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Mitochondrial STAT3 contributes to transformation of Barrett’s epithelial cells that express oncogenic Ras in a p53-independent fashion

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Yu C, Huo X, Agoston AT, Zhang X, Theiss AL, Cheng E, Zhang Q, Zaika A, Pham TH, Wang DH, Lobie PE, Odze RD, Spechler SJ, Souza RF. Mitochondrial STAT3 contributes to transformation of Barrett’s epithelial cells that express oncogenic Ras in a p53-independent fashion. Am J Physiol Gastrointest Liver Physiol 309: G146–G161, 2015. First published June 4, 2015; doi:10.1152/ajpgi.00462.2014.—Metaplastic epithelial cells of Barrett’s esophagus were transformed by the combination of p53 knockdown and oncogenic Ras expression, which known to activate signal transducer and activator of transcription 3 (STAT3). When phosphorylated at tyrosine 705 (Tyr705), STAT3 functions as a nuclear transcription factor that can contribute to oncogenesis. STAT3 phosphorylated at serine 727 (Ser727) localizes in mitochondria, but little is known about mitochondrial STAT3’s contribution to carcinogenesis in Barrett’s esophagus, which is the focus of this study. We introduced a constitutively active variant of human STAT3 (STAT3CA) into the following: 1) non-neoplastic Barrett’s (BAR-T) cells; 2) BAR-T cells with p53 knockdown; and 3) BAR-T cells that express oncogenic H-RasG12V. STAT3CA transformed only the H-RasG12V-expressing BAR-T cells (evidenced by loss of contact inhibition, formation of colonies in soft agar, and generation of tumors in immunodeficient mice), and did so in a p53-independent fashion. The transformed cells had elevated levels of both mitochondrial (Ser727) and nuclear (Tyr705) phospho-STAT3. Introduction of a STAT3CA construct with a mutated tyrosine phosphorylation site into H-RasG12V-expressing Barrett’s cells resulted in high levels of mitochondrial phospho-STAT3 (Ser727) with little or no nuclear phospho-STAT3 (Tyr705), and the cells still formed tumors in immunodeficient mice. Thus, tyrosine phosphorylation of STAT3 is not required for tumor formation in Ras-expressing Barrett’s cells. We conclude that mitochondrial STAT3 (Ser727) can contribute to oncogenesis in Barrett’s cells that express oncogenic Ras. These findings suggest that agents targeting STAT3 might be useful for chemoprevention in patients with Barrett’s esophagus.

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SIGNAL TRANSDUCER AND ACTIVATOR of transcription 3 (STAT3) is a key regulator of numerous biological processes, including inflammation and carcinogenesis (28). Persistent activation of STAT3 appears to play a major role in the development and progression of a number of human tumors, including breast, lung, and colon cancers (11). When Janus kinase phosphorylates STAT3 at tyrosine 705 [phospho-STAT3 (Tyr705)], it translocates to the nucleus to function as a transcription factor that induces the expression of target genes that can be involved in carcinogenesis (1). A number of inhibitors that target STAT3’s nuclear function have been shown to promote cancer cell death in vitro, but the therapeutic value of these agents has not been demonstrated (17). STAT3 had been assumed to contribute to carcinogenesis exclusively through its nuclear transcription factor function until recently, when investigators identified a pool of STAT3 in mitochondria (32). For mitochondrial localization, STAT3 must be phosphorylated at serine 727 [phospho-STAT3 (Ser727)] (28). This phospho-STAT3 (Ser727) is bound by gene associated with retinoid-interferon-induced mortality 19 (a component of complex I of the electron transport chain), which transports the serine-phosphorylated protein into mitochondria (7, 13, 16, 25, 35). Mitochondrial STAT3 has been shown to play a role in oncogenic H-RasG12V-dependent malignant transformation through mechanisms unrelated to nuclear transcription (9). Indeed, mouse embryonic fibroblasts that express oncogenic RasG12V require phospho-STAT3 (Ser727), but not phospho-STAT3 (Tyr705), for malignant transformation (9). In these transformed fibroblasts, mitochondrial STAT3 stabilizes mitochondrial function and membrane potential and augments the activity of complexes II and V of the electron transport chain (9). These findings suggest that mitochondrial STAT3 could be a novel target for cancer
Table 1. Oligonucleotide primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Location</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 primer 1-5’</td>
<td>AGA CTG CCT TCC GGG TCA</td>
<td>Sense</td>
<td>PCR &amp; sequencing</td>
</tr>
<tr>
<td>p53 primer 4-3’</td>
<td>ACG GGG GAA CAA GAA GGG GA</td>
<td>Antisense</td>
<td>PCR &amp; sequencing</td>
</tr>
<tr>
<td>p53 primer 2-5’</td>
<td>CAT AGG GGA CCA GCA CAC TA</td>
<td>Sense</td>
<td>Sequencing</td>
</tr>
<tr>
<td>p53 primer 3-3’</td>
<td>TGG CCA TCT ACA AGC ACT CA</td>
<td>Antisense</td>
<td>Sequencing</td>
</tr>
<tr>
<td>STAT3CA Sal-1-5’</td>
<td>AAA GTC GAC TCA CAT GGG GGA GGT AGG GCA</td>
<td>Sense</td>
<td>PCR &amp; subcloning</td>
</tr>
<tr>
<td>STAT3CA Sal-1-3’</td>
<td>AAA GTC GAC TCA CAT GGG GGA GGT AGG GCA</td>
<td>Antisense</td>
<td>PCR &amp; subcloning</td>
</tr>
<tr>
<td>STAT3CA Y705F-5’</td>
<td>GT AGG GGT GGA CCA TTT CTC AAG ACC AAG TTT</td>
<td>Sense</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>STAT3CA Y705F-3’</td>
<td>AAA CTG GCT CTT CAG AAA TGG GGC AGC GCC AGC</td>
<td>Antisense</td>
<td>Site-directed mutagenesis</td>
</tr>
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STAT3CA, constitutively active variant of human signal transducer and activator of transcription 3.

chemoprevention, but few data are available on the contribution of mitochondrial STAT3 to the malignant transformation of human epithelial cells.

To explore the contribution of mitochondrial STAT3 to the development of human epithelial tumors, we chose for our disease model Barrett’s esophagus, the condition in which a metaplastic, intestinal-type epithelium predispersed to adenocarcinoma replaces esophageal squamous epithelium that has been damaged by gastroesophageal reflux disease (27). Approximately 6% of adult Americans have Barrett’s esophagus, and the frequency of esophageal adenocarcinoma in the United States has increased more than sevenfold over the past several decades (12, 22, 23, 26, 30). Thus there is intense interest in understanding the molecular mechanisms underlying Barrett’s carcinogenesis to identify potential targets for chemoprevention to stem the dramatic rise in the frequency of this cancer.

