Specific microRNA–mRNA Regulatory Network of Colon Cancer Invasion Mediated by Tissue Kallikrein–Related Peptidase 6

Earphia Sells*, Ritu Pandey†,‡, Hwudaurw Chen†, Bethany A. Skovan†, Haiyan Cui† and Natalia A. Ignatenko‡,*Biochemistry and, Molecular and Cellular Biology Graduate Program, Department of Molecular and Cellular Biology, College of Science, University of Arizona, Tucson, AZ, USA; †University of Arizona, Cancer Center, University of Arizona, Tucson, AZ, USA; ‡Department of Cellular and Molecular Medicine, University of Arizona, Tucson, AZ, USA

Abstract

Metastatic colon cancer is a major cause of deaths among colorectal cancer (CRC) patients. Elevated expression of kallikrein 6 (KLK6), a member of a kallikrein subfamily of peptidase S1 family serine proteases, has been reported in CRC and is associated with low patient survival rates and poor disease prognosis. We knocked down KLK6 expression in HCT116 colon cancer cells to determine the significance of KLK6 expression for metastatic dissemination and to identify the KLK6-associated microRNAs (miRNAs) signaling networks in metastatic colon cancer. KLK6 suppression resulted in decreased cells invasion in vitro with a minimal effect on the cell growth and viability. In vivo, animals with orthotopic colon tumors deficient in KLK6 expression had the statistically significant increase in survival rates (P = .005) and decrease in incidence of distant metastases. We further performed the integrated miRNA and messenger RNA (mRNA) expression profiling to identify functional miRNA-mRNA interactions associated with KLK6-mediated invasiveness of colon cancer. Through bioinformatics analysis we identified and functionally validated the top two up-regulated miRNAs, miR-182 and miR-203, and one down-regulated miRNA, miRNA-181d, and their seven mRNA effectors. The established miRNA-mRNA interactions modulate cellular proliferation, differentiation and epithelial–mesenchymal transition (EMT) in KLK6-expressing colon cancer cells via the TGF-β signaling pathway and RAS-related GTP-binding proteins. We confirmed the potential tumor suppressive properties of miR-181d and miR-203 in KLK6-expressing HCT116 cells using Matrigel invasion assay. Our data provide experimental evidence that KLK6 controls metastasis formation in colon cancer via specific downstream network of miRNA-mRNA effectors.

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Introduction

Metastases present the main cause of mortality in cancer patients. The 5-year survival of patients with CRC drops from 90%, if cancer is detected at localized stage, to 68% if the cancer cells have spread regionally to involve adjacent organs and/or lymph nodes [1]. Despite improvements in early detection and screening, about 20% of patients with CRC present at the incurable metastatic stage.

Although significant progress has been made in identifying a number of key molecules contributing the process of tumor growth and metastatic invasion, the efforts to block cancer metastasis remain...
a challenging step in treating cancer. The invasion-metastasis cascade includes several important steps starting with local invasion of cancer cells from the primary site into surrounding tumor-associated stroma, following intravasation into the lumina of lymphatic or blood vessels, cell survival during circulation, and initiation of tumor cells parenchyma, and growth within blood vessel into the tissue (extravasation) [2]. The list of molecular mediators of the different steps in invasion-metastasis cascade has expanded significantly to include, in addition to the known genetic and epigenetic events, also the different classes of small non-protein coding regulatory RNAs, such as microRNAs (miRNAs), small nucleolar RNAs ( snoRNAs), short interfering RNAs (siRNAs) and small double-stranded RNAs [2–4]. MicroRNAs are 19- to 25-nucleotide-length non-coding RNAs, which negatively regulate gene expression post- transcriptionally through imperfect binding to the 3′ untranslated region (3′UTR) of target messenger RNA leading to inhibition of translation and mRNA destabilization [5,6]. MicroRNAs are dysregulated in many cancers, including colorectal cancer, and can act as tumor suppressors or oncogenes, depending on their targets [7–9]. A number of miRNA networks that function in a synergistic manner to control different cellular pathways have been under investigation [10,11].

Growing evidence indicates that the members of a human tissue kallikrein-related peptidases family of 15 serine proteases, which have diverse physiological functions, are associated with malignancy and have the diagnostic/prognostic applications in cancer [12–14]. The role of kallikreins in cancer pathogenesis is related to their ability to degrade the extracellular matrix proteins and promote angiogenesis [12]. Human kallikrein-related peptidase 6 (KLK6) gene (by old nomenclature protease M/zyme/neurosin) is a member of the human tissue kallikrein family [15]. Biochemical and structural studies show that human KLK6 protein is an active serine protease [16]. KLK6 mRNA expression is elevated in malignant colorectal tissues compared with non-malignant ones and correlates with serum invasion, liver metastasis, advanced Duke’s stage, and a poor prognosis [17–19]. The bioinformatics analysis of expression data from a cohort of colorectal cancer patients, identified the KLK6 gene as one of the top six up-regulated genes associated with the survival of colorectal cancer patients through metastasis related signaling pathways [20]. The mechanism of regulation of KLK6 expression in colon cancer is not fully investigated. Nevertheless, alterations in major cancer driver genes, such as K-RAS oncogene and APC tumor suppressor gene, may contribute to KLK6 up-regulation in colon cancer [21,22]. The possibility of post-transcriptional regulation of KLK6 by microRNAs, i.e., miRNA let-7f, has been also reported [11,23]. Here we investigated the miRNA-based mechanism of regulation of invasion in KLK6-expressing colon cancer cells with high metastatic potential. We have designed the specific KLK6-mediated miRNA-mRNA network which regulates the expression of genes controlling cellular proliferation, differentiation and epithelial-mesenchymal transition (EMT) through TGF-β signaling pathway and RAS-related GTP-binding proteins.

Materials and methods

Cell lines

The HCT116 parental cells, HCT116 Control and shKLK6 cell lines (described in more details below) were used in this study. HCT116 parental cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 mg/L glucose, 1-glutamine w/o sodium pyruvate, supplemented with 10% FBS and 1% penicillin/streptomycin. HCT116 Control and shKLK6 cells were maintained in DMEM media described above with addition of selection marker puromycin at the concentration of 0.5 μg/ml of media (Thermo Fisher Scientific, Inc.).

