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The *HMGA1-COX-2* axis: A key molecular pathway and potential target in pancreatic adenocarcinoma

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Abstract

Context—Although pancreatic cancer is a common, highly lethal malignancy, the molecular events that enable precursor lesions to become invasive carcinoma remain unclear. We previously reported that the high-mobility group A1 (HMGA1) protein is overexpressed in >90% of primary pancreatic cancers, with absent or low levels in early precursor lesions.

Methods—Here, we investigate the role of HMGA1 in reprogramming pancreatic epithelium into invasive cancer cells. We assessed oncogenic properties induced by HMGA1 in non-transformed pancreatic epithelial cells expressing activated *K-RAS*. We also explored the HMGA1-cyclooxygenase (COX-2) pathway in human pancreatic cancer cells and the therapeutic effects of COX-2 inhibitors in xenograft tumorigenesis.

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Results—*HMGA1* cooperates with activated *K-RAS* to induce migration, invasion, and anchorage-independent cell growth in a cell line derived from normal human pancreatic epithelium. Moreover, *HMGA1* and *COX-2* expression are positively correlated in pancreatic cancer cell lines ($r^2=0.93$; $p<0.001$). *HMGA1* binds directly to the *COX-2* promoter at an AT-rich region *in vivo* in three pancreatic cancer cell lines. In addition, *HMGA1* induces *COX-2* expression in pancreatic epithelial cells, while knock-down of *HMGA1* results in repression of *COX-2* in pancreatic cancer cells. Strikingly, we also discovered that Sulindac (a COX-1/COX-2 inhibitor) or Celecoxib (a more specific COX-2 inhibitor) block xenograft tumorigenesis from pancreatic cancer cells expressing high levels of *HMGA1*.

Conclusions—Our studies identify for the first time an important role for the *HMGA1*-*COX-2* pathway in pancreatic cancer and suggest that targeting this pathway could be effective to treat, or even prevent, pancreatic cancer.

Keywords

high mobility group A1 (*HMGA1*); cyclooxygenase-2; pancreatic adenocarcinoma; tumor progression

INTRODUCTION

Pancreatic cancer remains a highly lethal cancer that has not been effectively targeted by current therapies (1–8). With a five year survival rate of <5%, death rates closely mirror incidence rates. The overwhelming majority (~80%) of patients present with locally advanced or metastatic malignancies, rendering the cancers surgically unresectable (1–7). Unfortunately, even patients who undergo surgical resection eventually succumb to metastatic disease, including those cases where the primary lesion was extremely small at diagnosis. These abysmal statistics underscore the profound need for further research to identify better therapies.

Like other epithelial malignancies, pancreatic adenocarcinomas arise from non-invasive precursor lesions after a prolonged series of stepwise molecular genetic alterations that correspond with distinct histopathological entities (1–9). These precursor lesions, called pancreatic intraepithelial neoplasia or PanINs, are separated into three groups using an international classification system that is based on histopathologic features, including the degree of architectural and cytological atypia. PanIN-1 is a relatively common lesion that can be identified in approximately half of all elderly persons and is rarely associated with malignant lesions. The intermediate stage, PanIN-2, is more likely to be associated with pancreata harboring invasive carcinomas. PanIN-3 lesions, formerly known as “carcinoma-in-situ”, immediately precedes the development of invasive carcinoma and is often found in association with adjacent invasive cancer. Several studies have demonstrated that the histologic progression from PanIN lesions to invasive and metastatic adenocarcinoma is paralleled by a stepwise accumulation of molecular abnormalities (5–9).

While the early molecular events leading to the development of pancreatic intraepithelial lesions have been well-studied, little is known about the molecular pathways involved in tumor progression. For example, activating *K-RAS* mutations are found in the early, PanIN-1 lesions associated with pancreatic cancer (9). In fact, *K-RAS* mutations are present in 75–90% of cases of sporadic pancreatic cancer, making it the highest fraction of *K-RAS* mutations found in any cancer type (9). We recently reported high levels of *high mobility group A1* (*HMGA1*) mRNA in pancreatic adenocarcinoma cell lines derived from invasive tumors (10). In our study of 125 primary pancreatic tumors, we also found that *HMGA* protein levels are highest in invasive, metastatic tumors, and undetectable in normal pancreas. In addition, *HMGA1* was absent in most early PanIN-1 precursor lesions, but

