SPERMINE OXIDATION AND EPIGENETIC REGULATION: IMPLICATIONS FOR INFLAMMATION/INFECTION-ASSOCIATED CARCINOGENESIS

by

Tracy Murray-Stewart

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Abstract

Intracellular mammalian polyamine catabolism occurs through 2 distinct pathways, both of which culminate in oxidation reactions that generate highly reactive, potentially toxic byproducts. In the back-conversion to spermidine, spermine can either undergo direct oxidation by spermine oxidase (SMOX) or be acetylated by spermidine/spermine N^1-acetyltransferase (SSAT), followed by subsequent oxidation by acetylpolyamine oxidase (APAO). Polyamines are absolutely essential for cell viability and proliferation, and polyamine metabolism and intracellular concentrations are frequently dysregulated in hyperproliferative conditions such as cancer. As a result, many studies have successfully focused on the induction of polyamine catabolism as a rational target for antiproliferative chemotherapeutic intervention. However, it has also become apparent that chronically elevated levels of polyamine catabolism, particularly through SMOX activity, can have disease implications in non-tumorigenic cells. A variety of stimuli, including microbial pathogens and inflammatory signals, induce SMOX activity, generating the reactive oxygen species precursor hydrogen peroxide as a byproduct that, along with the reduction in protective spermine and spermidine levels, can have deleterious physiological effects resulting in the manifestation and promotion of multiple pathologies.

The studies presented in this dissertation investigate some of the mechanisms involved in the induction of SMOX in gastric epithelial cells exposed to *Helicobacter pylori*. This induction and subsequent oxidative DNA damage has been causally associated with the development of gastric cancer from chronic gastritis. We identify a miRNA (hsa-miR-

124) that directly targets the 3'-UTR of spermine oxidase, thereby repressing the expression of SMOX mRNA and protein. Importantly, this miRNA becomes silenced via DNA hypermethylation in several epithelial cancers that are associated with chronic inflammation and/or infection, including *H. pylori*-associated gastric cancer. We demonstrate that overexpressing miR-124 in gastric adenocarcinoma cells abrogates the induction of SMOX mRNA and activity that occurs upon exposure to *H. pylori.* Importantly, we present preliminary data from human samples suggesting that increased methylation of miR-124 identifies populations at high risk for gastric carcinogenesis through its modulation of SMOX expression.

Additionally, using a conditional SMOX overexpression system, we develop an *in vitro* model to investigate the molecular mechanisms through which SMOX-generated oxidative DNA damage contributes to tumorigenesis. We demonstrate that SMOX overexpression results in the down-regulation of the MGMT tumor suppressor gene and propose the recruitment of epigenetic chromatin modifiers to regions of oxidative DNA damage as a mechanism for SMOX-dependent tumor suppressor gene silencing. Combined, these studies suggest a mechanism by which pathogen-induced SMOX can result in epigenetic silencing of critical genes and possibly even feed-forward through a miRNA regulatory mechanism to exacerbate this phenomenon.

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3'-UTR: 3'-untranslated region

5-AC: 5-azacytidine

AdCre: recombinant adenovirus expressing Cre recombinase

APAO: acetyl polyamine oxidase

BER: base-excision repair

DFMO: α-difluoromethylornithine

DNMT: DNA methyltransferase

ETBF: enterotoxigenic *Bacteroides fragilis*

HAT: histone acetyltransferase

HDAC: histone deacetylase

H_2O_2: hydrogen peroxide

H. pylori: *Helicobacter pylori*

HPLC: high-pressure liquid chromatography

IBD: irritable bowel disease

IM: intestinal metaplasia

MDL72527: a polyamine oxidase inhibitor

MGMT: O^6-methylguanine-DNA methyltransferase

miRNA: microRNA

miR-NC: negative control miRNA mimic

ODC: ornithine decarboxylase

qRT-PCR: quantitative reverse transcription polymerase chain reaction

PUT: putrescine

ROS: reactive oxygen species

SMOX: spermine oxidase (previously PAOh1)

SNP: single nucleotide polymorphism

SPD: spermidine

SPM: spermine

SSAT: spermidine/spermine N^1-acetyltransferase

TNFα: tumor necrosis factor-α

TSG: tumor suppressor gene

UC: ulcerative colitis

Chapter 1:

General Introduction and Literature Review

The structure and function of polyamines in mammals

The naturally occurring mammalian polyamines, spermine, spermidine, and putrescine, are ubiquitous molecules that are absolutely essential for cellular proliferation and differentiation. Polyamines are present in millimolar concentrations in the cells of virtually all mammalian tissues [1], where their primary and secondary amino groups are protonated at physiologic pH (Fig. 1.1). This polycationic nature allows polyamines the ability to interact with negatively charged macromolecules, including DNA, RNA, and proteins, thereby contributing to their structural stability. In addition to electrostatically interacting with the phosphate groups of DNA, polyamines interact with nucleic acid bases through hydrophobic interactions and consequently play a significant role in DNA condensation and chromatin organization [2-5]. Polyamines can therefore play a role in gene regulation through inducing sequence-specific conformational changes in gene regulatory regions as well as by inducing DNA bending through charge neutralization, which can affect regulatory protein-DNA interactions [6-8]. Spermidine also has the unique potential to regulate protein translation, as it serves as the sole substrate for the post-transcriptional hypusination of eukaryotic initiation factor (eIF)-5A [9-11]. Consistent with their requirement for growth and differentiation, polyamines contribute significantly to cell cycle control through the regulation of specific cyclins, including B1, D1, and E [12, 13].

In addition to their effects on cell proliferation, polyamines contribute to the antioxidant capacity of the cell. Spermine in particular serves as an important free radical scavenger in the protection from reactive oxygen species (ROS)-induced DNA strand breaks [14, 15]. However, polyamine homeostasis is tightly regulated and an excess of polyamines is toxic. The resulting induction of catabolism of the higher polyamines (spermine and spermidine) produces elevated levels of an ROS precursor, hydrogen peroxide (H_2O_2), while diminishing the intracellular concentrations of these antioxidants. Consequently, the extreme or prolonged upregulation of polyamine catabolism can have detrimental cellular effects including apoptosis, oxidative DNA damage, and an increased potential for carcinogenesis [16-19].

Mammalian polyamine catabolic enzymes and their metabolites

In mammalian cells, the catabolism of spermine to spermidine occurs via one of two distinct pathways. As a substrate for spermidine/spermine N^1-acetyltransferase (SSAT), spermine can be converted to N^1-acetylspermine, which is subsequently oxidized by the FAD-dependent acetylpolyamine oxidase (APAO) to form spermidine. Conversely, spermine can be directly oxidized by spermine oxidase (SMOX) to form spermidine. Spermidine is then back-converted to putrescine through the 2-step SSAT/APAO reaction that includes an N^1-acetylspermidine intermediate (Fig. 1.2).

SSAT is the rate-limiting enzyme of the polyamine catabolic pathway that catalyzes the transfer of an acetyl group from acetyl coenzyme A to the N^1 position of spermine or spermidine [20-22]. The resulting molecule has a reduced positive charge that alters its

binding affinity for cellular macromolecules and facilitates its export from the cell. Additionally, N^1-acetylated spermine or spermidine can be oxidized by APAO, resulting in spermidine or putrescine, respectively, H_2O_2, and 3-acetamidopropanal [23-25]. H_2O_2 is a potential ROS; however, the peroxisomal localization of the APAO enzyme can protect the cell from its oxidative effects, as catalase is also located in the peroxisome.

The *SMOX* gene encoding spermine oxidase is alternatively spliced, and multiple isoforms have been characterized in both the human and mouse [26-29]. The catalytically active SMOX isoforms are FAD-dependent enzymes that directly oxidize spermine to yield spermidine, H_2O_2, and the aldehyde 3-aminopropanal (Fig. 1.3). Importantly, the active SMOX isoforms are found in significant amounts in both the cytoplasm and nucleus of the cell, resulting in the production of H_2O_2 as an ROS precursor in close proximity to DNA and chromatin, while catalyzing the oxidation of the free-radical scavenger spermine [29-31]. SMOX activity therefore has the potential to significantly contribute to cellular oxidative damage and subsequent disease development. In particular, the oxidative DNA damage resulting from SMOX induction is implicated in the initiation of infection/inflammation-associated carcinogenesis and will be the focus of this dissertation.

The induction of spermine oxidase

The oxidation of spermine via SMOX is inducible, and the majority of its regulation appears to be at the level of transcription [32]. The initial induction of SMOX was investigated using certain polyamine analogues intended for chemotherapeutic

intervention through the tumor-specific depletion of natural polyamines, generation of ROS, and induction of apoptosis [26, 32]. Since then, SMOX induction has been detected in response to certain pro-inflammatory cytokines, in tissues following ischemia-reperfusion injury [33, 34], in states of chronic inflammation, and upon microbial pathogen infection [17]. This upregulation of spermine oxidation in response to inflammatory stimuli suggests a common molecular mechanism linking inflammatory stimuli to the initiation of carcinogenesis. Several bacterial pathogens have now been identified to induce SMOX expression in host cells, the ultimate outcome of which is oxidative DNA damage, apoptosis, and an increased potential for neoplastic transformation. These pathogens include *Helicobacter pylori (H. pylori)* and enterotoxigenic *Bacteroides fragilis* (ETBF), both of which will be described in greater detail below [35, 36]. Additionally, endotoxin (LPS), a constituent of Gram-negative bacterial cell walls, induces SMOX transcription and activity in the kidney [37], and expression of an HIV1-encoded protein induces SMOX in neuronal cells [38]. Furthermore, in hepatocytes, the alcohol metabolite acetaldehyde was recently shown to induce spermine oxidation [39], suggesting a role for SMOX in alcohol-related liver damage.

Elevated spermine catabolism contributes to inflammation through the production of ROS; however, it is also directly regulated by certain inflammatory signals. In addition to infectious agents, SMOX is activated by the inflammatory cytokines tumor necrosis factor-α (TNFα), intereukin-1β (IL-1β), and interleukin-6 [40, 41]. Meanwhile, polyamines themselves are recognized as mediators of immune function by negatively

regulating the production of certain inflammatory cytokines, including TNFα and IL-1β, and nitric oxide [42-44]. Therefore, the transcriptional activation of SMOX by pro-inflammatory cytokines, such as TNFα, decreases the abundance of spermine that would normally repress further production of TNFα, thus having the potential to exacerbate the chronic inflammatory response.

The carcinogenic potential of chronically elevated spermine oxidase

The higher polyamines, spermine in particular, play important physiological roles in the protection from oxidative stress [14, 45]. Enhanced polyamine catabolism reduces this protection while concomitantly generating reactive oxygen species and toxic aldehyde byproducts. As a result, increased spermine catabolism, resulting from the stimuli mentioned above, has been implicated in several pathophysiological conditions, including neurological and liver disease, stroke, kidney failure, ischemia-reperfusion and toxin-induced injury, and cancer.

Nearly 25% of all human cancers can be causally linked to chronic inflammatory conditions, approximately 50% of which result from chronic infection with human pathogens, including bacteria, viruses, and parasites [46, 47]. In addition to infectious agents, certain intrinsic mediators of the inflammatory response, including pro-inflammatory cytokines, growth factors, and reactive oxygen and nitrogen species, contribute to the initiation of tumorigenesis through a variety of mechanisms. Certain pathogens known to invoke cancer predispositions through chronic inflammation are also potent inducers of spermine oxidase, as are certain pro-inflammatory cytokines, and

elevated levels of SMOX have been observed in several inflammation-associated, pre-malignant human conditions. These observations suggested a role for spermine oxidation in the generation of oxidative DNA damage and initiation of tumorigenesis that has since been confirmed in *in vivo* model systems of epithelial cancers. Furthermore, as well as generating DNA-damaging oxidative stress in epithelial cells, polyamine oxidase induction and subsequent apoptosis has been observed in infiltrating inflammatory cells as a means for immune response evasion and bacterial persistence [48, 49].

The predominant area of investigation regarding the role of spermine catabolism in infection and inflammation focuses on *H. pylori,* a Gram-negative, microaerophilic bacterium that colonizes the stomach mucosa and causes inflammation in the form of chronic gastritis and peptic ulcers [50]. Although eliciting acute and chronic immune and inflammatory responses, *H. pylori* evade the antimicrobial mechanisms of the immune response and often persist for the life of the host [51, 52]. Approximately two-thirds of the world's population is infected with *H. pylori*, the only bacterium to be classified a class I carcinogen by the World Health Organization (WHO). As such, *H. pylori* infection is now estimated to be the causal agent of up to 90% of gastric cancers [53, 54]. Infected individuals develop superficial gastritis and often remain asymptomatic; however, a subgroup of patients will progress through the histopathological cascade of multifocal atrophic gastritis (MAG), intestinal metaplasia (IM), dysplasia, and gastric cancer [55]. Data from the year 2012 estimate 951,600 new cases of gastric cancer and 723,100 deaths, making it the third leading cause of cancer death worldwide and a major public health concern [56, 57].

Gastric epithelial cells respond to *H. pylori* infection through an induction of ornithine decarboxylase (ODC), a polyamine biosynthetic enzyme, as well as increased polyamine catabolism that occurs predominantly through increased SMOX mRNA and activity. The generation of H_2O_2 that results specifically from SMOX induction by *H. pylori* has been causally linked to DNA damage and apoptosis in the gastric mucosae of humans and mice with active gastritis [35]. Importantly, a subpopulation of gastric epithelial cells in which *H. pylori* infection has induced SMOX activity and high amounts of DNA damage remains resistant to apoptosis, therefore increasing the likelihood that these cells will undergo malignant transformation [58]. Relative to normal gastric mucosal tissue, SMOX protein is elevated in human tissues spanning the spectrum from gastritis to carcinoma. However, the most profound induction is observed in IM tissues, suggesting a causal role for SMOX in the development and/or progression of this high-grade precursor lesion [59]. Most recently, SMOX was determined to be a key differentiating factor influencing the risk of progression to gastric cancer in *H. pylori*-induced gastritis patients inhabiting high-risk versus low-risk geographical regions of Colombia, South America [60], where the overall incidence of *H. pylori* infection is approximately 90%.

Chronic inflammation also predisposes one to the development of colorectal neoplasia. Induction of SMOX has been directly linked to carcinogenesis in a mouse model of infection with enterotoxigenic *Bacteroides fragilis*, an anaerobic colonic bacterium that in humans causes ulcerative colitis (UC), acute diarrheal disease, inflammatory bowel disease (IBD), and ultimately colon cancer. ETBF-mediated SMOX induction was

identified as the source of oxidative DNA damage in ETBF-infected mouse colonic epithelium; importantly, pretreatment with the SMOX inhibitor MDL72,527 decreased ETBF-induced DNA damage, colonic inflammation, proliferation, and tumorigenesis, thereby validating SMOX induction as a molecular process that links inflammatory stimuli with carcinogenesis [36]. In colonic epithelial cells obtained from human UC and IBD patients, intracellular levels of spermidine were enhanced, while spermine concentrations were decreased, also suggesting an upregulation of SMOX activity [61]. Additionally in human UC patients, SMOX protein expression in infiltrating mononuclear cells was found to correlate with the severity of inflammatory disease scoring, consistent with a role for SMOX in colitis pathogenesis [62].

Finally, chronic inflammation in the form of prostatitis is believed to contribute to the development of prostate cancer. In prostate tissue samples from patients with prostate disease spanning the spectrum from inflammation to prostate adenocarcinoma, SMOX protein expression was increased in comparison with individuals without disease [63]. When examining patient-matched samples, SMOX expression was increased in prostatic intraepithelial neoplasia (PIN) and prostate cancer samples relative to benign prostatic epithelium from the same individual, with the greatest increase observed in PIN lesions, which are recognized as precursors to prostate carcinoma. Consistent with the inflammation-associated conditions described above, these data imply a role for SMOX in precursor lesion development and carcinogenic initiation events. Furthermore, patients that developed prostate disease demonstrated significantly higher SMOX expression even in non-diseased areas of prostatic epithelium when compared to those without disease,

suggesting that increased SMOX expression is an important component early in the disease process.

The studies described above focus on the DNA-damaging effects of the ROS generated through elevated spermine oxidation; however, the resulting reduction in intracellular spermine concentration must also be considered, as its depletion can significantly exacerbate the toxic outcome. Spermine and spermidine function to protect DNA from oxidative damage, and spermine has been shown to act directly as a free-radical scavenger [14, 15, 45, 64-66]. Therefore, elevated polyamine catabolism, particularly through nuclear SMOX induction, not only generates reactive oxygen species but also functionally reduces the antioxidant levels of the cell.