Generation of HCT116 Isogenic Cells with KLK6 Knockdown

In order to knock down KLK6 expression, SureSilencing sh-hairpin RNA plasmids targeting four different regions of the KLK6 gene (shKLK6 plasmids), as well as a negative control plasmid expressing a scrambled shRNA sequence, were used (SureSilencing shRNAs, Qiagen, Inc.) (Supplementary Figure 1A). Plasmids were transfected into HCT116 colon cancer cell line using LipofectAMINE 2000 according to the manufacturer’s instructions. Forty-eight hours after transfection cells were switched to selection medium containing 0.5 μg/ml puromycin. Selection medium was changed every two days until only cells expressing control and shKLK6 plasmids grew. Initial screening of HCT116 shKLK6 isogenic cell lines was done using quantitative RT PCR (qPCR) and KLK6 ELISA.

Cell Growth

HCT116 control clone 1 and shKLK6 clones were plated in six-well plates at a concentration of 0.1 x 10⁶ cells/well and incubated for 37°C in 5% CO₂. All cells were plated in triplicate. The cells were grown for at least 10 days. The cells were harvested and total cell concentration and viability were determined using the trypsin blue dye exclusion method on a Vi-Cell Series Cell Viability Analyzer (Beckman Coulter, Inc.). Determination of population doubling time was performed by plotting viable cell number vs. time (days). Data were derived from three independent experiments.

RNA Isolation

Total RNA was extracted from HCT116 Control and shKLK6 clones 48 hours after subculture using miRVana miRNA isolation kit (Ambion, Austin, TX). RNA purity was assessed using a Nanodrop spectrophotometer (ND-2000; Thermo Scientific, Pittsburgh, PA). All RNA samples prepared for experiments had 260/280 absorption values between 1.8 and 2.0. Integrity of the isolated RNA was also evaluated by using a standard 1% formaldehyde agarose gel and confirmed by using the Agilent Technologies’ 2100 Bioanalyzer (Agilent Technologies). The RNA Integrity (RIN) Numbers generated were between 8.5 and 10 (Intact and non-fragmented RNA) (UA Genomic Core Facility). Microarray Hybridization

Affymetrix Human ST 1.0 array and Affymetrix miRNA 2.0 chips were used to quantify mRNA and miRNA in total RNA from the HCT116 Control clone 1 cells and shKLK6-3 clone 3 cells. RNA labeling and hybridization, as well as array processing and scanning were performed by Asuragen, Inc., using standard procedures with normalization performed using the Variance Stabilization Normalization (VSN) method.

mRNA and miRNA Data Processing

Raw data files from gene ST 1.0 array hybridization were processed using BioConductor [24] Affymetrix modules to perform background subtraction and quantile normalization using the Robust MultiChip Algorithm (RMA). Quality control analysis was performed on the chips that included correlation plots, density plots, box plots and
RNA degradation analysis. Analysis of Variance was used to estimate differential mRNA expression between samples using the BioConductor Limma module [25]. Limma analysis provides an empirical Bayesian method to improve variance estimation and corrects for multiple hypothesis testing by the Benjamini Hochburg False Discovery Rate method. mRNA with adjusted P values lower than .05 and log odds score provided by B-statistics greater than 3.0 were considered significantly changing. miRNA differential expression analysis between two groups was done using statistical analysis of variance test by Asuragen, Inc., and miRNAs with P value lower than .05 were considered significant.

**Quantitative Real-time PCR**

Total RNA was isolated as described above. Reverse transcription to produce cDNA template was completed using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Part #4368814). qPCR was performed using Taqman® probes (Applied Biosystems) specific for the miRNAs of interest: hsa-miR-181d (TM:001099), -miR-182 (TM:000483), and -miR-183 (TM:001119). Data were analyzed using a Student’s t-test when comparing two groups and one-way ANOVA. The significance level of statistics was set at $P = .05$.

**Western Analysis**

Cells were harvested by trypsinizing for 2 minutes and subsequently pelleting at 1000 X g for 5 minutes at 4°C. Cells were washed (3×) with ice-cold PBS, lysed on ice (30 minutes) in RIPA lysis buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with 1 tablet Complete Mini protease inhibitor and 1 tablet PhoshoSTOP (30 μg/ml aprotinin, 100 mM sodium orthovanadate and 10 mg/ml phenylmethylsulfonyl fluoride at 1 tablet each per 10 ml RIPA lysis; Roche), and scraped into microcentrifuge tubes. Total proteins in the supernatant were isolated by centrifugation (10,000g, 4°C) for 10 minutes and quantitatively analyzed by using the Bio-Rad DC protein assay kit. Equal amounts (50 μg) of the protein were separated on Mini-PROTEAN TGX Bio-Rad Precast Gels Any-kD (Criterion TGX Stain-free, Cat#5678123; Bio-Rad laboratories, Inc.) and transferred to Hybond-C nitrocellulose membrane (GE Healthcare Life Sciences) for 1 hour. After blocking with Blotto A (5% w/v non-fat milk, 0.1% Tween 20, and Tris-buffered saline (TBS) consisting of 10 mM Tris–HCl, pH 8.0, 150 mM NaCl) for 1 hour at room temperature. The primary and secondary antibodies used in this study and blotting conditions are presented in Supplementary Table 1. The primary antibodies were incubated with the membrane (blot) overnight at 4°C. The blots were washed in TBS/0.05% Tween-20. Proteins of interest were detected with an enhanced chemiluminescent detection reagent (Thermo Scientific Super Signal West Pico or West Femto Chemiluminescent Substrate, Cat#34077 or 34,095, respectively) and exposed to film (Denville Scientific Inc., HyBlot CL, Autoradiography film, Cat#E3012).

All Western blots were repeated at least two times. Image processing program, Image J (NIH) was used for evaluation of western blot bands intensity where specified (Image J, http://rsb.info.nih.gov/ij/docs/menus/analyze.html#gels). Bands of the protein of interest were normalized to β-actin.

**KLK6 ELISA**

An ELISA kits for the detection of human KLK6 in conditioned media were obtained from IBEX (Quebec, Canada) and, after this product was discontinued, from Boster Biological Technology Co., Ltd. (Pleasanton, CA, Cat# EK0818). The assays were performed according to manufacturer’s instructions. The detection range for these kits is: 0.2 to 20 ng/ml (IBEX) and 0.078 to 5 ng/ml (Boster Biological). KLK6 antigen levels are expressed as nanograms per milliliter. The plate was read at 490 nm on an EL800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). In each experiment samples were analyzed in triplicates and all experiments were repeated two or three times. Detection of KLK6 secreted in the serum of SCID animals injected with HCT116 control clone 1 and shKLK6 clone 3 was done by ELISA in the Laboratory of Dr. Eleftherios P. Diamandis, at the Advanced Centre for Detection of Cancer (ACDC) (www.acdclab.org, University of Toronto, Canada).

