Magnesium deficiency with high calcium-to-magnesium ratio promotes a metastatic phenotype in the CT26 colon cancer cell line

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Abstract. Magnesium (Mg) plays important roles in maintaining genomic stability and cellular redox. Mg also serves as nature's physiological calcium (Ca) channel antagonist, controlling intracellular Ca entry. Because Ca is the most important second messenger, its intracellular concentration is tightly regulated. Excess intracellular Ca can activate aberrant signaling pathways leading to the acquisition of pathological characteristics and cell injury. Several epidemiological studies have linked Mg deficiency (MgD) and increased Ca:Mg ratios with higher incidences of colon cancer and increased mortality. While it is estimated that less than 50% of the US population consumes the recommended daily allowance for Mg, Ca supplementation is widespread. Therefore, we studied the effect of MgD, with variable Ca:Mg ratios on cellular oxidative stress, cell migration, calpain activity, and associated signaling pathways using the CT26 colon cancer cell line. MgD (with Ca:Mg ratios >1) elevated intracellular Ca levels, calpain activity and TRPM7 expression, as well as oxidative stress and cell migration, consistent with observed degradation of full-length E-cadherin, β -catenin, and N-terminal FAK. MgD was accompanied by enhanced degradation of I κ B α and the transactivation domain containing the C-terminus of NF- κ B p65 (RelA). MgD-exposed CT26 cells exhibited increased p53 degradation and aneuploidy, markers of genomic instability. By contrast, these pathological changes were not observed when CT26 were cultured under MgD conditions where the Ca:Mg ratio was kept at 1. Together, these data support that exposure of colon cancer cells to MgD with physiological Ca concentrations (or increasing Ca:Mg ratios) leads to the acquisition of a more aggressive, metastatic phenotype.

Key words: calcium-to-magnesium ratio, calpain activity, cell migration, CT26 cell line, genomic instability, magnesium deficiency, focal adhesion kinase, oxidative stress

Introduction

Magnesium (Mg) and calcium (Ca) share several chemical properties. In biological systems, their functions are often antagonistic due to differences in the number of hydration shells surrounding each ion and their solubility constants [1]. Mg is more soluble than Ca and is the most abundant intracellular divalent cation. Ca is excluded from the intracellular environment and several pumps have evolved to either sequester Ca in specialized intracellular compartments or pump it outside the cells, as it can precipitate phosphate and organic anions, damaging cellular macromolecules [2]. Later in the course of evolution, this difference between intracellular and extracellular concentrations of Ca (10^4 X gradient) made it one of the most important second messengers for intracellular communication and signal transduction [3].

Like Ca, Mg participates in a wide range of biological functions. It acts as an essential cofactor in more than 300 enzymatic reactions in the body [3-5] and serves as a physiological Ca channel antagonist [6]. As such, Mg maintains or regulates cellular antioxidant status, cellular energetics, genomic stability, and immuno-inflammatory responses [7]. While the recommended dietary allowance (RDA) for Mg is 300-400 mg per day, it has been estimated that less than 50% of the U.S. population consumes the RDA for Mg [8] and certain populations (e.g. elderly, diabetics, and critically ill patients) are at increased risk [9]. As previously described by Castiglioni and Maier, because Mg participates in numerous metabolic and redox processes, it is not surprising that Mg contributes to cell growth and survival, and Mg deficiency may contribute to cancer initiation and progression [10].

Ca mediates numerous biological functions by directly binding to intracellular target proteins or by stimulating Ca sensors that respond to changing Ca concentrations by activating downstream responses such as calpains, a Ca^{2+} -dependent caspase [11]. Mg regulates intracellular Ca entry due to its larger size in aqueous environments [1]. In order to cross ion channels, ions must shed their hydration shells in an energy-consuming

process. Mg with two layers of hydration shells (making it 400X larger than its dehydrated form) is larger than Ca, which has only one layer (making it 25X larger than its dehydrated form) and thus, extracellular Mg is nature's physiological Ca channel antagonist by acting as a gate (*figure 1A*). Increases in intracellular Ca concentrations can activate various signaling pathways that promote cell growth, dedifferentiation, survival, angiogenesis, migration and invasion, oxidative stress, and genomic instability [1]. These processes, when sustained or dysregulated, may lead to acquisition of the hallmarks of cancer [12].

Cancer is the second most common cause of death worldwide and the incidence of cancer is increasing [13]. Colon cancer is the second most common cancer in women and third most common cancer in men [13]. In the US despite widespread colon cancer screening, it is the third leading cause of cancer deaths [14]. High rates of tumor recurrences and metastasis contribute to its high mortality rates. Improved understanding of factors that promote tumor progression and metastasis are warranted as low-grade primary tumors are amenable to surgical resection and these patients have better prognosis and survival.

Several epidemiological studies have linked Mg deficiency (MgD) with high Ca:Mg intake ratios to a higher incidence of colon cancer and mortality [15, 16]. In addition, higher Ca:Mg ratios promote the proliferation of prostate cancer cells and are associated with highly aggressive prostate cancer [17-19]. Similarly, an inverse association between total Mg intake and all-cause mortality was reported in older women with breast cancer and this was stronger among women with high Ca:Mg intake ratios [20]. While most Americans do not consume the RDA for Mg [8], calcium supplementation is prevalent, especially among the elderly [21]. Therefore, we investigated the effect of MgD with various Ca:Mg ratios on cellular oxidative stress, intracellular Ca levels, calpain activity, markers of genomic instability and cell migration, as well as signaling pathways related to these processes using the well-characterized CT26 colon cancer cell line, which shares numerous molecular characteristics with aggressive and undifferentiated human colon carcinoma cells.

Figure 1. Mg status regulates intracellular calcium levels, oxidative stress, TRPM7 expression, and calpain activity, but not calpain 2 protein expression in CT26 colon cancer cells.

