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Adrenomedullin is a therapeutic target in colorectal cancer

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Abstract

The *KRAS* oncogene influences angiogenesis, metastasis, and chemoresistance in colorectal cancers (CRC), and these processes are all enhanced in hypoxic conditions. In order to define functional activities of mutant *KRAS* in a hypoxic microenvironment, we first performed cDNA microarray experiments in isogenic DKs5 and DKO3 colon cancer cell lines that differ only by their expression of mutant *KRAS* (K-ras^{D13}). Adrenomedullin (ADM) was identified as one of the most significantly upregulated genes in DKs5 cells that express the *KRAS* oncogene in hypoxia (3.2-fold, $p=1.47 \times 10^{-5}$). Ectopic expression of mutant *KRAS* (K-ras^{V12}) in Caco-2 cells (K-ras^{WT}) induced ADM, while selective knockdown of mutant *KRAS* alleles (K-ras^{D13} or K-ras^{V12}) in HCT116, DLD1, and SW480 colon cancer cells suppressed the expression of ADM in hypoxia. Knockdown of ADM in colon tumor xenografts blocked angiogenesis and stimulated apoptosis, resulting in tumor suppression. Furthermore, ADM also regulated colon cancer cell invasion *in vitro*. Among 56 patients with CRC, significantly higher expression levels of ADM were observed in samples harboring a *KRAS* mutation. Collectively, ADM is a new target of oncogenic *KRAS* in the setting of hypoxia. This observation suggests that therapeutic targets may differ depending upon the specific tumor microenvironment.

Keywords

K-ras; Adrenomedullin; Hypoxia; Colorectal cancers

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Introduction

Hypoxic microenvironments invariably develop as solid tumors rapidly enlarge, and unique cellular adaptations ensure tumor survival in these conditions. These include selection of genotypes favoring survival under hypoxia and an anabolic switch in cellular metabolism.^{1, 2} Hypoxia not only promotes tumor angiogenesis, invasion, and metastasis but also enhances chemoresistance and radioresistance.¹⁻³ We have demonstrated that hypoxia-inducible factor-1 (HIF-1), vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) are induced in tumor cells with oncogenic *KRAS*.⁴⁻⁸ The biological properties of oncogenic mutations in normoxic conditions *in vitro* are well-established, but little is known about how oncogenes such as mutant *KRAS* may influence cellular behavior in a hypoxic microenvironment.

KRAS mutations occur in as many as 50% of colorectal cancers (CRC). Activating mutations in *KRAS* facilitate colon tumor invasion and metastasis and are considered a negative clinical prognostic factor.⁹⁻¹¹ In addition to its central role in promoting cell proliferation through activation of downstream effectors including Raf kinases and phosphoinositide 3-kinase (PI₃K), K-ras also regulates tumor angiogenesis, cell morphology, and cell adhesion.^{9, 12} Our previous studies have demonstrated a synergistic interaction between K-ras and hypoxia in upregulating VEGF in colon cancer cells.^{6, 8} Mutant K-ras can also enhance glycolysis and survival in colon cancer cells in low-glucose conditions through the upregulation of *GLUT1*.¹³ These data suggest that mutant *KRAS* may play a specific role in tumor survival in hypoxic environments. To systematically identify the full spectrum of downstream genes that are regulated by mutant *KRAS* in hypoxia, we performed cDNA microarrays using two isogenic colon cancer cell lines with either mutant or wild-type *KRAS*. We identified adrenomedullin (ADM) as one of the genes most significantly upregulated in mutant *KRAS* cells.

ADM was originally isolated from a human pheochromocytoma and modulates numerous physiological functions including vasodilation and cellular growth.¹⁴⁻¹⁶ ADM has also been shown to be involved in tumor survival and progression by promoting cellular proliferation and angiogenesis. Overexpression of ADM enhances vascular density and directed growth of blood vessels in pancreatic and breast cancer xenografts.^{17, 18} Exogenous ADM administration upregulates the expression of VEGF in a mouse hind-limb ischemia model and enhances VEGF-induced capillary tube formation.¹⁹ Overexpression of ADM can also inhibit hypoxic cell death by upregulation of the anti-apoptotic factor Bcl-2 in endometrial cancer cells.²⁰ However, the role of mutant oncogenes such as *KRAS* in the regulation of ADM in hypoxic conditions has not been previously described.