In earlier studies, we found that Barrett’s epithelial cells transformed through a combination of p53-knockdown and forced expression of H-RasG12V secrete interleukin-6 and activate STAT3 (34, 37). In human biopsy specimens of Barrett’s metaplasia, epithelial cell levels of phospho-STAT3 (Tyr705) and DNA sequencing. Retroviral particles were generated as previously described (15). All of the BAR-T epithelial cell lines [BAR-T, BAR-T H-RasG12V (clones R6 and R7) and the transformed BAR-T p53RNAi+ H-RasG12V-expressing cell line (clone R1)] for this present study (37). The nontransformed cells were co-cultured with a fibroblast feeder layer, as previously described (15, 37); the transformed cells did not require a fibroblast feeder layer. The OE33 esophageal adenocarcinoma cell line was purchased from Sigma. All cell lines were maintained in monolayer culture at 37°C in humidified air with 5% CO2 in growth media, as previously described (37). For individual experiments, nontransformed cell lines were equally seeded into collagen IV-coated wells (BD Biosciences, San Jose, CA), whereas transformed and esophageal adenocarcinoma cell lines were equally seeded onto standard culture dishes. Cellular morphology was documented using the Metamorph imaging system (Universal Imaging, Downingtown, PA).

Viral vectors and vector infection. The human STAT3-CA plasmid (pcDNA3.1-STAT3CA) is a constitutively active variant of STAT3 containing substitutions in cysteine residues (A661C and N663C of STAT3) that enable the protein to dimerize spontaneously [in the absence of tyrosine phosphorylation (Y705)] (2, 18). When transfected in serum-grown cells, STAT3CA is serine phosphorylated (Ser727), but is not always tyrosine phosphorylated (2). STAT3CA cDNA was amplified by PCR from the plasmid using primers designed to introduce a SalI site (Table 1). PCR conditions consisted of incubation at 95°C for 5 min, followed by 42 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 2.5 min. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. PCR products were purified by using Qiagen gel extraction kit (Qiagen, Valencia, CA) per the manufacturer’s instructions. The STAT3CA cDNA fragment was inserted into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), then subcloned into the pWZL-hygromycin B retroviral mammalian expression vector (obtained from Dr. Scott Lowe, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The retroviral vector pWZL-hygromycin B without the insert served as a control. The presence of the insert was confirmed by SalI digestion and DNA sequencing. Retroviral particles were generated as previously described (15). All of the BAR-T epithelial cell lines [BAR-T, BAR-T p53RNAi, and BAR-T H-RasG12V (clones R6 and R7)] were infected at ~50% confluence in the presence of 4 mg/ml of Polybrene (Sigma, St. Louis, MO) for 10–12 h. After recovery for 72 h, cells were selected in 30 μg/ml hygromycin B for 10 days. Cell clones were selected using cloning cylinders. We generated BAR-T, BAR-T p53RNAi, BAR-T H-RasG12V R6, and BAR-T H-RasG12V R7 cells containing pWZL-retro-hygromycin B (vector control) or pWZL-STAT3CA-hygromycin B.

We also introduced a tyrosine-to-phenylalanine mutation specifically in the phosphorylation site of Tyr705 (Y705F) of the pWZL-STAT3CA-hygromycin B construct using in vitro site-directed mutagenesis. The in vitro site-directed mutagenesis reaction was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) using primers designed to introduce the desired mutation (Table 1) per manufacturer’s instructions. PCR conditions consisted of incubation at 95°C for 1 min,
followed by 18 cycles at 95°C for 50 s, 60°C for 50 s, and 68°C for 8 min. The construct was sequenced to verify the mutation.

**Growth rate and population doubling time.** Growth rate was determined by cell counting using a Z1 particle counter (Beckman Coulter, Fullerton, CA). Population doublings and doubling times were calculated as previously described (37). All analyses were performed in two independent experiments.

**Cell-to-cell contact inhibition.** Cells were equally seeded into six-well plates and placed in the incubator for at least 10 days. Cell counts were performed using a Z1 particle counter at various time points. All analyses were performed in two independent experiments.

**Soft agar assay.** The soft agar assay was performed as previously described (37). In brief, 5,000 cells were added to 2.5 ml of Noble agar (Sigma), which had been kept at 45°C (final concentration of 0.33% [wt/vol] agar) and supplemented with 20% fetal bovine serum (FBS). For each cell line, the cell-agar mixture was plated in triplicate onto 12-well plates containing a solidified 0.7 ml layer of 0.5% agar-cell culture medium mix. Cells were fed twice weekly with growth media for 3 wk, after which the plates were imaged and colony counts performed with a Bio-Rad Molecular Imager (Bio-Rad, Hercules, CA). OE33 esophageal adenocarcinoma cells were used as a positive control. All analyses were performed in two independent experiments.

**Migration and invasion assays.** For both migration and invasion assays, cells were cultured in starvation keratinocyte growth medium (KBM-2; catalog no. 354165; BD Biosciences, Bedford, MA); KBM-2 growth media without and with coated Matrigel matrix (catalog no. 351152 and no. 354165; BD Biosciences, Bedford, MA); KBM-2 growth media containing 0.1% FBS. For each cell line, the cell-agar mixture was plated in triplicate onto 12-well plates containing a solidified 0.7 ml layer of 0.5% agar-cell culture medium mix. Cells were fed twice weekly with growth media for 3 wk, after which the plates were imaged and colony counts performed with a Bio-Rad Molecular Imager (Bio-Rad, Hercules, CA). OE33 esophageal adenocarcinoma cells were used as a positive control. All analyses were performed in two independent experiments.

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**Migration and invasion assays.** For both migration and invasion assays, cells were cultured in starvation keratinocyte growth medium (KBM-2; containing 0.5% FBS without growth factors) for 24 h, then stained with DIOC12(3) fluorescent dye (Corning, Tewksbury, MA; catalog no. 354218) at 10 μg/ml for 1 h. Cells were trypsinized and counted, and equal numbers of cells (3 × 10⁵) were seeded onto a BD Falcon FluoroBlok 24-Multiwell Insert plate with an 8.0-μm pore size and without and with coated Matrigel matrix (catalog no. 351152 and no. 354165; BD Biosciences, Bedford, MA); KBM-2 growth media containing 5% FBS was placed in the bottom of the wells as a chemoattractant. After 3 or 24 h, images of the cells on the bottom of the inserts were obtained from six separate high power fields (×10) per well; cells were counted and then averaged. All analyses were performed in three independent experiments.