**Cell Migration/invasion Assay**

HCT116 Control clone 1 and shKLK6 clones 1 to 3 were seeded in 24-well chambers with either control plastic inserts or Matrigel coated inserts with a 0.8 μm pore size (Corning Inc., Corning, NY).
In the lower chamber of each well 0.5 ml of media with 10% FBS was added as a chemoattractant. Cell suspension was prepared at a concentration of 0.5 × 10^6 cells/ml in serum-free medium. 200 μl of cell suspensions (1 × 10^5 cells) was plated into inserts. The cells were allowed to migrate/invade for 48 hours. The media were discarded and inserts were rinsed briefly with 1× PBS and swabbed gently with a cotton tip inside inserts to remove non-migrating or non-invading cells. Subsequently, migrating/invading cells were fixed in 100% methanol for 2 minutes, stained with 1% toluidine blue in 1% borax) for 2 minutes, rinsed twice in ddH2O, swabbed gently again inside with a cotton tip and air-dried. Control inserts were included following similar treatment. The stained membranes were cut from the inserts and the incorporated toluidine blue was dissolved in 200 μl of 0.1 M citric acid in a 96-well plate while shaking for 5 minutes on a high-speed titer plate shaker and quantified at 560 nm. The samples were transferred to a new 96-well plate and read at 560 nm on a Synergy 2 Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). Each migration/invasion experiment was carried out in sextuplet and was repeated twice.

**In Vitro KLK6 Rescue Experiment**

The pcDNA3.1 KLK6 plasmid, expressing enzymatically active KLK6 [26] was generously provided by Dr. Georgios Pampalakis and Dr. Georgia Sotiropoulou, Department of Pharmacy, University of Patras, Greece. Control clone 1 and shKLK6 clone 3 cells were seeded in 100 mm² Petri dishes at the concentration of 1.2 × 10^6 cells. Twenty-four hours after subculture cells were transfected with pcDNA 3.1 KLK6 plasmid or empty pcDNA3.1 using LipofectA-MINE 2000 reagent according to the manufacturer’s instructions. Twenty-four hours after transfection (48 hours after subculture) cells were trypsinized and seeded into the Matrigel invasion chambers as described above. Cells were allowed invade for another 48 hours and then were processed for colorimetric assay as described above in cell migration/invasion assay.

**miRNA Mimic Transfections Experiment**

HCT116 parental cells were seeded in 100 mm² Petri dishes at 1.5 × 10^6. The next day cells were transfected with specific miRNAs (miR-181d, miR-182-3p and miR-203) or scrambled miRNA control at the concentrations of 75 nM (SwitchGear Genomics, Menlo Park, CA) using DharmaFect Duo transfection reagent (GE Healthcare Dharmacon, Inc., Lafayette, CO) according to manufacturer’s instructions. The miRNA mimics sequences are listed in Supplementary Table 2. Forty-eight hours later, conditioned media was collected for KLK6 ELISA and cells were processes for Western blot analysis and migration/invasion assays as described above. For each miRNA mimic, migration and invasion assays were done in sextuplet and repeated twice.

**3’ UTR Luciferase Reporters with miRNA Mimic Analysis**

HCT116 parental cells were plated in 24-well plates at 0.12 × 10^6 cells per well and transfected with 300 ng/μl per well of GoClone 3’ UTR reporter constructs (PAM 3’ UTR, FOS 3’ UTR and EHF 3’ UTR (SwitchGear Genomics, Menlo Park, CA) and 50 nM of miRNA mimics (miR-181-3d and miR-182-3p) or non-targeting miRNA controls using DharmaFect Duo transfection reagent, according to the manufacturers’ instructions. The following controls were used: control plasmid encoding the 3’UTR sequence of the housekeeping gene glyceraldehyde 3’-phosphate dehydrogenase (GAPDH) and the Random 3’UTR plasmid, containing non-conserved, non-genic and non-repetitive human genomic fragments. Twenty-four hours after transfection, plates were removed from incubator, rinsed once with PBS and analyzed using the manufacturers’ recommended protocol (LightSwitch Assay, SwitchGear Genomics). Luminescence signal was detected using a luminometer (Synergy 2 Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). Each experiment included transfections of miRNA mimics and control plasmid in triplicates.

Normalization was done according to the manufacturers’ recommendations below: http://switchgeargenomics.com/sites/default/files/pdf/LightSwitch_3UTRnorm.pdf. Statistical analysis was done in each experiment independently.

**Orthotopic Colon Cancer Model**

In vivo experiments were conducted on 6 to 7 weeks old Severe Combined Immunodeficient (SCID) mice (CB-17/IcRAGcid, which were originally purchased from Taconic Inc. (Germantown, NY). The SCID mouse colony is housed at animal facility in the University of Arizona Cancer Center. The facility is AAALAC accredited, and holds an Animal Welfare Assurance # with the Public Health (File#A-3248-01). Mouse colonies were maintained under microisolation and were handled and manipulated only in laminar flow hoods. All procedures involving animals were conducted according to the University of Arizona Animal care and Use Committee policies.

For the animal survival study, Control clone 1 and shKLK6 clone 3 clonal cells were injected at the concentration of 2 × 10^6 cells in 50 μl of saline solution into the cecum between the mucosa and the muscularis externa layers of the cecal wall of anesthetized mice (15 animals per experimental group) by using a 30-gauge needle. Animals were observed twice weekly for adverse signs associated with tumor growth (e.g., >20% body weight loss, immobility, loss of grooming), at which time mice were euthanized. Metastatic colonization and tumors formed were counted postmortem.

**Statistics**

Statistical analysis of cell culture experimental data was performed using paired t-test or ANOVA (Microsoft Excel, MicrosoftCorp.). Statistical analyses comparing rates of tumor formation between the groups were determined using two-way ANOVA followed by Bonferroni tests. The Kaplan–Meier curve for survival was estimated by groups. The long-rank test was employed to test the difference in the survival curves. Fisher’s Exact test was employed to test the difference in incidence of metastases between genotype groups.