Schematic of Mg blocking intracellular Ca entry through Ca channels (A). CT26 cells were grown under various conditions for 48 h: 100% Mg media, 50% Mg media, 10% Mg media with 100% Ca (i.e. varying Ca:Mg ratios) or with varying Ca levels (100% Mg/100% Ca, 50% Mg/50% Ca, 10% Mg/10% Ca, i.e. keeping Ca:Mg ratio at 1), as indicated (B, C, D, E). In addition, CT26 cells were grown in 10% Mg media followed by 90% Mg supplementation (MgS) (10% Mg + 90% Mg or MgD/MgS) for 48 h (B, C). See Methods for details. (B) Intracellular Ca concentrations were analyzed using the intracellular Ca indicator Fluo-4, AM, as described in the Methods (n = 6 per condition). Data are shown as mean relative fluorescence units (RFU) ($\pm$ SD). (C) Oxidative stress ($\pm$ H₂O₂ stimulation, 100 μ M) was determined using the DCFH-DA assay, as described in the Methods (n = 6 per condition). Data are shown as mean percent control-oxidative stress ($\pm$ SD). CT26 expression of TRPM7 and β -tubulin proteins (D-E) and calpain 2 and β -tubulin proteins (F-G) were assessed by western blotting; representative western blots are shown with quantitation of western blots shown above (mean $\pm$ SD; n = 4-6 per condition). (H) Cell-associated calpain activity levels were assessed after treating CT26 cells with 100% Mg, 50% Mg, or 10% Mg with 100% Ca or 50% Mg/50% Ca and 10% Mg/10% Ca for 6 h or 24 h, as indicated. Data are shown as mean ($\pm$ SD) relative calpain activity corrected for cell protein (n = 4 per condition). *** = $p < 0.001$ vs. 100% Mg; ††† = $p < 0.001$ vs. 100% Mg; ††† = $p < 0.001$ vs. 10% Mg. * = $p < 0.05$ vs. 100% Mg.

Methods and materials

Cell lines, antibodies, and reagents

The CT26.WT (CT26) cell line (derived from N-nitroso-N-methylurethane (NNMU)-induced, undifferentiated mouse colon carcinoma [female]), the HCT116 cell line (human colorectal carcinoma [epithelial] [male]), and the MCF7 cell line

(human breast adenocarcinoma (epithelial) derived from a metastatic site [female]) were purchased from ATCC (Manassas, VA, USA). The A2780 human ovarian carcinoma cell line (epithelial [female]) was obtained from T.C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA, USA). Antibodies: FAK (rabbit anti-mouse), I κ B α (mouse anti-mouse), NF- κ B (p65) (rabbit anti-mouse), E-cadherin (rabbit anti-

mouse), β -catenin (rabbit anti-mouse), p53 (mouse anti-mouse), calpain 2 (rabbit anti-mouse), and β -tubulin (rabbit anti-mouse) were purchased from Cell Signaling Technology (Danvers, MA, USA). The TRPM7 (rabbit anti-mouse) antibody was purchased from Alomone Labs (Jerusalem, Israel). MgSO_4 (anhydrous) was purchased from MP Biomedicals (Irvine, CA, USA). Fluo-4, AM, and 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes/Invitrogen (Carlsbad, CA, USA). 1X MEM (Mg- and Ca-free) media (Cat# MT15015CV) was purchased from Corning (New York, USA). Fetal bovine serum (FBS) containing approximately 20 $\mu\text{g}/\text{ml}$ Mg was purchased from Atlantic Biologicals/Bio-Techne (Minneapolis, MN); note: 10% FBS-containing media contained approximately 2 $\mu\text{g}/\text{ml}$ or <0.1 mM Mg. CaCl_2 was purchased from Ricca Chemical Company (Arlington, TX, USA).

CT26 culture conditions for experimental studies

For experiments with varying Ca:Mg ratios, CT26 cells were plated in 1X MEM media containing 10%FBS, Pen/Strep/glutamine (PSQ), nonessential amino acids (NEAA), Ca (100%/1.35 mM) as CaCl_2 , and either 100% Mg (1.35 mM), 50% Mg (0.675 mM), or 10% Mg (0.135 mM) as MgSO_4 . For the Mg supplementation experiments, CT26 cells were first grown in 10% Mg (0.135 mM), as described above, for 24 h to mimic Mg deficiency and after 24 h, cells were supplemented with 90% Mg (1.215 mM) to simulate Mg replacement after deficiency (Mg supplementation, MgS, 100% final). Cells were harvested and analyzed as indicated below. The 100% values reflect Mg and Ca concentrations in mouse serum (CT26 cells are a mouse cell line) [22]. For experiments where the Ca:Mg ratio was kept at 1, CT26 cells were grown in 1X MEM media containing 10%FBS, PSQ, NEAA, and either 100% Ca (1.35 mM) and 100% Mg (1.35 mM), 50% Ca (0.675 mM) and 50% Mg (0.675 mM), or 10% Ca (0.135 mM) and 10% Mg (0.135 mM), as CaCl_2 and MgSO_4 , respectively.

Analysis of protein markers by western blotting

CT26 cells were harvested 6 h, 12 h, 18 h, 24 h, and/or 48 h after plating in 100% Mg, 50% Mg,

or 10% Mg media (with 100% Ca or varying Ca concentrations) or after Mg supplementation after MgD (MgS/MgD), as described above. CT26 cell pellets were homogenized in lysis buffer (Tris-buffered saline, pH 7.3 containing 0.25% Triton X-100 and protease and phosphatase inhibitor cocktail); protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Proteins (50 $\mu\text{g}/\text{lane}$) were separated by NuPAGE electrophoresis under reducing conditions (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's directions and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking for 1 h, the membranes were incubated with each primary antibody (FAK, $\text{I}\kappa\text{B}\alpha$, NF- κB (p65), E-cadherin, β -catenin, p53, calpain 2, and β -tubulin) overnight at 4 °C (one antibody at a time and according to the manufacturer's directions). After washing, the blots were then incubated with the appropriate HRP-linked secondary antibody, anti-rabbit or anti-mouse IgG (Cell Signaling, according to the manufacturer's guidelines) for 2 h at room temperature and washed. Blots were developed using the Clarity ECL reagent (Bio-Rad) and assessed on the ChemiDoc MP Imaging System (Bio-Rad) using the ImageLab Software. A representative image is shown for each set of proteins (FAK, $\text{I}\kappa\text{B}\alpha$, NF- κB (p65), E-cadherin, β -catenin, p53, calpain 2, and TRPM7). Blots were stripped with ReBlot Plus (Millipore) and reprobed with various primary antibodies and secondary antibodies (described above). Band loading was confirmed using β -tubulin protein; quantitation was determined using Image J Software (NIH). Data from blots are presented as ratios of each protein to total protein (mean $\pm$ SD).