**p53 sequencing.** Total RNAs were isolated using RNasy Mini kit (Qiagen, Valencia, CA) per manufacturer’s instructions and quantitated by spectrophotometry. Reverse transcription was performed using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) per manufacturer’s instructions. cDNA was amplified using PCR products were electrophoresed on 2% agarose gels and purified using the Qiaquick gel extraction kit (Qiagen, Valencia, CA) per manufacturer’s instructions. The purified PCR products (4–8 μg/μl) were sequenced using p53 primer 1 and p53 primer 4 (Table 1). PCR conditions consisted of incubation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min. After amplification, PCR products were electrohoresed on 2% agarose gels and purified using the Qiagen gel extraction kit (Qiagen, Valencia, CA) per manufacturer’s instructions. The purified PCR products (4–8 μg/μl) were sequenced using p53 primer 1–4 at the University of Texas Southwestern DNA Sanger Sequencing Core.

**UV-B irradiation.** Cells were irradiated with 200 J/m² of UV-B, and cell lysates were collected for Western blot analysis 24 h later. All analyses were performed in two independent experiments.

**Reporter gene assay.** αs-Macroglobulin luciferase reporter containing the −215 to +8 region of the rat αs-macroglobulin promoter cloned into pGL3 basic (Promega, Madison, WI) upstream of the firefly luciferase reporter (αsM) was used for transient transfection studies; renilla reporter pRL (Promega, Madison, WI) was used to equalize for transfection efficiency. Transcription of the αsM promoter requires STAT3 binding and has been used to determine constitutive transcription signaling by STAT3CA efficiency (2, 18). Cells were grown on 24-well plates to 60–80% confluence and were cotransfected with 500 ng of the αsM plasmid and 25 ng of pRL using 1.25 μl lipofectamine LTX (Invitrogen, Carlsbad, CA) per manufacturer’s instructions. After 48 h of transfection, cells were lysed, and luciferase assays were performed using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI) per manufacturer’s instructions. Data were expressed as relative light units for firefly luciferase normalized to renilla luciferase. All analyses were performed in three independent experiments.

**Isolations of mitochondrial-cytosolic-nuclear protein extracts.** Isolation of mitochondria and cytosolic protein extracts were prepared from cells using specific mitochondria isolation buffers and differential centrifugation (29). Briefly, cells were washed twice with ice-cold 1× PBS buffer, harvested in ice-cold mitochondria isolation buffer (220 mM d-mannitol, 70 mM sucrose, 2 mM HEPES, pH 7.4 with KOH), immediately transferred to a 2-ml Eppendorf tube, and centrifuged at 900 g for 10 min at 4°C. The supernatant was removed and transferred to a new Eppendorf tube and centrifuged at 10,000 g for 10 min at 4°C to obtain a soluble cytosolic fraction and a pellet containing the mitochondria. The pellet (containing the mitochondria) was suspended in 30–50 μl of sucrose/HEPES ice-cold buffer (250 mM sucrose, 10 mM HEPES, pH 7.5 with KOH).

The nuclear and cytosolic extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, Rockford, IL) per manufacturer’s instructions. Protein extracts were subjected to immunoblot analyses.

**Mitochondrial reactive oxygen species detection.** To measure mitochondrial reactive oxygen species (ROS), the fluorescent probe MitoSOX Red (Life Technologies, Grand Island, NY) was used according to the manufacturer’s instructions. In brief, cells were placed in two-well Lab-Tek II chamber slides (Nalge Nunc, Rochester, NY) with a chamber volume of 1 ml at 1 × 10⁵ cells per well. Cells were pretreated with or without 100 μM Mito-TEMPO (Enzo Life Sciences, Farmingdale, NY) for 60 min in Hank’s buffered salt solution (HBSS) containing calcium and magnesium (Sigma, St. Louis, MO), after which the cells were washed two times with HBSS. Cells were loaded with 5 μM MitoSOX in HBSS for 30 min and then washed two times with HBSS. For positive controls, BAR-T H-Ras(522V)R6 cells containing the vector were treated with 500 μM H₂O₂; STAT3CA-expressing BAR-T H-Ras(522V)R6 clone 2 cells were treated with 20 μM doxorubicin in HBSS (with Ca/Mg) containing 1% BSA (all of the chemicals were from Sigma, St. Louis, MO) for 30 min. Cells were fixed in 2% paraformaldehyde for 3–5 min and washed with PBS two times. Then the cells were stained with 4-diamidino-2-phenylindole for 1 min, and washed with PBS three times before laser excitation at 514 nm, and imaged by confocal microscopy (model TCS SP5, Leica Microsystems, Buffalo Grove, IL). Fluorescence was quantitated using National Institutes of Health image J 1.48 software from five separate high-power fields (×40) per well and then averaged. All analyses were performed in three independent experiments.

**Ras activity assays.** We used an Active Ras Pull-down kit ( Pierce, Rockford, IL) per the manufacturer’s instructions. Ras is active when bound to GTP, and active Ras binds specifically to the Ras-binding domain (RBD) of Rafl. The Active Ras Pull-down kit (Pierce, Rockford, IL) uses a glutathione S-transferase (GST)-fusion protein of the RBD of Rafl along with a glutathione agarose resin to specifically pull down active Ras from the protein lysate. For analysis of GTP-bound Ras, equally seeded plates of cells were cultured in starvation KBM-2 medium overnight and were washed twice by cold 1 × Tris-buffered saline buffer, followed by lysis in 0.5 ml lysis-binding-wash buffer per the manufacturer’s instructions. Supernatants (containing equal amounts of protein) were incubated at 4°C and agitated for 1 h with 80 μg of GST-Rafl-RBD and glutathione resin. Precipitated proteins bound to the resin were washed three times with lysis-binding-wash buffer, eluted in Laemmli sample buffer, and subjected to immunoblot analyses. All analyses were performed in two independent experiments.

**Immunoblot analysis.** For Western blotting, protein concentrations were determined using the BCA-200 Protein Assay kit (Pierce, Rockford, IL), and equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with 1:1,000 dilutions of
phospho-STAT3 (Ser727), total STAT3 (Cell Signaling Technology, Danvers, MA), p53 (Calbiochem, Billerica, MA), H-Ras (Santa Cruz Biotechnology, Dallas, TX), phospho- and total ERK1/2, or lamin A/C (Cell Signaling Technology); 1:500 dilutions of phospho-STAT3 (Tyr705), voltage-dependent anion channel, SOD-1 (Cell Signaling Technology), p21 (BD Pharmingen), or Mcl-1 (Santa Cruz); and 1:5,000 dilutions of β-actin or β-tubulin (Sigma). Horseradish peroxidase secondary antibodies were used, and chemiluminescence was determined using the Super Signal West Dura detection system (Pierce, Rockford, IL). Voltage-dependent anion channel and SOD-1 are specific markers for the mitochondrial and cytosolic fractions, respectively; lamin A/C was used as a specific marker for the nuclear fraction; β-actin or β-tubulin was used to confirm equal loading. All Western blots were performed on two independent experiments.