present in the majority of advanced precursor (PanIN-3) lesions (10). We also demonstrated that high levels of HMGA1 protein correlate with poor differentiation status and adverse outcomes, suggesting that HMGA1 drives tumor progression (10). The HMGA1 protein is a member of the HMGA superfamily that includes HMGA1 and HMGA2 proteins (11–33). The *HMGA1* gene encodes the HMGA1a and HMGA1b protein isoforms that result from alternatively spliced RNA and differ by an internal 11 amino acids, present only in the HMGA1a isoform (11–13), while HMGA2 is encoded by a separate gene (13, 16, 24, 27, 32–33). HMGA chromatin binding proteins are involved in diverse biological processes by virtue of their ability to regulate gene expression (11–33). HMGA proteins are AT-hook proteins that bind to the minor groove of DNA at AT rich regions (11–13, 33–35), recruit additional transcription factors, and in concert with these factors, alter gene expression (11). The downstream gene targets activated by HMGA1 are only beginning to emerge, and include genes involved in cell signalling, cellular motility, and inflammation (11–13, 20, 23, 25–26, 28, 36–38).

Here, we provide the first evidence that the HMGA1-COX-2 pathway is important in tumor progression in pancreatic cancer. Moreover, our preclinical studies indicate that this pathway could be targeted to treat, or possibly even prevent, this deadly cancer.

Results

HMGA1 Cooperates with K-RAS to Induce Anchorage-Independent Cell Growth in Human Pancreatic Epithelial Cells

To investigate the functional role of HMGA1 in pancreatic cancer, we assessed the effects of HMGA1 on transformation phenotypes in nestin-positive cell lines derived from normal pancreatic epithelium (HPNE cells) that were immortalized with human telomerase (hTERT) cDNA (39) and engineered to express an activated, mutated RAS protein (K-RAS^{G12D}), denoted HPNE-K-RAS^{G12D} cells. The HPNE-K-RAS^{G12D} cell line is a previously described, non-transformed HPNE cell line that expresses a mutant, activated K-RAS^{G12D} (39–40). These cells were used because mutated, activated K-RAS^{G12D} is present in most cases of pancreatic cancer (39–40). We engineered the HPNE-K-RAS cells to express high levels of *HMGA1a* by transduction with an *HMGA1a* lentiviral construct linked to Green Fluorescent Protein (GFP; ref. 23), called HMGA1-HPNE-K-RAS cells. As control cell lines without HMGA1, we also engineered the HPNE-K-RAS^{G12D} cells to express the same lentiviral vector linked to GFP (without HMGA1a), called control-HPNE-K-RAS cells. *HMGA1a* mRNA and protein were increased in the cells transduced with the HMGA1a lentivirus as shown by quantitative, reverse transcriptase real-time PCR (qRT-PCR) and Western analysis (Fig. 1A). To determine if HMGA1 promotes a transformed phenotype in these cells, we assessed anchorage-independent cell growth in soft agar. Strikingly, only cells expressing *HMGA1* and activated *K-RAS* (designated HMGA1) exhibited transformed foci in soft agar (Fig. 1B). Few colonies formed in the control-HPNE-K-RAS cells (designated control).

HMGA1 Enhances Migration and Invasion in HPNE-K-RAS Cells

Next, we assessed phenotypes that are involved in metastatic progression, including migration and invasion in the HMGA1 and control cells. We found that the HMGA1 cells had a significant increase in migration and invasion compared to the control cells (Fig. 1C). Specifically, HMGA1 enhanced migration by over 3-fold ($p=0.001$, student's t-test) and invasion by over 2-fold ($p=0.0001$, student's t-test). We also measured cellular growth rates in the HMGA1 and control cells and found no significant difference in growth rates as assessed by the MTS assay (Fig. 1D). We also assessed the levels of transduced HMGA1 protein (labeled exogenous) in the HMGA1 cells compared to HMGA1 protein levels

(labeled endogenous) in the human pancreatic cancer cell lines (BxPC-3, MiaPaCa-2). The exogenous HMGA1 protein levels in the HPNE-K-RAS cells engineered to express HMGA1 were slightly lower than that observed in the pancreatic cancer cell lines, suggesting that this is a reasonable model for the role of HMGA1 in the transformation of pancreatic epithelium. Moreover, the endogenous HMGA1 levels in the HPNE cell lines were much lower than that observed in the pancreatic cancer cell lines (Fig. 1E). Taken together, our results suggest that HMGA1 cooperates with activated K-RAS to promote anchorage-independent cell growth. Moreover, HMGA1 enhances both migration and invasion in HPNE-K-RAS cells.