In this study, we demonstrate that multiple genes are upregulated selectively in cells with a mutant *KRAS* oncogene under hypoxic conditions. We identified ADM as a novel target of mutant K-ras in colon cancer cells in hypoxia. Furthermore, the expression level of ADM is significantly higher in human CRC samples with a mutant *KRAS* oncogene. Knockdown of ADM suppresses tumor growth and impairs angiogenesis in colon tumor xenografts. Collectively, the enhanced activation of ADM by mutant K-ras may play a critical role in the adaptation of colon tumors to hypoxic stress conditions.

Materials and Methods

Cell cultures

Human colon cancer cell lines (SW480, HCT116 and DLD-1) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Isogenic cell lines of DKs-5 (K-ras^{D13}) and DKO-3 (K-ras^{WT}) are derived from DLD1 colon cancer cells, and HK2-10 (K-ras^{D13})

and HKe-3 (K-ras^{WT}) from HCT116 cells, in which WT or mutant *KRAS* alleles have been disrupted by targeted homologous recombination, as described previously.²¹ Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO-Invitrogen, Carlsbad, CA) or McCoy's 5A medium (Invitrogen, Carlsbad, CA) supplemented with FBS (HyClone, Ogden, UT) and antibiotics (Penicillin-Streptomycin, Invitrogen). Hypoxic conditions were achieved by culturing cell lines in a sealed hypoxia chamber (Billups-Rothenberg, Del Mar, CA) with a mixture of 1% O₂, 5% CO₂, and 94% N₂.

cDNA Microarray analysis

The isogenic cell lines DKs-5 (K-ras^{D13}) and DKO-3 (K-ras^{WT}) were cultured in normoxic (21% O₂) or hypoxic (1% O₂) conditions for 12 hours. Total RNA was extracted using the RNeasy kit (Qiagen). Duplicate samples were hybridized to probes and transcripts based on the Affymetrix U133A 2.0 microarray platform. Microarray data was analyzed using R version 2.4.0 and bioconductor modules. The data have been submitted to the GEO repository (accession number GSE35973). The transcripts were compared between normoxic and hypoxic conditions and a *p* value of <0.001 was considered statistically significant.

Establishment of stable cells

pCSGW^{K-ras V12} or empty vector were introduced with a lentiviral-based system into Caco-2 cells (K-ras WT) and stable cells were established as described previously.⁵ We obtained three lentiviral constructs containing 21-mer short hairpin RNAs (shRNA) directed to various coding regions of ADM from the plasmID repository (DF/HCC DNA Resource Core, Harvard Medical School, MA). A scramble target shRNA vector (pLKO.1) was purchased from Addgene Inc. (Cambridge, MA). Two shRNAs were verified as effectively silencing ADM and were used for further study (ADM target sequence #1, gtt tcg aaa gaa gtg gaa taa; ADM sequence #2, cct cac tat tac ttg aac ttt).

Xenograft tumor model

Nude mouse experiments (*BALB/c nu/nu* female; 6 weeks old; Charles River Laboratories) were carried out in accordance with the standards of the Institutional Animal Care and Use Committee at Massachusetts General Hospital. SW480 cells (5×10⁶) stably transfected with one of two shADM (#1 and #2) vectors or the pLKO.1 vector were injected subcutaneously (s.c) into the flanks of mice (n=10 each group). Tumor volume was assessed every three days according to the following formula: $V = L \times W^2 / 2$ where *V*, volume (mm³); *L*, largest diameter (mm); *W*, smallest diameter (mm). The mice were euthanized and tumors were weighed at the end of the experiments.