Determination of intracellular calcium concentrations

Forty-eight hours after plating in 100% Mg, 50% Mg, or 10% Mg media ($\pm$ MgS) with 100% Ca (for increasing Ca:Mg ratios) or varying Ca concentrations (keeping Ca:Mg ratio at 1), CT26 cells were washed with Mg and Ca-free HBSS and then were incubated with Fluo-4-AM (dissolved with DMSO; Molecular Probes/Invitrogen) and pluronic acid for 0.5 h at 37 °C, followed by 0.5 h at room temperature. The cells were then harvested, washed with HBSS, resuspended at 1×10^6 cells/ml, and plated in

96-well plates (100 µl/well). Intracellular Ca concentrations were measured with the Genios Pro Fluorescence plate reader (Tecan) at an excitation wavelength of 485 nm and emission wavelength of 535 nm. Data were collected as relative fluorescence unit (RFU) (mean $\pm$ SD).

Assessment of oxidative stress using the DCFH-DA assay

CT26 cells were harvested 48 h after plating in either 100% Mg or 10% Mg ($\pm$ 90% Mg, MgS post-MgD) with 100% Ca (to mimic increasing Ca:Mg ratios), washed with Mg and Ca-free HBSS, resuspended at 1×10^6 cells/ml in HBSS, and labeled with DCFH-DA (20 µM) for 20 min. Labeled cells were washed, treated with vehicle or H₂O₂ (100 µM), and analyzed for oxidative stress 0.5 h later in a quantitative manner using the Victor3 fluorescence plate reader (Perkin-Elmer) at an excitation wavelength of 485 nm and emission wavelength of 535 nm. This assay measures the conversion of nonfluorescent DCFH-DA into fluorescent dichlorofluorescein (DCF) by ROS intermediates [23]. Data are presented as percent control oxidative stress (mean $\pm$ SD).

Determination of cell-associated calpain activity

Cell-associated calpain activity was measured using a fluorometric-based Calpain activity assay kit (BioVision, Inc., Milpitas, CA), according to the manufacturer's guidelines and as previously described [24]. Briefly, CT26, A2780, HCT116, and MCF7 cells were plated in 1X MEM media containing 10% FBS, PSQ, NEAA, and either 100% Mg, 50% Mg or 10% Mg with 100% Ca (to mimic increasing Ca:Mg ratios) or 50% Mg/50% Ca and 10% Mg/10% Ca (for Ca:Mg ratios = 1), as indicated. After 6 h or 24 h (as indicated), cells were washed and processed to assess calpain activity. Samples were assayed in quadruplicate (with and without Z-LLY-FMK at 10 µM, calpain inhibitor). Total calpain activity was determined after subtracting the activity observed in the presence of Z-LLY-FMK; activity was corrected for total protein content, determined by micro-BCA assay (Pierce/thermoscientific). Data are shown as relative calpain activity/mg protein (mean $\pm$ SD).

Scratch assay for assessing cell migration

CT26 cells were plated in 1X MEM media containing 10% FBS, PSQ, NEAA, 100% Ca (1.35 mM), and 100% Mg (1.35 mM) in 10 cm dishes. At confluence a scratch was made in the cell monolayer using a sterile 200 µl pipette tip. Subsequently, the cells were washed twice to remove cell debris and the media was replaced with 1X MEM containing 10%FBS, PSQ, NEAA, 100% Ca, and either 100% Mg or 10% Mg, and photographed using a Zeiss Axiovert 200 M Apotome Microscope and then incubated at 5%CO₂/37°C, for 48 h and photographed again. The percent wound closure was measured by calculating the area under the scratch at t = 0 and at t = 48 h using Image J Software (NIH), as in [25].

Assessment of aneuploidy by flow cytometry

Following 48 h exposure of CT26 to the various Mg- and Ca-culture conditions described above cells were underwent cell cycle analysis of DNA content using flow cytometry based on a method developed by Carbonari *et al.* [26]. Briefly, cells were lifted with Accutase, and for each condition a total of 0.5×10^6 cells were centrifuged (300 g, 4 min, 20 °C) and washed with PBS. Cells were resuspended in PBS containing 10% FCS (PF10) and absolute formamide (Invitrogen, Carlsbad, CA) was added and gently mixed to obtain a final concentration of 50% (1:1 v/v) in PF10 (PF10/FA50). After 10 min at 20 °C, cells were centrifuged and washed with PF10 and centrifuged again. Cell pellets were resuspended in PF10 containing 7-AAD (10 µg/ml, Sigma Aldrich, USA) and samples were incubated for 60 min at 20 °C. Cells were washed with PF10 and resuspended in PF10 and then assessed by flow cytometry (Fortessa Flow Cytometer (BD)) and analyzed using FlowJo software (version 10.1r5; Ashland, OR).

Statistical analyses

Experiments were performed at least twice and data are expressed as mean $\pm$ SD. One-way ANOVAs were used for multiple comparisons followed by Bonferroni post-hoc testing using GraphPad Prism version 5.04 (GraphPad Soft-

ware, San Diego, CA, USA). P values <0.05 were considered significant.

Results

Mg status modulates intracellular Ca concentrations and oxidative stress in CT26 tumor cells

We examined the effect of MgD exposure on intracellular Ca concentrations using CT26 colon cancer cells. CT26 cells grown in Mg-deficient conditions (50% Mg or 10% Mg-containing media with 100% Ca) had significantly higher intracellular Ca concentrations after 48 h compared to cells grown with media containing 100% Mg (with 100% Ca) (*figure 1B*). Next, we examined the effect of Mg supplementation (MgS) after exposure to MgD (MgS/MgD) *vs.* MgD alone on intracellular Ca concentrations. The Ca concentrations in the MgS/MgD-treated cells were significantly lower than cells grown under continuous MgD conditions; Ca concentrations in MgS/MgD cells were comparable with control cells maintained in 100% Mg (*figure 1B*). When we examined the effect of MgD, when Ca was decreased proportionally (*i.e.* Ca:Mg ratios were 1), on intracellular Ca concentrations, we found that intracellular Ca levels decreased as the extracellular Mg and Ca concentrations declined (*figure 1B*). When the Ca:Mg ratio was maintained at 1, CT26 cells grown in 100% Mg/100% Ca media had the highest intracellular Ca levels, followed by CT26 cells grown in 50% Mg/50% Ca; CT26 cells grown with 10% Mg/10% Ca showed the lowest intracellular Ca concentrations (*figure 1B*).