Epigenetic factors affecting inflammation-associated carcinogenesis

Although the evidence described above clearly indicates a role for SMOX-mediated DNA damage in inflammation-associated carcinogenesis, the precise molecular mechanisms are unclear. One of the hallmarks of cancer development is the accumulation of genetic and epigenetic changes in the genome. Genetic alterations such as mutations in driver genes and various forms of somatic gene arrangements play a large role in cancer development and progression, and unrepaired DNA damage resulting from ROS can certainly cause mutations [67]. However, chronic inflammation such as that associated with *H. pylori* infection has also recently been shown to contribute to increased levels of DNA hypermethylation [68, 69], an epigenetic change associated with aberrant tumor suppressor gene silencing (Fig. 1.4). Furthermore, the increased methylation levels

apparent in gastric mucosae harboring *H. pylori* infection have been positively associated with the risk of progression to gastric cancer [70, 71], and overall methylation levels are observed to decrease following infection eradication in gastritis patients [72]. Notably, this aberrant silencing has been associated with the inflammatory environment triggered by *H. pylori* infection rather than the bacterium itself, as treatment of *H.* pylori-colonized gerbils with cyclosporin A significantly attenuated the levels of inflammation and DNA methylation, while not affecting colonization [69]. Methylation typically occurs at the 5' position of the cytosine ring within a region of CpG dinucleotides in a gene promoter. Importantly, epigenetic changes are heritable, and abnormally methylated DNA begins accumulating in normal-appearing tissues exposed to chronic inflammation/infection, forming what is known as an epigenetic field defect for cancerization [68, 73]. Aberrant DNA hypermethylation is, therefore, an early occurrence in gastric carcinogenesis that has been observed to increase throughout the multi-stage process from gastritis to malignancy [74].

ROS generated in response to inflammatory stimuli are believed to play a role in this accumulation of DNA methylation. Using the same mouse model of ETBF-induced colitis in which SMOX induction was implicated as the source of ROS responsible for tumorigenesis, it was determined that ROS-generated DNA damage induced the recruitment of epigenetic silencing complexes including DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) to regions of oxidative DNA damage. The result of this recruitment was a repressive chromatin architecture that was conducive to CpG island DNA hypermethylation. Importantly, those gene promoters to which these

repressive complexes were recruited demonstrate similar characteristics as those which frequently undergo aberrant gene silencing in cancer, namely, high GC content and low basal expression [75]. Taken together, these studies suggest the potential that the induction of SMOX-generated ROS that occurs in response to inflammatory stimuli is a key factor influencing aberrant epigenetic chromatin modifications and CpG island DNA hypermethylation in the development of chronic inflammation/infection-associated carcinogenesis. The development of a relevant *in vitro* system to test this hypothesis is one of the main focuses of the dissertation and will be discussed in depth in Chapter 3.

Epigenetic silencing via DNA hypermethylation is generally believed to target protein-coding genes that code for tumor suppressors or other regulatory proteins responsible for cell cycle progression, growth, apoptosis, invasion, or migration. However, certain non-protein-coding genes, particularly microRNAs (miRNAs), can also be negatively regulated by aberrant DNA hypermethylation. miRNAs are short, non-coding RNA molecules of approximately 22 nucleotides in length that exert their regulatory effects at the post-transcriptional level. Single-stranded miRNAs typically bind to the 3'-untranslated regions (3'-UTRs) of target mRNA molecules through sequence-specific base pairing, resulting in destabilization/degradation of the messenger RNA and/or translational repression. The targets of miRNAs include the transcripts of genes involved in essential processes such as differentiation, proliferation, and apoptosis, and expression profiling of miRNAs between tumors and normal tissues has revealed tumor-specific expression patterns, suggesting differential regulation of miRNA expression during tumorigenesis. Importantly, the expression levels of specific miRNAs are frequently

observed to be down-regulated in multiple tumor types relative to normal tissue controls, implicating tumor suppressive functions for these miRNAs. As with protein-coding genes, these down-regulated miRNA genes are frequently silenced via promoter region DNA hypermethylation (Fig. 1.5).

Human miR-124-3p is one such aberrantly silenced miRNA that plays a role in inflammation-associated carcinogenesis and contributes to the epigenetic field for cancerization in *H. pylori*-infected gastric mucosae [73, 76]. Hypermethylation-mediated silencing of miR-124 has since been detected in other inflammation/infection-associated conditions, including ulcerative colitis and cervical intraepithelial neoplasia (CIN) [77-80], and in many cancer types. As these are some of the same conditions in which increased SMOX activity is thought to play a role in disease etiology, we hypothesized that miR-124 could be a regulator of SMOX either directly or indirectly. As the focus of Chapter 2, we investigate and confirm a direct relationship between miR-124 and the potential for SMOX induction and determine the role of miR-124 methylation as a predisposing factor in the progression from *H. pylori*-gastritis to gastric cancer. We further analyze differences in methylation levels of the miR-124 genes between populations of *H. pylori* gastritis patients inhabiting high- versus low-risk geographical regions of the state of Nariño in Colombia, South America and associate these differences with SMOX protein expression levels, oxidative DNA damage, and risk of disease progression.

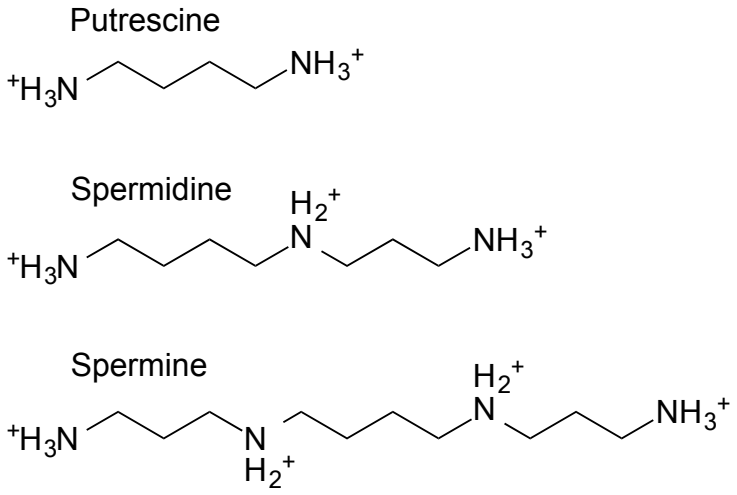

Figure 1.1. ***Chemical structures of the naturally occurring mammalian polyamines.*** The natural unmodified polyamines are shown protonated, as is the case at physiological pH.

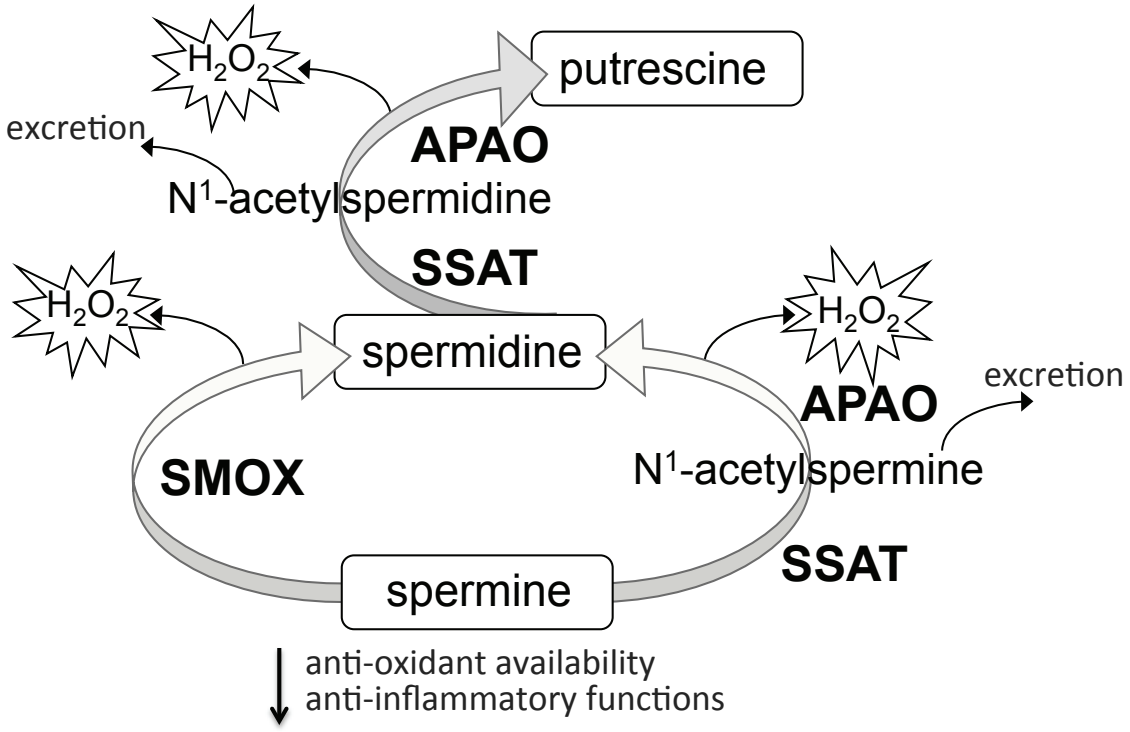

Figure 1.2. ***The mammalian polyamine catabolic pathway.*** Spermine is back-converted to spermidine either directly via spermine oxidase (SMOX) or through the combination of acetylation by spermidine/spermine N^1-acetyltransferase (SSAT) and oxidation by acetyl polyamine oxidase (APAO). Spermidine is further back-converted to putrescine through the same SSAT/APAO mechanism. Both oxidation reactions generate the reactive oxygen species (ROS) precursor H_2O_2 along with aldehydes as byproducts. The resulting reduction in spermine and spermidine pools implies diminished anti-oxidant and anti-inflammatory functions. Figure modified from Goodwin, *et al*. [81].

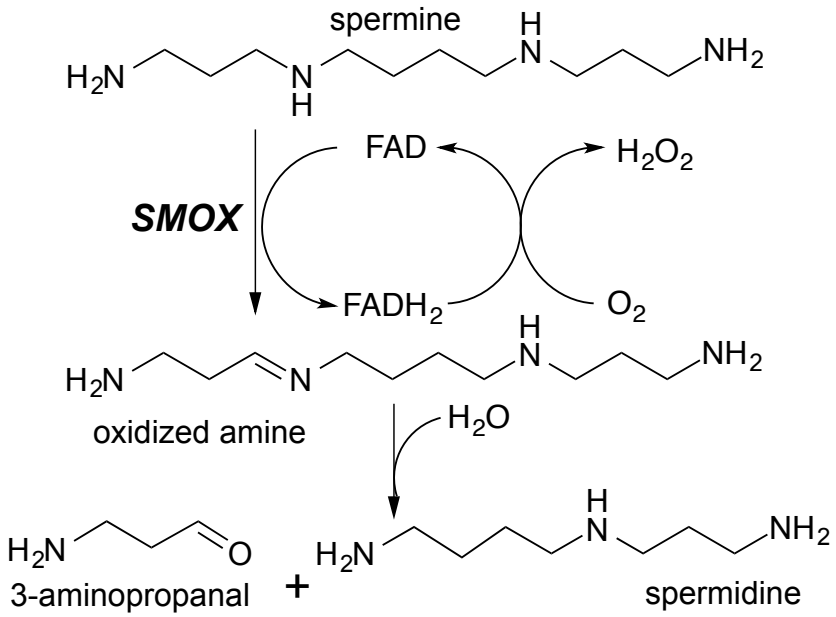

Figure 1.3. ***Reaction scheme of spermine oxidase.*** Spermine undergoes FAD-dependent oxidation by spermine oxidase (SMOX) to yield spermidine, H_2O_2, and the aldehyde 3-aminopropanal. Figure modified from Goodwin, *et al.* [82].

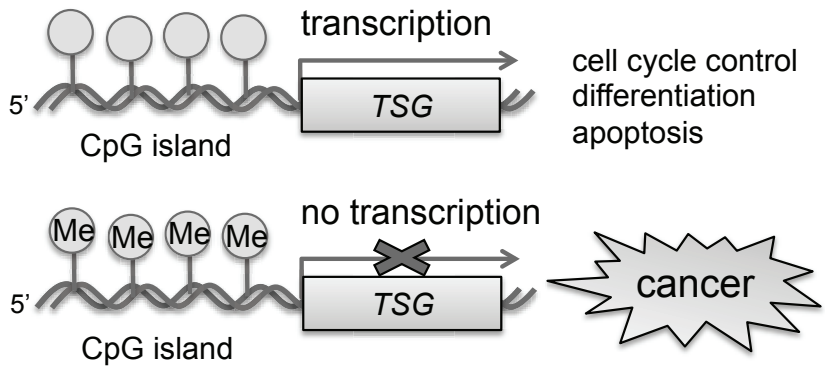

Figure 1.4. ***Schematic diagram depicting epigenetic gene silencing through CpG island DNA hypermethylation in cancer.*** Circles represent promoter-region CG dinucleotides with the potential for methylation (Me). When unmethylated, transcription of tumor suppressor genes (TSG) or other genes encoding anti-cancer-related proteins is permitted, resulting in the maintenance of normal cellular homeostasis. Methylation of promoter-region CG dinucleotides and the repressive, closed chromatin structure that it accompanies prevents the transcription of genes with anti-cancer functions, leading to cells with dysregulated proliferation and other hallmarks of cancer.

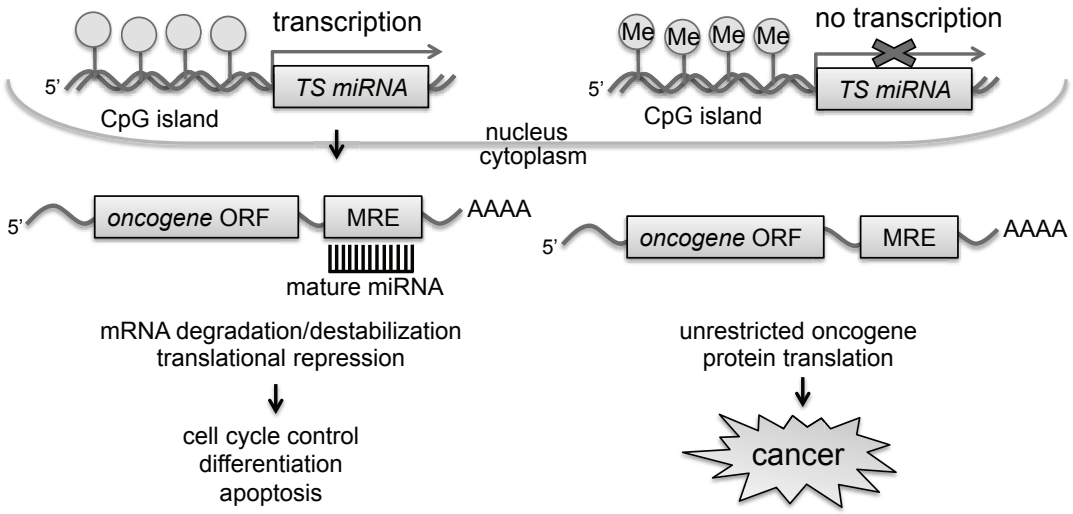

Figure 1.5. ***The effects of DNA methylation on miRNA-mediated tumor suppression.*** miRNA genes are transcribed as initial pri-miRNA molecules that, following processing and nuclear export, are eventually incorporated into an RNA-induced silencing complex (RISC) as a mature miRNA capable of interacting with the miRNA recognition element (MRE) of the target mRNA, typically located in the 3'-UTR. Like mRNAs, most miRNAs are transcribed by RNA polymerase II and are thereby affected by similar promoter-region gene regulatory features, including CpG island DNA methylation. As depicted in the left side of the figure, miRNAs can serve tumor suppressive (TS) functions by negatively regulating target mRNAs with potentially oncogenic functions, such as cell cycle control. Depending on the degree of perfect base-pairing, the mRNA is then degraded by cleavage, destabilized through de-adenylation, or translation is repressed. Promoter-region DNA methylation of a tumor suppressor miRNA (right column) prevents transcription of the pri-mRNA, eliminating production of the mature miRNA. Potentially oncogenic target mRNAs become unregulated, their gene products are produced, and cancer-related alterations result.

Chapter 2:

Expression of miR-124 suppresses spermine oxidase-mediated hydrogen peroxide generation associated with Helicobacter pylori infection of human gastric epithelial cells: Implications for infection/inflammation-induced carcinogenesis

Abstract

Chronic inflammation contributes to the development of various forms of cancer. The polyamine catabolic enzyme spermine oxidase (SMOX) is induced in chronic inflammatory conditions such as *Helicobacter pylori*-associated gastritis and colitis-associated colon cancer. As a generator of H_2O_2, elevated SMOX has the potential to contribute to DNA damage and the subsequent tumorigenesis associated with chronic inflammation. MicroRNA expression levels are also altered in inflammatory conditions. Specifically, the expression of miR-124, a potential negative regulator of SMOX, is silenced by DNA methylation in cases of ulcerative colitis and *H. pylori*-associated gastric cancer. We therefore sought to determine if the repressed levels of miR-124 expression that occur with inflammation are associated with the elevated levels of SMOX observed under these same conditions.

In the current study, miR-124-3p is overexpressed using a miRNA mimic or re-expressed using the DNA methyltransferase inhibitor 5-azacytidine in AGS human gastric adenocarcinoma cells. The expression levels of SMOX mRNA and protein were then measured by qRT-PCR and Western blot analysis, and changes in SMOX activity were

detected using a luminol-based assay to measure the production of H_2O_2. The resultant changes in intracellular polyamine levels were measured by HPLC. A bioinformatics analysis predicted the presence of a miR-124 recognition element in the 3'UTR of SMOX. To verify if miR-124 directly regulates SMOX mRNA, this portion of the *SMOX* 3'UTR was cloned downstream from the luciferase reporter gene, the resultant plasmid was cotransfected into AGS cells with the miR-124 mimic or a negative control, and luciferase activity was measured. The role of miR-124 expression in the regulation of SMOX induction resulting from *H. pylori* infection was investigated by infecting AGS cells with *H. pylori* in the presence or absence of miR-124. Finally, we analyze the degree of DNA methylation at the promoters of the three miR-124 gene loci in human gastric biopsies obtained from gastritis patient populations determined to be at low or high risk for the development of *H. pylori*-associated gastric cancer and associate this risk with miR-124-mediated SMOX expression.