Primary CRC samples and mutation analysis

A total of 56 sporadic CRC samples were collected retrospectively from 2001 to 2008 at the Massachusetts General Hospital. The clinico-pathological characteristics of patients and DNA microsatellite status have been described previously.²² Genomic DNA was extracted from paraffin-embedded blocks of CRC tissues and used as template for subsequent PCR amplifications for *KRAS* sequencing according to the protocol of TaKaRa DEXPATTM (Takara Bio Inc.). This study was approved by the Institutional Review Board of Massachusetts General Hospital.

Immunohistochemical staining

Immunohistochemical staining was performed as previously described. Primary antibodies against ADM (1:100), Ki-67 (1:500) and cleaved caspase-3 (1:100) were applied at 4° overnight, and a 1:500 dilution of the corresponding peroxidase-conjugated IgG was

incubated for 1 h at room temperature. Slides were incubated with the streptavidin-peroxidase complex with Vectastain Elite ABC Universal kit (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature, developed with 3,3-diaminobenzidine substrate, counterstained with Gill's hematoxylin, dehydrated with ethanol, fixed with xylene, and mounted. CD31 staining in xenograft tumors was performed as described above, with the exception that acetone-fixed frozen sections were used instead of paraffin-fixed sections.

The ADM intensity was graded using the scoring system by Oehler *et al*²³: 0, negative; 1, weak; 2, moderate; 3, strong. Ki-67-stained cells were counted in 5 randomly selected fields in each slide at 400× magnification. The Ki-67 labeling index was calculated as [Ki-67-positive cells / total cells] × 100. The microvessel density (MVD) was quantified by counting the number of CD-31 positive blood vessels at 100× magnification in five microscopic fields for each tumor sample.

Results

Identification of adrenomedullin (ADM) as a target of mutant *KRAS* in hypoxic conditions

In order to systematically define the downstream targets of mutant *KRAS* in hypoxic microenvironments, we utilized an Affymetrix oligonucleotide microarray in a set of isogenic colon cancer cell lines (DKs5, DKO3) incubated in hypoxic (1% O₂) or normoxic (21% O₂) conditions. DKO3 and DKs5 cells are derived from DLD1 colon cancer cells; in DKO3 cells, the mutant *KRAS* allele has been deleted through homologous recombination, while DKs5 cells express the parental mutant *KRAS* allele (D13) (21). We focused on targets that exhibited differential responses based on the presence or absence of a mutant *KRAS* allele and identified 7 such genes (Fig. 1A, Supplementary Table 1). 4 of these genes have been reported to play important roles in tumor angiogenesis (*VEGFA*, *ADM* and *P4HA2*) and cell metabolism (*PFKFB3*).^{24–27} Among these targets, adrenomedullin (ADM) exhibited the largest and most significant induction in DKs5 cells in hypoxic conditions (3.2-fold, $p=1.47\times10^{-5}$). In addition, microarray analysis revealed 31 genes that were differentially regulated in both DKs5 (K-ras^{D13}) and DKO3 (K-ras^{WT}) cells in response to hypoxia ($p<0.001$) (Fig. 1A). Most of these genes (19/31) have been reported as HIF-1 targets or possess high stringency HIF-1 binding sites (Supplementary Table 2, 3).

Our microarray findings were validated by qPCR and ELISA. ADM mRNA increased 12.0 ± 4.3 -fold in DKs5 cells subjected to 12 h of hypoxia, compared to a 3.7 ± 1.0 -fold induction in DKO3 cells ($p<0.01$) (Fig. 1B). We also found that the level of secreted ADM protein in the culture medium was significantly increased (4.2 ± 0.8 -fold) in DKs5 cells (Fig. 1C). In contrast, the level of ADM did not significantly change in DKO3 cells. Of note, basal levels of ADM mRNA and protein were not significantly different between these two cell lines in normoxic conditions ($p>0.05$). We also verified these findings by examining the expression levels of ADM mRNA in another set of isogenic cells, HK2-10 (K-ras^{D13}) and HKe-3 (K-ras^{WT}) cells that are derived from HCT116 cells. The expression of ADM by qRT-PCR was significantly higher in HK2-10 cells in hypoxia when compared with HKe-3 cells (4.1-fold increase, $p<0.01$).