Next, we examined the effect of Mg status on oxidative stress in CT26 cells using the DCFDA assay. Cells grown in 10% Mg/100% Ca media had significantly higher oxidative stress levels as compared to cells maintained in 100% Mg/100% Ca media, under basal and H₂O₂-stimulated conditions (*figure 1C*). CT26 cells treated with MgS (90% Mg) after MgD (10% Mg) exhibited significantly lower oxidative stress under baseline and H₂O₂-stimulated conditions compared with cells maintained in 10% Mg (*figure 1C*), supporting that MgS after MgD reverses the effect of MgD alone.

TRPM7 expression increases in CT26 cells following MgD in a Ca:Mg ratio-dependent manner

Next, we examined the effect of MgD, on the expression of the divalent cation ion channel kinase TRPM7, which is permeable to both Ca and Mg, and regulates tumor cell invasion and migration [27]. Cells grown in MgD/100% Ca media (with a high Ca:Mg ratio) for 48 h showed a dose-dependent increase in TRPM7 expression compared to cells grown in control 100% Mg/100% Ca media; this difference was significant with 10% Mg/100% Ca (*figure 1D*). When both Ca and Mg concentrations were decreased proportionately in the CT26 cell media (maintaining Ca:Mg ratio at 1), we found no significant change in TRPM7 expression with decreasing Mg concentrations after 48 h (*figure 1E*).

Calpain 2 expression is not dependent on Mg or Ca status in CT26 tumor cells

Next, we examined the effect of Mg and Ca status on the expression of calpain 2, a Ca-dependent protease that regulates downstream signaling proteins involved in cell motility. CT26 cells exposed to MgD with 100% Ca (with high Ca:Mg ratios) for 48 h showed no difference in calpain 2 expression (*figure 1F*). Similarly, when the Ca:Mg ratio was kept at 1 there was no change in calpain 2 expression following MgD (*figure 1G*).

Calpain activity is enhanced by Mg deficiency

Although MgD did not alter calpain 2 expression by CT26 cells, assessment of calpain activity revealed that 50% Mg and 10% Mg with 100% Ca (high Ca:Mg ratios) significantly enhanced calpain activity (*vs.* 100% Mg) when assessed 24 h later (*figure 1H*). However, 50% Mg appeared to induce more calpain activity than 10% Mg conditions (*figure 1H*). This enhancement in calpain activity was not found when CT26 cells were cultured in MgD media with decreasing Ca concentrations (*figure 1H*). When calpain activity was measured at an earlier time point (6 h post MgD), 10% Mg induced higher calpain activity when compared to 50% Mg (with 100% Ca) (*figure 1H*). Next, we

explored whether MgD promoted calpain activity in other tumor cell lines. We found that 10% Mg (with 100% Ca) significantly induced calpain activity in the HCT116 human colon carcinoma cell line ($96,132 \pm 8,188$ [100% Mg/100% Ca] *vs.* $143,493 \pm 12764$ [10% Mg/100% Ca] relative calpain activity/mg protein, $p < 0.001$), the A2780 human ovarian cancer cell line ($50,428 \pm 7,674$ [100% Mg/100% Ca] *vs.* $77,486 \pm 7,957$ [10% Mg/100% Ca] relative calpain activity/mg protein, $p < 0.005$), and the MCF7 human breast cancer cell line ($45,626 \pm 3,379$ [100% Mg/100% Ca] *vs.* $76,457 \pm 6,511$ [10% Mg/100% Ca] relative calpain activity/mg protein, $p < 0.0005$).

MgD promotes degradation of N-terminal FAK; MgS blocks FAK degradation

Ca-activated calpain 2 cleaves full-length FAK, important for cell motility, to produce a 90kDa N-terminal FAK and 35kDa C-terminal FAK [28]. Here, we examined the effect of Mg status on the degradation of N-terminal FAK (N-FAK). CT26 cells cultured in 100% Mg/100% Ca media for 48 h showed N-FAK expression, whereas CT26 cells cultured in MgD media (50% Mg/100% Ca and 10% Mg/100% Ca) for 48 h showed no N-FAK expression (*figure 2A*). Kinetics studies revealed that at 6 h, N-FAK was expressed by CT26 cells under both MgD (10% Mg or 50% Mg) and 100% Mg (control) conditions (*figure 2B*). At 12 h cells grown with 10% Mg expressed less N-FAK protein product when compared to 100% Mg (control cells) (*figure 2B*). After 18 h N-FAK expression in 10% Mg-exposed cells was significantly decreased (*vs.* 100% Mg, control cells), while N-FAK expression in 50% Mg-exposed cells was decreased to a lesser degree (*vs.* 100% Mg, control cells) (*figure 2B*). At 24 h, there was no expression of N-FAK found in either 10% Mg or 50% Mg-cultured cells (*figure 2B*) similar to that observed at 48 h (*figure 2A*).

We also investigated whether MgS after MgD (MgS/MgD) with 100% Ca reversed these effects in CT26 cells. At 24 h post-MgS/MgD, we observed no N-FAK protein expression; however, at 36h-post MgS/MgD, the N-FAK protein product was observed and its expression was further increased at 48 h (*figure 2A*).

MgD leads to p53 degradation in CT26 cells and this is rescued by MgS

Calpain also degrades p53 [29]. Therefore, we examined p53 expression by CT26 cells cultured under various Mg concentrations. Similar to N-FAK at 48 h, p53 expression was not observed (presumably degraded) under MgD conditions (10% Mg and 50% Mg with 100% Ca), whereas it was expressed when cells were in 100% Mg/100% Ca media (*figure 2A*). The kinetics study showed that p53 was expressed by cells maintained for 6 h in both MgD (containing 10% or 50% Mg) and control media (containing 100% Mg) with 100% Ca (*figure 2B*). Loss of p53 expression was first detected 12 h post-10% Mg (*figure 2B*) and it was almost undetectable by 18 h post-10% Mg, while CT26 cells maintained in 50% Mg showed reduced p53 expression when compared to 100% Mg (control)-exposed cells (*figure 2B*). At 24 h, no p53 expression was detected in cells exposed to either 10% Mg or 50% Mg, whereas cells grown in 100% Mg-media retained p53 expression (*figure 2B*). Also, we examined the effect of MgS/MgD on p53 expression; p53 expression was not restored after 24 h post-MgS/MgD but it was detected 36 h post-MgS/MgD and expression increased at 48 h post-MgS/MgD compared to MgD alone (*figure 2A*).

