Non-Muscle Myosin IIA Differentially Regulates Intestinal Epithelial Cell Restitution and Matrix Invasion

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Epithelial cell motility is critical for self-rejuvenation of normal intestinal mucosa, wound repair, and cancer metastasis. This process is regulated by the reorganization of the F-actin cytoskeleton, which is driven by a myosin II motor. However, the role of myosin II in regulating epithelial cell migration remains poorly understood. This study addressed the role of non-muscle myosin (NM) IIA in two different modes of epithelial cell migration: two-dimensional (2-D) migration that occurs during wound closure and three-dimensional (3-D) migration through a Matrigel matrix that occurs during cancer metastasis. Pharmacological inhibition or siRNA-mediated knockdown of NM IIA in SK-CO15 human colonic epithelial cells resulted in decreased 2-D migration and increased 3-D invasion. The attenuated 2-D migration was associated with increased cell adhesiveness to collagen and laminin and enhanced expression of $\beta 1$ -integrin and paxillin. On the 2-D surface, NM IIA-deficient SK-CO15 cells failed to assemble focal adhesions and F-actin stress fibers. In contrast, the enhanced invasion of NM IIA-depleted cells was dependent on Raf-ERK1/2 signaling pathway activation, enhanced calpain activity, and increased calpain-2 expression. Our findings suggest that NM IIA promotes 2-D epithelial cell migration but antagonizes 3-D invasion. These observations indicate multiple functions for NM IIA, which, along with the regulation of the F-actin cytoskeleton and cell-matrix adhesions, involve previously unrecognized control of intracellular signaling and

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In the human intestine, a single layer of polarized epithelial cells creates a protective barrier that separates the body interior from the intestinal lumen. This barrier is capable of withstanding a variety of mechanical, chemical, and biological stressors and represents a dynamic self-renewing entity. The dynamic nature of the normal intestinal epithelium is illustrated by the fact that this epithelial sheet is constantly moving at a speed of 5 to 10 $\mu\text{m}/\text{h}$.¹ This movement plays a vital role in the normal life cycle of intestinal epithelial cells. Indeed, epitheliocytes that originate from stem cells at the bottom of intestinal crypts differentiate and migrate along the crypt-surface axis and eventually shed from the intestinal surface.² In addition to its role in normal intestinal homeostasis, epithelial cell migration significantly contributes to the pathophysiology of intestinal disorders such as inflammatory bowel disease and colorectal cancer. In the former disease, a collective movement of the epithelial sheet plays a major role in closure of mucosal wounds, which is known as epithelial restitution.^{3,4} In the latter pathology, invasion of epithelial cancer cells into underlying tissues results in tumor dissemination.^{5,6} Thus, epithelial cell motility is a fundamental feature of normal intestinal mucosal physiology, mucosal repair, and cancer metastasis.

Cell migration is generally considered as a cyclic process initiated by extension of protrusions in the direction of migration and completed by the retraction of the trailing end of the cell.^{7,8} Reorganization of actin filaments

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drives the entire migration cycle by generating forces to extend membrane protrusions and to move the cell body forward. This reorganization of filamentous (F)-actin is mediated by two major mechanisms: the so-called F-actin "treadmilling" that involves actin polymerization and depolymerization at opposite filament ends, and contraction of filaments driven by the myosin II motor.⁷⁻⁹ Whereas F-actin treadmilling is known to mediate protrusions at the migrating cell front, the roles of myosin II in cell motility appear to be more diverse and involve regulation of protrusion dynamics, cell-matrix adhesions, and forward translocation of the cell body.⁷⁻¹⁰ Therefore, myosin II-driven contractility can be considered as a key mechanism integrating different steps of cell migration.

Myosin II is a motor protein that utilizes ATP to move actin filaments. This motor functions as a heterohexamer composed of two heavy chains and two pairs of light chains.^{11,12} The heavy chain consists of a globular head that binds to actin and hydrolyzes ATP and an extended tail that coils together with another heavy chain tail to form a rigid rod-like structure. The tails of multiple myosin II molecules readily self-associate, creating bipolar myosin aggregates that are crucial for actin filament movement and bundling.^{11,12} Epithelial cells express non-muscle myosin (NM) II, which is characterized by three different heavy chain isoforms: IIA, IIB, and IIC.^{13,14} These isoforms possess a high degree (64% to 80%) of sequence similarity, but have different enzymatic/biochemical properties.^{15,16} As a result, different NM II heavy chains may have either unique^{15,17-20} or interchangeable roles^{21,22} in regulating cell shape, cell adhesion, cytokinesis, and vesicular traffic.

Several recent studies have yielded conflicting data on the involvement of NM II in epithelial cell migration. Thus, pharmacological inhibition of NM II with blebbistatin was shown to attenuate migration of pancreatic and renal epithelial cells,^{23,24} but reportedly did not affect motility of mammary and prostate epithelial cells.²⁵ In other studies, small interfering (si)RNA-mediated knockdown of the NM II heavy chain A isoform (hereafter referred to as NM IIA) was found to suppress migration of mammary epithelial cells²⁶ but enhanced the motility of lung epithelial cells.¹⁹ These contradictory data may reflect peculiar behavior of different cell lines, as well as different experimental conditions used to study cell migration.^{24,27} Despite its biological importance, the role of NM II in migration of intestinal epithelial cells has not yet been studied.

The present study was designed to investigate the role of NM IIA in intestinal epithelial cell migration. This myosin II isoform was shown to be a major generator of traction forces in motile cells¹⁷ and it is abundantly expressed in both well-differentiated epithelial cells¹⁵ and their embryonic precursors.²⁸ The role of NM IIA was examined by using two different models of cell migration: a two-dimensional (2-D or planar) wound closure assay and a three-dimensional (3-D) Matrigel invasion assay, resembling restitution of injured epithelial sheets, and metastatic dissemination of colorectal tumors, respectively. We report that inhibition of NM IIA oppositely affects epithelial cell restitution and invasion via multiple mechanisms that involve alterations in cell-matrix adhesion and F-actin or-

ganization, as well as profound changes in intracellular signaling and protein expression.