**Results**

**Knockdown of KLK6 in HCT116 Cells**

To investigate the significance of KLK6 expression in metastatic colorectal cancer, we developed isogenic cell lines of HCT116 colon cancer cells with stable knockdown of KLK6 expression using the commercially available KLK6 silencing plasmids (Supplementary Figure 1A) as described in Materials and Methods. To confirm efficiency of knockdown of KLK6, we first examined mRNA levels in bulk transfections of HCT116 cells with negative control plasmid and shKLK6 plasmids 1 to 4 using quantitative real-time PCR (qRT-PCR). We found that KLK6 transcript levels in cells treated with shKLK6 plasmids 2 to 4, but not shKLK6 1, were significantly
lower compared to negative control plasmid (*P < .01, ***P < .0004 and **P < .002, respectively) (Supplemental Figure 1B). Because the shKLK6 plasmid 3 (shKLK6-3) produced the most significant inhibition of KLK6 transcript, we isolated individual clones from this cell line as well as from HCT116 cells transfected with negative control plasmid. Clonally selected negative control clones (Control clones 1 and 3) and shKLK6-3 clones (named as shKLK6 clones 1, 2, and 3) were evaluated further for expression of KLK6 at the level of transcription and protein secretion. At 48 hours after subculture, KLK6 transcript levels in shKLK6 clone 2 and clone 3 were suppressed by 80% compared to Control clones 1 and 3 (Figure 1A, **P < .02), while shKLK6 clone 1 showed approximately a 50% reduction (*P = .05). The suppression of KLK6 expression in shKLK6 clone 2 and shKLK6 clone 3 was efficient and prolonged since even at 7 days after subculture the levels of KLK6 in conditioned media from these shKLK6 clones remained significantly low (Figure 1B, *P ≤ .05, **P < .02). A significant decrease of the KLK6 intracellular levels in shKLK6 clones 2 and 3 compared to HCT116 parental cell line and Control clone 1 corroborated with the decrease in KLK6 transcription in shKLK6 clones (Figure 1C). These data validated silencing of endogenous KLK6 transcript in HCT116 cells isogenic clones expressing the shKLK6-3 plasmid.

Effect of KLK6 Knockdown on HCT116 Cell Growth, Migration and Invasion In Vitro

We assessed the growth of Control clone 1 and shKLK6 clones 1 to 3 by counting viable cells at different days after subculture using the trypan blue exclusion method. The statistically significant growth inhibition was noted for shKLK6 clone 2 on Days 4 and 6 after subculture but this clone grew comparable to others at later times (Supplementary Figure 2). Next, we evaluated the effect of KLK6 knockdown on cell migration and invasion using Boyden migration/invasion chambers with the basement membrane-like substrate Matrigel. Cells were seeded either on control or Matrigel-coated inserts and allowed to migrate/invasive for 48 hours. The migratory and invasive abilities were both suppressed in tested shKLK6 clone 2 and shKLK6 clone 3 compared to Control clone1 and Control clone 3 which expressed and secreted significant levels of KLK6 (Figure 2A). Migration through control inserts, *P < .05 and Figure 2B. Invasion through Matrigel-coated inserts, **P < .03. To confirm that KLK6 expression rescued the invasive phenotype of HCT116 cells, we re-expressed the full-length KLK6 plasmid (pcDNA3.1 KLK6) in shKLK6 clone 3 cells. The shKLK6 clone 3-transfected cells were seeded on Matrigel-coated inserts and allowed to invade for 48 hours. We observed a significant increase in the invasiveness of shKLK6 clone 3 cells transfected in comparison with the mock-transfected shKLK6 cells (Figure 2C). Further, we measured the levels of KLK6 protein secreted into the conditioned media by the mock-transfected or KLK6-transfected shKLK6 clone 3 cells seeded on Matrigel. The shKLK6 clone 3 transfected with pcDNA3.1 KLK6 plasmid secreted 10-fold more KLK6 than the mock transfected cells (Figure 2D). These results confirmed that KLK6 expression and secretion is necessary to restore the invasive phenotype of HCT116 cells.

Effect of KLK6 Knockdown in an Orthotopic Colon Cancer Mouse Model

The Control clone 1 and shKLK6 clone 3 were selected for testing the consequences of KLK6 knockdown in vivo. Cells were injected into the cecum of SCID mice, and animal survival rates, as well as the presence and location of metastases were assessed, when animals were sacrificed. The duration of this experiment was 125 days. Animals in Control clone 1 group were becoming morbid earlier during the course of the experiment and the majority of them had to be sacrificed before the shKLK6-injected group (median survival time of Control clone 1 group was half of shKLK6 clone 3 group, as shown by Kaplan-Meier survival curve, Figure 3A, P = .0005). Since KLK6 is a secreted protein and can be detected in CRC patients [17], we measured KLK6 levels in the serum of tumor-bearing animals at days 10 through 35 after cell injection with 7 to 10 days interval between samples collection (samples were collected retro-orbitally). Animals
injected with shKLK6 clone 3 had significantly lower levels of KLK6 in the serum compared to animals injected with Control clone 1 cells (Figure 3B, *P < .01). Upon sacrifice, no difference was found in the primary tumor weights between animals injected with Control clone 1 and shKLK6 clone 3 (average weight 0.46 ± 0.36 vs. 0.57 ± 0.24, respectively). At the same time, the analysis of the severity of metastatic disease showed that animals injected with Control clone 1 developed significant number of metastases in the abdominal wall, mesentery, diaphragm, and liver, showing the similar pattern of dissemination as observed in CRC cancer patients. In contrast, shKLK6 clone 3 injected animals developed less metastasis to the diaphragm and mesentery wall (Table 1, *P = .0068 and **P = .0 3 7 3 , respectively). No macroscopically visible metastasis was detected in the abdominal wall of shKLK6 clone 3-injected animals, although this difference did not reach statistical significance due to variability among injected animals. The tumor spreading to the colon, which is near the primary site of injection (cecum), was comparable in shKLK6 clone 3 and HCT116 control clone 1 injected animals (Table 1). Taken together, these data suggest that KLK6 mediates distant colon cancer metastasis.

Identification and Correlation of miRNAs and mRNAs Differentially Expressed in KLK6 Knockdown Cells

Since in vitro and in vivo analyses both suggest the direct role of KLK6 in invasion and metastasis, we sought to identify genes and miRNAs involved in KLK6-mediated colon cancer metastasis formation. We hypothesized that a subset of genes may regulate the metastasis in the presence of the elevated KLK6 level in colon cancer cells. To test this hypothesis, we performed concomitant miRNA and mRNA expression profiling of HCT116 control clone 1 and shKLK6 clone 3 using the Affymetrix Human Gene 1.0 ST (28,869 well-annotated genes) and Affymetrix GeneChip miRNA 2.0 arrays (1105 human mature miRNAs), respectively.