HMGA1 and COX-2 Expression Correlate Positively in Pancreatic Cancer Cells

The *COX-2* gene encodes a key enzyme involved in the conversion of arachidonic acid to prostaglandins and its expression is increased in diverse cancers. In cancer, *COX-2* is thought to promote proliferation, survival, angiogenesis, and metastatic progression (41). In previous studies, we showed that *COX-2* is a critical downstream gene target in uterine tumorigenesis (23, 26). To determine if the HMGA1-COX-2 pathway is involved in pancreatic carcinogenesis, we measured *HMGA1* and *COX-2* mRNA expression using quantitative RT-PCR in six pancreatic cancer cell lines and the HPNE-K-RAS cells. The six pancreatic cancer cell lines, included: 1.) BxPC-3 (from a 61 year old female with moderately well to poorly differentiated pancreatic carcinoma), 2.) HPAF-II (from peritoneal ascitic fluid from a 65 year old male with a primary pancreatic adenocarcinoma and metastases to the liver, diaphragm and lymph nodes; ref. 42), 3.) MiaPaCa-2 (from a 65 year old male with a primary, undifferentiated pancreatic carcinoma that involved the pancreatic body and tail and extended to the periaortic soft tissues), 4.) Panc1 (from a pancreatic ductal adenocarcinoma), 5.) PL45 (from a man with poorly differentiated pancreatic adenocarcinoma of ductal origin), and, 6.) XPA-3 (xenografted tumor cells from a 77 year-old male with primary pancreatic carcinoma that extended to involve the duodenum, peripancreatic soft tissues and metastasized to lymph nodes). We found a striking positive correlation between *HMGA1* and *COX-2* expression in these cell lines (Fig. 2A; $r^2 = 0.93$, $p < 0.001$).

HMGA1 Binds to the COX-2 Promoter by Chromatin Immunoprecipitation *in vivo*

Next, we investigated HMGA1 binding to the *COX-2* promoter *in vivo* by chromatin immunoprecipitation (Fig. 2B). For these studies, we used XPA-3, PL45, and HPAF-II cells. We previously reported high *HMGA1* expression in XPA-3 cells (10). Both PL45 and HPAF-II cells also express similar levels of *HMGA1* compared to XPA-3 cells (Supplementary Fig. 1). We found that HMGA1 binding is enriched in the *COX-2* promoter near the AT-rich, predicted HMGA1 DNA binding site in all three pancreatic cancer cell lines (XPA-3, PL45, and HPAF-II cells; Fig. 2B). Enrichment for binding to the *COX-2* promoter was greatest in the poorly differentiated XPA-3 and PL45 cells.

HMGA1 Induces COX-2 Expression

To determine if HMGA could regulate *COX-2* expression in pancreatic cancer cell lines, we assessed *COX-2* expression after knock-down of *HMGA1* using an siRNA interference approach (Dharmacon) in two poorly differentiated pancreatic cancer cell lines (XPA-3, PL45). Knock-down of *HMGA1* results in a significant repression in *COX-2* expression in these cell lines (Fig. 3A). Conversely, we also found that *COX-2* expression is induced in HMGA1-HPNE-K-RAS cells compared to control cells (Fig. 3B). Taken together, these findings suggest that HMGA1 binds to the *COX-2* promoter to induce its expression.

COX-2 Inhibitors Block Tumorigenesis in Human Pancreatic Cancer Xenografts Overexpressing *HMGA1*