Materials and Methods

Antibodies and Other Reagents

The following primary polyclonal (pAb) and monoclonal (mAb) antibodies were used to detect matrix adhesion, cytoskeletal, and signaling proteins by immunoblotting and immunofluorescence labeling: anti-NM IIA pAb (Covance, Berkeley, CA); anti-paxillin, anti- β 1-integrin, anti-focal adhesion kinase (FAK), and anti-vinculin mAbs (BD Biosciences, San Jose, CA); anti-phosphorylated (Tyr118) paxillin, anti-phospho-(Tyr397) FAK, anti-total extracellular signal-regulated kinase (ERK) 1/2, anti-phospho-ERK1/2, anti-total Raf-1, anti-phospho-(Ser338) Raf-1, and anti-matrix metalloproteinase (MMP)-2 pAbs (Cell Signaling Technology Inc., Beverly, MA); anti-calpain-1 and anti-calpain-2 pAbs (Santa Cruz Biotechnology, Santa Cruz, CA); anti-MMP-9 pAb (Upstate Biotechnology, Lake Placid, NY); anti-MMP-2 and anti-MMP-7 mAbs (EMD Chemicals Inc., Gibbstown, NJ); and, anti- β -actin pAb and anti- α -tubulin mAb (Sigma-Aldrich, St. Louis, MO). Alexa-488 or Alexa-568 dye-conjugated, donkey anti-rabbit and goat anti-mouse secondary antibodies, and Alexa-labeled phalloidin, were obtained from Invitrogen (Carlsbad, CA); horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). S(-)-blebbistatin was obtained from Sigma; U0126, PD 098059, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN), and calpeptin were purchased from EMD Biosciences; and GM-6001 was obtained from Biomol International (Plymouth Meeting, PA). All other reagents were of the highest analytical grade and were obtained from Sigma.

Cell Culture

SK-CO15, a transformed human colonic epithelial cell line,²⁹ was a gift from Dr. Enrique Rodriguez-Boulán (Weill Medical College of Cornell University, NY). Caco-2 and T84-transformed human colonic epithelial cell lines, IEC-6, a non-tumorigenic rat intestinal epithelial cell line and COS-7, green monkey kidney epithelial cells were purchased from the American Type Culture Collection (Manassas, VA). SK-CO15, Caco-2, and Cos-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 15 mmol/L HEPES, 1% nonessential amino acids, 40 μ g/ml penicillin, and 100 μ g/ml streptomycin, pH 7.4. For IEC-6 culture, 0.1% insulin was added to the Dulbecco's modified Eagle's medium. T84 cells were cultured in the 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 5% newborn calf serum, 10 mmol/L HEPES, 14 mmol/L Na HCO₃, 40 μ g/ml penicillin, and 100 μ g/ml streptomycin, pH 7.4. For immunolabeling experiments, epithelial cells were grown

on collagen-coated coverslips. For other assays, the cells were cultured on plastic plates.

Wound Closure Assay

SK-CO15 cells were grown to confluency on collagen-I-coated 24-well culture plates after which a single linear wound was created through the monolayers using a sterile pipette tip. Monolayers were washed to remove cellular debris and placed in complete media. Sites at which wounds were to be measured were marked on the undersurface of the wells to ensure that measurements were taken at the same place. Wounds were imaged at 0 and 6 hours on a Zeiss Axiovert microscope with an attached CCD-camera. Stock solutions of blebbistatin and other inhibitors were diluted in cell culture medium and added to cell monolayers immediately after wounding. SK-CO15 cells transfected with NM IIA-specific or control siRNA were wounded at 84 hours post-transfection. Wound widths were measured from the images using Scion Image software (Scion Image Corp., Frederick, MD). Ten measurements along the wound length were averaged to determine wound widths and the distance (μm) the wound edges migrated into the wound space. For each experimental group, the migrating distances of two different cell monolayers was measured and averaged in a particular experiment, and the same experiment was independently performed three times.

Matrigel Invasion Assays

Cell invasion assay was performed using commercially available modified Boyden chambers layered with growth factor-reduced Matrigel (BD Biosciences) according to the established protocol.³⁰ Chambers were hydrated and blocked in serum-free media containing 0.1% bovine serum albumin (BSA) for 2 hours at 37°C. SK-CO15 cells were trypsinized, washed in serum-free media, and counted. An equal number of cells (1×10^5 cells) were loaded into upper chambers in serum-free media containing 0.1% BSA. Compete media was placed in lower wells. After a 24-hour incubation at 37°C (5% CO_2), the upper chamber was cleared of cells using a cotton swab tip. The inserts were then fixed in a solution of 3.7% paraformaldehyde containing 0.1% crystal violet. The number of cells on the undersurface of membranes (ie, invaded cells) was counted using bright field microscopy. Cell number in three different $\times 100$ objective fields were counted and averaged for each group in a particular experiment, and each experiment was independently performed three times. In experiments involving pharmacological inhibitors, the inhibitors were added to both the upper and lower chambers. SK-CO15 cells transfected with NM IIA-specific or control siRNAs were loaded into invasion chamber at 72 hours post-transfection.

Cell Adhesion Assay

The cell adhesion assay was performed as previously described.³¹ Briefly, SK-CO15 cells were transfected

with siRNA and, 84 hours post transfection, were trypsinized and washed in HEPES-buffered Hanks balanced salt solution (HBSS). Cells were resuspended in HBSS containing 0.1% BSA and equal numbers of cells (2.5×10^4) were plated in collagen I or laminin-coated 96 well plates that had been blocked with the HBSS-BSA for 2 hours. Cells were then incubated at 37°C for 1 hour then gently washed three times with HBSS. Adherent cells were fixed and stained with 3.7% paraformaldehyde containing 0.1% crystal violet. Cell adhesion was then assessed using a microplate reader by analyzing absorbance at 570 nm.

