activities, in the presence of S100P using luciferase assay. Blockage of AP-1 and not NF-kappa B suppressed MIR155HG promoter luciferase activities in the presence of exogenous S100P (Figure 4A and 4B). The MIR155HG promoter does display activity in the tumor without S100P (Supplementary Figure 1). Inhibition of AP-1 activation with MEK inhibitor – U0126, resulted in decreased luciferase activities, whereas inhibition of NF-kappa B activation with NF-kappa B inhibitor – CAY10512, resulted in no appreciable changes in the MIR155HG promoter luciferase activity. These data imply that AP-1, not NF-kappa B may be responsible for the activation of the MIR155HG luciferase promoter upon S100P/RAGE receptor stimulation. The AP-1 family consists of Jun members (c-Jun, JunB, and JunD) and Fos members (c-Fos, FosB, Fra-1 and Fra-2) (reviewed in [41]). Because, c-jun has been shown to modulate gene expression in colon cancer cells, we chose to inhibit c-Jun with a dominant negative c-Jun construct (TAM67). Furthermore, genetic inhibition of the AP-1 family member c-Jun with TAM67, a dominant negative suppressor of c-Jun [42–44], attenuated miR-155 induction by exogenous S100P. Combined, these results suggest that AP-1 may be involved in the transcriptional regulation of miR-155.

Next, ChIP assays were performed to confirm that AP-1 regulates the miR-155 expression upon activation of S100P/RAGE receptor signaling. Results indicate that S100P treatment enhanced c-Fos occupancy on MIR155HG promoter region in two different colon cancer cell lines (Figure 5A and B). The c-Fos occupancy of the MIR155HG promoter was investigated following the interaction and activation of RAGE by exogenous S100P using chromatin immunoprecipitation assay analysis. The antibodies specific for RNA polymerase II were used as positive control. The immunoprecipitated chromatins were analyzed using primers for the GAPDH active locus. Relative levels of the chromatin were the same and unaffected by the exogenous S100P treatment. Non-immune mouse IgGs were used as a negative control. We observed increased chromatin precipitates using c-Fos antibodies in the exogenous S100P treated cells compare to the vehicle control, indicating c-Fos binding to the MIR155HG promoter. Non-immunoprecipitates were used as the input control for the

experiments. Overall, these results demonstrate that S100P/RAGE signaling pathway can stimulate miR-155 expression via AP-1 in colon cancer cells.

Down-regulation of miR-155 decreases cell growth, motility and invasion in human colon cancer cells

S100P is implicated in cancer cell proliferation, migration and invasion [15, 17, 18]. The significant elevation of both S100P and miR-155 expression in tumor specimens and our findings that S100P stimulates miR-155 expression prompted investigation into the functional importance of miR-155. The miRNA “sponge” approach provides a useful means of creating miRNA loss of function [45–47]. The miR-155 sponge or empty vector was transduced into colon cancers. Using the confocal microscope, we observed the expression of Green Fluorescent Protein (GFP) gene in the transduced cells indicating the successful transduction of the retroviral plasmids. Also, reduced miR-155 level was observed by performing the qRT-PCR analysis and comparing cells bearing the sponge to empty vector (Supplementary Figure 2). Next, cell growth, colony formation and migratory assays were performed using the transduced (SW480/S100P, DLD-1, LS174T) cells. Similar to LS174T cells, DLD-1 cells express both S100P and miR-155 [22, 48]. Knockdown of miR-155 significantly decreased cell growth (Figure 6A; also see Supplementary Figure 3). In the colony formation assay, miR-155 knockdown decreased the ability of the cells to form colonies (Figure 6B; Supplementary Figure 4). In addition, knockdown of miR-155 significantly reduced colon cancer cell motility and ability to invade into the transwells (Figure 6C and 6D; Supplementary Figure 5 and 6). Altogether, these results indicate that loss of miR-155 levels suppresses tumorigenic characteristics associated with the metastatic phenotype of colon cancer cells. These functions support our notion that the regulation of miR-155 by S100P/RAGE signaling is an important step by which cancer cells promote their growth and migration during carcinogenesis.

Discussion

Elevated levels of S100P have been detected in a variety of human tumors including, human colon cancers [14, 18, 49–52] and proven to play an important role in the pathogenesis of cancer since its discovery in 1992. It has become of special interest because it is associated with a poor prognosis and its ability to contribute to tumor invasion and metastasis. In the present study we further investigate the molecular mechanism by which S100P and RAGE receptor signaling contribute to metastasis.

We show for the first time that oncogenic miR-155 can be regulated by the S100P/RAGE pathway. Enforced S100P expression up-regulates miR-155 level in colon cancer cells. In addition, exogenous S100P protein stimulates miR-155 expression and that this stimulation is RAGE dependent. The blockage of RAGE with anti-RAGE antibodies suppresses miR-155 induction by exogenous S100P protein. The regulation of miR-155 was MAPK kinase, and to a lesser extent NF-kappa B dependent. These data also show that S100P/RAGE mediated regulation of miR-155 is AP-1 dependent. Thus these studies provide a molecular mechanism linking inflammation and oncogenic events important in colon cancer progression and metastasis.

To determine the biological relevance of miR-155 induction by S100P in colon cancer cells, we selectively inhibited miR-155 function using miR-155 sponge. The sponge RNAs contain complementary binding sites to a miRNA of interest and are produced from transgenes within cells [45–47]. Therefore, miRNA sponges have proven to be very useful tools for understanding miRNA functions in several experimental systems. We observed a decrease in cell growth, colony formation, motility and invasion in colon cancer cells

expressing the miR-155 sponge. This observation is in agreement with previous studies investigating miR-155 function in cancer cells [53–56].