Previous studies from our group demonstrated that COX-2 inhibitors block tumorigenesis in an *HMGA1* transgenic model of uterine cancer and in uterine cancer xenografts from poorly differentiated human cancer cells. We therefore investigated the anti-tumor effect of COX-2 inhibitors in pancreatic cancer cells using a tumor xenograft model with poorly differentiated XPA-3 cells. These cells were used because they express high levels of *HMGA1* (10) and *HMGA1* binds to the *COX-2* promoter *in vivo* by chromatin immunoprecipitation (Fig. 2B). We injected XPA-3 cells subcutaneously into nude mice fed control diet or diet supplemented with sulindac or celecoxib. Large subcutaneous tumors formed in all (6 of 6) nude mice fed the control diet after only 14 days (Table 1; Supplementary Fig. 4) with a mean tumor volume of $168.1 \pm 222.6 \text{ mm}^3$ (Fig. 4A–B). Strikingly, treatment with either sulindac or celecoxib significantly suppressed tumor growth. Only one small tumor formed (52.5 mm^3) in the sulindac arm ($p=0.0076$ by Fisher's exact test) and two small tumors formed in the celecoxib arm (44 and 20 mm^3 ; $p=0.03$ by Fisher's exact test). These results suggest that COX-2 inhibitors could be effective in the prevention or treatment of pancreatic cancers that up-regulate the *HMGA1*-COX-2 pathway.

Discussion

Pancreatic cancer is the fourth most common cause of cancer-related mortality in the United States and a leading cause of cancer death worldwide, claiming over 150,000 individuals each year (1–2). While molecular lesions that occur early in intraepithelial neoplasia are well-described, little is known about the molecular underpinnings that enable precursor lesions to progress to invasive carcinomas. For example, *K-RAS* mutations that result in a constitutively active protein are found in >90% of pancreatic cancers, making it the highest fraction of *K-RAS* mutations found in any cancer type (3–7). These activating *K-RAS* mutations are also present at high frequencies in premalignant PanIN-1 lesions and are thought to be responsible for initiating the early precursor lesions (9). Of note, the first experimental model of transformation was a co-transformation assay of rat embryo fibroblasts by *cMYC* and mutated *RAS* (43–44). In preliminary studies, we found that *HMGA1* can substitute for *MYC* and cooperate with *RAS* to transform rat embryo fibroblasts (Resar, unpublished data). In addition, we previously reported that *HMGA1* is a downstream transcriptional target of *cMYC* (15,45). We recently reported that the *HMGA1* protein is highly expressed in advanced pancreatic cancers with absent or low levels in early precursor lesions (10). We also discovered that *HMGA1* protein levels are positively correlated with poor differentiation status and decreased survival (10). Here, we found that *HMGA1* and mutated *RAS* cooperate to induce anchorage-independent cell growth in pancreatic epithelial (HPNE) cells. The pathways downstream of *RAS* and *HMGA1* are distinct, which could account for their cooperation in transforming pancreatic epithelium. Because *K-RAS* mutations occur in early PanIN lesions and *HMGA1* overexpression occurs in advanced disease, these results suggest that overexpression of *HMGA1* promotes neoplastic transformation in precursor lesions with *K-RAS* mutations. Interestingly, *HMGA1* (not shown) or *K-RAS* alone (40) were not sufficient to transform pancreatic epithelial cells.

A previous study found that knock-down of *HMGA1* blocks metastatic progression in an orthotopic murine model of pancreatic cancer (46). In the present study, we explored mechanisms through which *HMGA1* could function in pancreatic cancer progression. We found that *HMGA1* enhances both migration and invasion, two phenotypes involved in metastatic progression. In uterine cancer cells, we previously reported that *HMGA1* induces expression of *COX-2* during tumorigenesis (23), which led us to investigate this pathway in pancreatic cancer. In that study, we showed that *HMGA1* binds to the *COX-2* promoter *in*