Immunofluorescence Labeling and Confocal Microscopy

Cell monolayers were fixed/permeabilized in 100% methanol (-20°C for 20 minutes), blocked in HBSS containing 1% BSA (blocking buffer) for 60 minutes at room temperature, and incubated for another 60 minutes with primary antibodies diluted in blocking buffer. Monoclonal and polyclonal primary antibodies were used at final concentrations of 2.5 to 5 $\mu\text{g/ml}$ and 0.8 to 2 $\mu\text{g/ml}$ respectively. Cells were then washed, incubated for 60 minutes with Alexa dye-conjugated secondary antibodies, rinsed with HBSS and mounted on slides with ProLong Antifade medium (Molecular Probes). For fluorescent double-labeling of myosin II isoforms with F-actin, monolayers were fixed in 100% ethanol (-20°C for 20 minutes) and sequentially stained with primary anti-myosin II heavy chain and Alexa dye-conjugated secondary antibodies, whereas F-actin was labeled with Alexa-conjugated phalloidin. For tissue labeling, frozen sections (5 μm thickness) of normal human colonic mucosa were mounted on glass coverslips, air-dried, fixed in 100% ethanol (-20°C for 20 minutes), and immunolabeled as described above. In addition, H&E staining of colonic mucosa sections was performed to observe general tissue architecture and orientation. Stained cell monolayers and tissue sections were examined using a Zeiss LSM510 laser scanning confocal microscope (Zeiss Microimaging Inc., Thornwood, NY) coupled to a Zeiss 100M axiovert and $\times 63$ or $\times 100$ Pan-Apochromat oil lenses. The fluorescent dyes were imaged sequentially in frame-interlace mode to eliminate cross talk between channels. Images shown are representative of at least three experiments, with multiple images taken per slide.

Immunoblotting

Cells were homogenized in a RIPA lysis buffer (20 mmol/L Tris, 50 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 1% sodium deoxycholate, 1% TX-100, and 0.1% SDS, pH 7.4), containing a proteinase inhibitor cocktail (1:100, Sigma) and phosphatase inhibitor cocktails 1 and 2 (both at 1:200, Sigma). Lysates were then cleared by centrifugation (20 minutes at $14,000 \times g$), diluted with 2 $\times$ SDS sample buffer, and boiled. SDS polyacrylamide gel electrophoresis and immunoblotting were conducted by standard protocols with 10 to 20 μg protein per lane. Proteins

of interest were visualized after their transfer onto nitrocellulose membranes using appropriate primary and horseradish peroxidase-conjugated secondary antibodies. Monoclonal and polyclonal primary antibodies were diluted to a final concentration of 0.5 to 1 $\mu\text{g/ml}$. Results shown are representative immunoblots of three independent experiments. Protein expression was quantified by densitometric analysis of immunoblot images using UN-SCAN-IT digitizing software (Silk Scientific, Orem, UT).

Quantification of NM II Isoform Expression

Epithelial cells cultured in T75 flasks were scraped into RIPA buffer modified by the increased concentration of NaCl (250 mmol/L) and EGTA (5 mmol/L) and supplemented with 0.1 mmol/L phenylmethylsulfonylfluoride, 10 $\mu\text{g/ml}$ leupeptin, protease inhibitor cocktail (Sigma), 1 mmol/L dithiothreitol, and 5 mmol/L MgATP. After 10 minutes incubation on ice, the samples were centrifuged ($10,000 \times g$, 10 minutes) and the supernatant was separated by SDS polyacrylamide gel electrophoresis. The Coomassie Blue stained bands near the 205 kDa molecular size marker were excised, destained, reduced and alkylated, digested with trypsin, and submitted to the National Heart Lung and Blood Institute Proteomics Core Facility for analysis by liquid chromatography tandem mass spectroscopy. Peptide numbers for each of the NM II heavy chain isoforms were counted and the percent contribution to total amount of myosin II heavy chain peptides was calculated.

RNA Interference

siRNA-mediated knock-down of NM IIA, was performed using either isoform-specific siRNA SmartPools (Dharmacon, Lafayette, CO) or the individual siRNA duplex-2 (5'-GGCCAAACCUGCCGAAUAAUU-3') obtained from the same vendor. Cyclophilin B siRNA SmartPool or the individual cyclophilin B siRNA (5'-UCACCGUAGAUGCUCUUUCUU-3') were used as controls. SK-CO15 cells were transfected using the DharmaFect 1 transfection reagent (Dharmacon) in Opti-MEM I medium (Invitrogen) according to manufacturer's protocol with a final siRNA concentration of 100 nmol/L.

Expression of Enhanced Green Fluorescent Protein-Tagged NM IIA

A plasmid encoding full-length enhanced green fluorescent protein (EGFP)-tagged NM IIA³² was obtained from Addgene (Cambridge, MA). Control EGFP-C3 vector was provided by Dr. Kevin Bourzac (Oregon Health Science University, Portland OR). COS-7 cells were transfected with EGFP-NM IIA or control plasmid using Lipofectamine 2000 and analyzed at 48 hours post-transfection.

Calpain Activity Assay

The calpain activity assay was obtained from Biovision Research Products (Mount View, CA) and performed ac-

Table 1. Relative Levels (%) of Different NM II Heavy Chain Isoforms in Cultured Human Intestinal Epithelial Cells

Cell line	NM IIA	NM IIB	NM IIC	Number of experiments
Caco-2	85 $\pm$ 4	8 $\pm$ 4	7 $\pm$ 2	4
SK-CO15	66 $\pm$ 12	20 $\pm$ 6	14 $\pm$ 9	7
T84	80 $\pm$ 3	0	20 $\pm$ 3	3

cording to the manufacturer's protocol. Briefly, SK-CO15 cells were transfected with either NM IIA or control siRNAs, and 80 to 84 hours post-transfection, cells were trypsinized and counted. An equivalent number of cells (2×10^6) was pelleted, and the pellets were resuspended in supplied extraction buffer and incubated on ice for 20 minutes. After a brief centrifugation ($10,000 \times g$, 1 minute), the obtained lysates were transferred to a Costar flat-bottomed black polystyrene 96-well assay plate, mixed with a fluorogenic calpain substrate and incubated for 1 hour at 37°C. The fluorescence intensity at 400 nm excitation and 505 nm emission wavelengths was measured using a Fluostar plate reader (BMG Labtechnologies, Durham, NC).

Statistics

Numerical values from individual experiments were pooled and expressed as mean $\pm$ SEM throughout. Obtained numbers were compared by a single-tailed Student's *t*-test, with statistical significance assumed at $P < 0.05$.