MgD regulates key NF- κ B pathway proteins in CT26 cells

The NF- κ B pathway is implicated in tumorigenesis and tumor progression, as cancer cells exhibit constitutive NF- κ B activation. Next, we examined the effect of Mg status on the canonical NF- κ B pathway proteins, I κ B α and p65 (RelA). Full-length I κ B α protein (39kDa) that inhibits NF- κ B activation can be cleaved by calpain. At 48 h post-MgD (50% Mg and 10% Mg/100% Ca) CT26 cells expressed an I κ B α cleavage product when compared to 100% Mg/100% Ca-exposed cells, which only expressed the full-length protein (*figure 2A*). The time course experiment shows that the expression of full-length I κ B α began to decrease in 10% Mg-exposed cells after 12 h (*figure 2B*). By 18h-post MgD, the full-length I κ B α protein completely disappeared with 10% Mg, whereas two bands (one corresponding to the full-length I κ B α (39kDa)) and a lower band consistent with the 30-40 amino acid

Figure 2. Mg deficiency (MgD) promotes N-FAK, p53 degradation and cleavage of IκBα, p65 in CT26 cells; expression returns when Mg supplementation (MgS) follows MgD. No loss of p53, N-FAK, IκBα, or p65 expression occurs when Ca:Mg ratio is kept at 1. CT26 cells were grown in various Mg and Ca-containing media (n = 3-5 per condition) as indicated (and described in *figure 1* and the Methods section) for 6 h, 12 h, 18 h, 24 h, and 48 h, as indicated (A-B). Representative western blots for N-FAK, p53, IκBα, p65, and β-tubulin proteins are shown for CT26 cells analyzed at 48 h post 100% Mg, 50% Mg, 10% Mg, and MgD/MgS (A). Representative western blots for N-FAK, p53, IκBα, p65, and β-tubulin proteins are shown for CT26 cells analyzed at 6 h, 12 h, 18 h, and 24 h post 100% Mg, 50% Mg or 10% MgD (B). CT26 cells were grown in 100% Mg/100% Ca, 50% Mg/50% Ca, or 10% Mg/10% Ca (maintaining Ca:Mg ratio at 1) (n = 3-4 per condition) for 48 h and analyzed for N-FAK, p53, IκBα, p65, and β-tubulin protein levels by western blotting (C).

cleaved product were observed in cells grown under 50% Mg at 18 h (*figure 2B*). After 24 h, 100% Mg-exposed cells continued to express full-length I κ B α (*figure 2B*). At 24 h post-10% Mg- and 50% Mg-exposure CT26 cells expressed a single I κ B α band corresponding to the cleaved product (*figure 2B*). After 24 h of MgS/MgD only the cleaved I κ B α product was found and at 36 h both full-length I κ B α and some cleaved product was found (*figure 2A*). By 48 h post-MgS/MgD full-length I κ B α was prominently expressed (*figure 2A*).

The full-length p65 subunit of NF- κ B is 65kDa and its cleaved 40kDa product lacks the C-terminal transactivation domain required for the transcription of target genes [30]. We examined p65 protein expression (and its cleavage product) by CT26 cells under MgD/100% Ca conditions. At 48 h a single 65kDa NF- κ B band was expressed by cells grown in 100% Mg/100% Ca, whereas cells maintained in MgD media (50% and 10% Mg)/100% Ca expressed a single 40kDa NF- κ B cleavage product (lacking the C-terminal transactivation domain) (*figure 2A*). Our kinetics study showed that at 6 h post-MgD, there was no cleavage of p65, at 12h-post MgD less full-length p65 protein was observed in CT26 cells cultured in 10% MgD media (*vs.* 100% Mg media) and by 18h-post MgD the amount of full-length p65 was dramatically reduced in the 50% Mg-exposed cells (*vs.* 100% Mg-exposed cells); this was accompanied by an increase in the 40kDa cleavage product (*figure 2B*). CT26 cells maintained in 50% Mg-containing media for 18 h showed some expression of the full-length p65 protein, but this was weaker than 100% Mg-exposed cells and stronger than the expression levels observed with 10% Mg (*figure 2B*). In addition, the cleaved 40kDa NF- κ B band was weaker in intensity when compared to cells grown with 10% Mg (*figure 2B*). At 24 h under MgD conditions (10% Mg and 50% Mg) only a single 40kDa NF- κ B product was found (*figure 2B*). Similarly, at 24hr post-MgS/MgD only the 40kDa cleavage product was observed (*figure 2A*); at 36 h post-MgS/MgD the full-length p65 protein was found with some 40kDa cleavage product, whereas at 48 h post-MgS/MgD the full-length p65 band was prominently expressed with faint expression of the 40kDa cleavage product (*figure 2A*).

No loss of N-FAK, p53, I κ B α , or p65 when CT26 cells are cultured in MgD media when Ca:Mg ratios are maintained at 1

As we determined that TRPM7 expression by CT26 cells was not dependent on MgD but rather on the Ca:Mg ratio in media, we examined the expression of p53, N-FAK, I κ B α , and p65 when Ca was decreased in proportion to Mg in the media. When the Ca:Mg ratio was kept at 1, no loss of p53, N-FAK, full-length I κ B α , or p65 was observed when CT26 cells were cultured in 10% Mg/10% Ca or 50% Mg/50% Ca (MgD) media (*figure 2C*).