Mutant *KRAS* selectively regulates the expression of ADM

To confirm that ADM is regulated by mutant K-ras, we selectively knocked down the *KRAS* oncogene in three colon cancer cell lines (Supplementary Fig. 1A). Each of these cells has a mutant *KRAS* allele; HCT116 and DLD1 are both heterozygous for the K-ras^{D13} mutation, while SW480 is homozygous for the K-ras^{V12} mutation. In these cell lines, we found that the expression of ADM mRNA was significantly upregulated after 12 h of incubation under

hypoxic conditions (13.1–14.6-fold). Silencing of K-ras reduced ADM expression levels by 70–89% in hypoxia compared to cells transfected with a control siRNA (Fig. 2A). Moreover, the secretion of ADM protein significantly increased in response to hypoxia and decreased dramatically after knockdown of K-ras ($p<0.05$) (Fig. 2B).

To confirm the specific effects of mutant *KRAS* in the regulation of ADM expression, we utilized Caco-2 cells in which a mutant *KRAS* allele (K-ras^{V12}) was stably introduced (Supplementary Fig.1B). Consistent with prior observations, overexpression of the mutant K-ras^{V12} oncogene significantly enhanced the expression of ADM mRNA as measured by qPCR (Fig.2C). ELISA experiments also demonstrated that ectopic expression of K-ras^{V12} increased ADM protein expression 2.4-fold after 12 hours of hypoxia compared to those cells transfected with empty vector (Fig. 2D). Our results suggest that mutant K-ras is a key regulator of ADM in hypoxic conditions in colon cancer cells.

Knockdown of ADM suppresses colon tumor growth *in vivo*

To evaluate the functional role of ADM in colon tumorigenesis, we investigated the effects of ADM knockdown on colon tumor xenografts. We generated lentiviral-based stable knockdown of ADM in SW480 (with homozygous mutant *KRAS*, K-ras^{V12/V12}) cells using two independent shRNA constructs (shADM #1 and shADM #2). SW480 cells were utilized for these studies because they exhibit more aggressive growth patterns with respect to cell migration, invasion, and capacity for EMT.²² Consequently, they form larger tumor xenografts, and phenotypic differences attributable to ADM would be predicted to be more apparent in SW480 cells. In addition, basal levels of ADM in normoxic conditions are higher in SW480 cells compared to DLD1 cells (2.2-fold increase by qRT-PCR). SW480 cells stably transfected with shADM #1, shADM #2, or control pLKO.1 vector were subcutaneously injected into the flanks of immunodeficient mice (Supplementary Fig. 2A, B). Tumor volume was significantly decreased in shADM mice relative to the control group over a 3-week period (Fig. 3A). The mean volume and wet weight of tumors in shADM mice were also significantly lower than those from the shRNA control group ($p<0.05$) (Fig. 3B, C). Of particular note, 70% (7/10) of mice injected with shADM #1-transfected SW480 cells exhibited no detectable tumor when examined at the end of the experiment. These results indicate that silencing of ADM suppresses colon tumor growth *in vivo*.

Knockdown of ADM impairs tumor angiogenesis and stimulates apoptosis

We analyzed xenograft tumors to understand mechanisms of ADM activity. We first performed immunohistochemical staining for Ki-67. We found no significant differences in the Ki-67 labeling index between shADM and control tumors ($p>0.05$) (Fig. 4A, C). *In vitro*, silencing of ADM in SW480 cells resulted in only a slight reduction in cell proliferation by MTS assay in a 4-day time course ($p<0.05$) (Supplementary Fig. 2C). These data indicate that ADM may not regulate cell proliferation directly. ADM can function as an angiogenic factor, so we hypothesized that the knockdown of ADM would result in an impairment of angiogenesis. Immunohistochemical staining for the endothelial cell marker CD31 demonstrated that the mean number of micro-vessels in shADM #1 and #2 tumors was 47.7 ± 1.4 and 30.3 ± 9.8 , respectively, and this was significantly lower than the microvessel density in shRNA control mice (76.9 ± 14.0) ($p<0.01$) (Fig. 4A, D).