In vivo tumorigenesis. Experimental methods using mice were approved by the Institutional Animal Care and Use Committee at the Dallas Veterans Affairs Medical Center under ACORP no. 05-049. Female, 6-wk-old, specific pathogen free, NOD/SCID mice (deficient in T, B, and NK cells, as well as complement) were obtained (Charles River Laboratories, Wilmington, MA) and allowed to acclimate to the animal facilities at the Dallas Veterans Affairs Medical Center for 1 wk. An average of 5 × 10^6 cells suspended in growth media were mixed with equal volumes of Matrigel (BD Biosciences, Franklin Lakes, NJ) and implanted under the skin of the mouse in the dorsal flank; mice were assessed twice weekly for tumor formation and growth. At death, tumors were removed and processed by fixing in 4% formaldehyde solution, followed by immersion in 10% neutral buffered formalin, dehydration, and paraffin embedding. Five-micrometer paraffin-embedded sections were stained with hematoxylin and eosin for histological assessment.

Immunohistochemistry. Use of human esophageal cancer tissues for immunohistochemical studies was approved by the Internal Review Board at the Brigham and Women’s Hospital, Boston, MA. Sections from two human esophageal adenocarcinoma and six esophageal adenocarcinoma tissues were deparaffinized, treated with 10 mM sodium citrate buffer for antigen unmasking (pH 6.0, boiling temperature, 30 min), blocked in goat serum (Vectastain ABC kit, Vector Laboratories, Burlingame, CA), incubated with 1:25 dilution of phospho-STAT3 (Ser727) and 1:600 dilution of total STAT3 (Cell Signaling Technology, Danvers, MA) at 4°C overnight, rinsed, and incubated with anti-rabbit secondary antibody (Vectastain ABC kit). Signals were amplified using Vectastain ABC kit per manufacturer’s instructions. Targeted protein was visualized using diaminobenzidine as substrate. The tissues were evaluated by two gastrointestinal pathologists (ATA, RDO).

Statistical analyses. Quantitative data are expressed as means ± SE. Statistical analyses were performed using an unpaired Student’s t-test with the Instat for Windows statistical software package (GraphPad Software, San Diego, CA). For multiple comparisons, an ANOVA and the Student-Newman-Keuls multiple-comparisons test were performed using the Instat for Windows statistical software.
package (GraphPad). *P* values ≤ 0.05 were considered significant for all analyses.

**RESULTS**

Expression of STAT3CA alone does not induce malignant transformation of Barrett’s epithelial cells. Clones of BAR-T cells infected with STAT3CA exhibited a marked increase in total and phospho-STAT3 (Ser727) compared with BAR-T cells containing vector only (Fig. 1A). After reviewing these data, we selected clones 3 and 5 for all further experiments. To seek evidence of malignant transformation, we assessed cell numbers over 15 days of continued cell culture. Vector-containing control cells and STAT3CA-containing cells (clones 3 and 5) all demonstrated a plateau in cell number, reflecting intact cell-to-cell contact inhibition (a feature of nontransformed cells) (Fig. 1B). Next, we cultured vector-containing control cells and STAT3CA-containing clones in soft agar to see if they exhibited anchorage-independent growth (a feature of transformed cells). As a positive control, we used the OE33 esophageal adenocarcinoma cells, which form numerous large colonies by 3 wk of growth in soft agar (Fig. 1C). None of the vector-containing control cells or the STAT3CA-containing BAR-T cell clones formed colonies in soft agar (Fig. 1C).

Finally, we injected BAR-T STAT3CA-containing clones (3 and 5) subcutaneously into immunodeficient (NOD/SCID) mice. After 24 wk, we observed no tumor formation for either clone (Fig. 1D). These findings show that expression of STAT3CA alone does not induce malignant transformation of Barrett’s epithelial cells.

Expression of STAT3CA does not induce malignant transformation of Barrett’s epithelial cells with p53 knockdown. Barrett’s epithelial cells with p53 knockdown (BAR-T p53RNAi+) were infected with STAT3CA, and the STAT3CA-containing clones exhibited a marked increase in total and phospho-STAT3 (Ser727) compared with a vector-containing BAR-T p53RNAi+ cells (Fig. 2A). In continued cell culture, vector-containing control cells and STAT3CA-containing clones 2 and 3 all demonstrated a plateau in cell number characteristic of intact contact inhibition (Fig. 2B). No colonies formed in soft agar after 3 wk for vector- or STAT3CA-containing BAR-T p53RNAi+ cells, and we observed no tumor formation at 24 wk after injection of STAT3CA-containing BAR-T p53RNAi+ clones into NOD/SCID mice (Fig. 2D). These findings show that STAT3CA does not induce malignant transformation of Barrett’s epithelial cells with p53 knockdown.
Expression of STAT3CA does induce malignant transformation of Barrett’s epithelial cells that express oncogenic H-Ras G12V. We next infected Barrett’s epithelial cells expressing H-Ras G12V (BAR-T H-Ras(G12V) clones R6 and R7) with STAT3CA. The STAT3CA-containing clones 2 and 7 of BAR-T H-Ras G12V R6 exhibited a marked increase in total and phospho-STAT3 (Ser727) compared with vector-containing cells (Fig. 3A); we observed a similar increase in total and phospho-STAT3 (Ser727) in BAR-T H-Ras G12V R7 clones 1 and 6 (Fig. 4A). We determined population doubling time in the BAR-T H-Ras G12V R6 and BAR-T H-Ras G12V R7 cells and found a significant decrease in doubling time for the STAT3CA-expressing clones (Figs. 3B and 4B). These same clones exhibited a loss of contact inhibition characteristic of transformed cells (Figs. 3C and 4C). In contrast to the epithelial appearance of the vector-containing control cells by optic morphology, the STAT3CA-expressing clones of BAR-T H-Ras G12V R6 and BAR-T H-Ras G12V R7 had a spindle-shaped appearance typical of mesenchymal cells (Figs. 3D and 4D). Since the clones resembled mesenchymal cells, we studied their ability to migrate and invade. Compared with vector-containing controls, STAT3CA-expressing clones of BAR-T H-Ras G12V R6 and BAR-T H-Ras G12V R7 demonstrated a significant increase in cell migration (Figs. 3E and 4E) and invasion (Figs. 3F and 4F). After 3 wk in soft agar, STAT3CA-expressing clones of BAR-T H-Ras G12V R6 and BAR-T H-Ras G12V R7 formed a number of colonies, unlike the vector-containing control cells (Figs. 5A and 6A). The STAT3CA-expressing clones of BAR-T H-Ras G12V R6 and BAR-T H-Ras G12V R7 also formed tumors in NOD/SCID mice within 4 – 8 wk (Figs. 5B and 6B). Histological evaluation of the BAR-T H-Ras G12V R6 STAT3CA-expressing tumors revealed poorly differentiated carcinoma with sarcomatoid and squamous differentiation (Fig. 5C); BAR-T...
H-RasG12VR7 STAT3CA-expressing carcinomas showed mixed glandular and squamous features (adenosquamous carcinoma), with columnar portions of the tumors showing a combination of glands and signet ring cells (Fig. 6C). These findings demonstrate that expression of STAT3CA induces the neoplastic transformation of oncogenic Ras-expressing BAR-T cells.