Results

NM IIA is the Predominant NM II Isoforms in Human Intestinal Epithelium

By using reverse transcription-PCR, immunoblotting, and immunofluorescence analyses we recently demonstrated that human colonic epithelial cells express NM IIA, IIB, and IIC heavy chains.¹⁵ However, these analyses did not allow a direct quantitative comparison of the levels of each NM II isoform. Such quantification is important because NM IIA, IIB, and IIC might be functionally redundant, and the most highly expressed isoform could therefore play the most significant physiological role.^{21,22} In the present study, we determined the relative protein concentrations of NM IIA, IIB, and IIC in human colonic epithelial cell lines. Mass spectrometry analysis of NM II-derived polypeptides revealed that NM IIA is the most abundant isoform, comprising approximately 85%, 80%, and 66% of all NM II in Caco-2, T84, and SK-CO15 cells, respectively (Table 1). NM IIC was also detected in all three colonic epithelial cell lines, whereas no NM IIB was identified in T84 cells. This lack of NM IIB in T84 cells is consistent with our previous immunoblotting data¹⁵ and it validates the specificity of the mass spectroscopy analysis. Overall this quantification clearly shows that NM IIA is the predominant NM II isoform in intestinal epithelial

cells. Given these findings, our subsequent experiments were focused on the role of NM IIA in intestinal epithelial cell motility.

We next determined the localization of NM IIA in normal human colonic mucosa. Immunofluorescence labeling and confocal microscopy demonstrated abundant expression of NM IIA in epithelial cells lining the villous surface, as well as in colonic crypt epithelium (Figure 1, A and B). Interestingly, in actively migrating crypt epithelial cells, a significant fraction of NM IIA localized along the basal membrane where epithelial cells adhere to the underlying basement membrane (Figure 1A, arrows). By contrast, in less motile surface epithelial cells, NM IIA was predominantly localized in the apical plasma membrane domain (Figure 1A, arrowheads). Such differential localization of NM IIA in motile crypt and senescent surface epithelial cells is consistent with the involvement of this cytoskeletal motor in colonic epithelial cell migration.

Inhibition of NM IIA Function Attenuates 2-D Migration of Colonic Epithelial Cells

To characterize the functional role of NM IIA in different migration modes of intestinal epithelial cells, we first investigated its effect on 2-D migration during closure of large epithelial wounds. SK-CO15 and Caco2 cell monolayers growing on collagen-coated coverslips were scratch-wounded, and 6 hours later, double fluorescently labeled for NM IIA and either F-actin or paxillin, a protein involved in cell adhesion to the extracellular matrix. NM IIA was abundant in lamellae of migrating cells at the wound edge, where this motor protein was enriched along thick F-actin bundles (Figure 1C, arrows) and in paxillin-based cell-matrix adhesions (Figure 1C, arrowheads). To gain functional insight into the role of NM IIA in planar migration, the effect of NM IIA inhibition on wound closure in SK-CO15 cell monolayers was investigated. Activity of NM IIA was suppressed by either siRNA-mediated expressional down-regulation or pharmacological inhibition with blebbistatin.³³ Transfection of SK-CO15 cells with NM IIA-specific siRNA duplex-2 caused approximately 90% decrease in its protein expression (Supplemental Figure S1 available at <http://ajp.amjpathol.org>). This knockdown resulted in ~ 30% decrease in the migration distance of NM IIA-depleted cells when compared with control siRNA-transfected cells (Figure 2A and B). Similarly, wound closure was inhibited by approximately 50% in SK-CO15 cell monolayers treated with 100 μ mol/L of blebbistatin (Figure 2, C and D). These data indicate that NM IIA promotes planar migration of intestinal epithelial cell sheets.

Depletion of NM IIA Increases Cell-Matrix Adhesion but Impairs the Assembly of Focal Adhesions and F-Actin Stress Fibers

Since the velocity of cell migration depends on interactions with the extracellular matrix,^{7,34} we next examined whether inhibition of NM IIA affects cell-matrix adhesion.

Figure 1. Localization of NM IIA in normal human intestinal mucosa and in migrating cultured intestinal epithelial cells. **A:** Sections of normal human colonic crypt and surface epithelium were immunolabeled for NM IIA (green) and either occludin or Na^+/H^+ exchanger I to visualize the cell apex and lateral plasma membrane respectively (red). Position of individual cells was highlighted by nuclear staining (blue). Note a significant presence of NM IIA at the basal pole of crypt epithelial cells (**arrows**) and almost exclusive apical localization of NM IIA in surface enterocytes (**arrowheads**). Scale bar = 20 μm . **B:** Corresponding section of human colon was stained with H&E to show normal mucosal architecture. Note prominent crypts that contain progenitor cells that proliferate and mature as they migrate to the surface. **C:** Scratch-wounded Caco-2 and SK-CO15 cell monolayers were double fluorescence labeled for NM IIA (green) either paxillin or F-actin (red). Note the association of myosin filaments with paxillin-rich focal adhesions in Caco-2 cells (**arrowheads**) and the abundance of actomyosin fibers throughout the entire migrating lamellae in SK-CO15 cells (**arrows**). Scale bar = 10 μm .

SK-CO15 cells were transfected with either NM IIA-specific or control siRNA and their adhesion to collagen 1 and laminin was examined on day 3 post-transfection. NM IIA knock-down significantly increased cell adhesion

Figure 2. Inhibition of NM IIA attenuates 2-D migration of intestinal epithelial cells. Effects of siRNA-mediated down-regulation of NM IIA expression (**A, B**) or pharmacological inhibition of NM II with 100 μ M blebbistatin (**C, D**) on 2-D migration of SK-CO15 cells was examined using a wound closure assay. Note that either siRNA-mediated or pharmacological inhibition of NM IIA significantly decreased the distance migrated by intestinal epithelial cells during 6 hours of wound closure. * $P < 0.05$.