Histopathologic analysis revealed that large necrotic areas were more prevalent in shADM #1 and shADM #2 tumors when compared with control (Fig. 4A). In addition, a significantly higher number of cleaved caspase-3 positive cells were observed in tumors from the shADM #1 and shADM #2 group (Fig. 4A, B). These apoptotic cells were typically localized in the areas surrounding necrosis. Collectively, these observations suggest that the reduced vascularity seen in the absence of ADM may contribute to an increase in cell death.

ADM modulates colon cancer cell invasion *in vitro*

We further examined the role of ADM in colon cancer cell invasion *in vitro*. Cell invasion assays were performed in Biocoat Matrigel Invasion Chambers and DAPI-stained invading cells were counted. Stable knockdown of ADM (#1 and #2) suppressed cellular invasion in SW480 cells by 81.7% and 77.3%, respectively ($p < 0.01$) (Fig. 5A, B). Transient overexpression of ADM promoted SW480 cell invasion 1.8-fold (Fig. 5C, Supplementary Fig. 3A, B). In addition, administration of ADM (50 nm and 200 nm) to the invasion chamber as a chemoattractant significantly stimulated cell invasion by 1.54 and 2.11-fold, respectively ($p < 0.01$, Fig. 6D). These data suggest that ADM can also regulate colon cancer cell invasion.

ADM is associated with *KRAS* genotype in human colorectal cancers

Based on our findings that mutant K-ras regulates the expression of ADM in colon cancer cells, we sought to determine whether ADM expression is correlated with *KRAS* genotypes in human CRC samples. Among 56 CRC samples, 50 (89%) exhibited positive ADM staining by immunochemistry. The intensity of ADM staining was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong), as determined independently by a GI pathologist (Fig. 6A) (23). Normal colon tissues were negative for ADM expression, except for scattered positive staining observed at the base of the glands in neuroendocrine cells.²⁸

Expression levels of ADM were significantly higher in colorectal tumors with a *KRAS* mutation (Fig. 6B). 71% (10/14) of mutant *KRAS* tumors exhibited moderate to strong staining of ADM, compared to only 40% (17/42) of WT *KRAS* tumors ($p < 0.05$). Interestingly, the staining level of ADM was also higher in tumors with DNA microsatellite stability (MSS) and poor pathological differentiation ($p < 0.05$) (Fig. 6B, Supplementary Table 4). Our results are consistent with previous observations that ADM expression is associated with a more aggressive tumor phenotype in breast and ovarian cancers.^{23, 29} No significant correlation was observed between ADM staining level and TNM stage. These results demonstrate that ADM is expressed in human CRC and its expression is associated with the *KRAS* genotype.

Discussion

A critical role for K-ras in the adaptation to a hypoxic microenvironment is highlighted by our observation that oncogenic K-ras regulates multiple targets involved in tumor angiogenesis and cell metabolism during oxygen deprivation. We identified ADM as a new effector of oncogenic *KRAS* that regulates colon tumor growth and angiogenesis. In human CRC samples, higher levels of ADM expression were associated with a mutant *KRAS* genotype.

Our study provides new insights into functional properties of the *KRAS* oncogene by demonstrating that its oncogenic activity can depend upon specific microenvironmental conditions. ADM is one of these target genes that requires a mutant *KRAS* oncogene for maximal induction in hypoxia. The concept that oncogene activity may be modulated by different microenvironments is not well-described. We have previously demonstrated that hypoxia can activate wild-type *KRAS*, suggesting a key role for this proto-oncogene in conditions of low oxygen.⁶ In contrast, hypoxia can also lead to the downregulation of the C-myc oncoprotein.³⁰ Thus, the activity of a specific oncogene may fluctuate depending upon the local oxygen concentration. This has important implications for targeted therapies against specific oncogenes, as the underlying tumor genotype cannot be considered to have fixed molecular properties and functions.