vivo and activates its expression in transfection experiments (23). COX-2 is an enzyme responsible for the conversion of arachidonic acid to prostaglandins (41). *COX-2* is typically expressed at low levels in most tissues and its expression is induced by stressors such as pro-inflammatory cytokines, mitogens, growth factors, some hormones, and infectious agents (41). Deregulation of *COX-2* and up-regulation of the prostaglandin cascade is thought to play an important role in tumorigenesis in diverse cancers including breast (47), prostate (48), lung (49) and colon (50). In pancreatic cancer, *COX-2* up-regulation at the mRNA and protein levels has been reported in 74% to 100% of cases (51–57). High levels of *COX-2* were also found in some PanIN lesions (55–58) suggesting a potential benefit of targeting COX-2 in the prevention of tumor development and progression. In published preclinical studies, we found that inhibiting *COX-2* blocked uterine tumorigenesis in murine models characterized by high *HMGA1* expression (23, 26). To determine if this pathway contributes to pancreatic tumorigenesis, we first assessed HMGA1 binding to the *COX-2* promoter and found that HMGA1 occupies the *COX-2* promoter *in vivo* in pancreatic cancer cells, with the greatest enrichment for DNA binding in the poorly differentiated cells. In poorly differentiated pancreatic cancer cells, we also found that *COX-2* levels fall when *HMGA1* expression is inhibited, indicating that HMGA1 up-regulates its expression. Strikingly, in preclinical studies, we found that two COX-2 inhibitors, sulindac and celecoxib, significantly block tumor formation *in vivo*. Our results suggest that pancreatic tumors could respond to COX-2 inhibitors. Notably, a prior study in a conditional K-RAS transgenic model of pancreatic cancer showed that the COX-2 inhibitor, nimesulide, delayed progression of the PanIN lesions (58). These findings, together with our results, suggest that inhibiting *COX-2* and downstream pathways could be effective in the treatment or prevention of pancreatic cancer.

Work from our group and others also found that inhibition of *COX-2* blocks anchorage-independent cell growth in diverse cancer cell lines (23, 26). Previous studies also showed that inhibition of *COX-2* expression blocks invasion and/or migration in cultured cancer cells *in vitro* (59–65). In this study, we focus on *in vivo* tumorigenesis because this is more likely to be relevant to pancreatic cancer growth in humans. Although prior studies suggest that COX-2 inhibitors could be beneficial in diverse cancer models (26,47–70), including pancreatic cancer (63–65), these findings have not translated into major improvements in the clinic (66–69). Of note, prior clinical trials with COX-2 inhibitors in pancreatic cancer have been done in patients with advanced disease (66–69). Successful use of COX-2 inhibitors may require administration at an earlier time point in tumor progression or initiation. This potential explanation is supported by the finding that individuals exposed to anti-inflammatory agents have a lower incidence of diverse tumors (70). Despite the disappointing results with COX-2 inhibitors in clinical trials with advanced pancreatic cancer, our studies help to elucidate a previously unknown pathway that could drive tumor progression in pancreatic cancer. Future studies are needed to determine how best to target this pathway.

In conclusion, we report for the first time that HMGA1 cooperates with activated K-RAS to transform pancreatic epithelial cells. In addition, we found that HMGA1 binds to the *COX-2* promoter and up-regulates *COX-2* expression in pancreatic cancer cell lines. Although further studies are needed, we provide additional evidence that targeting the HMGA1-COX-2 pathway could be beneficial in the treatment, or possibly prevention, of pancreatic cancer.

Methods

Cell Culture

The human pancreatic ductal adenocarcinoma cell line XPA-3 (PX154) was grown as previously described (10). BxPC-3 (CRL-1687), HPAF-II (CRL-1997), MiaPaCa-2 (CRL-1420), Panc-1 (CRL-1469), PL45 (CRL-2558) cells were obtained from the American Type Culture Collection and grown as recommended. Human pancreatic nestin-expressing (hTERT-HPNE) cells are pancreatic duct-derived cells that were immortalized by ectopic expression of the catalytic sub-unit of human telomerase (39). The HPNE-K-RAS^{G12D} cell line was generated as previously described (40). The HMGA1-HPNE-K-RAS and control HPNE-K-RAS cells were cultured in 4 parts low-glucose DMEM (Invitrogen, Carlsbad, CA) to 1 part M3A base culture media (INCELL, San Antonio, TX) supplemented with 5% FBS (Sigma, St. Louis, MO), 10 ng/ml EGF (Sigma), 25 µg/ml Gentamycin (Quality Biological, Gaithersburg, MD) and 200 µg/ml Hygromycin B (Mediatech, Manassas, VA).

Generation of HPNE-K-RAS^{G12D}-HMGA1 and HPNE-K-RAS^{G12D}-GFP control cells

HPNE-K-RAS cells were infected with the pFG12 lentivirus encoding the green fluorescent protein (GFP) and containing the murine *hmga1a* cDNA with cells at 90% confluency as previously described (23, 71–72). Control cells were infected with the pFG12 lentiviral vector alone that also expresses GFP.

Methylcellulose Assays

HPNE-K-RAS^{G12D}-HMGA1 or HPNE-K-RAS^{G12D} control cells (10^3) were plated in six-well plates in methylcellulose medium according to the manufacturer's instructions (StemCell Technologies, Vancouver, Canada) and foci were allowed to grow for 14 days.