to collagen 1 and laminin by an average 1.6- and 1.5-fold, respectively (Figure 3A). Immunoblotting analysis was performed to examine if such an increase in cell adhesiveness reflects altered expression of proteins involved in the formation of cell-matrix adhesions. As shown in Figure 3, siRNA-mediated down-regulation of NM IIA increased expression of paxillin, Tyr118-phosphorylated-paxillin, and β 1-integrin by approximately 4.3-, 2.9-, and 2.7-fold, respectively, compared with the control siRNA-treated group. Interestingly, protein expression of vinculin, FAK, and Tyr397-phosphorylated FAK was not significantly changed (Figure 3, B and C). Since epithelial cell adhesion to the extracellular matrix is mediated by special multiprotein structures referred to as focal adhesions (FAs),^{10,34} we analyzed whether organization of FAs is altered in NM IIA-deficient cells. In control SK-CO15 cell monolayers, paxillin almost exclusively localized in large, elongated FAs that were particularly enriched at the front of lamellipodia (Figure 4A, arrows). In contrast, paxillin was diffusely distributed throughout entire lamellae after NM IIA-depletion and no obvious FAs were observed. Likewise, pharmacological inhibition of NM II caused complete disappearance of paxillin-based FAs in nontumorigenic IEC-6 cells (Supplemental Figure S2 available at <http://ajp.amjpathol.org>). To further verify the role of NM IIA in the formation of FAs, we expressed EGFP-tagged version of this protein in COS-7 cells that reportedly lack the expression of endogenous NM IIA.^{21,26} Non-transfected or control-EGFP-expressing COS-7 cells possessed very small peripheral FAs visualized by immunolabeling for pTyr118-paxillin (Figure 4B). Expression of EGFP-NM-IIA resulted in dramatic enlargement of FAs especially at the ends of prominent myosin-based filaments (Figure 4B).

Figure 3. Down-regulation of NM IIA expression enhances cell adhesiveness and increases expression of matrix adhesion proteins. **A:** SK-CO15 cells were transfected with either control (cyclophilin B) or NM IIA siRNAs and 84 hours later, cells were analyzed for their adhesion to either collagen 1 or laminin. Note a significant increase in adhesiveness of NM IIA-depleted cells to both extracellular matrices. * $P < 0.05$. **B, C:** Western blot analysis of control and NM IIA-depleted SK-CO15 cells, 84 hours post-siRNA transfection. Note a dramatic increase in expression of total paxillin, phospho-paxillin, and β 1-integrin in NM IIA-depleted cells.

Organization and dynamics of FAs are known to be regulated by NM II-dependent contractility.³⁴ These contractile forces are transduced to FAs via attached actomyosin bundles called 'stress fibers'.³⁴ Therefore, we next investigated whether the lack of morphologically defined FAs in NM IIA-depleted epithelial cells is a consequence of abnormalities in stress fiber formation. As shown in Figure 5A, lamellae of migrating control SK-CO15 cells contained prominent F-actin stress fibers, which were enriched in NM IIA. In a stark contrast, no such structures were observed after knockdown of NM IIA. Instead, NM IIA-depleted SK-CO15 cells formed broad, abnormally shaped protrusions with diffuse unstructured F-actin labeling (Figure 5A). Pharmacological inhibition of NM II with blebbistatin caused similar disappearance of basal stress fibers in migrating IEC-6 cells (Supplemental Figure S2 available at <http://ajp.amjpathol.org>). As expected, expression of EGFP-tagged NM IIA in COS-7 cells induced formation of prominent stress fibers (Figure 5B, arrows) whereas expression of control EGFP was without effects. Together, these data suggest that inhibition of 2-D migration in NM IIA-depleted colonic epithelial cells is accompanied by dramatic abnormalities in cell matrix adhesions manifested by increased substrate adhesiveness and impaired organization of FAs and stress fibers.

Figure 4. NM IIA regulates the assembly of focal adhesions. **A:** SK-CO15 cells plated on coverslips were transfected with control or NM IIA siRNAs and 84 hours post-transfection were double labeled for NM IIA (red) and paxillin (green). Note the assembly of paxillin-based focal adhesions at the migrating front of control cells (arrows) and lack of distinctive focal adhesions in NM IIA-depleted cells. Scale bar = 10 μm. **B:** COS-7 cells plated on coverslips were transfected with either EGFP-tagged NM IIA or control EGFP and 48 hours later immunolabeled for phospho-paxillin (red). Note that expression of EGFP-NM IIA increases the size of peripheral focal adhesions (arrows) whereas control EGFP expression is without effect. Scale bar = 20 μm.

Inhibition of NM IIA Enhances SK-CO15 Cell Invasion via Activation of the Raf-ERK1/2 Signaling Pathway

Accumulated evidence suggests that cellular machinery mediating migration over a 2-D surface is different from the machinery that drives cell invasion through 3-D matrices.^{35,36} Therefore we next analyzed whether NM IIA may also control matrix invasion of intestinal epithelial cells. Figure 6, A and B demonstrates that in contrast to its inhibitory effect on 2-D cell migration, siRNA-mediated depletion of NM IIA substantially increased SK-CO15 invasion through Matrigel-coated filters (~2.5-fold increase). Likewise, pharmacological inhibition of NM IIA with blebbistatin resulted in ~twofold increase in SK-CO15 cell invasion (Figure 6, C and D).

To define mechanisms underlying the enhanced invasive phenotype of NM IIA-depleted SK-CO15 cells, we next examined the role of the Raf-ERK1/2 signaling pathway, which is considered to be a key regulator of epithelial cell matrix invasion.^{37–40} As shown in Figure 7A, down-regulation of NM IIA expression induced significant activation of ERK as evidenced by increased phospho-

Figure 5. NM IIA is essential for the formation of stress fibers. **A:** SK-CO15 cells plated on coverslips were transfected with control or NM IIA siRNAs and 84 hours post-transfection were double labeled for NM IIA (red) and F-actin (green). Note thick actomyosin-based stress fibers in lamellae of control cells (arrows) and diffuse F-actin staining in abnormally shaped protrusions of NM IIA-depleted cells. Scale bar = 10 μm. **B:** COS-7 cells plated on coverslips were transfected with either EGFP-tagged NM IIA or control EGFP and 48 hours later labeled for F-actin (red). Note that expression of EGFP-NM IIA induces formation of prominent F-actin stress fibers (arrows), whereas control EGFP expression is without effect. Scale bar = 20 μm.

ERK1/2 levels (~threefold, Figure 7B). Furthermore, activation of an upstream member of the ERK signaling cascade, Raf-1 kinase, was also evident, as the amount of active Ser338-phosphorylated Raf-1⁴¹ was increased ~twofold in NM IIA-depleted SK-CO15 cells (Figure 7, A and B). No change in the expression of total ERK1/2 and RAF-1 proteins was detected. To determine the functional significance of this ERK stimulation, we next examined whether inhibition of ERK1/2 activity reverses the effect of NM IIA knockdown on intestinal epithelial cell invasion. Figure 7, C and D demonstrates that NMIIA-depleted SK-CO15 cells invaded approximately threefold less in the presence of U0126 (5 μmol/L), a cell-permeable compound preventing ERK1/2 activation,⁴² as compared with vehicle-treated cells. Similar attenuation of cell invasion was achieved by using another canonical inhibitor of ERK signaling, PD 098059 (data not shown). Together these data suggest that ERK1/2 activation is responsible for the increase in matrix invasion of SK-CO15 cells caused by down-regulation of NM IIA.