p53 is intact and functional in STAT3CA-induced malignant transformation of Barrett’s epithelial cells expressing oncogenic H-RasG12V. We have previously shown that expression of oncogenic H-RasG12V in combination with p53 knockdown can transform BAR-T cells (37). Therefore, we determined whether p53 was intact and functional in our STAT3CA-transformed, oncogenic Ras-expressing BAR-T cells. We detected no p53 mutations by Sanger sequencing in any clones of the STAT3CA-transformed BAR-T H-RasG12VR6 and BAR-T H-RasG12VR7 cells. We next irradiated the transformed cells with UV-B, a well-known inducer of p53 and p21, and determined expression of these proteins by Western blot. In response to UV-B, there was an increase in p53 and p21 expression in the vector-only containing cells and in the selected clones of STAT3CA-containing BAR-T H-RasG12VR7 cells (Fig. 7A). To confirm that the xenograft tumors maintained STAT3CA and p53 expression, Western blots for phospho-STAT3 (Ser727), total STAT3, and p53 were performed on tumors derived from the STAT3CA-containing clones of the BAR-T H-RasG12VR6 and BAR-T H-RasG12VR7 cells. Similar to our in vitro findings for these cells, the xenograft tumor tissues maintained expression of phospho-STAT3 (Ser727), total STAT3, and p53 protein (Fig. 7B). These data demonstrate that the combined expression of STAT3CA and oncogenic Ras can transform Barrett’s cells, even when their p53 is intact and functional.
STAT3CA-induced malignant transformation of Barrett’s epithelial cells that express oncogenic H-RasG12V is not associated with increased constitutive transcriptional signaling by spontaneously dimerized STAT3CA. The rat α2-macroglobulin promoter-driven luciferase reporter gene is highly responsive to spontaneously dimerized STAT3CA, which does not require phosphorylation at Tyr705 to induce transcription. To exclude the possibility that this spontaneously dimerized STAT3CA exerts transcriptional effects that contribute to the malignant transformation of our Ras-expressing Barrett’s cells, we transfected both BAR-T H-RasG12V R6 cells and the STAT3CA-containing clones of BAR-T H-RasG12V R6 cells with the rat α2-macroglobulin promoter-driven luciferase reporter gene. We found no significant difference in α2-macroglobulin promoter-driven luciferase expression between these cell lines, suggesting that increased constitutive transcriptional signaling by spontaneously dimerized STAT3CA does not contribute to oncogenesis in Ras-expressing Barrett’s cells (Fig. 8A).

STAT3CA enhances Ras expression and activity in STAT3CA-transformed Barrett’s epithelial cells expressing oncogenic H-RasG12V. Recently, it has been shown that oncogenic K-Ras is not constitutively active, but must be stimulated to become active. Once stimulated, however, the oncogenic Ras remains active for a protracted period and is slow to return to baseline (14, 21). We determined the expression of H-Ras and the phosphorylation of its downstream protein ERK1/2 as an indicator of Ras activity in clones of the STAT3CA-expressing, transformed BAR-T H-RasG12V R6 and BAR-T H-RasG12V R7 cells and in corresponding vector controls. As shown in Fig. 8B, the clone cells showed a marked increase in total H-Ras expression that was associated with an increase in phosphorylation of ERK1/2. We confirmed these findings using Western blotting for active, GTP-bound Ras (Fig. 8C), which showed that activity of oncogenic H-RasG12V indeed was increased in the STAT3CA-expressing, transformed cells.
Mitochondrial STAT3 expression and decreased mitochondrial ROS production are associated with malignant transformation of Barrett's epithelial cells expressing oncogenic H-RasG12V. Ras and other oncogenes are known to impair mitochondrial function and increase mitochondrial production of ROS, whereas mitochondrial STAT3 can restore mitochondrial function (9, 33), which would be expected to reduce ROS production. To explore a potential contribution of mitochondrial STAT3 to tumorigenesis in Barrett's metaplasia, we first studied the expression of mitochondrial STAT3 (Ser727) in our nontransformed and transformed, Ras-expressing BAR-T cells. In the mitochondria, minimal to no expression of phospho-STAT3 (Ser727) was detected in BAR-T H-RasG12V R6 and BAR-T H-RasG12V R7 cells that contain vector only (Figs. 9A and 10A). In contrast, a marked increase of phospho-STAT3 (Ser727) was found in the mitochondria of clones of the STAT3CA-transformed BAR-T H-RasG12V R6 and BAR-T H-RasG12V R7 cells (Figs. 9A and 10A). We used MitoSOX Red to measure levels of the ROS superoxide produced in the mitochondria. As expected, the transformed, STAT3CA-expressing BAR-T H-RasG12V R6 clone 2 cells showed significantly less MitoSOX Red immunostaining (i.e., less mitochondrial superoxide) than the nontransformed, vector-containing BAR-T H-RasG12V R6 cells (Fig. 9B). Mito-TEMPO, a mitochondrial-targeted antioxidant, confirmed the mitochondrial origin of the ROS (Fig. 9B).

In earlier studies using Barrett's cells transformed by a combination of p53 knockdown and forced expression of oncogenic H-RasG12V, our laboratory found tyrosine-phosphorylated STAT3 functioning as a nuclear transcription factor.