Although we have been able to establish that S100P/RAGE signaling can regulate the expression of miR-155, the targets of miR-155 important in colon cancer progression and metastasis are not well defined. There have been many miR-155 targets identified using both genomic and proteomic approaches [29, 38, 57, 58]. Most notably, suppressor of cytokine signaling 1 (SOCS1) [57] and RhoA [29] are two miR-155 targets implicated in migration and invasion in other cancers. Thus, the target genes controlled by the S100P/RAGE/miR-155 signaling axis remain to be identified.

To our knowledge, this is the first time it has been shown that S100P/RAGE receptor signaling regulates the expression of oncogenic miR-155. Previous reports have shown that inhibition of S100P, RAGE, or miR-155 suppressed colon cancer growth and metastasis [11, 21, 53, 59]. Our results support these observations and suggest that the newly identified S100P/RAGE/miR-155 pathway may help open up new avenues for understanding the relationship between inflammation and metastasis in colon cancer as well as aid efforts for the development of effective therapeutic strategies for the treatment of late stage colon cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. S100P regulates miR-155 expression levels in colon cancer cells

(A) SW480 cells with stable S100P expression shows increase S100P transcript. (B) Elevated miR-155 expression in S100P stably transfected SW480 cells. (C) Knockdown of S100P in LS174T cells with shRNA which targets S100P mRNA. (D) A marked decrease of miR-155 level in cells with shS100P knockdown. The results were normalized against the empty vector control. The vertical bar is the SEM. ** indicates $p < 0.01$, and *** also indicates $p < 0.001$.

Figure 2. S100P induces miR-155 expression through RAGE receptors

(A) Exogenous S100P treatment induced miR-155 in SW480 cells in time dependent response. Vehicle control was used for normalization. (B) miR-155 expression induced most at 4 hours in LS174T cells following exogenous S100P treatment. (C) Blockage of RAGE with anti-RAGE antibody suppresses miR-155 induction by exogenous S100P treatment of SW480 cells. The value of the results are present as the mean $\pm$ SEM (**p < 0.01, ***p < 0.001).

Figure 3. S100P stimulates miR-155 expression through the activations of MAPK kinase and NF-kappa B pathways

(A) Exogenous S100P treatment induces the activation and nuclear translocation of AP-1 family (c-jun and c-fos) and NF-kappa B p65 in SW480 cells in a time dependent fashion.

(B) Pre-treatment with MEK or NF-kappa B inhibitors (U0126 and CAY10512) blocks miR-155 induction by exogenous S100P treatments in SW480 colon cancer cells. The normalization unit is the value of the vehicle treated control. The data are means $\pm$ SEM of the experiments (**p < 0.01, ***p < 0.001).

Figure 4. Inhibition of MAPK kinase suppresses MIR155HG promoter activities

(A, B) One million SW480 or LS174T cells were seeded and incubated overnight at 37 °C. Next, the cells were transiently transfected overnight with MIR155HG promoter luciferase reporter gene or vehicle constructs. The cells were pre-treated with MEK inhibitor (U0126) or NF-kappa B inhibitor (CAY10512) for 4 hours and then treated with purified human recombinant S100P for 1 hour. Cells were harvested and analyzed. Pre-treatment with MEK inhibitor and not NF-kappa B inhibitor blocked MIR155HG luciferase activities. (C) TAM67 suppresses miR-155 induction by S100P. TAM67 or empty vector was transiently transfected overnight into SW480 cells. Cells were treated with S100P for 1 hour and harvested for miR-155 qRT-PCR analysis. The results were normalized against the empty vector (control). Value of the results are shown as mean $\pm$ SEM (** $p < 0.01$, *** $p < 0.001$).

Figure 5. S100P prompts c-Fos enrichment at AP-1 site in MIR155HG promoter

(A, B) SW480 or LS174T cells were treated with exogenous S100P for an hour. Following this, the cells were fixed with 1% formaldehyde for 10 minutes at room temperature. The chromatin was then fragmented by sonication. Next, c-Fos protein was immunoprecipitated with anti-human c-Fos antibody. Finally, the results were analyzed using the real time PCR. The result shows that c-Fos bind to the MIR155HG promoter following S100P treatment. The results were normalized against the vehicle treated cells (as control). Value of the results are represented as mean $\pm$ SEM (**p < 0.01, ***p < 0.001).

Figure 6. Knockdown of miR-155 suppresses cell growth, colony formation, motility and invasion

SW480 cells stably expressing S100P were transduced using miR-155 sponge or empty vector. (A) The cells were plated in triplicate at 250,000 cells/well in 6-well plates and cells numbers were counted every 2 days using trypan blue to monitor growth. (B) The sponge or empty vector transduced cells were seeded at 1,000 cells per 100 mm plate and incubated for 3 weeks to allow colony formation. Next, the culture medium was aspirated and colonies stained with methylene blue solution. The plates were then rinsed with water and stained colony numbers counted. (C and D) The same cells were plated at 50,000 cells per well in transwell and incubated for 24 hours. The bottom top of the insert was stained with crystal violet solution and the number of motile and invaded cells counted under a light microscope. The results were normalized against the empty vector control. Value of the results are shown as mean $\pm$ SEM (**p < 0.01, ***p < 0.001).