The results of these experiments clearly indicate that miR-124 is a negative regulator of ROS-generating SMOX that is induced in chronic inflammation-associated tumorigenesis. In the AGS gastric adenocarcinoma cell line, which harbors a highly methylated and silenced endogenous *miR-124* gene, treatment with 5-azacytidine yielded a decreased level of SMOX mRNA expression. Transfection of AGS cells with a miR-124-3p mimic repressed SMOX mRNA and protein expression as well as H_2O_2 production by >50% within 24 hours. Luciferase reporter assays indicated that miR-124 directly interacts with the 3'UTR of SMOX mRNA, resulting in decreased SMOX transcript levels. Importantly, overexpression of miR-124 prior to infection with *H. pylori*

abrogated the increase in SMOX mRNA and activity that was observed when non-transfected AGS cells or those carrying a negative control miRNA mimic were exposed to this pathogen. Finally, preliminary results indicate that the *miRNA-124* gene loci are more heavily methylated in a South American population at high-risk for gastric cancer, a population that also is characterized by high SMOX activity and DNA damage. Accumulating evidence has indicated that elevated SMOX activity arising from exposure to infectious agents and/or pro-inflammatory cytokines is a contributing factor in the development of inflammation-associated cancer; the results reported here indicate that this elevation is correlated with, and may result from, the loss of miR-124 expression that occurs during the progression from inflammation to cancer.

Introduction

Spermine oxidase (SMOX), a member of the polyamine catabolic pathway, is responsible for the direct back-conversion of spermine to spermidine [26]. In addition to spermidine, this FAD-dependent oxidase reaction generates significant amounts of the ROS precursor H_2O_2. Mammalian SMOX activity occurs in both the cytoplasm and nucleus [29, 30], where its production of H_2O_2 in close proximity to DNA can result in oxidative DNA damage that elevates the potential for neoplastic transformation. SMOX is an inducible enzyme that responds to various stimuli, including bacterial infection [35, 36, 48], pro-inflammatory cytokines [40], the natural polyamines, and certain polyamine analogues [26]. Importantly, significantly increased expression levels of SMOX protein have been detected in biopsied tissue from patients harboring multiple conditions that are characterized by chronic inflammation, including *Helicobacter pylori*-associated gastritis [59], prostatic intraepithelial neoplasia (PIN) [63], and ulcerative colitis [62]. Each of these conditions is associated with an increased risk of tumorigenesis, and the inhibition of SMOX activity in animal models representing these conditions decreases tumor occurrence [36, 58, 60]. Therefore, the increase in SMOX-generated H_2O_2 that occurs as a result of infection and/or inflammation plays a causal role in the development of chronic inflammation-associated tumorigenesis.

In this regard, much research has focused on *H. pylori*-mediated induction of SMOX in gastric epithelial cells as a mechanism linking chronic gastritis and gastric carcinogenesis. *H. pylori* is a Gram-negative, microaerophilic bacterium that inhabits the

gastric mucosa of greater than two-thirds of the world's population. The bacterium was first identified in 1982 [83], and chronic infection with *H. pylori* was subsequently determined to be the causative agent in gastritis and peptic ulcer disease [84]. *H. pylori* infection is now considered the predominant environmental risk factor for the development of gastric cancer: using incidence data from 2012, approximately 90% of newly diagnosed noncardiac gastric cancer cases were attributed to chronic *H. pylori* infection [54]. Gastric cancer has a 5-year survival rate of less than 15%, and is the third leading cause of cancer-related deaths in males worldwide [53, 56, 85].

The prevalence of *H. pylori* infection is greatest in developing countries, where it is generally acquired in childhood. Throughout the Department of Nariño, Colombia, approximately 80% of children are *H. pylori*-positive by the age of 5 [86]. However, the risk of eventually developing gastric cancer differs greatly between two geographically isolated regions: those inhabiting the rural Andes mountain villages have an approximately 25-fold greater risk of developing gastric cancer than those residing in the low-risk region along the Pacific coast (150 vs. 6 per 100,000). Importantly, gastric tissues biopsied from gastritis patients in the high-risk region demonstrated elevated SMOX expression and activity that resulted in increased oxidative DNA damage, relative to similarly staged patients from the low-risk region [60]. These results, combined with related studies in a Mongolian gerbil model, implicated SMOX as the mediator of increased *H. pylori*-associated gastric cancer risk in the high-risk Colombian population and suggested the use of aberrant spermine oxidase induction as an indicator of gastric cancer risk and target for chemoprevention.

The dysregulation of specific microRNAs (miRNAs) with tumor suppressive or oncogenic roles is becoming widely reported in association with cancer. miRNAs are short, ~22-nt-long, non-coding RNA molecules that negatively regulate target mRNA transcripts, typically through base-pairing with a region in the 3'-UTR. As tumor suppressor genes, miRNAs bind to the mRNA transcripts of genes with potentially oncogenic functions, such as proliferation or differentiation, down-regulating their expression via message destabilization or translational inhibition. These tumor suppressive miRNAs are frequently inactivated in various forms of cancer through multiple mechanisms, including DNA copy number changes, gene mutations, and epigenetic changes. Aberrant promoter-region DNA hypermethylation has been reported as an epigenetic silencing mechanism for several tumor suppressive miRNAs, including hsa-miR-124. Three genomic loci produce the mature miR-124: *miR-124-1* (8p23.1), *miR-124-2* (8q12.3), and *miR-124-3* (20q13.33), and each of these are associated with canonical CpG islands, resulting in dense DNA hypermethylation and transcriptional repression that was first identified in the HCT116 colon cancer cell line [87]. This tumor-specific epigenetic silencing of miR-124 has since been observed in many cancer types, including gastric, colon, bladder, kidney, breast, cervical, uveal melanoma, liver, pancreatic, and prostate as well as glioblastomas, leukemias, and lymphomas [73, 77, 80, 87-95]. In contrast, in normal tissues of various origins, miR-124 is nearly always unmethylated and expression is easily detected.

A chronic inflammatory microenvironment contributes to epigenetic silencing through DNA hypermethylation, which accumulates in non-cancerous tissues prior to the development of malignancy. Notably, several studies have implicated correlations between miR-124 epigenetic inactivation and a predisposition to tumorigenesis. In particular, miR-124 hypermethylation has been reported in several premalignant conditions that are associated with chronic inflammation and/or infection. Of note, miR-124 hypermethylation occurs more frequently in healthy volunteers with *H. pylori* infection, who have an increased risk of gastric cancer, than in uninfected volunteers [73]. In *H. pylori*-negative individuals, miR-124 methylation levels are higher in non-cancerous gastric mucosae biopsied from patients with gastric cancer than in gastric mucosae from healthy volunteers, suggesting that hypermethylation of miR-124 contributes to the epigenetic field defect associated with past *H. pylori* infection and the development of gastric cancer [73]. Similarly, miR-124 DNA hypermethylation was detected in colonic tissues of both pediatric and adult patients with active ulcerative colitis [77, 78], a colorectal cancer predisposition. Methylation-mediated silencing of miR-124 was also frequently detected in premalignant cervical lesions and has demonstrated potential as a biomarker for the detection of high-grade cervical intraepithelial neoplasia (CIN) precursor lesions [80].

As the above-mentioned infection/inflammation-associated conditions under which miR-124 becomes silenced through DNA hypermethylation mimic those in which SMOX becomes activated, we hypothesized that miR-124 is a negative regulator of SMOX that when expressed, prevents the DNA-damaging and tumorigenic effects of SMOX

induction. Using miRNA mimics and human gastric adenocarcinoma cells, we investigate the inverse relationship between miR-124 and SMOX expression levels and confirm the direct regulation of SMOX by miR-124 using a luciferase reporter assay. Furthermore, we identify a protective role for miR-124 through the inhibition of *H. pylori*-induced SMOX activity and H_2O_2 production in AGS cells and provide preliminary data extending this role to the previously studied Colombian population of gastritis patients considered to be at low risk for developing gastric cancer. Conversely, in the high-risk Colombian gastritis population, the data indicate that increased DNA hypermethylation of miR-124 correlates with elevated SMOX activity and oxidative DNA damage, thereby implicating the inactivation of miR-124 during *H. pylori*-associated gastritis as a key player in the induction of oxidative DNA damage and subsequent gastric carcinogenesis.

Materials and Methods

Cell lines, culture conditions, and chemicals

The AGS human gastric cancer cell line (CRL-1739, ATCC, Manassas, VA) was maintained in F12K medium containing 10% fetal bovine serum, penicillin, and streptomycin at 37°C, 5% CO_2. Custom primers for cloning, qRT-PCR, and sequencing were synthesized by Integrated DNA Technologies (Coralville, IA). Restriction and DNA modification enzymes for cloning were purchased from New England Biolabs (Billerica, MA). Treatments with the DNA methyltransferase inhibitor 5-azacytidine were conducted at the doses and times indicated and included a change of medium with or without the addition of freshly diluted inhibitor every 24 hours.

RNA extraction, gene expression, and miRNA expression studies

RNAiMAX (Life Technologies, Grand Island, NY) was used to transfect AGS cells (2.5×10^5 cells/well in 6-well plates) with *mir*Vana miRNA mimics (Life Technologies, Grand Island, NY) corresponding to hsa-miR-124-3p or a negative control. After 24 hours, total RNA was extracted using TRIzol reagent (Life Technologies) according to the provided protocol. RNA was quantified by spectrophotometry, and cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). SYBR green-mediated, real-time PCR was performed using primer pairs and annealing temperatures as previously reported for the predominant isoform of SMOX (SMOX1) and GAPDH [29], with SYBR green SuperMix for iQ (Quanta Biosciences). The optimum annealing temperature for each primer pair was determined on cDNA using

temperature gradients followed by melt curve analyses and visualization on 2% agarose gels with GelStar staining (Lonza, Walkersville, MD) and KODAK Digital Science Image Analysis Software (Rochester, NY). Amplification conditions consisted of a 5-minute denaturation step at 95ºC, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60ºC for 30 s, and extension at 72°C for 30 s. Thermocycling was performed on a BioRad MyiQ2 real-time PCR detection system with data collection by the iQ5 optical system software (Hercules, CA). For each of the qPCR experiments, samples were analyzed in triplicate, normalized to the *GAPDH* reference gene, and the fold-change in expression was determined relative to cDNA from untreated cells using the $2^{-\Delta\Delta Ct}$ algorithm.

For miRNA expression analysis, 1 mg of TRIzol-extracted RNA was converted to cDNA using the miScript II PCR System (SABiosciences, Frederick, MD). PCR was performed using a miR-124-specific sense primer [87] with a universal antisense primer (SABiosciences), according to the manufacturer's recommendations. U6 snRNA was amplified as the normalization control. Amplification products were electrophoresed on 2% agarose gels, stained with GelStar, and visualized and photographed using KODAK Digital Science Image Analysis software.

Analysis of spermine oxidase protein and activity

AGS cells were seeded at a density of 6.5×10^5 cells per 25-cm^2 flask and allowed to attached overnight prior to transfection with the hsa-miR-124-3p or negative control miRNA mimic. Following 24 or 48 hours of incubation, cells were collected and quick-

frozen for analysis. Spermine oxidase activity was measured using a luminol-based assay measuring the production of hydrogen peroxide, as previously described [96]. Assays were normalized relative to milligrams of total cellular protein determined using the method of Bradford [97] with interpolation on a bovine serum albumin standard curve.

For Western blots, total protein (50 μg per lane) was separated on pre-cast 4-12% Bis-Tris NuPAGE gels with 1 × MOPS running buffer (Invitrogen) and transferred onto Immun-Blot PVDF membranes (BioRad). Blots were blocked for 1 hour at room temperature in Odyssey blocking buffer (LI-COR, Lincoln, NE), followed by overnight incubation at 4ºC with antibodies specific to SMOX (1:1000 dilution), as previously described [29], and β-actin (Sigma). Blots were then incubated with species-specific, fluorophore-conjugated secondary antibodies to allow the visualization and quantification of immunoreactive proteins using the Odyssey infrared detection system and software (LI-COR).

Analysis of the 3'-UTR of spermine oxidase

Putative hsa-miR-124 binding sites were initially predicted using bioinformatic tools available at www.microrna.org [98] and TargetScan (version 6.2) to analyze the 3'-UTR of the human SMOX transcript. To experimentally verify that miR-124 could directly influence SMOX mRNA, an 86-bp region of the human SMOX 3'-UTR (+177 to +263) including the putative recognition element for miR-124 was PCR-amplified and inserted downstream of the luciferase gene in the pMIR-REPORT Luciferase expression vector (Life Technologies). Insertion was verified using Sanger-based sequencing methods [99].

Cotransfection of the resulting pMIR-Luc-SMOX plasmid, the *mir*Vana hsa-miR-124-3p mimic or negative control mimic, and the pMIR-REPORT-β-Gal reporter control into AGS cells was performed using Lipofectamine 2000 (Life Technologies) according to the protocol provided with the mimic. Cell lysates were collected 48 h later in reporter lysis buffer, and luciferase and β-galactosidase activities were measured using the Luciferase Assay System and the β-Galactosidase Enzyme Assay System, respectively (Promega, Madison, WI). Luciferase activity was measured as relative light units and normalized to β-gal activity measurements determined by absorbance at OD_{420} to account for transfection variability.

Helicobacter pylori culture and infection of AGS cells

H. pylori strain 60190 (ATCC) was maintained on trypticase soy agar plates containing 5% sheep blood at 37ºC, 5% CO_2. In preparation for the infection of AGS cells, the bacteria were transferred to 75-cm^2 tissue culture flasks containing Brucella broth supplemented with 10% fetal bovine serum and grown for 48 hours in an upright position with occasional agitation at 37ºC, 5% CO_2. Bacteria were then collected by centrifugation, washed with PBS, and cell number was estimated based on OD_{600}.

AGS gastric epithelial cells transfected with the miR-NC or miR-124-3p mimic, as described above, were divided and replated in antibiotic-free F12K medium supplemented with 10% fetal bovine serum for 24 h prior to the addition of *H. pylori* at an MOI of 100 bacteria per cell for 6 hours. Uninfected cells from the same transfected population were used as negative controls. Cells were harvested and aliquoted to prepare

lysates for SMOX activity and protein analysis as well as for total RNA extraction using Trizol reagent. Complementary DNA was generated and used for SYBR-green-mediated detection of SMOX mRNA expression level relative to GAPDH as described above.

Human subjects

The human gastric biopsies used in the current study were those previously described by Schneider and colleagues [100, 101]. Briefly, samples were obtained by upper endoscopy from male patients with dyspeptic symptoms who resided in either the high-risk Andean community of Tuquerres or the low-risk Pacific Coast city of Tumaco in the Department of Nariño, Colombia.

Quantitative DNA methylation analyses

DNA was isolated from frozen human gastric antral biopsies obtained from the high and low-risk regions and used for bisulfite modification and PCR amplification as previously described [100]. Multiple primer pairs including 1 biotinylated primer were designed specifically for each of the 3 gene loci of *hsa-miR-124* as follows: *miR-124-1* forward (mir124a-1PF) 5'-GGAGTTTTTTAGAAGTAGGTTTGATGTT-3' and reverse (mir124a-1PR) 5'-biotin-CTCCCCTCCCTAAACCCTCCAAC-3'; *miR-124-2* forward (mir124a-2G) 5'-AGGAGGGAATTATTGTTTTTTAGATAGTTG-3' and reverse (mir124a-2H) 5'-biotin-AAAAAAAACTCCTACTTTTCCATTACAAC-3'; and *miR-124-3* forward (mir124a-3F) 5'-AAAGAGAAGAGTTTTTATTTTTGAGTAT-3' and reverse (mir124a-3R) 5'-biotin-TCCTCCTCAACTACCTTCCCCTA-3'. The production of a single product for each primer pair was verified by electrophoresis in a 2% agarose

gel. Pyrosequencing was performed on the biotinylated strand of each amplification product along with AGS cell DNA as a positive methylation control and normal human blood DNA as a negative control. Sequencing primers for each amplification product were as follows: *miR-124-1* (mir124a-1PS) 5'-TAGAAGTAGGTTTGATGT-3'; *miR-124-2* (mir124a-2GS) 5'-AATTTTTTTAGGAGATT-3'; and *miR-124-3* (mir124a-3FS) 5'- GAGATTAGTTTTTTTAAT-3'.