Gene Expression Results. More than 300 genes were found to be significantly altered in KLK6 knockout cells when compared to Control clone 1 cells. We focused on top 50 genes that were changing significantly based on the adjusted P value as shown in Table 2 and depicted in Supplemental Figure 3A. In this set, 36 gene transcripts were significantly down-regulated and 14 were up-regulated.

miRNA Expression Results

Based on the statistical analysis we found 30 miRNAs altered in KLK6 knockout cells compared to Control clone 1 cells (Table 3). Among the differentially expressed miRNAs, 21 were significantly down-regulated and nine were up-regulated (Supplemental Figure 3B).

Correlation of miRNAs and Their Targeted mRNAs. Probes with significant expression changes from mRNA and miRNA array experiments were analyzed further to detect miRNA-mRNA target pairs and their inverse correlation. Multiple methods were used to detect the mRNA and miRNA target pairs. Bioconductor package Rmir was used to search TargetScan and pictar databases for the differential expressed mRNA and miRNA. Further predicted human
target mRNAs and miRNAs were extracted from TargetScan 5.2 [4] and microRNA.org (version 8-2010) [27]. The microRNA.org resource comprises predictions computed by the mirSVR-miRanda algorithm [28]. The only predictions that were considered, were those annotated as ‘conserved miRNA’ and ‘good mirSVR score’. For the analysis, the intersection between microRNA.org and TargetScan predictions were taken into account. The applied bioinformatics tools identified six of the most significantly altered miRNAs and 37 gene transcripts targeted by those miRNAs (Figure 4A).

### Table 1. The incidence of metastases in HCT116 control clone 1 (n = 15 mice) and shKLK6-3 clone 3 (n = 15 mice)

| Location     | Control clone 1 | shKLK6 Clone 3 |
|--------------|-----------------|----------------|
| Liver        | 53.33%          | 18.75%         |
| Diaphragm    | 40.00%          | 0.00%*         |
| Mesentery    | 40.00%          | 6.25%**        |
| Colon        | 13.33%          | 12.50%         |
| Abdominal wall | 26.67%          | 0.00%          |

* P = .0068. ** P = .0373 by Fisher’s exact test.

#### Experimental Validation of Bioinformatic Predictions

From the set of six altered miRNAs described above, we selected three the most significantly altered microRNAs, miR-181d, miR-182 and miR-203, to further validate and explore their selected targeted genes. We confirmed that miR-181d was significantly down-regulated and miR-182, and miR-203 were significantly up-regulated (*P < .0001) (Figure 4B). The genes predicted to be targeted by these miRNAs were identified using bioinformatics resources, such as microRNA.org and TargetScan.org, and are depicted in Figure 4C. The sequence alignments of selected miRNAs with the 3′UTR region of candidate target genes based on TargetScan.org search results are shown in Supplementary Figure 4A. The miR-181d is predicted to target the 3′UTR of FRα murine osteosarcoma viral oncogene homolog (FOS), peptidylglycine alpha-amidating monooxygenase (PAM), and chondroitin sulfate proteoglycan versican (VCAN), at single locations, and Ets Homologous Factor (EHF) miRNA at two locations. For miR-182, predicted targets are 3′UTRs of EHF at one location, and TGFβ2 at two locations. The putative sites of miR-203 targeting were identified in 3′UTRs of Ric-related gene expressed throughout the organism (RIT1), transforming growth factor beta 2 (TGF-β2) all at single locations and the Runt-related transcription factor 2 (RUNX2) 3′UTRs at two locations. The relative positions of the predicted seed sequences in 3′UTRs of target genes are depicted in Supplementary Figure 4B. The supplementary Figure 4B implies that one gene can be regulated by multiple miRNAs, which can produce either co-operative or counteractive effects on expression of the targeted genes. We further validated the alterations of selected transcripts in shKLK6 clone 3 by qPCR (Figure 4D). Table 4 summarizes the validated altered miRNA-mRNAs sets and their cellular functions.

#### Functional Validation of Selected miRNA-mRNA Interactions

**a. Validation of predicted microRNA-targeted genes using 3′UTR reporter constructs.** Co-transfection of miRNA mimics (Switchgear Genomics, Inc.) and commercially available 3′UTR luciferase reporter plasmids (Switchgear Genomics, Inc.) was done in the HCT116 parental cell line. The GoClone reporter plasmids carrying the full length 3′UTR sequences of EHF, FOS or PAM RNAs were co-transfected with miR-181d and miR-182 mimics into HCT116 cells and luciferase activity was measured 24 h later. The negative control plasmid encoding the 3′UTR of the glyceraldehyde 3′-phosphate dehydrogenase (GAPDH) sequence was used to normalize the luminescence values. The results showed a significant decrease in luciferase activity of the tested 3′UTRs that confirms the bioinformatics results that miR-181d can target the EHF, FOS and PAM 3′UTRs and miR-182-3p can interact with the 3′UTR of EHF (Figure 5A). However, the two putative miR-181d binding sites found in silico within the EHF 3′UTR (Supplementary Figure 4B) suggests that further assays need to be performed to determine which or if both predicted sites are targeted.

**b. Validation of selected miRNA-targeted proteins by Western blotting.** We measured levels of the selected proteins predicted to be targeted by miRNAs-181d, -182 and -203 after treatment with their respective miRNA mimics. We confirmed the altered expression (on average two-fold decrease in expression levels) of the following targeted proteins: FOS and VCAN, which are predicted to be targeted by miR-181d (Figure 5B, left panel); TGF-β2, RIT1, VCAN, RUNX2, which are predicted to be targeted by miR-203 (Figure 5B, right panel).
Because the TGF-β signaling pathway is one of the major signaling pathways inducing the EMT in cancer [29] and TGF-β2 was identified as a putative target of miR-203, we explored further the effect of miR-203 on TGF-β-mediated EMT. We measured the TGF-β2 protein level in HCT116 cells upon transfection with miR-203 mimic and observed miR-203-dependent suppression of the TGF-β2 protein level (~1.8-fold decrease on average) (Figure 5C). The other two proteins tested were transcription factors involved in the EMT program: Snail1 (also known as Snail) and the high-mobility group AT-hook 2 (HMGA2). Snail is the major transcription factor downstream of TGF-β that controls expression of other epithelial molecules, such as claudins, occludins, and mucin 1 and suppresses them during transition to the mesenchymal and invasive phenotype [30]. To demonstrate that the TGF-β signaling is intact in HCT116 cells we treated cells with TGF-β2 ligand, and showed more than 2-fold induction of Snail protein, which was reversed by miR-203 mimic (Figure 5C). MiR-203 has been reported to regulate the expression of Snail through two binding sites within its 3′UTR and also controls Snail expression via a specific double stranded miR-203-3p target site in the 3′UTR of VCAN mRNA, the level of VCAN protein was found to be suppressed by miR-182-3p mimic (Figure 5B). At the same time, the level of TGF-β2 protein, which is predicted to be targeted by miR-182, was found to be elevated. These observations reflect the complexity of interactions among miRNAs and their predicted targets and may be explained by changes in ratios of different miRNAs available for binding to the target mRNAs at the specific time.