Fig. 6. In vitro and in vivo tumorigenicity assays of STAT3CA-containing BAR-T H-RasG12V R7 cells. A: anchorage-independent growth in soft agar of containing BAR-T H-RasG12V R7-containing vector or STAT3CA (clones 1 and 6). No colonies are observed for the vector-containing cells, whereas a number of colonies are observed for the BAR-T H-RasG12V R7 cells containing STAT3CA (clones 1 and 6); OE33 esophageal adenocarcinoma cells served as a positive control. Values are means ± SE. B: the STAT3CA-expressing clones of BAR-T H-RasG12V R7 formed tumors in NOD/SCID mice within 8 wk. C: H&E staining of tumors (×200) from STAT3CA-containing BAR-T H-RasG12V R7 cells clones 1 and 6 demonstrate mixed glandular and squamous features (adenosquamous carcinoma), with columnar portions of the tumors showing a combination of glands (long arrows) and signet ring cells (short arrow).
to block apoptosis through the upregulation of Mcl-1, a member of the Bcl-2 family of anti-apoptotic proteins (34, 37). In the present study, we also found elevated levels of tyrosine-phosphorylated STAT3 in the nucleus of STAT3CA-transformed BAR-T H-RasG12VR6 and BAR-T H-RasG12VR7 cells (Figs. 9C and 10B). Thus these cells have elevated levels of both mitochondrial and nuclear phospho-STAT3. We also explored whether mitochondrial STAT3 might be involved in the aforementioned transformation of Barrett’s cells induced by the combination of p53 knockdown and forced expression of oncogenic Ras (BAR-T p53RNAi H-RasG12VR1 cells). Unlike STAT3CA-transformed BAR-T H-RasG12VR6 cells, BAR-T p53RNAi+ H-RasG12VR1 transformed cells had no phospho-STAT3 (Ser727) in the mitochondria and had higher levels of phospho-STAT3 (Tyr705) in the nucleus (Fig. 9D). These data suggest that STAT3 might contribute to the transformation of Barrett’s cells through effects in the nucleus, but do not establish whether mitochondrial STAT3 [independent of nuclear (Tyr705) STAT3] can transform Barrett’s cells that express oncogenic H-RasG12V.

**STAT3 Ser727 phosphorylation (without Tyr705 phosphorylation) supports malignant transformation of Barrett’s epithelial cells expressing oncogenic H-RasG12V.** As shown above, our STAT3CA-transformed BAR-T H-RasG12VR6 and BAR-T H-RasG12VR7 cells exhibit elevated levels of both nuclear and mitochondrial phospho-STAT3 (Tyr705) in mitochondrial phospho-STAT3 (Ser727). To minimize the contribution of mitochondrial phospho-STAT3 (Tyr705), we performed site-directed mutagenesis to the tyrosine phosphorylation (Y705F) site of the STAT3CA construct and then infected BAR-T H-RasG12VR6 cells with this mutated STAT3CA construct. Compared with the unmutated, STAT3CA-transformed BAR-T H-RasG12VR6 Clone 2 (CA2) cells, we found that the YF-mutant, STAT3CA-expressing clones of BAR-T H-RasG12VR6 had minimal (YF1, YF3, YF4) to no (YF2) tyrosine-phosphorylated STAT3 (Fig. 11A).

Western blotting of both mitochondrial and nuclear fractions confirmed that phospho-STAT3 (Tyr705) was eliminated in the YF-mutant, STAT3CA-expressing clones of BAR-T H-RasG12VR6 (Fig. 11B). Furthermore, Western blotting for Mcl-1, a downstream transcriptional target of STAT3, confirmed that there was no transcriptional activity of phospho-STAT3 (Tyr705) in YF-mutant, STAT3CA-expressing BAR-T H-RasG12VR6 (Fig. 11C). Unlike vector (V) control cells, we found that the YF-mutant, STAT3CA-expressing clones of BAR-T H-RasG12VR6 exhibited marked phosphorylation of STAT3 at Ser727 (Fig. 11A). The YF-mutant, STAT3CA-expressing BAR-T H-RasG12VR6 clone 2 cells showed significantly less MitoSOX Red immunostaining than vector-containing BAR-T H-RasG12VR6 clone 2 cells (Fig. 11D). We injected YF-mutant, STAT3CA-expressing BAR-T H-RasG12VR6 clones (YF1 and YF2) subcutaneously into NOD/SCID mice, and both clones formed tumors in the mice within 4 wk (Fig. 11E). Histological evaluation of these tumors showed a mixture of glandular and squamous differentiation (mixed adenosquamous carcinoma; Fig. 11F). These findings in the YF2-mutant clone that has no increase in tyrosine-phosphorylated STAT3 or in its transcriptional activity suggest that STAT3 tyrosine phosphorylation (Tyr705) is not required for tumor formation, and that STAT3 mitochondrial Ser727 alone can transform Barrett’s epithelial cells that express oncogenic Ras. A schematic model summarizing proposed mechanisms based on our findings is provided in Fig. 12.

**STAT3 Ser727 phosphorylation is expressed in primary human esophageal adenosquamous cancer.** To explore whether STAT3 Ser727 phosphorylation occurs in human esophageal adenosquamous carcinomas and adenosccarcinomas, we performed immunohistochemical staining for phospho- and total STAT3 in two human esophageal adenosquamous carcinomas and six esophageal adenocarcinomas (Fig. 13). In histologically normal squamous epithelium, cells in the basal...
layer exhibited strong nuclear staining for total STAT3 and weak nuclear staining for phospho-STAT3 (Ser727); very little phospho-STAT3 (Ser727) staining was seen in squamous cells above the basal layer. In histologically normal gastric foveolar mucinous epithelium, the columnar cells exhibited moderate cytoplasmic staining for total STAT3, but no staining for phospho-STAT3 (Ser727). Nondysplastic Barrett’s-associated metaplastic columnar epithelium (data not shown).

DISCUSSION

We have demonstrated that expression of STAT3CA, in a p53-independent fashion, can cause the malignant transformation of benign Barrett’s epithelial cells that express oncogenic H-RasG12V. Compared with nontransformed Barrett’s cells that express oncogenic H-RasG12V, the STAT3CA-transformed cells exhibit a greater and more sustained increase in the activity of oncogenic H-RasG12V, an increased expression of nuclear STAT3 (Tyr705), an increased expression of mitochondrial STAT3 (Ser727), a reduced mitochondrial production of superoxide, and no increase in transcriptional signaling of spontaneously dimerized STAT3CA. In contrast, Barrett’s cells transformed by a combination of p53 knockdown and forced expression of oncogenic H-RasG12V show no expression of mitochondrial STAT3 (Ser727) and high levels of nuclear STAT3 (Tyr705). Although expression of the STAT3CA construct increased cellular levels of both Ser727- and Tyr705-phosphorylated STAT3, we found that the construct could transform oncogenic RasG12V-expressing Barrett’s cells even when the tyrosine phosphorylation site was eliminated by site-directed mutagenesis. Finally, we have shown that human Barrett’s-related adenosquamous carcinomas and adenocarcinomas demonstrate increases in cytoplasmic phospho-STAT3 (Ser727). These observations support a role for mitochondrial STAT3 in the malignant transformation of Barrett’s metaplasia.