Results

Exogenous expression of miR-124 decreases SMOX expression

The AGS human gastric adenocarcinoma cells used in this study express nearly undetectable levels of miR-124 (Fig. 2.1A). Transiently transfecting these cells with a miRNA mimic corresponding to hsa-miR-124 significantly down-regulated the expression of SMOX mRNA within 24 hours, compared to cells transfected with a negative control miRNA mimic (miR-NC) (Fig. 2.1B). SMOX protein levels were also diminished upon miR-124 expression, as indicated by Western blot analyses (Fig. 2.1C) and SMOX activity assays, which verified decreases in functional SMOX protein as measured by the generation of H_2O_2 (Fig. 2.1D).

miR-124 directly targets the 3'UTR of SMOX

To evaluate whether the expression of miR-124 can directly reduce SMOX expression, the 3'-UTR of the human *SMOX* gene was examined for predicted miRNA recognition sites. Bioinformatic analysis revealed a miR-124 target site starting at position 249 of the human SMOX 3'-UTR that consisted of an exact match to positions 2-8 of the mature miRNA (the seed + position 8) (Fig. 2.2A). This region of the human *SMOX* gene, which is broadly conserved among vertebrates, was isolated and inserted downstream of the firefly luciferase gene to generate a reporter construct. Transfection of the resulting plasmid into AGS cells induced robust luciferase activity that was attenuated approximately 40% upon cotransfection with the miR-124 mimic (Fig. 2.2B), relative to

the negative control mimic. These results indicate that miR-124 regulates SMOX, at least in part, through direct targeting of its 3'-UTR.

miR-124 inhibits H. pylori-mediated induction of SMOX

Infection of gastric epithelial cells with *H. pylori* induces SMOX expression that results in increased DNA damage in association with gastric tumorigenesis. To determine if the expression level of miR-124 could influence *H. pylori*-mediated SMOX induction, AGS gastric epithelial cells were transfected with miR-124 or the negative control mimic 48 hours prior to infection with the pathogenic *H. pylori* strain 60190. Quantitative PCR results demonstrated significant (6-8-fold) increases in SMOX mRNA in wild-type AGS cells or those containing the negative control miRNA mimic following co-incubation with *H. pylori*, while the cells transfected with the miR-124 mimic maintained SMOX mRNA levels that were not significantly greater than basal levels (Fig. 2.3A). These results implied that miR-124 is capable of negatively regulating the increased SMOX transcript levels that occur with *H. pylori* infection, thereby potentially preventing the SMOX-mediated production of DNA-damaging H_2O_2.

To confirm this protective role for miR-124, SMOX activity and the associated generation of H_2O_2 that is induced upon the exposure of AGS cells to *H. pylori* was analyzed in cells expressing the miR-124 mimic (Fig. 2.3B). As with SMOX mRNA, significantly increased SMOX activity occurs following 6 h of co-incubation with *H. pylori* in either wild-type or negative control AGS cells. Importantly, the AGS cells overexpressing miR-124 generated diminished levels of spermine oxidase-specific H_2O_2,

regardless of the presence of *H. pylori*. These data verify that miR-124 is a negative regulator of SMOX and as such, serves to protect cells from the DNA-damaging effects of increased spermine oxidation in association with *H. pylori* infection.

Re-expression of endogenous miR-124 represses SMOX expression

Previous studies have demonstrated that DNA hypermethylation of the promoter region of the *miR-124* genes is responsible for the lack of expression in AGS cells [73]. We therefore treated AGS cells with the DNA methyltransferase (DNMT) inhibitor 5-azacytidine (5-AC) to re-express endogenous miR-124 and to determine if this re-expression altered the expression of SMOX. As depicted in Fig. 2.4, treatment of AGS cells with the DNMT inhibitor resulted in an increase in miR-124 expression that correlated with decreased expression of the SMOX transcript.

miR-124 DNA methylation in gastric biopsies from high-risk versus low-risk gastritis populations

To investigate the clinical implications of the miR-124 – SMOX interaction, DNA methylation levels of the 3 *hsa-miR-124* genes were quantitatively analyzed using pyrosequencing in human gastric mucosae biopsies obtained from *H. pylori*-positive gastritis patients from high- versus low-risk regions of Colombia, South America. This preliminary study included 7 samples from the low-risk region and 5 samples from the high-risk region. As depicted in Fig. 2.5, methylation levels for each of the 3 miR-124 gene loci tended to be higher in the high-risk population relative to those of the low-risk individuals. In particular, the increases in methylation levels at the miR-124-1 locus were

statistically significant in all primer pairs evaluated, despite the small sample size. Using a larger cohort of this same group of biopsies, we previously demonstrated that increased SMOX activity and oxidative DNA damage are mediators of the high tumorigenic risk of this population [60]. These data therefore suggest that aberrant epigenetic silencing of miR-124 is a critical factor in determining susceptibility to *H. pylori*-associated oxidative damage in the etiology of gastric cancer, and further experiments using the entire cohort (approximately 40 samples per region) are currently in progress.

Discussion

The chronic inflammation associated with *H. pylori* infection is perceived to play a causative role in epigenetic gene silencing through DNA hypermethylation, which is observed to increase as one progresses through the cascade from non-atrophic gastritis to cancer [69-71]. As with miR-124, methylation levels of other genes known to play tumor suppressive roles in gastric carcinogenesis are also increased in the high-risk Colombian gastritis patients [100, 101], suggesting a fundamental difference affecting the methylation process. Genetic differences also exist between the two populations that may contribute to cancer risk, including ancestral differences in both the human and bacterial lineages [102], and environmental factors related to their extremely different geographical locations as well as socioeconomic factors have influential roles [103]. Chaturvedi and colleagues isolated and characterized multiple *H. pylori* strains from individuals inhabiting either the high- and low-risk regions; interestingly, compared to those obtained from low-risk individuals, the strains obtained from high-risk patients were capable of inducing significantly higher levels of SMOX mRNA and activity with reduced apoptosis levels in AGS cells [60]. Therefore, patients who are already predisposed to induce SMOX due to hypermethylated miR-124 appear to also be colonized by *H. pylori* strains with a heightened ability to induce SMOX without triggering apoptosis.

The current study did not investigate the effect of miR-124 expression on cell proliferation, as this has been previously reported in AGS cells as well as in many other

tumor cell lines. However, as would be expected for a miRNA with tumor suppressive function, overexpressing miR-124 decreased the rate of proliferation in all tumor cell types examined, and mouse tumor xenografts originating from miR-124-overexpressing gastric carcinoma cells, including AGS cells, resulted in significantly slower tumor growth [104, 105]. As miR-124 has many mRNA targets, including regulators of proliferation such as CDK6 [87], EZH2 [105], and CDK4 [95], changing the expression level of miR-124 likely has multiple effects on multiple pathways. It is therefore important to acknowledge that in cells in which miR-124 is silenced, growth is significantly upregulated via these other potentially oncogenic targets in the presence of increased SMOX-mediated oxidative DNA damage and decreased apoptosis, thereby advancing the potential for malignancy.

The results of the current study suggest the aberrant epigenetic silencing of miR-124 as a potential risk indicator for *H. pylori*-associated gastric carcinogenesis. As illustrated in Fig. 2.6, when expressed in gastric epithelial cells, miR-124 targets and prevents the induction of SMOX-generated ROS that occurs in the presence of inflammatory stimuli, such as *H. pylori*-associated gastritis. As a result, gastritis patients expressing miR-124 maintain lower levels of SMOX and appear less likely to progress through the higher-grade, pre-malignant lesions that lead to gastric cancer. In contrast, patients at high risk for progressing to gastric cancer demonstrate significantly higher levels of SMOX and oxidative DNA damage that is associated with increased levels of miR-124 CpG island hypermethylation. It should be noted that, in general, the patients with the highest levels of methylation at one locus are the same patients with the highest levels at the other loci.

As each locus produces the same mature miRNA, a combination of the methylation levels at the three loci will likely provide a more accurate prediction of the true effects on expression, and it is possible that the results presented here are underestimated.

Our studies therefore suggest the potential use of methylated miR-124 as a valuable indicator of gastric cancer risk in *H. pylori*-positive individuals. As greater than half of the world's population is infected with *H. pylori*, antibiotic eradication of the bacteria is not feasible. In addition to cost prohibitions, antibiotic resistance is a factor, and eradication only reduces the risk of gastric cancer if conducted prior to the occurrence of pre-malignant lesions. Furthermore, inverse correlations have been observed between *H. pylori* infection and asthma, esophageal reflux disease, and eosinophilic esophagitis. As a biomarker, methylation of miR-124 could provide an indicator for those patients most likely to progress to the pre-malignant lesions and cancer, who could then receive more intensive monitoring with appropriate eradication and/or chemopreventive strategies. In fact, without regard to its downstream effects, methylation of miR-124-3 has shown utility as an accurate indicator of metachronous gastric cancers, in a multicenter cohort study [76].

Due to the limitations of eradication, strategies for the chemoprevention of *H. pylori*-associated gastric carcinogenesis are also needed to significantly reduce the risk associated with this pathogen. Pre-clinical data using a Mongolian gerbil model of *H. pylori*-associated gastric cancer has provided promising results by combining α-difluoromethylornithine (DFMO), an inhibitor of polyamine biosynthesis, with

MDL72,527, an inhibitor of spermine oxidase [60]. This combination inhibited the SMOX-mediated DNA damage and reduced the gastric dysplasia and carcinoma associated with *H. pylori* infection and is expected to enter clinical trials. In this regard, the detection of miR-124 hypermethylation could provide a feasible method for identifying the patients most likely to benefit from a therapy targeting activated spermine oxidation. Additionally, although not specifically targeting SMOX, the fact that the mode of miR-124 silencing is epigenetic indicates its reversibility and suggests the use of DNMT inhibitors such as 5-azacytidine, which have been extensively studied in the clinic and are currently being revisited for their lasting demethylating effects using transient dosing schedules at less cytotoxic concentrations [106]. Finally, the utility of miRNAs themselves as potential anticancer drugs is an area of active investigation currently limited by a lack of efficient delivery systems [107].

In addition to *H. pylori*-associated gastric cancer, hypermethylation of miR-124 has been observed in many other cancer types as well as in several pre-malignant conditions associated with chronic inflammation and/or infection. In particular, miR-124 methylation increases in ulcerative colitis and has been identified as a potential risk marker for colitis-associated cancer [77], a condition that, like gastritis, varies greatly among individuals. As we have previously reported that elevated SMOX activity in an ETBF-induced model of mouse colitis is a mechanism linking inflammatory stimuli with tumorigenesis, it is easy to speculate that miR-124 expression influences SMOX activity in this system as well. Moreover, the lack of miR-124 expression in multiple systems

further implicates a fundamental role for SMOX induction in the etiology of chronic inflammation/infection-associated carcinogenesis that warrants further investigation.

In conclusion, the current study linked the aberrant DNA methylation of miR-124 with the activation of spermine oxidation and consequential generation of oxidative DNA damage in the etiology of *H. pylori*-associated gastric cancer. Methylation levels of the *miR-124* genes were elevated in patients at heightened risk of progression to *H. pylori*-associated gastric adenocarcinoma, while low methylation levels in the low-risk population were associated with miR-124-mediated protection from SMOX induction. Therefore, the methylation or expression level of miR-124 could provide a useful marker for identifying those *H. pylori*-positive individuals at increased risk for developing gastric cancer.

Tables and Figures

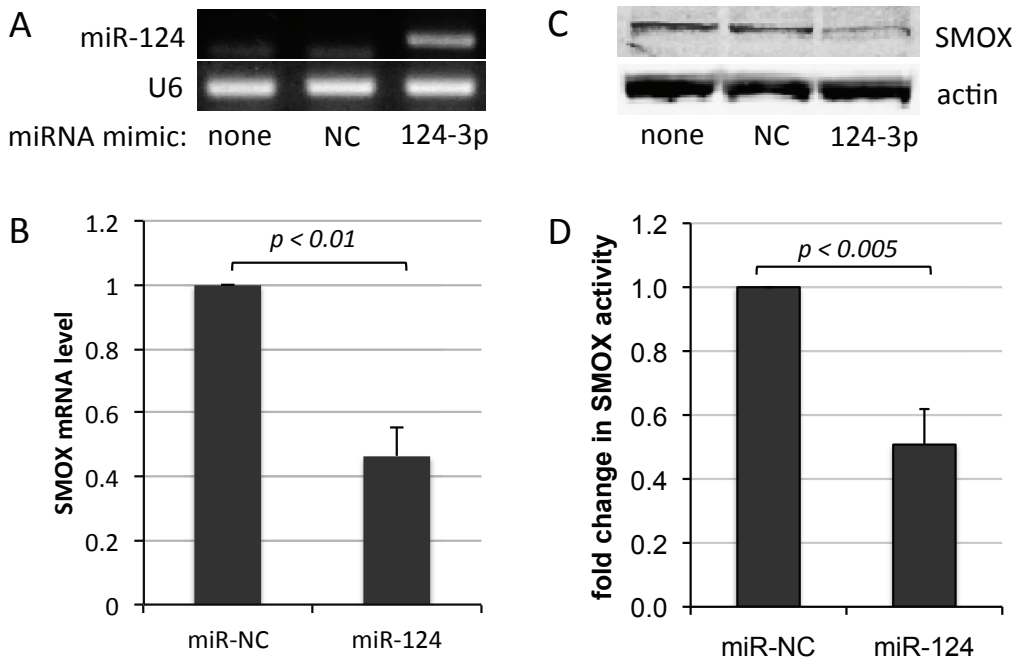

Figure 2.1. ***Exogenous expression of miR-124 decreases SMOX expression in gastric adenocarcinoma cells.*** AGS cells were transfected with an hsa-miR-124-3p mimic or a negative control mimic (miR-NC) for 24 h: **A**. RT-PCR of miR-124 expression in transfected AGS cells. U6 snRNA was amplified as a normalization control; **B**. Quantitative RT-PCR of SMOX mRNA, relative to GAPDH, following mimic expression (histograms represent the means of 4 independent experiments; error bars indicate SEM); **C**. Western blot using antibodies to SMOX and β-actin in miR-transfected AGS cells; **D**. Luminol-based SMOX activity assay demonstrating decreased H_2O_2 production in miR-124-transfected cells, measured as H_2O_2 produced/mg protein/minute (histograms represent the means of 4 individual experiments, each performed in triplicate; error bars indicate SEM). Where relevant, Student's t-test was used to calculate *p*-values.

A

hsa-miR-124: 3’ ccguaAGUGGCG - - CACGGAAu 5’
| : | : : | : | | | | | |
position 249 of SMOX 3’UTR: 5’ ccuugUUAUUGUAAGUGCCUUc 3’

B

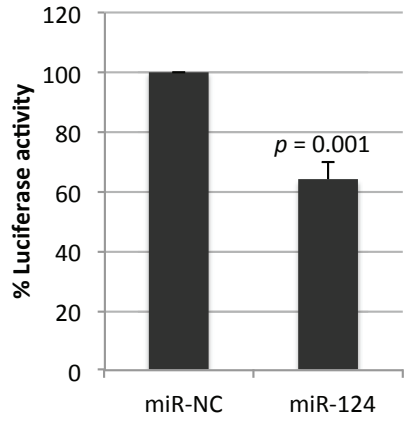

Figure 2.2. ***miR-124 directly targets the SMOX 3’-UTR.*** **A**. Bioinformatic analysis demonstrating the complementarity of the 3’-UTR of human SMOX mRNA with the hsa-miR-124 seed sequence (underlined). **B**. Luciferase assay indicating that expression of the miR-124 mimic in AGS cells attenuates reporter plasmid activity through direct interaction with the SMOX 3’-UTR. Luciferase activity was measured in RLUs and normalized to β-galactosidase activity. Data represent 3 independent biological experiments, each measured in triplicate. Error bars indicate SEM.

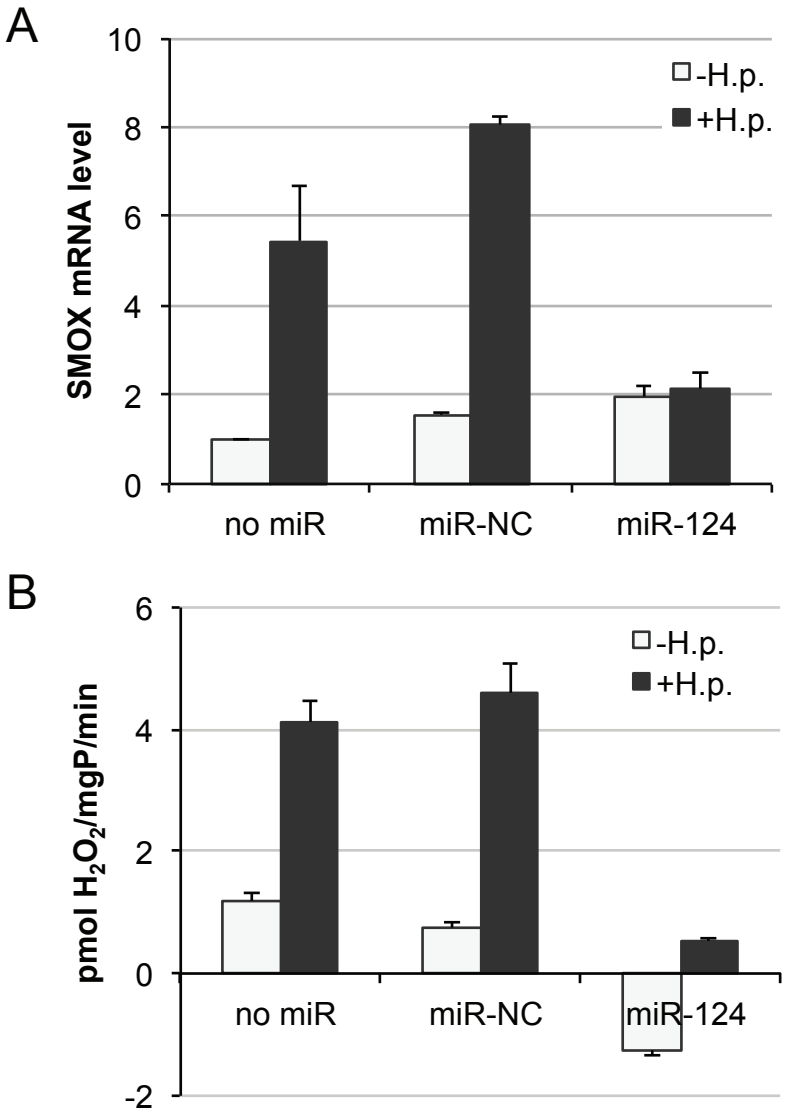

Figure 2.3. ***Overexpression of miR-124 inhibits H. pylori-mediated induction of SMOX.*** AGS cells expressing the miR-NC or miR-124 mimics were stimulated with *H. pylori* strain 60190 for 6 hours. **A**. Quantitative RT-PCR demonstrates that the presence of miR-124 represses the increase in SMOX mRNA that is typically observed in response to *H. pylori* infection. **B**. Spermine oxidase activity assay demonstrating a lack of *H. pylori*-mediated SMOX activity and H_2O_2 production with the overexpression of miR-124. Columns represent the means with error bars indicating SEM.