Because the TGF-β signaling pathway is one of the major signaling pathways inducing the EMT in cancer [29] and TGF-β2 was identified as a putative target of miR-203, we explored further the effect of miR-203 on TGF-β-mediated EMT. We measured the TGF-β2 protein level in HCT116 cells upon transfection with miR-203 mimic and observed miR-203-dependent suppression of the TGF-β2 protein level (~1.8-fold decrease on average) (Figure 5C).
binding sites for miR-203 within the 3′UTR of HMGA2 mRNA; therefore, we attributed the observed suppression to the downstream signaling through TGF-β, which was previously reported [33]. The phase-contrast images of HCT116 cells treated with miR-203 mimic at concentration of 75 nM demonstrate that miR-203 promotes epithelial-like features in HCT116 cells pre-treated with TGF-β ligand (Supplementary Figure 5).

**Transient Transfection of miR-181d or miR-203 Inhibits KLK6 Expression and Cell Invasion**

The above validation of miRNA targets was done in HCT116 colon cancer cells which express significant amount of KLK6 endogenously [34]. Here we investigated how the level of endogenous KLK6 is modulated by the selected miRNAs identified in KLK6 knockdown cells. We measured the intracellular and secreted levels of KLK6 protein upon treatment with miR-181d, miR-182 and miR-203 mimics. We found that the 48-hour treatment with the individual miRNA mimics at the concentration of 75 nM caused the decrease of both the intracellular and secreted KLK6 levels (Figure 6A and B (*P ≤ .02), respectively). The intracellular level of KLK6 protein was not affected when cells were pretreated with the TGF-β ligand alone, but increased slightly upon addition of miR-203, while the secreted KLK6 was significantly decreased in the presence of TGF-β ligand and miR-203 (**P = .002) suggesting that miR-203 may also inhibit KLK6 protein secretion. Next, we conducted Matrigel invasion assays with selected miRNAs and observed decrease in cell invasion after transfection with miR-181d and miR-203 mimics, but not the miR-182-3p mimic (Figure 6C, *P ≤ .002). The HCT116 cells migration was inhibited after transfection with miR-203 mimics at two different concentrations (Supplementary Figure 6C), but miR-181d and miR-182 did not alter cell migration (data not shown). In silico analysis identified a poorly conserved

| miRNA Symbol | Mean_HCT116_control | Mean_shKLK6 | LogFC  | P         |
|--------------|---------------------|-------------|--------|-----------|
| hsa-mir-718  | 5.294974654         | 6.750364124 | 1.45908847 | 0.045879181 |
| hsa-mir-203  | 7.029301149         | 8.020819799 | 0.99151865 | 0.008956982 |
| hsa-mir-182  | 10.2932168          | 11.1547809  | 0.86125933 | 0.015434767 |
| hsa-mir-548 b-4 | 2.884592843     | 3.622938972 | 0.73891133 | 0.04964134  |
| hsa-mir-183  | 7.582421649         | 8.317662777 | 0.734641128 | 0.008237583  |
| hsa-mir-3118-1 | 2.68656791         | 3.141930151 | 0.45527366 | 0.093966262  |
| hsa-mir-3118-2 | 2.68656791         | 3.141930151 | 0.45527366 | 0.093966262  |
| hsa-mir-3118-3 | 2.68656791         | 3.141930151 | 0.45527366 | 0.093966262  |
| hsa-mir-1278  | 3.244512972         | 3.6993644   | 0.454812428 | 0.032830919  |
| hsa-mir-648   | 5.27078224          | 5.085925032 | -0.41153919 | 0.038104121  |
| hsa-mir-548 b-3 | 3.327951758     | 2.866221129 | -0.61730863 | 0.045667792  |
| hsa-mir-4270  | 9.329300958         | 8.843975171 | -0.485325786 | 0.04746584  |
| hsa-mir-1301  | 6.74950824          | 6.18688073  | -0.55817751 | 0.04917560   |
| hsa-mir-3116-2 | 4.051619554        | 3.411182752 | -0.60466802 | 0.09766037   |
| hsa-mir-1276  | 4.90115372          | 3.834323045 | -0.55792327 | 0.053190054  |
| hsa-mir-214   | 8.95040913          | 8.237959651 | -0.68085379 | 0.027345777  |
| hsa-mir-668   | 4.73429596          | 4.015324807 | -0.718971153 | 0.03354443   |
| hsa-mir-330   | 5.78598752          | 5.09805273  | -0.720153479 | 0.03403607   |
| hsa-mir-1208  | 5.02469786          | 4.275301936 | -0.74934875 | 0.062634766  |
| hsa-mir-181B-1| 8.77301975          | 7.921179422 | -0.851842533 | 0.026985615  |
| hsa-mir-4277  | 3.988026543         | 3.029042193 | -0.958984351 | 0.017739471  |
| hsa-mir-181B-2| 8.917382218         | 7.948311081 | -0.969071138 | 0.016960484  |
| hsa-mir-4294  | 5.727053762         | 4.749509452 | -0.97547818 | 0.03354712   |
| hsa-mir-3163  | 5.57470523          | 4.545762998 | -1.028966525 | 0.03174295   |
| hsa-mir-1256  | 3.825993651         | 2.777575511 | -1.052418141 | 0.03926028   |
| hsa-mir-181d  | 8.103003726         | 6.894016758 | -1.20896967 | 0.018355356  |
| hsa-mir-3127  | 6.79589692          | 5.583243032 | -1.212638888 | 0.052905044  |
| hsa-mir-3161  | 5.188483579         | 3.915881448 | -1.272604131 | 0.010378268  |