Figure 6. Inhibition of NM IIA enhances 3-D matrix invasion of intestinal epithelial cells. Effects of siRNA-mediated down-regulation of NM IIA expression (**A, B**) or pharmacological inhibition of NM II with 100 $\mu\text{mol/L}$ blebbistatin (**C, D**) on matrix invasion of SK-CO15 cells was examined using a modified Boyden chamber assay. Averaged number of cells counted per $\times 100$ -objective field is shown. Note that either siRNA-mediated or pharmacological inhibition of NM IIA substantially increased invasion of colonic epithelial cells into 3-D Matrigel. * $P < 0.05$.

Enhanced Matrix Invasion of NM IIA-Deficient Cells Does Not Depend on Activity of MMPs but Is Reversed by Calpain Inhibition

Multiple downstream effectors have been shown to mediate the invasive behavior of epithelial cells caused by activation of the Raf-ERK1/2 signaling cascade.³⁸ Stimulation of different proteases appears to be particularly important. For example, ERK1/2 activation has been shown to increase expression/secretion of MMPs, which promotes cell invasion by accelerating degradation of the extracellular matrix.^{38,43} On the other hand, ERK-dependent stimulation of the intracellular protease calpain has been found to increase epithelial cell invasion by accelerating the remodeling of cell-matrix adhesions.⁴⁴ Based on these data we next investigated whether MMPs and calpain can be responsible for the increased invasiveness of NM IIA-depleted SK-CO15 cells. The role of MMPs was addressed by analyzing expression of their predominant epithelial isoforms and by pharmacological inhibition of MMPs. No significant changes in expression of MMP-9 in NM IIA-depleted SK-CO15 cells were detected by immunoblotting (Figure 8A) and no increase in the levels of MMP-2, MMP-7, or MMP-9 proteins was observed in these cells by immunofluorescence labeling and confocal microscopy (data not shown). Importantly, GM 6001 (10 $\mu\text{mol/L}$), a potent broad spectrum MMP

Figure 7. Activation of the Raf-ERK1/2 signaling cascade mediates the increased invasiveness of NM IIA-depleted colonic epithelial cells. **A, B:** Western blot analysis of SK-CO15 cells 84 hours post-transfection with control or NM IIA-specific siRNAs. Note the dramatic increase in the levels of phosphorylated (active) ERK1/2 and Raf-1, but unchanged total expression of these kinases after down-regulation of NM IIA. **C, D:** Matrigel invasion of control and NM IIA-depleted SK-CO15 cells was analyzed in the presence of either vehicle or a pharmacological inhibitor of ERK1/2 activation, U0126 (5 $\mu\text{mol/L}$). Note that ERK inhibition dramatically attenuated matrix invasion of both control and NM IIA-depleted cells. * $P < 0.05$.

Figure 8. NM IIA-depleted cells invade in a MMP-independent manner. **A:** Western blot analysis of SK-CO15 cells 84 hours after transfection with control or NM IIA-specific siRNAs. Note that NM IIA knock-down does not increase expression of MMP-9. **B, C:** Matrigel invasion of control and NM IIA-depleted SK-CO15 cells was analyzed in the presence of either vehicle or a general MMP inhibitor GM 6001 (10 μ mol/L). Note that the MMP inhibitor does not affect invasion of NM IIA-depleted cells.

inhibitor,^{45,46} failed to reverse the enhanced invasiveness of NM IIA-deficient cells (Figure 8, B and C). These data suggest that increased MMP activity is not responsible for acquisition of the pro-invasive phenotype in NM IIA-depleted epithelial cells.

To examine a possible role of calpain, two potent cell-permeable inhibitors of this protease, ALLN^{47,48} and calpeptin⁴⁹ were used. Figure 9 shows that treatment of SK-CO15 cells with 1 μ mol/L of ALLN completely abrogated the enhanced invasion of NM IIA-depleted cells through Matrigel-coated filters, which became indistinguishable from the invasion of control siRNA-treated cells. Similar results were obtained when the experiments were conducted in the presence of 0.1 μ mol/L calpeptin (data not shown). Since calpain inhibition blocked the increased invasion caused by NM IIA knockdown, we examined the effects of NM IIA depletion on calpain expression and activity. Protein expression of two isoforms, calpain-1 and -2, previously implicated in cell

Figure 9. Calpain inhibition blocks the enhanced invasion of NM IIA-depleted colonic epithelial cells. Matrigel invasion of control and NM IIA-depleted SK-CO15 cells was analyzed in the presence of either vehicle or a pharmacological inhibitor of calpain, ALLN (1 μ mol/L). **A, B:** Note that the calpain inhibitor dramatically attenuates the invasion of NM IIA-depleted cells. * $P < 0.05$.

migration,⁴⁴ was examined by immunoblotting, whereas an enzymatic activity of all 13 calpain isoforms was determined by using a fluorogenic substrate. Figure 10A and B shows a significant (~1.5-fold) increase in the expression of calpain-2 in NM IIA-depleted cells as compared with the control siRNA-treated group, whereas expression of calpain-1 was not changed. Furthermore, NM IIA-deficient SK-CO15 showed a small (~15%) but statistically significant increase in total calpain activity (Figure 10C). This small increase in calpain activity likely reflects upregulation of certain calpain isoforms (such as calpain-2 in this case) whereas expression of other isoforms contributing to the total calpain activity remains unchanged. Together these data suggest that the increased expression and activity of calpain-2 plays a role in the enhanced invasiveness of NM IIA-depleted SK-CO15 cells.