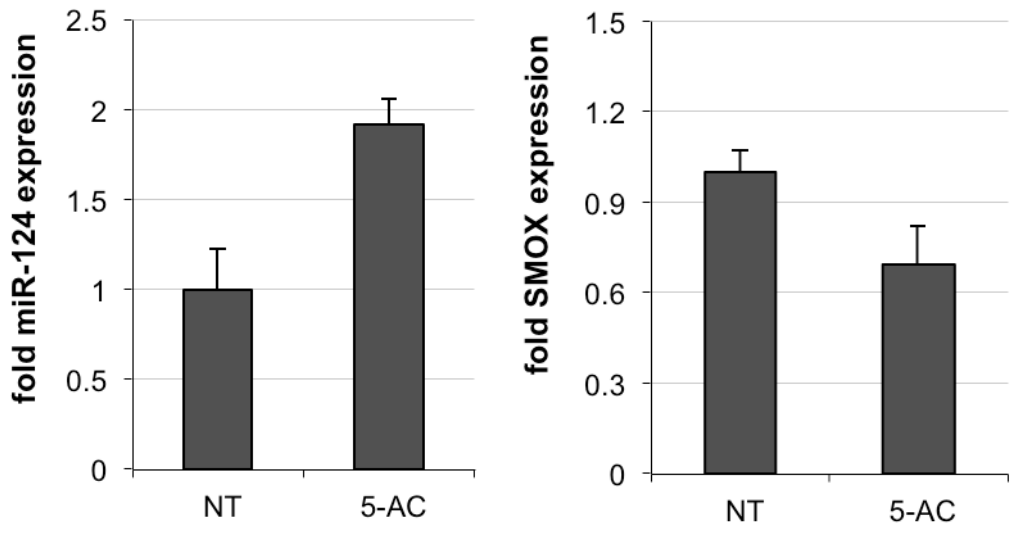

Figure 2.4. ***Re-expression of endogenous miR-124 represses SMOX expression.*** AGS cells were treated with 0.5 μM 5-azacytidine (5-AC) for 6 days (refreshed daily) and RNA expression levels were quantified using qRT-PCR with primers specific to miR-124 or SMOX. miR-124 transcript levels are relative to U6, and SMOX levels are relative to GAPDH. Columns indicate the means with standard deviations. NT: no treatment.

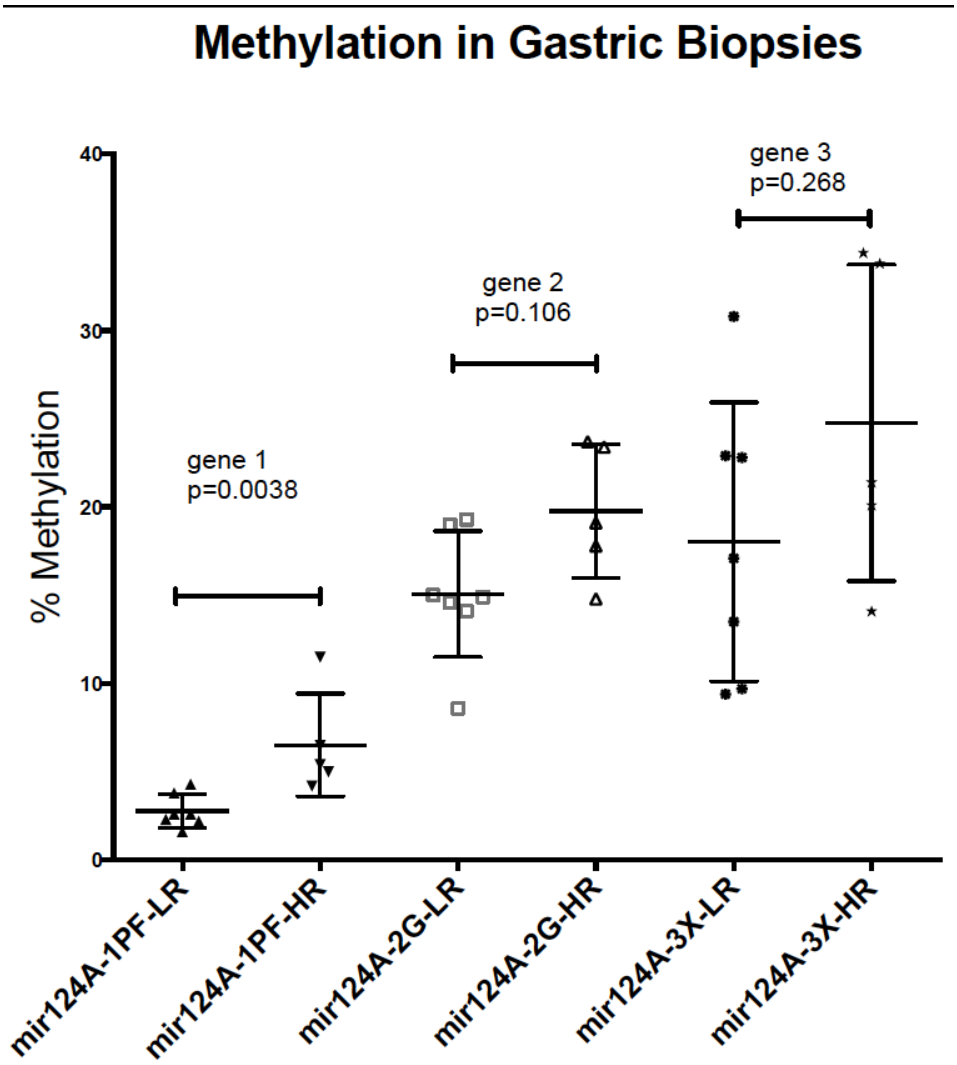

Figure 2.5. ***Methylation levels of miR-124 genes are elevated in gastritis patient biopsies from the high-risk region versus the low-risk region.*** The percent of methylation at specific CpG sites at each *miR-124* gene locus was determined using pyrosequencing. Each data point represents a patient from the low-risk (LR) or high-risk (HR) region of Colombia. The bars indicate the means. Statistically significant differences ($p < 0.005$) were determined using the Mann Whitney test and are as indicated.

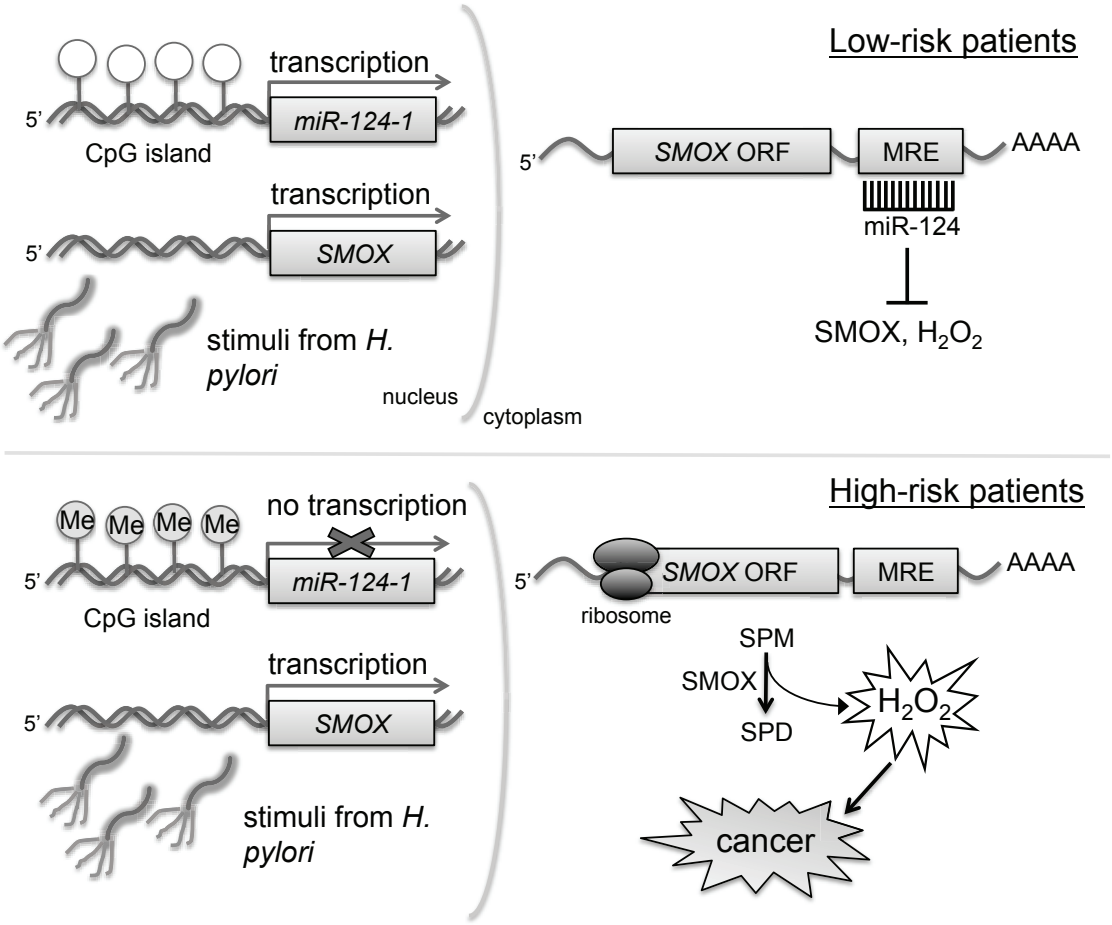

Figure 2.6. ***The proposed mechanism of miR-124-mediated protection in patients from low-risk versus high-risk regions of Colombia.*** *H. pylori*-positive gastritis patients representing the low-risk region (top panel) have low levels of DNA methylation at the *miR-124* gene promoters, allowing sufficient transcription to ensure post-transcriptional silencing of SMOX mRNA by mature miR-124. The result is a lack of SMOX protein induction in spite of transcriptional activation by *H. pylori*-associated inflammatory stimuli. In high-risk patients (bottom panel), CpG island methylation (Me) is increased in the miR-124 promoter, the abundance of mature miR-124 is reduced, and SMOX protein production is uncontrolled. This results in diminished spermine concentrations and persistent ROS production leading to oxidative DNA damage and cancer.

Chapter 3:

Spermine oxidase induction as a potential mechanism for aberrant epigenetic gene silencing in the etiology of chronic inflammation/infection-associated carcinogenesis: Development of an in vitro model system

Abstract

Chronic inflammation is a predisposing risk factor for epithelial cell carcinogenesis. Although the precise mechanisms are unclear, the increased generation of reactive oxygen species (ROS) and decreased antioxidant capabilities are major contributing factors. One such mechanism affecting both of these factors is the induction of spermine oxidation in response to inflammatory stimuli. Spermine oxidase (SMOX) catalyzes the back-conversion of the free radical scavenger spermine to spermidine while generating H_2O_2, an ROS precursor, as a byproduct. Considering that significant SMOX activity is localized in the cell nucleus, the increased ROS production combined with the decreased pool of protective spermine in close proximity to DNA increases the likelihood of oxidative DNA damage that can lead to carcinogenesis. In fact, SMOX activity is induced by the human pathogens *Helicobacter pylori* and enterotoxigenic *Bacteroides fragilis* (ETBF), and through its generation of ROS, consequently serves as a molecular link between inflammatory stimuli and epithelial cell tumorigenesis. However, the precise mechanisms governing this ROS-mediated carcinogenesis remain unclear.

A hallmark of cancer is the accumulation of genetic and epigenetic alterations in the genome, both of which can be attributed to oxidative DNA damage. Of the epigenetic changes, DNA CpG island hypermethylation plays a prominent role in cancer-related gene silencing and has recently been demonstrated to increase as a result of H_2O_2-induced DNA damage in a mouse model of ulcerative colitis – a model system in which SMOX induction also links inflammation to carcinogenesis. DNA methylation increases have been observed in additional conditions associated with inflammation/infection-mediated SMOX induction, including the infection of gastric epithelial cells with *H. pylori*, where levels of CpG island methylation are observed to increase with progression through the premalignant lesions leading to carcinoma. As overall DNA hypermethylation levels appear to correlate with the induction of SMOX, we sought to develop a model system with which to investigate the potential of SMOX induction as a molecular link between inflammatory stimuli and aberrant epigenetic gene silencing in the etiology of chronic inflammation/infection-associated cancer.

In this regard, we developed a conditional SMOX overexpression system in gastric epithelial cells that is designed to imitate the rapid induction of SMOX activity that occurs with *H. pylori* infection. An expression vector containing a LoxP-flanked "stop" sequence located upstream of the SMOX coding sequence was stably integrated into gastric epithelial cell lines. Upon co-incubation with a Cre-recombinase-expressing adenovirus, this "stop" sequence was excised and SMOX was overexpressed, resulting in altered intracellular polyamine concentrations and H_2O_2 generation comparable to those observed in *H. pylori*-associated gastric carcinogenesis. Preliminary studies using this

model to investigate a candidate tumor suppressor gene, MGMT, which becomes hypermethylated in *H. pylori*-positive patient samples, revealed its down-regulation in response to SMOX induction, exemplifying the potential utility of this novel model system. Furthermore, with the establishment of this model, changes in DNA damage response, chromatin modifications, CpG island methylation levels, and gene expression can be documented over the time of ROS exposure, thereby potentially providing proof of principle that SMOX-generated ROS are capable of inducing the epigenetic gene silencing associated with chronic inflammation/infection.

Introduction

The aberrant DNA methylation of promoter-region CpG islands is a well-established mode of epigenetic gene silencing associated with cancer in general. This hypermethylation is frequently detected in nearly all forms of cancer and often results in the loss of tumor suppressor gene transcription [108, 109]. However, DNA methylation levels are not only increased in established cancers; they are also evident in many premalignant lesions and in nontumor tissues of cancer patients, suggesting a fundamental alteration in methylation that occurs prior to the initiation of tumorigenesis. This phenomenon is referred to as an epigenetic field for cancerization and is the result of exposure to a carcinogen over an extended period of time, resulting in molecular changes that contribute to the development of carcinogenesis and increase the potential for metachronous tumors and reoccurrence [110]. Chronic inflammation and/or infection are factors suggested to contribute to this epigenetic field for cancerization, creating a predisposition for epithelial tumorigenesis [111].

Chronic inflammation is characterized by an infiltration of macrophages and other inflammatory cells resulting in continuous tissue damage through the production of reactive oxygen and nitrogen species (ROS and RNS, respectively) [112, 113]. The epithelial cells exposed to the infection or inflammatory stimulus also generate significant amounts of ROS, elevating the potential for significant oxidative DNA damage [114]. Furthermore, infiltrating immune system cells secrete growth factors and certain cytokines that support the proliferation of ROS-damaged cells [115]. Therefore,

tissue regions exposed to chronic inflammation are predisposed to the development of neoplasia. Infection of the gastric mucosa with *Helicobacter pylori* induces a chronic active inflammation that results in severe oxidative damage. In a subset of patients, this gastritis progresses through the "Correa cascade," a series of morphologically distinct lesions consisting of intestinal metaplasia (IM), dysplasia, and invasive carcinoma [55]. The carcinogenic potential of *H. pylori* infection has been well studied with regard to mutations and the activation of cell signaling and growth pathways (reviewed in [116]); however, epigenetic gene alterations are also major factors influencing the etiology of *H. pylori*-associated gastric carcinogenesis. In fact, certain tumor suppressor genes considered to be driver genes in gastric cancer, such as *MLH1* and *CDH1*, are more commonly silenced in association with aberrant CpG island DNA methylation than by mutation [68]. It is now known that the inflammation associated with chronic *H. pylori* infection is responsible for increasing levels of DNA methylation in gastric epithelial cells [68, 69], and higher DNA methylation levels are correlated with more severe inflammation and more advanced precancerous gastric lesions [101, 117, 118]. Importantly, using a Mongolian gerbil model of *H. pylori*-associated gastric cancer, Niwa and colleagues recently demonstrated the effectiveness of a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine, as a chemopreventive agent that decreased gastric tumorigenesis in response to *H. pylori* infection [119]. These studies implicated aberrant epigenetic silencing of tumor suppressor genes and its associated CpG island DNA hypermethylation as a contributing mechanism in the etiology of *H. pylori*-associated gastric cancer.