Figure 4. Validation of selected miRNAs and their predicted target genes by qRT-PCR. (A) Heat map of six miRNAs and their predicted targeted 37 genes. (B) Conformation of down-regulation of miR-181d and up-regulation of miR-182 and miR-203 in KLK6 knockdown cells. *P < .0001. (C) Relationship between validated altered miRNAs and their predicted target genes. (D) Conformation of altered expression of selected genes *P ≤ .05, **P ≤ .02, ***P = .002 by ANOVA.
7-mer seed binding region for miR-203, but not miR-181d or miR-182 in the KLK6 3′ UTR, (Supplementary Figure 6A). The ability of KLK6 3′ UTR to interact with miR-203 was tested in co-transfection experiment with the luciferase reporter vector carrying KLK6 3′UTR and miRNAs mimics. This functional analysis showed that miR-203, but not miR-181d and miR-182, is capable of binding to KLK6 3′UTR (Supplementary Figure 6B). Thus, decrease of the intracellular KLK6 protein in the presence of miR-181d and miR-182 mimics suggests rather indirect involvement of these miRNAs in KLK6 expression and secretion.

Discussion

We have previously shown that blocking of KLK6 expression in HCT116 cells by using small interference KLK6 RNA or KLK6-specific antibody decreased the ability of cells to migrate and invade though Matrigel [21]. The goal of the current study was to investigate the role and mechanism of KLK6-regulated cell invasion in colon cancer. To study KLK6 role in invasion we generated HCT116 isogenic cell lines with stable KLK6 knockdown (shKLK6 clones). Down-regulation of KLK6 in knockdown clones was confirmed at mRNA and protein levels (both expression and secretion). Further, we investigated the consequences of KLK6 inhibition on invasion in vitro, using the Matrigel invasion assay. Knockdown of KLK6 in colon cancer cells did not alter the cell growth, but suppressed the migration and invasion in vitro. Analysis of KLK6 knockdown in vivo was done using the SCID orthotopic colon cancer model. The animal study showed that the Control clone...
and shKLK6 clone 3-injected mice developed comparable colon tumors at the site of injection (cecum), but the shKLK6 tumors were not able to metastasize into distant organs (diaphragm and mesentery wall). As a result, the shKLK6-injected animals had doubled their survival rates, compared to the Control group ($P = .005$).

Metastasis is a complex process of interactions between transformed cells of the primary tumor, extracellular matrix components, blood and lymph vessels, and cells in the surrounding tissues, which provide growth factors and hormones. To understand the mechanism of KLK6-mediated metastatic progression in colon cancer we decided to focus on microRNAs, which have been linked to the different aspects of cancer pathology, including a metastatic disease [3,35,36]. MiRNAs regulate gene expression post-transcriptionally through binding to specific sites within the 3′UTR regions of targeted genes. Significant evidence collected in recent years demonstrates the essential role of mRNAs in the progression of colorectal cancer and suggests the use of miRNAs as possible CRC biomarkers [37]. In our study we applied Affymetrix mRNA and miRNA arrays to identify the miRNAs and their target genes which play an essential role in KLK6-driven cell invasion. Through bioinformatics analysis we initially selected a set of top six the most significantly altered miRNAs and 37 gene transcripts associated with the altered expression of KLK6. The miR-181d was found to be the most significantly suppressed miRNA in KLK6 knockdown cells (logFC $-1.209$, $P = .0183$). The miRNA-181d (Chr19p13.13) is located within the largest miRNA cluster of 43 miRNAs on chromosome 19. The most significantly up-regulated miRNAs in KLK6 knockdown cells were also identified: miRNA-182 (Chr7q32.2, logFC 0.861, $P = .015$) and miRNA-203 (Chr14q32.33, logFC 0.992, $P = .009$). All these miRNAs are transcribed from intergenic regions, where an individual miRNA gene or a cluster of miRNAs (in case of miR-181 and miR-182) form an independent transcriptional unit. We confirmed altered expression of miRNAs-181d, −182 and −203 and validated their effects on predicted target genes either by co-transfection of miRNA mimics with 3′UTR luciferase reporter constructs or by Western blot analysis of targeted proteins. Further, we evaluated the ability of these miRNAs to alter cell invasion and found that treatment with miR-181d and miR-203 mimics significantly suppressed HCT116 cells invasion through Matrigel.

The level of miR-181d was found to be suppressed in HCT116 cells upon KLK6 knockdown. We tested miR-181d status in another colon cancer cell line, Caco-2, which does not express KLK6 and does not form metastasis in vivo, and found that miR-181d level in this cell line was significantly higher than in HCT116 cells (data not shown).

Table 4. Summary of validated miRNAs and mRNAs and their metastasis-associated cellular functions

| miRNA    | Validated miRNA expression | Predicted RNA expression | Validated RNA expression | Involvement in cellular process                                                                 |
|----------|-----------------------------|--------------------------|--------------------------|-------------------------------------------------------------------------------------------------|
| miR-181d | Down                        | PAM Up                   | Peptidylglycin alpha-amidating monooxygenase. Involve in posttranslational modification of bioactive proteins. |
|          |                              | BAMBI Up                 | BMP and activin membrane-bound inhibitor. Involve in TGF-β signaling inhibition. |
|          |                              | FOS Up                   | FBF murine osteosarcoma viral oncogene. Nuclear phosphoprotein, regulator of cell proliferation, differentiation and transformation. |
|          |                              | VCAN Down                | Versican. Component of extracellular matrix, involve in cell adhesion, proliferation, migration. |
| miR-182  | Up                          | EHF Down                 | ETS homologous factor. Transcriptional activator of a subset of genes that control cell proliferation, transformation and apoptosis. |
| miR-203  | Up                          | RT1 Down                 | RAS-like without CAAX. RAS-related GTase, regulates p38 MAPK-dependent signaling. |
|          |                              | RUNX2 Down               | Runt-related transcription factor 2. Essential for osteoblastic differentiation and skelet morphogenesis. |
|          |                              | TGF-β2 Down              | As above. |
|          |                              | VCAN Down                | As above. |

Figure 4. (continued.)
This observation suggests a regulatory feedback relationship between KLK6 and miR-181d in colon cancer cells. Similarly, the levels of miR-181d were found to be low in human glioma samples [38], as KLK6 has been reported to be elevated in patients with the advanced stages of glioma [39]. The ectopic expression of miR-181d in glioma cell lines lead to suppression of cell proliferation, cell cycle arrest and apoptosis [38]. Together these data indicate that miR-181d has a tumor-suppressive properties and may act through regulation of KLK6 expression. Previously done bioinformatics analysis predicted that members of miR-181 family, miR-181b-1, -2/c/d can target...
transfection. Some cell culture plates were also treated with TGF-β ligand (5 ng/ml in a serum-free media) and/or miR-203 mimic. Cells were seeded onto Matrigel coated Boyden chambers 24 h after transfections and allowed to invade for 48 hours. Analysis was done as described in Material and Methods section.