One of most critical aspects of the response to hypoxia is the activation of the hypoxia-inducible factor-1 (HIF-1). Once activated, HIF-1 can amplify numerous target genes by binding consensus hypoxia response elements (HREs) (5'-RTGCG-3') in promoter regions. Previous studies have identified putative HREs in the ADM gene.³¹ In this study, we identified ADM as being significantly upregulated in mutant K-ras cells under hypoxic conditions. In several cancer types, the expression of ADM is stimulated by hypoxia and inflammatory cytokines such as IL-1 β .^{20, 31, 32, 33} It is reasonable to propose that oncogenic K-ras and HIF-1 α could modulate common targets that promote angiogenesis and metabolic shifts under hypoxic stress. This hypothesis was supported by a recent study by Chun *et al*,³⁴ in which several oncogenic K-ras targets were also induced by HIF-1 α . They suggested that activated K-ras and HIF-1 α may both regulate cancer cell metabolism via common targets.

The link between mutant *KRAS* and its regulation of ADM in hypoxia cells may have important therapeutic implications in colon cancer. The effects of ADM inhibition are primarily linked to anti-angiogenesis, although in some cases ADM may regulate cancer cells directly.^{17, 19, 35, 36} We found that exogenous administration or knockdown of ADM did not significantly affect colon cancer cell proliferation *in vivo* or *in vitro*. ADM can regulate tumor angiogenesis by promoting recruitment of hematopoietic progenitors, vascular morphogenesis, and blood vessel stabilization.¹⁶ Anti-angiogenic treatment targeting VEGF has proven effective in advanced metastatic colon cancer. However, resistance to single agents can emerge with continued treatment.³⁷ Inhibiting one angiogenic factor may result in the upregulation of other compensatory angiogenic factors, including ADM, angiogenin, and PAI-1.³⁸ We found that in addition to VEGF, the angiogenic factor ADM was also significantly induced in mutant *KRAS* cells subjected to hypoxia. Our data indicated that shRNA-directed knockdown of ADM effectively suppressed colon tumor growth and impaired tumor angiogenesis. *In vitro* studies suggest that blocking ADM may also limit cancer cell invasion. Several approaches have been explored to therapeutically target ADM, either by blocking ADM binding to its receptor (anti-ADM antibody or ADM receptor antagonist) or shRNA-mediated knockdown of ADM.^{17, 20, 35, 36} We propose that blockade of ADM may serve as a new therapeutic approach in CRC, particularly for patients whose tumor harbors a *KRAS* mutation. However, there may also be a small subset of tumors without a *KRAS* mutation that overexpress ADM, and we cannot rule out a potential therapeutic benefit for this subset in which ADM may be induced via K-ras independent mechanisms.

In conclusion, oncogenic K-ras regulates a unique set of genes to promote the survival of colon cancer cells in hypoxic conditions. We have identified ADM as one of these novel targets of mutant *KRAS* in hypoxia and demonstrated that it plays a key role in tumor angiogenesis. These studies reveal a unique interplay between tumor oncogenes and the tumor microenvironment in which oncogenic function can be modulated specifically by hypoxia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty and impact statements

We have identified a unique role for the K-ras oncogene, the most commonly mutated oncogene in human colorectal cancer, in the hypoxic response. K-ras cooperates with hypoxia to potentially induce the angiogenic factor adrenomedullin. These findings have important implications with respect to new therapeutic targets in colorectal cancer as well as the role of the microenvironment in the design of targeted therapies.

Figure 1. Adrenomedullin (ADM) is a target of mutant *KRAS* under hypoxic conditions
(A) Microarray flowchart. Gene expression profiles were analyzed in DKs5 (K-ras^{D13/-}) and DKO3 (K-ras^{WT/-}) cells after exposure to normoxia (21% O₂) or hypoxia (1% O₂) for 12 h in duplicate. Transcripts with a p-value of <0.001 were identified as being differentially expressed between hypoxic and normoxic conditions. Genes with high stringency HIF-1 binding sites were screened. The fold change was defined as gene expression under hypoxic compared to normoxic conditions. **(B)** The expression of ADM mRNA was measured by real-time PCR and expressed as relative fold change compared to DKs5 cells under normoxic conditions and normalized to 1. **(C)** The secreted level of ADM protein was analyzed by ELISA in DKs5 and DKO3 cells on 2, 4, 8, 12 and 24 h of hypoxia. Levels were compared to those in normoxic conditions. **p*<0.05, ***p*<0.01.