The factors underlying epigenetic gene silencing and DNA methylation in response to *H. pylori* infection are unclear, but the process appears to be a response to the chronic inflammatory microenvironment rather than the bacteria itself [69]. A major characteristic of this microenvironment is the presence of elevated levels of ROS. ROS cause oxidative DNA damage in the form of strand breaks and base adducts, such as 8-oxo-2'-deoxyguanosine (8-oxo-dG). Unrepaired DNA damage or mispairing during DNA replication due to adducts such as 8-oxo-dG can be a source of cancer initiating-mutations. However, O'Hagan and colleagues recently reported that this ROS-induced DNA damage is also a mediator of epigenetic gene silencing and CpG island DNA hypermethylation [75]. The repair of oxidative DNA damage occurs chiefly through the base excision repair pathway (BER) [120] and requires extensive chromatin remodeling in the form of histone modifications and nucleosome repositioning to repress transcription at sites of damage, increase accessibility for repair enzymes, and restore gene transcription once repair is complete. These changes in chromatin architecture are mediated by epigenetic modification enzymes such as DNA methyltransferases (DNMTs), histone deacetylases (HDACs), and histone acetyltransferases (HATs) – some of the same players responsible for cancer-related epigenetic gene silencing. In a mouse model of enterotoxigenic *Bacteroides fragilis* (ETBF)-associated colitis, oxidative DNA damage led to the recruitment of members of the epigenetic silencing machinery involved in DNA repair to chromatin regions containing damaged DNA. More specifically, DNMT1 and certain members of the polycomb repressor complex 4 (PRC4), including Sirtuin-1 (SIRT1), an HDAC, were enriched at CpG island-containing promoters of genes with relatively low basal levels of expression in inflamed colonic epithelial tissue

of ETBF-infected mice, relative to sham-infected mice. Importantly, PRC4 is known to exist exclusively in cancer cells as well as in embryonic and adult stem cells. Furthermore, the characteristics marking those genes to which the repressor complexes were recruited mimic those of genes identified to frequently undergo tumor-specific DNA methylation in a mouse model of inflammatory bowel disease [121]. Investigations using an *in vitro* system further determined that oxidative stress in the form of H_2O_2 caused the recruitment of DNMT1 to regions of DNA damage, where it complexed with DNMT3B and PRC4 and relocalized to GC-rich regions of the genome, including CpG islands, resulting in alterations in histone marks, DNA methylation, and gene transcription [75].

A potential source of this DNA-damaging ROS is spermine oxidase (SMOX). SMOX catalyzes the oxidation of spermine, an important free radical scavenger, while generating hydrogen peroxide (H_2O_2) and 3-aminopropanal as potentially toxic byproducts. H_2O_2 is rapidly converted through Fenton catalysis into a highly reactive ROS, the hydroxyl radical, which is estimated to cause approximately 70% of all DNA damage. Importantly, SMOX is rapidly and highly induced by several sources of inflammatory stimuli, including infection with *H. pylori* and ETBF as well as pro-inflammatory cytokines, such as TNFα [40]. In fact, using the same mouse model of ETBF-induced colitis as that described above to correlate ROS-induced DNA damage with epigenetic gene silencing, the induction of SMOX was demonstrated to serve as a molecular link between an inflammatory stimulus and tumorigenesis [36].

Likewise, accumulating evidence demonstrates that the induction of SMOX and its generation of ROS play a causal role in the progression from *H. pylori*-associated gastritis to gastric carcinoma, as SMOX expression levels are greatest in more advanced premalignant lesions, and gastritis patient populations at increased risk of developing gastric cancer demonstrate elevated levels of SMOX expression in gastric mucosal biopsies [59, 60]. As the instances of SMOX activation appear to correlate with circumstances of increased DNA hypermethylation, we hypothesized that the chronic induction of SMOX in epithelial cells exposed to inflammatory stimuli is a source of the oxidative DNA damage that contributes to aberrant epigenetic silencing through the recruitment of repressive chromatin modification complexes and CpG island DNA methylation. With the ultimate goal of testing this hypothesis, we herein describe the development of an *in vitro* expression system to conditionally activate SMOX-dependent ROS production in gastric epithelial cells and determine its effect on epigenetic modifications over time. We characterize this system with regard to SMOX mRNA, protein, and activity induction as well as fluctuations in intracellular polyamine pools. Additionally, we confirm the activation of DNA damage response proteins over time and conduct preliminary studies using a candidate tumor suppressor gene, *O^6-methylguanine DNA methyltransferase (MGMT)*, which is known to become aberrantly methylated in association with chronic *H. pylori* infection [122].

Materials and Methods

Cell lines, culture conditions, and reagents

The AGS human gastric cancer cell line (CRL-1739, ATCC, Manassas, VA) was maintained in F12K medium containing 10% fetal bovine serum, penicillin and streptomycin at 37°C, 5% CO_2. Recombinant adenovirus expressing Cre recombinase (AdCre) was purchased from SignaGen Laboratories (Rockville, MD). Custom primers for cloning, genotyping, qRT-PCR, and sequencing were synthesized by Integrated DNA Technologies (Coralville, IA). Restriction and DNA modification enzymes for cloning were purchased from New England Biolabs (Billerica, MA).

Conditional SMOX overexpression system

The LoxP-flanked STOP cassette was excised from the pBS302 plasmid [123] using restriction digest with NotI and ligated into the same site of the pCR2.1 vector (Life Technologies, Grand Island, NY) containing the SMOX1 cDNA, which had been amplified by PCR and inserted using TA-cloning. The resulting region containing the lox-squared STOP cassette followed by SMOX1 was subsequently amplified by PCR and ligated into the XhoI site of the pCAGGS expression vector (BCCM/LMBP Plasmid Collection, Zwijnaarde, Belgium) to create pCAGGS-STOP-SMOX (Fig. 3.1).

AGS cells were seeded at 0.2×10^6 cells per well of a 6-well plate and allowed to become approximately 80% confluent. Cells were cotransfected using the Continuum transfection reagent (Gemini Bioproducts, West Sacramento, CA) with either the pCAGGS empty

vector or the pCAGGS-STOP-SMOX vector along with the pcDNA3.1+ empty vector (Life Technologies) to provide a marker for antibiotic selection. Cells were transfected for 24 hours, at which time each well was equally divided into 3 100-mm dishes in complete medium and allowed to recover for 24 hours. Medium was then replaced with that containing 0.5 mg/mL G418 and cells were maintained until visible single colonies had formed. Colonies were isolated with cloning rings and moved to 24-well plates, from which they were divided and reseeded for maintenance or harvested for genomic DNA isolation. DNA from each colony was screened by PCR using the following 3 primer sets: 5'-GCTCACCTCGACCATGGTAATAGCG-3' (sense) and 5'-CGAAAAGTGCCACCTGGGTCGAC-3' (antisense), corresponding to the enhancer/promoter region (set A); 5'-GCCATCTAGTGATGATGAGGCTACTGC-3' (sense) and 5'-CGACCTGCAGCCCAAGCTTACTTACC-3' (antisense), corresponding to the STOP cassette (set B); and 5'-GATCCCGGCGGACCATGTGATTGTG-3' (sense) and 5'-CCTGCATGGGCGCTGTCTTTG-3' (antisense), corresponding to the SMOX1 cDNA (set C). Thermocycling conditions consisted of 2 minutes at 95ºC followed by 35 cycles of 30 seconds at 95ºC, 1 minute at 59ºC, and 30 seconds at 72ºC. Amplification products were separated on 2% agarose gels and visualized using GelStar staining (Lonza, Walkersville, MD) and KODAK Digital Science Image Analysis Software (Rochester, NY). Those AGS colonies generating robust amplification products of the correct size from all 3 primer pairs were kept for subsequent experiments.

To induce SMOX expression in the pCAGGS-STOP-SMOX-positive AGS clones, cells were incubated with recombinant adenovirus expressing Cre recombinase, AdCre, at an

MOI of 100. AGS pCAGGS-STOP-SMOX cells or those containing the empty expression and selection vectors were seeded at 3×10^5 cells/well of a 6-well plate and allowed to attach overnight. Cells were then incubated with or without AdCre for 24 h, at which time the virus was removed and cells were transferred to 25-cm^2 flasks. Following incubation for an additional 24 hours (unless otherwise noted), the cells were harvested as appropriate for mRNA and/or protein analyses.

Gene expression studies

Following AdCre exposure, total RNA was extracted from AGS cells using TRIzol reagent (Life Technologies) according to the manufacturer's protocol and quantified by spectrophotometry. Complementary DNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD), and SYBR-green-mediated, real-time PCR was performed using primer pairs as previously reported for SMOX and GAPDH [29]. Primers used for MGMT qRT-PCR were 5'-AGGAGCAATGAGAGGCAATCC-3' (sense) and 5'-ACAGAAAGGGCAGACACGCT-3' (antisense). The optimum annealing temperature for each primer pair was determined on cDNA using temperature gradients followed by melt curve analyses and visualization on 2% agarose gels with GelStar staining (Lonza, Walkersville, MD) and KODAK Digital Science Image Analysis Software (Rochester, NY). Amplification conditions consisted of a 5-minute denaturation step at 95ºC, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60ºC for 30 s, and extension at 72°C for 30 s. Thermocycling was performed on a BioRad MyiQ2 real-time PCR detection system with data collection by the iQ5 optical system software (Hercules,

CA). Samples in each qPCR experiment were analyzed in triplicate with normalization to the *GAPDH* reference gene, and the fold-change in expression was determined relative to cDNA from uninfected cells using the $2^{-\Delta\Delta Ct}$ algorithm.

Protein expression analyses

The induction of SMOX protein occurring with AdCre co-incubation over time was detected by Western blots. Cells were exposed to AdCre and harvested at the indicated time points up to 24 h; at 24 h, virus was removed and cells were replated for further incubation in the absence of virus. Cell pellets were lysed with 4% SDS followed by clarification through a QIAshredder homogenizer (Qiagen, Valencia, CA) and quantification using the method of Bradford [97]. Total protein (30 μg per lane) was separated on pre-cast 10% Bis-Tris NuPAGE gels with 1 × MES running buffer (Invitrogen) and transferred onto Immun-Blot PVDF membranes (BioRad). Blots were blocked for 1 hour at room temperature in Odyssey blocking buffer (LI-COR, Lincoln, NE), followed by overnight incubation at 4ºC with a custom rabbit primary antibody specific to SMOX (1:1000 dilution) [29]. A mouse-derived β-actin antibody was included for normalization (Sigma, St. Louis, MO). Blots were then incubated with species-specific, fluorophore-conjugated secondary antibodies to allow the visualization and quantification of immunoreactive proteins using the Odyssey infrared detection system and software (LI-COR).

Western blot analysis of MGMT protein levels was performed in parallel using an antibody against MGMT (Epitomics, Burlingame, CA). The presence of the DNA

damage response was verified using an antibody targeting the phosphorylated Ser/Thr substrates of ataxia telangiectasia mutated kinase (ATM) and ataxia telangiectasia and Rad3-related kinase (ATR) (Cell Signaling Technology, Danvers, MA) at a dilution of 1:1000.

Determination of inducible SMOX activity and intracellular polyamine concentrations

AGS cells infected with the Cre-recombinase-expressing adenovirus were collected and quick-frozen in 0.083 M glycine buffer for the analysis of spermine oxidase activity and the resulting changes in intracellular polyamine pools. SMOX activity was measured using a luminol-based assay that measures the production of hydrogen peroxide in the presence of saturating spermine concentrations, as previously described [96]. Intracellular polyamine concentrations were analyzed from the same sample using pre-column dansylation followed by reverse-phase, high-pressure liquid chromatography, as previously described [124]. Both assays were normalized relative to milligrams of total cellular protein determined using the method of Bradford [97], with interpolation on a bovine serum albumin standard curve.

Results

Conditional overexpression of spermine oxidase in AGS cells

A conditional overexpression model of SMOX induction (Fig. 3.1) was created in gastric epithelial cells with the goal of mimicking the activation of SMOX that occurs with *H. pylori*-associated chronic infection and inflammation. Insertion of the Cre-inducible pCAGGS-STOP-SMOX expression vector was verified in multiple stable subclones of the AGS gastric adenocarcinoma cell line, and following incubation with a recombinant adenovirus expressing Cre recombinase, an abundant increase in spermine oxidase protein was observed (Fig. 3.2A). The detection of SMOX protein via Western blotting was nearly undetectable in AGS cells that were not exposed to AdCre as well as in the empty vector-containing control cells. Subsequent analyses were conducted with multiple subclones with similar results; therefore, a single representative subclone (SMOX #5) was chosen to depict SMOX overexpression in subsequent analyses.

The Cre-mediated induction of SMOX was further investigated at the level of transcription. As shown in Fig. 3.2B, the basal level of SMOX mRNA expression was similar between the control (V) and pCAGG-STOP-SMOX cells. However, 48 h following AdCre exposure, SMOX transcript levels in the cells containing the LoxP-flanked STOP sequence were elevated approximately 40-fold, while those of the control cells remain unchanged.

SMOX exerts its DNA damaging effects principally through the generation of H_2O_2. To confirm that the SMOX protein induced in AGS cells following Cre-mediated recombination was functional, the generation of H_2O_2 was measured using a luminol-based reaction specific for the oxidation of spermine (Fig. 3.2C). In correlation with the mRNA levels, SMOX activity was significantly induced only in the pCAGGS-STOP-SMOX cells exposed to AdCre.

Alterations in intracellular spermine and spermidine pools following SMOX overexpression

As a polyamine catabolic enzyme, SMOX directly back-converts spermine, an important free radical scavenger, to its precursor spermidine. Aliquots of the cell lysates used for SMOX activity assays were acid extracted, labeled with dansyl chloride, and analyzed by HPLC. The results further demonstrated the functional activity of the Cre-induced SMOX: the pCAGGS-STOP-SMOX AGS cells exposed to AdCre displayed a reduced intracellular concentration of spermine, the substrate of SMOX, while spermidine pools were significantly elevated (Fig. 3.3). Notably, this decrease in spermine indicates a reduction in antioxidant pools, thereby exacerbating the potential for oxidative DNA damage resulting from SMOX-generated H_2O_2. The pCAGGS-STOP-SMOX cells and empty vector control cells possessed similar basal levels of polyamines in the absence of Cre induction, and levels were not significantly altered when vector control cells were incubated with AdCre.

SMOX overexpression stimulates increased expression of DNA damage response proteins

It has been well established in multiple systems, including AGS cells, that elevated levels of SMOX activity induces DNA damage [35, 36, 40, 58, 59, 125]. To clarify the time frame required for induction of SMOX and subsequent players in response to DNA damage, Western blot assays were performed using cell lysates collected following various incubation times with AdCre. As indicated in Fig. 3.4 (top), a detectable increase in SMOX protein begins in the pCAGGS-STOP-SMOX AGS cells after approximately 14 hours of exposure to AdCre and continues to increase through 48 hours (note that virus-containing medium was removed at 24 h, at which time cells were collected and replated for further growth). The blots were subsequently probed with an antibody that targets the phosphorylated Ser/Thr substrates of ataxia telangiectasia mutated kinase (ATM) and ataxia telangiectasia and Rad3-related kinase (ATR), kinases that regulate cell cycle checkpoints and DNA repair and are activated by ROS-induced DNA damage in gastric epithelial cells infected with *H. pylori* [126]. Basal levels of several of the proteins were quite high in AGS cells; however, an increase could be observed in the bands with lower background levels, and this increase appeared to correlate with the induction of SMOX (Fig. 3.4 bottom).

SMOX overexpression down-regulates the expression of the MGMT tumor suppressor gene

The induction of DNA methylation in specific gene promoter regions of gastric epithelial cells is enhanced in response to chronic *H. pylori* infection. Many genes have now been identified as aberrantly silenced as a result of CpG island hypermethylation in gastric cancers. Of these, methylation of the *O^6-methylguanine DNA methyltransferase* (*MGMT*)

gene has been observed in association with *H. pylori* infection and gastritis [122], and *MGMT* is one of the few genes that remains unmethylated and expressed in the AGS adenocarcinoma cell line used in the present studies. *MGMT* was therefore chosen as a candidate gene to investigate the potential that SMOX overexpression could contribute to epigenetic-mediated silencing of a tumor suppressor gene.

As shown in Fig. 3.5A, qRT-PCR analysis reveals a modest decrease in MGMT mRNA expression that is specific to the pCAGGS-STOP-SMOX cells following 24 hours of induction with Cre recombinase. Preliminary Western blot data also demonstrate a modest decrease in MGMT protein that correlates with the changes in mRNA (Fig. 3.5B). The modest decrease observed in these preliminary studies is promising, as it is as might be expected to result from repressive chromatin changes during this short time frame. Therefore, the repression of *MGMT* gene expression in response to SMOX overexpression indicates the promising potential of this model and warrants further investigation in terms of time course studies and alterations in chromatin architecture and DNA methylation levels in the *MGMT* promoter region.

Discussion and Future Directions

The current study described the development of an *in vitro* conditional SMOX overexpression system designed with the intent of interrogating the effects of SMOX activation on epigenetic gene silencing in the etiology of chronic inflammation/infection-associated carcinogenesis. With a focus on *H. pylori*-associated gastric carcinogenesis, we created a stable subclone of the AGS human gastric adenocarcinoma cell line that expresses a Cre-recombinase-inducible SMOX gene. Upon co-incubation with Cre-expressing adenovirus, these cells expressed significantly elevated levels of SMOX mRNA and functional protein (approximately 40-fold) that effectively altered the distribution of intracellular polyamine pools: specifically, levels of the free radical scavenger spermine were significantly reduced, while spermidine pools were elevated. Combined with the diminished availability of spermine as an antioxidant, the increased intracellular concentrations of the ROS precursor H_2O_2 that were generated through spermine oxidation correlated with the activation of DNA damage response pathways that included both ATM and ATR, and these responses increased over time.