**Figure 6.** Effect of selected miRNA mimics on KLK6 expression and/or secretion and invasion in HCT116 colon cancer cells. (A) Western blot analysis of KLK6 intracellular protein level in HCT116 cells treated with each of selected miRNA mimics at concentration of 75 nM for 48 h after transfections. Some cell culture plates were also treated with TGF-β ligand (5 ng/ml in a serum-free media) and/or miR-203 mimic. β-actin was used as a loading control. Protein bands quantitation was done using Image J software and presented as Relative Intensity Units (RIU, β-actin ratio normalized to scrambled miRNA sample). Figure is a representative of two independent experiments. (B) Concentration of KLK6 in the conditioned media of HCT116 cells upon treatment with selected miRNA mimics by ELISA. *P ≤ .02, **P = .002 by t-test. (C) Matrigel invasion assay of HCT116 cells treated with individual miRNA mimics. Cells were seeded onto Matrigel coated Boyden chambers 24 h after transfections and allow to invade for 48 hours. Analysis was done as described in Material and Methods section *P ≤ .002 by t-test. Another member of serine proteinase family, KLK13, in breast and ovarian cancer; however, there have been no follow-up study to confirm this prediction [23]. Recent miRNA expression profiling study in a breast cancer cell line stably transfected with preproKLK5 revealed a 25.6-fold increase in miR-181c-5p level with a concomitant decrease of ten miRNA targets encoding extracellular matrix proteins [40]. Thus, the regulatory signaling, probably, exists between kallikreins and the members of miR-181 family of miRNAs.

Our results also point to a significant effect of miR-203 on migration and invasion of colon cancer cells. We show that this effect is due to the pivotal role of miR-203 in regulating the EMT through direct targeting of TGF-β2. TGF-β2 stimulates EMT during the early stages of the transition via up-regulation of Snail following the subsequent expression of ZEB1/2, Snail2 (also known as SLUG) and TWIST1 which maintain the mesenchymal phenotype [41,42]. The double-negative feedback loop involving TGFβ, ZEB and miR-200 family, which plays a significant role in the establishment and maintenance of EMT, has been previously identified in invasive ductal carcinomas and also is important for embryonic development and tumor metastasis [43,44]. Recent study in xenograft mouse model of head and neck squamous cell carcinoma showed that treatment with miR-203 mimic can inhibit lung metastasis through suppression of the pro-metastatic proteins involved in extracellular matrix remodeling, cell metabolism and cytoskeleton [45].

HCT116 cell line is a microsatellite unstable colorectal cancer line and has a truncating mutation in the type II TGF-β receptor. Nevertheless, HCT116 cells maintain their sensitivity to the TGF-β signaling, as we showed in the TGF-β2 ligand-treated cells, similar to other microsatellite unstable cell lines [46]. Treatment of HCT116 cells with miR-203 resulted in suppression of TGF-β2, Snail and HMGA2 proteins, which suggests the existence of miR-203-TGF-β2–HMGA2 signaling axis. The HMGA2 expression is implicated in the EMT program through the TGF-β signaling pathway [32] and is associated with a poor survival in colorectal cancer patients [47]. All three miRNA mimics inhibited the levels of intracellular and secreted KLK6 protein when they were introduced transiently, but only miR-181d and miR-203 mimics suppressed the invasion of HCT116 cells through Matrigel. Our bioinformatics analysis shows that miR-203, but not miR-181d or miR-182 could target the KLK6 3′UTR. The posttranscriptional regulation of KLKs through specific miRNAs has been previously suggested [23]. The most conserved predicted miRNAs for KLK6 3′UTR are the members of the let-7 family of miRNAs and none of them were altered in shKLK6 cells in significant manner.

There is a growing interest to understand the complex molecular networks regulated by kallikreins in cancer. Previously done bioinformatics analysis of miRNAs dysregulated in human prostate cancer identified the inverse interactions between 29 miRNAs and 10 members of KLK family with potential clinical applications [48]. The important link has been recently established between KLK6 and specific miRNAs expressed in the luminal and basal-like molecular subtypes of breast cancer that can predict the patients survival outcome [49]. Particularly, KLK6 overexpression in breast cancer cells induced miRNA biogenesis and correlated with the expression of genes involved in cell-cycle control (e.g., CDKN1B, cyclin D), MAPK, Wnt signaling pathways and EMT (e.g., miR-34a). The KLK6-regulated networks reported in breast cancer cells [49] and identified here in the colon cancer cells demonstrate that KLK6 regulates common signaling pathways in epithelial tumors, although
the KLK6-mediated miRNA and mRNA targets within these pathways may differ due to the organ-specific expression and regulation.

In summary, our bioinformatics and experimental approaches provide the analysis of miRNA-mRNA networks regulated through KLK6 in colon cancer cells. In the present study, we profiled miRNAs and mRNAs in the colon cancer cells, which lost their invasive phenotype due to knockdown of expression of KLK6. We established and validated a network of two up-regulated miRNAs (miR-182, miR-203) and one down-regulated miRNA (miR-181d), that regulates expression of genes involved in proliferation, differentiation and post-translational modification (FOS, EHF, PAM), EMT (TGF-β2), RAS (RIT1) and invasion (RUNX2, VCAN) signaling pathways (Figure 7A).

We constructed a regulatory network of validated miRNA and mRNA which demonstrates the complexity of miRNAs interactions with their target genes in colon cancer cells expressing KLK6 (Figure 7B). Based on the predicted interactions, a few dysregulated miRNAs can have a significant effect on multiple signaling pathways, as has been postulated by Gurtan and Sharp [50]. The American Society of Clinical Oncology (ASCO) recommends the development and use of novel tumor marker with high specificity and sensitivity for the prevention, screening, treatment and surveillance of all gastrointestinal cancers and, particularly, colon cancer [51]. The miRNAs, identified in this study, present a promising targets for future anti-metastatic application.

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Appendix A. Supplementary Data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neop.2017.02.003.

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