Figure 2. K-ras regulates the expression of ADM under hypoxic conditions

(A) ADM transcript levels are expressed as relative fold change as compared to cells transfected with siRNA control (KD control) in normoxic conditions and normalized to 1. Cells were cultured under hypoxic or normoxic conditions for 12 h. Experiments were performed in triplicate. **KD**, knockdown; **hypoxia**, black bars; **normoxia**, white bars. (B) The level of secreted ADM was examined in HCT116 cells after knockdown of K-ras or a non-targeting control siRNA (KD control). Expression levels of ADM mRNA (C) and secreted protein (D) were detected in Caco-2 cells stably transfected with mutant K-ras (K-ras^{V12}) or empty vector (pCSGW vector) in hypoxic or normoxic conditions for 12 h. * $p < 0.05$, ** $p < 0.01$.

Figure 3. Knockdown of ADM suppresses growth of colon tumor xenografts

SW480 cells (5×10^6) stably transfected with one of two independent lentivirus-based shADM (#1 and #2) or shRNA non-targeting pLKO.1 vector were injected subcutaneously into the flanks of athymic nude mice (6 weeks old) mice ($n=10$ each group). (A) Representative pictures of gross appearance of xenografts are shown at the end of the observation period. (B) Representative pictures of tumor mass are shown after removal. Tumors from each group were weighed immediately after removal. The average tumor weight is indicated as mean $\pm$ SEM. (C) After 3 days of tumor cell implantation, tumor volume (mm³, mean $\pm$ SEM) was assessed every 3 days. ● shRNA control; ■ shADM #1; ▲ shADM #2. * $p<0.05$, ** $p<0.01$.

Figure 4. Knockdown of ADM induces apoptosis and impairs angiogenesis in colon tumor xenografts

(A) Representative examples of tumor sections stained with H&E, cleaved caspase-3, Ki-67, and CD-31. Insets are necrotic areas marked with lines at 400× magnification. Cleaved caspase-3 and Ki-67 positive stained cells are shown and were analyzed in five 400× magnification fields per section. The micro-vessel density was determined by immunohistochemical stains of the endothelial cell surface marker CD31 at 100× magnification. (B) The number of cleaved caspase-3 positive cells was expressed as relative fold change when compared to control tumors. (C) The percent Ki-67 labeling index was quantified by morphometric analysis and expressed as mean ± SD. (D) The mean number of micro-vessels visualized by CD31 staining was quantified from five microscopic fields in each mouse tumor sample. * $p < 0.05$, ** $p < 0.01$.

Figure 5. ADM regulates colon cancer cell invasion

(A) Cell invasion assays were performed in Biocoat Matrigel Invasion Chambers. 1×10^5 SW480 cells with stable silencing of ADM by two different vectors (shADM #1, shADM #2) were plated and incubated for 24 h. Invaded cells on the bottom of the membrane were fixed and nuclei were stained with DAPI (**left panel**). Cells were counted by fluorescence microscopy in five random high-power fields and are expressed as mean number of visible cells (**right panel**) (B). SW480 cells were transiently transfected with pCMV-3 $\times$ FLAG-ADM or empty vector (C), treated with or without exogenous different doses ADM (1–52) (50, 200 nM) in the lower chamber (D), and the mean numbers of invasive cells were calculated using the same methods described above.

A **B**

K-ras	ADM staining score (n)				p value
	0	1	2	3	
Mutant	0	4	4	6	0.031
WT	6	19	8	9	
Total	6 (11 %)	23 (41%)	12 (21%)	15 (27%)	

Figure 6. ADM is associated with mutant *KRAS* genotype in human CRC

(A) Immunohistochemical staining of ADM in human colorectal cancers. The intensity of ADM staining among primary CRC tissues samples was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) as independently reviewed by a GI pathologist. **Arrows**, ADM staining in the islets of the normal pancreas, normal colon, and colorectal cancer tissues. (B) The relationship between ADM staining level and *KRAS* genotype (Mutant or WT) was analyzed among 56 CRC tissues.