The mechanisms responsible for repairing oxidative DNA damage, chiefly the base-excision repair pathway (BER)[120], require the recruitment of chromatin-modification enzymes to regions of DNA damage. These enzymes, which include DNMTs, HDACs, and HATs, are many of the same players involved in aberrant epigenetic gene silencing, and recruitment of repressor complexes containing these modifiers to regions of oxidative DNA damage have been reported to result in repressive chromatin changes, aberrant CpG

island methylation, and tumor suppressor gene silencing [75, 127]. As we hypothesize that the generation of DNA-damaging H_2O_2 resulting from the persistent activation of SMOX in circumstances of chronic inflammatory stimuli is a mechanism contributing to these cancer-related epigenetic alterations, the model described above will allow us to precisely provide proof of principle of our hypothesis.

A limitation to the model described in the current study is the fact that the AGS parent cell line in which the system is established is a tumor cell line that has already undergone a multitude of both genetic and epigenetic changes, including a high degree of aberrant DNA methylation. For this reason, we chose a candidate gene approach using the *MGMT* tumor suppressor gene to investigate the effects of SMOX activation on epigenetic changes. The *MGMT* gene, which is itself a DNA-repair gene, is frequently methylated in gastric cancers, and methylation levels are significantly increased in gastric biopsies of patients with *H. pylori*-associated gastritis, compared with *H. pylori*-negative samples [122], indicating that methylation of its promoter is an early event occurring prior to the onset of gastric carcinogenesis. Furthermore, in these same studies, *H. pylori* infection of AGS cells *in vitro* resulted in decreased MGMT mRNA and protein levels that corresponded to increased CpG island methylation. These data supported the use of the *MGMT* gene as an indicator of epigenetic changes resulting from SMOX overexpression, and preliminary studies examining MGMT mRNA and protein levels in our AGS model system are promising. Additional studies are in progress to determine the exact epigenetic changes underlying this decreased expression. Specifically, time-course studies are being conducted to observe the changes in MGMT expression over time following the

induction of SMOX. Chromatin immunoprecipitation (ChIP) experiments are also being conducted using antibodies specific to certain chromatin-modifying enzymes, including DNMT1 and SIRT1 (a member of PRC4), as well as specific histone modifications that are indicative of either active or repressed chromatin (i.e., H3K27me3). Finally, methylation-specific PCR (MSP) studies have been initiated to validate whether the induction of SMOX is capable of invoking the epigenetic changes required to allow for CpG island hypermethylation and aberrant gene silencing. Assuming that the preliminary decreases observed in MGMT expression are due to the hypothesized mechanism, our SMOX-overexpression system also has the potential to provide deeper insight into the mechanisms involved in converting the intended repair of oxidative DNA damage into cancer-related epigenetic changes. Furthermore, a comparison of changes observed in our conditional SMOX overexpression system versus those that occur in our established *in vitro* model of *H. pylori* infection in AGS cells will allow the segregation of changes that result specifically from SMOX induction from those occurring from other aspects of infection.

The utility of the AGS cell model for investigating genome-wide changes in response to SMOX activation simulating that of chronic inflammation/infection is limited by its tumorigenic nature. The same Cre-inducible SMOX overexpression system has therefore been inserted into an immortalized stomach (ImSt) epithelium cell line and is currently being characterized. The ImSt cell line is conditionally immortal and was derived from the immortomouse, a transgenic mouse strain that carries a temperature-sensitive mutant of the SV40 large T gene [128]. Although unable to achieve the extremely high levels of

SMOX induction detected in AGS cells (~40-fold), the approximately 4-fold inductions that occur in the ImSt system are likely to be physiologically relevant in terms of what is observed with *H. pylori* infection in these cells. Once it is confirmed that the induction of SMOX that occurs following Cre-mediated recombination in ImSt cell system is sufficient to invoke oxidative DNA damage to an extent similar to that in response to *H. pylori* infection, we will be able to examine the resulting epigenetic changes over time on a genome-wide scale. In particular, ChIP-sequencing will be performed using the essential epigenetic modification enzymes (DNMT1 and SIRT1) as well as specific histone marks to determine general trends with regard to oxidative stress-mediated recruitment and relocalization of these repressor complexes. This system will also allow the detection of changes in DNA methylation levels on a genome-wide scale over time, thereby providing insight into which genes are most susceptible to silencing early in the course of inflammation-associated tumorigenesis and potentially providing new targets for intervention. Again, results from these experiments could be compared to those using *in vitro* infection of ImSt cells with *H. pylori* to identify SMOX-specific alterations.

As we described in Chapter 2, certain microRNAs (miRNAs) are expressed in "normal" cells that are inactivated in tumor cells such as AGS. As we have now identified that the SMOX transcript is a target for at least 1 of these miRNAs, miR-124, it could be that we are observing miRNA-mediated inhibition of high levels of SMOX induction in normal epithelial lines (similar results have been obtained using Beas2B and HBEC bronchial epithelial cell lines). Although the precise target region of the SMOX 3'-UTR that we studied with regard to miR-124 is not included in the pCAGGS-STOP-SMOX expression

vector, further bioinformatic analysis has revealed multiple less-conserved but potential binding sites for miR-124 in the included SMOX 3'-UTR. Using specific miRNA inhibitors might therefore enhance the inducibility of this system in ImSt cells.

The use of a Cre-inducible overexpression system allows the opportunity for tissue-specific SMOX induction in animal models. In fact, we previously established a pCAGGS-STOP-SMOX transgenic mouse line using the same expression system described herein. Crossing members of this line with a mouse expressing Cre recombinase under the control of a tissue-specific promoter results in progeny that overexpress SMOX specifically in that tissue, thereby providing a means to analyze the effects of persistent SMOX induction over time *in vivo*. These tissue-specific SMOX overexpression animal models will be valuable tools for interrogating the *in vivo* association between SMOX activation and epigenetic gene silencing in the etiology of cancer.

Overall, the AGS conditional SMOX overexpression system presented in the current study will provide important proof of principle whether SMOX overexpression can invoke epigenetic chromatin changes and DNA hypermethylation such as that associated with cancer. Although focusing on *H. pylori*-associated gastric cancer, it is likely that the correlations observed will translate to other chronic-inflammation-associated cancers, such as colitis-associated cancer and prostate cancer, thereby validating the induction of SMOX by inflammatory stimuli as a fundamental mechanism in the precancerous accumulation of repressive cancer-related epigenetic modifications.

Tables and Figures

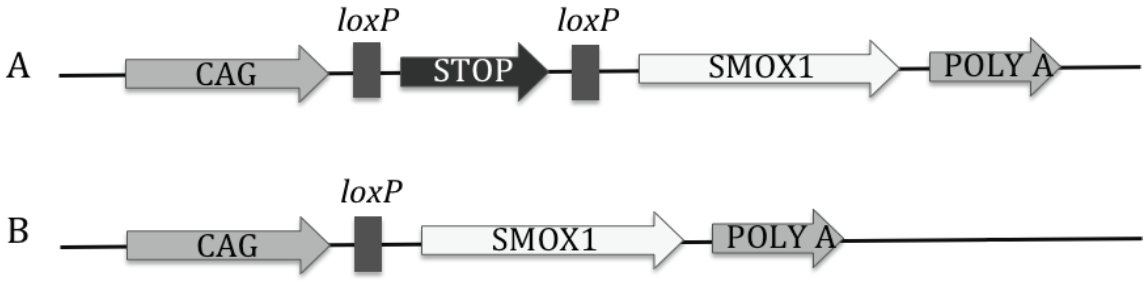

Figure 3.1. Schematic diagram of the conditional SMOX overexpression system. AGS human adenocarcinoma cells were stably transfected with the pCAGGS expression plasmid containing a LoxP-flanked stop sequence preceding the SMOX1 cDNA (shown in **A**) (CAG indicates the promoter/enhancer region). Following antibiotic selection, single colonies were isolated and screened for plasmid insertion by PCR on genomic DNA. Positive clones were then infected with Cre recombinase-expressing adenovirus, resulting in recombination, excision of the "STOP" sequence, and expression of SMOX1 (depicted in **B**).

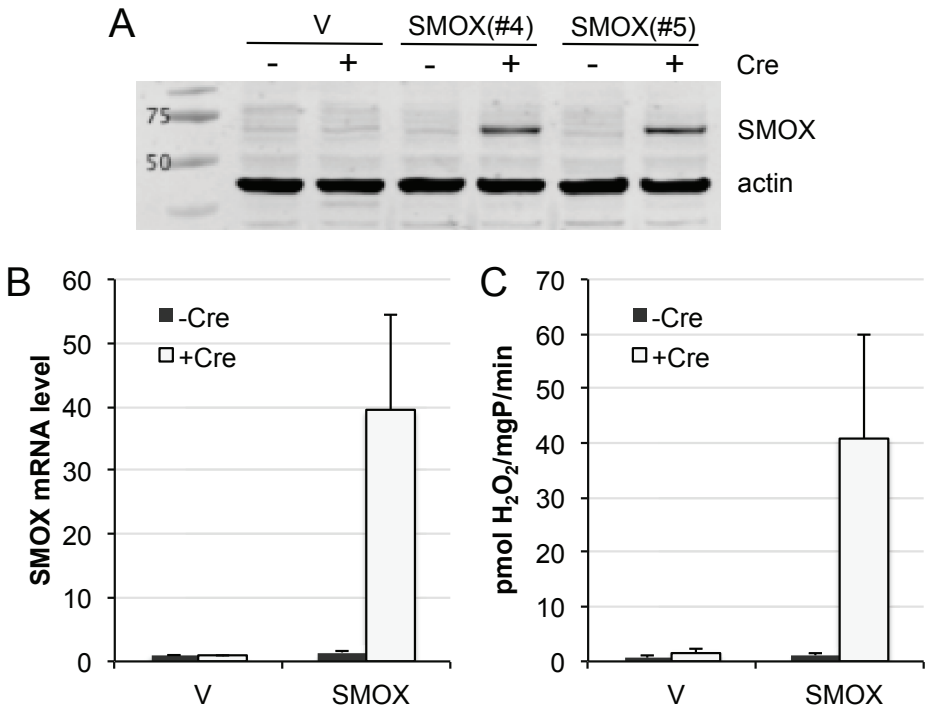

Figure 3.2. Adenoviral Cre-recombinase-driven conditional SMOX overexpression in AGS cells. In **A**, a representative Western blot displays the induction of SMOX protein in 2 individual AGS cell subclones containing the pCAGGS-STOP-SMOX expression plasmid following infection with Cre-recombinase-expressing adenovirus. Cells were infected for 24 h and harvested 48 h later for total cellular protein. **B.** qPCR results quantifying SMOX mRNA expression, relative to GapDH, following incubation with or without AdCre. **C.** SMOX activity following AdCre co-incubation as measured by the production of H_2O_2. In panels **B** and **C,** empty vector control cells (V) or pCAGGS-STOP-SMOX clone #5 (SMOX) were exposed to AdCre for 24 h followed by a 24-h recovery period. Histograms represent the means of at least 3 independent experiments, each performed in triplicate. Error bars indicate S.E.M.; (*) implies $p<0.05$ as determined by Student's t-test.

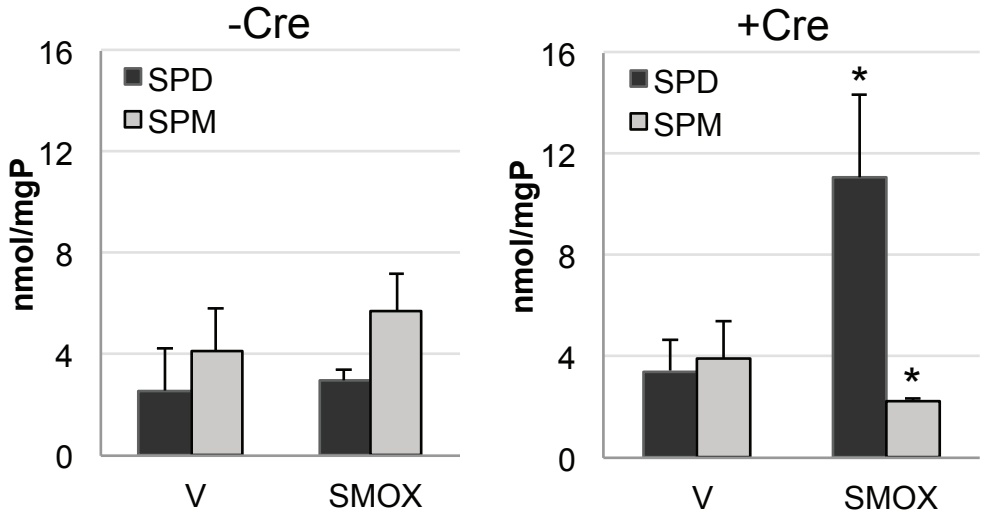

Figure 3.3. Intracellular polyamine alterations following Cre-mediated SMOX induction. Reflecting the induction of SMOX activity in the pCAGGS-STOP-SMOX-expressing AGS cells, intracellular spermine (SPM) levels are significantly decreased while spermidine (SPD) levels are increased following exposure to AdCre (right panel). Lysates from the same 3 independent experiments used in Fig. 12C were each measured in duplicate. (*) indicates a statistically significant change ($p<0.05$) in polyamine level following Cre exposure, relative to cells not exposed to AdCre.

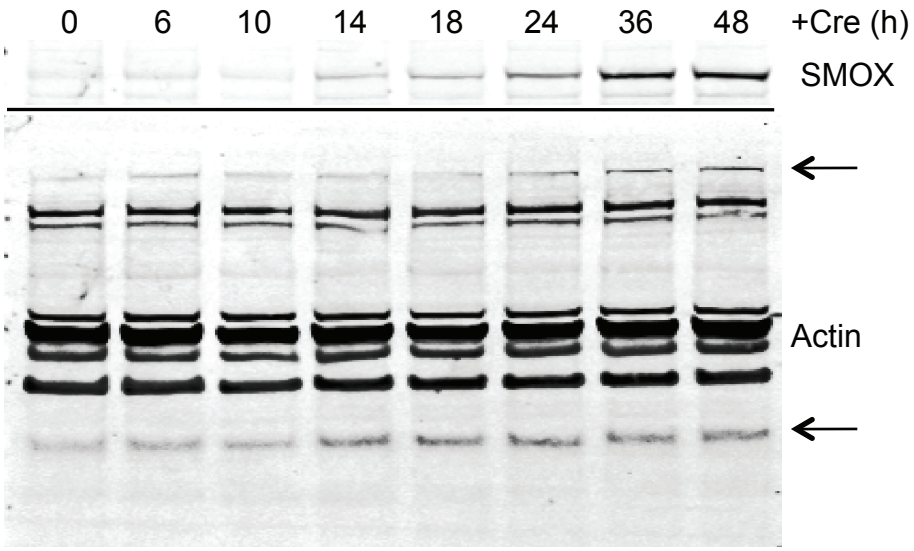

Figure 3.4. Induction of SMOX correlates with DNA damage response activation.

Western blot of pCAGGS-STOP-SMOX-containing AGS cells demonstrating the induction of SMOX protein over time following the addition of AdCre (top row). In the bottom panel, the same blot was probed with an antibody specific to phosphorylated Ser/Thr substrates of the DNA damage response kinases ATM and ATR. Arrows indicate increases in these substrates in a manner consistent with SMOX activation, suggesting activation of these pathways as a result of oxidative DNA damage. Actin was used as a loading control.

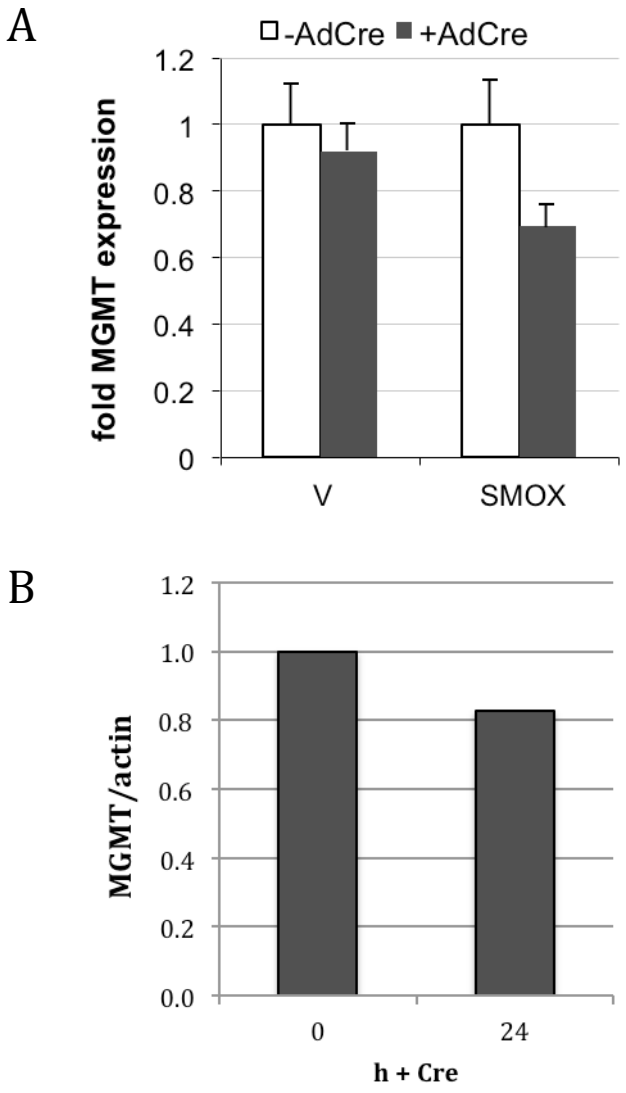

Figure 3.5. SMOX overexpression represses expression of the MGMT tumor suppressor gene. Panel **A** indicates the reduction in MGMT mRNA level in AGS cells following the Cre-mediated induction of SMOX overexpression, as determined by qRT-PCR relative to GAPDH. Panel **B** depicts the decrease in MGMT protein following 24 h of SMOX induction. Data was acquired from a representative quantitative Western blot.