Migration and Invasion Assays

Migration and invasion assays were performed in 24-well plates containing 0.8 µm pores cell culture inserts with polyethylene terephthalate membranes according to manufacturer's instructions (BioCoat Cell Culture Inserts, BD Biosciences). For invasion assay, membranes were coated with a uniform layer of BD Matrigel Basement Membrane Matrix (BioCoat BD Matrigel Invasion Chamber, BD Biosciences). The cells were seeded in 500 µl of HPNE media without FBS at 10,000 cells/well for migration and 50,000 cells/well for invasion into the upper chamber. The lower chamber was filled with 750 µl of complete HPNE media. After 24 h, migration or invasion was assessed by counting cells on the underside after fixing and staining the membrane with Diff-Quick Stain set (Imeb Inc, CA) according to the manufacturer's instructions.

Cellular Growth Rate Determinations

Cellular growth rates were determined as previously described (25) using the CellTiter Cell Proliferation Assay (Promega, WI).

Gene Expression Analysis

Cells were lysed in RLT buffer (Qiagen, CA) and total RNA was extracted with an RNeasy Mini Kit (Qiagen). *HMGA1a* and *COX-2* levels were analyzed by quantitative reverse transcription real-time PCR as previously described (23). The student's t-test was used to assess differences in gene expression between groups. Prism graphics software was used to assess correlations in gene expression.

Chromatin Immunoprecipitation Experiments

Chromatin immunoprecipitation experiments were performed as previously described (23) using XPA-3 cells. Proteins cross-linked to chromatin were immunoprecipitated with the following antibodies: HMGA1 (23), histone H3 (as a positive control), IgG, and the HMGA1 antibody with no chromatin (as negative controls). No DNA was also included as a negative control for the quantitative RT-PCR reaction (23). The *COX-2* promoter region with the consensus HMGA1 DNA-binding site was amplified from the immunoprecipitated protein-DNA complexes using the forward and reverse primers 5'-TAA GGG GAG AGG AGG GAA AA-3' and 5'-GAC GCT CAC TGC AAG TCG TA-3', respectively. Chromatin binding is assessed by the quantity of the amplified fragment determined by quantitative RT-PCR and expressed as percent of total chromatin as previously described (25, 28).

HMGA1 Knock-Down Experiments

Small interfering RNA (siRNA; Dharmacon) was used to knock down expression of *HMGA1* according to the manufacturer's instructions as previously described (23). *HMGA1a* and β -actin mRNA levels were assessed by qRT-PCR at 24, 48, and 72 h after transfection with each siRNA.

Animals and Pancreatic Xenografts studies

Poorly differentiated pancreatic cancer (XPA-3) cells (5.0×10^6) were injected subcutaneously into the flanks of nude mice as we previously described (15–16). Mice were fed mouse pellets supplemented with celecoxib, sulindac, or control food starting 2–3 days before injections. Mice were sacrificed when tumors reached a maximum diameter of 1.5 cm (after ~14 days) in the control group. celecoxib (800 ppm, LKT Laboratories) or sulindac (320 ppm; Sigma-Aldrich) were mixed with pelleted, purified rodent diet AIN-74 (Dyets). These doses were previously shown to be efficacious in other murine tumor models (26, 62). The experiment was performed twice with 3 mice in each experimental group (control, celecoxib, or sulindac; total n=6 in each arm). Tumor volumes were estimated as length X width²/2 (26). Fisher's exact test was used to compare the tumor frequency in each arm and student's t-test was used to compare tumor volumes in each arm. All animal studies were approved by the Animal Care and Use Committee at the Johns Hopkins University.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

<i>HMGA1a</i>	<i>high mobility group A1 gene</i>
RT-PCR	real time polymerase chain reaction

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FIGURE 1. HMGA1 cooperates with activated K-RAS^{G12D} to induce anchorage-independent cell growth, migration and invasion in pancreatic epithelial cells

A. qRT-PCR analysis shows that *HMGA1* expression is significantly increased in the HPNE-K-RAS^{G12D} cells transduced with the HMGA1-GFP lentivirus (labeled HMGA1) compared to the control HPNE-K-RAS^{G12D} cells transduced with the control GFP lentivirus (labeled control). The control HPNE-K-RAS^{G12D} cells were arbitrarily assigned a value of 1.0. All PCR reactions were done in triplicate and performed twice. The bars represent the mean ± the standard deviations. The Western blot shows the increased HMGA1 protein in the HPNE-K-RAS-HMGA1 cells (denoted HMGA1) compared to the control HPNE-K-RAS cells (denoted control).