Persistent activation of STAT3 appears to be essential for the progression of a number of human tumors, including Barrett’s-associated adenocarcinomas (6, 11). Tyrosine-phosphorylated STAT3 is well known as a nuclear transcription factor that can induce the expression of genes that promote carcinogenesis. Acid and bile salts, the major noxious components of gastric refluxate, have been shown to increase levels of phospho-STAT3 (Tyr705) in cancer cells in vitro. Thus the chronic gastroesophageal reflux of acid and bile might underlie the high levels of nuclear phospho-STAT3 (Tyr705) that have been found in Barrett’s dysplasia and adenocarcinoma (6). In earlier studies, our laboratory showed that Barrett’s cells transformed by the combination of p53 inactivation and oncogenic H-RasG12V activation secrete interleukin-6 and increase phospho-STAT3 (Tyr705), which enables the transformed cells to resist apoptosis (34). These findings support a role for the nuclear transcription factor function of STAT3 (Tyr705) in Barrett’s carcinogenesis, but little had been known about the role of mitochondrial phospho-STAT3 (Ser727) in this process.

Recently, it has been appreciated that a number of nuclear transcription factors (e.g., p53, NF-kB, STAT3) also can be found in the mitochondria, where they might influence activity of the electron transport chain, interact with apoptotic machinery, and modulate the expression of mitochondrial RNAs (28). A potential role for mitochondrial STAT3 in oncogenesis was demonstrated by Gough et al. (9), who showed that mitochondrial STAT3 mutants that could not be tyrosine phosphorylated and could not bind DNA, nevertheless, supported Ras-mediated transformation. To explore a role for mitochondrial
STAT3 in the malignant transformation of Barrett’s esophagus, we infected Barrett’s cells with a human, constitutively active variant of STAT3 (STAT3CA) that can be phosphorylated at Ser727 as well as at Tyr705.

We found that STAT3CA did not induce features of neoplastic transformation in unaltered BAR-T cells or in BAR-T cells with p53 knockdown, but did transform BAR-T cells that express oncogenic H-RasG12V. These transformed cells had an intact and functional p53 pathway, and the xenograft tumor tissues derived from these cells, which retained elevated levels of p-STAT3 (Ser727) and total STAT3, also expressed p53 protein. Thus the transformation process induced by STAT3 in Ras-expressing Barrett’s cells occurred independent of p53 pathway inactivation.

For decades, it had been assumed that oncogenic Ras is in a permanent, maximal “on” state. In transgenic mouse models, however, it has been found that inflammatory stimuli can cause a strong and prolonged increase in K-Ras activity, suggesting that unstimulated oncogenic Ras is not maximally “on”, and only a small proportion of cells that express oncogenic K-Ras develop cancer (3, 4, 14, 21). These findings are the basis of a new concept for Ras-induced oncogenesis in which oncogenic Ras remains largely inactive until it is stimulated, at which point the stimulated Ras triggers an inflammatory feed-forward loop, activating downstream inflammatory mediators like STAT3 that can further activate oncogenic Ras (4, 21). We found that infection of our oncogenic H-RasG12V-expressing Barrett’s cells with STAT3CA caused marked increases in total H-RasG12V and phospho-ERK1/2 expression, and in H-RasG12V activity, consistent with the notion that Ras activity levels are key to the efficiency of Ras-induced transformation (4). Furthermore, these finding suggest that the activity of oncogenic H-Ras, like oncogenic K-Ras, can
be enhanced by its downstream inflammatory mediators like STAT3.

Gough et al. (9) showed that phosphorylation of STAT3 at Ser727 was essential for the transformation of cells by Ras oncogenes. Ser727 lies within STAT3’s conserved PMSP sequence, which contains a mitogen-activated protein kinase consensus sequence (10). Thus STAT3 can be phosphorylated at Ser727 by ERK, JNK, and p38 members of the mitogen-activated protein kinase family. STAT3 Ser727 phosphorylation also can be accomplished by certain other kinases [including protein kinase C, phosphoinositide 3-kinase, and mammalian target of rapamycin (mTOR)] and by silencing suppressor of cytokine signaling 2 (5, 10, 19). Furthermore, different stimuli can elicit STAT3 Ser727 phosphorylation through different signaling pathways (5). In a subsequent study, Gough et al. (10) demonstrated that Ras isoforms required activation of MEK-ERK signaling, but not phosphoinositide 3-kinase or mammalian target of rapamycin signaling, for phosphorylation of STAT3 at Ser727, and that MEK-ERK signaling that targeted STAT3 to mitochondria (through Ser727 phosphorylation) was required to achieve malignant transformation. In earlier studies, our laboratory found that H-RasG12V-expressing Barrett’s cells exhibited an increase in phospho-MEK1/2 and phospho-ERK1/2, but expression of H-RasG12V alone did not cause malignant transformation (37). Now, we show that H-RasG12V-expressing Barrett’s cells transformed by STAT3CA exhibit high levels of phospho-STAT3 (Ser727) in their mitochondria, and that phospho-STAT3 (Tyr705) is not required for their transformation. We also show that these transformed cells have marked increases in H-RasG12V activity and in phospho-ERK1/2 expression, perhaps due to an inflammatory feed-forward loop initiated by infection with STAT3CA. Thus our findings suggest that the increases in oncogenic H-Ras activity and in ERK signaling caused by STAT3CA lead to Ser727 phosphorylation of STAT3, which targets it to the mitochondria where it mediates cellular transformation. Further studies will be required to establish this mechanism.

A number of oncogenes, including Ras, are known to impair mitochondrial function and increase mitochondrial ROS production (9, 33). Conversely, mitochondrial STAT3 has been shown to restore mitochondrial function and reduce ROS production in transformed mouse breast adenocarcinoma cells (36). Our Ras-expressing Barrett’s cells that were transformed by STAT3CA showed high levels of phospho-STAT3 (Ser727) in their mitochondria. We then measured levels of the ROS superoxide using MitoSOX red, and we confirmed its mitochondrial origin using Mito-TEMPO, an antioxidant that specifically targets mitochondrial ROS. Our human STAT3CA-transformed, Ras-expressing Barrett’s cells showed less mitochondrial superoxide production than our nontransformed, Ras-expressing Barrett’s cells.