MgD leads to loss of full-length E-cadherin and β -catenin; no loss when Ca:Mg ratio is 1

Full-length E-cadherin (120kDa) is a glycoprotein important for cell-cell adhesion. When we examined the effect of MgD on E-cadherin expression in CT26 cells after 48 h, we found that cells grown in 10% Mg/100% Ca and 50% Mg/100% Ca-media lacked expression of the full length E-cadherin protein, but showed increased expression of the cleaved 100kDa E-cadherin fragment (*figure 3A*). In contrast, 100% Mg/100% Ca-exposed CT26 cells expressed the full-length E-cadherin, as well as the cleaved 100kDa fragment (less when compared to MgD). This fragment, generated via calcium-calpain-dependent pathway, is unable to bind β -catenin and promotes metastasis [17]. Our kinetic studies revealed that at 6 h post MgD, the expression of full-length E-cadherin was observed under all Mg conditions, but CT26 cells cultured in 10% Mg/100% Ca media showed increased levels of the cleaved 100kDa fragment when compared to cells cultured in 50% Mg/100% Ca-media or 100% Mg/100% Ca-media (*figure 3A*). At 12 h post MgD there was loss of full-length E-cadherin in the 10% Mg/100% Ca-exposed cells, and at 24 h post MgD the level of E-cadherin was similar to that found at 48 h (*figure 3A*). In addition, we examined the effect of Mg status on β -catenin expression in CT26 cells at 48 h and found that full-length β -catenin (92kDa) expression was only present in the 100% Mg-exposed CT26 cells, whereas the MgD-exposed CT26 cells expressed a 75kDa β -catenin cleavage product (*figure S1A*). When CT26 cells were maintained in media where the

Figure 3. Mg deficiency (MgD) promotes E-cadherin cleavage in CT26 cells; no cleavage occurs when Ca:Mg ratio is kept at 1. MgD also promotes CT26 cell migration. CT26 cells were grown in various Mg and Ca-containing media (n = 3-4 per condition) as indicated (and described in *figure 1* and the Methods section) for 6 h, 12 h, 18 h, 24 h, and 48 h, as indicated (A-B). Representative western blots for E-cadherin and β -tubulin proteins are shown for CT26 cells analyzed at 6 h, 12 h, 18 h, 24 h, and 48 h post 100% Mg, 50% Mg, 10% Mg, and MgD/MgS (A). CT26 cells were grown in 100% Mg/100% Ca, 50% Mg/50% Ca, or 10% Mg media/10% Ca (keeping Ca:Mg ratio at 1) (n = 3-4 per condition) for 48 h and analyzed for E-cadherin and β -tubulin protein levels by western blotting (B). For scratch/migration assays CT26 cells were grown in 100% Mg with 100% Ca until confluence (n = 3 per condition), when the scratch was made and then photographed (t = 0 h); cells were washed, the media was replaced with 100% Mg media or 50% Mg media or 10% Mg media with 100% Ca (as indicated), and the cells were incubated and then photographed 48 h later (t = 48 h) (C). Quantification of the migration assay is shown as percent wound closure (mean $\pm$ SD)(D). *** = $p < 0.001$ vs. 100% Mg.

Ca:Mg ratio was kept at 1, no loss of E-cadherin (*figure 3B*) or β -catenin (*figure S1B*) was observed with any MgD conditions.

MgD (with a high Ca:Mg ratio) increases the migration of CT26 colon cancer cells

Based on our previous results showing that CT26 cells cultured under MgD/100% Ca conditions (with high Ca:Mg ratios) expressed stress-related signaling pathways associated with motility/migration, we examined the effect of MgD on the migration of CT26 cells using the scratch assay. While cells maintained in 50% Mg/100% Ca-media for 48 h showed slightly increased migration when compared to cells maintained in 100% Mg/100% Ca-media for 48 h, cells maintained in 10% Mg/100% Ca-media exhibited significantly enhanced migration when compared to cells maintained in 100% Mg/100% Ca-media (*figure 3C-D*).

CT26 cells maintained in MgD media (with a high Ca:Mg ratio) exhibit increased aneuploidy; MgD media is not associated with increased aneuploidy when Ca:Mg ratio is 1

We showed that MgD increases oxidative stress in CT26 cells and the loss of p53, the guardian of the genome. Therefore, we investigated the effect of MgD with varying Ca:Mg ratios on baseline aneuploidy, a phenotype that can lead to genomic instability. Baseline aneuploidy of CT26 cells grown in 100% Mg/100% Ca was denoted as 100%. When cells were cultured in 50% Mg/100% Ca-containing media aneuploidy increased to 134% and this was dramatically increased to 178% when the cells were cultured in 10% Mg/100% Ca (*figure 4A-D*). When the Ca:Mg ratios were maintained at 1, CT26 colon cancer cells exhibited 100% aneuploidy when maintained in 100% Mg/100% Ca-media, and 97% and 100.5% aneuploidy when maintained in 50% Mg/50% Ca-media and 10% Mg/10% Ca-media, respectively (*figure 4E-H*).

Discussion

Inadequate Mg consumption is widespread, while Ca supplementation is prevalent [31]. Consistent with this imbalance, several epide-

miological studies have linked higher dietary Ca:Mg ratios with increased incidences of cancers, including colon cancer [15, 17-19, 32, 33] and higher rates of cancer mortality [15, 16]. In this study we showed that culturing murine CT26 colon cancer cells in media containing insufficient Mg concentrations with a constant, normal (100%) Ca concentration (increased Ca:Mg ratios) dramatically affects calpain activity, oxidative stress, cell migration, and aneuploidy, as well as the expression of several components of signaling pathways leading to a metastatic-like phenotype. By contrast, decreasing Ca levels in proportion with decreasing Mg levels (keeping the Ca:Mg ratio at 1) had little or no effect on calpain activity, oxidative stress, cell migration, aneuploidy, or the expression of several components of associated signaling pathways.

Mg serves as nature's physiological Ca channel antagonist [6] (*figure 1A*). Using the CT26 colon cancer cell line we demonstrated that MgD (10% Mg, 50% Mg) when accompanied by higher Ca:Mg ratios increased intracellular calcium concentrations (*figure 1B*). In addition, MgS post-MgD (10% Mg, followed by 90% Mg [100% Mg final]) decreased intracellular Ca concentrations comparable to control cells maintained in 100% Mg (*figure 1B*). This likely occurs by preventing Ca entry and/or by controlling the efflux of Ca out of the cells possibly by regulating Ca-ATPase pumps in the plasma membrane and SER, as Mg is a cofactor for the activity of ATP. Due to the gating property of Mg on intracellular Ca entry when Ca:Mg ratios were maintained at 1, intracellular Ca concentrations decreased as the extracellular Ca (and Mg) concentrations in the media decreased (*figure 1B*).