Chapter 4:

Overall Discussion, Future Directions, and Conclusions

Overall Discussion and Future Directions

The studies described in this dissertation support two main hypotheses: (1) miR-124 is a negative regulator of SMOX that can be repressed by CpG island hypermethylation, thereby allowing persistent SMOX activation in response to inflammatory stimuli and creating a predisposition to carcinogenesis; and (2) chronically elevated SMOX activity can potentially recruit epigenetic repressor complexes that contribute to cancer-related aberrant DNA methylation and gene silencing.

The results presented in Chapter 2 provide convincing evidence that miR-124 directly regulates SMOX and can therefore provide protection against the high levels of SMOX activity and consequent ROS generation that are induced in response to chronic inflammatory stimuli, such as that associated with *H. pylori* infection. Preliminary results suggest that this protection is abrogated in gastritis patients with aberrant DNA methylation of the *hsa-miR-124-1*, *-2*, and *-3* gene promoters, predisposing them to SMOX-mediated DNA damage and subsequent carcinogenesis. Hypermethylation of the *miR-124* gene loci might therefore potentially serve as a risk marker for the development of gastric cancer and provide a means for patient stratification with regard to *H. pylori* eradication and/or chemopreventive intervention. The initial methylation studies presented here were obtained from only a small sampling of representative patient

biopsies from a much larger cohort of low- versus high-risk patients. Pyrosequencing assays of the entire cohort are currently underway and are anticipated to further increase the significance of our findings. Additionally, previous data measuring SMOX protein and the DNA adduct 8-oxo-dG in these same samples will allow us to further validate this correlation.

In addition to epigenetically mediated silencing of miR-124, studies in other systems have identified a genetic single nucleotide polymorphism (SNP), rs531564/pri-miR-124-1, which is involved in tumorigenesis. This G/C alteration occurs in the pri-miR-124 molecule and alters its predicted secondary structure, thereby having the potential to alter downstream processing into the mature miR-124 [129]. These initial studies in neuronal tissue suggested that this SNP increases the abundance of mature miR-124, and subsequent studies have identified associations between rs531564 and the risk for gastric [130], bladder [131], and esophageal cancers [132]. Specifically, protective roles for this SNP were reported in esophageal squamous cell carcinoma and cervical cancer [133, 134]. As this SNP has been reported to exist in gastric tissue, it will be interesting to investigate its role in the regulation of SMOX in the etiology of *H. pylori*-associated gastric carcinoma.

DNA methylation-mediated gene silencing of miR-124 has been detected in many other cancer types in addition to gastric cancer, suggesting the potential for elevated SMOX activity in the etiologies of these cancers as well. In fact, dysregulated polyamine homeostasis is a well-studied characteristic of tumor cells [135] in which miR-124 could

play a role. Moreover, miR-124 expression is decreased in premalignant lesions of other chronic inflammation/infection-associated cancers, including those of the colon and cervix [77, 80]. Future experiments further investigating the role of SMOX in these systems are warranted with regard to the elucidation of mechanisms contributing to the development of cancer. Furthermore, SMOX induction has also been implicated in other pathophysiological etiologies, including ischemia/reperfusion injury of the liver, kidney, and brain, as well as alcohol-induced liver damage (reviewed in [19]).

The SMOX-mediated ROS production in response to inflammatory stimuli is a major factor believed to contribute to carcinogenesis; however, the precise mechanisms involved are unclear. As inducers of oxidative DNA damage, ROS facilitate both genetic and epigenetic changes in the genome. In Chapter 3, we described the generation of a conditional SMOX-overexpression system with which to provide proof of principle that SMOX as a source of ROS can induce the repressive epigenetic changes, including DNA methylation, associated with chronic inflammation/infection-associated carcinogenesis. As described in detail in the Discussion and Future Directions section of Chapter 3, the expression system described is incorporated into the AGS gastric adenocarcinoma cell line and studies are thus limited to using a candidate gene approach to study epigenetic changes because of its tumorigenic nature. The conditional SMOX-overexpression system has therefore also been inserted into immortalized mouse stomach epithelial cells. Further experiments using this system of non-tumorigenic cells will allow the use of genome-wide approaches to examine the epigenetic gene expression changes resulting from SMOX induction as they occur over time. Finally, the use of this system in animal

models will enable tissue-specific SMOX induction in a more physiologically relevant microenvironment.

Combining the two main ideas of the current studies, it can be speculated that the regulatory mechanisms at play might feed forward to one another, thereby exacerbating the potential for epigenetically mediated carcinogenesis. Specifically, the aberrant epigenetic repression of miR-124 during chronic inflammation (through a mechanism that may or may not be SMOX-related) allows the translation of active SMOX protein that has been induced by inflammatory stimuli at the transcriptional level; this SMOX induction increases oxidative DNA damage, epigenetic repressor complex recruitment and aberrant epigenetic gene silencing including DNA CpG island hypermethylation at additional CpG-island-containing, cancer-related gene promoters; methylation at the miR-124 promoter may also be increased, thereby allowing even greater SMOX induction, and so on. In considering this potential feed-forward loop, the fact that the histone methyltransferase EZH2 has also been identified as a target of miR-124 in gastric cancer cells should not be ignored, as it is responsible for catalyzing the formation of H3K27me3, a repressive histone mark that precedes the establishment of DNA methylation [105]. Therefore, in cells with inactive miR-124, not only would activated SMOX create DNA damage that likely recruits epigenetic silencing machinery (including EZH2), but also the levels of EZH2 might be elevated.

In light of the accumulating evidence supporting a role for polyamine oxidation in the etiology of chronic inflammation/infection-associated cancer initiation, attention must be

given to attenuating this response as a means for chemoprevention. SMOX activity exists in the cell nucleus, and pharmacologically inhibiting SMOX induction in the presence of cancer-predisposing factors such as *H.* pylori infection provides protection from the oxidative DNA damage that leads to carcinogenesis [35, 58, 60, 136]. This inhibition of polyamine catabolism prevents the generation of ROS while maintaining the intracellular spermine pools capable of free radical scavenging. Although effective inhibitors for SMOX exist, including MDL72,527, they are non-specific and also inhibit APAO. The identification of specific inhibitors of the individual polyamine oxidases will provide both experimental and therapeutic benefit.

Recent studies using a Mongolian gerbil model of *H. pylori*-induced gastritis demonstrated increased antitumor efficacy when combining the SMOX inhibitor MDL72,527 with α-difluoromethylornithine (DFMO), an inhibitor of the polyamine biosynthetic enzyme ornithine decarboxylase (ODC) [60]. ODC is upregulated in response to *H. pylori* infection as well as in gastritis tissue samples [137], thereby increasing the overall concentrations of polyamines in the cell and providing additional substrate for SMOX-mediated catabolism. DFMO on its own has shown chemopreventive potential through inhibiting the colonization and growth of *H. pylori* in mice, resulting in decreased gastritis severity [137, 138].

The current data indicating the inverse relationship between miR124 and SMOX suggest the potential for targeting the miRNA as a means of chemopreventive intervention. As epigenetic changes are reversible, the fact that miR-124 is frequently silenced by DNA

hypermethylation suggests the use of DNMT inhibitors, such as 5-azacytidine (decitabine). Additionally, the potential exists for using modified miRNA molecules as anticancer agents. In the absence of miR-124 expression, other epigenetic inhibitors, such as those specific to EZH2, might also have utility in the prevention of SMOX-induced epigenetic changes.

Conclusions

Overall, the data presented here provide further valuable insight into the molecular pathogenesis of chronic inflammation/infection-associated cancer. Our identification of miR-124 as a direct negative regulator of SMOX expression provides fundamental knowledge of the regulation of polyamine homeostasis that will likely have significant implications in multiple fields of study, in addition to carcinogenesis. The protective role of miR-124 expression in preventing *H. pylori*-induced SMOX activity suggests the use of miR-124 expression or methylation levels as an indicator of gastric cancer risk that could provide a means for patients stratification in terms of bacteria eradication and/or chemopreventive measures. Lastly, preliminary studies in a conditional SMOX-overexpression model support the possibility that SMOX is a mediator of aberrant epigenetic changes contributing to gastric carcinogenesis.

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Tracy Murray-Stewart **March 23, 2015**

Curriculum Vitae

DEMOGRAPHIC INFORMATION

Current Appointments

Ph.D. Candidate, Johns Hopkins University School of Medicine Graduate Program in Cellular and Molecular Medicine, Baltimore, MD

Faculty Research Associate, Division of Cancer Biology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD

Senior Editor, American Journal Experts, Raleigh-Durham, NC

Personal Data

88 Hoyle Lane, Severna Park, MD 21146
(410) 544-2290 (home); (410) 733-6490 (mobile)
e-mail: tmurray2@jhmi.edu

Education and Training

Master of Arts, 2002, Johns Hopkins University School of Medicine, Dept. of Cellular and Molecular Medicine

Bachelor of Science, Magna Cum Laude, 1995, Salisbury State University, Biology

Professional Experience

1996, Quality Control Technician, Chesapeake Biological Laboratories

1996-1998, Senior Laboratory Technician, Division of Cancer Biology, Johns Hopkins University School of Medicine

1998-1999, Research Technician I, Division of Cancer Biology, Johns Hopkins University School of Medicine

1999-2000, 2002-2006, Research Technician II, Division of Cancer Biology, Johns Hopkins University School of Medicine

2006-2008, Research Specialist, Division of Cancer Biology, Johns Hopkins University School of Medicine

2009-present, Faculty Research Associate, Division of Cancer Biology, Johns Hopkins University School of Medicine

2012-2014, Junior Contact Editor, American Journal Experts

RESEARCH ACTIVITIES

Publications

Peer-reviewed original research articles:

1. Mank-Seymour, A, Murray, T, Berkey, K, Xiao, L, Kern, S, Casero, RA, Jr. Two active copies of the X-linked gene spermidine/spermine N1-acetyltransferase (SSAT) in a female lung cancer cell line are associated with an increase in sensitivity to an antitumor polyamine analogue. *Clin. Cancer Res.*1998; 4(8): 2003-2008.

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Review articles:

1. Casero, RA, Jr, Wang, Y, Stewart, T, Devereux, W, Hacker, A, Wang, Y, Smith, R, Woster, P. The role of polyamine catabolism in anti-tumour drug response. *Biochem. Soc. Trans.* 2003; 31(2): 361-365.

2. Casero, RA, Jr, Frydman, B, Stewart, T, Woster, P. Significance of targeting polyamine metabolism as an antineoplastic strategy: unique targets for polyamine analogues. *Proc. West. Pharmacol. Soc.* 2005; 48: 24-30.

3. Babbar, N, Murray-Stewart, T, Casero, RA, Jr. Inflammation and polyamine catabolism: the good, the bad, and the ugly. *Biochem. Soc. Trans.* 2007; 35(2): 300-304.

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Book chapters:

1. Casero, RA, Fraser, AV, Murray-Stewart, T, Hacker, A, Babbar, N, Fleischer, J, and Wang, Y. Recent advances in the understanding of mammalian polyamine catabolism: the regulation and potential role of polyamine catabolism in drug response and disease processes. In: *Polyamine Cell Signaling-Physiology, Pharmacology, and Cancer Research*. R.A. Casero and Y-J Wang, Editors. Totowa, NJ: Humana Press, Inc.; 2006: 205-231.

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2. Murray-Stewart, T, Applegren-Darling, N, Devereux, W, Casero, RA, Jr. Spermidine/spermine N^1-acetyltransferase activity in human small cell lung carcinoma following transfection with a genomic SSAT construct. *Proc. Amer. Assoc. Cancer Res.* 2001; 42: Abstract 2666.

3. Devereux, W, Stewart, TM, Casero, RA, Jr. The characterization of the interaction between human polyamine-modulated factor (PMF-1) and Nrf-2 in the transcriptional regulation of the spermidine/spermine N1-acetyltransferase (SSAT) gene. *Proc. Amer. Assoc. Cancer Res.* 2001; 42: Abstract 4817.

4. Wang, Y, Devereux, W, Stewart, TM, Casero, RA, Jr. Identification of human COP9 7a, a subunit of the COP9 signalsome that can bind to polyamine modulated factor-1 (PMF-1) and Nrf-2 transcriptional factor. *Proc. Amer. Assoc. Cancer Res.* 2001; 42: Abstract 4835.

5. Murray-Stewart, T, Wang, Y, Devereux, W, Casero, RA, Jr. Cloning and characterization of human polyamine oxidase splice variants. *Proc. Amer. Assoc. Cancer Res.* 2002; 43: Abstract 5552.

6. Devereux, W, Wang, Y, Stewart, TM, Hacker, A, Casero, RA, Jr. Differential induction of polyamine oxidase by polyamine analogues in various human lung carcinoma lines. *Proc. Amer. Assoc. Cancer Res.* 2002; 43: Abstract 4773.

7. Devereux, W, Wang, Y, Stewart, TM, Hacker, A, Smith, R, Ward, TD, Woster, PM, Casero, RA, Jr. Induction of polyamine oxidase by polyamine analogues in multiple human tumor lines. *Proc. Amer. Assoc. Cancer Res.* 2003; 44: Abstract 3634.

8. Wang, Y, Stewart, TM, Devereux, W, Hacker, A, Casero, RA, Jr. Biochemical properties of recombinant human polyamine oxidase. *Proc. Amer. Assoc. Cancer Res.* 2003; 44: Abstract 5477.

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12. Wang, Y, Hacker, A, Stewart, TM, Woster, PM, Casero, RA, Jr. Overexpression of N1-acetylpolyamine oxidase (PAO) in human lung cancer cells alters cellular response to specific antitumor polyamine analogues: Mechanism for drug resistance. *Proc. Amer. Assoc. Cancer Res.* 2005; 46: Abstract 5084.

13. Verghese, S, Hacker, A, Murray-Stewart, T, Casero, RA, Jr. Woster, PM. Polyaminohydroxamic acids and polyaminobenzamides as inhibitors of histone deacetylases. The 39th Mid-Atlantic Graduate Student Symposium (MAGSS), June 18-20, 2006, Columbus, OH.

14. Verghese, S, Hacker, A, Murray-Stewart, T, Casero, RA, Woster, PM. Polyaminohydroxamic acids and polyaminobenzamides that selectively inhibit histone deacetylase 6. The 232nd National Meeting of the American Chemical Society. September 10-14, 2006, in San Francisco, CA.

15. Huang, Y, Greene, E, Stewart, TM, Goodwin, AC, Baylin, SB, Woster, PM, Casero, RA, Jr. Re-expression of aberrantly silenced genes resulting from inhibition of lysine-specific demethylase 1 (LSD1) by polyamine analogs in human colon cancer cells. The 235th National Meeting of the American Chemical Society. April 6-10, 2008, in New Orleans, LA.

16. Murray-Stewart, T, Huang, Y, Woster, PM, Casero, RA, Jr. Polyamine analogue inhibition of lysine-specific demethylase 1 in human acute myeloid leukemia cell lines. *Proc. Amer. Assoc. Cancer Res.* 2008; 49: Abstract 2605.

17. Hazeldine, S, Crowley, M, Huang, Y, Murray-Stewart, T, Casero, RA, Woster, PM. Alkylamino- and aklylguanidinolysine analog inhibitors of lysine-specific demethylase 1. The 236th National Meeting of the American Chemical Society. August 17-21, 2008, in Philadelphia, PA.

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20. Murray-Stewart, T, Marton, LJ, Belinsky, SA, Casero, RA, Jr. Combination of a conformationally restricted polyamine analogue with DNA methyltransferase or histone deacetylase inhibition induces synergistic re-expression of aberrantly silenced tumor suppressor genes. *Proc. Amer. Assoc. Cancer Res.* 2010; 51: Abstract LB-97.

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22. Murray-Stewart, T, Sierra, JC, Chaturvedi, R, Wilson, KT, Casero, RA, Jr. Expression of miR-124 suppresses spermine oxidase-associated H_2O_2 generation in human gastric adenocarcinoma cells: Implications for infection/inflammation-induced carcinogenesis. *Proc. Amer. Assoc. Cancer Res.* 2015; 56: Abstract 201.

ORGANIZATIONAL ACTIVITIES

Institutional Administrative Appointments

Committee for Oncology Research Technicians (CORT), 2007-2008
CORT Newsletter Subcommittee and author of Cancer News column
CORT Website Subcommittee

Professional Societies

American Association for Cancer Research, Active Member, 2009-present
American Association for Cancer Research, Associate Member, 2000-2009

RECOGNITION

Awards, Honors

Double Helix Award, 2007, Oncology Research Technicians Poster Event
Sponsor: Dr. Robert A. Casero, Jr.