B. HMGA1 induces anchorage-independent cell growth or foci formation in HPNE-K-RAS cells (HMGA1) compared to the control cells. HMGA1-HPNE-K-RAS cells exhibited transformed foci in soft agar, compared with HPNE-K-RAS RAS^{G12D}-GFP cells. Experiments were done in duplicate and performed at least twice. The bars represent the mean ± the standard deviations.

C. HMGA1 enhances migration and invasion in HPNE-K-RAS cells. Experiments were done in duplicate and performed at least twice. The bars represent the mean ± the standard deviations.

D. Growth curves were similar for the HMGA1 and control cells. All standard errors were <20% (not shown). There was no significant difference in proliferation at all time points.

E. HMGA1 protein levels in HPNE-K-RAS-HMGA1 (HMGA1) cells compared to the HPNE-K-RAS-GFP (control) cells and human pancreatic cancer cell lines (BxPC3 and MiaPaCa). Exogenous HMGA1 is Myc-tagged and runs higher; endogenous HMGA1 is also indicated (upper panel and arrows). β-actin is shown as a loading control (lower panel and arrow).

FIGURE 2. HMGA1 binds to the COX-2 promoter *in vivo* and regulates COX-2 expression in poorly differentiated human pancreatic cancer cells

A. Correlation between HMGA1 and COX-2 in six pancreatic cancer cell lines (BxPC-3, HPAF-II, MiaPaCa Panc1, PL45, and XPA-3). Pearson's test (Prism graphics) was used to determine the correlation coefficient and p-value.

B. Chromatin immunoprecipitation shows binding of HMGA1 to the COX-2 promoter *in vivo* in the human pancreatic cell lines: XPA-3, PL45, and HPAF-II. The graphs show the quantity of immunoprecipitated DNA with the following antibodies: HMGA1, histone H3 (as a positive control), and IgG, no chromatin immunoprecipitation with the HMGA antibody, or no DNA for PCR (as negative controls). In the XPA-3 cells, HMGA1 binding to the COX-2 promoter was the highest, estimated to be enriched by over 10-fold compared to that observed for the negative controls. In the PL45 and HPAF-II cells, HMGA1 binding to the COX-2 promoter was enriched by 2 to 4-fold compared to the negative controls.

FIGURE 3. Knock-down of HMGA1 results in repression of *COX-2* mRNA in human pancreatic cancer cells

A. Silencing of *HMGA1* decreases *HMGA1* and *COX-2* mRNA levels in human pancreatic cells from poorly differentiated tumors (XPA-3 and PL45 cells). Pancreatic cancer cells were transfected with ON-TARGET^{plus} SMARTpool siRNAs to *HMGA1* or a siCONTROL Non-Targeting pool siRNA as negative control as we previously described (23). Expression of *HMGA1* (upper panel) and *COX-2* (lower panel) was assessed by qRT-PCR, also as we described (23). siRNA-mediated knockdown of *HMGA1* effectively decreased the levels of endogenous *HMGA1a* and *COX-2* transcripts 72 h. There was no decrease in *HMGA1* or *COX-2* mRNA with the siRNA controls. (Additional time points are shown in Supplementary Figures 3–4).

B. Forced expression of HMGA1 induces *COX-2* expression in HPNE-K-RAS cells.

FIGURE 4. Sulindac and celecoxib block tumorigenesis in human pancreatic cancer xenografts overexpressing *HMGA1*

A. Representative nude mice treated with control food or food supplemented with sulindac or celecoxib. Tumors formed in all 6 mice treated with control food (100%). Both sulindac (p=0.0076; Fisher's exact test) and celecoxib (p=0.03; Fisher's exact test) significantly inhibit xenograft tumorigenesis.

B. Only one small tumor formed (52.5 mm³) in the sulindac arm and two small tumors formed in the celecoxib arm (44 and 20 mm³). (Relative tumor volumes are shown in Supplementary Fig. 4)