Discussion

This study addresses the role of NM IIA in regulation of two different modes of intestinal epithelial cell migration: a 2-D migration of epithelial sheets, which models homeostasis and restitution of intestinal mucosa, and invasion of epithelial cells through a 3-D matrix, which mimics metastatic dissemination of colorectal tumors. We show that NM IIA inhibition has opposite effects on these motility processes by attenuating 2-D migration and enhancing 3-D matrix invasion of colonic epithelial cells. Such a dual effect of NM IIA inhibition reflects the involvement of this motor protein in multiple cellular events including assembly of cell-matrix adhesions, F-actin stress fiber formation, and regulation of the Raf-ERK1/2 signaling and calpain activity.

Figure 10. siRNA-mediated knock-down of NM IIA increases calpain-2 protein expression and stimulates total calpain activity. SK-CO15 cells were transfected with control or NM IIA siRNAs and 84 hours post-transfection were analyzed for calpain-1 and calpain-2 protein expression (**A, B**) and total calpain activity (**C**). Note a significant increase in calpain-2 expression and stimulation of calpain activity in NM IIA-depleted cells. * $P < 0.05$.

Role of NM IIA in Regulating Planar Migration and Matrix Adhesion of Colonic Epithelial Cells

Our data demonstrate that either pharmacological inhibition or siRNA-mediated knockdown of NM IIA, which is the predominant myosin II isoform in intestinal epithelium (Table 1), attenuates planar migration of SK-CO15 cells in the wound closure assays (Figure 2). This finding is consistent with published studies demonstrating impaired 2-D migration in blebbistatin-treated pancreatic and renal epithelial cells^{23,24} and NM IIA-depleted MDA-MB-231 breast cancer cells.²⁶ Importantly, our data suggest that this attenuated migration of myosin II-inhibited epithelial cells is due to their altered adhesion to the substratum. It is widely appreciated that adhesion to extracellular matrix plays a key role in cell migration,^{10,34,50} however the relationship between these two processes is not simple. Both insufficient and excessively strong matrix adhesion can impede cell movement.^{24,51} We observed that NM IIA-depleted SK-CO15 cells adhere more avidly to collagen and laminin matrices (Figure 3A) and we believe that the increased adhesiveness contributes to impaired 2-D migration of these cells. Interestingly, knock-down of NM IIA in SK-CO15 cells caused significant expressional up-regulation of major matrix adhesion proteins such as

β 1-integrin and paxillin (Figure 3B). Despite the increased expression of adhesion proteins, NM IIA-depleted SK-CO15 cells were unable to form morphologically defined FAs (Figure 4A), whereas introduction of exogenous NM IIA into COS-7 cells lacking this myosin isoform caused a dramatic enlargement of their FAs (Figure 4B). These data are consistent with several recent studies observing a loss of FAs after down-regulation of NM IIA expression in fibroblasts^{17,52,53} and lung epithelial cells.¹⁹ What is the mechanism of FA disappearance in NM IIA-deficient cells? Biogenesis of cell-matrix adhesions has been shown to consist of several steps including initial assembly of small focal complexes and their subsequently fusion into larger FAs.⁵⁰ The formation of focal complexes was found to be myosin independent,⁵⁴ whereas maturation of focal complex into FAs was shown to be regulated by contractility of actomyosin stress fibers.^{54,55} Consistent with the lack of mature FAs, NM IIA-depleted SK-CO15 cells also had no obvious stress fibers in their lamellae (Figure 5A). Furthermore, expression of NM IIA in COS-7 cells triggered formation of stress fibers, which paralleled the enlargement of FAs (Figures 4B and 5B). These data suggest that defects in stress fiber formation in NM IIA-depleted SK-CO15 cells prevent transformation of their initial adhesion complexes into mature FAs. These defects in FA and stress fiber formation are likely to have functional consequences. Contractile FA-anchored stress fibers were shown to generate traction forces,⁵⁵ which are necessary for forward translocation of the cell body, as well as for breakdown of cell-matrix adhesions at the cell rear and retraction of cell's trailing end.^{7,8,34,56} NM IIA-deficient cells that are unable to exert contractile forces are likely to have impaired detachment and retraction of the cell body,⁵² which impedes the velocity of their migration over the 2-D surface. Together our results suggest that NM IIA inhibition attenuates planar migration of intestinal epithelial sheets via two major mechanisms viz., increased adhesion to the extracellular matrix and decreased stress-fiber mediated detachment/retraction.

Role of NM IIA in Regulating 3-D Matrix Invasion of Colonic Epithelial Cells

In a stark contrast to the attenuation of 2-D migration, inhibition of NM IIA dramatically stimulated invasion of SK-CO15 cells through 3-D Matrigel (Figure 6). This surprising observation, however, is consistent with an emerging idea that cell behavior on 2-D surfaces and in 3-D matrices has fundamental differences.^{35,36} It is noteworthy that endothelial cells and fibroblasts embedded into 3-D gels have been shown to lack typical FAs and stress fibers.^{57–59} As a result, myosin-dependent contractility of stress fibers is not essential for cell invasion into 3-D matrices. This can explain why inhibition of NM IIA, which caused a loss of FAs and stress fibers, did not attenuate Matrigel invasion of SK-CO15 cells, as it did during 2-D migration. Furthermore, lack of involvement of stress fiber-mediated contractility unmasked strong promigratory mechanisms responsible for accelerated 3-D

invasion of NM IIA-depleted SK-CO15 cells. These mechanisms include stimulation of the Raf-ERK1/2 signaling pathway and up-regulation of calpain expression/activity.

The involvement of the Raf-ERK 1/2 pathway is supported by two lines of evidence. The first is significant increase in the phosphorylation of ERK1/2 and Raf-1 kinase after siRNA-mediated knock-down of NM IIA (Figure 7, A and B). Second is the reversal of increased invasiveness of NM IIA-deficient SK-CO15 cells by pharmacological inhibitors of ERK1/2 activation (Figure 7, C and D). Such stimulation of Raf-ERK1/2 signaling by NM II inhibition has not been previously reported. However this finding is consistent with a current idea that Raf-ERK1/2 signaling plays a central role in regulating tumor cell matrix invasion.^{37,39,40}

How can inhibition of NM IIA stimulate Raf-ERK1/2 activity? Although the exact mechanism remains unknown, recent data suggest the involvement of microtubules. Indeed, inhibition of NM IIA was shown to change the organization and to increase stability of cortical microtubules in fibroblasts⁵² and colonic epithelial cells.⁶⁰ On the other hand, pharmacological stabilization of microtubules is known to activate Raf-1 kinase⁶¹ and ERK1/2.^{62,63} Furthermore, a significant fraction of ERK1/2 was shown to be physically associated with microtubules in epithelial and neuroendocrine cells.^{64,65} Based on these data we suggest that microtubule stabilization can be responsible for stimulation of the Raf-ERK1/2 signaling cascade in NM IIA-depleted SK-CO15 cells.