In earlier studies, our laboratory found nuclear phospho-STAT3 (Tyr705) with transcription factor function in BAR-T cells transformed by the combination of p53 knockdown and forced expression of oncogenic H-RasG12V (34, 37). In the present study, we explored a role for mitochondrial STAT3 (Ser727) in those cells. We confirmed our earlier finding of high levels of nuclear phospho-STAT3 (Tyr705) in BAR-T cells with p53 knockdown and oncogenic H-RasG12V expression, but we found no mitochondrial STAT3 (Ser727) in those cells. In contrast, our BAR-T cells transformed by the expression of oncogenic H-RasG12V in combination with STAT3CA exhibited high levels of mitochondrial phospho-STAT3 (Ser727), but also showed elevated levels of nuclear phospho-STAT3 (Tyr705). Thus it was not clear whether mitochondrial phospho-STAT3 (Ser727), nuclear phospho-STAT3 (Tyr705), or both were required to transform the Ras-expressing cells. To address this issue, we performed site-directed mutagenesis to the tyrosine phosphorylation (Y705F) site of the STAT3CA construct and found that Ras-expressing Barrett’s cells with this mutant STAT3CA formed tumors in immunodeficient mice. This observation shows that phosphorylation of STAT3 Tyr705 is not required for tumor formation in Ras-expressing Barrett’s cells.

STAT3CA has substitutions in its cysteine residues that could enable the protein to dimerize spontaneously (without phosphorylation), bind to DNA, and activate the transcription of genes involved in malignant transformation (2). However, our Ras-expressing Barrett’s cells that were transformed by STAT3CA did not show higher levels of tran-
scriptional activity by spontaneously dimerized STAT3CA protein than their nontransformed, Ras-expressing Barrett’s cell counterparts (without STAT3CA). This finding indicates that transcriptional signaling by spontaneously dimerized STAT3CA protein was not the underlying mechanism for transformation.

Histological evaluation of the tumors in immunodeficient mice injected with Barrett’s cells that express oncogenic Ras and STAT3CA revealed carcinomas with sarcomatous and squamous differentiation or with mixed glandular and squamous differentiation. Although Barrett’s-associated cancers typically are pure adenocarcinomas, esophageal tumors with both adenomatous and squamous features are well described in patients with Barrett’s esophagus (8, 20, 24, 31). van Ress et al. (31) reported a Barrett’s cancer with both glandular and squamous components that, by molecular analysis and p53 sequencing, demonstrated a common clonal evolution from the patient’s Barrett’s metaplasia. Our findings support the concept that the cells giving rise to tumors in Barrett’s esophagus have the capacity to differentiate into disparate cell types. The small number of human tumors that we analyzed is a limitation of our study; nevertheless, our demonstration that the glandular portion of human Barrett’s-associated adenosquamous cancers and adenocarcinomas exhibit increases in cytoplasmic phosphorylation at Ser727 supports the relevance of our studies to human disease.

In conclusion, we have shown that Barrett’s epithelial cells that express oncogenic Ras can be transformed by the expression of STAT3CA. This transformation does not require p53 inactivation or nuclear phospho-STAT3 (Tyr705), and mitochondrial phospho-STAT3 (Ser727) appears to play a role in the tumorigenesis. Our findings suggest that mitochondrial STAT3 can contribute to the malignant transformation of Barrett’s metaplasia, and that agents targeting STAT3 might be useful for chemoprevention in patients with Barrett’s esophagus.

**Fig. 11.** YF mutant-containing, STAT3CA-expressing BAR-T H-RasG12VR6 cells. A: Western blot demonstrating STAT3 phosphorylation at Ser727, with minimal to no phosphorylation at Tyr705 in YF mutant-containing, STAT3CA-expressing BAR-T H-RasG12VR6 cells (clones YF1-YF4), STAT3CA-containing BAR-T H-RasG12VR6 clone 2 (CA2), which has no YF mutation, exhibits STAT3 phosphorylation at both Ser727 and Tyr705, while vector-containing control cells (V) show no STAT3 phosphorylation at either site. B: Western blot demonstrating nuclear p-STAT3 (Tyr705) in the STAT3CA-containing BAR-T H-RasG12VR6 clone 2 cells, but no nuclear p-STAT3 (Tyr705) in vector controls or in the YF mutant-containing, STAT3CA-expressing BAR-T H-RasG12VR6 cells. No STAT3 phosphorylated at Tyr705 was observed in the mitochondria of any of the cell lines. C: Western blot demonstrating Mcl-1 expression in STAT3CA-containing BAR-T H-RasG12VR6 clone 2, but not in vector controls or in YF mutant-containing, STAT3CA-expressing BAR-T H-RasG12VR6 cells clone 2. D: YF mutant-containing, STAT3CA-expressing BAR-T H-RasG12VR6 cells clone 2 shows significantly less MitoSOX red immunostaining for ROS than vector-containing BAR-T H-RasG12VR6 cells. Mito-TEMPO confirms the mitochondrial origin of the ROS. Scale bar, 10 μM. *P = 0.0002 compared with vector-containing cells. *P < 0.0001 compared with corresponding vector-containing cells. E: YF-containing, STAT3CA-expressing BAR-T H-RasG12VR6 clones 1 and 2 formed tumors in NOD/SCID mice within 4 wk. F: H&E staining of a tumor (×200) from YF-containing, STAT3CA-expressing BAR-T H-RasG12VR6 clone 2, demonstrating areas of glands (short arrow) and goblet cells intermixed with areas of squamous differentiation (long arrow).
Fig. 12. Schematic model demonstrating the effects of STAT3 phosphorylated at Tyr705 and Ser727 in oncogenic H-RasG12V-expressing Barrett’s cells. Phosphorylation of STAT3 on Tyr705 allows for its dimerization and nuclear translocation to increase transcription of target genes involved in cancer formation (i.e., Mcl-1) and, perhaps, to stimulate activation of a Ras inflammatory feed-forward loop. Phosphorylation of STAT3 on Ser727 allows it to enter mitochondria, where it reduces the production of reactive oxygen species (ROS). Thus phosphorylation of STAT3 both at Tyr705 and Ser727 triggers molecular events that might contribute to Barrett’s carcinogenesis.

Fig. 13. Representative photomicrographs (×200) of a human Barrett’s-related adenosquamous carcinoma showing a strong increase in cytoplasmic p-STAT3 (Ser727) in the glandular component and in the associated Barrett’s metaplasia. Histologically normal squamous epithelium and normal gastric foveolar mucinous epithelium are shown for comparison.
REFERENCES