The relationship between MgD and oxidative stress is well documented [34]. Mg deficiency has been implicated in the initiation and progression of cancer by promoting oxidative stress and inflammation [10]. MgD promotes oxidative stress by decreasing the antioxidant defense of cells and by increasing intracellular Ca concentrations leading to intracellular Ca overload. Using the DCFH-DA assay, we observed that MgD with 100% Ca increased oxidative stress in CT26 cells under baseline and H₂O₂-stimulated conditions and this effect was reversed by Mg supplementation following Mg deficiency (*figure 1C*). Oxidative stress can activate TRPM7, which plays an important role in regulating intracellular Mg, Ca, and Zn homeo-

Figure 4. Culturing CT26 cells in MgD media with high Ca:Mg ratios increases aneuploidy. CT26 cells were grown in 100% Mg media (A), 50% Mg media (B), or 10% Mg (C) media for 48 h, with 100% Ca-containing media (i.e. increasing Ca:Mg ratios, $n = 2-3$ per condition). CT26 cells were analyzed for aneuploidy after 48 h by flow cytometry (A-C). Quantification % aneuploidy from A-C is shown as mean ($\pm$ SD) in (D). A similar set of CT26 cell cultures was assessed for aneuploidy after exposure to 100% Mg/100% Ca (E), 50% Mg/50% Ca (F), and 10% Mg/10% Ca (G), keeping Ca:Mg ratio at 1 ($n = 2-3$ per condition). CT26 cells were analyzed for aneuploidy after 48 h by flow cytometry (E-G). Quantification of aneuploidy from E-G is shown as mean ($\pm$ SD) in (H). *** = $p < 0.001$ vs. 100% Mg.

Figure 5. Summary: Mg deficiency with high Ca:Mg ratios increases intracellular Ca levels, calpain activity, oxidative stress, genomic instability, and cell migration by activating various signaling pathways in CT26 colon cancer cells.

stasis. This channel pore preferentially permits the flow of Mg and to a lesser extent Ca [35-38]. Cancer cells exhibit increased TRPM7 expression [39], which has been shown to promote cell migration and epithelial to mesenchymal transition (EMT). In CT26 colon cancer cells, TRPM7 expression was dependent on the Ca:Mg ratio in the media, with enhanced TRPM7 expression under MgD conditions with higher Ca:Mg ratios (*figure 1D*), whereas no change in TRPM7 expression was found when the Ca:Mg ratio was kept at 1 (*figure 1E*). Our findings are consistent with Sun *et al.* who reported similar results (higher TRPM7 with higher Ca:Mg ratios) using human prostate cancer specimens [17] and Santos and Hussain, who showed that exogenously added Mg significantly reduced prostate cancer cell migration [40]. However, they are not consistent with Baldoli *et al.*, who reported that Mg deficiency (0.1 mM) reduced TRPM7 protein expression and decreased human microvascular endothelial cell migration [41]. The exact reasons for these differences are not known. It is possible that primary endothelial cells *vs.* cancer cell lines exhibit different gene expression profiles and motility differences. The CT26 cell line harbors a Kras mutation, expresses several cancer-relevant genes (e.g. *Nras*, *Vegfa*, and *Myc*), and has lost tumor suppressor gene expression (e.g. *Cdkn2a*) [42, 43]. This gene expression profile is very different from primary endothelial cells and may impact cell motility. In addition, cancer cells may be able to better tolerate high stress environments and utilize increased intracellular Ca to promote pathways that enhance their motility. For example, intracellular Ca in cancer cells may modulate classical receptor kinase signaling pathways leading to changes in cell matrix adhesion and myosin contraction that promote migration (as described by [44]). Future studies are needed to explore the cell specificity of our findings.

Because epidemiological studies support that MgD and increased Ca:Mg intake ratios enhance mortality among patients with colorectal cancer [15], we studied the effect of MgD on cell migration and signaling pathways/markers associated with aggressive, metastatic-like phenotypes using CT26 colon cancer cells. Culturing CT26 cells in 10-50% MgD/100% Ca media increased cell migration when compared to cells maintained in 100% Mg/100% Ca media

(*figure 3C-3D*). These findings are consistent with studies by Nasulewicz *et al.* showing increased metastases in mice following an MgD diet (with high Ca:Mg ratio) [45]. Increased intracellular Ca is implicated in cell motility, an important aspect of tumor invasion and metastasis [46], and therefore, we examined the effect of Mg and Ca status on the expression of calpain 2, a Ca-dependent cysteine protease that catalyzes limited proteolysis of several proteins involved in cell motility. Increased calpain expression has been reported in numerous types of cancer, including colorectal carcinomas [47-49]. We found that in CT26 cells, calpain 2 protein expression was independent of extracellular Ca or Mg status (*figure 1F-1G*), suggesting that calpain activity is modified by increasing intracellular Ca concentrations. Using a fluorescence-based calpain activity assay, we found that 50% Mg and 10% Mg (with 100% Ca, high Ca:Mg ratios) significantly induced calpain activity in the CT26 cell line (*figure 1H*); the earlier (6hr) time point showed a dose-response curve, suggesting that compensatory pathways and Ca utilization may occur by the 24hr time point. By contrast, calpain activity was not altered when both Mg and Ca concentrations were reduced equally, to maintain Ca:Mg at 1 (*figure 1H*). Furthermore, we observed that 10% MgD/100% Ca treatment of other cancer cell lines (A2780 human ovarian cancer, MCF7 human breast cancer, and HCT116 human colon cancer) also significantly promoted calpain activity levels over 100% Mg/100% Ca-treated cells; these findings show that our results are not restricted to CT26 mouse colon cancer cells.

Next, using CT26 cells we examined the effects of MgD on downstream targets of calpain. Since calpain proteolyzes both FAK [28] and p53 [29] and we demonstrated that MgD/100% Ca increases intracellular Ca and oxidative stress, we examined the effect of MgD/100% Ca on N-FAK and p53 degradation. MgD/100% Ca-exposed CT26 colon cancer cells exhibited reduced expression of N-FAK in a time-dependent manner (*figure 2A-B*). By 24hr post-MgD/100% Ca the degradation of N-FAK was complete. We also observed the time-dependent reappearance of N-FAK when the cells were treated with MgS/MgD (*figure 2A*). FAK plays an important role in various cancer processes, such as migration and invasion [50-52]. Although not demonstrated, we propose that MgD leads to the

formation of a truncated FAK, lacking the N-terminal FERM domain, which displays higher catalytic activity, as described by Jacamo [53]. Similarly, we found that MgD/100% Ca-exposure of CT26 cells reduced p53 expression in a time-dependent manner, with complete loss by 24 hr. Consistent with our finding with FAK, we also observed the time-dependent reappearance of p53 following MgS for 36-48 h (after MgD, *figure 2A*). Studies by Lim *et al.* showing that FAK enters the nucleus, where its N-terminus binds p53 and degrades it, while FAK binds Mdm2 (an E3 ubiquitin ligase that negatively regulates p53) leading to the ubiquitination and degradation of the FAK complex [54] provide a potential mechanism for the loss of p53 and FAK expression by MgD-exposed CT26 cells.