Calpain Activation Contributes to Increased 3-D Invasion of NM IIA-Depleted Epithelial Cells

The Raf-ERK1/2 cascade is one of the major intracellular signaling pathways, which can activate multiple downstream effectors. Recent studies identified several major downstream mechanisms mediating ERK-dependent stimulation of cell motility.^{38,44} These mechanisms include: 1) phosphorylation of myosin light chain kinase resulting in increased actomyosin contractility; 2) phosphorylation of FAK which alters FA assembly and dynamics; 3) stimulation of MMP expression to promote extracellular matrix degradation; and, 4) phosphorylation and activation of the calpain family of proteases, leading to remodeling of cell-matrix adhesions. The first two mechanisms are unlikely to be involved in ERK-dependent stimulation of 3-D invasion of NM IIA-depleted SK-CO15 cells because these cells have greatly diminished NM II activity and do not form FAs. This leaves the latter two downstream mechanisms for testing.

The role of the MMP family of enzymes in 3-D invasion of epithelial cells has been extensively studied. These secreted enzymes have been shown to promote cell invasion by degrading extracellular matrix and therefore eliminating a physical barrier that restricts cell movement.^{43,66,67} The invasive/metastatic behavior of many epithelial tumors including breast carcinomas and gastric adenocarcinomas has been attributed to increased activity of different members of the MMP family.^{68,69} However, we were unable to detect an increase in protein

levels of MMP-9, MMP-7, and MMP-2 in NM IIA-depleted SK-CO15 cells by either immunoblotting or immunofluorescence analysis (Figure 8A and data not shown). More importantly, enhanced 3-D invasion of these cells was not sensitive to MMP inhibition (Figure 8, A and B). These data are consistent with a recent report demonstrating that inhibition of Rho-dependent kinase (which is likely to inactivate NM II) did not stimulate MMP-2, MMP-9, and MMP-13 activity in several human colonic epithelial cell lines.⁷⁰ Overall these data suggest that activation of MMP-dependent remodeling of the extracellular matrix is not responsible for the enhanced invasiveness of NM-IIA-depleted SK-CO15 cells.

However, our results did implicate activation of the intracellular protease, calpain, in the increased invasion of NM IIA-depleted colonic epithelial cells. This conclusion is based on the blockade of enhanced invasion of NM IIA-depleted SK-CO15 cells by two different calpain inhibitors (Figure 9 and data not shown), enhanced calpain-2 expression, and increased total calpain activity after NM IIA knockdown (Figure 10). These findings are significant in that they provide evidence that NM II regulates calpain expression/activity and indicate a critical role of calpain in matrix invasion of intestinal epithelial cells.

The calpain family of cysteine proteases consists of 13 members, two of which, calpain-1 and 2 have been implicated in regulating motility (see for review,^{44,71}). Our results suggesting the involvement of calpain-2 in increased invasiveness of NM IIA-depleted SK-CO15 cells are consistent with published studies that demonstrated a pro-migratory role of this calpain isoform in other cell types. For example, calpain-2 was found to mediate growth factor-stimulated fibroblast motility^{72,73} and to promote matrix invasion of fibrosarcoma and lung epithelial cells *in vitro*.^{74,75} In addition, calpain-2 was recently implicated in invasion of prostate epithelial cells *in vivo*.⁴⁸ Since calpain-dependent proteolysis involves multiple intracellular substrates⁴⁴ this enzyme can promote cell motility via several mechanisms. The most characterized mechanism is acceleration of cell detachment due to cleavage of matrix adhesion proteins such as paxillin, talin, β 3-integrin, and FAK.^{44,71,76} Another less understood mechanism involves the regulation of cellular protrusions that are associated with calpain-mediated cleavage of cortactin and other components of actin-polymerization machinery.⁷⁷ It remains to be determined which mechanism underlies calpain-dependent enhancement of matrix invasion in NM IIA-depleted colonic epithelial cells.

Calpain activity is regulated by several factors including intracellular calcium, phospholipids, and its endogenous inhibitor calpastatin.⁴⁴ Importantly, calpain-2 is known to be activated by ERK-dependent phosphorylation at the Ser50 residue.⁴⁴ Therefore stimulation of the Raf-ERK1/2 signaling can be partially responsible for the enhanced activity of calpain-2 in NM IIA-deficient SK-CO15 cells. Another mechanism for calpain-2 activation involves its increased expression induced by NM IIA knockdown (Figure 10). It is noteworthy that down-regulation of NM IIA in SK-CO15 cells resulted in increased

expression of at least three different proteins: calpain-2, paxillin, and β 1-integrin. This effect of NM II inhibition on protein expression has not been previously described and its mechanisms remain unknown. The stimulation of calpain-2, paxillin, and β 1-integrin expression can be a consequence of ERK1/2 activation because the Raf-ERK1/2 signaling pathway is commonly involved in transcriptional regulation of many different genes.^{78,79} Alternative mechanisms may involve specific myosin-dependent transcriptional factors. The best example of such myosin-dependent transcriptional regulation is a serum response factor.^{80,81} How NM II regulates protein expression in epithelial cells is an important point of future investigations.

In conclusion, this study demonstrates a dual role for NM IIA in regulating migration of intestinal epithelial cells. This cytoskeletal motor appears to promote migration of epithelial cells over 2-D surfaces and inhibits cell invasion through 3-D matrices. The pro-migratory function of NM IIA is based on its ability to decrease epithelial cell adhesiveness by limiting expression of matrix adhesion proteins and stimulating the assembly of the basal contractile system involving stress fibers and FAs. The antagonistic effect of NM IIA on 3-D cell invasion appears to be mediated through suppression of the Raf-ERK1/2 signaling cascade and calpain activity. Based on these results, we predict that pharmacological strategies aimed to activate NM II in intestinal mucosa may have therapeutic potential to accelerate restitution of epithelial wounds during intestinal inflammation and to inhibit the development of colorectal cancer metastasis.

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