NF- κ B can be activated by reactive oxygen species (ROS), DNA damage, and oncogenic stress from inside the cells [55]. *In vitro* studies have demonstrated that both I κ B α and NF- κ B p65 are substrates of calpain [56, 57]. Here, MgD/100% Ca-exposed CT26 cells showed cleavage of full-length I κ B α to a smaller fragment, corresponding to the loss of approximately 30-40 amino acids, in a time-dependent manner and cleavage was complete by 24hr (*figure 2A-B*). MgS post-MgD resulted in the re-expression of full-length I κ B α and loss of the cleaved fragment by 36hr post MgS (*figure 2A*). I κ B α cleavage allows nuclear translocation of the p65 subunit of NF- κ B. We found that MgD-exposed CT26 cells expressed a cleaved fragment of p65 corresponding to the 40kDa fragment lacking the transactivation domain (required for gene transcription) (*figure 2A-B*). MgS post-MgD/100% Ca led to expression of full-length p65 at 36 h, which was further enhanced 48 h-post MgS (*figure 2A*).

E-cadherin expression or its cell surface localization is often lost in aggressive or advanced tumors and this loss is associated with increased metastasis and tumor recurrence [58, 59]. The loss of E-cadherin expression is associated with metastasis and worse prognosis in colon cancer; this is more often due to post-translational modifications rather than genetic mutation [60]. Rios-Doria and Day *et al.* reported that cleavage of full-length E-cadherin to its 100kDa-fragment occurs in a calcium-calpain-dependent manner [61]; this E-cadherin fragment is unable to bind to β -catenin, γ -catenin, or

p120, which are essential for the cell adhesion and signaling functions of E-cadherin [61, 62]. In one study, all metastatic prostate tumors expressed the 100kDa E-cadherin fragment, whereas localized prostate tumors did not express it [61]. Accordingly, we found that MgD-exposed CT26 cells (with increasing Ca:Mg ratios) showed loss of full-length E-cadherin in a time-dependent manner, accompanied by increasing levels of the 100kDa cleaved product (*figure 3A*). As cleaved E-cadherin is unable to bind β -catenin, we also examined the effect of MgD on β -catenin expression and found that MgD-exposed CT26 cells did not express full-length β -catenin, but expressed a 75kDa cleaved product, with highest expression following 10% Mg (*figure S1A*).

Our results support that Mg status regulates intracellular Ca concentrations and oxidative stress leading to altered signaling pathways via calpain-mediated cleavage of target proteins and/or oxidative stress-induced changes that are associated with more aggressive tumors. Our next objective was to assess similar pathways and proteins after maintaining CT26 cells in MgD-media where Ca:Mg ratios were kept at 1. Exposure of CT26 cells to decreasing concentrations of Mg and Ca (with a constant Ca:Mg ratio of 1) maintained the levels of p53, N-FAK, I κ B α , p65 (*figure 2C*), full-length E-cadherin (*figure 3B*), and β -catenin (*figure S1B*). We propose that MgD relative to Ca in the extracellular media may promote Ca influx, with release of Ca from intracellular stores followed by activation of the store operated calcium entry mechanism contributing to sustained intracellular Ca concentrations. MgS following MgD exposure likely promotes the extrusion of cytosolic Ca levels in CT26 cells possibly by activating the ATP-dependent Ca pumps.

We observed enhanced oxidative stress and loss of p53 in MgD-exposed CT26 cells (*figure 1C* and *figure 2A-B*). Since oxidative stress leads to DNA damage and p53 stimulates various DNA repair mechanisms [63] and serves as the guardian of the genome by triggering growth arrest following DNA damage [64], we examined the effect of MgD exposure of CT26 cells on aneuploidy, a marker of genomic instability. MgD with 100% Ca (high Ca:Mg ratios) significantly enhanced aneuploidy in G₁ cells (*figure 4 A-D*). By contrast, CT26 cells main-

tained in media with a Ca:Mg ratio at 1, showed no change in baseline aneuploidy with decreasing Mg and Ca levels (*figure 4 E-H*). An alternate explanation for this finding is that the aneuploid cell population is proliferating more rapidly under conditions that increase intracellular Ca relative to intracellular Mg. Our previously published data using the CT26 model of colon cancer revealed that mice fed a MgD diet had larger tumors than mice fed the 100% Mg diet [65]. We were unable to elucidate the molecular mechanisms due to limited tumor tissue available for analysis; in hindsight it is possible that the aneuploid cell population in the tumor proliferated more with sustained, elevated intracellular Ca with the MgD diet, as compared to the diploid cell population.

In conclusion, we show for the first time that MgD (with high Ca:Mg ratios) promotes oxidative stress and increases cell migration using CT26 colon cancer cells. We propose that MgD exposure (with high Ca:Mg ratios) increases intracellular Ca levels in part by increasing TRPM7 expression and unblocking the gating effect of Mg on intracellular Ca entry. Increased intracellular Ca levels promote ROS generation and MgD likely blunts cell-associated antioxidant capacity to further promote oxidative stress. We further show that high intracellular Ca levels and elevated oxidative stress: 1) activate calpain signaling leading to downstream activation of E-cadherin, FAK, p53, NF- κ B, and β -catenin signaling pathways and 2) lead to p53 loss and genomic instability, which in combination promote an aggressive and metastatic phenotype (*figure 5*). This study also sheds some insight on the epidemiological findings that link high Ca:Mg ratios with increased incidence of cancer [15, 17-19, 32, 33] and increased mortality among colon cancer patients [15, 16]. Finally, it provides a potential mechanism to explain prior studies reporting increased metastasis and higher mortality among tumor-bearing mice fed an MgD diet *vs.* tumor-bearing mice fed a 100% Mg diet [45]. Future studies should further explore the roles of Mg status and Ca:Mg ratios using additional tumor cell lines and primary colon epithelial cells, more closely assess serum Ca:Mg ratios among patients with cancer, including colon cancer, and investigate Ca:Mg ratios in animal models of cancer and tumor metastasis.

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